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Foreword

The active application of physical and chemical approaches in various areas of physiology and biochemistry is the remarkable phenomenon of the end of the twentieth and the beginning of the twenty-first centuries. From this position, this book brings to the reader's attention a sample of the uses of advanced fluorescence and spectroscopic analytical methods in combination with computer processing of data for the solution of ecological and biochemical problems.

A considerable part of the book describes and discusses results of research on the scientific theme 6.50.2 of the Russian Academy of Sciences: "The further development of energetic bases of resistance of animal and green cells of *Procaryota* and *Eucaryota* to extreme influences of environmental factors."

The book is written by biochemists and physiologists who are interested in modern methods of physics and physical chemistry and are actively applying them in physiological and biochemical experiments. The scientific value of the first book published by the authors' collective from two institutes, *Derivative Spectrophotometry and ESR Spectroscopy for Solution of Ecological and Biological Problems* (2010), was assessed by foreign colleagues and by the world-famous publishing house Springer Verlag (Wien, Heidelberg, New York, Dordrecht, London). As a consequence of the positive reaction from the publishing house, this book was release in English under the title *Derivative Spectrophotometry and Electron Spin Resonance* (*ESR*) *Spectroscopy for Ecological and Biological Questions* (2013). In this uneasy time for Russian science, such recognition is very valuable.

The amazing idea of the known English physicist Prof. E. Rutherford about registration of a derivative of the usual absorption spectrum conquered the analytical world. Leading world manufacturers of electrooptical equipment now produce commercial devices for which Russia pays in currency. However, representatives of our industry, by virtue of inertness, indifference, or lack of understanding of the importance of this question and despite available individual domestic engineering designs, have not considered it necessary to show an initiative in producing this equipment. This happened even though the range of method applications has

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enabled the solution of analytical problems from nuclear physics to microbiology and even though this method can be applied to ecological monitoring of the oceans and the surface of the Earth.

The method of pulse-amplitude modulated fluorescence (PAMF method) appeared in the late 1980s and has quickly earned a world reputation in the international competition against other analytical fluorescence methods. Analytical devices of this scientific direction are made by Walz (Effeltrich, Germany) and have spread worldwide (http://www.walz.com). To our regret, our country does not produce such devices, and application of the method is limited to two or three laboratories in Moscow and St. Petersburg, despite its excellent references.

The original feature of this book is the consideration of combining the possibilities of PAMF as a tool for the *functional* estimation of the cellular state with the results obtained by the derivative spectroscopy method, which characterizes the *structural* reorganization of a cell under the influence of external factors. Similar methodological approaches are absent from publications in the scientific literature. Moreover, this is the first report in the world on the large range of PAMF applications.

In the book there are many original conclusions and sometimes disputable points of view; significant attention is given to the description of questions of evolutionary biochemistry and carotenoid metabolism. Quite difficult phenomena are described in an understandable fashion and are beautifully illustrated.

Consolidation of the authors' experience on expansion of the application range of analytical methods considered in the book has promoted a profound knowledge of the evolution of biochemical mechanisms and an understanding of the molecular and energetic basis of cellular stability under extreme influences.

An abundance of illustrative material is a doubtless advantage of this book. The book is written in a good scientific style and contains a large number of the major literature references from different areas of the science.

The material offered by the authors, and their interpretation, will be of interest to a wide audience of biologists, evolutionists, geneticists, physicians, biophysicists, biochemists, analytical and pharmaceutical chemists, and radiochemists. It will also be valuable to postdoctoral students and students of biological, physical, chemical, and medical profiles of research, and also for any inquisitive readers wishing to familiarize themselves with features of biological work in the physical and chemical field and to enlarge their understanding of this interesting area of knowledge.

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Acknowledgments

First, our authoring team expresses appreciation to Springer publishing house for its many years of efficient work on questions of scientific literature publishing.

Preliminary (previously for a number of months) Senior Editor Dr. Claudia Panuschka has co-operated and agreed with our treatment concerning this second project. Dr. Panuschka is an excellent and intelligent specialist, a beautiful woman able to figure out an escape from any situation to the benefit of the publishing house and authors of published books. Her lovely soul and physical beauty harmonize with her logical consideration and insistence on observing the rules of the publishing house. The splendid administrative skills of Dr. Panuschka have promoted the employment of a highly professional team. We are extra thankful to Editorial Assistant Tatiana Grabner for accurate, quick, and kind work. We are delighted by the professional approach and kind communication of all staff members of Springer publishing house Production dept. for attention to our book and separately the group of Mr. Paramasivam Nagarajan responsible for professional accomodation the illustrations in the book. Additionally, we express our appreciation to Dr. Paula Antony, the translator of the Springer publishing house for careful correction of the English translation of the book initially prepared by I.G. Danilova and acknowledge the collective efforts of the team of Springer Wien, New York.

When working with the authors of this book, all mentioned and unmentioned employees created a well-intentioned atmosphere of help and readiness to give advice on the better appearance of the monograph. It was the collaborative friendly job of representatives of various countries.

We are very thankful to the OriginLab Corporation and its Customer Care Manager Barbara Tobias for the kind permission to publish some images and descriptions illustrating the application of the *Origin* package in our analytical work.

We cannot leave unacknowledged our scientific colleagues of the older generation from Germany and Austria: Prof. Dr. Wolfgang Klose (Karlsruhe, Essen); Prof. Dr. Hartmut K. Lichtenthaler (Karlsruhe) for the permanent interest in our work and for the kind permission to use interesting pictures illustrating the new

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pathway of carotenoid biosynthesis; Prof. Dr. Achim Hager (Tübingen); Prof. Dr. U. Heber (Würzburg); and Prof. Dr. Harald Bolhar-Nordenkampf (Wien) for help in the work and productive discussions encouraging the qualitative consideration of scientific material.

Our gratitude is extended to colleagues who retired from science because of age or other reasons but who worked together with us: Dr. E. Brecht (currently Bürgermeister der Stadt Quedlinburg/Harz) and Dr. Doz. Armin Meister (Gatersleben/Aschersleben).

Finally, we express deep appreciation to the academician V.L. Svidersky for attention to the manuscript during book writing and to the academician Yu.V. Natochin for critical and constructive recommendations on the monograph text. We also express gratitude to the academician N.P. Vesyolkin for the constant support and timely remarks and counsel.

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Chapter 1
Introduction. Development of the Methodological Base, Disputes, and Conclusions

In the period of writing our previous books and this present monograph, a group of authors was formed, every one of whom has worked hard for the development of modern methodological approaches in different areas of physical and chemical biology. This has led to a productive working symbiosis of representatives of physiological and physical schools and experts in the field of organic chemistry and the biochemistry of pigments and also in the field of computer data processing.

After publication of the book *Derivative Spectrophotometry and ESR Spectroscopy for Solution of Ecological and Biological Problems* (Saakov et al. 2010), and then of its expanded edition in English (Saakov et al. 2013) by Springer (Vienna), there was some question by readers and colleagues about the reasonability of the great number of coauthors. We answer: Members of the author collective have performed collaborative investigations with St. Petersburg Electrotechnical University (LETI) for the past 45 years. It is sufficient for readers to familiarize themselves with the large lists of publications by these authors, which are extensive enough that such questions need not be asked.

Furthermore, as a counterquestion to curious people, we ask: Have you written at least one monograph? If yes, how much did its technical illustrative preparation cost, what were the publishing expenses, and for how long after writing did the book stay with the publishing house? Times have changed and Russia has changed. Can one person pay all publishing expenses? Think about and compare the salaries of current professorates with the price of a modern edition of this book in Russia. If you can easily get funds for book publication, please impart your experience. If you have any additional questions, please write. We will respond in person to everyone.

Behind these questions there is a certain invisible nihilism towards the writing and work of colleagues, inherent in modern scientists. It is a kind of envy in the absence of creative initiative. Please count in this book the number of publications by each author of the senior generation. Each of them has already written at least a number of monographs, but such an association of authors with differently directed interests in the combination of two modern methods of physical and chemical biology is simply not present in world biochemistry.

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In addition, the great psychological pressures during the writing of a book require a like-minded person who can listen and discuss what has been written, share doubts, give qualified council or criticism, be in friendly disagreement, or be glad to read a successful text. The characters of the coauthors are different, and disputes promoted the authors to state the most paradoxical opinions in the course of the work. Some were good; others were various. However, at the moment of intense creative work, mutual respect and friendliness won through.

In this book we have tried to state the bases of analytical methods that have become classic for professionals, but are still unclear, unfamiliar, and not friendly for those scientists who do not work directly in these areas of physical and chemical biochemistry and cell physiology.

On introducing the terms "fluorescence" and "spectrophotometry" into the title of the book, we realized that to consider two large problems of world science in one publication is simply impossible.

Therefore, from first lines of this book, we *focus the reader's attention* on the widely used approaches to the fluorescence analysis of cells (Kautsky and Hirsch 1931; Govindjee and Papageorgiou 1971; Papageorgiou 1975; Govindjee 1995) and its variations (Lichtenthaler 1992; Lichtenthaler 1988a,b; 1996, 2000; Lang and Lichtenthaler 1991; Buschmann and Lichtenthaler 1988; Lichtenthaler and Rinderle 1988). We pause at the method of pulse-amplitude modulated fluorescence, known in the world literature as the PAM fluorescence or PAMF method. This method appeared in analytical work because of the Würzburg school of biophysicists and biochemists (Schreiber 1983, 1986; Schreiber et al. 1986; Krause and Weis 1984) and allows the quick and reliable characterization of *functional* changes in cell reactions in response to an external influence (Schreiber and Bilger 1987; Lichtehthaler and Rinderle 1988; Strasser and Govindjee 1991) and is used in the process of ecological monitoring (Bolhar-Nordenkampf et al. 1989; Mohammed et al. 1995). The PAMF method was quickly recognized in leading laboratories of the world.

On the other hand, from the variety of existing spectroscopic analytical approaches, we chose *the derivative spectrophotometry method*. Ideas making up the body of this approach were formulated by the known English physicist Lord Prof. E. Rutherford in the 1920s (Dymond 1924). As a rule, expressed opinions are not met with great evaluation; the result is much more welcome. In this book, we decided to combine the original, possibly even invaluable, thought of Prof. E. Rutherford with its real implementation in technical decisions in different countries. After this decision, a question was posed: How can the original idea be realized in a real research experiment to obtain new data on the secrets of nature?

The active conquest by the specified method of analytical positions in biochemistry and analytical chemistry happened a bit later (French et al. 1954; French and Church 1955; Meister 1966a, b; Saakov 1971a–d; Marenko et al. 1972; Saakov and Shpotakovskii 1973; Fell 1979, 1980; Fell and Smith 1982; Perfil'ev et al. 1985) and is described in reviews (Spitsyn and L'vov 1985; Talsky 1994; Ojeda et al. 1995; Ojeda and Rojas 2004; Rojas Sanchez and Ojeda Bosch 2009; http://chemanalytica.com/book/novyy_spravochnik_khimika_i_tekhnologa/04_analiticheskaya_khimiya_chast_III/4016; Saakov et al. 2010, 2013). The last reference is

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accessible as separate chapters of a whole book at Springer's website (http://dx.doi.org/10.1007/978-3-7091-1007-2).

We specify that, unlike the PAMF method, which characterizes *functional* changes, the derivative spectrophotometry method allows *structural* changes occurring in a cell or in molecular structures to be analyzed (Talsky 1994; Saakov et al. 2010).

We pay attention of the reader that exactly these two basic features of research methods considered in the book *should be taken into account in the process of the further familiarization with materials of the present monograph.*

Our book *Derivative Spectrophotometry and ESR Spectroscopy for Solution of Ecological and Biological Problems* (2010) was devoted to aspects of the application of derivative spectrophotometry and electron spin resonance (ESR) spectroscopy in scientific and applied research in biology, medicine, and ecology. The material was discussed with emphasis on the *interpretation* of derivative signals of biological structures. However, we said almost nothing about the *evolution* of circuit schemes for derivative spectra registration.

The book was noticed and positively assessed by Western researchers and publishers. The oldest European publishing house, Springer Verlag (Wien, Heidelberg, New York, Dordrecht, London) decided to publish and enlarge the book with some additions. The monograph was published in English in 2013 with the title *Derivative Spectrophotometry and Electron Spin Resonance* (*ESR*) *Spectroscopy for Ecological and Biological Questions*. This present book includes some historically interesting electronic schemes for reception of the derivative spectrum signal published earlier in a number papers.

As the distinguished German physicist Prof. Wolfgang Klose wrote to us: "The publication of this book . . . is the international acclamation."

When posing research problems and in the conclusions of separate chapters, we have remembered and considered the statement of the known Austrian physicist Ludwig Eduard Boltzmann (1844–1906) that there is nothing more practically valuable than good theory (translated from Russian). Therefore, the descriptions of our experiments are always based on a working hypothesis, which we then deny or confirm with research experience, translating experimental data into a theoretical position.

In the introduction to this book, we focus the reader's attention in advance on some important questions and positions that are discussed in later chapters. We have done this to acquaint the reader step by step with topical issues of the theory and with some elements of scientific practice in areas of biochemistry close to us.

The fluorescence of chlorophyll in chloroplasts is an important and inherent indicator of photosynthetic reactions in green cells. Following the publications by (Kautsky and Hirsch 1931, 1934; Kautsky and Frank 1943, Kautsky et al. 1960) describing results on the relation between chlorophyll fluorescence and photosynthetic reactions (the Kautsky effect), a large body of literature appeared describing the interrelationship of chlorophyll fluorescence with the mechanism of photosynthetic reactions. We will not repeat these results and so refer to the most significant reviews of this problem and of the method, which even after 80 years is still attracting

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attention and is the main research tool in physical and chemical biology for investigation of the primary processes of photosynthesis and ecological biochemistry (Lavorel and Etienne 1977; Krause and Weis 1984, 1991; Lichtenthaler 1988a, b).

In Chap. 2, the authors describe their own experience with the PAMF method as an instrumental tool for assessment of the cell state and assess the possibility of combination of the information obtained with the data set registered using an instrumental method for assessment of the structural changes in a cell and its molecular structures (i.e., using derivative spectrophotometry). Moreover, taking into account the feedback of readers to the first and second books, the authors have expanded the material presenting electronic schemes of signal transformation of serial registering spectrophotometers for derivative spectra registration. We have also added a description of operations for programs on data processing and visualization and some information on radiochemical purification (see Chap. 3). Chapter 2 also presents material on changes in PAM signal harmonics under the influence of different extreme factors of the environment (EFEs). The kinetics of these harmonics and the kinetics of coefficients of fluorescence quenching are functions of EFE intensity and do not show specificity for the nature of the EFE. The damaging effect of an EFE is localized in the part of the primary–secondary acceptor of electrons of reaction centers on photosystem-2 and does not depend on the specificity of the external influence. Damage to systems in the Calvin cycle and to the metabolic reactions of carbohydrates and other cellular metabolites is secondary. It was concluded that there is a primary dependence of the stability and reparational abilities of a green cell in the maintenance of its autotrophy on the stability of the step $P_{680} \rightarrow Phe \rightarrow Q_A \rightarrow Q_B \rightarrow PQ$ of the electron transport chain of photosynthesis and on additional interconnections of these processes with mitochondrial energetics.

Development of scientific research in the second half of the twentieth century was mainly dependent on the introduction of new analytical approaches, in particular radioisotope methods. These approaches allowed, for example, establishment of data identity in calculations of CO_2 absorption intensity with gazometric and radiometric methods (Saakov 1959; Voznesensky et al. 1960. Fulfilling unwritten rules existing then in our country, this work was included in the PhD dissertation of the communistic doer of the local scale (Voznesensky 1960). But here it is necessary to consider the time at which specified events took place. On the agenda there was a question “to be or not to be”, could the young scientist develop his carrier in case of objection to the party functionary. These features of Russian science might be unfamiliar to the modern reader.

We ask the reader to bear with criticism presented in this book. The criticism of a number of articles is not disavowal of particular people, but is an attitude towards events prevailing at those times (and sometimes now). Times change, but the mentality of leaders remains as before.

At the same time, experiments in other scientific areas forced us to reconsider the earlier established view of the methodological correctness of application of buffer systems proposed at the beginning of the twentieth century by Prof. Otto Warburg (Saakov 1960, 1961). Infringement of the rules of use of buffer mixes promoted a

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rather cautious attitude to data obtained with help of the manometric method in a set of articles on ecological and physiological investigation of the assimilation intensity of carbonic acid during photosynthesis (Semikhatova and Chulanovskaja 1965). There were two reasons: The first concerned the use of ill-considered, large areas of leaf cuttings. The second was that the manometer reading was started earlier than 70–80 min before achievement of gas phase saturation in an analytical vessel. Thus, the main requirement of the Warburg method concerning the constant concentration of a gas during the course of the experiment was not fulfilled. The standard approach to features of the new method led to errors. The advantages of this method manifested in studies of photosynthesis intensity in the steady state when the potential intensity of the process, under the conditions of the experiment, was being assessed. This should not be confused with the greatest possible potential value.

Further perfection of the technique allowed derivation of the formula used to calculate the time needed to achieve dynamic equilibrium of the carbonic acid concentration between air and buffer phases in a container, depending on the container's geometry (Saakov and Shiryaev 2000). The formula can be useful in other experiments when, in a closed system, the leveling of the concentration between liquid and gas phases occurs.

The work on derivation of the time calculation formula for dynamic equilibrium of carbonic acid concentration was performed with the use of the PAMF method, application of which is considered in Chaps. 2 and 5 of this book. Sad to say, for reasons of no scientific sense at all, the domestic instrument engineering facilities did not start to produce corresponding analytical equipment. However, because it was the method in demand, its development and commercial introduction were promoted and a number of peripheral laboratories in our country bought analytical devices made by the firm Walz (Effeltrich, Germany).

The combination of radioactive labeling of preparations with tritium or ^{14}C assisted in the availability of radiochemically pure biological preparations of pigments (Shlyk 1971; Saakov 1963b, c; Saakov and Saidov 1965; Saakov and Shiryaeva 1967; Saakov and Nasarova 1970a). Against the background of the often thoughtless use of labeled preparations in experiments and, as a result, the registered "questionable" or incorrect data, fierce disputes appeared. The material on this topic is partly presented in Chaps. 3 and 5.

In the second half of the twentieth century, after works by Ruben et al. (1941) and Vinogradov and Teys (1941), the theoretical question of the intermediate stages of water oxygen oxidation to molecular oxygen of air was actively discussed (Vinogradov and Teys 1947). The problem was in the sight of academician N.M. Sisakyan, who at that time was supervising one of the directions of space research in the USSR. The push for new experiments was made in response to an article by the future Nobel Prize winner Prof. Melvin Calvin (Radiation Laboratory, University of California, UCRL) and coworkers. There were three questions in their scheme, one of which was connected to the possibility of using oxygen migration from xanthophyll epoxy groups in the process of its oxidation to the molecular state

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(Dorough and Calvin 1951; Anderson et al. 1960; Shneour 1961, 1962a; Shneour and Calvin 1962):

$$H_2O \rightarrow ? \rightarrow ? \rightarrow ? \rightarrow O_2.$$

This conception interested academician N.M. Sisakian, who promoted contact with the director of the A.F. Ioffe Physical and Technical Institute (PhTI) of the USSR Academy of Sciences. After specification of research, the decision to carry out this work at the cyclotron in PhTI AS USSR was reached. At first in the discussion and then in the performed work, Prof. D.G. Alkhazov (former employee of academician I.V. Kurchatov and at that time head of the PTI cyclotron laboratory), his coworker Prof. I.H. Lemberg, and A.B. Girshin (chief engineer of the cyclotron) took part. According to the preliminary concept, one of the question marks in Calvin's scheme could be the epoxy groups of xanthophylls. Calculations by physicists showed that the mass spectrometry method was insufficiently sensitive for detection of isotope O^{18} quantities in oxygen-containing groups of xanthophylls at the used initial concentration of isotope O^{18} (23 % in water) and at the quantity of xanthophylls available for analysis (10–15 μg). This was because not all 23 % oxygen groups of xanthophylls will have the isotope O^{18} label. Therefore, they decided to use the radioactivation method of analysis for registration of isotope O^{18} presence in carotenoids containing atoms of oxygen in *hydroxyl* and *epoxy* groups. These reasons agreed with the point of view of researchers from Switzerland, who had investigated the oxygen exchange between molecules of ATP and water with the help of the radioactivation method (Fogelstrom-Fineman et al. 1957; Fleckenstein et al. 1959, 1960; Marmier et al. 1959). Initially, the decision was made to use the nuclear reaction of O^{18} bombardment by protons, the reaction $O^{18}(p,n)\ F^{18}\beta+$.

In order for the reader to follow the course and logic of the discussion on application of the method for O^{18} analysis, without having to read textbooks on nuclear physics, we will briefly describe the methodological basis.

Isotope F^{18} was obtained by Snell in 1937 (Snell 1937), and the reaction $O^{18}(p,n)F^{18}\beta+$ was studied by DuBridge (Barnes et al. 1937; DuBridge et al. 1937, 1938). Radioactive F^{18} spontaneously turns into O^{18} with half-decay time $t_{½} = 112 \pm 8$ min, and this is accompanied by emission of positrons with energy $E_{\beta+} = 0.65$ MeV (DuBridge et al. 1938; Blaser et al. 1949, 1951, 1952). As a result of positron annihilation, γ-quanta with energy of 0.510 MeV are emitted. As the spontaneous radioactive decay $F^{18} \rightarrow O^{18}$ is an exoergic process, the reaction $O^{18}(p,n)F^{18}\beta+$ should be endoergic and, therefore, possesses a threshold. The threshold of this reaction is about 2.6 MeV (DuBridge et al. 1938; Blaser et al. 1949, 1951, 1952; Mark and Goodman 1955). The excitation curve of reaction $O^{18}(p,n)\ F^{18}\beta+$ was registered in the range from 2.6 to 7 MeV and showed maximal reaction cross section at a proton energy of about 5.0 MeV (DuBridge et al. 1938). In the work of Ajzenberg-Selove and Lauritsen (1959), the nuclear reactions and isotopes appearing as a result of the bombardment of organic substances by protons were considered and analyzed. All radioactive isotopes arising in reactions of protons with stable isotopes of *C, H, O,*

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and P and having a 2.5 min half-life period possess insignificant activity by the time that hardware measurements of F^{18} can begin. Thus, from the analysis of possible reactions, it follows that detection of O^{18} isotope is not masked by extraneous activity when using protons with energy below 4.2 MeV (Hornyak et al. 1950). The activation method is of a very high degree of sensitivity and allows detection of quantities of (we underline!) isotope O^{18} of the order of 10^{-7} to 10^{-9} g.

Results obtained for the first time in Russian biochemical research had considerably greater reliability than those reported in foreign papers because, apart from careful radiochemical purification of initial preparations (we especially emphasize this stage of the experiment), for the first time the quantity of particles bombarding a target was strictly estimated at each exposure and the support material was cooled in order to avoid target burnout. In this approach, the substantial double novelty of the technology of method application meant that, unlike foreign works, the exit of annihilation quanta was proportional to the quantity of protons falling on the target. Stability of the irradiation mode was checked by separate experiments (Saakov 1963a–d).

Improvement of the physical part of the experiment promoted application of other nuclear reactions to irradiation of targets by alpha particles, $O^{18}(\alpha, n\gamma)Ne^{21}$ (Saakov 1963a–d, 1965a, b; Vartapetian 1963, 1970; Vartapetian 1967; Lemberg et al. 1966; Saakov et al. 1969, 1970a).

Soviet researchers obtained unique data that reliably proved inclusion of water oxygen in epoxy groups of xanthophylls, in particular, of violaxanthin and some other compounds (Sapozhnikov et al. 1961, 1964; Saakov 1963a–d, 1965a, b, 1990a, b; Vartapetian 1963; Vartapetian et al. 1967; Saakov et al. 1970b). The above-listed works described the first experiments on the use of nuclear activation reactions in biochemical research in the Soviet Union. It is quite natural that research did not always go smoothly and that only theoretically expected unambiguous answers would be found. Nevertheless, the inclusion of water oxygen in xanthophyll epoxy groups was absolutely reliably established (i.e., the main problem set for the experiment was solved).

In those already distant times, our works (the order of authors' names in articles was determined by their administrative status, the laboratory leader and senior scientists at first place, instead of proportionality of participation in experimental work. For example, N.V. Bazhanova performed the communistic management of the experiment from another institute and did not visit PhTI AS USSR) attracted the attention of the US Commission studying atomic energy, and so the summary of an article from *Botanical* magazine (1961) translated by Shewchuck (University of California Lawrence Radiation Laboratory, UCRL, Berkeley) was published in *Radioisotopes in the biological sciences. An annotated bibliography of selected literature* by the US Atomic Energy Commission (Sapozhnikov et al. 1967a).

Data on water oxygen inclusion into xanthophylls are considered in more detail in Chap. 5.

However, Goodwin (1971b, p. 600), having no more than superficial knowledge of details of the radioactivation analysis technique, allowed himself to write in a very serious monograph (Isler 1971) about the research (Sapozhnikov et al. 1961,

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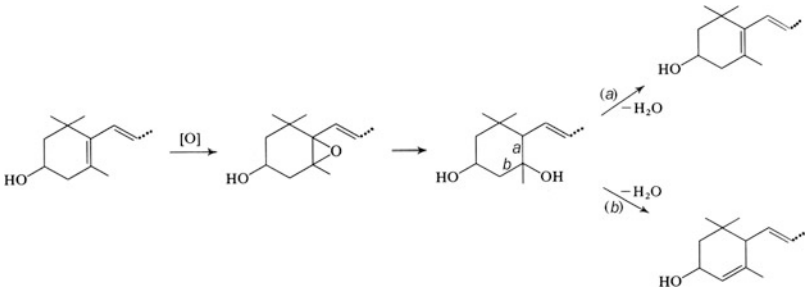

Fig. 1.1 First ideas on the interconversion of xanthophylls in green tissues. The shaky and free interpretation by Goodwin (O. Isler (ed) Carotenoids, 1971, pp. 602–603, scheme 24 and 19 and also (as well) Goodwin 1969, p. 672, scheme 4) of the results and points of view of Sapozhnikov et al. (1957, 1958, 1959, 1962) and Saakov (1964, 1965a, b)

1964), "... but the techniques used are open to criticism." There was a situation scornful arrogance regarding Russian works when a scientist had insufficient knowledge of the heart of the problem. Probably, the personal incompleteness of knowledge promoted Goodwin to be wary of having a dig at the results of other publications (Saakov 1965a, b; Saakov and Konovalov 1966; Saakov et al. 1969, 1970a, b). However, Goodwin did not oppose the reliability of the conclusion on the main point of this experiment: the inclusion of water oxygen into epoxy groups of violaxanthin and then of neoxanthin. Furthermore, on pages 602–603 of his monograph, Prof. Goodwin stressed that he and his coworkers showed the impossibility of interconversion of lutein and zeaxanthin with the help of an improved (*???*) technique. Citing Sapozhnikov's papers (1957, 1958, 1959), Goodwin completely perverts the meaning. We quote Goodwin: "Sapozhnikov and his colleagues (1957, 1958, 1959a, b, 1962) were first to observe reversible epoxidation but it was claimed that the reaction resulted in the conversion of *zeaxanthin into lutein*" (see Fig. 1.1).

Two errors are hidden in this seeming impersonal comment from Goodwin.

First, Sapozhnikov and colleagues were not the first to note the possible violaxanthin de-epoxidation (see Moster and Quackenbush 1952a, b; Chap. 5). However, after Goodwin, not delving into phrases written by him, scientists began to repeat and remark in Western works on the antecedence of the research of Sapozhnikov et al. (1957, 1959a, b).

In the democratization period of our society, the fierce hunting for priority by Russian (Soviet) scientists stopped. Goodwin's statement only underlines his indifferent and thoughtless attitude to the problem. What matters is not who first described the phenomenon, but the accuracy of the scientific description of facts. However, the absurdity of the Russian reality meant that many years later D.I. Sapozhnikov and his former employees advocated for recognition of this reaction as *their discovery* (Maslova and Markovskaia 2012).

Second, Goodwin did not understand which substance transforms into which and in what way (see Fig. 1.1). We will explain for readers unfamiliar with the details of

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the problem. Conventional or great scientist (researcher) can be the believer or non-beliver (irreligious, atheist) person. Each big researcher had mistakes (Galileo Galilei, Sir Isaac Newton, J.B.P.A Lamarck, Ch. Darwin, C. Linnaeus, K. Marx etc), but they belief in the postulates at them was kept. Mistakes were corrected by time, development of a science and colleagues is a normal scientific process.

In the articles of D.I. Sapozhnikov et al. (1957, 1958, 1959a, b, 1962), such cyclic trend of reactions of xanthophyll interconversions (lutein ↔ zeaxanthin, b ↔ a) (see Fig. 1.1) is not discussed at all and is not the subject of research. The question of lutein transformation into zeaxanthin and back was never within D.I. Sapozhnikov's eyeshot and attention, and it was not investigated by his former employee (Saakov 1964, 1965a, b), whose data are cited by Goodwin (see Goodwin 1971b, p. 602, references 177 and 178). The authors considered only alternative possibilities and ways of violaxanthin transformation into lutein under the inducing influence of light on a leaf and ways of the back reaction in the dark (Sapozhnikov and Saakov 1962). Only this point is common for the positions of D.I. Sapozhnikov and V.S. Saakov. Divergences of opinions of these researchers began with the determination of intermediate and end products of the de-epoxidation reaction. Later (Saakov 1966), it was found that after ^{14}C-lutein infiltration into a leaf (preparations of a high degree of radiochemical purity), the radioactive label was discovered in carotene, epoxylutein, X-xanthophyll (it is probably antheraxanthin), and neoxanthin. The latter could be the predecessor of zeaxanthin, but research into this question was not performed. Therefore, Goodwin (1971a, b) had no basis for speaking about the presence of well-founded interconversion of lutein into zeaxanthin in our works.

Prof. Goodwin is held in our respect, but he did not understand at all the sense of D.I. Sapozhnikov's (et al.) concept and rather imprudently continued to think that the main event of this concept is zeaxanthin transformation into lutein (see Goodwin 1971b, p. 602, scheme 24, fig. 1.1.). Whether the language barrier caused the incorrect understanding and wrong interpretation of the root of the discussed concept, or it happened due to pompous neglect, let experts judge.

Most likely these statements of Goodwin were based on his publications concerned with (as he thought) different ways of biosynthesis of α- and β-ionone rings of carotenoids.

First of all we will specify the experimental conditions. The duration of Goodwin's experiments was 69 h, and they were carried out on tomato cuttings (slices taken from the central portion of a ripe tomato) and on cut circles of carrot (carrot root slices 1–2 mm thick). Sapozhnikov investigated only the activating action of light (10 min) on the induction of violaxanthin metabolism in a leaf and chloroplasts. Goodwin and coworkers used labeled preparations of mevalonic acid, DL-[2-^{14}C, 4R-^{3}H], and these and similar experiments required rather careful radiochemical purification of newly synthesized labeled preparations, which Goodwin and coworkers did not perform (Goodwin and Williams 1965a, b; Williams et al. 1967). We analyzed the techniques of Goodwin and colleagues and noticed that the technique (two steps of column chromatography and sometimes two steps of thin-layer chromatography) did not lead to radiochemical purity of the

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preparations. Goodwin and coworkers ignored such important parameters as an assessment of constancy of the specific activity of pigments and radioautographs for an assessment of the presence of colorless compounds.

This, in turn, calls into question the conclusions made by them. Unfortunately, Goodwin did not convincingly prove the advantages of his technique for radiochemical purification of preparations. However, all this did not confuse Goodwin, and at the congress on photosynthesis (in Tübingen, 1968), he expanded his ideas on lutein and zeaxanthin synthesis (Goodwin 1969) and continued to support this point of view in the Isler monograph (Goodwin 1971b). Note that in the listed and later works, Goodwin does not demonstrate the *improved* technique for the proof of his opinion and so he should put forward his statements with less positiveness.

For accuracy, it should be noted that a decade later in the monograph *The Biochemistry of the Carotenoids* (Goodwin 1980), Goodwin again emphasized the primacy of the works of Sapozhnikov et al. (1957–1959a, b) when he describes the level change of xanthophyll epoxy groups in leaves under light (see Goodwin 1980, Section 3.4, pp. 86–89), but focuses attention only on the violaxanthin → zeaxanthin (Viol → Zea) reaction under the influence of light.

The words written by us in the previous four paragraphs only partially describe the true state of our thoughts and opinions on Goodwin's behavior, with whom we are personally familiar.

Another aspect is surprising. It is impossible to assume that D.I. Sapozhnikov did not carefully read Goodwin's review, because D.I. Sapozhnikov spent weeks in the Saltykov–Shchedrin Public library (now Russian National Library) searching the new information. Also, he received fresh reprints of articles from colleagues working in leading countries, rare issues for the USSR, and made the acquaintance of many of them personally (including Goodwin) or by correspondence. However, his employees read almost nothing and relied on the opinions and propositions of the chief.

Neither he nor any of his coworkers or evident followers and doctors under his supervision raised in the scientific press a voice for the truth and justice of their chief, or for his concept and methodological approach. Even in their anniversary article, the authors Maslova and Markovskaia (2012) bypass Goodwin's passage with their usual way of "concealment–suppression," which was unfriendly and generally incorrect.

In Chap. 5 we will consider some of Goodwin's errors.

The above-mentioned experiments at the cyclotron were expensive. Therefore, the number of repeated series of experiments was limited. But 50 years have passed since the beginning of this research, and the *basic* results of those times have still not been criticized. The obtained work experience was used by other researchers (Vartapetian 1963, 1970; 1967). Here, Goodwin (1971a, b) was obviously overzealous with his criticism.

Unfortunately, in pursuit of methodological sensationalism, articles containing a conglomerate of self-confident but not reliably well-founded ideas of rather doubtful scientific character tried to be included into the ensemble of above-listed publications (Sapozhnikov et al. 1965a, b). It is not necessary to speak about the

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first article; it is enough to attentively look at the figure showing results of the experiment (Fig. 1.2).

Reading this work, it is enough to point out the fact of nonobservance of the criteria of radiochemical purity of samples and the problematic character of real enrichment of samples with isotope O^{18} in order to not discuss the presented results at all. The extremely original positioning of experimental points in the graph, the absence of confidential intervals, and the snake form of the curve functionally proved by authors demonstrate the fruits of their imagination, underline the illogical sequence of dynamics of the radioactivity values of preparations, and call into question their general scientific reliability. Contrary to the authors' statement in the article and according to the figure caption (see Fig. 1.2), the abscissa is a logarithmic scale, but in this case, to mark a scale simultaneously with seconds and tens of minutes is not correct. From the caption of Fig. 1.2, it is not clear which radioactivity is mentioned: the activity of the irradiated target or the *specific activity* of the investigated sample unit.

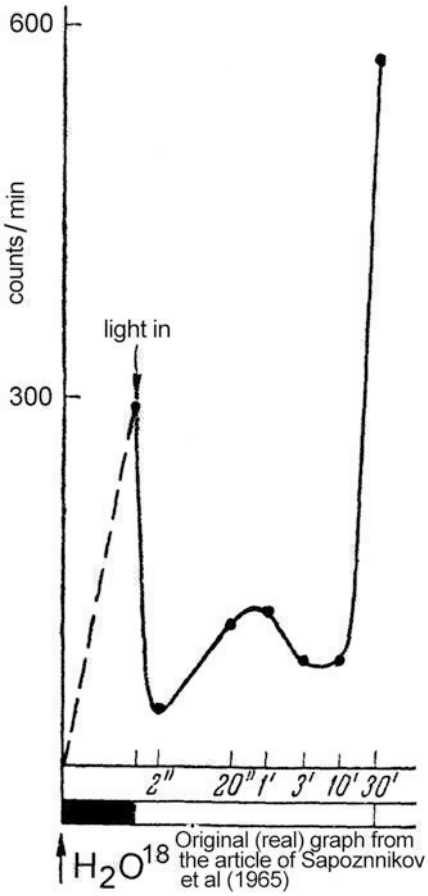

Fig. 1.2 Dependence of O^{18} inclusion into violaxanthin on illumination time. *Ordinate axis*: activity in counts/min; *abscissa*: illumination time on a logarithmic (*???*) scale. Along the *bottom line* the *dark part* corresponds to algae staying in the dark

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Generally speaking, and to avoid the appearance in the press of similar "scientific" works, it would be useful for authors of the article to write a more detailed discussion of the methodological part of the preparative work for experiments and, especially, give more details on their quite original explanation of results. Over the last few years, nobody from the scientific environment has commented on the details of the rare analytical technique used in that article. People have blindly trusted the authors. These details become unexpectedly notable to readers looking from the outside at the work and at the presented data. The inconsistent "visibility" was ignored by authors during manuscript preparation. This work and the illustrative showing of its results would be worthy of Goodwin's criticism (Goodwin 1971a, b).

For good reason, the names of leading scientists of the cyclotron laboratory of the A.F. Ioffe Physical Technical Institute of the USSR Academy of Sciences are absent from the list of coworkers, names commonly found in works from this research establishment on radioactivation analysis.

In the first and following of our works, the employees of the Physical-Technical Institute were equal coauthors. Similarly, Prof. B.B. Vartapetian (1963–1967) published his data in this way. Full participation of project members of the cyclotron laboratory in collaborative publications was included in their contract conditions.

It is remarkable that none of the authors of the criticized article (Sapozhnikov et al. 1965a, b) had ever earlier used radiochemical approaches nor had worked with radioisotope techniques with their own hands (i.e., independently). But they suddenly started such delicate work. Instead of organizing an emotional competition, they needed to describe methodological events in all completeness of reliability and to work without excessive hysterics.

The fact is that during the preparation and writing of this book, we critically analyzed a number of works from previous years and found striking discrepancies between data presented in these articles, as illustrated in Figs. 1.2 and 1.3.

Here, in Fig. 1.3 we present data discussed in the article, choosing as the abscissa the *true logarithm* of the number of seconds of algae exposure to light in H_2O^{18}.

The soundness of the points at 2 and 600 s raises doubts, not only because of the absence of confidence intervals, but also because of the *logic* of the dynamic change in radioactivity induced in violaxanthin molecules as a function of time of exposure of algae to labeled water. The preparation from the sample placed for 600 s in heavy-oxygen water had significantly smaller radioactivity than the sample after 60 s exposure (Fig. 1.3). Explanations suggested by authors do not hold water, but other arguments are not presented. However, the article describes events of improbable completeness of reliability. This allowed assessment of the background level of these researchers and their inexplicable negligence in description of experimental data.

If one is to accept that the initial, dark level corresponds to 100 % enrichment of O^{18}, then, according to the authors, the exposure to light for 2 s reduces this level by almost 80 %. Such an instant reaction of oxygen emission from violaxanthin molecules is functionally improbable and contradicts earlier statements by

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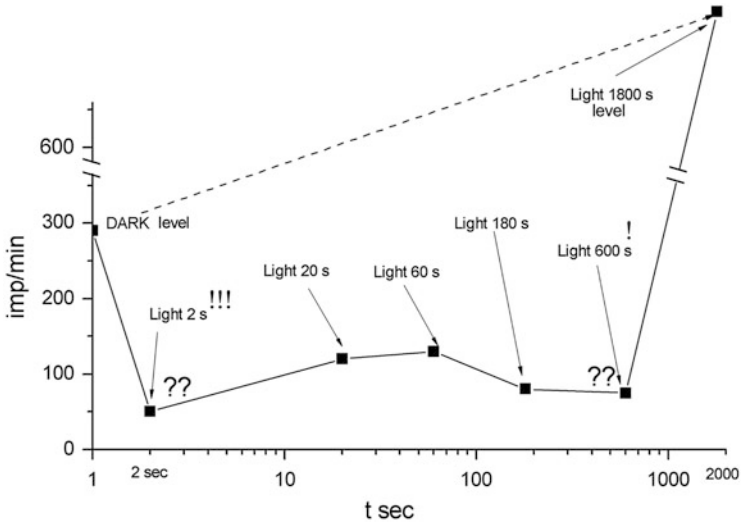

Fig. 1.3 The corrected *curve* showing the dynamics of O^{18} isotope inclusion from heavy oxygen water in the violaxanthin fraction, depending on time of exposure to light of an alga suspension in water. *Abscissa*: the logarithmic scale of exposure time in seconds. *Ordinate*: the specific activity of the preparation, expressed as the number of impulses per minute, as in the article by Sapozhnikov et al. (1965a)

D.I. Sapozhnikov et al. (1957, 1959a, b) about the time period of the de-epoxidation reaction.

In the time range from 2 to 600 s in light, there is no significant increase in violaxanthin molecule content, and the O^{18} content remains at the level of 29 % from the initial, dark level. The authors did not explain the radioactivity decrease at points 180 and 600 s. This implies that oxygen losses in violaxanthin as a result of 2 s illumination were not compensated for over 600 s and that the presented dynamics of the O^{18}-induced radioactivity change indicate substandard techniques for the experiment.

It would have been possible to increase the sample number for measurements at the initial, dark level and after 1,800 s of light and to obtain the desired data with correct observational statistics.

We blink our eyes and scratch our heads: Why did the authors of the criticized article, numerous readers of *Biophysics* (*Biofizika*), and we (authors of the present monograph, very familiar with the radioactivation method) fail to notice earlier these points? Maybe it is trifle, but it negatively characterizes the methodological background of the discussed group of researchers.

It is strange that nobody paid attention to these "super-original" data. We suppose that it could be caused by readers' misunderstanding or possibly by blind faith in the experimenters' decency. In 1965, the journal *Biophysics* (*Biofizika*) already had scientific authority. It was read or looked through by hundreds of researchers. But, as academician Ambartsumyan once wrote, after article

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publication it will be read by 100 persons, and only 10 of those will understand the idea's originality. It is unlikely that scientists reading the given issue of *Biophysics* (*Biofizika*) thought of the values of the points in the graph presented by authors of the article. Nobody made a protest about the super-originality of this research or, to be more exact, about its unscientific character. Because of indifference or misunderstanding, the errors were overlooked. As often, experimenters do not always have enough of the necessary mental acumen. We suppose that the eminent physicists taking part in the experiment, but not knowing biochemistry, intuitively felt the methodological disqualification of Sapozhnikov's group and stepped back from confirmation of their participation in "the experiment of the century." People began to clearly see after 48 years. However, the discovered issues were not a limit to inadmissible scientific unconscientiousness.

In spite of the fact that D.I. Sapozhnikov was regularly published in the *Proceedings of Academy of Sciences of the USSR*, the article, which is a copy of the above-discussed opus of Sapozhnikov et al. (1965a) from the journal *Biophysics* (*Biofizika*), also appears in *Reports of Academy of Sciences of the Tadjik Soviet Socialist Republic* (Sapozhnikov et al. 1965b). Obviously, the authors used the principle that people do not read articles, but count their number. On 23 January 1965 (i.e., 11 months after sending the above-discussed publication to *Biophysics* (*Biofizika*) on 25 February 1964), the new article (practically a copy of the above-mentioned one) was received by *Reports of Academy of Sciences of the Tadjik Soviet Socialist Republic*. In it are the same errors; the positions of experimental points in the plot are almost indistinguishable, and the discussion and data explanation are of the same indistinctness, already habitual for this group of authors. The conclusion is that this work was written by researchers of very low qualification. They do not explain the graph quality with specific printing in Tajikistan. The climate of relations, thoughts, and ideas prevailing in this group of researchers excludes any other alternative.

Until now nobody has and is unlikely to publish material reliably refuting these "scientific" articles. To protest the nonsense is the most difficult. The nonsense should be found out. Our success, our main small "discovery," consists of managing to see small but biased discrepancies in experimental points, so that they will not be repeated in future during work with this unique analytical method. The large heap of methodological mistakes in the works of N.V. Bazhanova, T.G. Maslova, and D.I. Sapozhnikov does not impress only incompetent peoply.

The pseudoscientific balancing act of Sapozhnikov and colleagues described in the cited articles (Sapozhnikov et al. 1965a, b), and, more precisely, the trivial rashness, implies that similar discoveries are an unsteady mirage in that the very desirable phenomenon conflicts fundamental laws of physics in the absence of a reasonable demonstrative base.

Also, in these times, being the laboratory leader of a communistic figure could prevent the healthy critical discussion, because scientists who talked about mistakes could be eliminated from the staff as "non-supporting the line of Communistic party". So sometimes investigators preferred not to ask questions even if it was safe for their career, but they waited until future experiments will clarify the problem.

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D.I. Sapozhnikov, as the project head, set a problem. To find a solution, executors in the persons of T.G. Maslova, N.V. Bazhanova, and O.F. Popova had to understand the essence of the work. And this understanding required a certain level of special knowledge, with which the research assistants were not overloaded, neither during experiments nor during preparation of the manuscript for publication. For a long time it has been known that errors always surface, but they are not always recognizable, and the majority usually accept them instead of correcting things. But regretfully, the opinion of the majority is often erroneous and can influence scientific politics. It is enough to remember the sad glory of the session of the V.I. Lenin All-Union Academy of Agricultural Sciences (on formal genetics in biological science) in 1948, which threw away the Soviet biological sciences by a majority of votes (Saakov SG (senior) ed. 1948/1949).

The independently running experiments on radioactivation analysis required more profound knowledge and understanding of the problem than the authors of the articles can be credited with. But that special knowledge of the radioactivation analysis technique and of radiochemical purification of pigment preparations did not overload the researchers possessed of rare intellectual qualities, N.V. Bazhanova and T.G. Maslova.

Documentary traces of this vigorous rush of Sapozhnikov's group remain in the great set of already completely forgotten PhD dissertations. New articles of researchers from this group are no less odious and pretend to be "discoveries." But we will talk more about it in Chap. 5.

Nevertheless, against the background of scientific talentless coworkers and of the impudent mediocrities expressing aggressive ignorance and incompetence and surrounding D.I. Sapozhnikov, he was an unordinary researcher endorsing his own scientific position, although not always a correct one. Without sufficient knowledge, he introduced something new into scientific analytical everyday routine.

As a result, the world scientific community came to a final decision, both on violaxanthin transformation and on the dynamics of oxygen O^{18} inclusion in violaxanthin molecules - the plots in these works are fantastic.

The preceding remarks concerning the omissions of the research technique are also relevant for consideration of another article (Sapozhnikov et al. 1967b), which is certainly not free of a number of methodological failings caused by absence of fulfillment of criteria of radiochemical purity of preparations, of their quantitative estimation, and especially of a way of accumulation of preparations for experiment. This means that the published data should be examined with great vigilance and calls into question their validity. A detailed description of the experimental technique is reasonably missed out by the authors of the article. Detailed analysis of this publication requires a multipage polemic and is not necessary for discussion of the questions raised in present monograph.

The example of the considered articles illustrates how the desire to show application of new progressive research methods in the absence of knowledge and objective thinking leads to trivial scientific mistakes. The question is whether it is possible to trust the scientific results of the mentioned group of researchers who publish and duplicate similar scientific bosh. Researchers, prove your correctness

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experimentally; present exact experiments and graphically fine plots. Then, the doubts can be cleared and we will be ready to apologize.

It was necessary to give our author's opinion on the above-mentioned material in this introductory chapter. Otherwise, the reader would puzzle over our criticism of some articles and of the scientific concepts of D.I. Sapozhnikov's group that are described in Chap. 5.

The reader should clearly understand why in the introduction we concentrated on these works. The content of our book is concerned with the use of two new methods of physical and chemical biochemistry, and the reliable methodological background of experiments is the basis of authenticity of the experimental results discussed in the book. Therefore, in our works we have fulfilled all methodological requirements and tried to cite the results of domestic and foreign papers whose methodological basis does not raise doubts about the results or scientific decency of their authors.

Also, we remember that there are lies, white lies, and statistics. Besides these three kinds of lies, there is a fourth, nondisclosure or deliberate silencing of facts. According to Antoine de Saint-Exupery, "what is essential is invisible to the eye" (from *The Little Prince*). It is necessary to know the kernel of the problem.

Perhaps, here it would be appropriate to underline the behavior of some research assistants, who were already old enough but still remained young in their virgin unfamiliarity with the basics of scientific discussion and material presentation. Pseudoscience is rather artful and under different faces arises again and again.

Recently, a tendentiously written one- side approach (Maslova and Markovskaia 2012) appeared in which well-known material was ignored and, we underline, distorted. There was spinning of facts, and ideas obtained by D.I. Sapozhnikov from other authors are accounted to him. Quoting the works of the 1980s and 1990s, Maslova and Markovskaia did not condescend to refer to publications of the end of the 1950s and beginning of the 1960s performed at good methodological level, but not according with the ideas of these coauthors. This non-acquaintance during insight into the problem source has compromised the authors of the cited work.

In that article, consciously or maybe due to basic unfamiliarity with the initial scientific literature, there are missing references to Dutton and Manning (1941), Dutton et al. (1943), Blinks (1954), and Goedheer (1957, 1959, 1969a, b, 1972); to articles and reviews (Krinsky 1962, 1964, 1966, 1968, 1971, 1972, 1979, 1984), to group works (Claes 1957, 1958, 1961; Claes and Nakayama 1959a, b); to the Stanier school (Griffits et al. 1955; Griffits and Stanier 1956; Sistrom et al. 1956; Cohen-Bazire et al. 1957; Cohen-Bazire and Stanier 1958; Jensen et al. 1958; Stanier 1960; Stanier and Cohen-Bazire 1957); to publications of known researchers of this theme (Sager and Zalokar 1958; Anderson and Robertson 1960; Anderson and Krinsky 1972; Lundegardh 1963a, 1966, 1967; Mathews 1963, 1964a, b); and also to the material of Mathews-Roth and Krinsky (1970), Mathews and Krinsky (1965), Mathews and Sistrom (1959, 1960), Foote (1968), Mathis (1969, 1979), Foyer et al. (1990), Schubert et al. (1994), etc.

Maslova and Markovskaia (2012) ignored many foreign authors, but they also forgot Russian authors of the recent past (Borisov 1974; Ozolina and Mochalkin

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1975; Paramonova 1984; Karnaukhov 1988, 1990, 2000). So the position of most of these, and other researchers not mentioned here.

Regarding a review of the work (Ozolina and Mochalkin 1975), there are detailed discussions of the positions, hypotheses, and opinions of some domestic (academician of the USSR Academy of Sciences A.N. Bach, 1937; corresponding member of the USSR Academy of Sciences V.L. Kretovich, 1971) and foreign authors concerning the cellular role of carotenoids and their ability to be catalysts or inhibitors of oxidation depending on influencing conditions. This carotenoid function is denoted by their chemical non-saturation (the presence of many conjugated double bonds), a heterogeneous localization in protein–lipid membranes, and their affinity to lipoproteins. Unfortunately, Maslova and Markovskaia do not seem to be familiar with these problems (points of view).

We are writing about this because when there is disagreement over a scientific position or criticism of another researcher, it is generally a sore subject for science. It is difficult to find a correct line without a thorough knowledge of the history of the problem, sources of origin of this or that scientific concept, opinions in the world literature, and the positions of dominant professionals of the scientific community. In this regard, hiding and blacking out of some parts of the problem, unjustified emphasis of other data points or excessive bias, and superficial and lopsided understanding of other problem items are all beneath notice.

Such a state of affairs is not surprising. Biologists know well the world information base, the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov), containing over 22 million significant and interesting scientific references. For almost 60 years of scientific activity up to the first of January 2013, only two of Maslova's works (published in 1965 together with D.I. Sapozhnikov) are contained in this database and also two articles published 39 years later in 2004, where she was not the first, leading author (according to Russian rules of author placement) but only third or fourth.

So that there is no ambiguity in interpretation of our position, we would like to emphasize that the professor and the doctor of biological sciences D.I. Sapozhnikov is a well-known figure in his area of science. He possessed good knowledge of the world literature and used it extremely skillfully. He did not feel himself to be too important a person for self-education in the necessary areas of physical chemistry. Whenever possible, he aspired to study at the highest level the methodological approaches in pigment biochemistry. But when he made errors, it is a thousand pities that they were not noticed on time. One of the "unfailing" ladies, wishing to use the collective material of the whole group in her thesis for the professor degree, "shook herself with large amplitude" because of indignation caused by publication of each new foreign or domestic paper that contrasted with her point of view.

One should not forget that D.I. Sapozhnikov was an iconic figure in science, and we should not allow his underestimation nor, in particular, biased overestimation in the question of his scientific merits. Another matter is that, desiring to strengthen his scientific positions, he, in the spirit of those hard times, resorted to violent discussion with his opponents. Also, in the 1950s and 1960s, there was insufficient evidential base and calculation in the experimental works of his group.

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Unfortunately, his argumentative methods for introduced concepts were not always scientific and legal.

In this scientific monograph, we will not carry out diplomatic maneuvering and insincere glorification. But it is necessary to remember that D.I. Sapozhnikov was the first to suggest the use of the radioactivation method in biochemical works in the USSR for the analysis of microquantities of O^{18} isotope. One detail has stuck sharply in memory but was not published in any publication. Sometimes D.I. Sapozhnikov spent nights at the cyclotron performing initial experiments and checking the registration of half-decay curves that showed the presence of induced radioactivity emitted by F^{18}. Night experiments were required by the radiating safety service on the grounds of notable neutron radiation.

The reader familiar with this problem should be vigilant with regard to the correctness of the review by Maslova and Markovskaia (2012) because of the absence of references to and a description of the position found in publications by leading American and German researchers on xanthophyll metabolism (their interconversion) and the opinions of the first opponents of Sapozhnikov (Hager 1955, 1966, 1967a, b, 1969, 1980; Yamamoto et al. 1962a, b; Anderson et al. 1960; Blass et al. 1959; Bamji and Krinsky 1965; Krinsky 1964, 1966).

In the article (Maslova and Markovskaia 2012), T.G. Maslova highlights herself more than D.I. Sapozhnikov but she has only dealt with the carotenoid subject rather sporadically. For an example, we will again refer to the NCBI database, where at the moment of our manuscript preparation, there were five works with Markovskaia as coauthor, published in the magazine *Ontogenez* in Russian. None of them was devoted to the carotenoid system. Dissembling of unwanted facts is inherent in Talanova's dissertational work performed in this department (Talanova-Sher 2004), which is seen from its literature review.

Probably therefore, Maslova and Markovskaia discussed their results with a known professional on plant cell respiration, Prof. O.A. Semikhatova, and they expressed gratitude "for the help in article writing". But carotenoid cell function and metabolism was not the object of Prof. O.A. Semikhatova's extensive investigations.

In order for the reader to clearly understand the core of the dispute, we need to explain. Sapozhnikov et al. (1957, 1959a, b) showed that, under light influence, there is a decrease in violaxanthin content and a simultaneous increase in the content of the main xanthophyll of green cells, lutein.

We call the reader's attention to the fact that the paper chromatography technique used did not allow careful separation of fractions of violaxanthin and lutein. So the lutein fraction contained zeaxanthin that was not detected with this technique. Therefore, the authors took the sum of violaxanthin and lutein pigments to be equal to 100 %. According to a slippery technique of their calculation, the conclusion followed that violaxanthin turns into lutein with separation of two epoxy groups and reorganization of one β-ionone violaxanthin ring into α-ionone lutein ring. The authors of the criticized article (Maslova and Markovskaia 2012) meaningly leave out the significant discrepancy.

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We quote: "Later this discovery was termed the 'violaxanthin cycle' (VC)." The authors go on to say that upon illumination of leaves with intense light, there was a reduction in the leaf content of epoxy-xanthophyll (violaxanthin) and an increase in the de-epoxy-xanthophyll, lutein (in the combined fraction of lutein and zeaxanthin (*??*) (*italics here and below are ours*). They state that this is the direct reaction of the cycle and that the back reaction of the cycle consists in conversion of *zeaxanthin* into *violaxanthin* (*!!!*) and can occur in the *dark* and in *light*.

This is where the supposition about the enzymatic component in the reactions of xanthophyll interconversions lies. Sapozhnikov and coworkers did not notice it, because it could not be included in their conception.

The authors state: "*Antheraxanthin* (Ax) is an intermediate product of both reactions" (*!!!?*). But this thesis and point of view were published in 1962 by Yamamoto, Nakayama, and Chichester (Yamamoto et al. 1962c). For many years Sapozhnikov had another interpretation. Where is the lutein fraction mentioned in explanation of results in the article (Maslova and Markovskaia 2012)? The lutein fraction is 12–15 times more than the zeaxanthin one. For many years Sapozhnikov's group spoke only about lutein (and only under the effect of light), and only lutein was taken into consideration; antheraxanthin and zeaxanthin were not even heard about until the end of 1962. On what basis did the authors of the article (Maslova and Markovskaia 2012) forget about lutein and speak only about the back reaction of zeaxanthin oxidation? The authors of that article did not present any data from their own experiments and did not have any. For many years, Sapozhnikov ignored the work of Yamamoto et al. (1962a–c) and forbade members of his science group to speak about the presence of intermediate products in the direct and back reactions. It was over this issue that the points of view of D.I. Sapozhnikov and V.S. Saakov disagreed with each other.

Consider, reader, that if violaxanthin (Viol) *quickly* transforms into lutein (Lut) then:

1. How are Viol funds replenished?
2. Which metabolic reactions are fast enough to be involved in the regeneration of Viol funds in a chloroplast?
3. The back reaction of Lut (Zea) transformation is very slow and cannot replenish funds of earlier reduced Viol. Maybe this return reaction does not take place at all, and Viol funds are replenished in the dark (and in light) due to processes of biosynthetic renewal. Nobody has proved it by performing direct experiments with transformation of the labeled Zea skeleton. Only an increase in Viol content in the dark has been shown.
4. If the reaction Viol → Lut is one short-term event and finishes after 10 min of illumination, then what does it functionally mean and what is its role in a chloroplast?
5. If the transformation Viol → Lut (Zea) is a functionally necessary process, then Viol stores should renew (regenerate) because of some very fast biosynthetic processes. This main question Maslova and Markovskaia (2012) not only dissemble, but do not even think of at all, repeating the decayed postulates from

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50 years before. This implies that they do not know the new literature of the last 20 years, which overturns Goodwin's theory. It seems that they forgot about this trifle.

Earlier reactions of Viol biosynthesis were assigned, according to Goodwin's concept (Goodwin 1955, 1958a, b, 1959, 1961, 1965, 1969; Porter and Anderson 1967), to the consequences of secondary reactions of carbon metabolism. Prof. C. Costes (1963a, b, 1965a, b) was the first to publish experimental data placing into question Goodwin's ossified position (see Goodwin 1965, Chapter 5, pp. 143–173), but this will be discussed later in Chap. 5.

Without knowing and maybe not thinking of this, the out-of-date data was published (Maslova and Markovskaia 2012) even though the problems of antecedence in carotenoid biosynthesis were formulated and partially solved at the second international congress on carotenoids in Cluj, Romania, in 1972.

The authors of the article (Maslova and Markovskaia 2012) do not cite the works of Yamamoto et al. (1962a–c) and put emphasis on the activity of Dorothea Siefermann-Harms (BRD-Germany) when estimating possible xanthophyll functions. It was fate that one of the authors of this book worked for 14 months in the same office as Siefermann-Harms. After cutting leaves in the mountains where an experimental station was located, she could hold them in the dark for 2 days without thinking of the passing metabolic processes. Only after this were objects fixed with liquid nitrogen and used for analysis. Despite the use of the modern method of high-performance liquid chromatography (HPLC) for the analysis of pigment structure for qualitative analysis and quantity, the obtained picture of pigment content was far from true to that inherent in an initially natural material. Having completed doctoral studies under the guidance of Prof. Dr. H. Metzner (Tübingen, BRD-Germany), Siefermann-Harms did not stay in his laboratory, but over a number of years migrated through different laboratories (including that of Prof. H. Yamamoto in Hawaii). She held contracts at the Institute for Genetics and Toxicology at the Nuclear Research Center, Karlsruhe, and then was dismissed.

Unfortunately T.G. Maslova did not create an independent backbone scientific theme, although her surname from time to time appeared in group articles of doubtful methodological validity.

It is known that much will be completely lost to human memory and, what is even worse, deformed (it is good if not to truckle to the time moment or the scientific head). Possibly such memory aberration occurs because the experienced and focused researcher brings to mind first those memories that are important and touch a nerve. But in writing the review (Maslova and Markovskaia 2012), T.G. Maslova did not and could not have such articles in her memory because she did not creatively work in this scientific direction and did not know the material and sources of discussion, silently listening to D.I. Sapozhnikov's instructions.

In one section of Chap. 5, we will return to the consideration of reactions and rates of carotenoid biosynthesis, and we will remind the reader about the current state of this question.

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D.I. Sapozhnikov et al. (1957, 1959a, b) *insisted only on the direct conversion of Viol molecules into Lut under the effect of light*. At the beginning, he did not know anything about any zeaxanthin, and later, for many years, he did not recognize its participation in pigment interconversion reactions. Concerning the back, dark reaction, D.I. Sapozhnikov agreed only with the reversible direct interconversion of lutein into violaxanthin in the dark. During this interconversion, in the back reaction, there is reorganization of the lutein α-ionone ring into the violaxanthin β-ionone ring. This idea is counter to Goodwin's data quoted above (Goodwin 1971a, b). We remind the reader that D.I. Sapozhnikov learnt about antheraxanthin participation in the back reaction only later from the publication by Yamamoto et al. (1962a–c) and then from Hager's publications (1966, 1967a, b). Further, Sapozhnikov had to agree with foreign treatment of the interconversion reactions (Sapozhnikov 1973a, b).

Here, it is useful to remember that Einstein thought highly of the ability to forget the theories of predecessors that had become unnecessary or denied by experience, so that they did not prevent the deduction of one's own new concepts. But Maslova and Markovskaia are not very successful in the creation of new concepts.

As to the name of this cyclic reaction, the term "violaxanthin cycle" is sometimes used in the scientific literature, but more often the term "xanthophyll cycle" is applied as having a fuller sense.

The term "violaxanthin cycle" was used earlier by Lee and Yamamoto (1968) when investigating the spectrum of de-epoxidation and epoxidation reactions in spinach leaves.

The term "xanthophyll cycle" is accepted in Wikipedia (http://de.wikipedia.org/wiki/Xanthophyllzyklus) and is used in the majority of European and US works by experts in the field (Yamamoto et al. 1962a, b; Hager 1966, 1967a, b, 1980; Hager and Stransky 1970a–c; Foyer et al. 1990; Gilmore and Yamamoto 1992, 1993; Schubert et al. 1994; Niyogi et al. 1997a, b; Gross et al. 1998; Bungard et al. 1999; Latowski et al. 2000, 2002; García-Plazaola et al. 2007, 2012; Grouneva et al. 2006, 2009; Saakov et al. 2013; etc). The specified term is also recognized in an English-language Wiki page (www.freebooknotes/wiki/Xanthophyll).

In the works of Sapozhnikov and coworkers, the term "violaxanthin cycle" can be found for the first time in the summaries of these by L.V. Ivantsova (1969) and O. Ja. Koroleva (1973), in papers by Sapozhnikov (1973a, b) and by Popova and Sapozhnikov (1973). Its use is more inherent in articles from Sapozhnikov's group, as can be seen from the cited works in the article of Maslova and Markovskaia (2012).

In this case, the dispute about the name of the cycle is absolutely not principal, but the term "xanthophyll cycle" is fuller, covers a large group of pigments, and can be applied to various evolutionary systematic groups. This is exemplified by the use of the various terms by the same author: *violaxanthin* cycle (Havaux and Niyogi 1999), *xanthophyll* cycle (Havaux et al. 2000), and *xanthophylls* cycle (Havaux et al. 1991). Maslova and Markovskaia on page 474 of their article (Maslova and Markovskaia 2012) use the term "xanthophyll cycle," and in the list of the literature

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quoted by them, the considered terms are mentioned nine times each. Also, the term "xanthophyll cycle" is more frequently used in European and US literature.

As to the position that D.I. Sapozhnikov with "scientific intuition inherent in him suggested the universality of this cycle in flora and its interrelation with photosynthesis and presence in leaves of the fermentative systems regulating carotenoids interconversions," then the authors did "distort cards" (Maslova and Markovskaia 2012). Thorough experimental research in this direction was carried out by Hager and Stransky, who investigated a wide set of representatives of seaweed of different families and classes (Hager and Stransky 1970a–c; Stransky and Hager 1970a–c).

We specify that D.I. Sapozhnikov initially held the opinion that the reactions of two xanthophyll interconversion are of photochemical nature instead of enzymatic (*Bulletin of Academy of Science of the Soviet Socialist Republic*, 1960, Vol 5. P.101, Vladimirov YuA, and Litvin FF; comments to report). The enzymatic nature of the direct and back reactions became known from a number of Hager's experimental works (Hager 1967a, b, 1969, 1975, 1980; Hager and Perz 1970) and from series of research by Yamamoto and coworkers (Yamamoto et al. 1962a–c, 1967, 1972a, b, 1979; Yamamoto and Chichester 1965; Yamamoto and Takeguchi 1971; Yamamoto and Bangham 1978; Yamamoto and Higashi 1978; Siefermann and Yamamoto 1975a–c), and this knowledge was later developed (Hieber et al. 2000).

We direct the reader's attention to the fact that, according to the data of Sapozhnikov and coauthors, the back reaction (i.e., the conversion Lut–Viol) can occur both in the dark and in light. It is necessary to emphasize that the phenomenon of the dark reaction implies the enzymatic nature of this reaction. In their usual manner, the authors did not see and did not understand this fact. Hager and Yamamoto have priority for this.

We also consider it necessary to emphasize that in all discussions, the sequence of scientific achievements and successes should be stated with absolute accuracy. This is absent, to great regret, in materials glorifying D.I. Sapozhnikov. If Maslova and Markovskaia stand for Sapozhnikov's concept, then they should show methodologically and statistically reliable experimental data and prove them with conscientious explanations. They should not deform the scientific figure of D.I. Sapozhnikov with mythical findings. If there were "findings," why was material on their registration not submitted? At that time, merely sending material to the State Committee on Science and Technologies put competitors forward into the first scientific rows. In the laboratory, D.I. Sapozhnikov told himself about a possible nomination for the State award for the considered phenomenon.

However, for some reason, they did not fill the application form for the invention or finding. As a *clever person*, but with foibles, D.I. Sapozhnikov understood that at the official registration of documents on findings, there would not be an unofficial discussion accompanied by euphoric chatter about the genial leader, but a very serious patent check, which the work of this group of hasty discoverers simply would not pass. Battle with Goodwin's writing if you do not agree with him.

So that the reader can in future critically regarded the data presented, we will return again to a more detailed consideration of this problem in one of the sections

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of Chap. 5 and end this discussion of the errors of representative of Sapozhnikov's group.

It is a pity that the assessment of scientific material described by researchers cannot always be verified by time and by international experts and that articles become assigned to a tray of marked-down ideas, concepts, or books.

Our more strict approach to the radioactivation analysis technique and to sample preparation was the reason for some divergence from the material of foreign researchers. Because of the complexity of the isolation of radiochemically pure pigments in amounts sufficient for analysis, we were limited to tiny quantities, which complicated obtaining the necessary amount of substance for a number of parallel targets. We underline the fact that parallel tests in experiments were absent both in some domestic and foreign works (Sapozhnikov et al. 1965a, b, 1967b; Yamamoto and Chichester 1965; Yamamoto and Takeguchi 1971), which complicated checking the correctness of the methodological part of the experiment. Additional complexities included the following: In the specified works, the necessary radiochemical purification of studied samples was not reached. One or two pigment fractions enriched by isotope O^{18} were studied in an experiment, whereas the analysis required several derivative forms of a substance differing in quantity and in arrangement of oxygen groups in the molecule (Saakov 1963a–d, 1965a, b). After all, such comparison serves as a mutual additional control and as the source of information about the place of oxygen inclusion in various parts of investigated molecules (Hornyak et al. 1950; Hornyak and Lauritsen 1948; Ajzenberg-Selove and Lauritsen 1959; Sapozhnikov et al. 1961, 1964; Saakov 1963a–d, 1964, 1965a, b; Saakov et al. 1969, 1970a).

In foreign experiments, the control fractions of preparations not enriched with isotope O^{18} were not checked (Yamamoto et al. 1962a–c; Yamamoto and Chichester 1965; Yamamoto and Takeguchi 1971). In experiments with molecular O^{18}, there was no necessary control with H_2O^{18} and with H_2O (Sapozhnikov et al. 1965a, b, 1967b). In the majority of foreign works, conclusions were made on the basis of one measurement in a variant without additional parallel measurements. At the same time, experience showed the necessity of additional parallel registrations (Saakov 1963a–d, 1964, 1965a, b). In the works of one scientific establishment, there was rather considerable variability of enrichment of the initial preparations labeled with O^{18} and H_2O^{18} and also in the duration of exposure to the labeled substrate. In foreign works, this caused significant discrepancies in evaluation of the transferred number of protons, that is, the incorrectness of the physical part of the oxygen isotope detection technique dictated the resulting exit intensity of the induced reaction (Dorough and Calvin 1951; Anderson et al. 1960; Shneour 1961, 1962a, b; Shneour and Calvin 1962).

The specified methodological faults or theoretical artificiality in works applying the new analytical approaches caused a skeptical attitude to this new, sensitive, and reliable method of research. Therefore, academician V.A. Engelgardt gave a timely warning about careful management of the newly introduced methods, about the possibility of unforeseen dangers caused by hasty and erroneous conclusions, and about the requirement for circumspect and careful data processing and advocated

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the critical analysis of results (Engelgardt 1955). It is possible to familiarize oneself with the O^{18} detection method with help of radioactivation analysis in more detail (DuBridge et al. 1938; Saakov 1963a–d, 1965a, b, 1990a, b; Saakov and Konovalov 1966; Saakov et al. 1969, 1970a, b).

Excessive piety towards Western research, caused by their splendid equipment and accompanying support, can lead to inexact assessments on the essence of the investigated processes.

We meaningly brought back to life the history about the application of the radioactivation method due to its promising usage in modern works with nano-quantities of substance, and today the temporary oblivion of this method is caused not by scientific reasons, but economical ones. In this connection it is important to underline mistakes made earlier by reserchers experimenters, mistakes as in methodology as in interpretation of their published results. This brightly illustrates the position in Rozengart's review (2012) about the adequacy of methodological approaches to the stated research task.

In the process of describing the radioactivation method and further techniques, we have tried not to use mathematical language so that the reader does not require any special knowledge apart from general culture and an inner interest in new modern approaches to analysis.

In 1990–1991, when working in the Nuclear Research Center in Karlsruhe, Germany (Kernforschungszentrum, later known as Forschungszentrum, and now the Karlsruhe Institute of Technology), one of us began investigations on the use of PAM fluorescence. In Europe, the method had appeared 4 years earlier. The cluster of works on this topic carried out in the period 1995–2005 are described in Chap. 2. On the basis of these data, the theory on the energetic basis of green cell stability was proved. The material of this research was included into the theme of the Russian Academy of Sciences as theme 6.50.2.

Spectrophotometry allows the analysis of true and colloidal solutions of various substances or of their mixes, and research on such difficult systems as intact leaves, chloroplasts, suspensions of seaweed, erythrocytes, mitochondria, etc. Spectral methods are successfully applied for study of the physiological state of autotrophic and mixotrophic organisms, for selection of pigment genetic mutations, for elucidating the metabolic pathways of various substances, for research on the damageability and stability of the plastid machinery under different physical, chemical, and biological influences, and for research on the kinetics of oxidation–reduction reactions (Rubin 1974, 1975, 2000, 2004; Morton 1975).

For modern molecular photobiology, it is established that the final effect of a photobiological process is the consequence of an initial photochemical reaction. At the heart of the process is a certain sequence of reactions and of conformational reorganizations organized in time and subcellular space. The cluster of mentioned processes depends on functional features of the biological object and on its evolutionary and ecological specificity. This promotes the specific space organization of an animal cell and of the plant plastid machinery; it assumes a certain packing order of the molecular complexes of the membranes of erythrocytes, mitochondria, and chloroplasts.

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The high analytical value of absorption spectrophotometry methods, in particular of derivative spectrophotometry, lies in their ability to decipher the *structural* specificity of the state and reactions of different native biological structures of leaf plastid machinery and of blood elements, which enables characterization of the optical parameters responsible for the course of photobiological and biochemical processes; importantly, this is without loss of intactness of their membrane structures (Litvin and Gulyaev 1969; Litvin et al. 1973a, b; Konev and Volotovskii 1974; Kvitko et al. 1976; Saakov et al. 1978a, b; Fell 1979, 1980; Fell and Smith 1982; Ojeda et al. 1995; Ojeda and Rojas 2004; Rojas Sanchez and Ojeda Bosch 2009).

Despite many detailed manuals on application of spectrophotometric, photocolorimetric, and ESR methods in various areas of biology, organic chemistry, and medicine (Litvin and Gulyaev 1969; Litvin et al. 1973a, b; Saakov et al. 1978a, b; Williams and Willson 1975; Freifelder 1976), the expansion of biochemical and biophysical research practice requires the creation of a short and understandable book describing the analytical approaches of absorption spectrophotometry, taking into account the tendencies of their modern development and the introduction of new analytical techniques in the last few decades.

In Chap. 3, we describe the basis of spectrophotometric analysis in a compressed and simple form, emphasizing the new (appearing in the last few decades) methodological and informative capabilities of absorption spectrophotometry, briefly summarizing the main approaches and requirements for the analysis of spectra of solutions and native cell structures (intact leaves, erythrocytes and blood plasma, chloroplasts and mitochondria), and also describing an interpretation of their changes in response to external environmental factors in physical and chemical biology experiments.

We realize that not all the problems of applied spectrophotometry considered in this book can satisfy the requirements of experts of different research directions. So the authors have relied on their own experience of the development of derivative spectrophotometry as accepted by professionals in various scientific fields (Marenko et al. 1972; Marenko and Saakov 1973; Kvitko et al. 1976) and the school of academician N.S. Poluektov (Aleksandrova et al. 1982; Nazarenko et al. 1982; Kucher et al. 1983; Perfil'ev et al. 1985; Mishchenko et al. 1987), and on the recommendation of known scientists of our country and foreign researchers (Litvin and Gulyaev 1969; Skujins 1986; Talsky 1994; Ojeda et al. 1995; Ojeda and Rojas 2004; Rojas Sanchez and Ojeda Bosch 2009; Karpinska 2012).

An unfailing interest and familiarization with the literature describing the application of derivative spectrophotometry in analytical studies in different scientific fields has revealed the close attention of foreign researchers to publications on this subject in the USSR and Russia. For example, while Talsky's monographic report was being written in 1992–1993 (Talsky 1994), its author had access to the information system covering not only physical and chemical publications, but also including successes in Soviet science in the field of physical and chemical biology. Talsky received articles from the whole world, and there were means for delivery of

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this information to his institute. Such a state of affairs allowed him, for example, to cite our works published in the journals of ordinary Russian institutes or in magazines of the Academy of Sciences of the Moldavian Soviet Socialist Republic.

We would like to explain. When working in the Nuclear Research Center in Germany, one of the authors was using the kind library services of this center for one and a half years and was receiving copies of world publications on required research subjects. This work was quickly and efficiently performed by library staff, without giving research personnel any search troubles.

More than 20 years have passed. The Internet has become a general powerful information tool, but even so we must pay to receive the appropriate information. Not only must scientists spend a lot of time looking through hundreds or thousands of works to select those desired, but they must sometimes pay money for own publications.

Larceny in various structures, about which Russian mass media inform almost every day, and the lifelong money shortage for science are not looked at seriously in Russia. Having summarized billions of stolen means, it is possible to construct and support tens of information systems for different scientific fields and to come to advanced world positions in science.

Because the present edition is mainly intended for biologists, physicians, physiologists, biochemists, and biophysicists, who still traditionally use a restricted mathematical language for the description of biological and biochemical processes, we have reduced the use of mathematical symbols to a minimum. Instead, we have used more accessible graphic material obtained on the basis of scientific data visualization programs.

Careful study of derivative spectrophotometry databases at the Central Institute of Genetics and Cultural Plants, CIGCP, DAdW GDR (Zentralinstitut für Genetik und Kulturpflanzenforschung, renamed after reunion of GDR and BRD as the Institut für Pflanzengenetik und Kulturpflanzenforschung, IPK) (during work in 1968−1970, 1976, 1989) promoted the development of an original apparatus for registration of the second- and the fourth-order derivative spectra on the basis of a Russian technique. The methodological part of this question is described in the first part of Chap. 3, and experimental data registered using the discussed technique are presented in Chaps. 4 and 5.

In the early 1980s, the application in biochemical research practice of the derivative spectrophotometry method attracted the attention of researchers investigating the roles of Ca^{2+} and Co^{2+} in the change in the conformational properties of animal blood proteins during blood osmolality regulation (Natochin et al. 1985). Adaptation of animals to conditions of various environmental salinities is caused by formation of osmotic regulator systems, by the ability to control the volume of liquid in cells and organisms. After addition of cobalt ions, the revealed system of urgent osmolar regulation can serve as an adaptation tool during an acute change in physical and chemical parameters in internal liquids or under a change in environmental conditions (Saakov et al. 1987).

Studies of ligand formation due to interactions of guanidine preparations with calcium and lanthanum ions, led by E.V. Rozengart, the known methodologist and

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an expert in cholinesterase biochemistry (Sadykov et al. 1976; Brestkin et al. 1997; Moralev and Rozengart 2007; Rozengart 2012), lasted many years. This material forms a significant part of Chap. 4. The subsequent sections of Chap. 4 consider the characteristics of derivative spectra of aromatic amino acids and proteins and also the features of spectral change under the influence of EFEs such as γ-radiation and temperature.

In Chap. 5, the authors discuss both methodological questions of cell pigment spectroscopy and a number of debatable and evolutionary questions of pigment metabolism in representatives of various systematic groups of *Eucaryota* and *Procaryota* domains. In a separate section, the data of the last 20 years on features of carotenoid biosynthesis are presented. In one section of Chap. 5, there is a detailed description of experiments on isotope O^{18} application and on its detection with the help of protons and α-particles. At that distant time, there were the first biochemical experiments on O^{18} incorporation into molecules of components of a chlorophyll–protein complex and experiments discrediting the theory about active participation of epoxy-xanthophylls in the main line of transport and the oxidation of water oxygen to air molecular oxygen.

Application of derivative spectrophotometry to blood state analysis, begun by us in the early 1970s (Saakov et al. 1973, 2010, 2013), gained practical acceptance (see Chap. 5) in different countries. According to the proposal of Prof. A.I. Krivchenko, research into blood spectral properties was carried out and laid the basis for hemoglobin (heme) interaction with metal ions of groups VII and VIII of Mendeleev's table and with a number of toxic substances.

The technique of derivative spectrophotometry of high orders also allowed the establishment of new approaches for the solution of problems of hyperbaric physiology and biochemistry (Sokolova et al. 1991, 1992; Leontyev and Saakov 1989; Panov et al. 1989). This was important because most analytical work on assessment of the effect of high pressure on physiological and biochemical characteristics was carried out after removal of investigated objects from the hyperbaric environment because of explosive or gradual stepwise decompression, which blurred the picture of true changes.

Pressures of 10–20 atm pressure cause specific changes in the state of biologically active substances and in the structure of enzymes and other proteins (Balny et al. 1984, 1987, 1996; Ragone et al. 1984; Mach and Middaugh 1994; Kornblatt et al. 1995, 1999; Kunugi et al. 1997; Lange et al. 1996a,b; Balny and Lange 1999; Lange and Balny 2002). This means that for experimental integrity, transducers or flow cells should be in the same hyperbaric environment and signals should be transmitted through communication lines or by noncontact ways to the analytical devices. This is especially important for the analysis of object states at extreme environmental pressure.

The tendency of the members of the authors' group towards methodological development has, of course, affected both the book composition and the style of its writing. The authors of this book do not fear to argue and indicate the points of view that have been dominant for a long time and actively obstructed by antiscientific efforts. We do not lapse into cheap sensationalism and uncompromising publicism.

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We think that the present book will help orientate the reader during an assessment of phenomena and positions in the discussed scientific areas.

The authors do not doubt that professional spectroscopists and experts in fluorescence analysis will discover an absence of some professional details in the book. Therefore, we wish to thank readers in advance for any benevolent instructions and remarks.

Finally, the authors express deep appreciation to the academician V.L. Svidersky for 40-year collaborative work, for his attention to the manuscript during book writing and to the academician Yu.V. Natochin for critical and constructive recommendations on the monograph text. We express gratitude to the academician N.P. Vesyolkin for the constant support and timely remarks and council.

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Chapter 2
Successes in Application of Pulse-Amplitude Modulated Fluorescence

Contents

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2.1 Fluorescence Spectroscopy in Amino Acid Analysis

Fluorescence is used not only in studying the *functions* of molecules, as demonstrated by the example of photosynthetic reactions (see Chap. 1), but also for an assessment of changes in molecular *structure* as a consequence of protein interactions with other molecules (e.g., with other proteins or DNA) or caused by the influence of damaging agents such as temperature, light, chemical agents, and radiation (see Chap. 4).

We remind the reader that the amino acid residues tryptophan, tyrosine, and phenylalanine fluoresce, although the phenylalanine quantum yield is very low and usually its fluorescence is not detectable (Cantor and Schimmel 1984). Also, the fluorescence of tyrosine residues included in proteins is much weaker than that of tryptophan ones, not only because of the lower yield of pure tyrosine compared with tryptophan but also because the incorporation of a tyrosine residue in a peptide bond reduces the tyrosine fluorescence by approximately 32 % (Chen and Edelhock 1976). Tyrosine residues, hydroxyl groups of which are involved in the formation of the hydrogen bond (TyrOH), cannot fluoresce at all (Chen and Edelhock 1976), and, at last, under favorable conditions, excited tyrosine residues ("donor residues") transfer energy to acceptor residues of tryptophan and TyrOH through non-radiative dipole–dipole coupling. This phenomenon takes place when, simultaneously, the donor *emission* spectrum overlaps with the acceptor *absorption* spectrum, chromophores are located not farther than a certain distance from each other, and their dipole moments are in proper relative orientation. The phenomenon is called Förster resonance energy transfer (FRET), after the German physicist T. Förster (in the case of tyrosine and tryptophan, it is singlet–singlet transfer) (Cantor and Schimmel 1980).

Therefore, although the spectra of tyrosine and tryptophan fluorescence have maxima at different wavelengths, the tyrosine fluorescence spectrum cannot be separately registered because of the tryptophan residues in the protein. Thus, at an excitation wavelength of 275 nm, the total spectrum of tryptophan and tyrosine emission is recorded. Nevertheless, the spectrum of tyrosine fluorescence can be obtained from the total spectrum of tyrosine and tryptophan fluorescence with the help of the method described by Isaev-Ivanov et al. (2000).

The principle of the method consists of subtracting two spectra from the total spectrum recorded in the range of 285–450 nm at an excitation wavelength of 275 nm: (1) the tryptophan emission spectrum in the range 305–450 nm at the excitation wavelength 295 nm and (2) the emission spectrum of *N*-acetyl-L-tryptophan amide (NATrpA) in the range of 300–320 nm at an excitation wavelength of 275 nm, shifted to the maximum of the total spectrum (see Fig. 2.1).

The use of data from X-ray crystallographic analysis, nuclear magnetic resonance, or electron microscopy, which can be found, for example, in the database of the RCSB Protein Data Bank (PDB), containing more than 72,000 structures (http://www.pdb.org/) (Berman et al. 2000), considerably helps in the analysis of results

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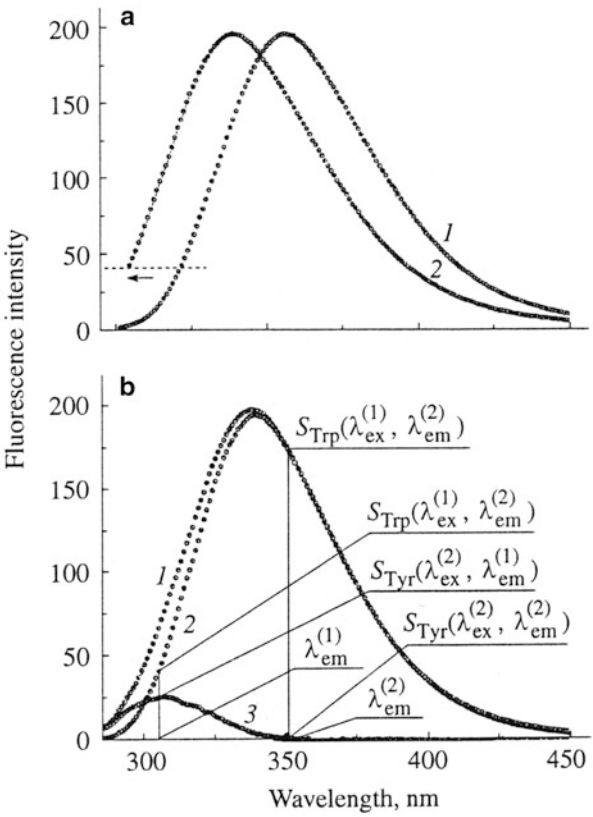

Fig. 2.1 (**a**) Line shapes of emission spectra of *N*-acetyl-L-tryptophanamide (*1*) and tryptophan (*2*) at $\lambda_{ex} = 275$ nm. (**b**) Examples of the recorded total emission spectrum of DNase I (*1*), the reconstructed tryptophan emission spectrum (*2*), and the separated tyrosine emission spectrum (*3*)

from fluorescence spectroscopy. The programs for viewing molecular structures (e.g., *Swiss-PdbViewer*, http://www.expasy.org/spdbv/; Guex and Peitsch 1997) allow, in particular, TyrOH residues to be found. Also, using the coordinates of atoms from PDB files, it is possible to calculate the parameters and efficiency of resonance energy transfer using known formulae (Cantor and Schimmel 1980):

$$E = \frac{R_0^6}{R_0^6 + r^6} \quad R_0 = 9.7 \times 10^3 \left(k^2 n^{-4} \Phi_D J\right)^{1/6}$$

$$k^2 = \left(\cos\theta_T - 3\cos\theta_D \cos\theta_A\right)^2 \quad J = \int S_F^D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda$$

where E is the FRET efficiency; R_0, the Förster radius; r, the distance between the donor and the acceptor; k^2, the geometric orientation factor characterizing the mutual orientation of the donor and acceptor dipoles; n, the refractive index of the medium; Φ_D, the fluorescence quantum yield of the donor in the absence of the acceptor; J, the overlap integral for the donor emission spectrum and the acceptor absorption spectrum; θ_T, the angle between the emitting dipole of the donor and the

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absorbing dipole of the acceptor; θ_D and θ_A, angles between these dipoles and the vector directed from the donor to the acceptor; S_F^D, the donor emission spectrum normalized to the area under this spectrum; and $\varepsilon_A(\lambda)$, the extinction coefficient of the acceptor, which depends on the wavelength λ.

It should be noted that in protein, for one tyrosine donor residue, there can exist several (n) acceptor residues, each of which (ith) is characterized by the FRET efficiency E_i and the energy transfer rate constant $k_T^i (i = 1 \ldots n)$.

The transfer rate constants k_T^i are calculated according to the formula (Cantor and Schimmel 1980)

$$k_T^i = \frac{1}{\tau_D}\left(\frac{R_{0i}}{R_i}\right)^6 = \frac{E_i}{\tau_D(1-E_i)},$$

where τ_D is the excited-state lifetime of the donor in the absence of acceptors.

So the total energy transfer constant K is calculated as the sum:

$$K = \sum_{i=1}^{n} k_T^i = \sum_{i=1}^{n} \frac{E_i}{\tau_D(1-E_i)}.$$

The total efficiency E of the transfer from one tyrosine residue to several acceptor residues can be calculated using the formula (Danilova et al. 2005)

$$E = \frac{\sum_{i=1}^{n} \frac{E_i}{1-E_i}}{1 + \sum_{i=1}^{n} \frac{E_i}{1-E_i}}.$$

As an illustration, we consider the protein bovine pancreatic deoxyribonuclease I (DNase I), the enzymatic activity of which, as well as of many other endonucleases, is activated by divalent cations (Mg^{2+}, Ca^{2+}, Mn^{2+}) and inhibited by physiological concentrations of monovalent cations (K^+, Na^+, Cs^+, Cd^+, Li^+) (Junowicz and Spencer 1973; Oshima and Price 1974). As shown by the results of dynamic light scattering, in the absence of any salts, this protein binds to DNA, and in the presence of monovalent cations, the dissociation of the protein–DNA complex takes place (Danilova et al. 2005).

From the primary structure of DNase I, it follows that the fluorescence of this protein molecule is defined by three tryptophan and 15 tyrosine residues. Analysis of the tridimensional DNase I structure (its identification number in the protein database RCSB PDB is 3DNJ) (Oefner and Suck 1986) and of DNase I complexes with model turns of DNA (1DNK and 2DNJ) (Weston et al. 1992; Lahm and Suck 1991) showed that, with high probability, five of the 15 tyrosine residues cannot fluoresce because they are TyrOH residues. The fluorescence of two other tyrosine

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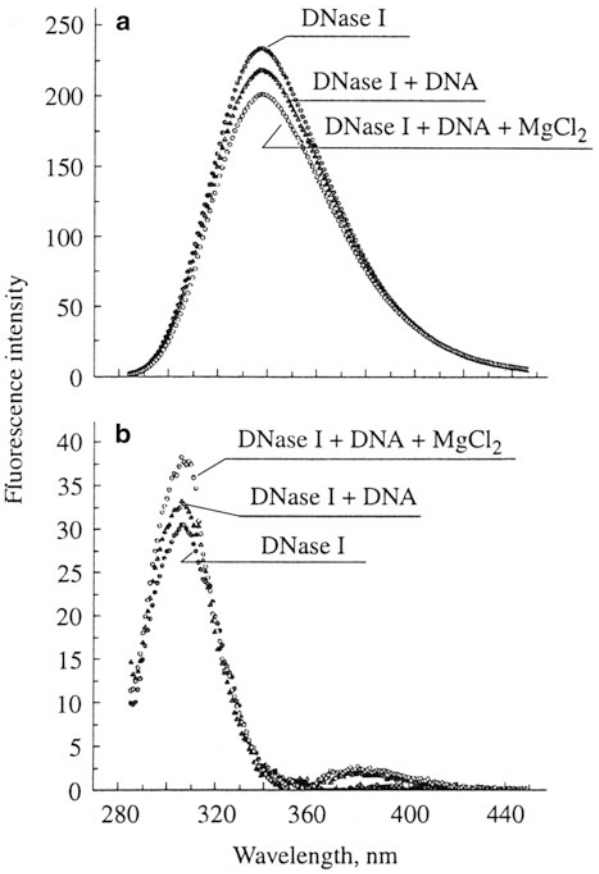

Fig. 2.2 Examples of (**a**) tryptophan and (**b**) tyrosine fluorescence emission spectra obtained by separation of the protein emission spectra recorded after sequential addition of phage M13 single-stranded DNA and $MgCl_2$ to the protein solution

residues is significantly quenched because of the high FRET efficiency (93–95 %) to tryptophan residues. Furthermore, four tyrosine residues are not quenched at all (efficiency, 0 %), but they are located not only far from acceptors but also far from the enzyme active center (AC) and from the enzyme DNA-binding site (DBS). So 11 tyrosine residues cannot contribute to the fluorescence changes caused by protein binding to DNA and by enzymatic activity.

Residues Tyr175 and Tyr253 are closest to the AC and DBS. Also, the DBS includes the tyrosine residue Tyr211 and one of the TyrOH residues, Tyr76. Positioned 20 residues from Tyr76 is Tyr97, which manifests some mobility in crystals with model DNA turns. No tryptophan residues are present in the AC and DBS (Danilova et al. 2005).

Figure 2.2 shows examples of emission spectra of the tyrosine and tryptophan fluorescence of DNase I, obtained by separation from the protein emission spectra recorded after consecutive addition of 12 μM circular single-stranded DNA of phage M13 and 5 mM $MgCl_2$ to a solution of 1.6 μM DNase I in Tris buffer. In Table 2.1, the numerical values of fluorescence changes are presented for cases when DNA and

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Table 2.1 Changes in tryptophan and tyrosine fluorescence of DNase I after addition of salts and/or DNA to the protein solution

Sample	Changes in fluorescence with respect to Fl of protein solution	
	Quenching of tryptophan Fl (%)	Change in tyrosine Fl (%)
Protein + $MgCl_2$	6.6 ± 1.3	Not changed
Protein + NaCl	4.9 ± 1.5	Not changed
Protein + $MgCl_2$+NaCl	8.5 ± 1.2	Not changed
Protein + DNA	5.0 ± 1.6	Increase by 9.4 ± 1.8
Protein + DNA + $MgCl_2$	11.1 ± 1.7	Increase by 17.8 ± 1.3
Protein + DNA + NaCl	11.3 ± 1.1	Increase by 16.9 ± 2.1
Protein + DNA + $MgCl_2$ + NaCl	15.2 ± 1.5	Increase by 22.3 ± 1.6
Protein + DNA + NaCl + $MgCl_2$	14.6 ± 1.2	Increase by 13.5 ± 1.4

monovalent and/or bivalent cations and their mixes were added to the protein solution. Fluorescence changes were calculated as the difference between the areas under spectra, expressed as a percentage of the protein solution fluorescence.

The simultaneous quenching of tryptophan fluorescence and the increase in intensity of tyrosine fluorescence means that the change in FRET efficiency is caused by conformational modifications (1) in DBS, when DNA was added to protein or when NaCl was added to the protein–DNA mixture and caused the dissociation of protein–DNA complexes; (2) in AC, after addition of $MgCl_2$ to the protein–DNA mixture that promoted the enzymatic activity; and (3) in AC, after addition of NaCl to protein–DNA or protein–DNA–$MgCl_2$ mixtures, because addition of $MgCl_2$ to the protein–DNA–NaCl mixture did not cause an increase in tyrosine fluorescence (in contrast to the situation when the protein–DNA mixture was without NaCl). Consequently, Na^+ ions influenced the AC conformation and not only the conformation of DBS. Thus, Mg^{2+} affects the AC conformation, and Na^+ affects DBS and AC.

Nevertheless, because protein complex structures containing amino acid residues are collisional quenchers of tyrosine and tryptophan fluorescence (Lakowicz 1983; Chen and Edelhock 1976), and because of the probability of breaking TyrOH hydrogen bonds as a result of conformational changes, it is impossible to determine conformational changes unequivocally and exactly and to conclude what part of the fluorescence change is caused by variations in FRET efficiency without using additional special fluorescent probes and mutagenesis.

For example, the addition of salts to the protein solution results in only the reliable change in tryptophan fluorescence; therefore, a change in FRET efficiency does not occur. So, because salts influence tryptophan fluorescence, the observed effects in mixtures of protein, DNA, and salt cannot be explained only by changes in FRET efficiency. The effects of salt in protein–salt mixtures are very probably caused by an increase in the influence of collisional quenchers of tryptophan fluorescence, namely, in pairs Trp158–Asp93 and Trp181–Asp198. In crystals

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grown at the same concentration of NaCl as used in experiments on fluorescence (150 mM), the distance between these residues in pairs is as little as approximately 5 Å.

The other case is protein binding to DNA, that is, in the protein–DNA mixture. One of the DBS residues, asparagine (Asn) 170, is a collisional quencher of two tyrosine residues, Tyr175 and Tyr211. After formation of the protein–DNA complex, the level of collisional quenching probably decreases, which additionally contributes to the increase in tyrosine fluorescence. Also, the TyrOH residue Tyr76 is included in DBS. However, breaking the hydrogen bond for Tyr76 seems improbable because then the increase in the intensity of tyrosine fluorescence would be more considerable (approximately 16 %) because of its zero FRET efficiency (Danilova et al. 2005).

Because the fluorescence of tyrosine and sometimes of tryptophan residues in protein is quite difficult to analyze, some special synthetic aromatic and polyaromatic, polyunsaturated, condensed compounds that are able to fluoresce are often used in research and are being applied as probes for research in molecular dynamics (Nytek Instruments 2004). Probes can be introduced even if the direct fluorescence of the studied substance is not registered. The fluorescent probe specifically binds or is covalently attached to a molecule of this substance. As a rule, when choosing a probe, the wavelength of its maximum absorption λ_{max} should differ from λ_{max} of the studied molecule, so all received information belongs to the probe (Cantor and Schimmel 1980).

Widely applied probes are (1) dansyl chloride and 1,5-I-AEDANS, which covalently bind to Lys and Cys residues of proteins; (2) fluorescein isothiocyanate (FITC), which covalently attaches to Lys; (3) 8-anilino-1-naphthalene sulfonate (ANS), which non-covalently binds to proteins; (4) ethenoadenosine and its derivatives, which are analogues of nucleotides and bind to both proteins and nucleic acids; (5) ethidium bromide, which non-covalently binds to nucleic acids; (6) proflavine mono-semicarbazide, which covalently joins to the 3′ end of RNA; and (7) pyrene and its derivatives, which are used in polarization measurements in large systems (Cantor and Schimmel 1980).

Some probes, for example, ANS, fluoresce only after binding with proteins, which is convenient for studying protein–protein interactions. For example, the fluorescence of ANS bound to apomyoglobin is easily noticeable. After adding heme, the fluorescence decreases because ANS is competitively replaced from the protein (Dobretsov 1987).

Also, probes are used for research into cellular membranes, namely, the determination of lipid affinity to a membrane, calculation of ligand penetration speed through a membrane, registration of ligand accumulation in cell organelles, and estimation of surface and transmembrane fields. Changes in probe fluorescence allow conclusions to be made about changes in the probe–membrane binding. The competition between a probe and a ligand for membrane-binding centers depends on the charge similarity of these molecules. After adding the ligand, the quantity of

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membrane-bound probes, which have the same charge as the added ligand, decreases and the number of oppositely charged probes increases (Dobretsov 1987).

To determine protein location in a membrane, probes serving as acceptors of tryptophan fluorescence are used. The higher the extent of protein fluorescence quenching, the closer the protein is to a membrane surface. Another method is the application of donor probes quenched by studied proteins. A universal probe is terphenyl, with an emission spectrum in the region above 300 nm. Registering the quenching speed of terphenyl fluorescence after adding the studied substance to model liposomes containing a terphenyl probe allows conclusions to be made about the speed of substance penetration through a membrane (Dobretsov 1987).

The use of membrane probes in medical research is of great value. With the help of ANS and pyrene, it was possible to demonstrate considerable structural damage to cellular membranes and changes in membrane potential at hypertensive disease, experimental hyperthyrosis and myocarditis, and chemical carcinogenesis, as well as structural damage to liver microsomes in cases of vitamin A deficiency (see review by Ivanova and Kirpichenok 2008, and the literature cited within it). In other experiments, the application of tetracycline as a probe demonstrated abnormalities of calcium transport through membranes of lymphocytes in patients with bronchial asthma and other pulmonary diseases.

The possibility of using a fluorescent probe to study Alzheimer's disease and malignant neoplasms was also shown, and fluorescence tests were developed for diagnosis of myocardial infarction, unstable stenocardia, hypertensive cardiopsychoneurosis, chronic alcoholism, and liver disease; an early prognosis of peritonitis development; an assessment of the severity and prognosis of acute pancreatitis; and identification of allergens (Ivanova and Kirpichenok 2008).

Fluorescent probes have been intensively developed in the Laboratory of Biophysical Methods of Diagnostics of the Department of Biophysics, the Scientific Research Institute of Physical and Chemical Medicine (PSBI SRI PhChM FMBA of Russia, Moscow). The main achievements of this laboratory are presented on the website http://niifhm.ru/nauchnye-issledovanija/otdel-biofiziki/laboratorija-biofizicheskih-metodov-diagnostiki/.

Thus, in this section we have described the possibility of a wider range of fluorescence analysis applications than was presented in the introduction to this book (Chap. 1).

2.2 Theory of the Energetic Basis of the Resistance of Green Cells to Abiotic Environmental Factors

Another type of analytical approach is applied when using a new variant of fluorescence analysis, namely, pulse-amplitude modulated fluorescence (PAMF). The method gained especially wide application in ecological and physiological research in cases of intravital studies (including in situ) of plant tissues of *Procaryota* and *Eucaryota*.

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The research into plant resistance to the influence of environmental stress factors of anthropogenous and natural origin is of paramount importance for Russia because the combination of the most diverse set of climatic zones, features of soil covering, and the complex ecological factors of negative targeting have created a danger to the harvest of cultivated plants and to the capability of forests, water pools, and the ocean to renew oxygen stocks in the atmosphere.

To understand the adaptation or failure mechanisms, there is a need to consider the set of in-depth mechanisms forming the basis of green cell adaptive reactions to the action of extreme factors of the environment (EFEs). Speaking of tolerance stability, it is necessary to remember and to accurately differentiate between enzymatic–biochemical reactions and energetic reactions in chloroplasts, protoplasma and their organelles, separate organs of plants, and plant productivity in toto. Only by performing such investigations based on modern techniques and theoretical constructions is it possible to create a general picture of the resistance of a plant organism to the influence of EFEs (Chen and Edelhock 1976; Bilger et al. 2001).

Development of modern molecular spectroscopy methods was interconnected with successes in improving the hardware base of electrooptical instrumentation and, by the end of the twentieth century, allowed plant research in vivo, often with contactless techniques, without interruption of the physiological processes inherent in plant cells. The possibility of this type of research is substantially caused by the existence of chlorophyll *a*, the presence of which unites autotrophic organisms such as *Cyanophyceae* in *Procaryota* with the *Eucaryota* kingdom.

Research on a number of barley mutants lacking chlorophyll *b*, investigations of the large variety of *Chlamydomonas* and *Scenedesmus* mutants with different light sensitivities, and studies of the cultivar variety collection in the N.I. Vavilov Institute of Plant Industry have laid the foundation for ideas on the localization of the damaging effect of environmental factors in the electron transport chain (ETC) of the photosynthetic apparatus. The independence of quantitative variability of a pigment spectrum on the plant manifestation of destruction reactions was shown. As a result, it was hypothesized that there exist centers in eukaryotic chloroplast thylakoids in which the influence of EFEs is localized and the activity of which determines the degree of leaf stability against the action of EFEs. These centers coincide with the reaction centers (RCs) of photosystems and with centers of pigment biosynthesis (Saakov 1971a, b; Baranov et al. 1974–1976; Saakov et al. 1975). Change in F shows to functional damage to the state of reaction centers of PSII (Saakov 1971a, b).

Imbalance of the sequence of ETC reactions leads to the impossibility of capturing light energy from the antenna chlorophyll in RCs; oxidized P_{680}^{+} does not catch energy and the RCs are closed and characterized by the maximum yield of fluorescence. Research carried out in subsequent years promoted an accumulation of facts in favor of the suggested hypothesis (Saakov 1987, 1990; Saakov and Leontjev 1988; Saakov et al. 1993).

Ideas developed by us found support and were expanded by works that studied fluorescence changes in response to a broad set of damaging effects of

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environmental factors. Success in this direction was connected with introduction of the method of PAM fluorescence registration (Saakov et al. 1993; Saakov 1993a–d, 1998a, b; Lichtenthaler and Rinderle 1988; Rubin 2000a) and was published in a number of works in specialized monographic and journal editions. Experimental data from the past 15–25 years (Saakov 1987, 1990; Saakov and Leontjev 1988; Saakov and Barashkova 1999; Saakov and Shiryaev 2000) have supported and developed our concepts. The conclusions of Russian and European researchers on the response of the photosynthetic ETC to various influences gave confidence in the correctness of the chosen direction (Lichtenthaler and Rinderle 1988; Saakov and Barashkova 1999; Saakov and Shiryaev 2000; Saakov 2000a–e). It should be noted that studying the action of an EFE is closely connected with the manifestation of its aftereffect because assessment of the aftereffect is conveniently related to a prognosis of the development of adaptive or lethal situations when a green leaf performs the phototrophic function.

The midday depression of intensity of carbon dioxide fixation by green cells under conditions of increased temperature reduces plant productivity not only in arid zones but also under conditions of moderate climate areas in a hot season. Along with structural changes in the photosynthetic apparatus, significant damage to chloroplast functional activity is registered.

The range of temperatures at which photosynthesis breaks down was identified by us (Semikhatova and Saakov 1962) and later confirmed in European research (Armond et al. 1978; Weis 1981a, b; Havaux 1988; Heber and Santarius 1973; Fork et al. 1985; Gounaris et al. 1983; Monson et al. 1982; Snel and van Kooten 1990). The boundary temperature, T^0, was +35 °C, at which significant suppression of CO_2 assimilation was observed. The effect of temperatures of 39–41 °C was a 60–80 % decrease in photosynthesis from control values. After exposure to +41 °C, the reparation of photosynthesis intensity in 17–19 h back to 70 % of the initial level (Semikhatova and Saakov 1962) was possible. Higher temperatures (42 °C) resulted in irreversible failure of photosynthesis.

Years later, in 1999, the work of Bungard et al. (1999) was published. These authors found the range of temperatures for which the reversible recovery of the photosynthesis process can take place. Their range exactly corresponds to those temperatures about which we wrote in 1962, 37 years before. This fact serves as example that sometimes scientific information can't be easily found or accessed. It's regrettable, because, more precisely, in 1958, these data were reported at a master's thesis defense (Saakov 1959) at the Leningrad State University, and the usual delay with publications in Russia allowed us to print our material only in 1962 (Semikhatova and Saakov 1962).

Acclimatization or adaptation to the influence of temperature means an increase in the reliability of the interaction system between light-harvesting complexes of pigments and the RCs of photosystems (Armond et al. 1978) because the dissociation of complexes induced by elevated temperatures manifests as a decrease in electron

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transport and change in energy distribution between photosystems PS-1 and PS-2 (Weis 1981a, b). The registered decrease in electron transport in PS-2 is noted at +35 °C. Photophosphorylation becomes unstable and is progressively inhibited from +35 °C (Monson et al. 1982). Thus, functional damage to CO_2 assimilation and the simultaneous decrease in the electron transfer speed in PS-2 at incubatory temperatures above +35 °C are not in doubt, but Bungard et al. (1999) do not mention it.

In the process of creating theoretical constructions, we carried out a comparative study of the character of signal harmonic kinetics (PAMF) under the influence of γ-radiation, various temperatures, drought, fumigation, and herbicides, hoping to find answers about the specificity or non-specificity of the response of the ETC photosynthetic apparatus to the chosen EFE and to limit questions regarding the possible localization of these influences.

Since the end of the 1980s, researchers have been registering stationary (F_0), variable (F_V), and maximum (F_m) fluorescence with the help of the pulse fluorometer Walz 101-103 (Effeltrich, Germany). The developments by Prof. Schreiber and the device manufactured by Walz brought about a methodological revolution in scientific research. The convenience of the method consisted in the speed of obtaining results about the changes in the main measured parameters directly from an intact leaf and the possibility of working not in darkness but in light. The properties of this method were tested in details and checked in many laboratories in the USA and Europe. The calculation of photochemical (q_q), non-photochemical (q_E), and other coefficients of fluorescence quenching and the interpretation of their changes were carried out using the accepted technique (see Schreiber and Neubauer 1990 and the special issue of *Photosynthesis Research* edited by van Kooten and Snel 1990a).

An example of experimental results on research into PAMF kinetics is presented in Fig. 2.3, and calculations of fluorescence coefficients are shown in Table 2.2. The level of F_0 arising after the dark period of leaf adaption, which is preceding and necessary for the experiment, when photosynthetic membranes are in the non-energized state and all RCs of PS-2 are "open," is caused by the radiation of excited chlorophyll *a* molecules. This takes place before the energy migration into RCs and when the first stable acceptor of PS-2, Q_A^+, is completely oxidized; at that point, the coefficients $q_q = 1$ and $q_E = 0$. F_0 is determined when the measured light flux of approximately 0.1 μE/(m^2 s) is switched on. From the data in Fig. 2.3, it follows that the F_0 value (F_0^t is the stationary fluorescence in any interval of time) increases with action of EFEs, which causes a change in the state of PS-2 pigments and leads to photoinhibition (PhIn). The data obtained over many years show that the influence of ionizing radiation, temperature, dehydration, and herbicides significantly changes the F_0 level after light induction.

In the experiment, after switching on the light impulse (3,000−3,500 μE/(m^2 s)) or the actinic light flow, the maximum fluorescence, F_m, increases, and its first increase (the variable fluorescence F_V) corresponds to the reduction $Q_A^+ \rightarrow Q_A^-$ and results in closing of RCs in PS-2. The F_V level shows that all non-photochemical extinguishing processes are minimal and that both q_q and q_E are equal to zero. The F_V value (including values of variable fluorescence F_V^t at any

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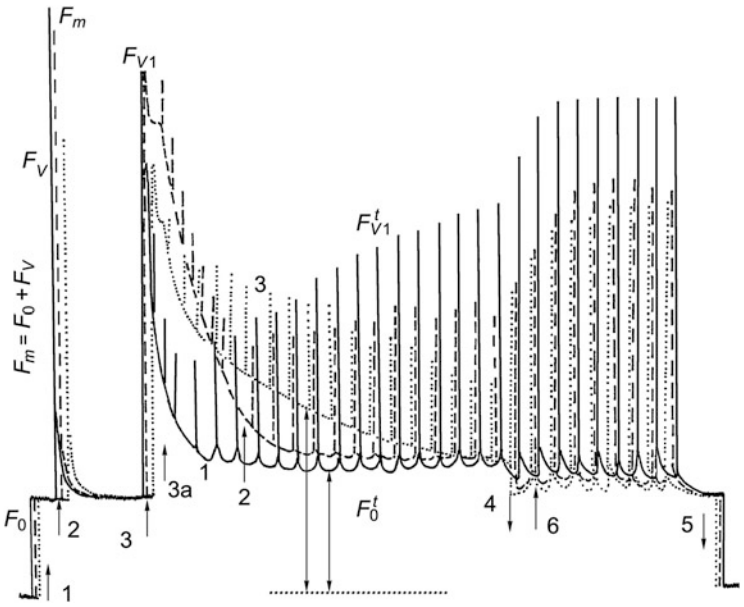

Fig. 2.3 The character of signal harmonics change in PAMF of a *Nicotiana tabacum* leaf in response to dehydration. *Curves*: *1* control; *2* experiment: dehydration to 45 % of the initial state; *3* dehydration to 30 % of the initial state. *Arrows*: *1* and *5* indicate, respectively, the input and shutdown of the modulating light flux of frequency of 1.6 kHz (5 μE/(m^2 s), $\lambda < 670$ nm); *2* input of the saturating 1 s impulse of white light (2,500 μE/(m^2 s)) for finding of F_m and F_V values; *3* and *4* input and shutdown, respectively, of actinic light (1,200 μE/(m^2 s)); *3a* input, 30 s later, of 1 s impulses of saturating light against the background of the actinic light for determination of F^t_{V1} values; *6* input of 1 s impulses of saturating light after shutdown of the actinic light (Saakov 2003a, b; Saakov et al. 2010)

time *t*, called "spikes" in the English literature) defined by a low value of coefficient q_E emphasizes the active utilization of energy in the Calvin cycle and a simultaneous decrease in the membrane proton gradient caused by ATP formation. The suppression of PS-2 activity defined by F_V suppression is a result of inhibition of the charge transfer between P_{680} and pheophytin (Pheo), that is, a result of damage localized in PS-2 RCs, which corresponds to earlier-stated ideas.

Because the open RC gets energy from antenna chlorophyll, oxidized Q_A^+ acts as the quencher of fluorescence; so in the state $P^*_{680}Q_A$, the photochemical transformation of energy ($P^+_{680}Q_A^-$ formation) is a convenient way for de-excitation because the presence of Q_A^- P^* cannot transfer an electron (e^-) to it and the probability of Fl emission from the state $P^+_{680}Q_A^-$ is increased, that is, the RC possesses a high quantum yield of Fl. The decrease in F_V yield is explained by manifestation of the photoinhibition effect. With an increase in influence intensity and aftereffect of EFEs, the F_V/F_0 ratio decreases, which is one more substantial criterion speaking for the damage in PS-2 of ETC and is explained by reduction of the $P^+_{680}PheoQ_A^-$ link. The e^- transport in PS-2 RCs starts with charge separation between P_{680} in the first excited singlet state and pheophytin and is expressed in the

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Table 2.2 The character of changes in the coefficients of modulated fluorescence quenching under the influence of low temperatures[a]

Object	Variant of experiment	Measured coefficient (average of $n = 4$, $p \leq 0.08$)											
		F_V/F_m	$F_{V\ exp}/F_{V\ cont}$	Level of PS-2 reduction (%)	F_{max}/F_0	F_V/F_0	F_{V1}/F_V	F_{V1}/F_0	F_0^5/F_0	$q_E^{5\,min}$	$q_q^{5\,min}$	Spikes 5 min (mm)	Spikes 6 min (mm)
Robinia pseudoacacia	Control	0.832	–	93.9	4.34	3.60	0.89	3.2	1.11	0.30	0.94	92	–
	(−10 °C) 2 min	0.652	0.462	43	2.57	1.69	0.814	1.37	1.62	0.27	0.43	21	–
Taxus baccata	Control	0.801	–	91	4.8	3.79	0.93	4.1	1.21	0.32	0.917	68	79
	(−15 °C) 15 min	0.66	0.49	38	3.15	1.79	0.81	1.62	1.67	0.73	0.375	22	36
Fraxinus ornus	Control	0.806	–	92.4	5.17	4.17	0.89	3.71	1.114	0.472	0.92	72	83
	(−13 °C) 13 min	0.736	0.517	52	3.1	2.28	0.854	1.95	1.51	0.801	0.48	26	32
Fagus sylvatica	Control	0.829	–	94.6	5.04	4.05	0.92	3.97	1.13	0.33	0.95	71	80
	(−14 °C) 10 min	0.723	0.64	53	3.12	2.3	0.83	1.9	1.54	0.47	0.46	26	33
Hedera taurica	Control	0.825	–	88	5.6	4.65	0.92	4.28	1.29	0.42	0.89	67	70
	(−12 °C) 20 min	0.694	0.51	32	3.86	2.33	0.86	2.02	1.83	0.72	0.32	12	27
Laurus nobilis	Control	0.817	–	89	5.1	3.83	0.91	3.81	1.22	0.38	0.91	88	93
	(~10 °C) 20 min	0.754	0.488	39	3.21	1.81	0.8	1.54	1.62	0.66	0.39	29	34

[a]The calculation of coefficients F_0^5, $q_E^{5\,min}$, $q_q^{5\,min}$ and values of spike 5 min were performed 5 min after switching the actinic light off; spike 6 min: 6 min after switching the actinic light off

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e^- transfer from the secondary donor Z to the primary and secondary acceptors Q_A and Q_B of the quinone type, as shown in Eq. (2.1):

$$e^{-\underline{h\nu}} \rightarrow ZP_{680}PheoQ_AQ_B\underline{^1} \rightarrow ZP^*{}_{680}PheoQ_AQ_B\underline{^2} \rightarrow ZP^+{}_{680}Pheo^-Q_AQ_B\underline{^3} \rightarrow ZP^+{}_{680}Pheo^-Q_A{}^-Q_B \rightarrow PQ^{2-} \quad (2.1)$$

When PhIn is induced by intense light, damage to e^- transport at stages 2 and 3 occurs and is caused by the disorder of charge separation between $P^*{}_{680}$ and Pheo. In the state $P_{680}Q_A{}^-$, there is no photochemical transformation of energy. The intermediate Pheo acts as the mediator between P_{680} and Q_A and promotes the primary charge separation (forming $P^+{}_{680}Pheo^-Q_A{}^-$). It is assumed that F_V should be "recombinant luminescence" during the transition $P^+{}_{680}Pheo^-Q_A{}^- \rightarrow P^*{}_{680}PheoQ_A{}^-$. Consequently, the decrease in F_V value directly indicates the suppression of PS-2 activity and the damage of e^- transport from P_{680} to $Q_A{}^+$ because of inhibition of charge migration between P_{680} and Pheo. The same follows from observation of the absorption signal at $\lambda = 540-560$ nm.

The ratio F_V/F_m is an indicator of the high efficiency of primary reactions in PS-2, and for intact chloroplasts, it is close to 0.832. Deviation of the experiment value from the control by 0.03−0.04 relative units is considered significant and testifies to the PS-2 response to the influence of external stress factors. A decrease in this ratio emphasizes the existence of a negative reaction of the photosynthetic apparatus to the action of EFEs, which is connected with the PhIn effect (illustrated by the necessary evidence as literature data). The transition from PhIn to the reversible restoration of ETC activity directly correlates with an increase in the ratio F_V/F_m. A change in F_V yield is also caused by energy transformation in PS-2 and indicates the presence of minimal non-photochemical processes. As can be seen in Fig. 2.3, the damaging influence of environmental factors on leaves usually reduces the F_V yield, which speaks for the existence of primary damage and its localization near the PS-2 RCs. A decrease in the ratio of $F_{V\ experiment}$ to $F_{V\ control}$ (Table 2.2) also emphasizes the suppression of PS-2 activity in the experiment and corresponds to the earlier-stated proposition (Baranov et al. 1974, 1975; Saakov et al. 1975; Saakov 1987, 1990; Saakov and Leontjev 1988). A high F_V value (including the F_V^t value) is connected to a low value of q_E and high utilization of the energy in the Calvin cycle, correlating with a decrease in the membrane proton gradient due to ATP formation.

After F_V decrease (3 min), the actinic light flux (180−1,100 μE/(m^2 s)) was switched on and induced the F_{V1} level; after 30 s a number of saturating impulses (F_V^t) of 1 s duration were also switched on (Fig. 2.3). These impulses together with actinic light flux promoted complete reduction of the $Q_A{}^-$ acceptor and RC closing. The higher the F_V^t amplitude and the closer it is to the F_{V1} level, the larger the probability of $Q_A{}^-$ pool reduction, of high ETC activity, of a decrease in q_E value, and activation of the Calvin cycle. It is especially necessary to emphasize the very similar levels of F_V^t under the influence of temperature and γ-radiation. Because the q_E value correlates with energizing of the thylakoid membrane, that is, with a light-excited proton gradient, it will be higher for a higher proton gradient and lower ATP

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synthesis. The influence an EFE on a leaf results in an increase in q_E value, together with a decrease in the yield of F_V, F_{V1}, and $F_V{}^t$ (Fig. 2.3), which proves the existence of primary damage and its localization near PS-2 RCs.

It is possible to speak about the presence of a functional link between the activities of PS-2 and other components of the photosynthetic ETC with a certain degree of confidence; the changes in F_V, F_{V1}, $F_V{}^t$, and F_m^t show the activity of the photosynthesis process as a whole. This supports the idea that increased temperature leads to an electron transport decrease at saturating light flux intensities. The damage to energy transmission between PS-2 and PS-1 is the primary event correlating with electron transport inhibition, termination of photophosphorylation, suppression of reducer formation, and suppression of photosynthesis intensity. The influence or aftereffect of an EFE reduces F_V/F_m for all investigated objects of different resistance degree, depending on the depth of the damage and efficiency of adaptive reactions at F_V quenching (Saakov 1971a, b, 1987, 1990, 1993a–d, 1998a, b, 2000a–e, 2001a, b; Baranov et al. 1974–1976; Saakov et al. 1975, 1993; Saakov and Leontjev 1988; Saakov and Baranov 1987; Saakov and Barashkova 1999; Saakov and Shiryaev 2000; van Kooten and Snel 1990a).

We have described the essence of the technique in detail to acquaint the reader from the outset with the principles of the analysis and methods of calculating results. Furthermore, in the course of describing experiments, we will repeatedly but briefly backtrack to features of obtaining and interpreting experimental data; the reader should not consider this to be distrust concerning their understanding of the research.

2.2.1 *Influence of Negative Temperatures on the Kinetics of Pulse-Amplitude Modulated Fluorescence Parameters (F_0, F_m, F_V)*

In Russia, plant cell tolerance to the influence of environmental stress factors of anthropogenous and natural origin is a problem of paramount importance because a combination of the very diverse set of climatic zones, soil features, and climatic and technogenic factors of the negative orientation create a constant threat to cultivated and wild plants. To understand the processes of plant green cell tolerance and adaptation, there have been numerous studies of the deep mechanisms at the heart of green cell adaptive reactions to the influence of EFEs.

It is necessary to emphasize and remind that, in speaking of (plant resistance) tolerance and adaptation, it is necessary to (1) differentiate very accurately and carefully between the *energy reactions* and *fermentative–biochemical reactions* of metabolism in chloroplasts; (2) characterize the mitochondrial energy reactions of a protoplasma and its organelles; (3) define the morphological changes in separate bodies of plants; and (4) describe the character of plant productivity as a whole, distinguishing the economic crop and the usual increase in vegetational mass.

We point out that in articles and discussions, all four of the above-listed problems often form a heterogeneous conglomerate of confused theoretical assertions, without division of the primary and secondary processes forming the basis of green cell's

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relations with environment, and this results in miscommunication (Schindler and Lichtenthaler 1990) because of uncoordinated positions and confused terminology.

Any theoretical constructions in science that are not characterized by strict mathematical formalization need much and many-sided experimental evidence. From this point of view and due to the attractive universality, the concept of the energetic basis of the *Procaryota* and *Eucaryota* green cell's resistance (Saakov 2000a–e) also needs its evidential value strengthening, especially when considering the influence of negative temperatures on photosynthesizing cells. This problem is important in Russia in connection with spring and autumn morning frosts, when the short-term impact of negative temperature on vegetational bodies and leaves can lead to the death of plants.

The definite answer, which would exclude the possibility of contradictory interpretation of the influence of negative and low positive temperatures on the yield character of signal harmonics of PAMF, was not obtained in vivo (Krause and Somersalo 1989: Schindler and Lichtenthaler 1990; Snel and van Kooten 1990). From this point of view, we began to analyze a number of herbaceous and woody plants, investigating the response of their photosynthetic device to a short influence of negative temperatures in the range −7 to −18 °C, which can be inherent in morning frosts. The work was carried out under late autumn conditions in regions of the Schwarzwald and southern Germany, when in nature and in a botanical garden there were enough objects with unfallen foliage.

Great success in studying the response of green cells of higher and inferior plants to EFE influence became possible due to the development of the pulse-amplitude modulated fluorescence method, allowing results to be obtained about the reaction of the photosynthetic device to the chosen influence type quickly and in field conditions (Schreiber and Bilger 1987; Schreiber 1986; Saakov et al. 1993). The possibility of this research is caused by the existence of chlorophyll *a*, the presence of which unites autotrophic organisms such as *Cyanophyceae* in *Procaryota* and *Vegetalia* in *Eucaryota*.

The stationary (F_0), variable (F_V), and maximum (F_m) fluorescence associated with those *functional* changes of the photosynthetic device, which take place *before structural* changes (Saakov 1993a–d), were registered with the pulse fluorometer Walz 101-103 (Effeltrich, Germany). The convenience of the method and the speed of obtaining results about changes in main measured parameters for an intact leaf were checked in detail in many laboratories of the USA and Europe. The calculation of photochemical (q_q), non-photochemical (q_E), and other coefficients of fluorescence quenching and the interpretation of their changes were carried out with the help of the accepted technique (Saakov 2000a–e; Schreiber and Bilger 1987; Schreiber et al. 1997a; Saakov et al. 1993). Separation of fluorescence from the much stronger excitation light is attained by the application of primary optical filters transmitting the excitation light ($\lambda < 670$ nm) and of secondary filters transmitting the long-wave spectral area ($\lambda > 680$ nm) and protecting the photodetector from parasitic diffused light from the excitation source. Owing to the design features, detectors of the device are insensitive to external light and are recommended for field ecological research because they allow efficient comparison of the influence of many anthropogenous and natural EFEs using only one

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characteristic sign. In addition, the received results are easily digitalized and easily entered into information computer systems for further processing.

Objects of research different in tolerance were chosen: leaves of haricot *Phaseolus vulgaris*, tobacco *Nicotiana tabacum*, dandelion *Taraxacum officinale*, acacias *Robinia pseudoacacia*, nettles *Urtica dioica*, ivy *Hedera taurica*, Chinese sumac *Rhus succedanea*, beech *Fagus sylvatica*, yew *Taxus baccata*, monastery *Laurus nobilis*, ash tree *Fraxinus ornus*, etc.

The results of experiments on kinetics of signal harmonic change and the change in corresponding coefficients of fluorescence quenching under the influence of negative temperatures demonstrated functional damage of the photosynthetic device (see Fig. 2.4a–d and Table 2.2). The F_0 level arising after the dark period of leaf adaptation, when photosynthetic membranes are in the non-energized state and all PS-2 RCs are open, is caused by radiation of excited chlorophyll *a* molecules before energy migration into RCs and when the first stable acceptor in PS-2, Q_A^+, is completely oxidized; at that point, coefficients $q_q = 1$ and $q_E = 0$ (Snel and van Kooten 1990; Saakov 2000a–c; Schreiber and Bilger 1987; Schreiber et al. 1986; Saakov et al. 1993).

From the data in Fig. 2.4 and Table 2.2, it follows that the F_0 value (F_0^t is the stationary fluorescence in any interval of time under actinic light influence) significantly increases with the action of negative temperature, which causes the change in the state of PS-2 pigments and leads to photoinhibition. At more intensive influence of the negative temperature or in the aftereffect process, F_0 recession to a level below the stationary value is observed (Fig. 2.4b–d, designated by arrows with an asterisk) that, probably, is the result of the deep inhibition of Q_A reduction and accumulation of its oxidized store, caused by damage of the e^- transport to Q_B. The character of base fluorescence kinetics changes under the influence of negative temperatures after induction with actinic light (Fig. 2.4a–d) and is similar to that seen in response to the influence of ionizing radiation, dehydration, and high temperature (Saakov 2000c).

An impulse of saturating light of 2,500−3,000 μE/(m^2 s) induces the maximum fluorescence, that is, F_m increases and its first increase, the variable fluorescence (F_V), corresponds to the reduction $Q_A^+ \rightarrow Q_A^-$ and results in closing of RCs in PS-2. Imbalance in the sequence of ETC reactions results in the impossibility of light energy capture from the antenna chlorophyll in RCs. Oxidized P_{680}^+ does not catch energy, and the RC is closed and is characterized by the maximum fluorescence yield. Decrease in the F_V yield is connected with manifestation of the photoinhibition effect. With an increase in intensity of the EFE and its aftereffect, the F_V/F_0 ratio decreases, which is one more substantial criterion speaking for damage in the PS-2 ETC and explained by reduction of the $P^+_{680}PheoQ_A^-$ link. The e^- transport in PS-2 RCs starts with charge separation between P_{680} in the first excited singlet state and pheophytin and is expressed in e^- transfer from the secondary donor Z to primary and secondary acceptors Q_A and Q_B of the quinone type. This is shown in Eq. (2.1), to which we will repeatedly return:

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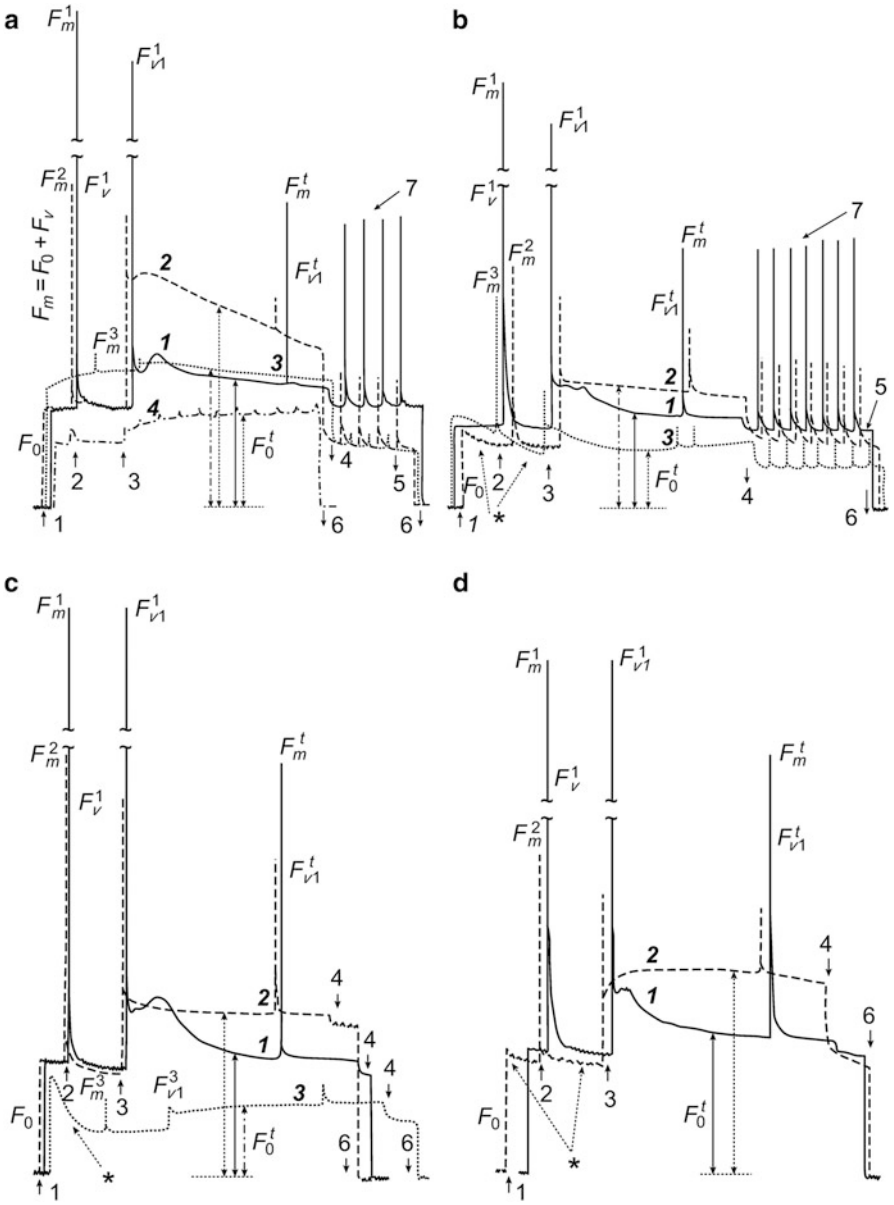

Fig. 2.4 Character of signal harmonics change in PAMF under the influence of negative temperatures on leaves of (**a**) *Hedera taurica* (−12 °C), 20 min; (**b**) *Fraxinus ornus* (−13 °C), 13 min; (**c**) *Fagus sylvatica* (−14 °C), 10 min; (**d**) *Robinia pseudoacacia* (−10 °C), 2 min. *Curves*: *1* control; *2* experiment; *3* aftereffect of the negative temperature in 2 h; *4* the same, but after a day. The F_0 level decreased almost to the initial value and rudiments of spikes appeared. *Arrows*: *1* input and *6* shutdown of the modulating light flux of frequency of 1.6 kHz (5−6 μE/(m^2 s), $\lambda < 670$ nm) for determination of the dark base fluorescence F_0; *2* input of the saturating impulse of the white light for determination of the F_m level; *3*, *4* input and shutdown of the actinic light (1,500 μE/(m^2 s)); *5* shutdown of impulses of saturating light; *7* the spikes after shutdown of the actinic light. *Arrows* marked with an *asterisk* point to F_0 decrease right after shutdown of the modulating light flux of 1.6 kHz, which confirms the damage to the antenna chlorophyll *a* pool

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$$e^{-\underline{h\nu}} \rightarrow ZP_{680}PheoQ_AQ_B\underline{^1} \rightarrow ZP^*{}_{680}PheoQ_AQ_B\underline{^2} \rightarrow ZP^+{}_{680}Pheo^-Q_AQ_B\underline{^3}$$
$$\rightarrow ZP^+{}_{680}PheoQ_A{}^-Q_B \rightarrow PQ^{2-}$$

When PhIn is induced by intense light, damage to e^- transport at stages 2 and 3 is found and caused by the disorder of charge separation between $P^*{}_{680}$ and Pheo. In state $P_{680}Q_A{}^-$ there is no photochemical transformation of energy. The intermediate Pheo acts as the mediator between P_{680} and Q_A and promotes primary charge separation (forming $P^+{}_{680}Pheo^-Q_A{}^-$). It is assumed that F_V should be "recombinant luminescence" during the transition $P^+{}_{680}Pheo^-Q_A{}^+ \rightarrow P^+{}_{680}Pheo^-Q_A{}^- \rightarrow P^*{}_{680}PheoQ_A{}^-$. Consequently, the F_V value decrease shown in Fig. 2.4 directly indicates the suppression of PS-2 activity and damage of e^- transport from P_{680} to $Q_A{}^+$ because of inhibition of the charge migration between P_{680} and Pheo and retardation of the transformation of light energy into the energy of ATP chemical bonds, that is, there is disorder of the phototrophic function in RCs of PS-2, which corresponds to earlier-stated conceptions (Saakov 1976, 1987, 2000a–e; Saakov and Shiryaev 2000).

The ratio F_V/F_m is an indicator of the high efficiency of PS-2 primary reactions, and for intact chloroplasts, it is close to 0.832. The deviation of the experimental value from the control by 0.03−0.04 relative units is considered to be significant and testifies to the PS-2 response to the influence of external stress factors (Schreiber and Bilger 1993, 1987; Schreiber et al. 1994, 1997a, b). The transition from PhIn to the reversible reduction of ETC activity directly correlates with an increase in F_V/F_m (Table 2.2) and indicates the high efficiency of charge separation (Genty et al. 1989). A decrease in the ratio $F_{V\ experiment}/F_{V\ control}$ (Table 2.2) also emphasizes the suppression of PS-2 activity in experiment, which corresponds to the earlier-stated proposition on localization of the EFE damaging influence (Saakov 1976, 1987, 2000c; Saakov and Shiryaev 2000). A high F_V value (including the F_V^t value, designated in English-language literature as "spikes"; Fig. 2.4, Table 2.2) is connected to a low q_E value. The high utilization of energy in the Calvin cycle correlates with a decrease in the membrane proton gradient due to ATP formation. The higher the F_{V1}^t amplitude and the closer it is to F_{V1}, the larger is the probability of a more complete $Q_A{}^-$ pool reduction, of high ETC activity, of a q_E value decrease, and Calvin cycle activation. It is especially necessary to emphasize the very similar levels of F_V^t under the influence of negative temperatures and other EFEs (Saakov 2000c; Saakov and Shiryaev 2000). Because the q_E value correlates with the thylakoid membrane energizing (i.e., with the light-excited proton gradient), it will be higher for a higher proton gradient and lower ATP synthesis. The low level of PS-2 reduction unambiguously indicates its damage (Table 2.2). The influence of an EFE on a leaf results in an increase in q_E value, together with a decrease in the yield of F_V, F_{V1}, and $F_V{}^t$ (Fig. 2.4), which proves the existence of primary damage and its localization near to PS-2 RCs. The q_E relaxation is accompanied by the simultaneous activation of the Calvin cycle, coupled with ATP usage and a proton gradient decrease. The quick decrease in q_q in the first minutes of light exposure occurs due to ETC reduction. The change in

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this coefficient by 0.03−0.04 relative units is significant and indicates the response of the photosynthetic device (Schreiber and Bilger 1993, 1987; Schreiber et al. 1997a, b). In the stationary state, q_q is usually approximately 0.8−0.9 relative units. When $q_q = 0$, there is no light energy transformation, and at $q_q = 1$, it is optimal. The increase in q_q takes place because of *re-oxidation* of Q_A^-. The low level of q_q shows the accumulation of electrons in the acceptor part of PS-2 and shows the high level of Q_A^- reduction and of the PQ^{2-} pool, that is, ETC rupture (Table 2.2, experimental variants). The high q_q value for well-illuminated plants indicates high levels of electron transport, $NADPH/H^+$, and intensity of photosynthesis (Table 2.2, controls). With the damaging influence of negative temperatures, q_q decreases, emphasizing suppression of the ETC activity, increase in the proton current, and e^- accumulation in the acceptor part of PS-2.

For a long time, there have been ambiguous ideas concerning the influence of low temperatures and frosts on the character of PAMF kinetics change (Snel and van Kooten 1990; Schreiber and Bilger 1987). Data presented in Fig. 2.4 and in Table 2.2, and also the results of our previous works (Saakov 2000a, b; Saakov and Shiryaev 2000), give grounds to suggest the similarity of damage mechanisms in ETC links in the region $P^+_{680}Pheo^-Q_A^- \rightarrow P^*_{680}\ PheoQ_A^-Q_B$ under the impact of negative temperatures and other EFEs of natural and technogenic origin. It allows us to reliably eliminate from discussion the statement on the exclusive PAMF invariance with the action of low temperatures and frosts, and to remove the problem of EFE damage specificity for separate components of PS-2 RCs. Serious support to our concepts was given by a work (Bukhov et al. 2001a) in which, with a method similar to ours, similar data were obtained on the change in variable fluorescence in spinach (*Spinacia oleracea*) under the influence of a scale of low positive and negative temperatures. There was a difference between our results and those of Bukhov et al. (2001a) only in the kinetics of the base, dark fluorescence values, which remained at the initial dark level with small fluctuations, whereas we observed F_0 changes under the influence of actinic light. Probably there were also distinctions in experimental conditions because in the work of Bukhov and colleagues, measurements of Fl kinetics were carried out against a background of temperatures (duration of thermal exposure was not specified) in the presence of the herbicide diuron, which impeded Q_A^+ re-oxidization by the secondary acceptor Q_B. At the same time, rather numerous measurements performed in different laboratories (Snel and van Kooten 1990; Schreiber and Bilger 1987; Schreiber et al. 1997a, b) emphasized the high lability of the F_0^t level under EFE influence of natural and anthropogenous origin. The correct explanation for the found discrepancies demands additional experimental support.

Thus, in the described work, after investigation of a large variety of plant material, reliable data were obtained on the change in PAMF kinetics under the influence of negative temperatures. They can be taken as the basis for the diagnostics of green cell resistance to the impact of an extreme factor. The presented material gives an additional proof of rightness of the stated reasons concerning the primacy of the energetic basis for the resistance of *Procaryota* and *Eucaryota* green cells to extreme environmental effects and also about localization of the

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damage induced by negative temperatures in PS-2 RCs (Saakov 2000a–e). The data presented can, to a certain degree, serve as the methodological management for works on selection of cultivars of cultivated plants tolerant to negative temperatures by analyzing the stability of their photosynthetic device, especially when using genetic engineering approaches.

2.2.2 *Association of High Temperature Stress to the Signal Harmonics Change in Pulse-Amplitude Modulated Fluorescence (F_0, F_m, F_V)*

To discover the possible localization of thermal influence in the system of interactions between photosystems and ATP synthesis (Weis 1981a, b), we carried out research on herbaceous and woody plants that were different in resistance to the influence of physiologically high temperatures. Our task was the profound application of the PAMF method for demonstration in vivo of the localization of stress-induced damage in a link of the ETC of photosystems. It should be noted that the great sensitivity of isolated preparations of PS-2 to EFEs is beyond doubt. It is necessary to emphasize that early experiments assessing the stability of photosystems were not performed in vivo (i.e., with a leaf) but with fragmentary preparations isolated as a result of a differential centrifugation of a chloroplast suspension.

We relied on earlier research (Semikhatova and Saakov 1962) and investigated the character of signal harmonic change in PAMF in the range of temperatures +35 to +45 °C. The registration of parameters F_0, F_m, and F_V was carried out using the fluorometer Walz 101-103 (Effeltrich, Germany) (van Kooten and Snel 1990a; Schreiber et al. 1997). The method was proven in leading laboratories of Europe and the USA as accurate and fast for obtaining results about changes in the key measured parameters of an intact leaf or of suspensions of chloroplasts or algae. The calculation of photochemical (q_q), non-photochemical (q_E), and other coefficients of fluorescence quenching and the interpretation of their changes were performed using the accepted technique (van Kooten and Snel 1990b; Schreiber et al. 1997a, b). Detectors of the analytical device are insensitive to external light, and therefore, the device is recommended for field ecological research because it allows comparison of the influence of many technogenic and natural EFEs after analysis of only one characteristic feature. Obtained results are easily digitalized, which provides their easy input into computer systems for further processing. We performed experiments in Europe, because at that time there were stagnation and disorganization of this scientific area in Russia.

Objects of research were chosen to simultaneously compare leaves resistant and intolerant to high temperatures: the cultivar Saratovskaya 29 of wheat (*Triticum vulgare*), known for its resistance to drought and thermal influence; haricot *Phaseolus vulgaris*; tobacco *Nicotiana tabacum*; acacia *Robinia pseudoacacia*;

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nettle *Urtica dioica*; pea *Pisum sativum*; bay laurel *Laurus nobilis*; ash *Fraxinus ornus*; clover *Trifolium* sp.; oak *Quercus robur*; etc.

In Figs. 2.5a–c and 2.6 and in Table 2.3, the block of data on features of PAMF change and coefficients of fluorescence suppression under the influence of physiologically high temperatures is presented.

The level of base dark fluorescence F_0 is characterized by the open RC state before migration of electrons into open RCs due to excited chlorophyll *a* emission and further reduction of the first stable acceptor of PS-2 $Q_A^+ Q_A^-$; at this time, thylakoid membranes of the leaves adapted to darkness are in a non-energized state.

The initial saturating light impulse of 1 s duration and intensity of 2,500–3,000 μE/(m^2 s) (Figs. 2.5 and 2.6) leads to an increase in maximum fluorescence (F_m). Its first increase, the variable fluorescence (F_V), corresponds to the level of Q_A^- reduction (Table 2.3) and is accompanied by RC closing. The change in F_V yield is caused by energy transformation in PS-2 and points to minimal non-photochemical processes (Snel and van Kooten 1990; Schreiber et al. 1997a, b). The intermediate Pheo works as the mediator between P_{680} and Q_A and promotes the primary charge separation (forming $P^+_{680}Pheo^- Q_A^-$). It is assumed that F_V should be a "recombinant luminescence" during the transition $P^+_{680}Pheo^- Q_A^- \; P^*_{680}PheoQ_A^-$. From this, the decrease in F_V directly indicates the suppression of functional activity of RCs of PS-2 and the damage of e^- transport from P_{680} to Q_A^+, coupled with the inhibition of charge migration between P_{680} and Pheo. This can be explained as follows: The quantum yield of the linear electron transport depends on the concentration of the RCs of opened PS-2, on the efficiency of acceptance of excitation energy by these open centers, and on the usage of the accepted excitation energy by the PS-2 complexes.

As follows from Figs. 2.5 and 2.6, the damaging impact of high temperature on leaves usually reduces the F_V yield, that is, indicates the existence of primary damage and its localization in PS-2 RCs (Snel and van Kooten 1990; Schreiber et al. 1997a, b). The decrease in the ratio $F_{V\ experiment}/F_{V\ control}$ (Table 2.3) also emphasizes suppression of PS-2 activity in experiment. With a certain degree of confidence, it is possible to speak about the existence of a functional link between the activity of PS-2 and other photosynthetic ETC components; therefore, the changes in F_V, F_{V1}, and F_V^t are coupled to changes in the photosynthesis process activity as a whole (Fork et al. 1985; Snel and van Kooten 1990). In favor of this statement is the fact that increased temperature results in a decrease in the electron transport at saturating intensities of light flux. The damage to energy transfer between PS-2 and PS-1 is the primary event correlating with inhibition of electron transport, termination of photophosphorylation, suppression of reducer formation, and photosynthesis intensity (Armond et al. 1978; Monson et al. 1982; Saakov 2000a–e). The value of the ratio F_V/F_m is extremely important and is considered to be an indicator of photosynthetic function. It fluctuates for intact chloroplasts around 0.83 relative units (Snel and van Kooten 1990; Schreiber et al. 1997). A deviation of this coefficient from the control by 0.03–0.04 relative units significantly emphasizes the existence of a negative reaction of the photosynthesis machinery to external influences, as illustrated by the data in Table 2.3. The effect

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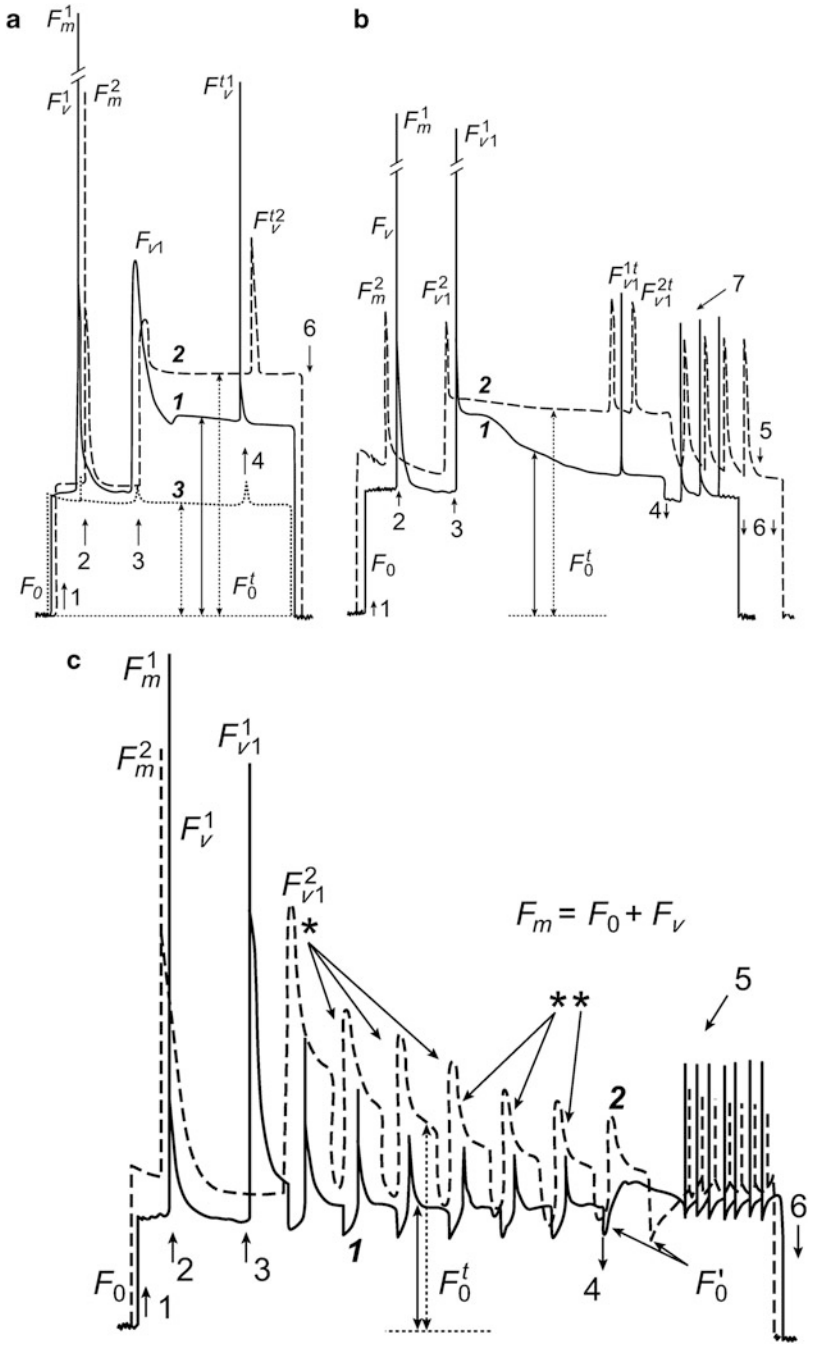

Legend to Fig. 2.5 see p.70

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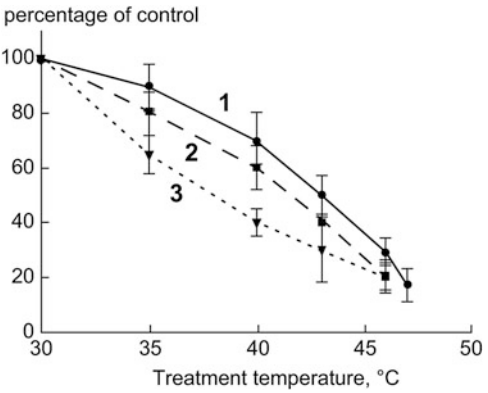

Fig. 2.6 Dynamics of the change in F_m (*curve 1*, $n = 12$), F_{V1} (*curve 2*, $n = 8$), and F'_V (*curve 3*, $n = 8$) for *Nicotiana tabacum* leaves relative to control in response to a change in the influencing temperature. *Abscissa*: temperature in degrees; *ordinate*: percentage decrease relative to control

or aftereffect (Saakov 1999, 2000a–e) of high temperature reduces F_V/F_m for all investigated objects, differing in tolerance degree, depending on the depth of damage and effect of adaptive reactions at F_V quenching.

Suppression of the RC activity in PS-2 determined from F_V suppression is linked, first of all, with inhibition of the charge transfer between P_{680} and pheophytin (Saakov 2000a, b, 2001a, b; Saakov and Shiryaev 2000), that is, with the primary change in excited energy distribution between PS-1 and PS-2 during thermal influence, which is localized in RCs of PS-2. This moment is especially emphasized because we, for the first time, connected the *functional stability* of the photosynthetic device with the *activity* of the RCs and *damageability* of their link in the area $P^+_{680}Pheo^-Q_A{}^-P^*_{680}PheoQ_A{}^-Q_B$. This idea corresponds to the conceptions stated earlier (Saakov 2000a, b, 2001a, b; Saakov and Shiryaev 2000). Moreover, the F_V value of the control (including values of variable fluorescence at any time $t = F_V{}^t$, designated in English-language literature as "spikes") and the index F'_m (Schreiber et al. 1997a, b) interrelated with the change in q_E value indicate the active utilization of energy in the Calvin cycle and simultaneous decrease in the membrane proton gradient interfaced to ATP formation (Saakov 2000c). From Table 2.3, it is seen that

Fig. 2.5 (continued) Character of signal harmonics change in PAMF during the influence of high temperature on leaves of (**a**) *Solanum lycopersicum*, (**b**) *Robinia pseudoacacia*, and (**c**) *Laurus nobilis*. *Curves*: *1* control, *2* experiment for (**a**) +42 °C, 7 min; (**b**) +43 °C, 5 min; (**c**) +45 °C, 5 min. *Arrows* for **a**–**c**: *1* and *6*, respectively, input and shutdown of the modulating light flux of frequency 1.6 kHz (0.5–0.6 μE/(m^2 s), $\lambda \leq 670$ nm); *2* input the saturating 1 s impulse of white light (2,500 μE/(m^2 s) for determination of F_m and F_V values; *3* and *4* (except **a**), respectively, input and shutdown of the actinic light (600 μE/(m^2 s)); for **a**: 4 input of the 1 s impulse of the saturating light against actinic light; *6* simultaneous shutdown of the actinic and modulating light fluxes; for **b**: *5* shutdown of 1 s impulse of white light (2,500 μE/(m^2 s)); *7* the level of 1 s spikes at switched shutdown of actinic light; for **c**: *3* and *4*, respectively, input and shutdown of the actinic light with periods of light/darkness of 60/20 s (800 μE/(m^2 s)); *5* the level of 1 s spikes after shutdown of the actinic light. *Arrows*: *single asterisk* indicates the start, *double asterisks* the development and recession of the slow component (Saakov 2000a–e, 2001a, b, Saakov et al 2012/2013), noticeable in **b** in the form of spike bifurcation. F_0 is the base F_0 level right after shutdown of the actinic light

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Table 2.3 Effects of high temperature (43°, **10 min**) treatment on quenching coefficients of PAM fluorescence from leaves of various species[a]

Species	Experimental conditions	Coefficient (mean value; $n = 4–5$, $p \leq 0.08$)											
		F_V/F_m	$F_{V\ exp}/F_{V\ cont}$	PS-2 reduction (%)	F_{max}/F_0	F_V/F_0	F_{V1}/F_V	F_{V1}/F_0	F_0^5/F_0	$q_E^{5\,min}$	$q_q^{5\,min}$	Spikes 5 min (mm)	Spikes 6 min (mm)
T. vulgore cultivar Saratovskaya 29	Control	0.827	0.893	94	5.1	3.57	0.93	4.30	1.10	0.326	0.940	91	102
	Exp	0.802		83	4.5	2.70	0.82	2.70	1.24	0.52	0.830	83	89
U. dioica	Control	0.817	0.477	91	5.2	3.79	0.89	3.70	1.24	0.40	0.912	77	83
	Exp	0.679		72	3.15	1.90	0.79	1.90	1.70	0.72	0.715	45	53
P. sativum	Control	0.835	0.521	92.7	4.9	4.10	0.92	3.80	1.03	0.37	0.930	79	84
	Exp	0.737		76	3.7	3.10	0.81	1.95	1.63	0.79	0.770	49	51
N. tobocum	Control	0.812	0.534	89.3	5.3	4.60	0.88	3.71	1.11	0.47	0.888	81	86
	Exp	0.694		64	4.3	3.40	0.77	1.87	1.57	0.81	0.645	43	59
Trifolium	Control	0.832	0.645	92.4	5.2	4.05	0.92	3.90	1.07	0.42	0.930	75	81
	Exp	0.656		72	4.1	2.70	0.75	1.89	1.48	0.73	0.715	46	52
Solanum lycopersicum	Control	0.841	0.783	90.3	5.0	4.65	0.90	3.92	1.21	0.39	0.900	79	88
	Exp	0.779		80.1	4.3	3.40	0.79	2.21	1.32	0.52	0.801	63	69
R. pseudoacacia	Control	0.821	0.672	94	4.8	3.70	0.89	3.30	1.09	0.38	0.940	88	94
	Exp	0.784		56	3.6	1.90	0.78	1.88	1.50	0.49	0.570	46	71
Fraxinus excelsior	Control	0.819	0.691	89	5.4	4.2	0.91	3.74	1.12	0.41	0.891	78	83
	Exp	0.767		72	4.6	3.5	0.85	2.20	1.41	0.56	0.725	43	67
Q. robur	Control	0.824	0.911	88	4.9	3.95	0.87	3.90	1.20	0.40	0.884	76	83
	Exp	0.795		80	4.3	2.70	0.79	2.66	1.37	0.52	0.810	53	73

[a] F_0^5, $q_E^{5\,min}$, $q_q^{5\,min}$ and spike 5 min were calculated 5 min after switching the actinic light on; spike 6 min—6 min after switching the actinic light off

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with an increase in the intensity of the influence of extreme factors, the q_E value also increases and points to suppression of the Calvin cycle activity. The F_V/F_0 ratio decreases, which is one more criterion proving the damage in ETC of PS-2 and is a result of the reduction in the link $P^*_{680}PheoQ_A^-$ (Weis 1981; Saakov 2000c). Simultaneously, the level of PS-2 reduction (Table 2.3) decreases.

Switching on the actinic white light flux of intensity 1,100 μE/(m^2 s) induces the Fl F_{V1} level arising after F_V recession, and the ratios F_{V1}/F_V and F_{V1}/F_0 (Table 2.3) seem to be reliable indicators characterizing the activity of RCs of PS-2 ETC (Saakov and Shiryaev 2000). The higher the amplitude of F_V^t impulses and the closer it is to the F_{V1} level, the higher the probability of more complete reduction of the Q_A^- pool and the high activity of ETC and the Calvin cycle. The calculation of the Fl coefficient at the fifth minute of the PAMF signal harmonics registration, when the most significant difference in F_0 and ${F_0}^5$ levels appears, gives the q_E value in control and experiment (Table 2.3), which also indicates a decrease in the Calvin cycle activity with EFE influence.

From the figures and Table 2.3, it follows that, with an increase in the negative impact of external factors, the coefficient ${F_0}^{5(t)}/F_0$ increases, that is, the ${F_0}^{5(t)}$ level (to the fifth minute of the measurement of PAMF signal harmonics) becomes significantly higher than F_0. In close connection with the value of coefficient, F_{V1} is the change in ${F_V}^{5(t)}$ (the value of spikes at the fifth minute) induced by the saturating light impulse against the actinic light flux (Figs. 2.5, 2.6 and Table 2.3) or after switching off the actinic light flux (spikes at sixth minute). The F_V^t value is sensitive to thermal processing and is already suppressed in response to 70 % relative humidity and γ-radiation (Monson et al. 1982; Saakov 2000a, c). The effect and aftereffect of γ-radiation correlate with the influence of temperature, fumigation, dehydration, and herbicides (Snel and van Kooten 1990; Schreiber et al. 1997a, b; Saakov 2000c), which reduce F_V^t and q_E levels, that is, decrease the activity of the Calvin cycle and q_q. This F_V^t change demonstrates the depth of the time-evolved damage of energy links of PS-2 RCs. Thus, the manifestation of non-specificity of the kinetics of the change in PAMF signal harmonics to the impact of various EFEs, and features of the changes in coefficients F_m, F_V, F_{V1}, F_V^t, F_0, and F_0^t point to the relation of the found disorders to damage of specific components of PS-2 RCs (Saakov 2001a, b).

The high F_V value (including spikes at F_V^t) is defined by the low q_E value and high utilization of energy in the Calvin cycle. The high temperature leads to an increase in q_E (to 0.6–0.75) that causes the F_V^t decrease and an energized thylakoid membrane. In this case, ETC inhibition occurs, probably when the Calvin cycle is unable to work as an electron acceptor. The q_E relaxation is accompanied by simultaneous switching on of the Calvin cycle and the ATP use coupled to it and by a decrease in the proton gradient. At thermal processing, the q_E relaxation is suppressed (Table 2.3), testifying to restriction of the Calvin cycle activity. This restriction probably precedes damage at the level of PS-2 RCs and is expressed as an increase in F_0, F_V^t reduction, and F_m^t decrease with a corresponding increase in q_E.

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It is reasonable that EFE influence affects the orientation of the photosynthesis carbon metabolism, but these are already stages of secondary reactions because the initial non-specificity is defined by primary reactions of the damage of the ETC RC functional state, arising ATP deficiency, and coupled accumulation of ADP and orthophosphate in chloroplasts. It is possible to prevent these phenomena by introduction of exogenous ATP in a suspension of chloroplasts. Earlier we wrote about pre-lethal increasing of the respiration intensity together with a simultaneous decrease in photosynthesis in a leaf (Saakov 1994) in response to radiation damage. Probably, it is defined by the intake of phosphoglyceric acid funds into the cytoplasm, and during the processes of glycolysis and respiration, these funds form pools of phosphoenolpyruvate and pyruvic acid, the synthesis of which is connected to ATP formation. These reactions couple together the metabolic and energy pathways of photosynthesis and respiration. The known activation of the intensity of glycolysis and respiration in response to a decrease in illumination correlates well with the increase in respiration in response to radiation impact on plants and also under the influence of other EFEs (Saakov and Shiryaev 2000) and can be considered as a special compensatory replacement of the ATP deficiency that arises with a break in the photosynthetic ETC.

From this point of view, the role of glycolysis is more significant. With an increase in the intensity of the influence of EFEs, glycolysis becomes the dominating ATP supplier, suppressing the role of oxidative phosphorylation. The manifestation of alternative pathways of energy supply in a cell under EFE influence has important biological value, because it allows maintenance of cellular energy resources at a stationary level during the midday depression of photosynthesis and under the influence of other environmental factors (Semikhatova and Saakov 1962). This implies the biological expediency of activation of energy processes in mitochondria and cytoplasm to supply damaged chloroplasts with ATP funds for the realization of carbon metabolism reactions in the Calvin cycle. Data on the use of exogenous funds of ATP and its penetration in chloroplasts has already become axiomatic material and does not raise doubts.

Thus, the presented results and some of our other works (Saakov 2000a–c, 2001a, b; Saakov and Shiryaev 2000) and reviews of European literature (Havaux 1988; Heber and Santarius 1973; Fork et al. 1985; Gounaris et al. 1983; Monson et al. 1982; Schreiber et al. 1997) are aligned in that the tolerance and reparation reactions of a green cell to the effect of high temperature depend, mainly, on primary damage to the chain $P_{680}\,Q_AQ_BPQ^{2-}$ in the link $P^{+}{}_{680}Pheo^{-}\,Q_A{}^{-}\,P^{*}{}_{680}PheoQ_A{}^{-}$. This conclusion is of general meaning in the sense that it interfaces the formation of ATP, formation of the reducer, and activity of the Calvin cycle, damages to which are secondary. Available data also suggest the possibility of close interrelation of vegetational cell resistance and the energy chain of mitochondria. The presence of chlorophyll *a* defining the Fl yield of blue-green algae (*Cyanobionta*, *Procaryota*) suggests that ETC disorder mechanisms in response to the damaging influences of environmental factors of natural and technogenic origin are similar to those in *Phycobionta* and *Embryobionta* (*Vegetalia* from *Eucaryota*) and also in *Procaryota*.

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Thus, there is the high probability that any factor influencing the efficiency of the excitation energy capture by RCs of open PS-2 will affect the speed of the electron transport through the PS-2 centers and be manifested as changes in Fl parameters F_m and F_V. The conclusion about the primacy of damage to the link $P^+_{680}Pheo^-Q_A$ $P^*_{680}PheoQ_A^-$ of the PS-2 RCs under the influence of an EFE can be used in practice for the selection of cultivars genetically modified with polygene systems and with transformed electron transport systems according to the tolerance of the considered characteristic.

2.2.3 *The Coupling of Mechanisms of Green Cell Resistance to Changes in the Pulse-Amplitude Modulated Fluorescence Parameters Under the Influence of Atmospheric Drought*

The ways that plants adapt to conditions of soil and atmospheric drought are very various. It is necessary to remember the coupling of the influence of atmospheric drought with, as a rule, a simultaneous temperature increase in a lamina and accompanying overheating and dehydration (Saakov 2002a–c). The point of view of researchers on the drought resistance of plants changed with developments in the methodological basis for experimental studies and with scientific successes in adjacent areas of the physiology and biophysics of plants. In this regard, in this section we will focus on changes in the functional reactions of a native leaf in connection with the main processes of light energy transformation into the energy of chemical bonds, that is, with the performance of or damage to the phototrophic reactions inherent in all green cells (Saakov 1993a–d, 2000a–e, 2001a, b). Emphasis will be placed on the assessment of the possibility and primacy of damage to the electron transfer link in the system $P_{680} \rightarrow Q_A$, since the results of earlier research suggested that this is the link most subjected to EFEs of natural and anthropogenous origin (Schreiber et al. 1997a, b; Saakov 2000a–e, 2001a, b, 2002a–c).

During short atmospheric drought, the considered functional reactions can be defining. Considering the connection between changes in the parameters of PAMF signal harmonics and the processes of carbonic acid fixation by a leaf, there was an attempt to connect the known midday minima of photosynthesis intensity with the processes of damage to the activity of the ETC, arising as a result of dehydration of a lamina. We considered the variant of *acute* (coming quickly and lasting for a short time) atmospheric drought, when radical changes in cell metabolism processes and in the synthetic activity of a green cell have not yet occurred, and the regulation and restoration of changes are still possible through repair of ETC energetics with formation of ATP and a reductant.

As objects of research, we chose leaves different in their tolerance to atmospheric drought: the cultivar Saratovskaya 29 of wheat (*Triticum vulgare*), a barley mutant lacking chlorophyll *b* (*Hordeum vulgare*, cultivar Donaria №. 3613), oak (*Quercus robur*), bay laurel (*Laurus nobilis*), tomato (*Lycopersicon esculentum*),

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nettle (*Urtica dioica*), acacia (*Robinia pseudoacacia*), clover (*Trifolium* sp.), haricot (*Phaseolus vulgaris*), tobacco (*Nicotiana tabacum*), and some others.

The specifics of the change in the character of stationary dark Fl (F_0), the maximum Fl at saturating pulse light flash (F_m), variable Fl (F_V), Fl in response to the action of actinic light (F_{V1}), and in a combination of actinic light with a saturating light impulse (F'_{V1}) in response to atmospheric drought were registered with the pulse fluorometer Walz 101-103 (Effeltrich, Germany). We consider it necessary to note the great convenience of this method, the speed of obtaining data on changes in key measured parameters of an intact leaf, and the possibility after a specified time interval to come back to registration of *repeated* characteristics of this leaf. The calculation of photochemical (q_q), non-photochemical (q_E), and other coefficients of Fl quenching and the interpretation of their changes were carried out by the accepted technique described earlier.

We generally follow the nomenclature for signals of PAMF suggested by van Kooten and Snel (1990a) and supported by other experts (Schreiber and Bilger 1993, 1997). The separation of the Fl signal from the much more intensive excitation light is achieved by the application of primary optical filters with the excitation light transmission ($\lambda < 670$ nm) and secondary filters with transmission in the long-wave spectral area ($\lambda > 680$ nm) to protect the photodetector from parasitic diffused light from the excitation source. Owing to design features, the detectors of the device are insensitive to external light. The device is recommended for field ecological researches because it allows comparison of the influence efficiency of many technogenic and natural EFEs using *only one parameter*. In addition, obtained results are easily digitalized and entered into information computer systems for further processing. The performance of large-scale and easily comparable research in different laboratories on all continents is possible, thanks to the existence of chlorophyll *a*, the presence of which unites autotrophic organisms, such as *Cyanophyceae* in *Procaryota* and *Vegetalia* in *Eucaryota*.

The measurement of photosynthesis intensity was carried out with a radiometric method using $^{14}CO_2$ (Saakov 1959, 1987) and in some cases with the Warburg method using vessels designed to allow introduction of the fiber-glass cable of the Walz 101-103 device directly into the vessel (Saakov and Shiryaev 2000). During the period of influence of a warm air current and a "dry wind," the leaves were kept in the dark. After a 1 s impulse of light, they were irradiated with actinic light or by a combination of the actinic light with a subsequent series of 1 s impulses of white light (Saakov 1993a–d). As a result, the relative number of light quanta effectively used in PS-2 by virtue of ETC saturation and active reduction of Q_A^+ and Q_B^+ increases. Experimental plants reacted to the influence of the dry wind by moisture reduction in leaf tissues, which was registered as a percentage of the initial weight of the leaf (Table 2.4). The plants reacted differently; most significant moisture loss was from *U. dioica*, *Trifolium* sp., and *N. tabacum*, with smaller water loss from *L. nobilis* and *Q. robur* (Table 2.4). Results of the experiments are presented in Figs. 2.7, 2.8, 2.9, and 2.10 and in Table 2.4.

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Table 2.4 Effect of atmospheric drought (10 min) on the changes in coefficients of quenching of PAM fluorescence[a]

Object	% of leaf humidity in exp.	Coefficient (mean value; $n = 4–5$, $p \leq 0.01$)												
		F_V/F_m	F_{V1} $h/2$ (mm)	$F_{V\ exp}/F_{V\ cont}$	PS-2 reduc. (%)	F_m/F_0	F_V/F_0	F_{V1}/F_V	F_{V1}/F_0	F_0^5/F_0	$q_E^{5\,min}$	$q_q^{5\,min}$	Spikes 5 min (mm)	Spikes 6 min (mm)
Q. robur	Control	0.827	5	0.893	90	5.1	3.57	0.91	4.30	1.10	0.426	0.880	92	101
	70	0.812	16		77	4.5	2.90	0.83	2.90	1.24	0.53	0.810	82	87
L nobilis	Control	0.817	9	0.827	89	5.2	3.79	0.87	3.70	1.23	0.40	0.90	77	85
	73	0.779	14		72	4.1	2.06	0.79	2.90	1.72	0.48	0.725	66	65
T. vulgore № 29	Control	0.835	4	0.791	87	4.9	4.10	0.94	4.10	1.13	0.37	0.880	78	87
	69	0.797	15		73	3.9	3.29	0.79	2.80	1.23	0.49	0.760	69	56
L. esculentum	Control	0.841	5	0.778	90.3	4.9	4.56	0.88	3.92	1.21	0.39	0.900	76	88
	61	0.789	18		68	4.1	2.96	0.72	2.41	1.32	0.64	0.70	66	73
R. pseudoacacia	Control	0.821	6	0.664	92	4.7	3.92	0.87	3.50	1.09	0.41	0.920	88	92
	45	0.789	20		56	3.2	2.10	0.72	1.78	1.50	0.49	0.575	54	73
Ph. vulgaris	Control	0.819	7	0.691	89	5.2	4.37	0.86	3.74	1.12	0.46	0.891	79	85
	40	0.758	19		62	3.7	2.35	0.72	2.20	1.65	0.66	0.625	44	65
H. vulgare № 3613	Control	0.815	9	0.622	84	5.1	3.1	0.89	3.9	1.37	0.37	0.79	80	96
	42	0.770	19		59	3.6	1.8	0.67	3.21	1.47	0.71	0.50	63	71
N. tobocum	Control	0.812	8	0.544	87	5.2	4.62	0.88	3.71	1.12	0.47	0.818	81	96
	39	0.578	21		46	3.4	2.32	0.70	1.67	1.57	0.81	0.645	40	57
U. dioica	Control	0.814	7	0.561	88	4.9	3.95	0.83	3.90	1.22	0.40	0.884	72	89
	34	0.656	25		50	3.2	1.70	0.66	1.66	1.87	0.68	0.690	49	72
Trifolium sp.	Control	0.830	8	0.545	90	5.1	4.05	0.92	3.94	1.18	0.42	0.910	73	87
	36	0.645	24		61	3.0	1.53	0.61	1.79	1.56	0.74	0.700	42	49

[a] F_0^5, $q_E^{5\,min}$, $q_q^{5\,min}$ and spike 5 min were calculated 5 min after switching the actinic light on; spike 6 min—6 min after switching the actinic light off

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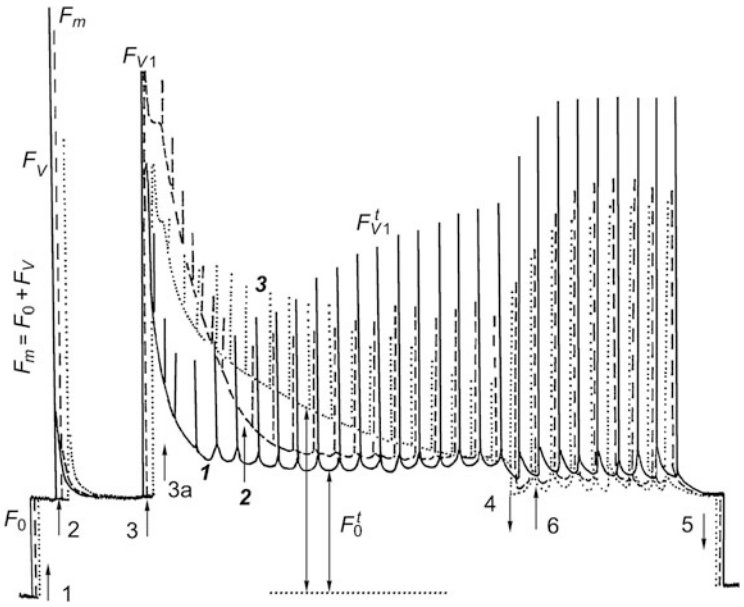

Fig. 2.7 The character of signal harmonics change in PAMF of a leaf of *Nicotiana tabacum* in response to dehydration. *Curves*: *1* control; *2* experiment: dehydration to 45 % of the initial state; *3* dehydration to 30 % of the initial state. *Arrows*: *1* and *5*, respectively, input and shutdown of the modulating light flux of frequency of 1.6 kHz (5 μE/(m^2 s), with $\lambda < 670$ nm); *2* input of the saturating 1 s impulse of white light (2,500 μE/(m^2 s)) for determination of F_m and F_V values; *3* input, *4* shutdown of the actinic light (1,200 μE/(m^2 s)); *3a* input after 30 s of 1 s impulse of the saturating light against the actinic light for determination of $F_{V1}^t = F_{V1}^{'}$ values; *6* input of the 1 s impulses of the saturating light after shutdown of the actinic light

It is necessary to remember that the parameters and PAMF configuration are substantially defined by the physiological features of a leaf and depend on its genotypic and phenotypic features. The PAMF method enables the same leaf to be used to compare PAMF contour changes caused by the EFE effect or aftereffect in control and experimental conditions. One such induced PAMF contour change is the change in band half-width, described by the height of F_{V1} and course of the descending curve F_0 (F_0^t), induced by actinic light flux under the influence of the stress factor (Saakov 1993a–d, 2000a–e).

We can see the characteristic change in the considered coefficient (F_{V1} $h/2$) under the influence on the green leaf of γ-radiation, high and low temperatures, and also under the influence of excess Na^+, Cl^-, and SO_4^{2-} ions (Saakov 1993a–d, 2000a–e, 2001a, b, 2002a–c; Saakov and Shiryaev 2000). We can recommend with a high degree of confidence the coefficient F_{V1} $h/2$ as a criterion to characterize the level of damage of the photosynthetic device of a green cell under the influence of EFEs of natural or technogenic origin.

The F_0 level of base (dark) Fl indicates the openness of RCs of PS-2 before electron migration into open RCs under the influence of the light impulse and the

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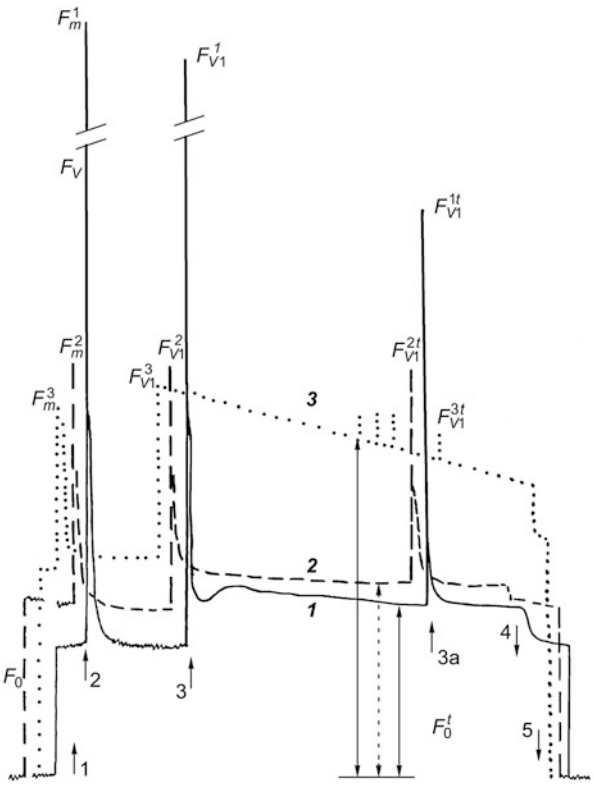

Fig. 2.8 The character of signal harmonics change in PAMF of a leaf of *Lycopersicon esculentum* in response to dehydration. *Curves*: *1* control; *2* experiment: dehydration to 55 % of the initial state; 3 experiment, dehydration to 38 % of the initial state (17 min of the "dry wind" influence. *Arrows*: *1*, *2*, *3*, *3a*, *4*, *5*, *6* as in Fig. 2.7

emission of excited chlorophyll *a* and before the reduction of the first stable acceptor PS-2 $Q_A^+ \rightarrow Q_A^-$ caused by this migration. F_0 quenching corresponds to a decrease in the absorbed light energy directed to PS-2. From the data in Table 2.4 and the figures, it follows that, with an increase in the negative influence of the dry wind, the value of coefficient F_0^5/F_0 increases, that is, the height of F_0^5 after the cell damage tends to increase, emphasizing PS-2 inactivation accompanied by increasing $F_0(F_0^t)$. In this regard, there is a direct dependence between the increase in coefficient F_{V1} $h/2$ and coefficient F_0^5/F_0.

The dependence of the F_0^5 height on the dose of γ-radiation impacting on the photosynthetic device (Saakov 1993a–d, 2000a–e) was confirmed for the influence of atmospheric drought. Taking into account data on the influence of fumigation and herbicides (Saakov 1993a–d), there is a high probability of the reliable application of coefficient F_0^5/F_0 for characterization of the openness state of RCs of PS-2 and the *oxidization degree*. The ratio F_m/F_0, similarly to the ratio F_V/F_m, allows conclusions to be made about PS-2 activity. For undamaged (control) plants, F_m/F_0 is approximately four to five times as in experiment much (Table 2.4); for slightly damaged plants, the coefficient F_m/F_0 on average is reduced by 15–22 % compared with controls; and for the damaged plants, it decreases by 30–40 % (Table 2.4).

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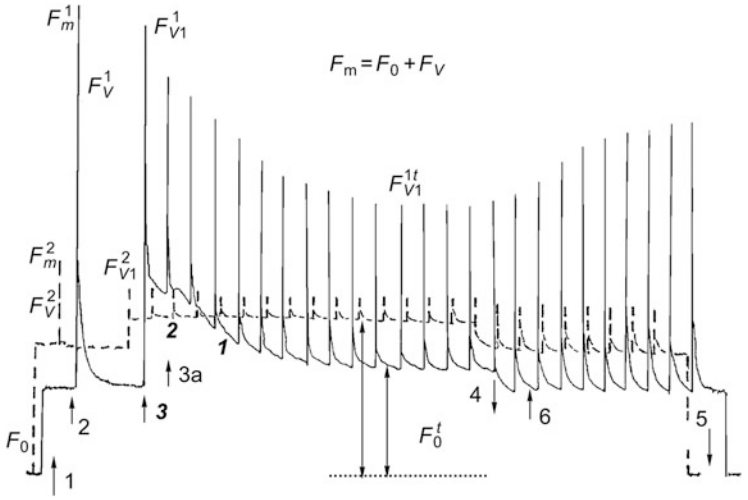

Fig. 2.9 The character of signal harmonics change in PAMF of a leaf of *Laurus nobilis* in response to dehydration. *Curves*: *1* control; *2* experiment: dehydration to 65 % of the initial state; *3* experiment: dehydration to 33 % of the initial state (25 min of the "dry wind" influence. *Arrows*: *1*, *2*, *3*, *4*, *5*, *6* as in Fig. 2.7

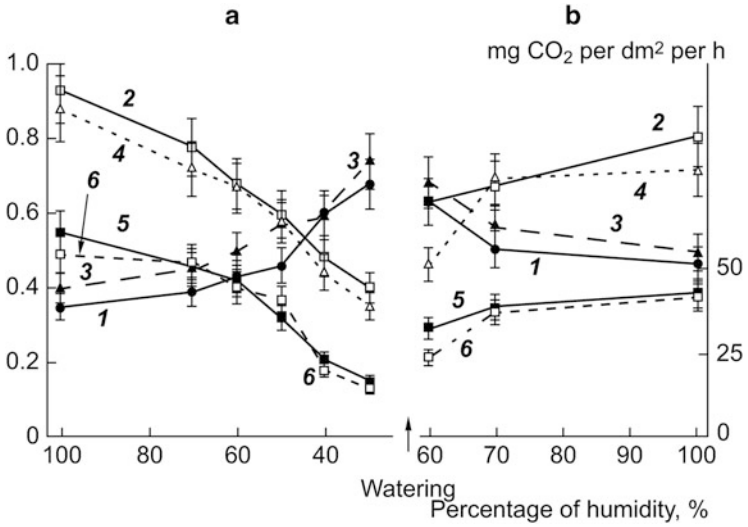

Fig. 2.10 The dynamics of change in (**a**) q_q and q_E coefficients and (**b**) in the photosynthesis intensity (mg CO_2/dm^2 h) during the "dry wind" period and at the tidal drop in water level for *Phaseolus vulgaris* (*curve 5*) and *Robinia pseudoacacia* (*curve 6*). *Curves*: *1* and *2*, respectively, demonstrate the dynamics of q_q and q_E for *Ph. vulgaris* and *3* and *4* for *R. pseudoacacia*. *Left ordinate*: coefficient change in relative units; *right ordinate*: values of the photosynthesis intensity

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Variable Fl, F_V, defines the level of Q_A^- reduction and is accompanied by RC closing. Research experience shows that the damaging influence of an EFE reduces the F_V level. This decrease indicates disorder of energy transformation in PS-2 and the incompleteness of RC closing, with partial damage of their function of light energy conversion into chemical potential. The inhibition of energy transmission between PS-2 and PS-1 is the primary event induced by dry wind and results in imbalance of the reaction ensemble of electron transfer in the link $P^*_{680} \rightarrow Q_A^+ \rightarrow Q_B^+$. This is caused by photophosphorylation damage and interruption of the phototrophic function of the leaf. There are bases for the belief that suppression of PS-2 activity is connected with suppression of the F_V value and is caused by inhibition of charge transfer between P_{680} and Pheo (Schreiber and Bilger 1993; Schreiber et al. 1997a, b; Saakov 2001a, b, 2002a–c), that is, directly by damage to the function of PS-2 RCs. The F_V value (especially important, the F_V level at any time t is F^t_{V1}, which is the same as F'_{V1}, designated in the literature as "spikes") is coupled with fluctuations in the coefficient q_E. The low value of q_E emphasizes a decrease in the proton gradient interfaced to ATP formation and simultaneous active use of the energy in the Calvin cycle. The correctness of this statement is illustrated by data in Table 2.4, from which it follows that an increase in the dry wind impact on a leaf results in an increase in the value of the coefficient q_E. At the same time, the ratio F_V/F_0 decreases, which is also evidence for ETC damage connected with a reduction of the link $P^*_{680}PheoQ_A^+$ (Saakov 2000a–e).

A decrease in the ratio $F_{V\ experiment}/F_{V\ control}$ also indicates suppression of PS-2 activity (Table 2.4). Obtained results on the influence of atmospheric drought correlate well with published data (Saakov 2000a–e) and are additional evidence for the link between the photosynthetic device stability and RC activity, and the damageability of their link at the stage $P^+_{680}Pheo^-Q_A^- \rightarrow P^*_{680}PheoQ_A^-Q_B$ (Saakov 2000a–e, 2001a, b, 2002a–c).

From the figures it follows that, after the decrease in F_V, the actinic light generates the Fl signal F_{V1}. Experience shows that the ratio F_{V1}/F_V and coefficient F_{V1}/F_0 (Table 2.4) can be considered as reliable indicators of PS-2 ETC activity (van Kooten and Snel 1990a; Saakov et al. 1992; Saakov 1993a–d; Schreiber and Bilger 1993, 1994). The higher the amplitude of impulses $F^t_{V1} = F'_{V1}$ and the closer they are to F_{V1}, the more significant is the probability of complete reduction of the Q_A^+ pool and of high activity of ETC and the Calvin cycle.

As can be seen from Table 2.4, in parallel with the increase in coefficient q_E, the coefficient q_q decreases. Values of q_q receding from 1 to 0 emphasize the decrease in light energy conversion and disorder of leaf phototrophic ability. A value of $q_q = 1$ indicates high levels of electron transfer, photosynthesis intensity, and $NADPH/H^+$. Figure 2.10 presents the dynamics of changes in q_q (curves 2 and 4) and q_E (curves 1 and 3) as a function of the decrease in leaf water content under the influence of a dry wind, together with the dynamics of CO_2 absorption intensity change (curves 5 and 6). After a tidal drop in water level, the opposite effect is seen. Simultaneously with a decrease in coefficient q_q and increase in q_E, the intensity of

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photosynthesis falls off and reaches a minimum at the minimum water content in a leaf; the respiration intensity increases 2.5- to 4-fold. We reported earlier on the change in PAMF parameters, photosynthesis, and respiration in response to γ-radiation for similar dehydration of a leaf (Saakov 1987, 1993a–d). The presented data correlate well with the described results of spectrophotometric research on the damage and regeneration of functional and structural features of thallomes of the red alga *Porphyra perforata* in the case of its predrying and a tidal drop in water level (Saakov 1987; Fork and Hiyama 1973).

Here it is possible to include the character of change in the photosynthetic processes of bryophytes (bryophytes are poikilohydric plants) when adapting to inconstancy of the water content in the surrounding environment. Among the most known consequences of drying for these plants is the termination of photosynthesis if the water content is lower than the critical level; this phenomenon is characteristic for a large number of liverworts (Tuba et al. 1994). A decrease in photosynthesis intensity is accompanied by an excess of light energy that cannot be transformed through ETC and leads to photoinhibition, suppression of PS-2, and non-photochemical quenching. The decrease in PS-2 activity under these conditions can be explained by the inability of RCs to carry out photochemical quenching. Scientists assume the existence of mechanisms protecting the photosynthetic device from photoinhibition in the period of dehydration. The xanthophyll zeaxanthin has been considered as such a possible quencher of the non-radiating dissipation energy, appearing under the influence of light on violaxanthin together in the process of its reduction through antheraxanthin (Schindler and Lichtenthaler 1996). The contribution of non-radiating energy transformations is rather great; its intensity decrease is caused by the acceptor–donor properties of the pigment system and a quencher. The quencher serves as an electron acceptor and the pigment system as the electron donor. However, the exact mechanism of such photoprotection is not known, and a number of facts place the reality of this hypothesis in the category of insufficiently convincing and not at all the only one possible (Saakov et al. 1993; Schindler and Lichtenthaler 1996). Moreover, in many publications describing the protective role of zeaxanthin (see Chap. 1), researchers fail to mention the numerous works and reviews of the 50–60 years of work by H. Claes, G.W. Cohen-Bazire, M. Griffiths, R.Y. Stanier, T. W. Goodwin, and N.I. Krinsky on the protective role of carotenoids in response to an excess of light (see Chap. 1).

We consider the increased respiration noted above and the decreased photosynthesis at deep dehydration to be a result of the manifestation of the *compensatory replacement* of cellular energy systems under the extreme life conditions discussed by us earlier (Saakov 2000a–e). We explained the manifestation of compensatory replacement by the possibility of cytoplasmic intake of phosphoglyceric acid funds that, under the influence of an EFE (dehydration) and via processes of glycolysis and respiration, form pools of phosphoenolpyruvate and pyruvic acid, the synthesis of which is connected with ATP formation. It is not excluded that a similar increase in respiration is coupled with the formation of glyoxalic and glycolic acids and

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glycolaldehyde. These substances are inherent in chloroplasts and mitochondria and also in animal cells. It is probable that, with an increase in dehydration, the role of glycolysis is more significant and it becomes the general supplier of endogenous ATP. These reactions show the *link between photosynthesis and respiration* under extreme living conditions, during the attempt to maintain the energy resources of the cell during the midday depression of photosynthesis, and under the influence of an EFE in its broadest terms. Obviously, in a green cell, there are mechanisms for certain oxidation–reduction reactions of metabolism that occur with light participation in chloroplasts to be replaced by the dark reactions of mitochondria. The specified reactions show the biological expediency of the activation of processes separated in space, that is, the energy processes in mitochondria and cytoplasm that supply damaged chloroplasts with ATP to guarantee the reactions of the Calvin cycle.

Thus, the results of the present section and of previous works (Schreiber et al. 1997; Saakov and Shiryaev 2000; Saakov 2000a, b, 2001a, b) provide material for a reliable conclusion about the primary influence of an EFE on the damage to the ETC at light energy conversion between P_{680} and Q_A. Existing data allow us to speak about localization of the damage at the link $P^{+}_{680}Pheo^{-}Q_A^{-} \rightarrow P^{*}_{680}PheoQ_A^{-}$. The dependence of photosynthesis intensity on the character of the dynamics of changes in q_q and q_E is shown and offers a possible explanation for the link between the midday depression of photosynthesis and increased dehydration of leaf tissue. The considered regularities could be general for a wide range of representatives of *Vegetalia* from *Eucaryota*. They should be taken into account during target selection of genetically modified cultivars for zones suffering from atmospheric drought. The material presented shows the correctness of our ideas about the cooperative interaction of three factors (Saakov 2000a–e) providing the necessary adaptation and resistance of a green cell to the influence of stress factors of natural and anthropogenous origin and coupled with structurally functional features of the photosynthetic device.

2.2.3.1 Coefficient Dynamics of Pulse-Amplitude Modulated Fluorescence Under the Long-Term Influence of Soil Drought and High Temperature

Different points of view of researchers on the nature of the stability of vegetational organisms determine the strategic outlook of scientists and are the basis for further development of experiments in different areas of physiology and biotechnology. At the same time, for many years the level of studies of plant tolerance to EFEs and the mechanisms explaining it fluctuated from extremely average to catastrophic (G.V. Udovenko) in their artificiality. The reason was both the methodology used and the very mediocre methodological and intellectual basis (potential). An example of a typical report on scientific activities in the field of research of plant resistance is the collection edited by G.V. Udovenko 1976. The content of this

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collection is shameful for science of the end of the 1970s. It dramatically contrasts with the well-grounded statements of P.A. Henckel (1954). Regretfully, under the pressure of the administration of the Institute of Plant Industry and for reasons of an unscientific nature, one of authors of this book had to publish twice his own theoretical and methodological developments, the coauthor of which was the scientific editor of that collection. It appears that European researchers are also familiar with analogous situations.

The statement that the primary localization of damaging influences of EFEs of natural and anthropogenous origin is in the RCs of photosystems was published by us in 1974–1975 on the basis of studies of *structural* changes in the photosynthetic device by methods of high-order derivative spectrophotometry (Baranov et al. 1974; Saakov et al. 1975; Saakov 1976; Saakov and Leontjev 1988). The harbinger of these works was research performed on mutant collections of green algae *Scenedesmus* and *Chlamydomonas*, which possess varying sensitivity to light and differ in nutrition type (Baranov et al. 1975).

The above-mentioned material was reported at a number of international and all-USSR forums with international participation. Works on the investigation of structural changes in the photosynthetic device under the influence of EFEs emphasized that primary damages of this mechanism are connected, not with quantitative disorders of pigment content, but with the qualitative reorganization caused by damage to chloroplast's autotrophic ability to convert light energy into the energy of ATP chemical bonds. Serious support for these conclusions followed from research on the character of fluorescence change under the influence of EFEs (Krause and Weis 1984; Lichtenthaler 1996a, b).

The introduction in leading laboratories of the world of the method of registration of the harmonics of pulse-amplitude modulated fluorescence (PAMF) (Schreiber and Bilger 1987) and the conclusions of leading European experts unequivocally showed the interconnection of PAMF signal changes induced by EFEs with functional damage of PS-2 RCs. The development of ideas about the localization of EFE damaging influences at a qualitatively new stage of research on the functional activity of the photosynthetic device is summarized in reviews (Lichtenthaler 1988a; Lichtehthaler and Rinderle 1988; Lichtenthaler 1988b, 1996a, b; Schreiber and Bilger 1987; Bolhar-Nordenkampf et al. 1989) and completely agrees with conceptions stated by us earlier. Plus, a strong point of European research consists in the speed of realization of new methodological advantages, so the wide range of various experiments covers a variety of EFEs and specific particular characteristics of biological objects.

The new material on the influence of various EFEs on vegetational objects different in their tolerance level allowed us to formulate the theory of the energetic basis of green cell tolerance to EFE impact (Saakov 2000a–e), which was further developed into the theory of energetic basis of the stability of phototrophic cells of *Procaryota* and *Eucaryota* to the influence of abiotic factors of the environment (Saakov 2004). As the basis of the green cell tolerance, energy theory was a complex of research such as ours and that of a number of European authors on

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changes in both the *structural* state of the chlorophyll–protein complex (ChPC) of RCs and its *functional* activity under the EFE influence. In formulating these theses, the main emphasis was on the existence of the most sensitive links in the ETC connected with the transformation of light energy into the energy of ATP chemical bonds. The deficiency of the latter does not say anything about the Calvin cycle activity or about other ways of further synthesis of endocellular products.

Thus, the disorder of light energy *conversion* into the energy of ATP chemical bonds is *primary* and is typical under the influence of various types of stress, that is, it is *nonspecific*. Changes in the content of other cellular metabolites (quantity of pigments, sugars, proteins, etc.) are *secondary* and demonstrate the time process of deeper functional damage induced by EFEs in an autotrophic cell (Saakov 2004). The plentiful material presented in reviews (Krause and Weis 1984; Schreiber and Bilger 1987; Lichtenthaler 1988a, 1996a, b; Bolhar-Nordenkampf et al. 1989), and also our own data, gave assurance during creation of these theories and supported our ideas on the identity of chloroplast ETC responses to the influence of various EFEs. This corresponds to the thesis of the well-known biochemist Prof. E.M. Kreps that "nature seldom invents new biochemical mechanisms; it widely uses earlier created functional systems, combining them in different variants throughout evolution" (Kreps 1976).

The mass application of the PAM method by known schools of Western Europe and the USA defined the readiness of the scientific audience to understand and interpret the obtained results. There were also plenty of skeptics, but it is almost impossible to oblige *urbi et orbi*. At the end of the twentieth century, there was a bulk of correct research in the field of physiology and biophysics on the stability of the photosynthetic device. This material was a result of the PAM method being able to provide in vivo data acquisition about the functional state of the photosynthetic device, allowing use of the same leaf to investigate in situ the long-term dynamics of the primary reactions of photosynthesis in connection with damage to the light energy conversion processes.

We investigated for the first time the reaction of the same phototrophy tissues of a set of plants differing in degree of tolerance to the influence of high temperature and drought, at increasing levels of EFE influence over a long interval of time. This response was estimated with the change in dynamics of the photochemical (q_q) and non-photochemical (q_E) coefficients of fluorescence quenching.

The specifics of PAMF change was measured with the Walz 101-103 device (Effeltrich, Germany) (Schreiber and Bilger 1987; Bolhar-Nordenkampf et al. 1989; Lichtenthaler 1996a, b). Unfortunately, the realities of life are such that we could get access to modern equipment only by working in Europe. The positive features of the method are defined, in particular, by its exclusively high sensitivity, and specialties of the interpretation of registered data are described in reviews (Krause and Weis 1984; Lichtenthaler 1988a; Schreiber and Bilger 1987; Bolhar-Nordenkampf et al. 1989).

The experimental points obtained at 7–9 min of PAMF registration and presented in Figs. 2.11, 2.12, 2.13, and 2.14 show the average values of studied

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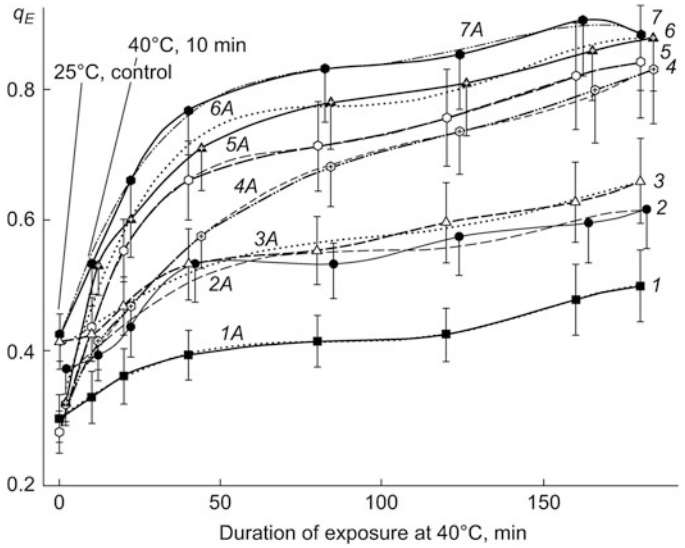

Fig. 2.11 The dynamics of change in the q_E coefficient of energy quenching of PAMF in response to increasing duration of a thermal influence of 40°C for different types of plants: *1 Artemisia* sp., *2 Laurus nobilis*, *3 Quercus robur*, *4 Phaseolus vulgaris*, *5 Nicotiana tabacum*, *6 Trifolium* sp., *7* the mutant № 3613 of *Hordeum vulgare*, lacking chlorophyll *b*, the cultivar Donaria. *Curves 1A–7A*, corresponding to *curves 1–7*, are nonlinear regressions of the fifth order

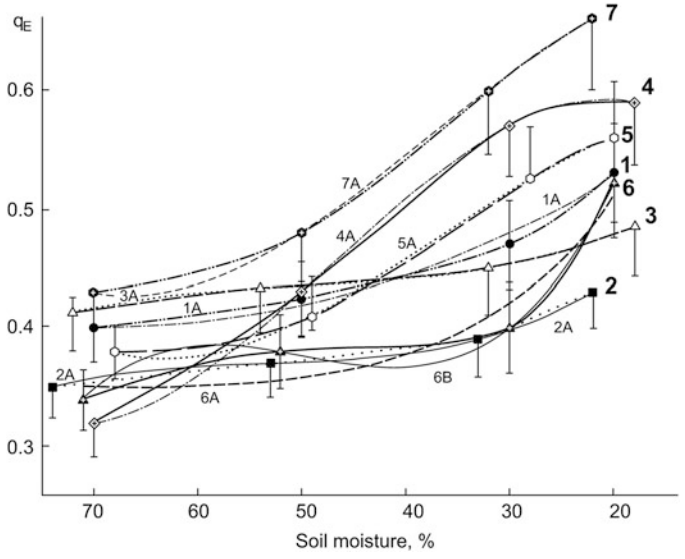

Fig. 2.12 The dynamics of change in the q_E coefficient of energy quenching of PAMF in response to increasing soil drought for plants: *curves 1–5* and *7* areas in Fig. 2.11; 6 *Triticum* sp. the cultivar Saratovskaya 29. *1A* and *6A* are nonlinear regressions of the second order for *curves 1* and *6*, respectively; *2A–5A*, *6B*, and *7A* are nonlinear regressions of the third order for *curves 2–7*, respectively. *Abscissa*: the decrease in soil humidity

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coefficients of 9–12 parallel experiments. Processing and visualization of data and regression curves were carried out with the software package *SigmaPlot* 2000.

From the previous works, it follows that the level of base fluorescence, F_0, arising after the dark adaptation of a leaf, when all RCs are open, membranes are in the non-energized state, and the first stable acceptor of PS-2 (Q_A^+) is completely oxidized, corresponds to $q_q = 1$ and $q_E = 0$. The influence or aftereffect of EFEs results in an increase in F_0^t (in any time interval t) and to the simultaneous change in the state of PS-2 pigments, inhibition of Q_A reduction, and accumulation of its oxidized funds caused by the rupture of the electron transport onto Q_B, that is, by the destruction of RCs of PS-2. An increase in intensity of the EFE influence reduces the F_V/F_0 ratio (F_V is variable Fl, indicating the change in q_E of the non-photochemical quenching), which is one more significant criterion speaking for the damage of the ETC of PS-2 and occurs due to reduction of the link $P^*_{680}PheoQ_A^-$. The e^- transport in RCs of PS-2 starts from the charge separation between P_{680} in the first excited singlet state and pheophytin and represents the e^- transfer from the secondary donor Z to primary and secondary acceptors Q_A and Q_B of the quinone type. To illustrate our line of reasoning, we again show Eq. (2.1) of the ETC:

$$e^{-\underline{h\nu}} \rightarrow ZP_{680}PheoQ_AQ_B\underline{^1} \rightarrow ZP^*_{680}PheoQ_AQ_B\underline{^2} \rightarrow ZP^+_{680}Pheo^-Q_AQ_B\underline{^3}$$
$$\rightarrow ZP^+_{680}PheoQ_A^-Q_B \rightarrow PQ^{2-}$$

At the photoinhibition induced by intensive light, damage to the e^- transport at stages 2 and 3 is found, that is, disorder of charge separation between P^*_{680} and Pheo. In the state $P_{680}Q_A^-$, there is no photochemical transformation of energy. The intermediate Pheo works as a mediator between P_{680} and Q_A, promoting the primary charge separation (forming $P^+_{680}Pheo^-Q_A^-$). The scientists suppose that F_V should be the "recombinant luminescence" at the transition $P^+_{680}Pheo^-Q_A^+ \rightarrow P^+_{680}Pheo^-Q_A^- \rightarrow P^*_{680}PheoQ_A^-$. From here, the decrease in F_V value directly indicates the suppression of PS-2 activity and the damage of the e^- transport from P_{680} to Q_A^+, caused by the inhibition of charge migration between P_{680} and Pheo and retarding the light energy transformation into the energy of ATP chemical bonds, that is, damage to the phototrophic function in PS-2 RCs occurs. The EFE influence on a leaf reduces the yield of F_V, F_{V1}, and F_{V1}^t and increases the q_E value, which proves the existence of the primary damage and its localization close to RCs of PS-2 (Krause and Weis 1984; Lichtenthaler 1988a, b; Schreiber and Bilger 1987; Bolhar-Nordenkampf et al. 1989; Lichtenthaler 1996a, b; Saakov 2000a–e, 2004). In this case, it is necessary to carefully distinguish between the reduction of the specified coefficient and the F_0 increase (Bolhar-Nordenkampf et al. 1989).

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Figures 2.11 and 2.12 show the dynamics of the coefficient q_E value change depending on the intensity of chosen EFEs. From consideration of Fig. 2.11, the following conclusion can be made: The dynamics of curves 1–7 is approximated by lines of the nonlinear regression of the fifth order (curves 1A–7A) and can be reconstructed with the corresponding equations provided by the program.

The chosen objects can be divided into two groups according to the character of their q_E dynamics (curves 1–3 and 4–7) and to the initial inclination angles of curve dynamics. The q_E increase is accompanied by a decrease in the activity of dark reactions. On the basis of ETC activity invariance, it is possible to draw a conclusion on the degree of tolerance of object leaves to the prolonged influence of high temperature. Taking into account that wormwood and laurels can be considered as thermosteady objects, the character of q_E dynamics is no surprise. In this case, RCs of PS-2 subjected to EFE impact turn into fluorescence quenchers, but also, probably, trap excitons and convert the accepted energy into thermal radiation, q_E. The increase in dissipation of the excited thermal energy reduces the primary photosynthetic efficiency and the activity of ETC. The q_E value corresponds to the *trans*-thylakoid ΔpH. From consideration of the dynamics regularity of the difference $1 - q_E$, it follows that a decrease in the yield of fluorescence is defined by an accumulation of protons in thylakoids.

Different dynamics of q_E change is observed in the case of prolonged soil drought (Fig. 2.12). In the experiment, the same objects were chosen as in the case of high temperature influence, but tolerant wheat Saratovskaya 29 was added. Curves 2A–7A (Fig. 2.12) are approximated by the nonlinear regression of the third order, and curve 1A is of the second order. They can also be reconstructed using the corresponding equations. It follows from the q_E dynamics that the barley mutant 3613, tobacco, and haricot suffer the effects of soil drought comparatively much worse than laurel and oak; wormwood and Saratovskaya 29 were intermediate. At 30 % soil drought, laurel, Saratovskaya 29, wormwood, and oak showed values of coefficient q_E only 10–20 % different from the initial reference level (70 %). The value of q_E for Saratovskaya 29 especially increased (jump-like) at the transition to 20 % soil humidity. For other objects, essential differences in q_E values in comparison with the control were registered in the interval between 40 and 30 % soil drought. Thus, the prolonged influence of high temperature and soil drought led to an increase in non-photochemical quenching of Fl caused by accumulation of reduced $Pheo^-$, decrease in the quantum yield, and inactivation of the Calvin cycle. Data on the q_E dynamics under the EFE influence increasing in time correspond well to results of researchers Schreiber and Neubauer (1990).

The dynamics of q_q under the prolonged influence of high temperature and soil drought is presented in Figs. 2.13 and 2.14, respectively. The data of Fig. 2.13 shows that the chosen objects can be subdivided into two groups

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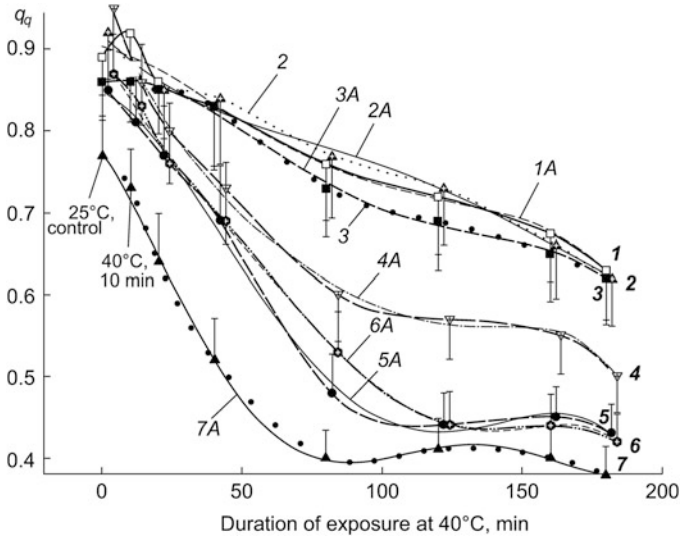

Fig. 2.13 The dynamics of change in the PAMF photochemical quenching coefficient q_q in response to increasing duration of the thermal influence of 40°C for plants: *curves 1*, *4–7* are as in Fig. 2.11; *2 Q. robur*; *3 L. nobilis*. *1A–3A* nonlinear regressions of the fourth order for *curves 1–3*, respectively; *4A–7A* nonlinear regressions of the fifth order for *curves 4–7*, respectively

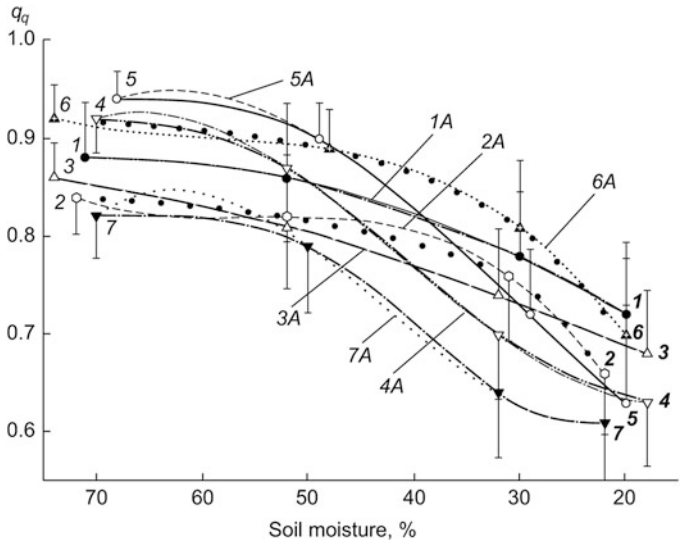

Fig. 2.14 The dynamics of the PAMF photochemical quenching coefficient q_q at the growing soil drought. *1–5, 7* as in Fig. 2.11; *6*: *Triticum* sp., the cultivar Saratovskaya 29. *Curves 1A–7A* are, respectively, nonlinear regressions of the third order for curves *1–7*. *Abscissa*: decrease in the soil humidity

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(curves 1–3 and 5–7). Objects of the first group manifested higher tolerance to temperature influence compared with the second group. Haricot is intermediate, but, possibly, is closer to the second group.

In Fig. 2.13, the dynamics of curves 1–3 is approximated by lines of nonlinear regression of the fourth order (curves 1A–3A); at the same time the dynamics of curves 4–7 is approximated by lines of nonlinear regression of the fifth order (curves 4A–7A). The decrease in q_q values observed in all variants emphasizes the increasing reduction of ETC (the primary acceptor is Q_A^-; the difference $1 - q_q$ points at the relative level of the Q_A reduction). The reduction of PQ^{2-} is coupled with the increase in the proton current, showing energy accumulation (electrons) in the acceptor part of PS-2, and the inhibition of ETC re-oxidation. These data correlate with results on CO_2 fixation obtained earlier.

The other dynamics of q_q can be seen under the increasing influence of soil drought (Fig. 2.14). Sharp differences in q_q values between the experiment and the control start in the region of 40 % soil humidity, reaching a maximum at 25–20 %. In this regard, wormwood, oak, and the wheat Saratovskaya 29 possess similar and higher q_q values, whereas haricot, tobacco, and the barley mutant 3613 are all in the group with much lower q_q. Laurels were intermediate, although it is necessary to emphasize that the relative recession of q_q values compared with the control point (70 %) is not lower than in the first group of objects. Curves 1, 3, and 7 of q_q dynamics are approximated by the nonlinear regression of the second order (curves 1A, 3A, 7A), whereas curves 2, 4, 5, and 6 are approximated by the nonlinear regression of the third order (curves 2A, 4A, 5A, and 6A). Unlike the data in Fig. 2.13, the results in Fig. 2.14 indicate the differentiated change in q_q within several days for the same objects during increasing soil drought and the corresponding reduction in the PQ^{2-} pool. Such work was performed for the first time, and its results reliably emphasize the ability of the PAM method to make numerous measurements of q_E and q_q, and, consequently, monitor the oxidation–reduction state of the chloroplast's energy status using the same vegetational material in situ.

Earlier, we found the identity of q_q and q_E changes for leaves when dehydration was induced by γ-irradiation and atmospheric drought, with analogous parameters for algae with changeable water content (poikilohydric). The same concerns the short-term influence of high temperatures. Data, brought to the reader's attention in this book, from studies of the long-term dynamics of q_E and q_q change correspond well to material obtained for drying mosses and lichens. Rehydration of these objects restores photochemical transformation of the energy, causes fluorescence growth, increases the q_q value, and leads to q_E relaxation (Heber et al. 2001).

The considered q_E and q_q dynamics are tightly interfaced to the change in other coefficients of fluorescence quenching. The level of base fluorescence (F_0) arising after dark adaptation of a leaf, when all RCs are open, membranes are in the non-energized state, and the first stable acceptor of PS-2—Q_A^+ is completely oxidized, corresponds to $q_q = 1$ and $q_E = 0$. The influence or aftereffect of EFEs results in an increase in F_0^t (in any time interval t) and to a simultaneous change in the state of PS-2 pigments, inhibition of Q_A reduction, and accumulation of its oxidized funds caused by rupture of the electron transport onto Q_B, that is, by

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destruction of PS-2 RCs. An increase in the intensity of EFE influence reduces the F_V/F_0 ratio (F_V is variable Fl, indicating the change in q_E of the non-photochemical quenching), which is one more significant criterion speaking for the damage of PS-2 ETC and occurs due to reduction of the link $P^*_{680}PheoQ_A^-$. The e^- transport in PS-2 RCs starts from the charge separation between P_{680} in the first excited singlet state and Pheo and represents the e^- transfer from the secondary donor (Z) to the primary and secondary acceptors Q_A and Q_B of the quinone type (see Eq. (2.1), shown again here for convenience):

$$e^{-\underline{h\nu}} \rightarrow ZP_{680}PheoQ_AQ_B\underline{^1} \rightarrow ZP^*_{680}PheoQ_AQ_B\underline{^2} \rightarrow ZP^+_{680}Pheo^-Q_AQ_B\underline{^3}$$
$$\rightarrow ZP^+_{680}PheoQ_A^-Q_B \rightarrow PQ^{2-}$$

During photoinhibition induced by intense light, damage to the e^- transport at stages 2 and 3 is found, that is, at the disorder of charge separation between P_{680} and Pheo. In the state $P_{680}Q_A^-$, there is no photochemical transformation of energy. The intermediate Pheo works as a mediator between P_{680} and Q_A, promoting the primary charge separation (forming $P^+_{680}Pheo^-Q_A^-$). It is supposed that F_V should be the "recombinant luminescence" at the transition $P^+_{680}Pheo^-Q_A^+ \rightarrow P^+_{680}Pheo^-Q_A^- \rightarrow P^*_{680}PheoQ_A^-$. A decrease in F_V value directly indicates suppression of PS-2 activity and damage of e^- transport from P_{680} to Q_A^+, caused by inhibition of charge migration between P_{680} and Pheo and retarding of the transformation of light energy into the energy of ATP chemical bonds (i.e., damage to the phototrophic function in PS-2 RCs occurs). The EFE influence on a leaf reduces the yield of F_V, F_{V1}, and F^t_{V1} and increases the q_E value, which proves the existence of the primary damage and its localization close to RCs of PS-2 (Krause and Weis 1984; Lichtenthaler 1988a, b, 1996a, b; Schreiber and Bilger 1987; Bolhar-Nordenkampf et al. 1989; Saakov 2000a–e, 2004). In this case, it is necessary to carefully distinguish between the reduction of the specified coefficient and the F_0 increase (Bolhar-Nordenkampf et al. 1989).

Thus, the functional mechanism of the light energy conversion created by nature simultaneously defines the tolerance of phototrophic cells to EFE influence, which corresponds to the position of academician E.M. Kreps (1976) and to the idea of interpenetration of all evolutionary processes on the Earth and their involvement in a uniform and eternal energy flux of life existence that assumes different shapes.

Our experiments and the data of European scientists using the PAM method provide a new orientation to the development of research on plant tolerance to the influence of various EFEs, connecting these studies with target works on biotechnology for use in the process of selection of tolerant cultivars genetically modified by polygene systems.

At the end of this section, the authors consider it their pleasant duty to express great thanks to Prof. Dr. Hartmut Lichtenthaler (University of Karlsruhe) and to Prof. Dr. H. Bolhar-Nordenkampf (University of Vienna) for the fruitful discussion on questions discussed in the section.

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2.2.4 The Influence of Na^+, Cl^-, and SO_4^{2-} Ions on the Change in Pulse-Amplitude Modulated Fluorescence Kinetics. Resistance Features of the Phototrophic Function of Photosystem 2 at Salification

The salt tolerance of photosynthesizing green cells is linked to features of ion flux changes in higher and lower organisms and takes a special place in the development of the resistance theory of *Procaryota* and *Eucaryota* because of the existence of a special physiologically unique group of halophytes. Despite long-term study of the causes and effects of salt tolerance, the variety of works ascertaining the appearance of secondary effects as a result of the salt stress do not mention the primary, deep reactions of the salt tolerance mechanism. Because of methodological insufficiency, cornerstone questions arise about the interrelation between ionic transport and its influence on the realization of general energy processes in a green cell.

European experiments of the last 70 years performed at the appropriate methodological level with spinach chloroplasts have provided assumptions about the association of activity of the PS-2 RCs with primary electron transfer and with the aggravation of competition between primary oxidizing equivalents in response to salt stress (Diner 1974). Meanwhile, an increase in the fluorescence yield of chloroplast suspensions was shown, indicating disorder of ETC activity in response to salification, in many respects similar to the influence of the herbicide diuron. As a working hypothesis, the authors assumed inhibition of the RCs by salification, which they noted for Mg^{2+} ions (Malkin and Siederer 1977). McSwain et al. (1976) came to similar conclusions. They showed the kinetics of change in levels "O" and "P" of the Kautsky curve under the joint influence of Na^+ and Mg^{2+} ions on the RCs of PS-2 and the energy distribution between the two photosystems. By investigating membranes of fragments of blue-green alga *Nostoc muscorum*, they found inhibition of reductant formation. These data were supported by the work of Wydrzynski et al. (1975), who also determined the decrease in levels "O" and "P" of the Kautsky curve of spinach chloroplasts. On the basis of changes in the amplitude ratio F_{695}/F_{685}, a conclusion was made about the influence of ionic agitation on PS-2 RCs. At the same time, for the halophyte green alga *Dunaliella tertiolecta*, the tolerance of thylakoid membrane preparations under the NaCl influence (1.5 M) was manifested as an increase in oxygen outflux, that is, as the effective work of photosynthetic ETC (Aoki et al. 1986). Studies on rice (*Oryza sativa*) with the codA incorporated gene for choline oxidase from the bacteria *Arthrobacter globiformis* and able to synthesis glycine betaine showed that this transgenic plant is more tolerant to photoinhibition and quicker to adapt to salt stress (Sakamoto et al. 1998, 1999). Transgenic cells of the rice mutant exhibited tolerance in 150 mM NaCl solution, whereas the control manifested complete disorder of PS-2 activity (Hoshida et al. 2000). Similarly, transgene cells of the *Synechocystis* sp. mutant PCC603 with damage to the mono-unsaturated fatty acid content lose

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PS-2 activity in comparison with the wild type in the presence of 0.5 M NaCl or LiCl (Allakhverdiev et al. 1999).

Thus, analysis of literature suggests that damage to the physiological functions of green cells under salt stress is probably caused by inhibition of the energy processes. We have previously published a review of structural changes in leaf chloroplasts under the influence of salt stress (Udovenko and Saakov 1976).

For the successful adaptation of a green cell to stress, our data (Saakov 2000c, 2001a, 2002a, b) indicate the necessity for the combination and cooperative interaction of three features:

1. An optimum structural state of the chlorophyll *a*–protein complex
2. The ETC activity inherent in the object and photophosphorylation
3. The activity of fermentative systems providing the habitual volume of CO_2 fixation and the activity of the Calvin cycle

From the above-cited material, it seems that, until recently, a number of studies were carried out with chloroplast fragments or other preparative structures. The conclusions drawn from them about the higher PS-2 lability became axiomatic, although they could not fully describe the true functional reactions and physiological processes in vivo. In this regard, we would like to rebuff with all clarity the statements about the lack of novelty of in vivo experiments with a leaf or integral suspensions of algae in comparison with those on chloroplasts.

Such a position only emphasizes the misunderstanding of some researchers of the experimental essence of a study carried out with contactless methods and *without damage* to the structure inherent in the intact photosynthetic device. Furthermore, contactless methods allow the researcher to come back after a specified time interval to the same leaf and to again obtain information on the course of its physiological processes. One such method is that of PAMF, which was acknowledged as possessing important informativeness, speed, and short response time. Since the end of the 1980s, this method has proved itself in leading laboratories of Germany, the USA, and France, but due to financial difficulties, it is only minimally available in Russia (Saakov 2000b, 2001a, 2002a).

In spite of that, details of the method have been published (Saakov 2000c, 2001a, 2002a), and it is necessary to describe some of its features.

The stationary, dark Fl (F_0) (or as it is sometimes called the base Fl) and the variable Fl (F_V), showing the activity of PS-2 at reduction $Q_A^+ \rightarrow Q^-$ and leading to closing of PS-2 RCs, can be registered with the impulse fluorometer Walz 101-103 (Effeltrich, Germany). The peculiarity of this device is the possibility of separation of an experimentally induced Fl signal from the significantly more intense excitation light, which is possible due to the application of primary ($\lambda < 670$ nm) and secondary ($\lambda > 680$ nm) optical filters. The latter protect the photodetector from the diffused light of the excitation source. Emission detectors and the selective amplifier allow fluorescence measurements to be carried out *in the light*. This feature of the device promotes its active application in ecological field research studying the influence of a variety of stress factors of natural and technogenic character. If necessary, the data digitalization or their input in packages

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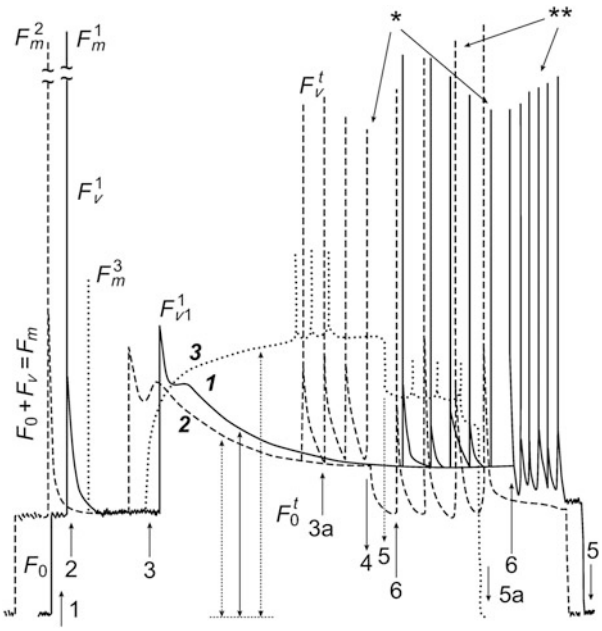

Fig. 2.15 The character of PAMF signal harmonics change for native cells of halophyte *Dunaliella salina* in response to salt stress. *Curves*: *1* control; *2* experiment: influence of 2.5 % $MgSO_4$ solution for 20 min; *3* experiment: the same as *2*, but showing the influence of 6 h of exposure. *Arrows*: *1* and *5*, respectively, input and shutdown of the modulating stream of light flux of frequency of 1.6 kHz (5 μE/(m^2 s), $\lambda < 670$ nm); *2* input of the saturating 1 s impulse of white light (2,500 μE/(m^2 s)) for determination of F_m and F_V values; *3* input and *4* shutdown of the actinic light (600 μE/(m^2 s)); *3a* input of 1 s impulse of the saturating light against the actinic light for determination of F_V^t values; *6* input of 1 s impulses of the saturating light after shutdown of the actinic light. *Arrows* with *one asterisk* show the F_V^t level recession; *two asterisks* indicate the growth of "spikes 6 min" (Saakov 2000b, 2001a, b, 2002a–c) after shutdown of the actinic light

Photoshop CS3 or *Origin* 6.0–8.0 can be performed with the help of the program *Graph Digitizer* 2.14 according to N. Rodionov (see http://nick-gd.chat.ru).

The choice of research objects was dictated by the desire to investigate vegetational cells of various plant systematics and plants that were either salt tolerant or easily damaged by this factor. As experimental plants, we used suspensions of a halophyte alga *Dunaliella salina*, clover leaves (*Trifolium* sp.), tobacco (*Nicotiana tabacum*), haricot (*Phaseolus vulgaris*), beech (*Fagus sylvatica*), acacia (*Robinia pseudoacacia*), etc. Salt solutions of necessary concentration were introduced into the culture or infiltrated in a leaf.

Figures 2.15, 2.16, 2.17, and 2.18 and in Table 2.5 show the results of experiments on the influence of salification on the change in PAMF signal harmonics and the corresponding coefficients of Fl quenching. From the provided data, it can be seen that salt stress causes a change in F_0 and F_0^t levels (F_0^t is the F_0 value in any interval of time t). It is necessary to remember that F_0 arising under the influence of a modulating flux of 1.6 kHz (5 μE/(m^2 s), $\lambda < 670$ nm) is characterized by the open

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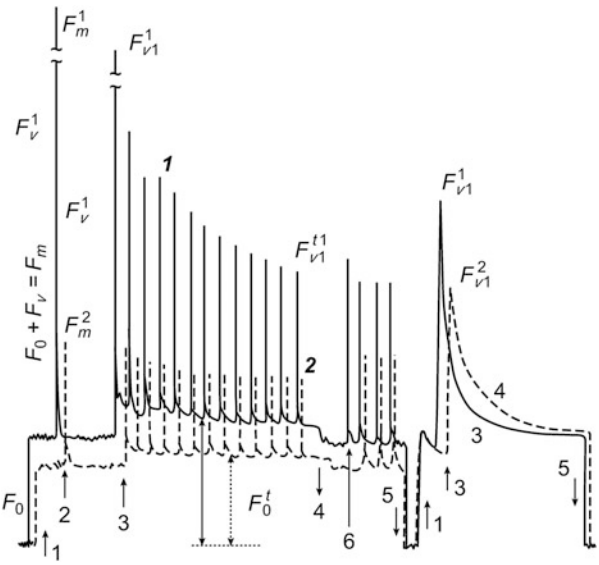

Fig. 2.16 The character of PAMF signal harmonics change for *Trifolium* sp. leaves in response to salt stress. *Curves*: *1* control; *2* experiment: effect of a solution of 0.5 % $MgSO_4$ for 15 min; *3* induction of F_{V1}^1 of the control; *4* the same experiment (F_{V1}^2) under the influence of only actinic light. *Arrows 1*, *2*, *4*, *5* as in Fig. 2.15. *Arrow 3* for *curves 1* and *2*: the simultaneous input of actinic light and the 1 s pulse light. *Arrow 3* for *curves 3* and *4*: input of only actinic light

RC state before migration of electrons into open RCs due to excited chlorophyll *a* emission and further reduction of the first stable acceptor of PS-2 $Q_A^+ Q_B^-$. At this time, thylakoid membranes of leaves are in a non-energized state and coefficient $q_q = 1$ and $q_E = 0$ (Saakov 2000c, 2001a, 2002a).

The prolonged influence of salification is accompanied by an increase in the F_0^t level. There is considerable similarity between the F_0 dynamics observed and the influence of other factors of extreme impact (Saakov 2000c, 2001a, 2002a). The induction of the maximum Fl (F_m) and its component (the variable F_V) by an impulse of saturating light is accompanied by RC closing. The decrease in the F_V yield corresponds to manifestation of the photoinhibition effect and shows the impossibility of capture of light energy from the antenna chlorophyll in RCs. With the increase in the impact of the influencing factor, and especially in the process of the aftereffect, a decrease in the ratio F_V/F_0 occurs that can be considered as the significant feature characterizing damage to the $P^*_{680}PheoQ^+_A$ reduction process. Intermediate Pheo acts as the mediator between P_{680} and Q_A and promotes the primary charge separation, with the formation of $P^*_{680}Pheo\ Q_A^-$. It follows that the decrease in the F_V level shown in Figs. 2.15, 2.16, 2.17, and 2.18 (experimental curves 2 and 3) suggests the suppression of PS-2 activity. More exactly, there is suppression of its link $P^*_{680}Pheo\ Q_A^+ \rightarrow P^*_{680}PheoQ_A^-$ connected with the e^- transfer that promotes the inhibition of the light energy into the energy of ATP chemical bonds. As a consequence, there is a significant change in the ratios F_V/F_m and F_m/F_V, indicating damage to PS-2 activity and its response to the influence of salt stress (Table 2.5). The specified values are widely used to explain the adaptive mechanisms during photoinhibition. However, discussion of the large amount of literature on this matter is beyond the scope of this book.

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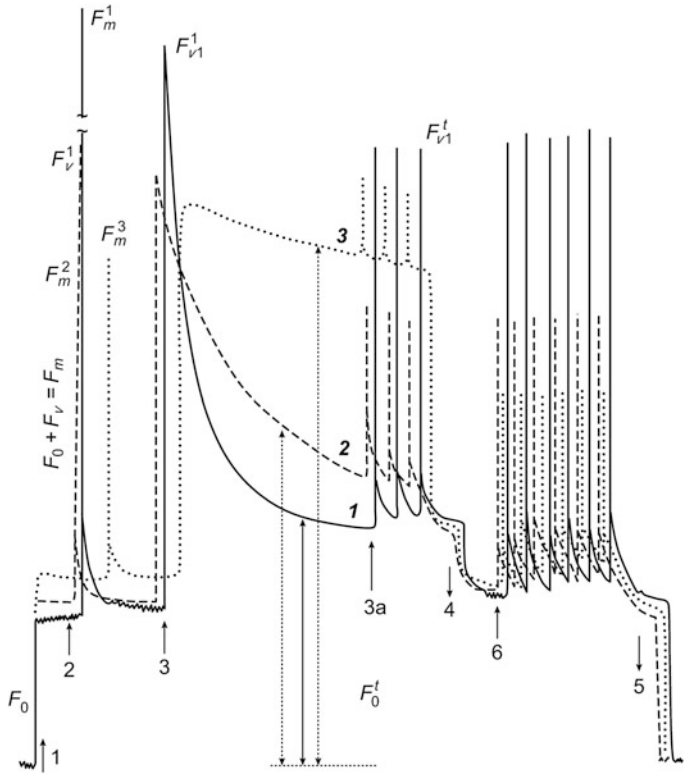

Fig. 2.17 The character of the signal harmonics change in PAMF for haricot leaves in response to salt stress. *Curves*: *1* control; *2* experiment: influence of 1.4 % NaCl solution for 15 min; *3* experiment: under the same influence after 24 h. *Arrows*: *1*, *2*, *3*, *3a*, *4*, *5*, *6* as in Fig. 2.15

The significant decrease in the ratio $F_{\text{V experiment}}/F_{\text{V control}}$ similarly indicates the suppression of PS-2 activity in the experimental variant and corresponds well to the earlier-stated reasons about the place of localization of the damaging influence of EFEs in RCs of PS-2 (Saakov 2000c, 2001a, 2002a).

The experimental curves in Figs. 2.15, 2.16, 2.17, and 2.18 show the reliable change in F_{V1} amplitude (including the F_{V1}^t amplitude) that is accompanied by a simultaneous increase in the value of q_{E}, which indicates the suppression of energy utilization in the Calvin cycle during the corresponding increase in the proton gradient coupled with a decrease in intensity of ATP store formation. The low value of the level of PS-2 reduction (Table 2.5) also testifies convincingly to its damage.

Thus, the yield decrease in F_{V}, F_{V1}, and F_{V1}^t in response to salt stress is similar to the change in these PAMF parameters in response to diverse extreme influences (Saakov 2000c, 2001a, 2002a) and rather reliably points to the existence of primary damage and its localization in the RCs of PS-2. This means that, obviously, the

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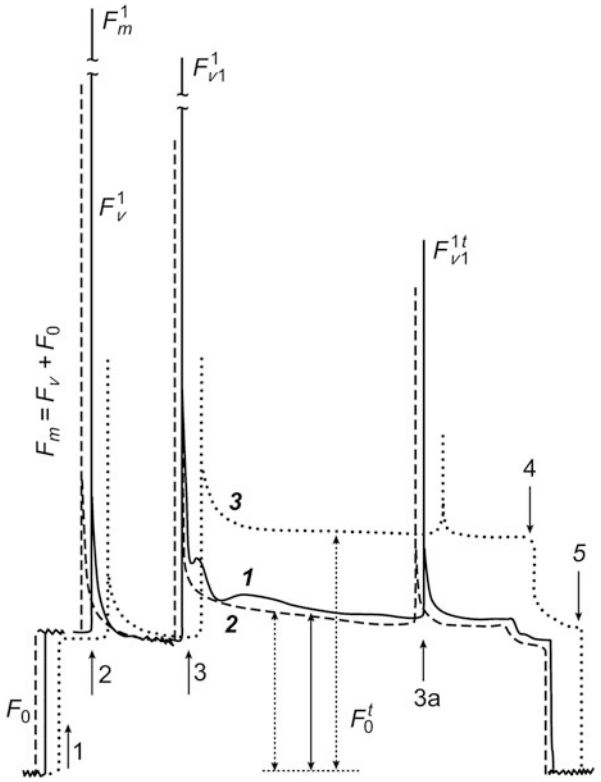

Fig. 2.18 The character of the signal harmonics change in PAMF for *Fagus sylvatica* leaves in response to salt stress. *Curves*: *1* control; *2* experiment: influence of 1.5 % NaCl solution for 15 min; *3* experiment: under the same influence after 24 h. *Arrows*: *1*, *2*, *3*, *3a*, *4*, *5*, as in Fig. 2.15

inhibition of energy transmission in the links of PS-2 RCs is the primary event damaging electron transport, uncoupling of photophosphorylation, and formation of a reductant. The integrity of exactly this link can be the defining factor during an assessment of adaptive and tolerant reactions of the photosynthetic device under the influence of stress.

In the steady state of photosynthesis, the value of coefficient q_q is close to 0.8–0.9, and its change by 0.03–0.04 relative units reliably proves the response of the photosynthetic device (Saakov 2001a). At $q_q = 0$, there is no transformation of light energy into the energy of chemical bonds, and at $q_q = 1$, this transformation is optimal. The fast decrease in the q_q value during the initial moments of illumination of the object is caused by ETC reduction, and the increase in this coefficient indicates Q_A^- re-oxidization. The low level of q_q is the result of electron accumulation in the acceptor part of PS-2 and the high level of reduction of Q_A^- and PQ^{2-} pools.

In other words, low values of q_q indicate the functional damage of ETC (Table 2.5, experimental variants), and a high q_q value emphasizes the active transport of electrons and a high level of NADPH/H^+.

Stress arising under the influence of Na^+, Cl^-, Mg^{2+}, and SO_4^{2-} ions naturally induces changes in the reactions of carbon metabolism and in the synthesis of

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Table 2.5 The pattern of salinization-induced changes in the quenching coefficients of modulated fluorescence[a]

Object	Variant of experiment	Measured coefficient, arithmetic mean of $n = 6$, $p < 0.085$)											
		F_V/F_m	$F_{V\ exper}/F_{V\ contr}$	PS 2 reduction (%)	F_m/F_0	F_V/F_0	F_{V1}/F_V	F_{V1}/F_0	F_0^5/F_0	$q_E^{5\,min}$	$q_q^{5\,min}$	Spikes 5 min (mm)	Spikes 6 min (mm)
Dunaliella salina	Control	0.841		94	4.44	3.57	0.87	3.3	1.15	0.38	0.94	94	106
	2.5 % Na_2SO_4, 15 min	0.811	0.935	91	4.1	3.45	0.81	3.15	1.12	0.40	0.91	92	101
Trifolium sp.	Control	0.839		89	4.8	3.61	0.84	4.1	1.23	0.33	0.89	67	87
	0.5 % Na_2SO_4, 15 min	0.673	0.802	58	3.12	1.86	0.73	2.0	1.54	0.73	0.42	34	37
R. pseudoacacia	Control	0.824		91	5.1	3.78	0.91	4.2	1.21	0.41	0.93	72	89
	0.6 % NaCl, 10 min	0.675	0.819	67	2.9	1.52	0.68	2.89	1.62	0.68	0.66	33	34
Artemisia sp.	Control	0.826		90	4.7	3.71	0.90	3.71	1.23	0.42	0.91	72	87
	1 % NaCl, 20 min	0.805	0.503	88	4.6	3.65	0.88	3.68	1.21	0.51	0.89	70	85
Artemisia sp.	Control	0.829		94	5.14	4.06	0.92	3.92	1.13	0.35	0.94	74	85
	1 % NaCl, 6 h	0.661	0.804	49	3.12	2.3	0.72	1.57	1.65	0.67	0.49	25	33
Fagus sylvatica	Control	0.817		89	5.1	3.83	0.93	3.84	1.20	0.38	0.90	89	93
	0.8 % NaCl, 25 min	0.665	0.813	41	3.24	1.71	0.82	1.64	1.63	0.67	0.42	31	36
Solanum lycopersicum	Control	0.826		91	5.19	4.27	0.891	3.72	1.14	0.46	0.921	72	83
	1.3 % NaCl, 15 min	0.727	0.880	52	3.71	2.28	0.754	1.93	1.31	0.78	0.48	36	33
Fraxinus ornus	Control	0.825		87	5.5	4.71	0.88	4.34	1.3	0.39	0.87	73	75
	0.8 % Na_2SO_4, 15 min	0.634	0.768	39	3.12	2.63	0.76	2.1	1.87	0.76	0.40	29	34
Nicotiana tabacum	Control	0.816		90	4.7	3.81	0.94	3.83	1.17	0.34	0.90	78	89
	0.4 % NaCl, 20 min	0.799	0.979	37	2.75	1.78	0.77	11.4	1.43	0.73	0.375	24	37

[a] $q_E^{5\,min}$, $q_q^{5\,min}$ and spike 5 min were calculated 5 min after switching the actinic light on; spike 6 min—6 min after switching the actinic light off

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proteins, pigments, and other components of a cell. However, these are the stages of deeper, secondary reactions of *adaptation processes* or of a lethal outcome, whereas the *primary processes* of damage to the functional state of PS-2 RCs define the *non-specificity* of the reactions inherent in *Procaryota* and *Eucaryota*.

In the course of manuscript preparation, we became aware of the work by Chauhan et al. (2001), the results of which are indicative. The mutant of blue-green alga *Anabaena variabilis* is tolerant to NaCl influence, characterized by slow inclusion of Na^+ ions and also by the high content of glycine betaine (Sakamoto et al. 1999), and showed inhibition of the Na^+ ion flux under the influence of diuron. This allowed us to interpret the results of the work (Chauhan et al. 2001) as the evidence of connection between the ion transport and the PS-2 energetics. The inhibition of the Na^+ ion flux was found under the influence of KCN, dinitrophenol, and sodium azide. The effect of the last two inhibitors suggests a link between the Na^+ ion flux and the photophosphorylation activity. At the same time, the authors of the work (Chauhan et al. 2001) suggested the possibility of utilization of oxidative phosphorylation energetics for the regulation of the Na^+ flux, which corresponds to the point of view of our work on the existence of compensatory replacement of energy funds under the influence of stress (Saakov 2002a). On the other hand, we showed earlier the destructive effect of inhibitors of photosystems, of uncouplers of photophosphorylation, and of KCN on structures of the ChPC (Saakov 1973). From this point of view, the restriction of the Na^+ flux can be explained by the damage to ChPC integrity, which is the necessary condition of ETC functioning and of normal adaptation of a green cell to a stress.

We have not yet clarified the dependence of the suppression of F_m, F_V, and F^t_{V1} on the anion valency. However, from the data in Table 2.5 and Figs. 2.15 and 2.16, it is possible to suppose a larger decrease in the ratio F_m/F_V in the case of SO_4^{2-} ions. This position corresponds well to the data in the work by Jajoo et al. (one of the authors, Govindjee, is a known expert in fluorescence analysis), in which the generalization was made that the valency increase in an anion row (Cl^-, SO_4^{2-}, PO_4^{3-}) reduces the ratio F_m/F_V and the electron transport in PS-2 and adequately increases the energy flux in PS-1 (Jajoo et al. 1998).

Thus, the material of the present work together with a literature analysis (Diner 1974; Malkin and Siederer 1977; Wydrzynski et al. 1975; McSwain et al. 1976; Udovenko and Saakov 1976; Aoki et al. 1986; Allakhverdiev et al. 1999; Sakamoto et al. 1999; Chauhan et al. 2001) allows us to make a conclusion about the primary localization of the negative influence of ions Na^+, Cl^-, Mg^{2+}, and SO_4^{2-}, substantially defining the manifestation of a salt stress, in the link of PS-2 RCs connected with the energy transfer $P^*_{680}PheoQ_A^+ \rightarrow P^*_{680}PheoQ_A^-$. The generality of the presence of chlorophyll *a* in *Procaryota* and *Eucaryota* and also data from the above-discussed works (McSwain et al. 1976; Aoki et al. 1986; Allakhverdiev et al. 1999; Chauhan et al. 2001) confirm our statement on the close interconnection of tolerance and reparation reactions of a green cell and the functional stability of the link $P_{680} \rightarrow Q_A \rightarrow Q_B \rightarrow PQ^{2-}$ and expand the idea of the energetic basis for green cell tolerance to the influence of stress factors.

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The ideas stated by us (Saakov 2000c, 2002a) about the change in the qualitative orientation of works on the target-intensive selection of cultivars genetically modified by polygene systems, transformed in their systems of electron transport in PS-2, have found support in studies of tolerant transgenic plants (Allakhverdiev et al. 1999; Sakamoto et al. 1998, 1999; Hoshida et al. 2000; Chauhan et al. 2001), although it is still necessary to specify the role of glycine betaine in salt tolerance.

2.2.5 *The Concept of the Energetic Basis of Green Cell Resistance to the Influence of Extreme Environmental Factors*

The working hypothesis suggested by us about the localization of damaging influences of environmental factors in the RCs of photosystems and in the centers of pigment biosynthesis in the photosynthetic device (Baranov et al. 1974; Saakov et al. 1975; Saakov 1976; Saakov and Leontjev 1988) was supported by European works on fluorescence changes under the influence of EFEs of natural and anthropogenous origin (Krause and Weis 1984; Lichtenthaler 1988a; Briantais et al. 1986). Subsequent years promoted the accumulation of facts in support of the put-forward hypothesis (Saakov 1987; Saakov 1990, 1992, 1996). Successes in this direction were connected first of all with the improvement in electron-optical methods of diagnostics of the stability of chloroplasts and cells, especially after introduction of the PAM fluorescence method (Lichtenthaler 1988a), which manifested as a stream of works in specialized journals and monographs (Lichtenthaler 1988a). Experimental data of the recent past (Saakov 1993a–d, 2000a–e; Saakov and Shiryaev 2000) supported our position and the conclusions of European researchers (Lichtenthaler 1996b, 1988b; Lichtenthaler and Rinderle 1988; Schreiber et al. 1994; Schreiber and Bery 1977) and gave confidence about the correctness of the chosen research direction.

Our task consisted in the comparative study of PAM kinetics under the influence of γ-radiation, temperature , drought, fumigation, and herbicides to obtain information on the *specificity* or *non-specificity* of the response of the ETC of the photosynthetic device to the impact of chosen EFEs. We emphasize the use of simultaneous comparative investigation of different factors using the same objects in a specified time interval.

The work was carried out with the help of a PAM fluorometer (Walz 101-103; Effeltrich, FRG) by the signal harmonics registration method tested in different countries. The calculation of photochemical (q_q), non-photochemical (q_E), and other coefficients of Fl quenching and the interpretation of their changes were performed by the accepted technique (Krause and Weis 1984; Briantais et al. 1986; Snel and van Kooten 1990; Lichtenthaler 1996a, b), and the results are presented in Table 2.6. As objects of research, we used the leaves of plants *Phaseolus vulgaris* L., *Robinia pseudoacacia* L., and *Nicotiana tabacum* L., which are different in tolerance.

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Table 2.6 Effects of environmental stressors on quenching coefficients of PAM fluorescence from leaves of various species[a]

Species and actinic light intensity	Stressor	Coefficient (mean value; $n = 4$, $p < 0.05$)											
		F_V/F_m	$F_{V\ exp}/F_{V\ control}$	Degree of PSII restoration (%)	F_V/F_0	F_{V1}/F_V	F_{V1}/F_0	F_0^5/F_0	$F_{V1}\ h/2$ (mm)	$q_E^{5\,min}$	$q_q^{5\,min}$	Spikes 5 min (mm)	Spikes 6 min (mm)
Robinia, 185 μE/m^2 s	Control	0.823	–	61	4.96	0.95	4.7	1.31	5	0.786	0.70	25	117
	Irradiation 8 kGy	0.804	0.880	28	4.24	0.935	3.96	2.16	45	0.45	0.26	20	74
	2 h after irradiation 8 kGy	0.800	0.880	42	4.0	0.935	3.96	1.93	27	0.62	0.40	21	68
	Low humidity (30 % of the normal)	0.808	0.827	14	4.29	0.877	3.77	2.26	18	0.74	0.117	5	28
	5 % SO_2 for 3 h	0.8	0.830	49	4.3	0.930	3.8	1.8	18	0.7	0.31	17	97
Phaseolus, 185 μE/m^2 s	Control	0.837	–	86	5.32	0.848	4.5	1.29	4	0.612	0.843	54	125
	Irradiation 3.5 kGy	0.83	0.927	74	5.1	0.88	4.51	1.5	17	0.64	0.71	38	94
	117 h after irradiation 3.5 kGy	0.789	0.703	54	3.74	0.922	3.45	2	16	0.43	0.53	34	93
	Heat stress (45 °C for 10 min)	0.751	0.751	25	3.45	0.958	3.31	1.68	14	0.74	0.212	7	41
Nicotiana, 590 μE/m^2 s	Control	0.850	–	62	5.7	0.906	5.16	1.46	5	0.842	0.46	15	91
	17 h after irradiation at 0.6 kGy	0.811	0.754	59	4.3	0.837	5.16	1.87	6	0.577	0.49	26	97
	200 h after irradiation at 0.6 kGy	0.821	0.807	29.4	4.33	0.949	4.3	2.6	20	0.51	0.24	16	102
	5×10^{-3} g/l methyl viologen	0.837	0.933	32.4	5.16	0.831	4.29	1.70	4.5	0.786	0.29	10	61

[a]F_0^5, $q_E^{5\,min}$, $q_q^{5\,min}$ and spike 5 min were calculated 5 min after switching the actinic light off; spike 6 min—6 min after switching the actinic light off

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Results of experiments on the kinetics of the change in PAMF signal harmonics under the influence of and during the aftereffect of various EFEs are presented in Figs. 2.19 and 2.20.

The F_0 level of the base dark Fl characterizes the open RC state before electron migration into the open RC due to the excited chlorophyll *a* emission and, therefore, before reduction of the first stable acceptor PS-2 $Q_A^+ \rightarrow Q_A^-$. At this time, the thylakoid membranes of leaves *adapted for darkness* are in the non-energized state. The F_0 quenching shows the decrease in the absorbed energy directed to PS-2. Even the first saturating impulse of light of 1 s duration and 3,000 μE/(m^2 s) intensity (Figs. 2.19 and 2.20, point 2) leads to an increase in maximum Fl (F_m). Its first increase, the variable Fl (F_V), defines the degree of Q_A^- reduction (Table 2.6) and is accompanied by RC closing.

The F_V yield change corresponds to the energy transformation in PS-2 and points to the stay at minimal non-photochemical processes (Krause and Weis 1984; Briantais et al. 1986; Saakov and Shiryaev 2000). It follows from Figs. 2.19 and 2.20 that the damaging EFE influence on thylakoids usually reduces the F_V yield, which proves the existence of the primary damage and its localization close to the RCs of PS-2 (Lichtenthaler 1988a, b, 1996a, b; Saakov 1993a, b; Snel and van Kooten 1990). The decrease in the ratio $F_{V\ experiment}/F_{V\ control}$ (Table 2.6) also emphasizes the suppression of PS-2 activity in the experiment. With a certain level of confidence, it is possible to speak about the existence of a functional link between the PS-2 activity and other components of the photosynthetic ETC and the change in F_V, F_{V1}, and F_V^t coupled to changes in the activity of the photosynthesis process as a whole (Krause and Weis 1984; Lichtenthaler and Rinderle 1988; Briantais et al. 1986; Lichtenthaler 1988a, b, 1996a, b; Saakov 1993a, b, 2000c).

This is supported by the fact that increased temperature leads to a decrease in electronic transport with the increased intensity of the saturating light flux. The disorder of energy transmission between PS-2 and PS-1 is the primary event correlating with the inhibition of electron transport, termination of photophosphorylation, suppression of reducer formation, and suppression of photosynthesis intensity (Saakov 1976, 1996; Krause and Weis 1984; Lichtenthaler and Rinderle 1988; Briantais et al. 1986; Lichtenthaler 1988a, b, 1996a, b; Saakov et al. 1993).

The value of coefficient F_V/F_m is extremely important because it is considered to be an indicator of photosynthetic function and is equal to 0.832 relative units for intact chloroplasts (Krause and Weis 1984; Lichtenthaler and Rinderle 1988; Briantais et al. 1986; Lichtenthaler 1988a, b, 1996a, b; Saakov 1993a, b, 2000c). The deviation of this coefficient from the control value by 0.03–0.04 relative units is considered significant and emphasizes the existence of a negative reaction of the photosynthetic device to the external influence (as illustrated by the data in Table 2.6). The effect or aftereffect of EFEs reduces F_V/F_m for all investigated objects (of different levels of resistance), depending on the depth of damage and the effect of adaptive reactions at F_V quenching.

It is known that the suppression of PS-2 activity (as defined by the F_V suppression) is coupled with inhibition of the charge transfer between P_{680} and Pheo

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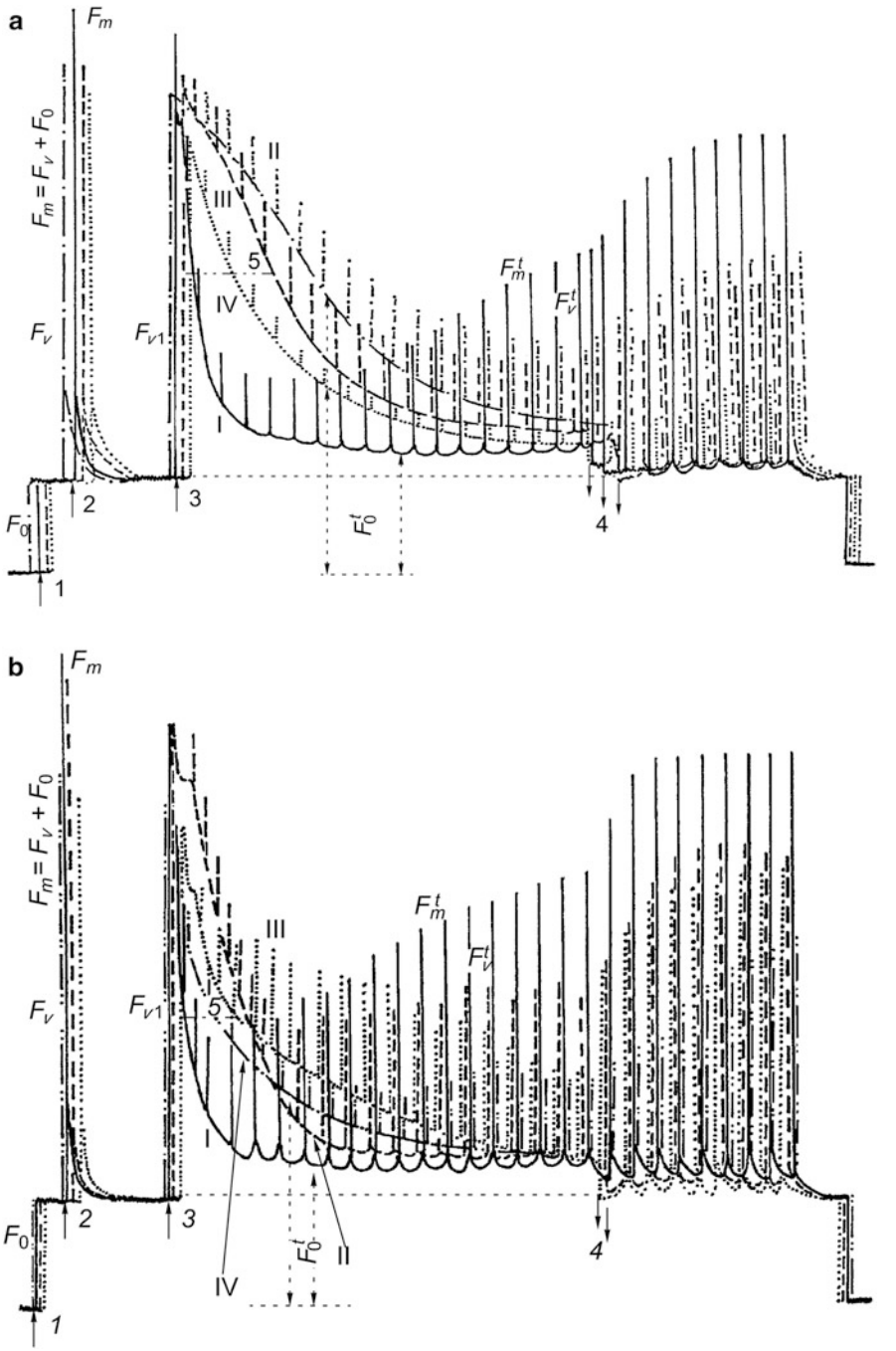

Fig. 2.19 (continued)

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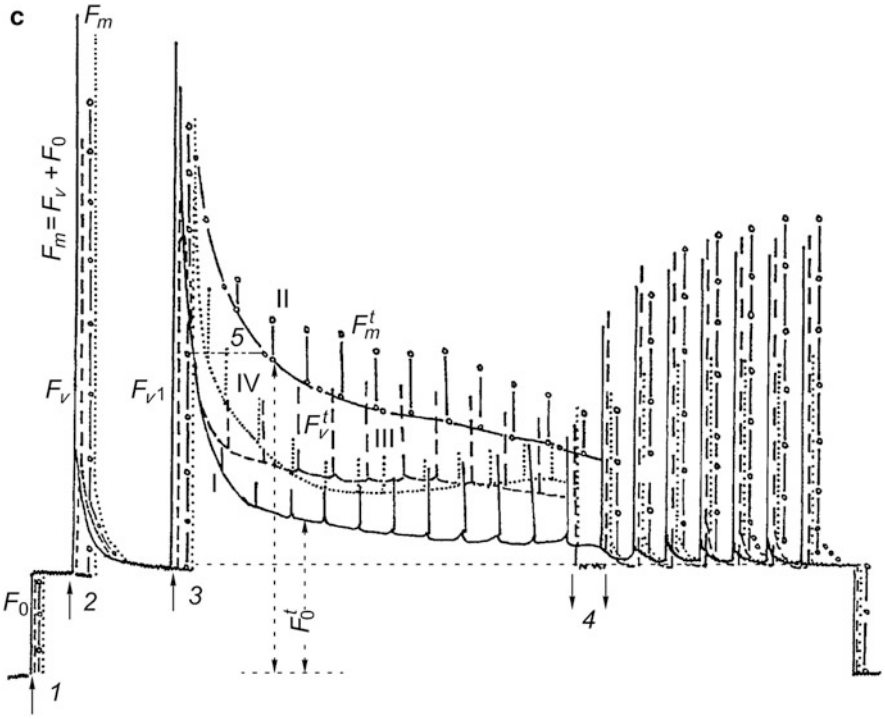

Fig. 2.19 The character of signal harmonics change in PAMF for the chlorophyll *a* of leaves adapted to darkness: (**a**) *Robinia* under the influence of γ-radiation and dehydration. *Curves*: *I* control; *II* influence of a γ-radiation dose of 8 kGy; *III* 2 h of the aftereffect of γ-radiation dose of 8 kGy; *IV* influence of 30 % relative humidity; (**b**) *Phaseolus* under the influence of γ-radiation and high temperature. *Curves*: *I* control; *II* influence of a γ-radiation dose of 3.5 kGy; *III* 117 h of the aftereffect of the γ-radiation dose of 3.5 kGy; *IV* influence of the temperature 45 °C for 10 min; (**c**) *Nicotiana*, the aftereffect of a γ-radiation dose of 0.6 kGy and methyl viologen (paraquat). *Curves*: *I* control; *II* 200 h of the aftereffect of a γ-radiation dose of 0.6 kGy; *III* influence of a γ-radiation dose of 0.6 kGy; *IV* influence of methyl viologen (solution concentration 5 mg/L). For determination of F_0^t and F_V^t values, a combination was used (for **a** and **b**) of a 1 s impulse of white light flux (1,100 μE/(m^2 s)) and 40 s of actinic light (185 μE/(m^2 s)); for **c**, the actinic light influence was 590 μE/(m^2 s) for 60 s, followed by a 1 s impulse of white light (1,100 μE/(m^2 s)). *Arrows*: *1* input of the modulating light flux of frequency of 1.6 kHz (~5 μE/(m^2 s), $\lambda \leq 680$ nm,) for determination of the dark (base) fluorescence value F_0; *2* input of the saturating white light impulse (3,000 μE/(m^2 s)); *3* input of the actinic light flux of frequency of 100 kHz and after 30 s input of the white light impulses (1,100 μE/(m^2 s)) for induction of spikes (F_V^t is the variable fluorescence during the time *t*); *4* shutdown of the actinic light flux for determination of $F_0{}^{11-12\ min}$ values and at the same time input of the light flux of frequency 1.6 kHz; *5* the value F_{V1} *h*/2 introduced by us (Saakov 1993a) and characterizing the F_0^t level at the half-height F_{V1} and the distance F_0^t from F_{V1} (in millimeters or seconds)

(Krause and Weis 1984; Briantais et al. 1986; Saakov and Shiryaev 2000; Lichtenthaler 1988a, b, 1996a, b), that is, with the localization of damage as being in the RCs of PS-2, which corresponds to ideas stated by us earlier (Baranov

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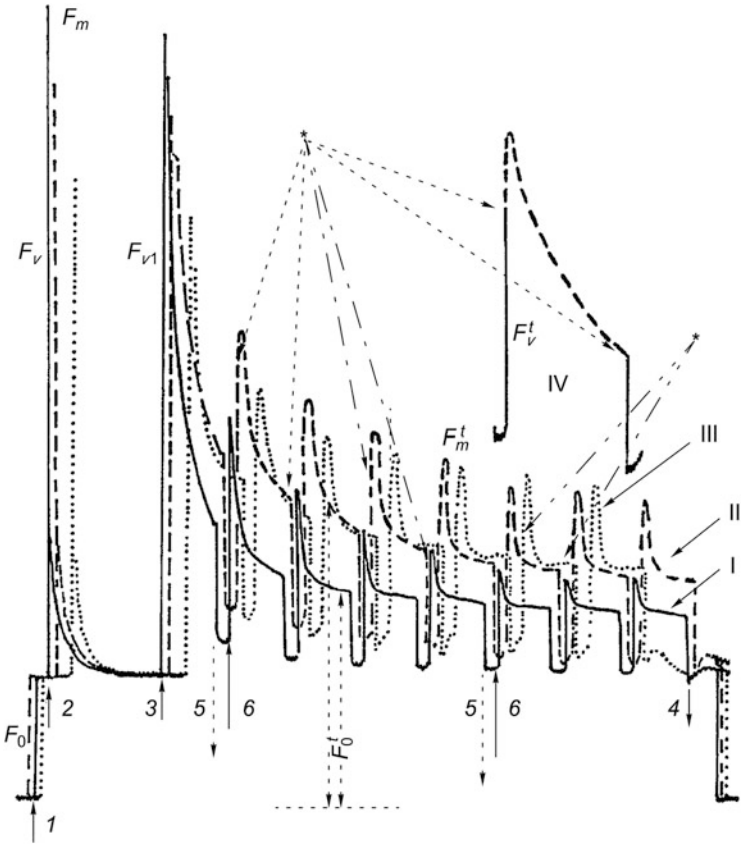

Fig. 2.20 The character of PAMF signal harmonics change for *Phaseolus* leaves adapted to darkness during cycles of light/darkness (80 s/20 s) and light impulses of 1,100 μE/(m^2 s). *Curves*: *I* control; *II* influence of γ-radiation of 1.3 kGy; *III* influence of temperature of 45 °C for 10 min; *IV* closeup view of the impulse 80 s/20 s with the expressed slow component arising in kinetics of PAMF signal harmonics for damaged leaves. *Arrows* with *asterisk*s specify the start of appearance and the termination of the slow component; *5* input and *6* shutdown of the 80 s light impulse. *Arrows 1–4* and other designations as in Fig. 2.19

et al. 1974; Saakov 1976, 1990, 1996; Saakov and Leontjev 1988). Moreover, the F_V value (including values of variable fluorescence at any time t, F_V^t, designated in English literature as "spikes") connected with the low q_E value indicates the active utilization of the energy in the Calvin cycle and the simultaneous decrease in the membrane proton gradient coupled with the ATP formation (Krause and Weis 1984; Saakov and Leontjev 1988; Lichtenthaler 1996a, b). From Table 2.6, it can be seen that during the increasing intensity of influence and aftereffect of extreme factors, the ratio F_V/F_0 decreases, which is one more criterion speaking for the damage in ETC of PS-2 caused by the reduction of the link $P^*_{680}PheoQ_A^-$ (Saakov and Shiryaev 2000).

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After the F_V decrease, switching on an actinic white light flux of intensity 1,100 μE/(m^2 s) induces the level of Fl F_{V1} and F_{V1}/F_V, as well as F_{V1}/F_0 (Table 2.6), which seem to be reliable indicators for characterizing the activity of PS-2 ETC (Saakov et al. 1992; Lichtenthaler 1988a, b, 1996a, b; Saakov and Shiryaev 2000). The higher the amplitude of the F_V^t impulse and the closer it is to the F_{V1} level, the higher the probability of more complete reduction of the Q_A^- pool, and higher activity of ETC and the Calvin cycle.

In this work, the calculation of the coefficient Fl after 5 min of PAMF signal harmonics registration is performed, when there is the most distinction between F_0 and F_0^5. By this time, an increased initial q_E value in the control is noted (Table 2.6), as defined by that after dark exposure (6–24 h); the photosynthesis process has not yet passed the light adaptation; and the stationary activity of the Calvin cycle has not increased. This is emphasized by the corresponding changes in q_E^{11-12} and q_q^{11-12} after 11–12 min of PAMF signal harmonics registration of control leaves (Fig. 2.19a, curve I 0.54–0.52 and 0.86–0.85; Fig. 2.19b, curve I 0.44–0.42 and 0.9–0.905; Fig. 2.19c, curve I 0.758–0.776 and 0.763–0.756), that is, showing complete de-epoxidation of the xanthophyll cycle (Saakov et al. 1992; Saakov 1993a–d), and confirmed by the degree of the PS-2 reduction in the control, the level of which decreases in the damaged objects.

From Figs. 2.19 and 2.20 and Table 2.6, it follows that, with the increase in the negative impact of external factors, the ratio F_0^5/F_0 increases, that is, the F_0^5 level (after 5 min of PAMF signal harmonics measurement) becomes significantly higher than F_0. The change in F_{V1} $h/2$ introduced by us (Lichtenthaler 1988a, b; Saakov 1993a) correlates well with this indicator and is characterized by the band half-width of the PAMF signal harmonic contour, which is described by the F_{V1} height and the course of the F_0^t curve induced by the actinic light flux (Fig. 2.19, point 5). The units of this coefficient can be expressed in seconds or in millimeters. From Table 2.6 and Fig. 2.19, it can be seen that F_{V1} $h/2$ in a certain way corresponds to the level of the reaction of the photosynthetic device caused by the damaging effect of the influencing factor. Earlier we determined the dependence of F_0 or F_0^t on the radiation dose (Saakov et al. 1993; Saakov 1993b). Our obtained results allow us to extend this dependence to the influence of high temperatures, dehydration and fumigation levels, and also herbicide concentration (methyl viologen, diuron, etc.) and agree well with European data (van Kooten and Snel 1990a, pp. 199–211; 241–248; 279–293).

In close connection with the value of coefficient F_{V1}, there is a change in F_V^5 (the value of "spikes 5 min") induced by the saturating light impulse superimposed with the activic light flux (Fig. 2.19, Table 2.6) or at shutdown of the latter ("spikes 6 min"). The F_V^t value is sensitive to thermal processing and is already suppressed by 70 % relative humidity and γ-radiation (Saakov et al. 1993; Lichtenthaler 1996a, b; Saakov and Shiryaev 2000). The effect and aftereffect of γ-radiation correlate with the influence of temperature, fumigation, dehydration, and herbicides and decrease the F_V^t and q_E levels (i.e., reduce the Calvin cycle activity and q_q). This change in F_V^t corresponds to the damage depth of PS-2 energy links that develops in time. It is noted that with an increase in the actinic light flux intensity from 185 to

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590 μE/(m^2 s), the value of the spikes (equal to F_V^t) decreases. Separately, we wish to draw attention to research on the kinetics of changes in F_V^t/F_0^t (Saakov 1993a), because this parameter is also closely coupled with the damage to PS-2 energetics and changes from 3 to 6 relative units for the control and from 1.3 to 0.1 relative units for the experiment (Saakov et al. 1992; Lichtenthaler 1996a, b; Saakov and Shiryaev 2000).

Thus, there is a clear manifestation of the *non-specificity* of the kinetics of PAMF signal harmonics in response to the influence of various EFEs, and features of the change in the coefficients F_m, F_V, F_{V1}, F_V^t, F_0, and F_0^t indicate the interconnection of determined disorders with the damage to components of PS-2 RCs.

The high F_V value (including spikes at F_V^t) is a result of the low q_E value and the high utilization of energy in the Calvin cycle. High temperatures result in increased q_E (to 0.6–0.75), which indicates F_V^t decrease and energizing of the thylakoid membrane. In this case, ETC inhibition occurs, probably when the Calvin cycle is unable to work as an acceptor of electrons and the suppression of its activity is much more probable than the inhibition of ATPase. The q_E relaxation is accompanied by the simultaneous switching on of the Calvin cycle, coupled ATP use, and decrease in the proton gradient. At thermal processing the q_E relaxation is suppressed, testifying to the restriction of Calvin cycle activity. This restriction probably precedes the damage at the PS-2 level and is expressed as an increase in the F_0 level, F_V^t reduction, and F_m^t decrease at the simultaneous growth of q_E. So the low q_E value (0.3–0.4 relative units) indicates the high activity of the carbon cycle reactions. Increases in q_E will not be observed after addition of uncouplers of the proton gradient. Also, after addition of methyl viologen (the electron acceptor PS-1), ATP and $NADPH/H^+$ will not be formed because methyl viologen is a better electron acceptor that directly or indirectly influences the proton gradient and CO_2 fixing.

The fast decrease in coefficient q_q in the first minutes of light exposure indicates ETC reduction. The change in this coefficient by 0.03–0.04 relative units is significant (Krause and Weis 1984; Lichtenthaler and Rinderle 1988; Briantais et al. 1986). The increase in q_q is caused by re-oxidization of Q_A^-. At values $q_q = 0$, there is no transformation of light energy, and at $q_q = 1$, it is optimal. In the steady state, q_q is usually approximately 0.8–0.9 relative units The high q_q value for well-lit plants indicates high levels of electron transport, $NADPH/H^+$, and photosynthesis. Before the leaf falls under the negative influence of environmental factors (Table 2.6), q_q decreases, pointing to suppression of the ETC activity (Krause and Weis 1984; Lichtenthaler and Rinderle 1988; Briantais et al. 1986; Lichtenthaler 1988a, b, 1996a, b; Snel and van Kooten 1990).The high reduction of the PQ^{2-} plasoquinone pool is accompanied by a low q_q value (Lichtenthaler 1988a, b). The q_q decrease to 0.4 indicates ETC reduction, with subsequent re-oxidization and increase in q_q value to 0.9; the decrease in q_q is also accompanied by an increase in the proton current and shows the accumulation of electrons in the acceptor part of PS-2 (Briantais et al. 1986; Lichtenthaler 1988a, b).

The unusual form of spikes obtained during the light/darkness cycles of 80 s/20 s allows tracking of the process of PS-2 reduction with a great degree of reliability

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and to take notice of the *two-phase* or sometimes *three-phase* structure of spikes for damaged leaves (Saakov 1993a–d; Saakov and Shiryaev 2000; Rubin 1987, 1997, 2000a, b, 2004) when the initial fast F^t_V increase slows (Fig. 2.20, point 5) and then starts to decrease smoothly, showing the transition from F_V to F_{V1}. The presence of three components in such a signal was also observed by other researchers (Bukhov et al. 2001b; Egorova et al. 2001). Shutdown of the actinic light flux reduces F^t_V. We suppose that the fast component of PAMF signal harmonics demonstrates the Q_A^- quenching, because Q_A^+ acts as a Fl quencher and in the state $P^*_{680}PheoQ_A$ the transformation of photochemical energy (i.e., formation of $P^*_{680}PheQ_A^-$, of the integral part of RCs) is a convenient opportunity for the de-excitation (Saakov and Shiryaev 2000). The second, short and slower, component (Fig. 2.20, arrows with asterisks) shows the delay of Q_A^+ reduction due to the electron transfer from P_{680} to Pheo. The third, slow, component suggests that ETC damage arises during the e^- transfer from Q_A^- to Q_B^+ (Krause and Weis 1984; Lichtenthaler and Rinderle 1988; Briantais et al. 1986; Lichtenthaler 1988a, b, 1996a, b; Snel and van Kooten 1990). Such a many-component decrease in the fluorescence coefficient underlines the complexity of mechanisms and pathways of Q_A^- re-oxidization. The slow component possibly corresponds to the inhibition (as under the influence of diuron) of the e^- transfer between Q_A^- and Q_B^+ or PQ^{2-} that leads to the q_q decrease, because all quenchers of Fl are reduced and the subsequent re-oxidization is absent. This is supported by the gradual decrease (relaxation) of the slow component after input of the actinic light flux. Shutdown of the light flux results in the fast re-oxidization of Q_A and Q_B and in the e^- transfer to PS-1 and the Fl signal decrease to F^t_0.

Acquaintance with the literature (Lichtenthaler and Rinderle 1988; Briantais et al. 1986; Lichtenthaler 1988a, b, 1996a, b; Snel and van Kooten 1990) and our own data (Saakov 1976, 1987, 1990, 1993a, b, 2000a–e; Saakov and Shiryaev 2000; Saakov et al. 1993) allow the conclusion to be made that the influence of natural EFEs (intensive light exposure, high and low temperatures, dehydration, ionic deficiency, flooding, salification (Na^+, Cl^-, SO_4^{2-}), pathogenic viruses and bacteria) and anthropogenous stressors (herbicides, fungicides, fumigation (SO_2, NO, NO_2), ozone and photochemical smog, active oxygen (radicals and peroxides), photo-oxidizers, acid rains, fogs, acid pH soils and the related deficiency of K^+, Mg^{2+}, Ca^{2+}, $Mn^{2+\,(4-7+)}$, and Zn^{2+}; surplus of nitrogen and NH_4^+, heavy metals, UV and γ-radiation, and high concentration of CO_2) is coupled in overwhelming majority to the primary influence on the state of PS-2 RCs, which manifests as changes in the coefficients of PAMF signal harmonics quenching and in the suppression of the re-oxidization of e^- transfer links in PS-2 RCs. This is illustrated by the following scheme, the vertical arrows of which emphasize the possible sites of the influence of various EFEs on PS-2 RCs:

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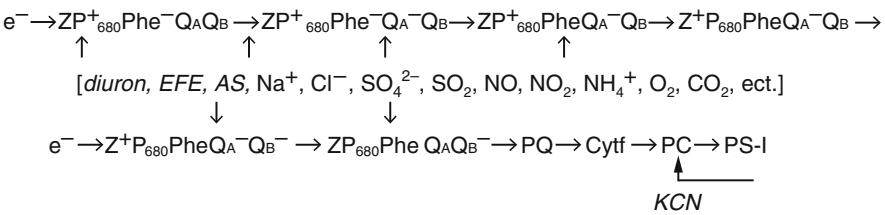

Z^+ and Q_A^- do not interact with each other because they are separated by the P_{680} complex. Therefore, damage to one of the links of redox transformations P_{680}, Q_A, or Q_B leads to the functional imbalance of the energy transformation; reduced Q_A^- loses its ability to accept e^-, and the Fl intensity increases, which is visible under the influence of diuron, temperature, and other harmful factors of the external impact; and processes of oxidation of the primary acceptor of the quinone type Q_A are retarded. The Fl growth is explained by the reduction of electron carriers, and the e^- transfer from Q_A to Q_B (PQ) opens RCs and so at the same time reduces the Fl yield.

Thus, the results of this section and previous works (McSwain et al. 1976; Saakov 1976, 1987, 1990, 1993a–d, 1996, 2000a–e; Saakov and Leontjev 1988; Saakov and Shiryaev 2000; Saakov et al. 1993) and the analysis of literature are combined in the harmonious chain of proofs that the stability and reparation reactions of a green cell mainly depend on primary damage of the link $P_{680} \rightarrow Q_A \rightarrow Q_B \rightarrow PQ^{2-}$. This conclusion is of a general character in the sense that it couples the formation of ATP, formation of a reducer, and the Calvin cycle, damage to which is secondary.

Overcoming the inertia of thinking and following new facts instead of settled dogmas, we suggest for the first time the concept of the energetic basis of green cell resistance. Available data also indicate the possibility of the close connection between vegetational cell tolerance and the energy chain of mitochondria (Lichtenthaler 1988a, b, 1996a, b). The existence of chlorophyll *a* defining the Fl yield of blue-green algae (*Cyanobionta*, *Procaryota*) (McSwain et al. 1976) suggests the similarity of mechanisms of ETC disorders, in response to damaging influences of environmental factors of natural and anthropogenous origin, to be similar to those in *Phycobionta* and *Embryobionta* (*Vegetalia* from *Eucaryota*) and also in *Procaryota*.

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2.2.6 *Additional Material for Substantiation of the Energetic Basis of the Theory of* Procaryota *and* Eucaryota *Phototrophic Cell Tolerance to the Influence of Abiotic Environmental Factors*

2.2.6.1 Problems of Chloroplast Resistance

In the 1980s and 1990s, there was mass development of works on the PAMF of chlorophyll, which allowed registration in vivo of the dynamics of the leaf state in direct sunlight and of the photosynthetic primary reactions. This directed the interest of researchers to biophysical aspects of assessment of the electronic transport efficiency and of photosynthesis regulation in situ, because they correspond to the chloroplast's functional activity under various ecological conditions of the plant state (Briantais et al. 1986; Bolhar-Nordenkampf et al. 1989; Schreiber and Bilger 1993; Mohammed et al. 1996; Schreiber et al. 1997a, b). The nomenclature of Fl levels and coefficients of Fl quenching, and also ways of their calculation and formulae for assessment of the PS-2 quantum yield, have been established (Schreiber and Bilger 1993; Schreiber et al. 1997a, b; van Kooten and Snel 1990a; Snel and van Kooten 1990). These have proved useful for the practical application of this technique in various areas of biophysics, physiology, and plant ecology under the influence of stress on a vegetational cell regardless of its systematic origin. The main factor is the presence of chlorophyll *a*. The stress influence of the EFE does not necessarily lead to a long-term decrease in the photosynthetic activity (Semikhatova and Saakov 1962), which also can be determined after analysis of the coefficients of Fl quenching.

Thanks to the regulatory mechanisms of compensatory replacement, adaptive repair of the process occurs and neutralizes the influences of stress and the induced photoinactivation (photoinhibition, PhIn), that is, the decrease in the photosynthetic activity in response to light excess (Saakov 2003a, b). Study of PAMF signal harmonics is useful for the quantitative assessment of characteristics of the photosynthesis change in response to light excess under the conditions of EFE stress on a cell. From the previous sections, it is seen that the ratio F_V/F_m (F_V is the variable Fl and F_m the maximum Fl) is a reliable indicator for assessment of the PS-2 quantum yield, which decreases at PhIn. With photoinhibition, the ability of PS-2 to manage the electron flux is broken, and the active RCs become photochemically inactive, dissipating the de-excitation energy in the form of heat. The research on non-photochemical quenching (q_E) opened the way for a wide range of investigations into adaptive and protective mechanisms against the stress influence of EFEs. Low-temperature Fl is applicable for study of theoretical aspects of the fluorescence phenomenon, such as the change in the excitation energy distribution between PS-1 and PS-2 and for the analysis of changes in the system of photosynthetic pigments caused by EFEs (Briantais et al. 1986; Bolhar-Nordenkampf et al. 1989). Still, it is important to note that the physiological interpretation of obtained practical data would be impossible without works in this direction. At room temperature, most of

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the fluorescence is emitted by PS-2, whereas at −196 °C there is a significant contribution of PS-1 to the far red spectral area.

Thus, there are two types of possible research into Fl, theoretical and practical. Until now, theorists treat practical conclusions deduced from the analysis of coefficients of Fl quenching with a certain snobbery. However, Fl measurement gives useful practical information on the state of cultured and wild plants and on algae and their communities in the ocean. Progress in the development and in the breadth of application of the method is possible due to the close connection of theoretical interpretation with practical conclusions about the functional state of an organism. Their combination with precision methods such as spectrophotometry in vivo also allows conclusions to be made about damage to the structural organization of the photosynthetic device (Saakov 1993a–d, 2002a–c, 2003a, b). The large volume of the world literature, partially summarized in reviews (Briantais et al. 1986; Bolhar-Nordenkampf et al. 1989; Schreiber and Bilger 1993; Mohammed et al. 1996; Schreiber et al. 1997a, b), gives severe rebuff to theorists haughtily looking at the practical use of the PAM method and at the conclusions made on the basis of its application.

It is necessary to emphasize that the Ministry of Agriculture and Sciences of Austria sponsored an international research program for the comparative assessment of the equipment made by different firms and for the assessment of their possibilities for the rapid diagnosis of plant states in field conditions when damage to biological objects is not yet visually detectable (Bolhar-Nordenkampf et al. 1989). Due to the high practical advantage of Fl research, many European firms produce various highly demanded models of fluorometers, the comparative analysis of which (Briantais et al. 1986; Bolhar-Nordenkampf et al. 1989; Mohammed et al. 1996) showed their high precision and good reproducibility of results and accented the prospective applications. This experience should doubtlessly be adopted not only for Russian scientific institutions; but the PAMF method could also be used by the Ministry of Emergency Situations and other State services in cases of ecological terrorism and technogenic accidents. The long experience of Russian experts working abroad in this direction is, as usual, unclaimed in Russia.

As in the majority of published works, the influence of different factors on different objects was studied with the use of different equipment and different conditions of measurements. In the present work, we summarize the experience of registration of PAMF signal harmonics changes under influence of various EFEs in one laboratory, in approximately the same season, and with the same device (101-103 Walz, Effeltrich, FRG) (Saakov 1993a–d, 2000a–e, 2002a–c, 2003a, b; Saakov et al. 1993). The calculation of quenching coefficient q_E and the photochemical coefficient q_q was carried out at 7–8 min of PAMF registration. We again emphasize that the analysis of q_q and q_E is of great diagnostic value because it helps to localize the biological link of EFE influence (Saakov 1993a–d; Saakov et al. 1993; Saakov and Shiryaev 2000). In this section, we limited ourselves to a review of high and low thermal influence on the character of the dynamics change in F_0, q_q, and q_E (Figs. 2.21, 2.22, 2.23, and 2.24) as a function of the stress factor

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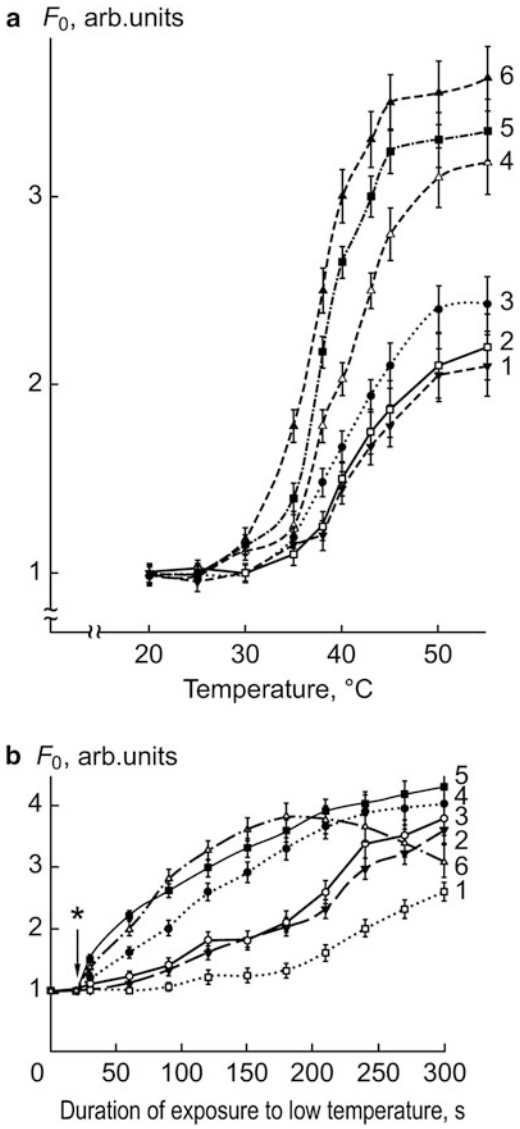

Fig. 2.21 (**a**) The character of the change in base fluorescence F_0 dynamics under the influence (15 min) of increased temperature for woody and herbaceous plants different in resistance. *1* laurel (*Laurus nobilis*); *2* wheat Saratovskaya 29 (*Triticum* sp., the cultivar Saratovskaya 29); *3* barley (*Hordeum* sp.); *4* clover (*Trifolium pratense*); *5* nettle (*Urtica dioica*); *6* barley mutant № 2807 lacking chlorophyll *b* (Donaria cultivar). *Abscissa*: the influencing temperature. *Ordinate*: relative F_0 values. (**b**). The character of the change in base fluorescence F_0 dynamics under the influence of low temperature (−5 °C) for woody and herbaceous plants different in resistance. *1* burdock (*Arctium* sp.); *2* laurel (*Laurus nobilis*); *3* plum (*Prunus domestica*); *4* clover (*Trifolium pratense*); *5* nettle (*Urtica dioica*); *6* barley mutant № 2807 lacking chlorophyll *b* (Donaria cultivar). *Abscissa*: duration of thermal influence; *ordinate*: relative F_0 values. The *asterisk* indicates the F_0 value at 20–22 °C

impact. The latter became possible due to the ability of the PAM technique to repeatedly measure the functional characteristics of an object in vivo (Mohammed et al. 1996; Saakov 2003a, b).

Usually in the experiment, 8–12 leaves were taken for one variant, and the value for each point (Figs. 2.21a, b and 2.22a, b) represents the average of the response of the object to the influence of the EFE, which corresponds to recommendations and our research experience (Bolhar-Nordenkampf et al. 1989; Mohammed et al. 1996). European researchers investigated plants that were

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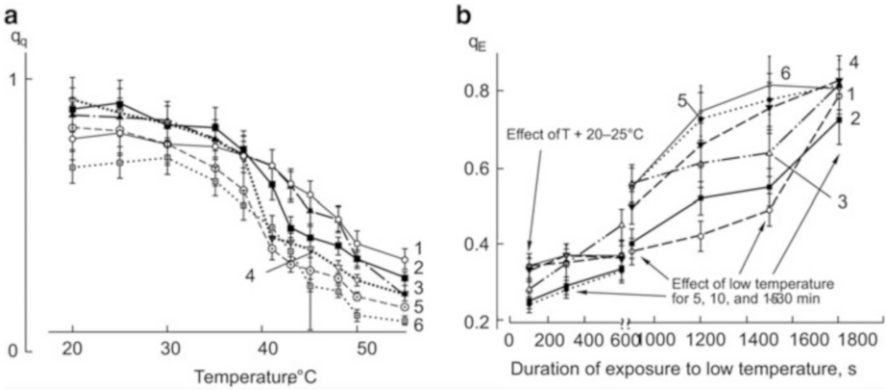

Fig. 2.22 (a) The dynamics of the change in PAMF photochemical quenching coefficient (q_q) under the influence (15 min) of high temperatures for woody and herbaceous plants: *1* oak (*Quercus robur*); *2* laurel (*Laurus nobilis*); *3* Sakhalin buckwheat (*Polygonum sachalinense*); *4* clover (*Trifolium pratense*); *5* nettle (*Urtica dioica*); *6* barley mutant № 2807 lacking chlorophyll *b* (Donaria cultivar). *Abscissa*: duration of thermal influence; *ordinate*: relative q_q values. (**b**) The dynamics of the change in PAMF non-photochemical quenching coefficient (q_E) under the influence of low temperature (−4 °C for 10 min) for woody and herbaceous plants. *1* ivy (*Hedera helix*); *2* oak (*Quercus robur*); *3* Sakhalin buckwheat (*Polygonum sachalinense*); *4* barley (*Hordeum* sp.); *5* barley mutant № 2807 lacking chlorophyll *b*; *6* clover (*Trifolium pratense*)

already adapted for higher summer temperatures owing to the climatic conditions of the countries. In this regard, we tried to perform experiments during the early autumn period.

The constant Fl after the *dark period* of object adaptation when all PS-2 RCs are open is designated as F_0 (the minimum Fl yield), which is induced by a modulated light beam ($\lambda \leq 670$ nm) of flux of 0.1 μE/(m^2 s); its intensity is small enough to change the oxidation–reduction potential of the primary acceptor of Q_A and leaves it in the Q_A^+ state (Saakov et al. 1993; Saakov and Hoffmann 1974; Schreiber et al. 1997a, b; Saakov 2003a, b). The q_q value is maximum and equal to 1.0. The F_m/F_0 ratio between the maximum yield of Fl (in this case Q_A^- is reduced) and the minimum yield of Fl (Q_A^+ is oxidized) fluctuates approximately from 5 to 6 relative units. The F_0 increase occurs at the damage of PS-2 RCs and the disorder of the light energy transfer from antenna complexes. It is shown that the F_0 change can be used for determination of the critical borders of phototrophy realization in *Procaryota* and *Eucaryota*, depending on the influence of high and low temperatures, radioactivity, and other EFEs (Mohammed et al. 1996). The ratio F_0/F_m indicates the temperature tolerance of PS-2 when cultivating plants at high temperature. The increase in F_0 under increased thermal impact correlates with the irreversible thermal influence on leaf tissue (Schreiber and Bilger 1993; Schreiber et al. 1997; Saakov 2003a, b). The soft influence of temperature does not cause the irreversible damage to tissues that is noted at midday depressions of photosynthesis. In this regard, we observed the change in F_0 over a wide range of physiological temperatures, but they were lower than those resulting in tissue

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necrosis (Fig. 2.21a, b); this caused the significant complexity of the present work and its difference from many European publications.

When analyzing F_V/F_m, it is especially necessary to take into consideration and to distinguish the F_0 level increase from the F_V reduction. The change in the F_0 level alters the usual ratio F_V/F_0 (3.1–3.5 relative units), reducing it to 0.9–1.0 relative units. The F_0 value increase characterizes the destruction of PS-2 RCs, whereas the decrease in F_V is coupled with the q_E change; PhIn causes both these changes (as illustrated by the data in Figs. 2.21 and 2.22). From Fig. 2.21, it follows that the changes in F_0 with increasing temperature are of an S-shaped character and already start to manifest in the region 35–38 °C. Exactly this area of temperature was determined by us many years ago (Semikhatova and Saakov 1962). A further increase in temperature results in a jump-like change in F_0 values. These data correlate well with studies of the suppression of photosynthesis intensity under similar conditions (Semikhatova and Saakov 1962; Schreiber and Bilger 1987; Schreiber and Neubauer 1987; Saakov 2001a, b). A conclusion about the possibility to distinguish the resistance of objects to the influence of high temperature by the change in their F_0 (Fig. 2.21a, curves 1, 2, 3 and the group of curves 4, 5, 6) is not excluded. The final validity of this conclusion demands the study of a wide set of plants different in stability and high statistics.

The stress influence of low temperatures also leads to an increase in F_0 (Fig. 2.21b). The degree of the F_0 increase directly depends on duration of the thermal influence. It should be noted that under the soft influence of low temperature, there is no reduction in the F_0 level, which was registered by us and other authors in the case of a shock influence of low temperature in the process of its aftereffect (Mohammed et al. 1996; Saakov 2001a, b), resulting in tissue necrosis. In rough leaves of *Arctium* sp. and also in woody leaves of stiff-leaved *Laurus nobilis* and *Prunus domestica*, the F_0 changes are smoother and less intensive than in herbaceous plants. At the same time, for the mutant of № 2807, which is the least resistant to thermal influence (Fig. 2.21b, curve 6), a rather early tendency for F_0 decrease was noted.

Serious and methodologically competent works established the satisfactory correlation between the level of CO_2 assimilation, O_2 exhalation, and the coefficient q_q (Schreiber and Bilger 1993; Schreiber et al. 1994, 1997a, b). With the increase in q_q, oxygen release linearly increases; the reduction of the coefficient q_q is accompanied by an increase in F_0 and q_E, and a decrease in F_V. The data obtained by us on the q_q dynamics as a function of the thermal stress influence are presented in Fig. 2.22a. The decrease in q_q corresponds to an increase in q_E (Fig. 2.22b) induced by other EFEs. Note that the obvious q_q decrease starts in the area (38–40 °C) of positive temperature influence. This agrees with data earlier obtained by us on the change in the photosynthesis intensity under the thermal influence (Semikhatova and Saakov 1962) and correlates with the opposite character of the F_0 change presented in Fig. 2.21. The suggested explanation for the q_q decrease is the high level of the PQ^{2-} reduction, the increased protonation, and the accumulation of electrons in the acceptor part of PS-2. Under the conditions of the reduced PQ^{2-} pool, the electron accessibility of the Q_B^+ acceptor is strongly limited, and the overall performance of the ETC of photosynthesis is broken. Thus, the obstacle to

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Q_A^- re-oxidization appears. In this state, the probability of Fl emission from the state $P^*_{680}Q_A^-$ increases.

From the data in Fig. 2.22b, it follows that the constant increase in the effect of the low-temperature influence leads to the increase in q_E values. In the steady state of the photosynthetic device, the fluctuation of this coefficient is in the range of 0.25–0.4 relative units. In damaged plants, the q_E level increases and points to inhibition of the ATP synthesis, an increase in the membrane proton gradient, and disorder of the Calvin cycle activity. Removal of the EFE promotes the relaxation of q_E values to 0.3–0.4, a decrease in the membrane proton gradient, and activation of dark reactions of the Calvin cycle and of the ATP use correlating with them.

Coefficients q_E and q_q are very sensitive parameters to the EFE influence, and their change is accompanied by structural damage in the photosynthetic device, which is registered with the help of the derivative spectrophotometry method (see below Sect. 2.2.9). In contrast to F_0 and F_V, the recovery of these coefficients requires a longer time after removal of the EFE, which was observed for drought (Saakov 2003a, b).

Thus, in this section, using leaves that varied in their level of EFE resistance, the dynamics of stationary values of F_0, q_q, and q_E as a function of the intensity of the stress influence was investigated, and statistically valid limits of their change under the influence of low and high temperatures were determined. We showed that changes in q_q and q_E appear a little earlier than changes in F_0 and F_V. The results of our previous investigation of the dynamics of F_m and F_V as a function of the high temperature influence (Saakov 2002a–c) do not contradict the results of this work.

This experimental work was the first of this kind to be reported in the world scientific literature. We invite any skeptic to perform control measurements and to enjoy the labor intensity of such measurements.

The accepted interpretation (Briantais et al. 1986; Bolhar-Nordenkampf et al. 1989; Schreiber and Bilger 1993; Mohammed et al. 1996; Schreiber et al. 1997a, b) of PAMF suppression coefficients indicates the link between these coefficients and the stability of phototrophy systems of a green cell. Together with the material published earlier (Saakov 1993a–d, 2000a–e, 2002a–c, 2003a, b), the results of this section support the correctness of the point of view about the primacy of the damage to the link $P^+_{680}Pheo^-Q_A^- \rightarrow P^*_{680}PheoQ_A^-$ and the interrelation of processes responsible for the *Procaryota* and *Eucaryota* green cell tolerance to EFE influence, with the optimization of the conversion of light energy into the energy of ATP chemical bonds.

2.2.7 *Features of the Fluorescence Change in F_0 and F_m in Response to Dithiothreitol Inhibition of Zeaxanthin Formation*

The xanthophyll cycle (sometimes called the "violaxanthin cycle") (see Chap. 1) is a complex reaction system consisting of the light-induced de-epoxidation of

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violaxanthin to zeaxanthin (Zea) via the monoepoxide antheraxanthin and of the reversible epoxidation of the latter, lower in speed and occurring in response to the photon flux density limiting the CO_2 fixing (Yamamoto 1995; Yamamoto et al. 1962; Saakov 1963, 1965). Considerable information has accumulated on the properties of the xanthophyll cycle and on the association of these reactions with photosynthesis and photophosphorylation processes (Saakov 1971a, b; Baltscheffsky 1971; Saakov and Hoffmann 1974; Saakov et al. 2013). The interrelation between the stability of the cycle activity and the stability of the plant plastid device system has been shown (Saakov 1973). Over a number of years, there has been active discussion on the correlative dependence between the Zea content and the type of chlorophyll Fl quenching in response to photoinhibiting stress, when Fl is characterized by a decrease both in the minimum dark stationary Fl (F_0) and in the maximum Fl (F_m) at the different photon flux density (Demming-Adams 1990; Demming-Adams and Adams 1990, 1992, 1996; Demming-Adams et al. 1987, 1989, 1990). The authors supposed that Zea is involved in the process of non-radiative dissipation of energy, which takes place in the complex of chlorophyll molecules and allows transfer of the excited chlorophyll into its ground state by means of thermal emission. The F_0 quenching is the key point of the dissipation process coupled with Zea, that is, in the absence of a change in Zea, changes in F_0 should not be observed.

At the same time, the first data were published (Foyer et al. 1990a, b) that showed the ambiguity of such an interpretation and were further developed in later works (Saakov et al. 1993; Richter et al. 1994).

In this section, we will concentrate on the research into the dependence of the Fl change on the Zea content. For this purpose, we used the inhibition of the Zea formation reaction with dithiothreitol (DTT) according to the work of Demming-Adams et al. (1990).

As the investigated object, the compound leaves of *Robinia pseudoacacia* L. and *Taraxacum officinale* L. were used. Their choice was defined by the possibility of the selection of physiologically homogeneous leaflets. After cutting, the leaves were left for 24 h in the dark, on water. This allowed a stationary level of Zea and F_0 to be reached in leaves adapted to darkness, when the RCs of PS-2 are completely open. Unlike previous methods of DTT introduction (Demming-Adams et al. 1990), we softly infiltrated the inhibitor solution (3 or 6 mM) in the half-light (Saakov 1965, 1971a, b). Control leaves were infiltrated with water. The measurement of fluorescence was carried out by means of the pulse-amplified modulated method (Schreiber 1986), using the PAM fluorometer M-101-103 (Walz, Effeltrich, Germany). We emphasize that the method of Fl registration applied by us was identical to the published method (Demming-Adams 1990; Demming-Adams and Adams 1990, 1992). The top part of the leaflet was placed on the frontal part of the light guide, which delivered both the white actinic light and the excitation red light ($\lambda \approx 670$ nm) to the leaf, and passed the fluorescence emission to the photodiode detector protected by a filter ($\lambda = 700$ nm). The selective amplifier perceived only pulse signals of Fl induced by the excitation light. Upon

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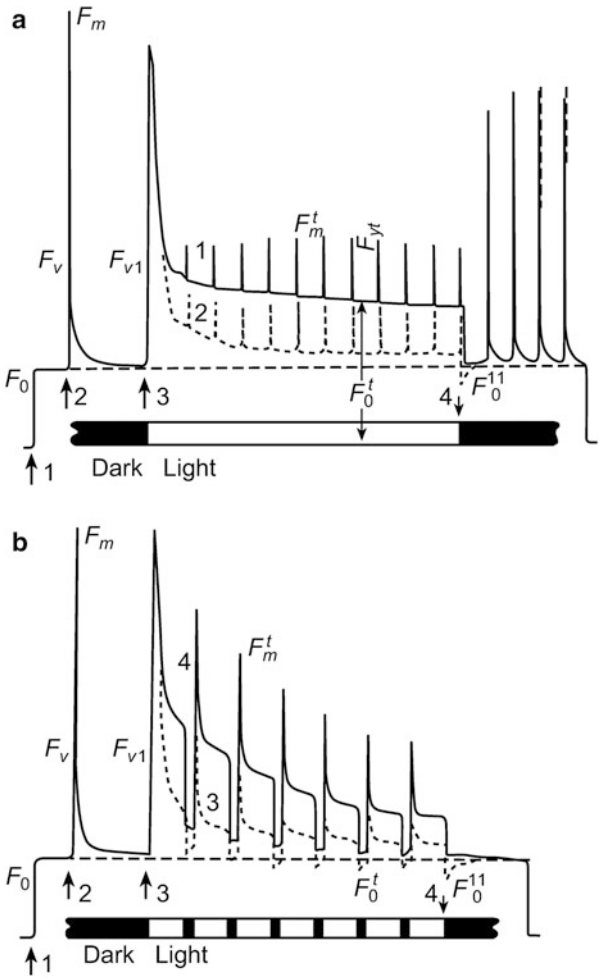

Fig. 2.23 The character of the change in modulated fluorescence harmonics for the chlorophyll *a* of *Robinia* leaflets adapted to the darkness and infiltrated with water (*curves 2* and *3*) or with 6 mM DTT solution (*curves 1* and *4*) in response to light exposition of 11 min. (**a**) The combination of a 1-s saturating white light impulse (3,000 μE/(m^2 s)) followed by 60 s of darkness and then by11 min of actinic light of 590 μE/(m^2 s) and frequency 100 kHz for the determination of F_m^t values. (**b**) Seven cycles of 80 s light (non-saturating light impulses)/20 s darkness for the F_0^t determination at the start of dark periods. The used actinic light was 1,550 μE/(m^2 s). *Arrows*: *1* input of modulating light of frequency of 1.6 kHz for the determination of the F_0 level of the minimum dark fluorescence; *2* the saturating light impulse (3,000 μE/(m^2 s)); *3* input of actinic light of frequency of 100 kHz; *4* shutdown of the actinic light for the F^{11} determination. F_0 the minimum dark fluorescence of leaves adapted to the darkness; F_m the maximum fluorescence; F_V the variable fluorescence ($F_V = F_m - F_0$); F_0^t the minimum fluorescence at the start of the 20-s dark period; F_0^{11} the minimum fluorescence at shutdown of the actinic light of 100 kHz

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termination of the exposure, the leaf material was fixed with liquid nitrogen and analyzed using high pressure liquid chromatography (Saakov et al. 1993).

Results on the character of the change in Fl yield harmonics after the DTT processing of leaves were obtained under conditions of actinic light radiation (590 μE/(m^2 s)) with a 1 s red light impulse and a dark interval of 60 s (Fig. 2.23a). A second approach used a light/dark cycle of 80 s/20 s and actinic light intensities of 560 and 1,550 μE/(m^2 s) (Fig. 2.23b). The second approach is optimum because of the different speeds of the light and dark reactions of the xanthophyll cycle and also due to the re-oxidization of acceptors Q_A, Q_B, and PQ.

From the data in Fig. 2.23, it follows that DTT does not significantly influence the F_V/F_m ratio (usually 0.825–0.830), and its high value emphasizes the increase in the PS-2 reduced state and the RC closing. At the moment of maximum dark Fl (F_m), the coefficient of the Fl photochemical quenching $q_q = 0$ and non-photochemical processes are minimized (i.e., $q_E = 0$). The F_m level after DTT addition increased for both investigated objects, pointing to an increase in the variable Fl, F_V, ($F_m - F_0 = F_V$). When exposing leaves to cyclic lighting (80 s/20 s), it follows that the 80-s non-saturating impulse does not significantly influence the F_m^t change. At the same time, the illumination of plants by actinic light considerably changes F_0, which suggests the incomplete work of the cycle of re-oxidization of electron acceptors in RCs of PS-2. The change in the ratio F_m/F_0 emphasizes the increase in the PS-2 reduction state. The high level of $F_0 + F_V^t$ corresponds to the small q_E value and points to activation of the energy utilization in the Calvin cycle. Further, from the data of Fig. 2.23, it follows that F_0^t values (F_0 in the specific interval of time t) increase in the presence of DTT. In response to DTT infiltration, the increase in F_0 correlates well with the coefficient $F_{V1}\ h/2$, characterizing the half-width of the Fl harmonic contour band and described by the height F_{V1} and the run of the curve F_0^t (Saakov 1993a–d), induced by the actinic light. The F_0^t level reached after 11 min of light exposure in the experiment after shutdown of the light was lower than the initial F_0 of the leaves adapted to darkness in the absence of DTT. So both approaches to the lighting of samples yielded identical results that agreed with the data (Demming-Adams 1990; Demming-Adams and Adams 1992; Demming-Adams et al. 1990).

In the absence of DTT, the character of the dynamics of the content change in the xanthophyll cycle components at pulse lighting (80 s/s) did not differ from the well-known dynamics (Yamamoto et al. 1962; Saakov 1963, 1965; Demming-Adams and Adams 1990, 1992, 1996). DTT infiltration inhibited Zea formation (Fig. 2.24, curve 3), in agreement with reported data (Demming-Adams et al. 1987, 1989, 1990).

At the same time, we found inhibition of violaxanthin de-epoxidation (Table 2.7). Other pigments of chloroplasts do not react to DDT infiltration.

The results presented in Fig. 2.24a, b show the considerable distinction between the kinetics of F_0 curves and the Zea content. The first curve (F_0) reaches the

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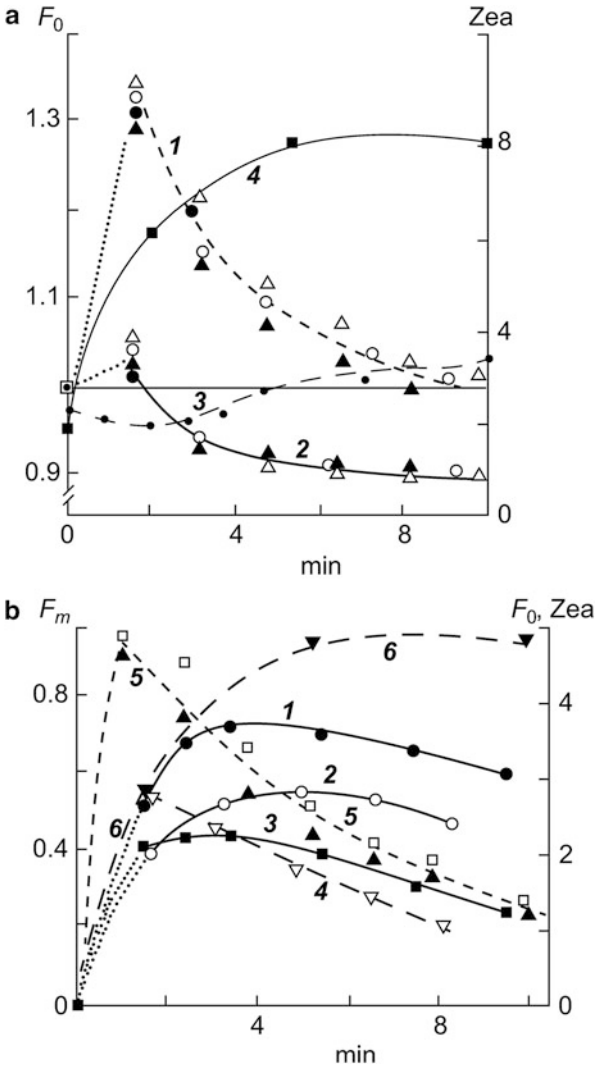

Fig. 2.24 (**a**) The kinetics of the change in the ratio F_0^t/F_0 and of the zeaxanthin content in *Robinia* leaves infiltrated with the DTT solution (6 mM) (*curve 1* $\left(F_0^t/F_0\right)^{+\mathrm{DTT}}$; *3* $\mathrm{Zea}^{+\mathrm{DTT}}$) and in the *Robinia* control leaves (*curve 2* $\left(F_0^t/F_0\right)^{-\mathrm{DTT}}$; *4* $\mathrm{Zea}^{-\mathrm{DTT}}$) at light/dark illumination of 80 s/20 s with actinic light of 1,550 μE/(m^2 s). *Abscissa*: time of light exposure in minutes; *left ordinate* relative units for *curves 1* and *2*; *right ordinate* Zea content in mol/100 mol of chlorophyll *a*. *Symbols* on *curves 1* and *2* show data for separate leaflets of the *Robinia* compound leaf. (**b**) The dynamics of F_m fluorescence quenching (*curves 1–3*), fluorescence F_0 (*curve 4*) of the additional fluorescence F_0^t (*curve 5*) at the DTT infiltration and in the presence of the additional quantity of the formed Zea (*curve 6*) in the absence of DTT. *Abscissa*: time in minutes; *left ordinate* for *curves 1–3* $\left(F_m^{t(+\mathrm{DTT})}/F_m^{t(-\mathrm{DTT})}\right) - 1$; *right ordinate* for *curve 4* $\left(F_0^{t(+\mathrm{DTT})}/F_0^{t(-\mathrm{DTT})}\right) - 1$; *curve 5* $\left(F_0^t/F_0\right)^{+\mathrm{DTT}} - \left(F_0^t/F_0\right)^{-\mathrm{DTT}}$, all in relative units; *curve 6* $\mathrm{Zea}^{-\mathrm{DTT}} - \mathrm{Zea}^{+\mathrm{DTT}}$, in mol/100 mol of chlorophyll *a*. *Curves 1* and *3* correspond to actinic light of 1,550 and 590 μE/(m^2 s), respectively (as in Fig. 2.23a), in combination with saturating impulses. *Curve 2* pulse period 80 s/20 s with actinic light of 1,550 μE/(m^2 s) (as in Fig. 2.23b). Data are averaged for 2–3 leaves

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Table 2.7 Inhibition of de-epoxidation of violaxanthin (mol/100 mol chlorophyll *a*)

	Violaxanthin content			
		Light (min)		
Inhibitor	Dark	2	5	10
−DTT	10.1	4.6	3.4	2.5
+DTT	9.75	9.3	8.0	7.0

maximum in 80 s of the light period, and the Zea content becomes maximal after three light periods (see curves 2 and 4). The difference curves for the kinetics $\left(F_0^t/F_0\right)^{+\mathrm{DTT}} - \left(F^t/F_0\right)^{-\mathrm{DTT}}$ and for the Zea content ($\mathrm{Zea}^{-\mathrm{DTT}} - \mathrm{Zea}^{+\mathrm{DT}T}$) are presented in Fig. 2.24b and demonstrably illustrate the discrepancy between the maxima of the curves for F_0 and Zea.

From data in Fig. 2.24, it follows that the intensity of the F_0 change induced in the presence of DTT (curve 1) is much higher than in the absence of DTT (curve 2). The very insignificant Zea content increase corresponds to the F_0^t yield increase at 8–10 min (curve 3). Note the mirror course of curves 2 and 4, which could be interpreted by some stretch of imagination as the existence of a correlation between the F_0 quenching and the increase in Zea quantity, although the positions of their maxima are different in time. Thus, under these experimental conditions, the F_0^t yield increase *does not correlate* with the Zea content increase.

So the illuminated *Robinia* leaves adapted to darkness are characterized by differing kinetics of the *F*l F_0 quenching and of Zea accumulation. Different time intervals were determined for the curves to reach the maximum in the range from 80 to 240 s. The data allowed us to come to the conclusion that the F_0 change develops quicker than Zea formation as a result of violaxanthin de-epoxidation and quicker than the F_m quenching (Fig. 2.24b). This suggests small probability that, after the transfer of leaves adapted to the darkness to the light, Zea acts as a *quencher* of Fl F_0 induced in the presence of DTT.

The different character of the F_0 and F_m quenching kinetics suggests the existence of various mechanisms of their quenching. In the assumption that the DTT influence on photosynthetic membranes is limited to Zea formation, our data would mean that the F_0 quenching is *not caused* by the Zea emergence. At the same time, considering the modulation of H^+-ATPase by DTT and the sensitivity of PS-2 protease to DTT, it is possible to assume that the F_0 effective quenching is the consequence of the DTT influence and is not related to the inhibition of Zea formation.

The presented materials demand that caution is taken in accepting the unambiguity of conclusions in the works of Demming-Adams and colleagues (Demming-Adams 1990, Demming-Adams and Adams 1992; Demming-Adams et al. 1990) about the role of Zea in the quenching of the excited chlorophyll fluorescence.

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2.2.8 *Specifics of γ-Radiation Influence on the Stability of Energetics and the Pigment System of the Photosynthetic Device*

Despite plentiful publications concerning the resistance of vegetational and animal organisms to the influence of environmental factors, for many years the theoretical aspect of the problem was not established and required additional experimental data about the energetic part of the resistance and the development of new approaches (Saakov 1976, 1987).

A confused picture is also observed regarding the influence of ionizing radiation on the photosynthetic device of higher plants and algae. This is of special concern for atomic proving grounds and accident situations at nuclear power stations and other technogenic sites (Gonzalez and Moreno 1983; Ignacimuthu and Babu 1989).

Analysis of incidents has shown the absence of appropriate methods. Obtained results were considered as the model for further research on the reliable diagnostics of the flora photosynthetic device state after exposure to ionizing radiation (USSR State Committee on the utilization of atomic energy: the accident at the Chernobyl nuclear power plant and its consequences. Working document for the IAEA post-accident review meeting. Vienna, 1086. P. 101; Gesellschaft für Reaktorsicherheit (GRS): Neuere Erkentnisse zum Unfall im Kernkraftwerk Tschernobyl. GRS-S-40 (Februar, 1987). ISBN 3-923875-13-4. P. 74; Gesellschaft für Strahlen und Umweltschutz: Umweltradioaktivität und Strahlenexposition in Südbayern durch Tschernobyl-Unfall. Bericht des Instituts für Strahlenschutz. München, Nueherberg: GSF-Bericht, 1986. Bd.16/86. S. 76.; International Atomic Energy Agency: Summary report on the post-accident review meeting on Chernobyl accident. IAEA safety ser. № 75- INSAG-1. Vienna, 1986. P.96.)

The tragic case at Fukushima demonstrated that appropriate conclusions from the Chernobyl accident were not made.

The method of pulse-amplitude modulated fluorescence (Schreiber 1983) was successfully used for the assessment of the damage to the photosynthetic device caused by temperature and radiation of radar devices (Rinderle et al. 1988; Schreiber and Bilger 1987). Taking into account the convenience of the method, we applied the modification of this method for characterization of the damage level of the photosynthetic device under the influence of high doses of γ-ionizing radiation using smaller doses of radiation. It is necessary to emphasize the significant difference in the order of effective dose values for vegetational and animal cells (Timofeev-Ressovsky et al. 1981). This was taken into consideration in choosing the radiation doses.

As objects of study, we chose the leaves of acacia (*Robinia pseudoacacia* L.) and haricot (*Phaseolus vulgaris* L.). The source of γ-radiation was a shielded capsule containing the isotope ^{57}Co. During the experiment, the source power was 670 Gy/h. PAMF spectra were recorded with the Walz 101-103 device (Effeltrich, Germany). Absorption spectra of intact leaves were registered with

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the spectrophotometer DW-2000 (Aminco, Germany). The computer built into the device carried out the calculation of derivative spectra.

The data in Figs. 2.25 and 2.26 show the comparative influence of different doses of γ-radiation on the yield and contours of the PAMF signal. In Table 2.8 the calculated coefficients on the change in initial (F_0), variable (F_V), and maximum (F_m) fluorescence are presented along with data on the photochemical (q_q) and non-photochemical (q_E) coefficients of the fluorescence quenching.

The fluorescence arising after the dark period of adaptation (F_0), when all RCs of PS-2 are open, is the emission of molecules of excited chlorophyll *a* taking place before the energy migration in RCs and when the first stable acceptor of PS-2, namely, Q_A, is oxidized. F_0 is defined at the input of the measured light flux of 0.1 μE/(m^2 s). The F_0 level depends on the EFE influence that causes the change in the state of PS-2 pigments. Thermal influence and photoinhibition lead to an increase in the F_0 level.

Low temperatures do not cause such a sharp change (in the opinion of Schreiber and Bilger 1987). The data in Fig. 2.25 and Table 2.8 show that the influence of ionizing radiation significantly changes the F_0 level after the induction with light (F_0^{10}). After the measurement of F_0, a saturating light flux impulse (3,000–3,500 μE/(m^2 s)) was applied for the determination of F_m. Its level defines the reduction of the primary electron acceptor Q_A. After the F_V decrease (2–3 min), actinic light (180–1,100 μE/(m^2 s)) was turned on to induce the F_{V1} level. After 30 s, a number of saturating impulses (1 s duration, light flux of 3,000 μE/(m^2 s)) were fed, with intervals between impulses of 20–60 s. This set of impulses in addition to the actinic light promotes the complete reduction of the acceptor Q_A. The higher the amplitude of these induced impulses, that is, the closer they are to the F_{V1} value, the larger is the quantity of the reduced acceptor Q_A. The ratio F_V/F_m is considered to be an indicator of the photosynthetic function, and for intact chloroplasts, the value of this ratio fluctuates as approximately 0.8–0.85. A deviation of 0.03–0.04 is considered significant and points to the response of the photosynthetic device to the external influence (Schreiber and Bilger 1987).

From Table 2.8, it follows that a dose of 3.5–4 kGy is boundary; exceeding that value leads to considerable changes in photosynthetic function.

It is established that the q_E value correlates with the energization of thylakoid membranes, that is, with a light-excited proton gradient (Schreiber and Bilger 1987). The q_E value will be the higher, the higher the proton gradient and the lower the ATP synthesis. The high activity of dark reactions reduces the q_E value. Under the EFE influence, q_E usually increases. At the same time, in response to the radiation of objects with 12 kGy, the q_E value decrease suggests the pre-lethal surge of ATP synthesis caused, probably, by the critical increase in respiration intensity (James 1953). The low q_q level indicates the accumulation of electrons in the acceptor part of PS-2 and the high level of reduction of Q_A and of the PQ pool, also as the value $1 - q_q$ characterizes the level of PS-2 reduction.

After accumulation of research experience with this method, we introduced a *new parameter*, which was not considered earlier in the scientific literature, namely,

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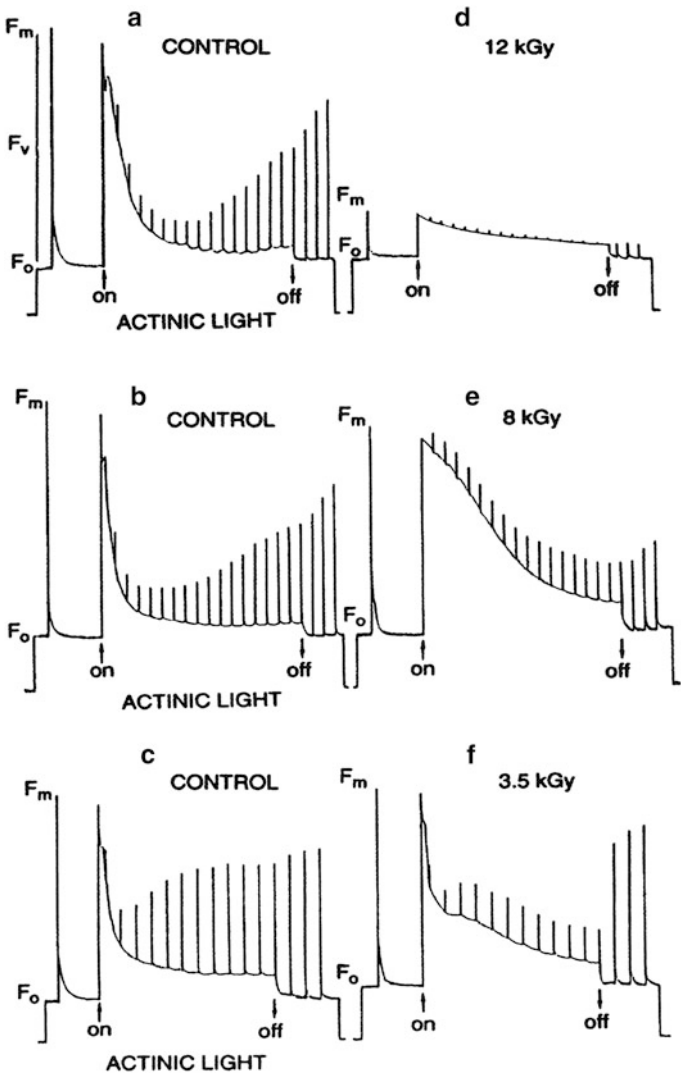

Fig. 2.25 Influence of γ-radiation of 12, 8, and 3.5 kGy (**d**–**f**) on the PAMF spectra change of Phaseoulus leaves compared with the corresponding controls (**a**–**c**). F_0 initial stationary fluorescence; F_m maximal fluorescence; F_V variable fluorescence; F_{V1} fluorescence induced by actinic light; F_0^{10} F_0 level after 10 min of actinic light influence. *Arrows* indicate the input (*on*) and shutdown (*off*) of the actinic light

F_{V1} $h/2$ (see Fig. 2.19), characterizing the half-width of the contour *band of the PAMF spectrum* (SPF), which is described by the height F_{V1} and the run of the curve F_0 induced by the actinic light. From the data in Table 2.8 it can be seen that this parameter describes, to a certain degree, the level of damage to the photosynthetic device caused by the influence of ionizing radiation.

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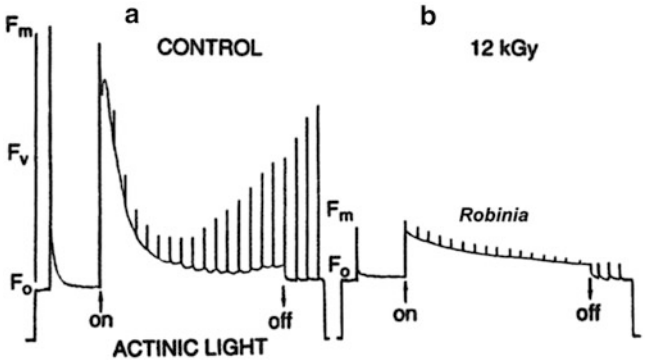

Fig. 2.26 The influence of gamma-radiation of 12 kGy on PAMF spectra change of *Robinia pseudoacacia L.* compared with corresponding control. Robinia has leaves with more xeromorphic structure

Table 2.8 Determination of borders of photosynthetic malfunction

Coefficient	Control	Experimental levels of irradiation, kGy, $p < 0.05$					
		12	8	4	3.5	2.5	1.3
Robinia							
	$n = 14$	$n = 4$	$n = 2$	$n = 4$	$n = 3$	$n = 3$	$n = 3$
F_V/F_m	0.823	0.776	0.776	0.791	0.817	0.801	0.809
q_E	0.695	0.250	0.660	0.670	0.710	0.857	0.710
q_q	0.839	0.080	0.530	0.786	0.772	0.860	0.859
$1 - q_q$	0.161	0.917	0.470	0.191	0.228	0.170	0.140
F_0^{10}/F_0	1.130	2.660	1.670	1.220	1.270	1.210	1.142
F_{V1} $h/2$	8.7	98.5	44.0	5.0	15.0	10.5	8.0
Phaseolus							
	$n = 4$	$n = 2$	$n = 4$		$n = 5$	$n = 4$	$n = 3$
F_V/F_m	0.826	0.47	0.79		0.788	0.79	0.792
q_E	0.552	0.68	0.66		0.714	0.67	0.485
q_q	0.847	0	0.53		0.727	0.90	0.861
$1 - q_q$	0.153	1	0.47		0.273	0.10	0.139
F_0^{10}/F_0	1.139	1.285	1.67		1.31	1.2	1.216
F_{V1} $h/2$	11	35–60	32–44		15	8.5	12

Thus, the change in coefficients calculated on the basis of SPF characterizes the damage to the energetics of the photosynthetic device and points to the damage localization in the RCs, resulting in damage to the coordinated character of reactions of components of the ETC of photosynthesis. This thesis can be also extended to the influence of other EFEs.

At the same time as SPF registration, the changes in the intensity of photosynthesis and respiration were assessed. Five hours after irradiation of haricot with 8 kGy, the photosynthesis intensity reduced by 75 %, and in 10 h it was 80% less or

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one fifth than the control. Correspondingly, the intensity of respiration sharply *increased* 11- and 18-fold. A smaller dose of radiation (3.5 kGy) at once reduced the intensity of photosynthesis to 0.34 of initial value and 0.14 of initial value after 5 days; the respiration intensity increased 6.1 times, and 5 days later, it fell and was only 1.6 times higher than the control. It is probable that the drop in respiration intensity corresponds to the attempt at compensatory replacement of the basic energy system of cells with the accessory system. We wrote earlier about such replacement.

It is possible to suppose, but not to state, that this phenomenon is the manifestation of the beginning of the repair of the photosynthetic device activity.

For irradiated leaves, but not cut off from plants, the characteristic feature is their water loss. At 24 h after a dose of 8 kGy, leaves contained 60 % less water than the control and 20 % less at 5 days after a dose of 3.5 kGy. After irradiation (8–12 kGy), acacia leaflets easily fall from a leaf stalk and dry up 30–50 min later; it is remarkable that they do not lose green color. In the experiment and in the control, the content of pigments *did not significantly differ*. It, apparently, masks changes in the *qualitative* state of the pigment complex of the photosynthetic device.

The *in vivo* research on derivative absorption spectra of control and irradiated leaves did not reveal considerable distinctions between the experiment and the control. Also, there was no hypsochromic shift of the red maximum inherent in the disaggregation of the chlorophyll–protein complex. Only the analysis of spectra with help of derivatives of higher orders (Fig. 2.27) allowed discovery of

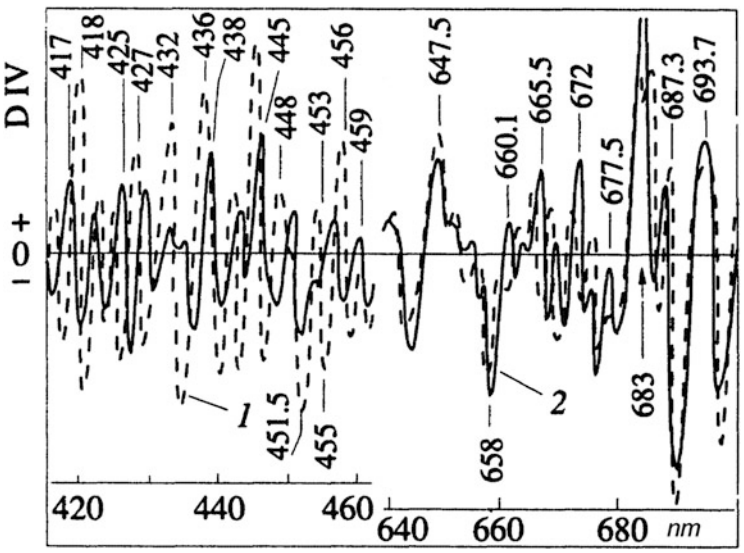

Fig. 2.27 The course of change in the fourth derivative (D^{IV}) of the absorption spectrum of intact leaves under the influence of γ-radiation (12 kGy). *1* experiment; *2* control. *Numbers* indicate the positions of maxima

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characteristic shifts in the absorption spectrum of the fourth derivative (D^{IV}) of intact irradiated leaves, more localized in the blue spectral area (namely, in the Soret band) and in the area of carotenoid absorption. In the red part of the spectrum, distinctions were insignificant and manifested, mainly, as changes in the positions of the spectrum of short-wave forms of the chlorophyll–protein complex. The analysis of spectra of the eighth derivative and the twelfth derivative did not reveal significant new additions about the character of damage to the chlorophyll–protein complex under the conditions of this experiment.

The set of native forms of chlorophyll is a system of energetically interacting elements participating in electron transfer. Even small changes in the spectral curve induced by ionizing radiation correspond to the disorder of stationary transitions of the energetic step. It is not excluded that ionizing radiation breaks the link of the electron transfer from P_{680} to the neighboring Pheo and thus prevents Q_A reduction. This can be extended to the long-term impact of powerful radar systems on the environment.

The material presented in this section indicates that the PAMF method can be considered a prospective technique for assessment of radiation damage to the photosynthetic device. We have presented data on the targeting of radiation damage to RCs of PS-2. The separate moments of spectral changes of the photosynthetic device induced by extreme influences are considered in more detail in the following sections. These results support our concept (Saakov 1976) of the interrelation of the resistance of a plant cell to EFE influence with the stability of RCs and the primary non-specific localization of the damage in the energy links of the photosynthetic device (Saakov 1987, 1996; Saakov and Leontjev 1988).

2.2.9 *Features of the Structural Stability of the Light-Harvesting Complex of Photosystem 2 Under the Influence of γ-Radiation*

In more recent years, we began research on the coupling of the changes in the structure and function of the photosynthetic device under the influence of γ-radiation (Saakov 1992; Saakov 1993a–c, 1996). It was shown that the impact of high doses of γ-radiation reduces the intensity of photosynthesis, promotes the manyfold increase in respiration intensity, but for a long time does not visibly changes the structural state of the chlorophyll–protein complex of the in vivo leaf, as assessed by the method of derivative spectrophotometry of high orders (D^{IV}, D^{VIII}, D^{XII}) (Saakov 1993a). In the process of the *aftereffect* of γ-radiation (see Sect. 2.2.8), the gradual dehydration of leaf tissues manifests, the processes of lamina growth (Saakov et al. 1993) are damaged, and the S-shaped suppression of the activity of the violaxanthin de-epoxidation cycle takes place (Saakov 1993a, b). It was noted that old leaves and leaves with a pronounced xeromorphous structure

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distinguished themselves by higher radioresistance (Saakov et al. 1992; Saakov 1993a). Further investigations showed the dependence of the photosynthetic function on the state of the ETC, in particular on the damage to the electron transfer links of PS-2; when the energy reaches the complex of the PS-2, the pigment P_{680} initiates the primary transfer of an electron into the RCs of PS-2 (Saakov and Shiryaev 2000). Here we again recall the electron transport scheme at the first stages of photosynthesis:

$$ZP_{680}PheoQ_AQ_B \xrightarrow{1} ZP^*{}_{680}PheoQ_AQ_B \xrightarrow{2} ZP^+{}_{680}Pheo^-Q_AQ_B \xrightarrow{3} ZP^+{}_{680}PheoQ_A{}^-Q_B$$

$$\xrightarrow{4} Z^+P_{680}PheoQ_A{}^-Q_B \xrightarrow{5} ZP_{680}PheoQ_A{}^-Q_B \xrightarrow{6} ZP_{680}PheoQ_AQ_B{}^-$$

The electron appearing at oxidization of water is transferred through the secondary donor (Z), through the pigment P_{680} and the primary acceptor of an electron, Pheo, to the acceptor of the quinone type Q_A. In stages 1 and 2, Q_A and Q_B are open; in stages 4 and 5, Q_B is open (Saakov 1993a; Saakov and Shiryaev 2000); and in stage 5, Q_A is again open. Components of Eq. (2.1) are known as integral parts of PS-2 RCs. When $Q_A{}^-$ is reduced, the RC is closed, and the electron transport and the ETC functioning are broken.

The high functional radioresistance of plant cells and of their protein-synthesizing systems (Saakov et al. 1992; Goncharova and Sheverdov 1993), and also the weak variability of ChPC in vivo under the influence of high doses of γ-radiation, promoted us to investigate the radioresistance of its separate components, namely, protein structures (Saakov 2000a–e), aromatic amino acids (Saakov 1993a–d, 1994, 1998a, b), and a number of pigment solutions (Saakov 1993a, b). The simple elements of the ChPC structure, taken apart, possess lower radioresistance than the compound biological ChPC of chloroplasts in vivo providing the basis of the photosynthetic device.

In this section, the influence of high doses of γ-radiation on the antenna light-harvesting complexes of PS-2 extracted from the plants *Phaseolus vulgaris* L. and *Spinacia oleracea* L., with differing radioresistance, is considered.

For the extraction of antenna light-harvesting complexes, we used the method of Bassi and Machold (Bassi et al. 1985), modified according to our objects of research. Leaves (2–3 g) were homogenized in 25 mL of cool medium (250 mM sorbitol; 42 mM HEPES buffer, pH 7.5; 15 mM $MgCl_2$ solution; 10 mM sodium ascorbate; 8 mM NaCl; 28 mass% polyvinylpyrrolidone 15). The homogenate was mixed with 10 mL of cooled hexane and filtered through nylon tissue. The filtrate was centrifuged for 3 min at $500 \times g$; the supernatant was again centrifuged for 5 min at $12{,}000 \times g$. The precipitate was resuspended in the wash medium (300 mM sorbitol; 50 mM HEPES buffer, pH 7.5; 10 mM NaCl) and was again centrifuged at $12{,}000 \times g$. The precipitate was subjected to osmotic shock in distilled water, centrifuged for 10 min at $40{,}000 \times g$, and resuspended in water. Samples of volume of 350–400 μL containing approximately 300 μg of chlorophyll were frozen and kept in liquid nitrogen. For the experiment, samples were defrosted and mixed with

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Triton X-100 for 5 min on a shaker (the ratio of chlorophyll to Triton X-100 was 1:30 by weight using 2 % Triton X-100) and again centrifuged for 10 min at $40{,}000 \times g$. The supernatant was added to polyacrylamide gel plates containing digitonin (0.5 mass%) and subjected to electrophoresis. The antenna light-harvesting complexes of PS-2 were washed from the plates using tricine buffer (50 mM, pH 7.8). The γ-radiation and spectrophotometry of samples were carried out as described previously (Saakov 1993a–d, 1994, 1996, 1998a, b, 2000a, b). Spectra were processed with the *Graph Digitizer* 1 program, digitalized, and, where necessary, differentiated with smoothing of the next to last derivative with the help of the Microcall *Origin* 5.0 program (Saakov 2000a, b).

It can be seen from the kinetics of change in optical density (OD) values in absorption spectra of the antenna light-harvesting complexes of PS-2 that within 24 h of exposure to dispersed light (control), there is a decrease of 6–10 % (Fig. 2.27). The γ-radiation of preparations of the light-harvesting complexes of PS-2 promotes a gradual decrease in the kinetics of the OD value change, and at high doses of γ-radiation, this reduction does not exceed 30 % in 24 h. Thus, the influence of γ-radiation on the light-harvesting complexes of PS-2 is a little deeper in comparison with a native leaf (Saakov et al. 1993; Saakov 1993a). The short-term influence of temperature on a complex causes considerably greater change in the OD values in comparison with γ-radiation, and their additive influence quickly destroys the antenna complex of PS-2. The total extract of pigments is notable for the lowest resistance to the influence of γ-radiation (Fig. 2.28).

So the antenna light-harvesting complex (Saakov 1994) shows significantly higher resistance than its separate constituent components of protein or pigment nature (Saakov 1993a, 1998a, b, 2000a, b). The consecutive simplification of ChPC or of its components inevitably leads to the loss of functional properties of the chloroplast system responsible for the energy supply of the photosynthetic device. In this regard, the antenna light-harvesting complex of PS-2 occupies an intermediate place in the resistance to γ-radiation, converging closer to the resistance of the native ChPC.

The data shown in Figs. 2.29a, b and 2.30 reveal fine changes in the spectral structure of the light-harvesting complex of PS-2, manifested as the influence of γ-radiation on the red and blue areas of its spectrum. Figure 2.29a shows, for the first time, the eighth derivative of the absorption spectrum (D^{VIII}) of a native leaf of haricot and clearly shows specific changes in the spectral structure that arise during extraction of the complex. The hypsochromic shift of the main maximum of absorption ($\lambda = 682$ nm) is specific to simpler structures or for ChPC in vivo, exposed to EFE influence. The fine structure of spectra D^{IV} and D^{VIII} allows confirmation of the data in Fig. 2.29a, b on the OD decrease and also connects this OD reduction with specific bands of the spectrum range of the antenna light-harvesting complex of PS-2. First of all, one can distinguish the more stable bands of the spectrum of the complex, unchanging during its extraction ($\lambda = 641.2$, 655.3, 666–667.1, 671–672, 676.6, 681.2–682, 692.7, and 694.7 nm), and the spectral bands of high instability ($\lambda = 659.8$, 674.2, 687.7, and 691.6 nm). At γ-irradiation, the location of spectral bands changes only a little, but OD decreases at $\lambda = 641.2$, 648.3, 652, 655.3, 658.3, 662.4, 666, 669.6–672, 676.6, 681.2, 690.8, and

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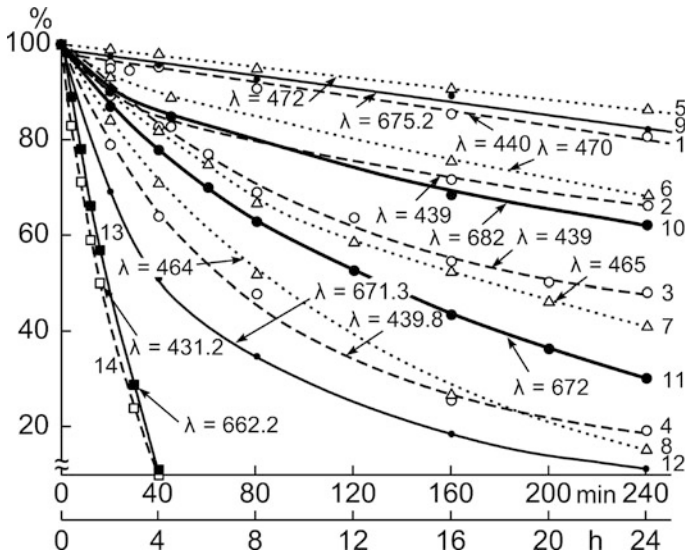

Fig. 2.28 Kinetics of change in OD value in absorption spectra of the antenna light-harvesting complex under the influence of γ-radiation (630 Gy/h) and temperature (55 °C, 6 min). *Curves*: *1*, *5*, *9* control samples (*1* $\lambda = 440$, *5* $\lambda = 472$, *9* $\lambda = 675.2$ nm); *2*, *6*, *10* γ-irradiated preparations (*2* $\lambda = 439$, *6* $\lambda = 470$, *10* $\lambda = 682$ nm); *3*, *7*, *11* thermally processed preparations (*3* $\lambda = 439$, *7* $\lambda = 465$, *11* $\lambda = 672$ nm); *4*, *8*, *12* under the simultaneous influence of temperature (6 min) and γ-radiation doses as in *2*, *6*, *10* (*4* $\lambda = 439.8$, *8* $\lambda = 464$, *12* $\lambda = 671.3$ nm); *13*, *14* the same for the acetone extract of pigments from samples of light-harvesting complexes after their processing by γ-radiation (*13* $\lambda = 431.2$, *14* $\lambda = 662.2$ nm). *Ordinate*: OD as a percentage of control value. *Abscissa*: time of action of γ-radiation or temperature aftereffect (*bottom abscissa* for *curves 1–3*, *5–7*, *9–11*; *upper abscissa* for *curves 4*, *8*, *12–14*)

694.7 nm. For some spectral bands, OD increases after γ-irradiation ($\lambda = 652$, 655.3, 666, and 672 nm). The OD increase in listed bands is coupled both with hypsochromic and with bathochromic shifts of initial bands of the spectrum of the light-harvesting complex. The general decrease in OD depends more on the OD value of the band $\lambda = 681.2$ nm.

The data obtained for haricot are supported by results of the γ-radiation impact on the antenna light-harvesting complex of PS-2 of spinach (Fig. 2.29b). The extraction of the antenna complex from *Spinacia oleracea* also leads to hypsochromic shift of the main maximum of absorption ($\lambda = 682.5$ nm). Spectral bands that do not change during the extraction of the antenna light-harvesting complex are $\lambda = 636.4$, 642.2, 645.6, 657, 667.6, 671.3, 674, 678.3, 687.5, and 693.6 nm. There are significantly changes in the position of spectral bands $\lambda = 657.4$, 660.8, 664.7, 674, 678.3, 685, and 689 nm. The γ-radiation results in an increase in the differentiation of spectral bands, sometimes in the OD increase

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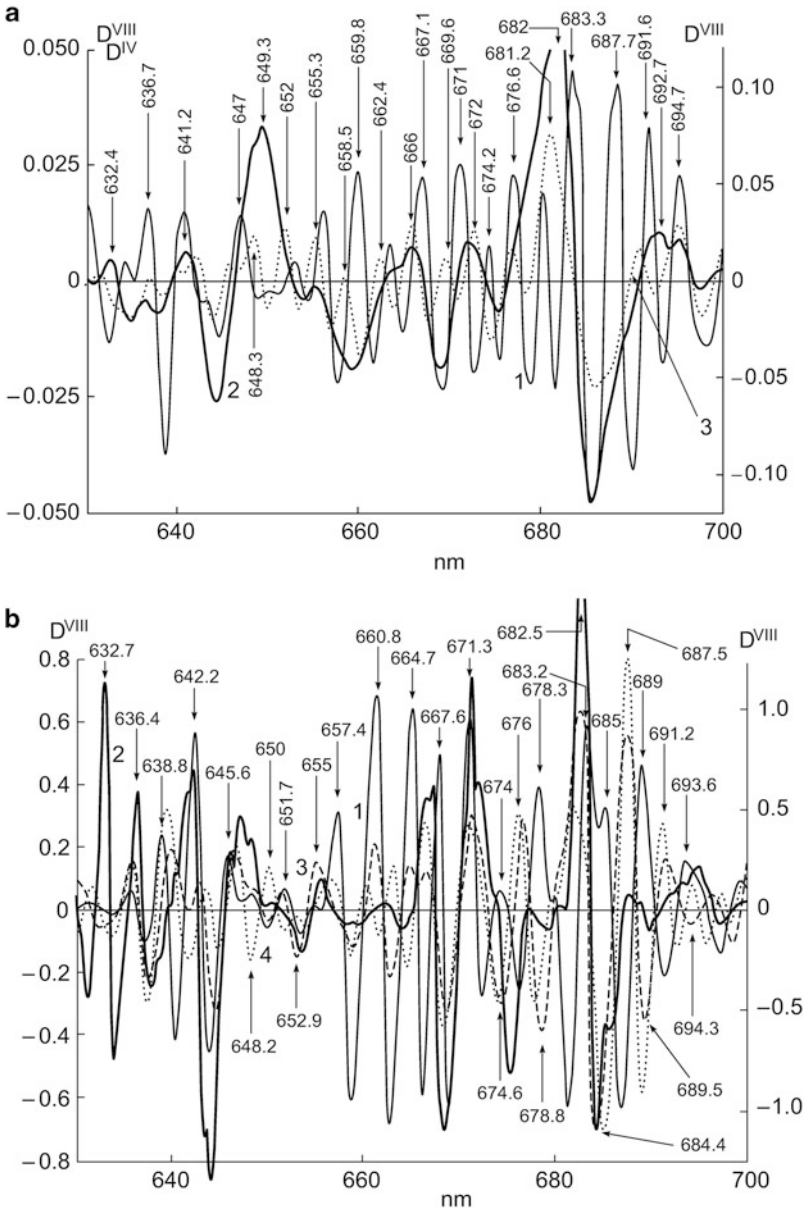

Fig. 2.29 Changes in the red area of the absorption spectrum of the antenna light-harvesting complex of PS-2 for *Phaseolus vulgaris* (**a**) and for *Spinacia oleracea* (**b**) in response to γ-irradiation. (**a**)*1* The eighth derivative of the absorption spectrum (D^{VIII}) of the intact leaf of *Ph. vulgaris* (control *1*); *2* the fourth derivative of the absorption spectrum (D^{IV}) of the sample of the antenna light-harvesting complex of PS2 (control *2*); *3* D^{VIII} of the light-harvesting complex of PS2 after irradiation by 24 kGy. *Left ordinate*: OD relative units for *curves 2* and *3*; *right ordinate*: the same for the *curve 1*. (**b**)*1* D^{VIII} of the intact leaf *S. oleracea* (control *1*); *2* D^{VIII} of the sample of the antenna light-harvesting complex of PS2 from spinach leaves (control *2*); *3* D^{VIII} of the antenna light-harvesting complex of PS2 after γ-irradiation by 2.8 kGy; *4* the same as *3* after 12 kGy. *Right ordinate*: D^{VIII}, OD relative units for *curve 1*; *left ordinate*: the same for *curves 2*, *3*, and *4*

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and very frequently in the hypsochromic shift in areas $\lambda = 648.2$, 650, 660.8–664.7, 674.6–678.8, 689.5, and 694.3 nm, with appearance of minima. The most stable bands of the spectrum of the antenna light-harvesting complex of PS-2 of spinach at γ-irradiation were $\lambda = 636.4$, 645.6, 651.7, 655, 664.7–667.6, 671.3, 682.5, 687.5–689, 691.2, and 693.6–694.5 nm. At γ-radiation, the OD increase is noticed for some bands in the spinach spectrum ($\lambda = 639$, 676, 687.5, and 691.2 nm).

Summing up the consideration of features of structural changes in the red spectral area of the light-harvesting complex of PS-2, we can separate spectral bands common for considered objects, some bands are resistant and others are non-resistant to the influence of γ-radiation.

In Fig. 2.30 changes in the blue area of the spectral structure of the PS-2 antenna complex of *S. oleracea* in response to the influence of γ-radiation are shown. In Fig. 2.30 it is seen that a dose of 2.5–2.6 kGy induces only about 10 % of damage to the kinetics of the change in OD values and that these changes can serve as an additional control for assessment of the influence of high doses of γ-radiation. The extraction of the antenna light-harvesting complex leads to disappearance of spectral bands $\lambda = 441.8$, 459.6, 463.5, 479.5, and 489.5 nm. Bands at $\lambda = 408.4$, 448.3, 450.9, 476.6, and 487.5 nm on the curve D^{IV} (curve 2) are not sufficiently resolved. Under the influence of γ-radiation, the most stable bands are still $\lambda = 404.4$, 408.4, 411.7, 420, 424.5, 429, 433.6, 444.3, 448.3, 454.8, 461.5, 465.5, 481, 487.5, and 495 nm. Similarly to the red area, the OD increases for some spectral bands ($\lambda = 404$, 411.7, 415, 420.8, 424.5, 429, 444.3, 448.3, 450.9, 451.4, 454.8, 461.5, 465.5, and 487.5 nm). An increase in the dose of γ-radiation to 12 kGy does not reduce the OD of spectral bands $\lambda = 411.7$, 415, 444.3, 448.3, 454.8, 461.5, 476.6, 487.5, and 495 nm.

The data presented lead to the conclusion that there is a higher radioresistance of spectral bands of the blue region in the structure of the spectrum of the antenna light-harvesting complex of PS-2. The stability of the Soret band and of carotenoids is especially outstanding. Lower stability characterizes the spectral bands interfaced to the violaxanthin de-epoxidation cycle in the area $\lambda = 467$–476.6 nm; the same is also characteristic of the influence of high temperatures and inhibitors of ETC of the photosynthetic device. Data from previous work (Saakov et al. 1978) allow us to conclude that changes in bands of the spectrum of the light-harvesting complex of PS-2 at $\lambda = 411.7$, 415, 419, 427–429, 433.6, and 444.3 nm, and also of the bands absent in Fig. 2.30 ($\lambda = 515$–516, 518–521, 540–541, 547–550, and 566–569 nm), and in the red spectral region (Fig. 2.29) ($\lambda = 623$–625, 632–635, and 663–666–669.6 nm) correspond to damage in the state of the submicroscopic structure of the pheophytin complex.

Changes in spectral bands $\lambda = 623$–625, 632–636, and 664–669.6–672 nm are closely coupled with changes in spectral bands at $\lambda = 678.3$–682.5 nm and indicate the localization of the damage of interaction in elements of the submicroscopic

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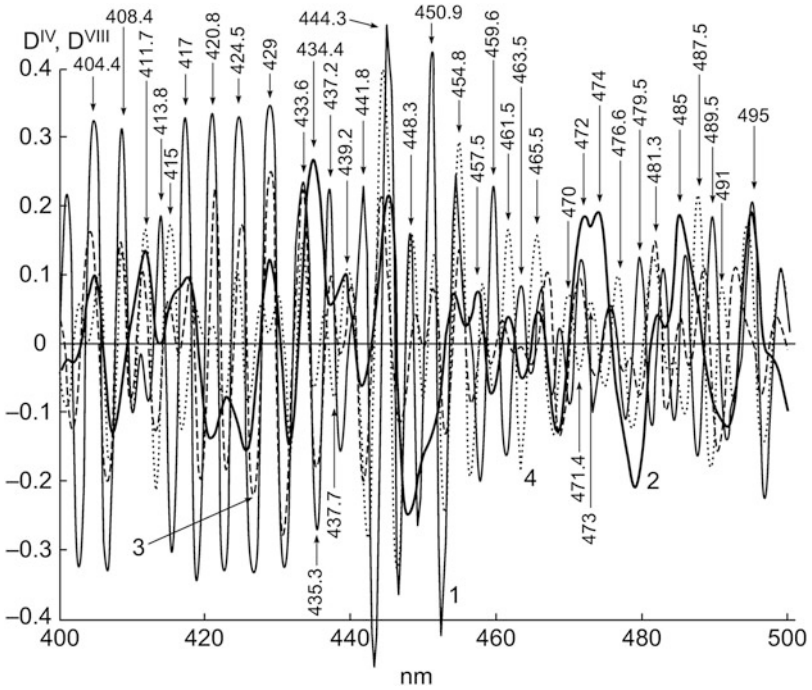

Fig. 2.30 Changes in the blue area of the absorption spectrum of the antenna light-harvesting complex of PS-2 extracted from *Spinacia oleracea* after γ-irradiation by 2.6 and 12 kGy. *1* The eighth derivative of the absorption spectrum (D^{VIII}) of the intact spinach leaf (control 1); *2* the fourth derivative of the absorption spectrum (D^{IV}) of the preparation of the antenna light-harvesting complex of PS-2 (control 2); *3* changes in D^{VIII} of the PS2 complex under the influence of 2.6 kGy; *4* the same as *3*, but under the influence of γ-radiation (12 kGy). The *ordinate* for D^{IV} and D^{VIII} is OD relative units

structure of PS-2 RCs (the complex P_{680}Pheo with acceptors Q_A and Q_B; see Eq. (2.1)) under the influence of γ-radiation, which also agrees with the published point of view (Saakov and Shiryaev 2000). Complexes of xanthophylls with chlorophyll require deeper study (Peterman et al. 1997). Forms of lutein ($\lambda = 494$ nm) and neoxanthin ($\lambda = 486$ nm) combine in the complex with chlorophyll ($\lambda = 675$ nm), causing quenching of the chlorophyll triplet state in the light-harvesting complex of PS-2. Spectral bands with $\lambda = 670$–672 nm are known as a manifestation of the presence of ChPC monomeric forms and correspond to the formation of the chlorophyll–violaxanthin complex. Possibly, under the influence of γ-radiation, the changes in the spectral structure in this area are caused by damage to the protective function of the xanthophyll cycle (Saakov 1998a, b) at the non-radiative dissipation of the light energy and the transfer of the excited chlorophyll to its ground state by means of thermal emission.

Thus, in this section we have presented for the first time comparative research on the antenna light-harvesting complexes of PS-2 from plants that differ in

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radioresistance. We have described their native spectra D^{VIII}, the set of spectral bands stable at the extraction of the complex, and the similarity of some bands for different objects. We have also established that the influence of high doses of γ-radiation on the antenna light-harvesting complex of PS-2 induces considerably smaller changes in the OD value kinetics than a short-term temperature shock or their combined impact. We found a set of stable spectral bands that are uncoupled from hypsochromic or bathochromic shifts under the influence of γ-radiation. We described the lower resistance of the antenna light-harvesting complex of PS-2 to the influence of γ-radiation than that of the native ChPC. We found spectral bands that corresponded to the realization of functional properties of ChPC during the energy transformation in RCs of ETC, which are aligned with the spectral characteristic of the link P_{680}Pheo (see Eq. (2.1)) in ETC of the photosynthetic device. The higher radioresistance of the functionally active ChPC in comparison with the resistance of its constituent structural elements was also emphasized.

2.2.10 *New Data on the Development of the Hypothesis on the Localization of Damaging EFE Influences in a Green Leaf; After-effect of γ-Radiation on the Energetics of Chloroplasts*

In the previous sections, we considered material in favor of the hypothesis on the existence of centers of localization of the EFE damaging influence in chloroplast thylakoids, the activity of these centers defining the level of leaf resistance to the EFE impact. It was supposed that these centers coincide with centers of pigment biosynthesis and with the RCs of photosystems (Baranov et al. 1974; Saakov et al. 1975; Saakov 1976). Further research provided additional material for developing ideas about coupling of the resistance of the plant photosynthetic device with the entirety of its ETC and with the photophosphorylation process (Saakov 1987, 1992, 1996; Saakov and Baranov 1987; Saakov and Leontjev 1988). Our point of view was also developed abroad (Schreiber and Bilger 1987; Krause 1988; Krause and Weis 1991, 1984; Dietz et al. 1985).

Research on the influence of γ-radiation and of its aftereffect is a convenient approach for studying the damage and reparation of energy links of the photosynthetic device and assessing the localization of this damage (Saakov 1993a, b; Saakov et al. 1993). Because biological systems respond with a delay to the influence of an EFE, the assessment of the influence of the *aftereffect* of γ-radiation is connected with the prognosis of situation development.

The objects of our researches were 2- to 3-week-old leaves of *Phaseolus vulgaris* and *Nicotiana tabacum* and compound leaves of *Robinia pseudoacacia*. We used the new methodological possibilities of the pulse fluorescence to look at the aftereffect of γ-radiation on the change in PAMF signal harmonics registered

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with the fluorometer Walz 101–103 (Effeltrich, Germany). The experimental technique is described in Sect. 2.2 (Fig. 2.3). Simultaneously with PAMF registration, we used the Warburg method to measure, at the same time, the intensities of photosynthesis and respiration. Of the 20 vessels of the device, two served as thermobarometers, nine for determination of photosynthesis intensity, and nine shielded vessels for assessment of respiration intensity. We obtained individual illumination of the leaf cuttings by the introduction of fiber-glass optical light guides in each vessel and were thus able to avoid the thermal influence of a light source on the object. The light flux from halogen lamps was transferred through the system of condenser lenses and the divider to an entrance end face of an optical bundle, which was fastened on the top part of a vessel with a Teflon gasket, creating a light flux within the range 180–1,400 μE/(m^2 s). The chosen method of lighting allowed the simultaneous measurement of the intensities of photosynthesis and respiration with the same device. To obtain correct data on the fluorescence intensity, the fulfillment of a number of conditions is necessary (Saakov 1960, 1961).

The formulae concerning theoretically expected areas of saturating concentrations of CO_2 when using Warburg's buffers for any geometry of a vessel are presented here.

Considering the interacting system "liquid–gas," let Δn be the number of molecules evaporating from the liquid in the time Δt; then

$$\Delta n = S \cdot V \cdot \Delta t \cdot n_g$$

where S is the area of the phase contact surface, V the speed of molecules in the liquid, and n_g the initial concentration of the gas in the liquid. Analogically, the number ($\Delta n'$) of molecules returned back to the liquid is

$$\Delta n^{'} = S \cdot V^{'} \cdot \Delta t \cdot n$$

where n is the concentration of the gas molecules above the liquid and V' the speed of molecules in the gas. Suppose that the number of evaporating and returned molecules in a unit time are equal to each other, and $V = V^{'}$. Then the comprehensive change in the number of molecules is described as

$$\Delta N = \Delta n^{'} - \Delta n = S \cdot V \cdot \Delta t \cdot \left(n - n_g\right)$$

At $\Delta t \rightarrow 0$, we will get the following system of equations:

$$\begin{cases} N_g^{'} = S \cdot V \cdot \left(n - n_g\right) \\ N_g^{'} = -N^{'} \end{cases}$$

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But $n_g = N_g/V_l$, where V_l is the volume of liquid and N_g is the number of molecules in the liquid. Similarly, $n = N_c/V_c$, where V_c is the volume of the upper space and N_c is the number of gas molecules in the upper space. Then the system becomes

$$\begin{cases} n_g^{'} = \dfrac{S \cdot V \cdot (n - n_g)}{V_l} \\ n^{'} V_c = -n_g^{'} \cdot V_l \end{cases} \tag{2.2}$$

Solving this system and adding E, the comparison error, we find the time t of the achievement of equilibrium of gas partial pressure in the system to be

$$t = \frac{\ln\left|\left(\frac{E}{n_g(0) - n(0)}\right)\right|}{-S \cdot V \cdot \left(\frac{1}{V_c} + \frac{1}{V_l}\right)}$$

and $E \to -0+$.

Thus, the formula for determination of equalization time of gas concentrations in the buffer system for any geometry of Warburg's vessel is deduced. This allows avoidance of mistakes connected with the time of gas phase saturation during determination of photosynthesis intensity. Besides, it is necessary to find the optimum size of a lamina to maintain the constancy of carbon dioxide concentration in the vessel volume, predefined by the chosen buffer mix.

The results of performed experiments are presented in Fig. 2.31a, b, c and 2.32 and also in Table 2.9, which show the character of changes in key parameters and in coefficients characterizing the fluorescence intensity during the aftereffect of γ-radiation. The F_0 level corresponding to the dark, base Fl, when photosynthetic membranes are in the non-energized state, is characterized by the emission of the excited antenna chlorophyll *a* before the migration of excitation in the open RCs. At this time, the first stable acceptor of PS-2, Q_A^+, is completely oxidized, $q_q = 1$, and $q_E = 0$. In response to an impulse of light or the actinic light flux, the maximum Fl (F_m) increases, and F_V shows the Q_A^- reduction that leads to closing of the PS-2 RCs (q_q and q_E equal to 0). The F_V level shows that all non-photochemical quenching processes are at a minimum (Schreiber and Bilger 1987; Krause 1988; Krause and Weis 1984). The suppression of F_V is coupled to the suppression of PS-2 activity and damage to the electron transport from P_{680} to Q_A^+, connected with the inhibition of the charge transfer between P_{680} and Pheo (Figs. 2.30 and 2.31; Table 2.9). The same follows from the analysis of the absorption signal at $\lambda = 540$–560 nm (Saakov 1976, 1987, 1992, 1996).

The ratio F_V/F_m is an indicator of the high efficiency of primary reactions of PS-2 and for intact chloroplasts is close to 0.832. The experimental deviation from the control by 0.03–0.04 is considered significant and points to the PS-2 response to

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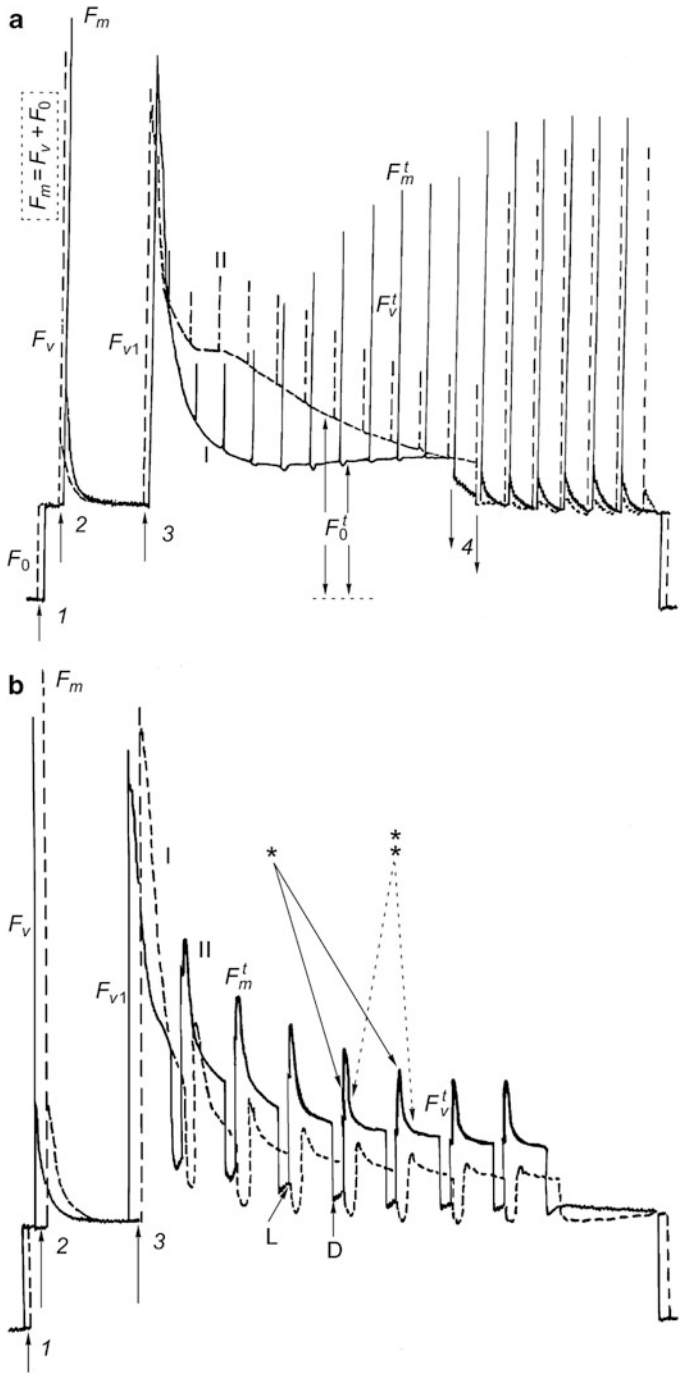

Fig. 2.31 (Continued)

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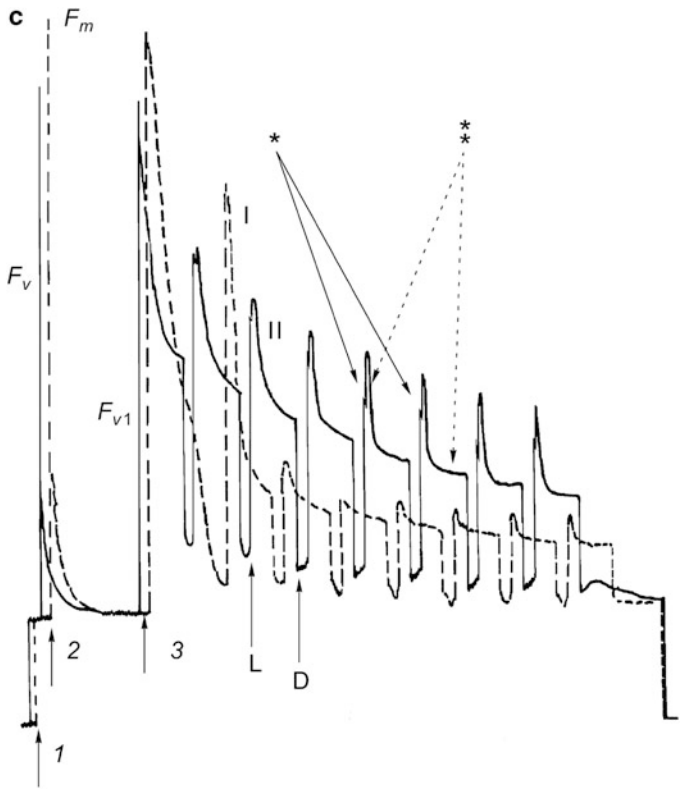

Fig. 2.31 The character of PAMF harmonics change for the chlorophyll *a* of *Phaseolus vulg. Forti* leaves adapted to darkness, in the process of the γ-radiation after-effect. (**a**) 20 hours after the influence of 3.5 kGy; input of1-s saturating white light impulse (1,100 μE/(m^2 s)) followed by 60 s of darkness and then by actinic light of 590 μE/(m^2 s) and frequency 100 kHz for the determination of F_0^t and F_V^t. (**b**) The kinetics of PAMF change in response to cycles of light (*L*)/dark (*D*) of 80 s/20 s. The actinic light used was 1,100 μE/(m^2 s) (40 h of the after-effect of 0.6 kGy). (**c**) The same as **b**, but 200 h of the after-effect of 0.6 kGy. With the increase in duration of the after-effect, the kinetics of the slow Fl component manifests more clearly. *Single asterisk* indicates the increase and *double asterisk*s the decrease in the slow component. *I* experiment; *II* control. *Arrows*: *1* input of the modulating light of 1.6 kHz (6 μE/(m^2 s), $\lambda = 660$ nm) for the determination of the level of the minimum (base) dark Fl, F_0; *2* the saturating impulse of light (3,000 μE/(m^2 s)), causing the reduction of the acceptor Q_A; *3* input of actinic light of frequency of 100 kHz; *4* shutdown of actinic light of 100 kHz for the determination of the $F_0^{11-12\ (min)}$ value and input of light of 1.6 kHz; *C* input of actinic light for 80 s; *T* shutdown of actinic light for 20 s of darkness. F_0 minimum dark Fl; F_V variable Fl; F_{V1} variable Fl at input of actinic light of 100 kHz; F_V^t variable Fl (spikes values) during *t*. The course of PAMF harmonics for *Robinia pseudoacacia* and *Nicotiana tabacum aurea* is similar

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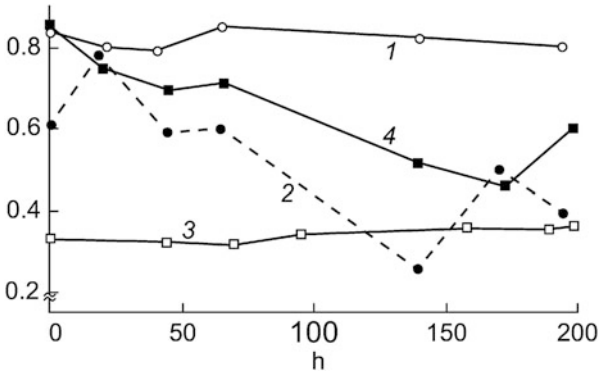

Fig. 2.32 The dynamics of the change in coefficients of fluorescence quenching in the process of the aftereffect of γ-radiation (3.5 kGy) for *Robinia*. *1* The change in the coefficient q_q, photochemical quenching, control; *2* the same, experiment; *3* the change in the coefficient q_E, non-photochemical quenching, control; *4* the same, experiment (indicates a decrease in the activity of dark reactions of the Calvin cycle). *Ordinate*: relative units; *abscissa*: the aftereffect time in hours

the EFE influence and to photoinhibition (Schreiber and Bilger 1987; Krause 1988; Krause and Weis 1984; Dietz et al. 1985) (Table 2.9).

EFEs and PhIn cause a decrease in F_V, indicating the existence of the primary damage and its localization close to PS-2 RCs (Schreiber and Bilger 1987; Krause 1988; Krause and Weis 1984; Dietz et al. 1985), which corresponds to the statement earlier formulated by us (Baranov et al. 1974; Saakov et al. 1975; Saakov 1976, 1987; Saakov and Baranov 1987).

The high F_V value (including values of spikes at F_V^t) is coupled with the low q_E value and indicates the high utilization of energy in the Calvin cycle and the decrease in the membrane proton gradient due to ATP formation (Schreiber and Bilger 1987; Krause 1988). After the F_V decrease (3 min), the light flux (1,100 μE/(m^2 s)) inducing the F_{V1} level is switched on. After 30 s, there is an additional input of a number of saturating impulses of 1 s duration and flux of 3,000 μE/(m^2 s) (Fig. 2.31a). These impulses in combination with the actinic light flux promote the complete reduction of Q_A^- and RC closing. The higher the amplitude of these impulses and the closer they are to the F_{V1} value, the higher the quantity of the reduced acceptor Q_A^-, and the higher the probability of high ETC activity, decreased q_E value, and Calvin cycle activation. The aftereffect of γ-radiation correlates with the influence of high temperature, reducing values of F_V^t and increasing the q_E value.

This phenomenon precedes the damage at the PS-2 level and is accompanied by the F_0^t increase and F_V^t decrease. The impulse of saturating light, as well as in the case with F_0, leads to closing of PS-2 RCs. Fluorescence F_0 depends on the EFE influence (except at low temperature) (Schreiber and Bilger 1987; Krause 1988; Krause and Weis 1984; Dietz et al. 1985). Earlier we found that the levels of F_0 or F_0^t (the base Fl at the input of actinic light flux for the separate time *t*) changed as a

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Table 2.9 The character of changes in the quenching of Fl of photosynthesis and respiration intensities in the process of aftereffect of γ-irradiation of *N. tabacum aurea*

Variant	Measured parameter	Value of control	Immediately after irrad, 0 h	Aftereffect of γ-irradiation (time, h), $p < 1$ %					
				20–24	40–44	68–72	138–140	160–170	190–200
		$n = 4$	$n = 3$	$n = 3$	$n = 3$	$n = 3$	$n = 3$	$n = 2$	$n = 2$
I 0.6 kGy	Int. of ph. synth. (mg CO_2/dm^2 h)	42	29	32	33	37	26	27	13
	Int. of respirat. (cm^3 CO_2/dm^2 h)	0.6	0.9	0.7	0.9	0.9	1.9	2.8	3.1
	F_V/F_m	0.843	0.815	0.805	0.816	0.785	0.740	0.735	0.720
	F_0^{10}/F_0	1.220	1.322	1.656	1.870	2.250	2.453	2.551	2.620
	q_q	0.808	0.705	0.525	0.517	0.472	0.463	0.431	0.390
	q_E	0.431	0.482	0.644	0.595	0.681	0.746	0.776	0.812
	F_{V1} $h/2$	24	30	36	42	120	146	156	224
II 1.3 kGy	Int. of ph. synth. (mg CO_2/dm^2 h)	45	27	29	25	21	16	12	9
	Int. of respirat. (cm^3 CO_2/dm^2 h)	0.8	1.0	1.6	1.8	2.3	2.8	4.2	5.6
	F_V/F_m	0.891	0.842	0.810	0.787	0.743	0.716	0.711	0.690
	F_0^{10}/F_0	1.120	1.320	1.390	1.732	1.923	2.541	2.634	2.742
	q_q	0.827	0.693	0.681	0.593	0.560	0.482	0.459	0.438
	q_E	0.430	0.671	0.613	0.690	0.746	0.873	0.879	0.889
	F_{V1} $h/2$	18	29	37	56	129	138	178	236
III 2.5 kGy	Int. of ph. synth. (mg CO_2/dm^2 h)	39	18	27	23	12	8	–	–
	Int. of respirat. (cm^3 CO_2/dm^2 h)	0.6	1.3	1.9	3.0	5.6	7.8	–	–
	F_V/F_m	0.836	0.792	0.800	0.810	0.760	0.681	0.661	0.660
	F_0^{10}/F_0	1.280	1.380	1.667	2.232	2.345	2.675	2.696	0.273
	q_q	0.863	0.800	0.814	0.606	0.511	0.390	0.370	0.320
	q_E	0.462	0.656	0.671	0.683	0.774	0.821	0.863	0.900
	F_{V1} $h/2$	22	36	48	59	126	152	192	243

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function of the radiation dose (Saakov 1992; Saakov et al. 1992). The ratio F_0^{10}/F_0 serves as one indicator of damage to the photosynthetic device (Fig. 2.31, Table 2.9). This means that a rise in F_0^t corresponding to the increase in the aftereffect of γ-radiation shows the depth of the radiation damage to PS-2 RCs evolving in time. We pay attention to the prospects of more detailed study of F_V^t/F_0^t because it is closely coupled with the damage to PS-2 energetics and with the change in ratio values from 3–6 to 1.4–0.1 relative units (Saakov 1992).

The data in Fig. 2.31b, c show, for the first time, the influence of the aftereffect of γ-radiation on the change in signal amplitude in response to periods of light and dark (80 s/20 s). From these data, it can be seen that input of the light flux for 80 s causes the amplitude of the induced signal of F_V^t to split into three components. The first, fast, component is connected with the so-called Q quenching, that is, the oxidized Q_A^+ acts as an Fl quencher because in the state $P^*_{680}PheoQ_A$ the transformation of the photochemical energy (i.e., the formation of $P^*_{680}PheoQ_A^-$ of the integral part of RCs) is a convenient opportunity for the de-excitation (Klimov and Krasnovskii 1981). The second component is short and slower (Fig. 2.31b, c, arrows with an asterisk) and emphasizes the delay in Q_A reduction due to the link damage of the transfer of e^- from P_{680} to the proximate Pheo (Saakov 1993a–d). The third , slow, component (SC) noted in Fl signals of damaged plants (Fig. 2.31b, c, curve II) most likely demonstrates damage in ETC arising during the e^- transfer from Q_A^- to the secondary acceptor Q_B^+ (the part of RCs) (Krause 1988; Krause and Weis 1984). The equilibrium $Q_A^- Q_B^+ = Q_A^+ Q_B^-$ can exist when the plastoquinone PQ^{2+} pool is completely oxidized (Schreiber and Bilger 1987; Krause and Weis 1984). Probably, the SC indicates the inhibition (as in the case with diuron) of e^- transport to the acceptor part of PS-2 between Q_A and Q_B, which leads to the q_q decrease (Table 2.9, Fig. 2.31), because all quenchers of Fl are reduced and there is no subsequent re-oxidization. In favor of this is the gradual decrease in the SC at the input of light flux (Fig. 2.31b, c, arrows with two asterisks). The shutdown of the light flux results in fast re-oxidization, e^- transfer to PS-1, and a decrease in the Fl signal to the F_0^t level. The gradual decrease in the SC shows the kinetics of q_q noted in response to pulse lighting, which is explained by the beginning of ETC reduction. The high q_q value (0.8–0.9) emphasizes the active work of ETC, the high level of $NADPH/H^+$, and the active assimilation of CO_2 (Fig. 2.31). The decrease in q_q before the leaf falls is coupled with ETC suppression (Schreiber and Bilger 1987; Krause and Weis 1984). The high reduction of the PQ pool is accompanied by the low q_q value, and PQ^+ re-oxidization leads to its increase.

Thus, the decrease seen in curve 2 in Fig. 2.32 specifies that the aftereffect of γ-radiation results in the impossibility of PQ re-oxidization and in the accumulation of electrons in the acceptor part of PS-2. The q_E increase occurs due to the increase in the proton gradient on membranes and the decrease in ATP synthesis. According to the change in q_q and q_E (Table 2.9), the intensity of photosynthesis changes. With its decrease during the aftereffect, the dynamics of the respiration intensity increases in agreement with the conformity noted before (Saakov 1992; Saakov et al. 1993).

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The provided results underline the similarity of damage to ETC energy links under the influence of various EFEs on a chloroplast and indicate that the most vulnerable areas of ETC are links of the e^- transfer chain to primary and secondary acceptors of PS-2 RCs.

The data presented in the section show, for the first time, that in the process of the aftereffect of γ-radiation, there is an increase in F_0 and a decrease in the level of spikes F_V^t and in the ratio F_V/F_m. The change in coefficient of Fl quenching indicates the damage to the dark reaction of the Calvin cycle, the decrease in photosynthesis intensity, and the increase in respiration intensity. The discussed data confirm our hypothesis about the localization of EFE damaging influences in the energy link of the photosynthetic ETC responsible for the reduction of primary and secondary acceptors of PS-2 RCs. It allows the supposition that thermal stress (Schreiber and Bilger 1987; Krause and Weis 1984), dehydration (Saakov et al. 1993; Schreiber and Bilger 1987), salification, various regimes of CO_2 and O_2 (Schreiber and Bilger 1987), intense light, and γ-radiation are characterized by identical mechanisms of the damaging influence on the redox state of Q_A of the ETC of photosynthesis.

2.2.11 *Specifics of Change in the Coefficients of Pulse-Amplitude Modulated Fluorescence Quenching (q_q and q_E) During the after-effect of γ-Irradiation*

At the modern level of research, green cell resistance to the influence of an EFE, identification of resistance mechanisms, and elucidation of the mechanisms for reparation and adaptation of the phototrophic ability are all closely connected to the choice of objects (within the possibilities of the biotechnological experiment) during the target selection of cultivars genetically modified by polygene systems (Saakov 2002a). There is a large volume of European literature, partially considered in reviews (Briantais et al. 1986; Schreiber et al. 1986, 1997a,b; 1998; Bilger et al. 1988; Schriber and Bilger 1987; Bolhar-Nordenkampf et al. 1989; Mohammed et al. 1996), on the results and interpretation of experiments using various ways of studying the Fl character change for leaves, suspensions of chloroplasts, and algae under the influence of the wide set of natural and anthropogenous EFEs.

In European works, together with the modern techniques and competent theoretical interpretation of obtained data, there is a common fault. The effect of only one, and occasionally two, stress factor is considered rather than combining the sets of results for chloroplasts to separate influences in a unifying concept that describes the universal character of the damage to the phototrophic reaction ensemble in a green cell. Most strikingly, this is manifested in the identity of fluorescence changes, especially of PAMF harmonics under the influence of various EFEs

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(Schreiber et al. 1986; Schreiber et al. 1975, 1994, 1997a,b; Bilger et al. 1988; Schriber and Bilger 1987; Bolhar-Nordenkampf et al. 1989; Mohammed et al. 1996). A good analytical approach has led to the statement of facts and to the explanation of separate findings that summarizes, without creation of new theoretical constructions, the complex of appeared knowledge in a new way that is applicable to the requirements of studying the tolerance of plant cells and the problems of biotechnology of transgene samples.

We have summarized the results of our research, linking them together in the concept of the energetic basis of the resistance of the phototrophic ability of prokaryotic and eukaryotic cells to the influence of various EFEs (Saakov 2000a–e). One EFE is the impact of γ-radiation, the effects of which on green (phototrophic) cells were reported at a conference at the Joint Institute for Nuclear Research, Dubna, Russia, 2000 (website http://www.jinr.ru/drrr/Timofeeff/conference/index_r.html and http:www.jinr/news00.html). Thus, the hypothesis about the localization of EFE damaging influences in the RCs of photosystems was further developed (Saakov 1987, 1990, 1996).

We consider it necessary to emphasize that during formulation of the energetic basis of the theory of green cell tolerance, we always considered the variety of earlier accumulated knowledge of European colleagues (Briantais et al. 1986; Schreiber et al. 1986; Schreiber et al. 1975, 1994, 1997a,b; Bilger et al. 1988; Schriber and Bilger 1987; Lichtenthaler 1988a,b; 1992, 1996a,b; 1998a,b; 2000; Bolhar-Nordenkampf et al. 1989; Bolhar-Nordenkampf 1997; Mohammed et al. 1996), whose experimental and theoretical approaches were trustworthy.

Theoretical positions on the energetic basis for the resistance of phototrophic cells to EFE influence were formulated by us on the basis of the set of experimental research on the localization of damaging influences and *structural* changes of the chlorophyll–protein complex in RCs (Saakov 1987, 1990, 1993a–d, 2000a–e, 2002a–c), and also of investigations of the *functional* damage to ETC activity (Saakov 1972, 1973, 1975, 2001a, b, 2000a–e, 2004; Bilger et al. 1988; Schriber and Bilger 1987). This research indicated the most sensitive points of the ETC connected with the transformation of light energy into the energy of chemical bonds. Our own material and the works of other groups gave confidence during the development of our theoretical concepts, especially the similarity of chloroplast reactions to the influence of the most diverse EFEs on taxonomically various phototrophs of *Procaryota* and *Eucaryota*. Such a similarity of reactions allowed us to state the idea about the adequacy of primary mechanisms of chloroplast RC damage under the influence of an EFE set (Saakov 1987, 1990, 1996, 2000a–e, 2001a, b, 2002a–c, 2003a, b, 2004).

The introduction of modern experimental techniques for the determination of the Fl yield and the subsequent emergence of new theoretical constructions was not always met with positivity. For the purpose of verification of both new types of technical decisions and scientific recommendations on the basis of experiments, programs were set up in different countries to find the optimum conditions suitable for practical application with simultaneous economy of material resources (Bolhar-Nordenkampf et al. 1989). It is very regretful that in Russia, except for declarations

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on the necessity of modernization of the experimental technique, no real steps towards such modernization were taken.

It should be noted that investigation of the influence of the EFE, in particular of γ-radiation or temperature, is closely coupled with the manifestation of its aftereffect, which is a convenient approach for investigation of the damage and reparation of the energy links of the photosynthetic device and assessment of the localization of this damage. The aftereffect is defined by the fact that biological systems respond to the EFE influence with some delay and, therefore, the assessment of aftereffect, in particular of γ-radiation, is conveniently connected with the prognosis of the development of adaptable or lethal situations during realization of the phototrophic function in a green leaf. For this purpose, we, for the first time in the world, analyzed changes in the PAMF coefficients over time for a long period, after the radiation influence and before the lethal outcome and possible necrosis of tissues.

The research on the influence of ionizing radiation allowed us to establish that PAMF changes in response to the impact of different doses of radiation (including lethal doses) are not accompanied by radical structural changes in the ChPC at the initial stages of EFE influence. References to these works are available in the bibliographic database of the National Center for Biotechnology Information USA or the Institute for Scientific Information (ISI) (http://www.ncbi.nlm.nih.gov/; http://wokinfo.com/) or, for example, on the site http://bashedu.ru/str_n_col/vestnic/magaz1_2/S1_31/html.

It is important to assess the processes of the aftereffect of γ-radiation doses by trying to track the possible reparation of the biological system or the occurrence of a lethal outcome and to assess the mechanisms of reactions. For this purpose, in this section, we first analyzed our own data on changes in PAMF coefficients over time for a long period, after the radiation influence. The aftereffect of γ-radiation on the specifics of the PAMF change was measured as earlier with the Walz 101-103 device (Effeltrich, Germany) (Saakov 1993a–d, 2000a–e).

Positive aspects of the method and features of the interpretation of obtained data are described in detail in reviews (Briantais et al. 1986; Lichtenthaler 1988a, b; Lichtenthaler and Rinderle 1988; Schreiber et al. 1986, 1994, 1997a,b; Schreiber 1983, 1986, 1998; Bolhar-Nordenkampf et al. 1989; Lichtenthaler 1996a, b).

The overwhelming amount of correct research of the end of the twentieth century in the field of resistance physiology is connected with the application of the PAMF method. This method provides the acquisition of data in vivo about the functional state of the photosynthetic device and allows, in the same leaf over a long time period, the investigation and comparison of the in situ dynamics of the processes connected with the damage to the primary reactions of photosynthesis induced by EFEs. It also allows tracking of the reparation processes or the death of an object. In our first works on the assessment of the influence of irradiation on phototrophic cells, we successfully applied this method (Saakov 1993a–d; Saakov et al. 1993) and obtained results that were repeatedly proved later. These results were taken as basis of our ideas on the energetic nature of the resistance of phototrophic tissues to EFE influence.

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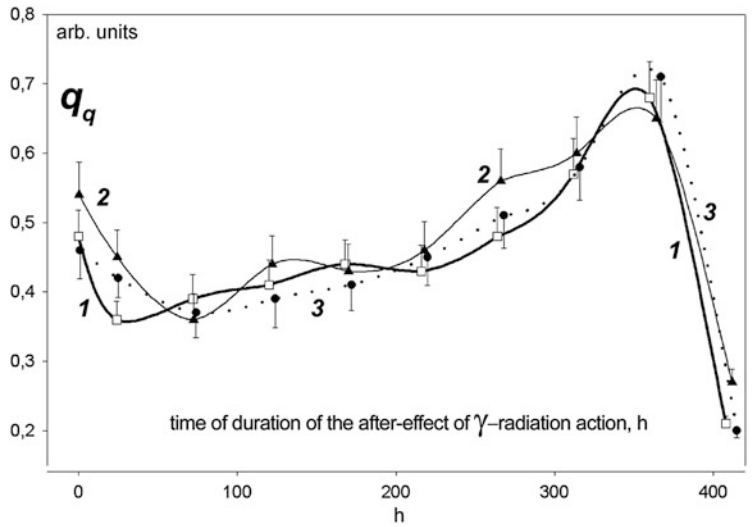

Fig. 2.33 The dynamics of the change in the coefficient q_E of the PAMF energetic quenching after the influence of γ-radiation of 0.3 kGy on leaves of *1* haricot; *2* pea; and *3* oak. *Ordinate*: q_E values, relative units; *abscissa*: the time of aftereffect of γ-radiation, in hours

The objects of the present research were 2-week-old plants of haricot (*Phaseolus vulgaris* L.) and pea (*Pisum sativum*) and oak seedlings (*Quercus robur*). The choice of objects was defined by radiation conditions in the experiment and by the need for assessment of the long-term aftereffect of γ-radiation. In the experiment, we took 8–10 leaves of one variant, and the data in Figs. 2.33, 2.34, 2.35, and 2.36 correspond to the average value of the variability induced by the radiation. The isotope ^{57}Co was used as the source of γ-radiation. The power source in experiments was 670–650 Gy/h. The design of the isotope chamber allowed us to bring objects in pots down to the γ-source and then to lift them out after exposure.

Two main types of Fl quenching are distinguished, the photochemical quenching (q_q) caused by the light energy transformation in the energy of chemical bonds in PS-2 RCs and non-photochemical quenching (q_E), representing other non-radiative ways of the de-excitation by means of the dissipation of energy and the activity of the xanthophyll and Calvin cycles. Results of experiments are presented in Figs. 2.33, 2.34, 2.35, and 2.36 and show the dynamics of the change in q_q and q_E during the aftereffect of various doses of irradiation. The calculation of the specified coefficients was based on the knowledge of a number of Fl parameters. The level of the dark, base Fl (F_0), is theoretically the Fl emission when all RCs are open, the q_q value is maximal (i.e., $q_q = 1$), and the primary acceptor Q_A^+ is oxidized. Just the F_0 level provides the basis for calculation and standardization of other signals and of Fl coefficients. The F_0 level is not constant; its increase occurs in response to damage of PS-2 RCs and disorder of the excitation energy transfer from antenna complexes to RCs. In this regard, the correct determination of

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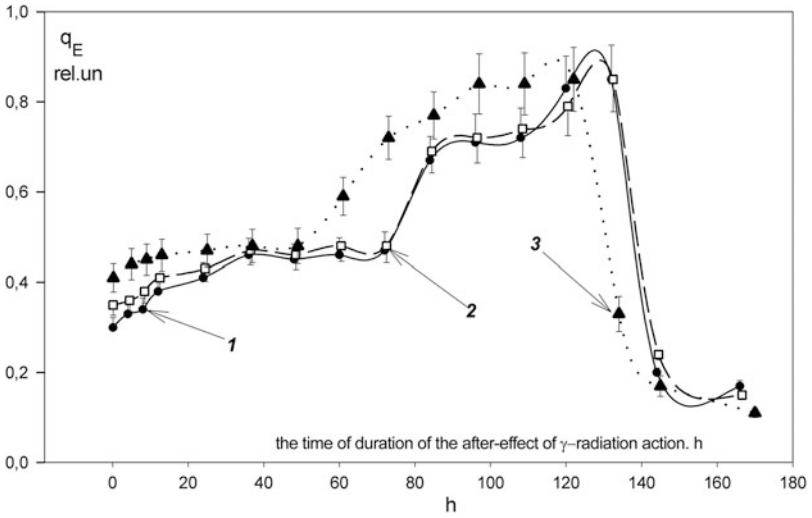

Fig. 2.34 The dynamics of the change in the coefficient q_q of the PAMF photochemical quenching as aftereffect of the influence of γ-radiation of 0.3 kGy on leaves of *1* haricot; *2* pea; and *3* oak. *Ordinate*: q_q values, relative units; *abscissa*: time in hours

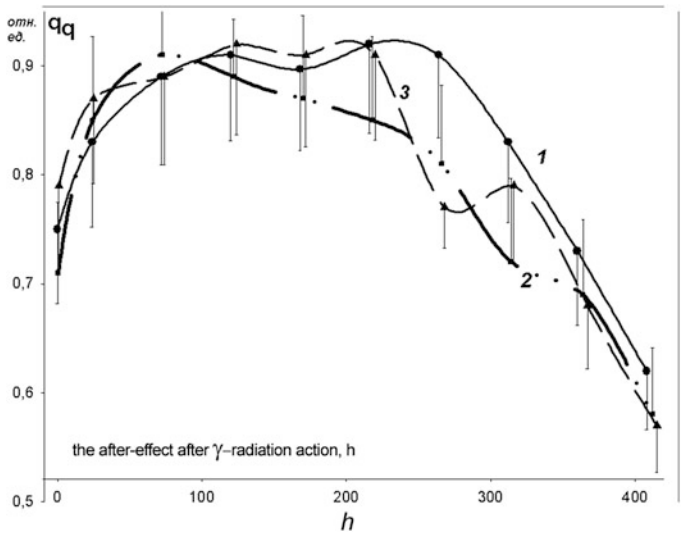

Fig. 2.35 The dynamics of the PAMF coefficient q_E change as aftereffect of the influence of γ-radiation of 2.5 kGy on leaves of *1* haricot; *2* pea; and *3* oak. *Ordinate*: q_E values, relative units; *abscissa*: the time of aftereffect of γ-radiation, in hours

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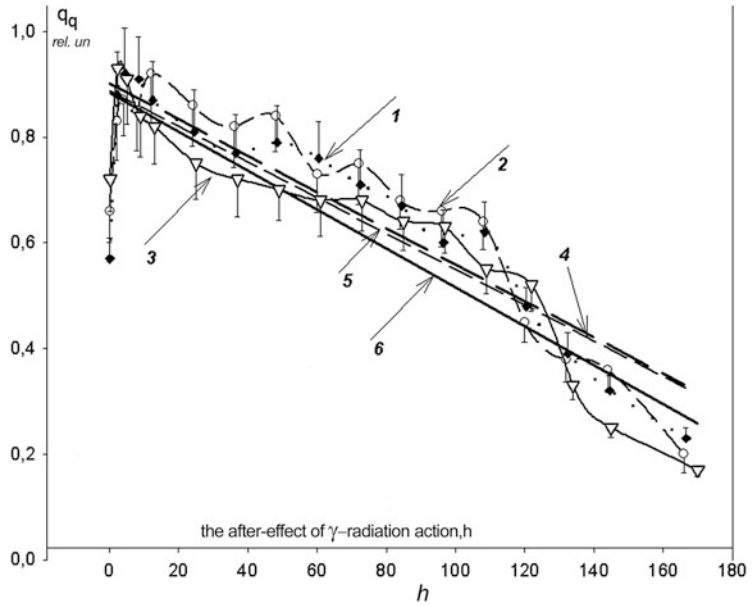

Fig. 2.36 The dynamics of the coefficient q_q change after the influence of γ-radiation of 2.5 kGy on leaves of *1* oak, *4* the regression line of the first order of the *curve 1*, $q_q = 0.845$; *2* haricot, *5* the regression line of the 1st order of the *curve 2*, $q_q = 0.947$; *3* pea, *6* the regression line of the first order of the *curve 3*, $q_q = 0.924$. *Ordinate*: q_q values, relative units; *abscissa*: the time of aftereffect of γ-radiation, in hours

F_0^t values induced by EFEs requires the initial alignment of F_0 in the experiment and a control at input of the modulating beam of 1.6 kHz. The highest point of Fl (F_m), induced by a light impulse of 3,000 μE/(m^2 s), corresponds to the complete reduction of the primary acceptor Q_A^-, and the photochemical quenching of Fl is equal to zero (i.e., $q_q = 0$). At this point, there is maximal closing of RCs and of traps of the excitation energy. The difference between F_m and F_0 is defined by the variable component F_V. In this regard, close attention to the change $F_V = F_m - F_0$ is necessary.

The ratio F_V/F_m is a sensitive parameter of the state when a leaf is subjected to EFEs. After the F_V decrease (2–3 min), actinic light (1,100–1,800 μE/(m^2 s)) inducing the F_{V1} level was put in. After 30 s, a number of saturating impulses (1 s duration, light flux of 3,000 μE/(m^2 s)) were put in (Bolhar-Nordenkampf et al. 1989; Mohammed et al. 1996; Saakov 1987, 1993a–d, 2000a, b, 2001a, b, 2002a–c, 2003a, b, 2004). These impulses added to the actinic light to promote the complete reduction of the acceptor Q_A. To obtain correct data on the q_q and q_E values, identically determined around the world (Saakov 1987–2004; Briantais et al. 1986; Schreiber et al. 1986, 1994, 1997a,b; Schreiber 1983, 1986, 1998; Bolhar-Nordenkampf et al. 1989; Mohammed et al. 1996), it is *necessary to distinguish precisely and carefully* between the increase in the F_0 value and the

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decrease in the F_V value. The increase in F_0 is characteristic of the destruction of PS-2 RCs, whereas the decrease in F_V shows the change in energetic quenching and depends on the accuracy of the F_0 determination. For all measurements of Fl coefficients, we used 7–9 min of PAMF signal harmonics, when the efficiency of the xanthophyll cycle reached the maximum.

The energy-dependent quenching of q_E (Fig. 2.34) is explained by the internal protonation of thylakoids, and its increase shows the increased energization of membranes, defined by the low speed of ATP utilization. For some time, the value of coefficient q_E remains at a plateau. Then, it increases and there is a short plateau with a further premortal peak in the q_E value. The increase in q_E to 0.5–0.6 indicates inactivation of the Calvin cycle. The secondary changes in the ChPC structural organization are less manifested (Saakov 2001a, b, 2003a, b). Specifically, because some defects of the photosynthetic device can be found only by analysis of Fl quenching coefficients, we investigated, for the first time, changes in the q_q and q_E dynamics in the process of the long aftereffect of γ-radiation (Figs. 2.33, 2.34, 2.35, and 2.36).

It is necessary to emphasize that the analysis of Fl quenching is essential and, at the qualitatively new level, supplements data on gas exchange to provide information on the specific sites of RC exposure to the stress influence.

The following conclusions result from the data in Figs. 2.33, 2.34, 2.35, and 2.36:

1. The initial stages of the curves specify that after removal of the radiation source, there is a tendency for the coefficient of Fl quenching to go to the level inherent in the intact plant (i.e., a decrease in q_E values and an increase in the coefficient q_q).
2. The process of influence of the aftereffect of γ-radiation starts when, for seemingly healthy plants, the level of q_q and q_E is similar to that of non-γ-irradiated plants, that is, the visible reparation of phototrophy functions previously suppressed by the influence of γ-radiation can be supposed. A similar phenomenon was noted earlier (Saakov 1996, 2000a–e).
3. This process is functionally connected with the influence dose.
4. For a seven- to eightfold increase in the dose of radiation, the time to lethal outcome is halved.
5. An increase in the dose of γ-radiation causes irregularity in the level change in q_E. The sharper increase and decrease in curve 3 in Fig. 2.33 show the lower resistance of pea leaves.
6. At radiation in high doses, the decreased dynamics of coefficient q_q values reduces by the law of the 1st-order regression.
7. The influence of rather small doses causes a plateau zone (Fig. 2.33, 50–80–150–200 h) in the dynamics of coefficient q_q. This plateau can be interpreted as the attempt of objects to maintain the normal level of functional reactions of phototrophy. In this case, the level of the PS-2 reduction is close to the control. This period coincides in time with the course of the curves in Fig. 2.33. Two plateaus in the dynamics of coefficient q_q probably reflect the pre-lethal state of the object.

The relaxation of curves in Fig. 2.35 indicates that the aftereffect of γ-radiation leads to the impossibility of the re-oxidization of PQ^{2-} and to the accumulation of

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electrons in the acceptor part of PS-2. Further, the decrease in all curves shows the death of plants and is accompanied by the drying of laminae, but not their necrosis. In this case, the lethal reaction of objects to the aftereffect of γ-radiation is identical to the reaction of leaves to high doses of irradiation of approximately 8–12 kGy (see our publications in the databases of NCBI Pub Med, ISI, Google).

Earlier we showed the high functional radioresistance of green cells and their protein-synthesizing systems, and small structural changes in various ChPC that differed in the complexity of their structural organization. Simplification of the ChPC structure resulted in damage to its radioresistance.

Thus, the influence of high doses of γ-radiation led to a considerable decrease in the photosynthetic intensity and a sharp increase in the level of respiration. Research on the influence of atmospheric drought and high temperatures (Saakov 2003a, b, 2004) revealed the correlation between the decrease in photosynthetic intensity and the reduction in q_q and increase in q_E.

The interpretation of these data allowed us to find the correlation between the reparation of photosynthetic intensity in response to watering and during a decrease in the day temperature.

Simultaneously, the close link between the decrease in coefficients F_m, F_{V1}, and $F_V^{'}$, the damage to the ETC functional activity, and the drop in CO_2 fixing was shown. The data in Figs. 2.33, 2.34, 2.35, and 2.36 on the influence of the aftereffect of γ-radiation on the dynamics of the change in Fl quenching coefficients confirm the early works studying the influence of other EFEs (Saakov 1993a–d, 2000a–e, 2001a, b, 2002a–c, 2003a, b, 2004) and allowed us to develop the assumption of the adequacy of mechanisms of response to the damage and the reparation of leaf autotrophy.

Scientists suppose that F_V should be "recombinant luminescence" during the $P^+_{680}Pheo^-\ Q_A^-\rightarrow P^*_{680}PheoQ_A^-$ transition. The decrease in F_V or F_V^t values under the influence of γ-radiation or its aftereffect is coupled with the changes in q_q and q_E and indicates the suppression of PS-2 RC function and of the e^- current from P_{680} to Q_A^+. Similar to the case of the influence of other EFEs, the phototrophy function of the photosynthetic device during the influence of the aftereffect of γ-radiation is connected with the RC activity and damage to the ETC in the link $P^+_{680}Pheo^-Q_A^-\rightarrow P^*_{680}PheoQ_A^-Q_B$, which is accompanied by a decrease in the level of PS-2 reduction. Thus, the specifics of the long-term dynamics of coefficients q_q and q_E are a convincing criterion for identification of the functional state of phototrophic systems when visual signs of damage to a green cell are absent. This should be taken into consideration in the case of ecological accidents of anthropogenous origin.

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2.2.12 *The Specifics of the Fine Structural Changes in the Photosynthetic Device Under the Influence of γ-Radiation*

2.2.12.1 The Character of the Changes in the Derivative Spectra of a Green Leaf In Vivo in the Red Spectral Area

Pigment–protein complexes of chloroplasts are responsible for the absorption of light energy and its transformation and conversion to other forms of energy, in particular, into the energy of chemical bonds. Significant progress in understanding the organization of photosynthetic membranes of eukaryotes and cyanobacteria (prokaryotes) responsible for the specified processes of energy transformation occurred after research on the ChPC composition by modern methods of the analysis (Green and Durnford 1996). The pigment complex consists of two photosystems, possessing a number of cofactors necessary for the charge separation and for the electron transfer. The organization and composition of the pigment complexes are rather conservative both for higher and lower eukaryotes and for prokaryotic cyanobacteria (Green and Durnford 1996). The state and functional activity of pigment complexes define the phototrophic ability of a leaf and correspond to its resistance to the EFE influence.

For the earlier assessment of the functional state of the photosynthetic device under the influence of an EFE, we used the method of PAMF (Saakov et al. 1993; Saakov 1993a–d, 1996). These studies allowed us to reveal the very peculiar character of the damage to the activity of the xanthophyll cycle, that is, some shifts in the character of derivative spectra in blue and red spectral areas (Saakov 1993a–d, 1996) indicating structural damage of the pigment complex. The improvement in research methods and the possibility of application of computer equipment for the processing of spectrophotometric research in vivo have allowed us to return, at a qualitatively new stage, to questions regarding the study of structural damage to the photosynthetic device under extreme influences (Saakov 2002a–c).

In this section we investigate features of the change in derivative absorption spectra of high orders in red and dark blue spectral areas using a native leaf under the influence of high doses of γ-radiation. Previous research showed that green cells of plants and algae are distinct in their rather high radioresistance, without essential disorder of their phototrophy function, and are much more tolerant to the influence of γ-radiation than animals (Saakov et al. 1992; Saakov 1993a, b, 1996).

As objects of research, we used the leaves of acacia (*Robinia pseudoacacia* L.), haricot (*Phaseolus vulgaris* L.), tobacco (*Nicotiana tabacum*), and the barley *Hordeum vulgaris* L. variety Donaria mutant № 2807 lacking chlorophyll *b*. The source of γ-radiation was the isotope ^{57}Co mounted into a lead chamber with a sliding glass for the placement of vessels with leaves inside. The power source for the period of the experiment was equal to 670 Gy/h. Absorption spectra of intact leaves were registered with the spectrophotometer DW-2000 (Aminco, Germany). In some cases, spectra were recorded by the UV-VIS-Specord device (Carl Zeiss, Jena,

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Germany) supplied with a differentiating facility (Saakov 2000a–e; Saakov et al. 2012/2013). The digitalization of spectral curves was carried out with the *Graph Digitizer* 2.14 program according to N. Rodionov (see http://nick-gd.chat.ru). Further processing of the digital material to obtain derivative spectra of the fourth (D^{IV}) and the eighth (D^{VIII}) orders was performed with the help of programs *Spectra Calc* and *Origin* 5.0-8 (Saakov 2000a, b; Zhukovskii and Saakov 2002). The reproducibility of results was proved or not proved by the record of five to seven parallel spectra. The available software easily allows the calculation of derivatives of high orders and determination of the integral of the area of a peak and on this basis to make conclusions about the change in the quantitative composition of components (*Peak Explorer* 1.0; *PeakFit* v.4), to perform statistical calculations (*SigmaPlot* 2000), to modify the view of any spectra to a uniform scale, and to carry out the addition of several spectra and calculate the average spectrum.

For more precise determination of substances possessing spectra found in registered spectral curves of native leaves (Figs. 2.39 and 2.40), we carried out careful purification of solutions of chlorophyll *a* and Pheo with the help of the technique (Saakov et al. 1978) described in Chap. 3 and performed the graphic presentation of their spectral properties with the corresponding derivative curves (Fig. 2.37).

Derivative spectra of high orders for Pheo are shown for the first time, as well as the eighth derivative of the absorption spectrum of chlorophyll *a*. In the spectral area *D* with $\lambda \approx 530$ nm for the spectrum D^{VIII} Pheo, there are six bands (from 522.7 to 539.5 nm); in the area with $\lambda \approx 565$ nm (from 556 to 576 nm), there are also six bands; in the area with $\lambda \approx 600$ nm (from 585 to 620.5 nm), there are nine bands; in the main maximum of Pheo absorption of $\lambda = 653$ nm (from 629.1 to 678.2 nm), 13 bands can be distinguished. The advantages of registration of D^{VIII} spectra are revealed when, on curve 4, bands appear with $\lambda = 529.9$ and 552.6 nm; peak bifurcation at 560.7, 568.8, 576.30, and 586.7 nm; the shoulder at 592.5 nm; and peaks at 612.80, 632.40, 648.80, and 652.2 nm. The registration of Pheo derivative spectra of higher orders is inexpedient and can be only defined by special research, which should be accompanied by an essential increase in the recording scale of the abscissa axis. The location of spectral bands of the D^{VIII} chlorophyll *a* spectrum substantially coincides with bands of the D^{VIII} Pheo spectrum, although sometimes bands have bathochromic or hypsochromic shifts along the abscissa. A number of bands do not show such coincidences: $\lambda = 650.4$, 652.3, 658.8, 668.1, and 671.20 nm.

To have the possibility to obtain information about the presence or absence of changes in the red area of the registered fine spectral structure of the ChPC for leaves in vivo subjected to radiation, we used the known selectivity of derivative spectrophotometry of higher orders. We also used properties of difference derivative spectra of the fourth (ΔD^{IV}) order (Figs. 2.38 and 2.39).

Because the set of ChPC forms is a system of energetically interacting elements participating in the electron transfer, even small changes in the spectral curve of a native leaf induced by ionizing radiation correspond to the disorder of stationary transitions of the step-like pathway and of the conversion of light energy into the energy of chemical bonds. Earlier, we supposed that ionizing radiation can break the function of the electron transfer link from P_{680} to Pheo and so prevent Q_A

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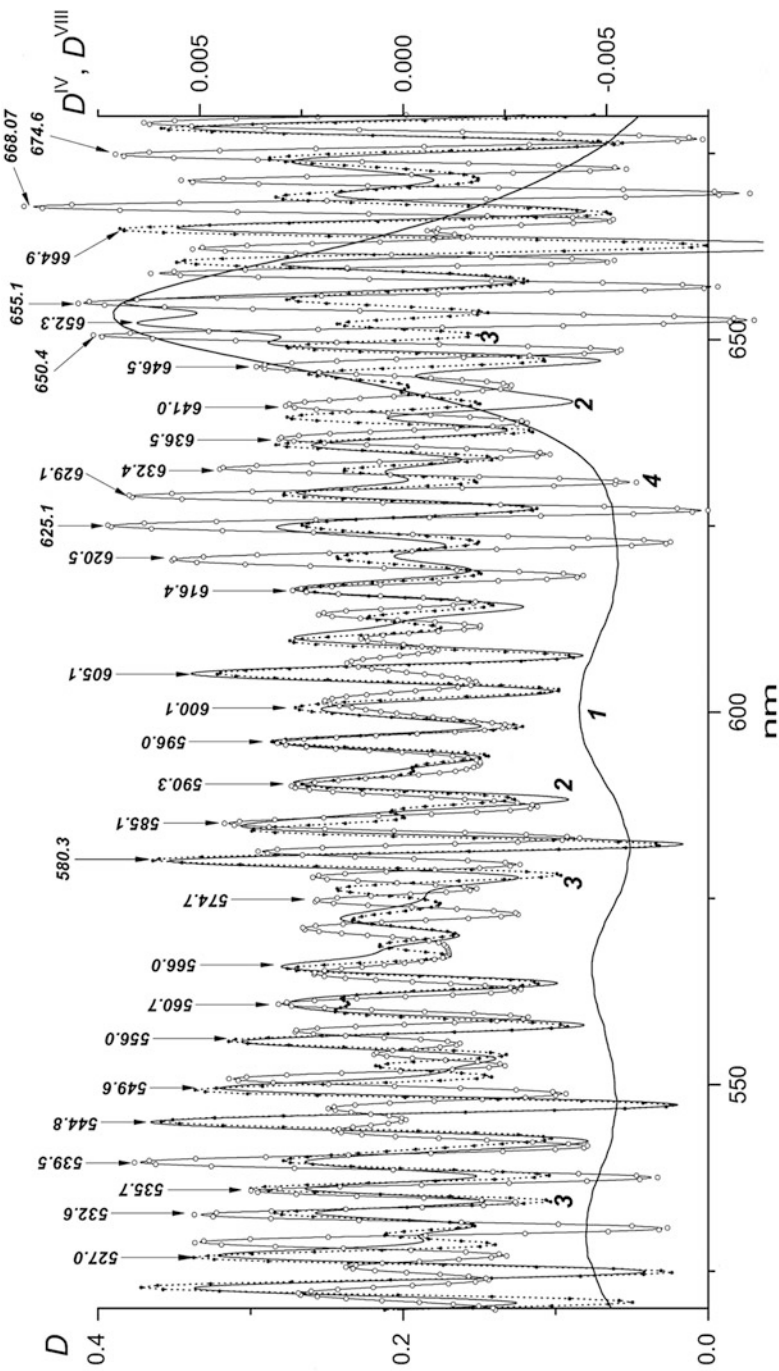

Fig. 2.37 The absorption spectrum of the pheophytin solution (*1*) and its fourth (*2*) and eighth derivatives (*3*). The eighth derivative of the absorption spectrum of chlorophyll in ethanol (*4*). Preparations passed fourfold chromatographic purification

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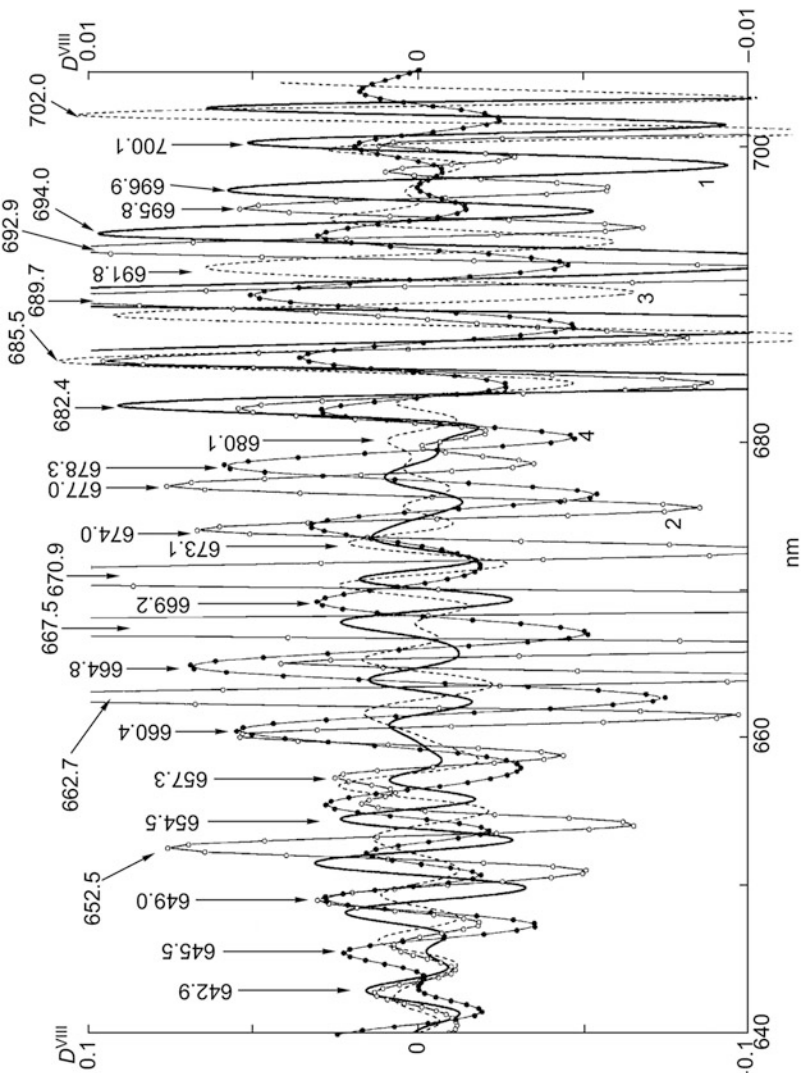

Fig. 2.38 The character of the change in the eighth derivative of the absorption spectrum of a native leaf of the barley mutant № 2807 lacking chlorophyll *b* (*1*) and under the influence of chloro carbonyl cyanide phenylhydrazone (*2*) or potassium cyanide (*3*). The eighth derivative of Pheo solution (*4*)

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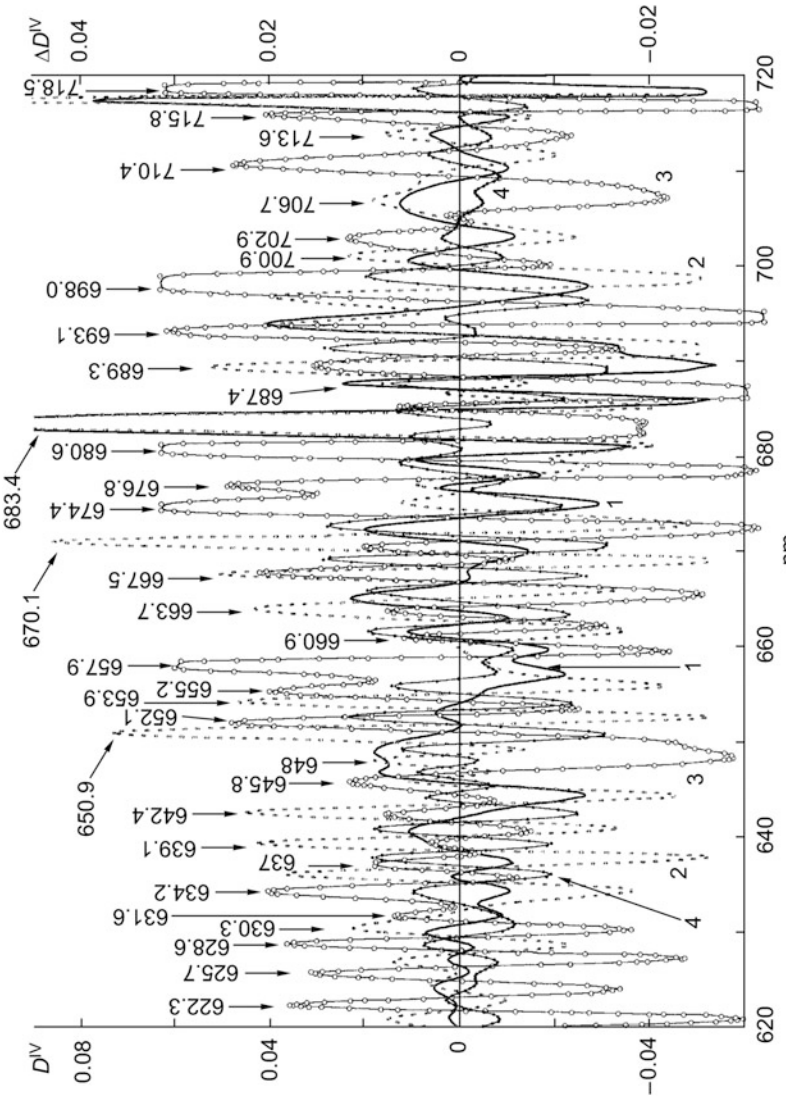

Fig. 2.39 The specifics of the fourth derivative change in the absorption spectrum of a native leaf of *Robinia pseudoacacia* under the influence of 8 kGy γ-radiation. *1* control; *2* the experiment after 8 kGy irradiation; *3* the difference spectrum (ΔD^{IV}) "control minus experiment"; *4* D^{IV} of the Pheo solution

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reduction (Saakov 1993a–d, 2000a–e). In assessing the functional damages to ETC caused by ionizing radiation and other EFEs, this situation was the subject of rather serious argumentation (Saakov 2000a–e, 2002a–c, 2003a, b; Bukhov et al. 2001a, b). The experiment showed that the short-term influence of EFEs, followed by the reparation of functional reactions of the photosynthetic device or the adaptation of the object to the influence of the EFE, is not necessarily accompanied by a quantitative change in the pigment content (Saakov 1993a–d; Semikhatova and Saakov 1962). From this point of view, the detection of minor changes in the state of the fine structure of the photosynthetic device becomes of significant value.

For the determination of separate spectral bands that are not disguised by the presence of chlorophyll *b* and can be with a high degree of reliability considered as a ChPC of chlorophyll *a*, we plotted the spectral curves of an in vivo leaf of the barley mutant № 2807 lacking chlorophyll *b* (Fig. 2.38). For this purpose, we digitalized graphic data from earlier years (Saakov 1973) and made the necessary operations of further differentiation to obtain the derivative spectra of the eighth order. This allowed us to conclude with sufficient confidence that the bands of positive extrema ($\lambda^{DVIII}{}_{max}$) presented in Fig. 2.38 (curve 1) are not caused by the contribution of absorption bands of chlorophyll *b*, and therefore they can be attributed to the absorption of native complexes of chlorophyll *a*. The absorption bands $\lambda^{DVIII}{}_{max}$ of the Pheo *a* solution (Fig. 2.38, curve 4) make it easier to understand the changes in the spectral structure of a leaf under the influence of chloro carbonyl cyanide phenylhydrazone (CCCP) and potassium cyanide (KCN).

With a high level of confidence, it is possible to say that the shifts of positive extrema of spectral bands (Fig. 2.38, curves 2 and 3) are also not caused by the contribution of absorption bands of chlorophyll *b*. There are bases to assume that the extrema $\lambda^{DVIII}{}_{max}$ induced by the influence of CCCP and KCN can be attributed to the absorption of new compounds formed after destruction of chlorophyll *a* complexes. Data presented in Fig. 2.38 on the influence of CCCP and KCN on the structural state of ChPC enable detection of new components with much higher accuracy than in previous work (Saakov 2003a, b). At the same time, these data confirm the correctness of measurements performed 30 years ago (see Saakov et al. 2013, p. 245, Fig. 4.22 and p. 254, Fig. 4.26).

The little-changed experimental design is significant because of the choice of the more informative technique for observation of phenomena. The harmonics of D^{VIII} spectra under the influence of CCCP and KCN (Fig. 2.38, curves 2 and 3) allows two conclusions to be made. First, in chloroplasts an increased content of pheophytinized products is formed from the ChPC composition, which was supported by bringing the optical densities of variants in the experiment to the same scale.

Second, the part of aggregated funds of chlorophyll *a* essentially decreases, and the part of short-wave forms with $\lambda_{max} = 677$, 670.9, 667.5, and 662.7 nm increases. The presented data create preconditions for the comparison of damage to the ChPC fine structure caused by the influence of chemical agents and radiation. The peak with $\lambda_{max} = 650$ nm in the spectrum D^{II}, induced by KCN, was decomposed into bands with $\lambda_{max} = 649.3$ and 653.1 nm. The CCCP impact induced the appearance of peaks in the spectrum D^{VIII} with $\lambda_{max} = 649.0$, 652.5, 655.5, 657.3, 659.9, 662.7,

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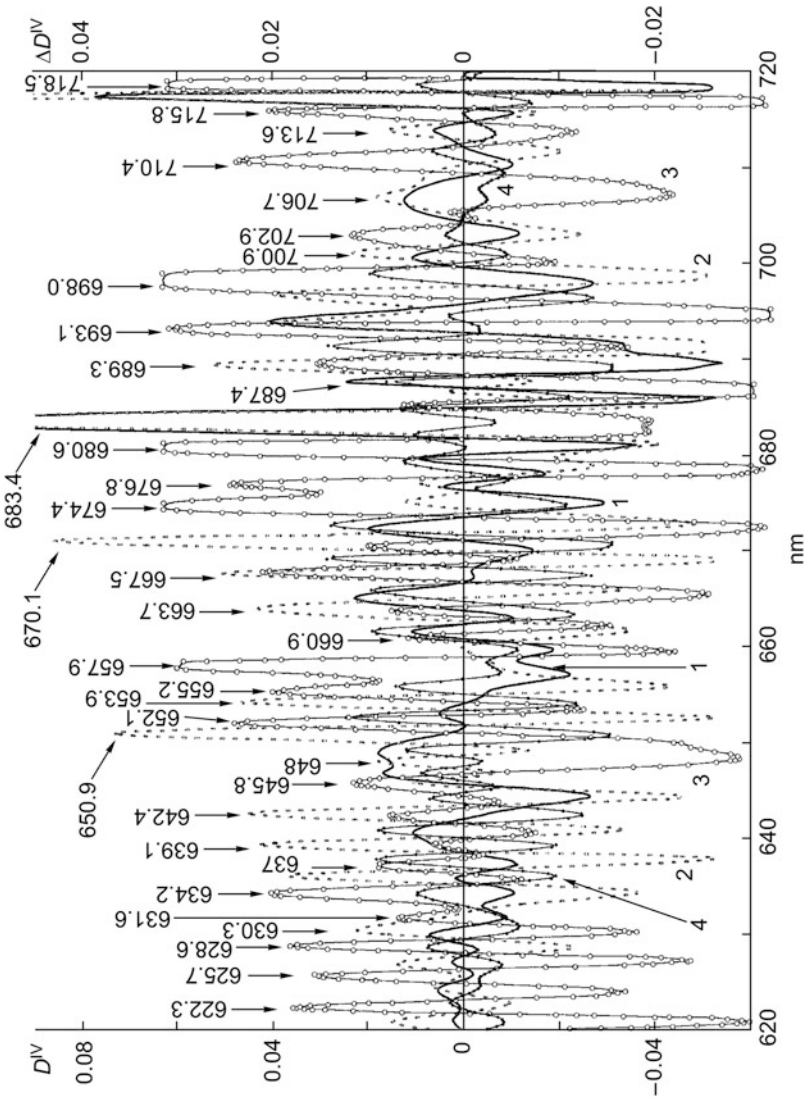

Fig. 2.40 Features of the fourth derivative change in the absorption spectrum of a native leaf of *Phaseolus vulgaris* under the influence of 12 kGy γ-radiation. *1* control; 2 the experiment just after 12 kGy irradiation; 3 and 4 curves the difference spectrum (ΔD^{IV}) "control minus experiment" 24 h after termination of irradiation

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665.0, and 667.5 nm, the location of which was not revealed in the spectrum D^{II}. The hypsochromic shift of maxima relative to the control was found at $\lambda_{max} = 674.0$, 677.0, 682.1, 685.4, 692.9, 695.8, and 698.2 nm.

Figures 2.39 and 2.40 present results on the influence of high doses of γ-radiation on the change in the ChPC structure in in vivo leaves of *R. pseudoacacia* and *Ph. vulgaris*. From the data in Fig. 2.39, it follows that the influence of γ-radiation leads to the formation of new absorption bands, namely, $\lambda_{max} = 620.7$, 623.9, 627.2, 635.70, 653.7, and 687.0 nm, and that these maxima are hypsochromically shifted relative to initial bands, the control. Bands with $\lambda_{max} = 630$, 648.1, and 665.2 nm have the bathochromic shift. A number of new extrema have no or very insignificant shift, but in them the domination of the hyperchromic effect is detectable: 632.4, 635.7, 643.8, 648.1, 650.4, 653.7, 655.9, 659.4, 662.1, 665.2, 669.2, 672.3, 678.3, 687, and 694.5 nm. The manifestation of this effect (e.g., for protein structures) suggests the formation of a less-ordered structure than the initial one, and it is also the criterion of the denaturation degree, that is, of the transition of ChPC protein molecules to the unfolded state (Rozengart and Saakov 2002). In favor of this idea is the hyperchromia of bands with $\lambda = 678.3$ and 672.3 nm, which correspond to non-aggregated ChPC forms. The difference between non-irradiated and irradiated leaves manifests very clearly in the difference spectrum "control minus experiment" (Fig. 2.39, curve 3). Because the same leaf is often used as both control and experimental object, the difference spectrum shows the difference in the structural state of a leaf *before and after irradiation*.

It is necessary to remember that, according to the basic rule of difference spectrophotometry, the orientation of difference spectra cannot coincide with the orientation of the initial spectra, and, as a rule, values of λ_{max} of difference spectra are not equal to λ_{max} of absorption spectra; the appearance of new extrema in positive and negative areas is possible. This is illustrated with curve 3 in Fig. 2.39. To the found hyperchromia in the experimental variant correspond negative extrema on this curve at $\lambda = 620.95$, 624.2, 627.4, 635.7, 643.7, 640.7, 648.50, 653.5, 659.6, 662.2, 665.5, 672.2, 678.5, 686.9, 682.9, and 694.7 nm. Taking into account the fact that the location of positive extrema of curve 1 in Fig. 2.39 substantially coincides with the position of positive extrema of curve 1 in Fig. 2.38, it is possible to attribute the absorption band with $\lambda_{max} = 644.7$, 651.3, 654.7, 659.3, 664.4, 669.39, 672.3, 675.3, 683.4, 688.0, 692.0, and 694.2 nm to bands of chlorophyll *a*.

In the experiments carried out earlier, the bathochromic shift of the main maximum of Pheo during the transition from the polar to nonpolar solvent was equal to 16–17 nm (Saakov and Hoffmann 1974). With a certain level of probability, it is possible to assume that a similar shift of Pheo absorption bands takes place in vivo. Then, it is possible to suppose that a number of extrema of curve 3 in Fig. 2.39 are caused by the formation of Pheo. For example, the band 678.3 in vivo probably corresponds to the in vitro band of the Pheo solution 660.1 nm and the band 672.3 in vivo to 665 nm of the solution. However, the initial polycomponent media in vivo points to necessary carefulness in the final conclusions, although these conclusions are very tempting.

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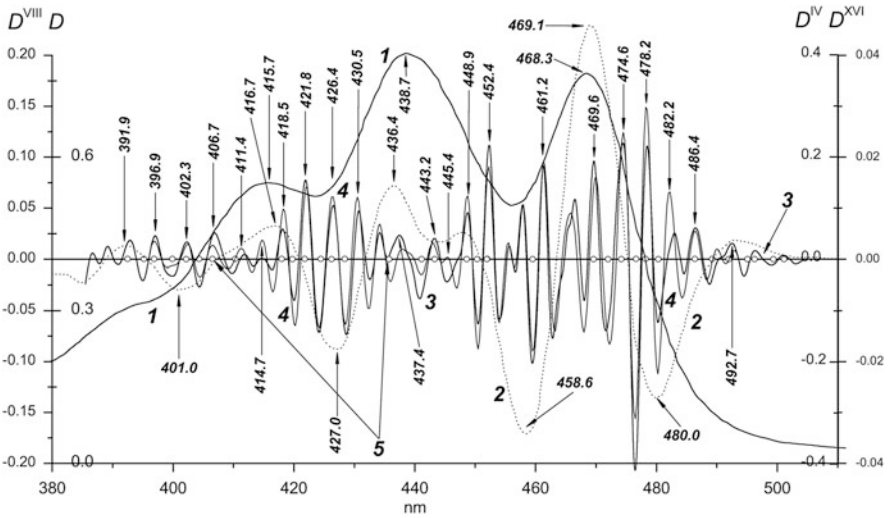

Fig. 2.41 Derivative spectra of sixfold purified and saponified solutions of violaxanthin in acetone of the 4th (*2*), 8th (*3*), and 16th (*4*) orders; *1* the absorption spectrum; *5* points of intersection of the zero axis by the D^{VII} of the absorption spectrum are shown on the zero axis. *Ordinates*: OD in relative units; *abscissa*: wavelength in nanometers

With the increase in the dose of γ-radiation (Fig. 2.41, curve 2), we specified the level of the change in ChPC separate bands for haricot leaves. It is necessary to emphasize that γ-radiation promoted high dehydration of the lamina and an increase in respiration (Saakov et al. 1993; Saakov 1993a–d, 2002a–c). We could expect functional and structural changes of the photosynthetic device, inherent in the response to atmospheric drought (Saakov 1973). Probably, the high dehydration of a leaf also promotes the manifestation of the hyperchromic effect, which manifests in the OD increase in spectral bands with $\lambda_{max} = 620.8$, 624.5, 630.3, 636.1, 639.1, 642.4, 645.7, 648, 650.9, 653.9, 659.9, 663.7, 667.5, 670.1, 675.09, 677.1, 683.4, 689.3, 696.8, 700.9, and 706.7 nm. For one band, the OD at 693.60 remained invariable; the hypsochromic effect was found for bands $\lambda = 679.8$ and 703.1 nm.

Thus, there should be no doubts about the hyperchromia induced by radiation, with appearance of the less-ordered structure and domination of non-aggregated ChPC forms. The course of the curve 3 ΔD^{IV} in Fig. 2.40 testifies to this as does the orientation of its extrema.

At the same time, not all extrema of the experimental curve 2 in Fig. 2.40 have the corresponding hypsochromic shift, the presence of which also points to ChPC de-aggregation. For example, bands 630.3, 650.9, 653.9, 659.8, and 696.7 nm have manifested bathochromic shift. Bands 620.8, 626.9, 636.0, 648.0, 667.5, 677.0, 683.4, 693.8, 700.9, 706.7, and 713.6 nm possess neither hypsochromic nor bathochromic shifts. Manifestation of the aftereffect starts 24 h later, accompanied by a reduction in OD values for the majority of bands of curves 2 and 3 down to

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values close to or even below the values of the initial control (Fig. 2.40, curve 4). At the same time, for bands with $\lambda_{max} = 622.7$, 628.7, 634.10, 637.65, 640.7, 644.1, 646.8, 649.0, 650.9, 652.4, 655.7, 658.3, 661.5, 665.9, 669.1, 672.6, 679.1, 682.1, 684.7, 687.5, 691.2, 698.9, 702.9, 711.7, and 718.5 nm, residual hyperchromia is observed that substantially coincides with extrema of curve 3. Simultaneously, during the aftereffect of radiation, the hypochromic effect of the OD decrease manifests for separate spectral bands $\lambda = 620.8$, 626.7, 630.7, 636.0, 639.2, 642.45, 648, 653.9, 663.0, 667.3, 670.5, 674.8, 676.3, 683.3, 688.8, 696.4, 700.8, 709.2, 713.1, and 716.6 nm. The location of the bands is sometimes accompanied by the bathochromic shift, showing the complexity of the structure.

In other words, during the aftereffect of radiation, the mirror course of curves 2 and 4 is observed in a quite large number of bands. It is improbable to expect the complete coincidence of the reversible dynamics of extrema positions on the specified curves. But the existence of such a fact, repeatedly registered in experiments, attracts attention and requires further experimental investigation and theoretical grounds. Due to its complexity, the final solution of the question about the possibility of the regenerative aggregation of ChPC during the aftereffect of radiation will be the subject of further research.

Thus, the material presented in this section allows us to draw certain conclusions. Under the influence of γ-radiation on intact leaves, the destruction of the photosynthetic device takes place, and this process is in many respects similar to that taking place under the influence of inhibitors of photosystems, many of which are known herbicidal substances. The structural damage to the photosynthetic device is in many respects similar to disorders arising in response to the impact of deep atmospheric drought (Saakov 2003a, b) and differs only slightly from the damage caused by temperature shock. Obviously, both in the case of the effect of inhibitors (Saakov 1973) and under the influence of ionizing radiation, the coupling of the energy transfer to and migration through ChPC forms is damaged. The appearance or the prevalence of some ChPC intermediate forms over others increases their acceptor properties at the expense of donor properties, which leads to decoupling of the energy conversion chain. The appearance of newly formed absorption bands cannot be unequivocally explained by the formation of pheophytin or of other products of chlorophyll transformation. The presence of the hyperchromic effect and of the hypsochromic shift in a number of bands reliably indicates the presence of mechanisms of de-aggregation and simplification of the structure of the chlorophyll–protein complex. As a result of the registration of derivative spectra of high orders, it became possible to consider the change in separate bands of the ChPC structure and to suggest ways of their reparation. The presented data are the evident illustration of the statement about the close interrelation between the structure and function of the photosynthetic device during the conversion of light energy into chemical potential energy and emphasize the correctness of our early ideas on the generality of primary reactions of photosynthetic device damage under the influence of EFEs. In our work, the eighth derivatives of absorption spectra of chlorophyll *a* and of pheophytin are presented for the

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first time, which is interesting for further investigation of their analytical biochemistry and physiological properties.

2.2.12.2 Features of the Influence of γ-Radiation on the Fine Structure of the Photosynthetic Device; the Assessment of the Character of In Vivo Damage in the Blue Spectral Area by Means of Derivative Spectra of High Orders

The correlation between the structure and function of the photosynthetic device is the cornerstone of the elucidation of plant phototrophic function. It especially manifests under extreme influences of the environment coupled with natural or anthropogenous stress. Earlier, we found features of structural change in the photosynthetic device of barley mutants lacking chlorophyll *b* under the impact of inhibitors of photosystems and herbicides (Saakov and Nazarova 1972; Saakov 1971a, b, 1973, 2001a, b; Saakov and Hoffmann 1974). The data pointed to links between structural changes in the photosynthetic device, the activity of the xanthophyll cycle, the ETC activity, and ATP synthesis during the photophosphorylation process (Saakov 1971a, b, 1973; Saakov and Hoffmann 1974). The development of research on the assessment of functional damage under the influence of natural and technogenic stress suggested that the reason for this damage is damage to the ETC link in the area $P^{+}_{680}Phe^{-}O_A^{-} \rightarrow P^{*}_{680}PheQ_A^{-} \rightarrow P^{*}_{680}\ PheQ_A{}^{-}Q_B$ (Saakov 2000a–e, 2002a–c). The inhibition of the phototrophic function of leaves and earlier registered changes in the fine structure of the photosynthetic device were shown (Saakov et al. 1993; Saakov 1994). It was proved that the photoinhibition of the phototrophic function of leaves begins earlier than registered changes in the fine structure of the photosynthetic apparatus (Saakov et al. 1993; Saakov 1994). The creation of conditions of artificial heterotrophy over a long time period does not result in significant changes in the structural organization of the pigment–protein complex of the photosynthetic apparatus (Saakov 1972).

The ambiguous picture in research on the influence of ionizing radiation on states of photosynthetic devices of *Procaryota* and *Eucaryota*, and also the analysis of accidents of technogenic origin, showed the insufficient reliability of the material on changes arising in the fine structure of the photosynthetic device under the irradiation of green cells of plants and algae. In this regard, in our work we decided to investigate the influence of ionizing radiation on live intact leaves at a statistically reliable level using the new possibilities of derivative spectrophotometry of high orders in combination with the computer analysis of obtained curves. We investigated the change in the fourth to eighth (D^{IV} and D^{VIII}) derivatives of absorption spectra of intact leaves for the possibility to judge about the change in the fine structure of the photosynthetic device in the blue spectral area manifested as damage of the Soret band of the chlorophyll and absorption bands of the oxidization–reduction cycle of xanthophylls (Saakov 1971a, b, 1973; Saakov and Hoffmann 1974). We also compared the obtained data with the results of previous

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sections of this chapter and with results obtained by application of the PAM method. Works of such type are unknown in experimental practice.

The objects of research were intact leaves of haricot (*Phaseolus vulgaris* L.) and barley (*Hordeum vulgaris* L., the Donaria variety, the mutant № 3613 lacking chlorophyll *b*). As the source of γ-radiation, a capsule containing the isotope ^{57}Co, of radiation power of 670 Gy/h, and shielded with lead was used. The absorption spectra of intact leaves were registered with the spectrophotometer DW-2000 (Aminco, Germany) with a built-in computer and also with the spectrophotometer UV-VIS Specord (Car1 Zeiss, Jena, Germany), supplied with an analogue differentiating device (Saakov et al. 1987). Solutions of pigments were measured in the spectrophotometer Specord-40 (Car1 Zeiss, Jena, Germany). If necessary, the digitalization of spectral curves of pigments or intact leaves was carried out with the help of the *Graph Digitizer* 2.14 program, according to N. Rodionov (see http://nick-gd.chat.ru). Further processing of the digital material was performed in *Spectra Ca1c* and *Origin* 8.0 programs.

In order to obtain information about the presence or absence of changes in the fine structure of the native chlorophyll–protein complex of irradiated leaves, we used the known selectivity of higher orders of derivative spectrophotometry and also the properties of differential derivative spectra of the fourth (ΔD^{IV}) order (Figs. 2.43 and 2.44). Here we will break the order of figure citation, and for the sake of logic, we will first refer to higher figure numbers. Because the set of ChPC forms is a system of energetically interacting elements participating in electron transfer, even small changes in the spectral curve of a native leaf, induced by ionizing radiation, correspond to the disorder of stationary transitions of the step-like pathway of the energy. Earlier, we supposed that ionizing radiation can break the function of the electron transfer link from P_{680} to Pheo and so prevent Q_A reduction (Saakov 1994). The assessment of functional damage in ETC induced by ionizing radiation and other EFEs supported this statement (Saakov 2000a–e, 2002a–c; Bukhov et al. 2001a, b).

To more exactly refer the studied curves of native spectra of leaves to specific compounds, in particular to the most important component of the xanthophyll cycle, violaxanthin (5,6,5′,6′-di-epoxy-3,3′-di-hydroxy-β-β-carotene) (Viol), the radiochemical purification of violaxanthin preparations and their spectrophotometry in two solvents of opposite polarity (acetone and in carbon tetrachloride) (Figs. 2.41 and 2.42) were performed. The spectral contour of the pigment solution in the nonpolar solvent (CCl_4) mostly corresponds to the spectrum of its native state, which allows consideration of separate bands of the blue spectral area with higher conviction (Figs. 2.42 and 2.43).

From the data (Fig. 2.41, curve 2), it follows that even on the spectrum D^{IV} the spectral bands specific to Viol, which are not detected by the usual spectrophotometry and are absent from absorption spectra, are revealed: $\lambda^{DIV}{}_{max} = 391.9$, 416.7, 436.4, and 448.9 nm; the position of the extremum is specified at 469.1 nm. The measurement of the D^{VIII} spectrum (curve 3) shows the existence of additional extrema at $\lambda^{DVIII}{}_{max} = 386.7$, 389.3, 392.7, 396.9, 406.7, 411.4, 414.7, 418.5, 421.8, 430.5, 434.3, and 437.4 nm; it also decomposes at $\lambda.^{DIV}{}_{max} = 469.1$ nm into four

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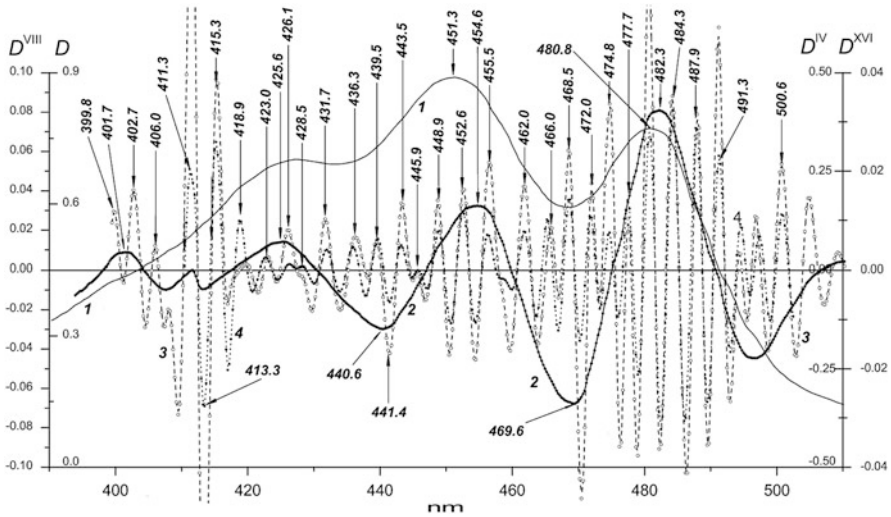

Fig. 2.42 Derivative spectra of saponified and sixfold purified violaxanthin solution in carbon tetrachloride of the fourth (*2*), eighth (*3*), and twelfth (*4*) orders; *1* the absorption spectrum. *Ordinates*: OD in relative units; *abscissa*: wavelength in nanometers

maxima. At the registration of $\lambda^{DXVI}{}_{max}$ (curve 4), the position of bands 414.7, 438.9–439.5, 445.4, 465.6, 482.2, and 489.9 nm is specified.

The spectra of Viol in CCl_4 have bathochromic shift comparative to the spectra in acetone (Fig. 2.42, curves 1 and 2). Bands with $\lambda^{DIV}{}_{max} = 401.0$, 411.5, 425.6, 454.6, and 482.3 nm are found. The double-hamped curve in the area 436.4–448.9 nm is characteristic for spectra D^{IV} of Viol in acetone (noted also in methanol and in ethanol and also for neoxanthin in these solvents) but is absent in D^{IV} spectra in carbon disulfide. The registration of D^{VIII} (curve 3) revealed the new harmonics of extrema with $\lambda^{DVIII}{}_{max}$ at 399.0, 402.7, 411.4, 415.3, 419.7, 423.0, 426.1, 431.7, 436.3, 439.5, 443.5, 448.9, 452.6, 455.5, 462.0, 466.0, 468.5, 472.0, 474.8, 477.7, 480.8, 484.3, 487.9, 491.3, 494.6, 497.3, 500.6, and 504.9 nm. We omit the presentation of curves of derivative spectra of 10–14 and 18–22 orders from the illustrative field. For example, spectra of D^{XVI} differ from spectra D^{VIII} only by small changes in the curve contour 4 at 426.2–428.5 and 459.3 nm. The consideration of the second ten orders of derivative spectra in this spectral area for the set problems of research does not provide essential additional information. The experimenter can be confined to the eighth order of derivative spectra for sufficient accuracy. Thus, for the first time in the scientific literature, derivative spectra of high orders are described for Viol; their value for the analytical chemistry of carotenoids is shown; and the expediency of restricting the routine analysis to the registration of derivative ranges of the eighth order is proved.

The quantitative content of chlorophylls in the control and in the experiment after γ-irradiation of leaves does not significantly differ. Such a situation,

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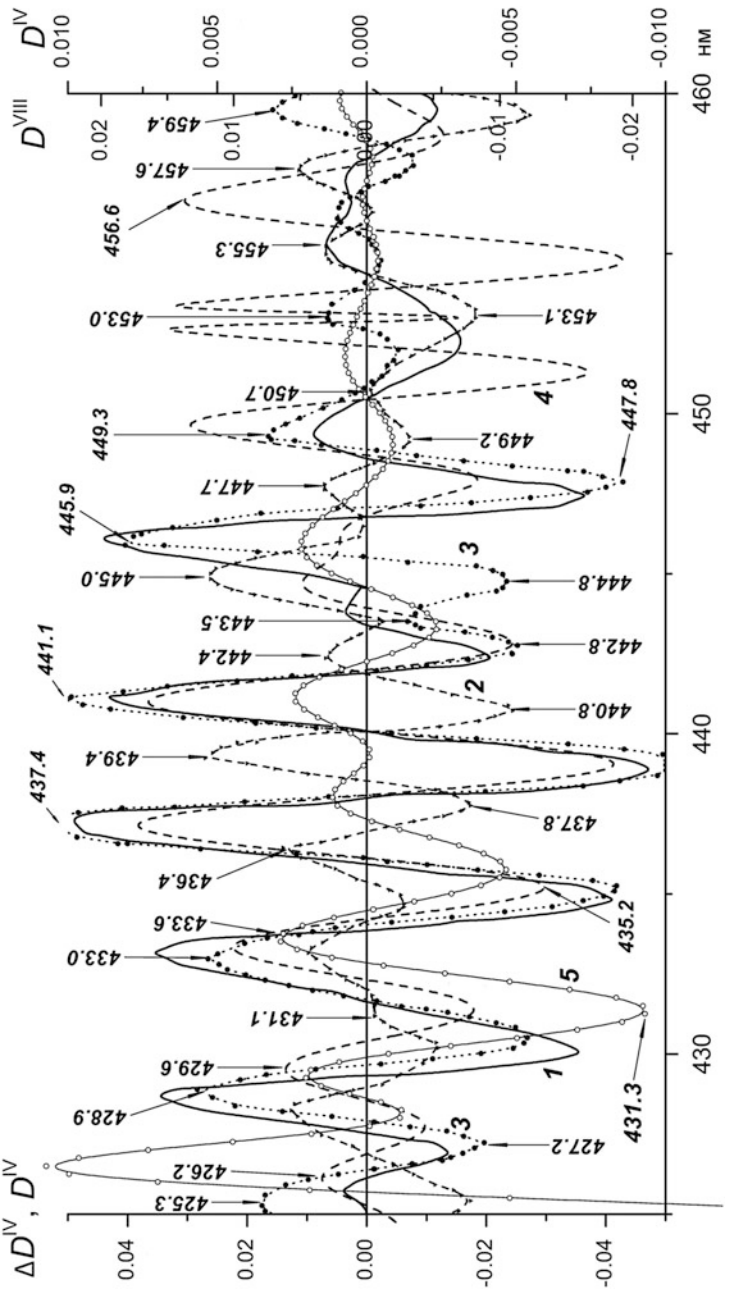

Fig. 2.43 The influence of γ-radiation (8 kGy) on the change in the spectral structure of a native leaf of *Phaseolus vulgaris* in the blue region of the spectrum (420–460 nm). *Curves*: *1* the fourth derivative of the absorption spectrum of a leaf (the control); *2* the influence of γ-radiation (the experiment); *3* the fourth derivative of the difference spectrum "control minus experiment"; *4* the eighth derivative of the spectrum of a native leaf of the barley mutant *Hordeum* lacking chlorophyll *b*, as described (Saakov 1971a, 1973); *5* the fourth derivative of the absorption spectrum of the pheophytin solution. *Abscissa*: wavelength in nanometers; *ordinates*: OD in relative units

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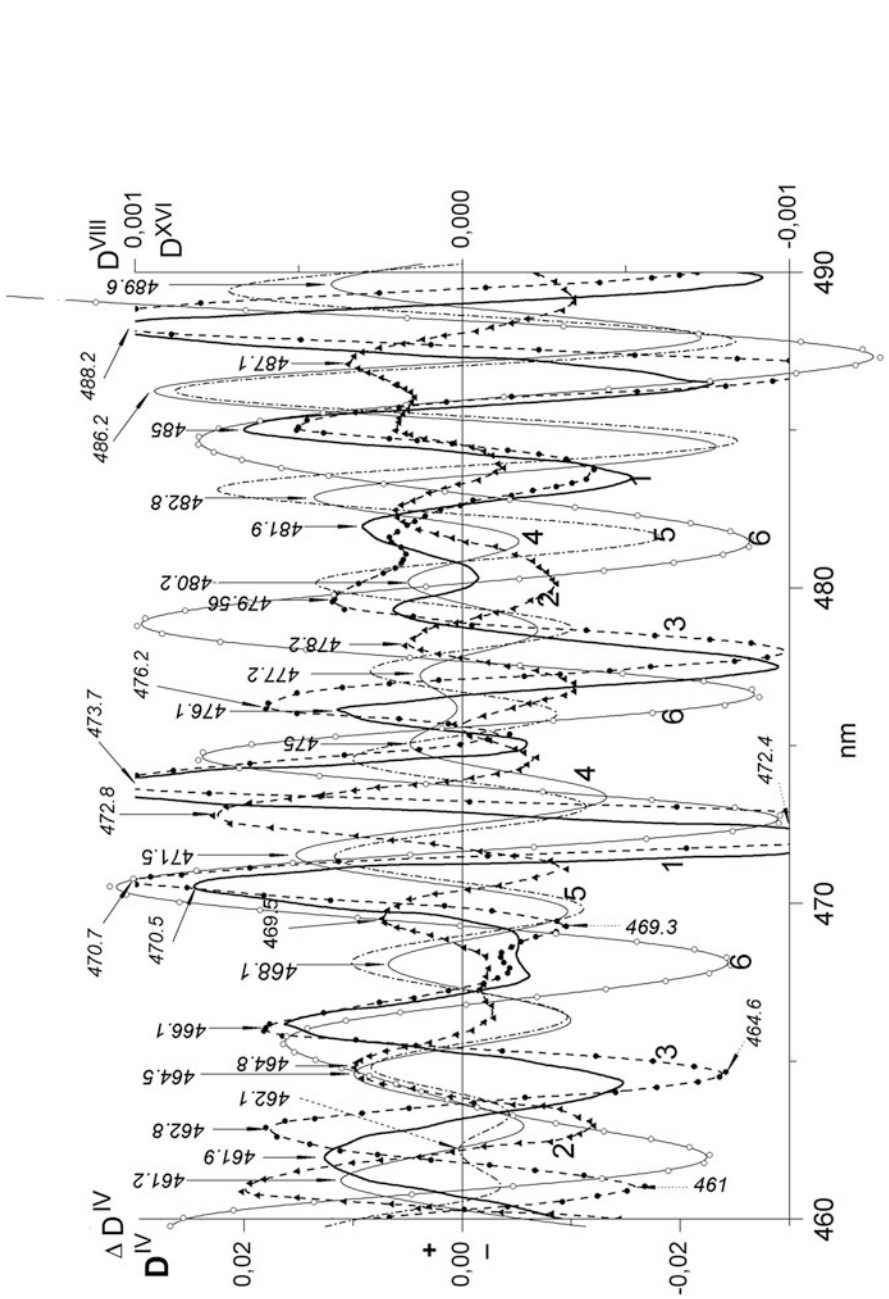

Fig. 2.44 The influence of γ-radiation (8 kGy) on the structural change in the spectrum of a native leaf of *Phaseolus vulgaris* in the blue area (460–490 nm). *Curves*: *1* the fourth derivative of the absorption spectrum of a leaf (the control); *2* the influence of γ-radiation (the experiment); *3* the fourth derivative of the difference spectrum "control minus experiment"; *4* the eighth derivative of the spectrum of a native leaf of the *Hordeum* mutant lacking chlorophyll *b*, as described (Saakov 1971a, b; 1973; Saakov and Hoffmann 1974); 5 D^{XVI} spectrum of the native leaf of *Hordeum* mutant treated with chloro carbonyl cyanide phenylhydrazone (CCCP) as described (Saakov 1973); 6 the fourth derivative of the absorption spectrum of pheophytin solution within the spectral range from 460 to 490 nm

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apparently, conceals changes in their *qualitative* state. In experiments with inhibitors, xanthophylls were the most labile pigments (Saakov 1993a–d). It is necessary to especially emphasize that in studies of control and γ-irradiated leaves by methods of registration of the first and the second derivatives of absorption spectra, the reliable differences in the run of spectral curves were not found.

A native leaf has a complex heterogeneous structure of components. In this regard, for the purpose of reliable determination of separate spectral bands that are not masked by the presence of chlorophyll *b* and can be considered as ChPC of the chlorophyll *a*, we have plotted the spectral curves of a leaf of a barley mutant lacking chlorophyll *b* (Figs. 2.43 and 2.44). To do this, we transferred the data on spectral curves D^{II} into numerical form by means of their digitalization and performed the necessary operations of differentiation.

We have designated the absorption maxima in derivative spectra of the fourth, the eighth, and further orders, as $\lambda^{DIV}{}_{max}$, $\lambda^{DVIII}{}_{max}$, etc. Then the following areas of spectral bands substantially coincide for curves 1 and 4 $D^{IV}{}_{max}$ and $\lambda^{DVIII}{}_{max}$ in Figs. 2.43 and 2.44: 428.9–429.6, 433.0–433.4, 437.1–437.4, 441.1, 449.3–449.7, 461.2–461.9, 470.5–471.5, 479.6–480.2, and 488.2–489.6 nm. With a certain level of confidence, it is possible to say that the above-listed bands of positive extrema are not caused by the contribution of absorption bands of chlorophyll *b*. It would be excessive to require the absolute coincidence of present data and the results of past years to fractions of nanometers (Saakov 1971a, b). However, it is possible to assume that the specified extrema $\lambda^{DIV}{}_{max}$ or $\lambda^{DVIII}{}_{max}$ can be attributed to the absorption of complexes of chlorophyll *a* and carotenoids (Figs. 2.42 and 2.43).

A similar comparison of absorption bands $\lambda^{DIV}{}_{max}$ of pheophytin solutions (Figs. 2.43 and 2.44, respectively, curves 5 and 6) with $\lambda^{DIV}{}_{max}$ of curve 1 (Figs. 2.43 and 2.44) reveals close areas of extrema, namely, 426.5, 429.3, 433.6, 438.0, 441.1, 445.9, 451.8, 456.7, 462.1, 465.6, 470.5, 474.7, 478.85, 484.6, and 490.1 nm. One should not expect the complete coincidence of extrema of bands of the considered curves 1, 5, and 6 because the arrangement of spectral bands of the solution is always hypsochromic, that is, they are shifted in the short-wave area relative to spectral bands of native complex structures.

In other words, absorption bands in solution are shifted towards the shorter wavelengths relative to the corresponding spectral bands in complex native structures.

Curve 2 in Figs. 2.43 and 2.44 shows the change in the D^{IV} absorption spectrum of a native leaf when it is processed with ionizing γ-radiation. The decrease in optical density (the hypochromic effect) is observed for the majority of registered bands, namely, $\lambda^{DIV}{}_{max}$: 425.5, 428.3, 433.2, 436.4, 446.5, 449.2, 453.1, 456.4, 459.3, 469.5, 472.8, 475.6, 477.0, 478.2, 480.1, 482.4, and 485.1–487.1 nm. Some spectral bands manifest hyperchromia coupled with the OD increase, namely, $\lambda^{DIV}{}_{max}$: 430.2, 431.1, 434.7, 437.8, 442.4, 444.95, 447.7, 450.7, 456.6, 457.6, 460.9, 464.8, and 467.3 nm. This gives the grounds to suppose that under the influence of γ-radiation, there is the formation of less-ordered structures than for the initial control. Taking into account that we deal with the native structure of

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ChPC, we can suppose the formation of its less-ordered structure as a result of denaturation processes induced by the ionizing radiation and the transition of the protein part of ChPC to a new conformational, unfolded state.

The appearance of the hypsochromic shift of spectral bands in the short-wave part under the influence of ionizing radiation, and most EFEs, also indicates the formation of less-ordered ChPC forms. However, sometimes it is difficult to determine whether there was a shift coupled with conformational changes or the formation of new bands under the influence of a studied factor, for example, $\lambda^{DIV}{}_{max}$: 431.1, 439.4, 442.4, 447.7, 460.9, 464.8, 472.8, and 478.2 nm.

The run of the curve of the differential spectrum (ΔD^{IV}) "control minus experiment" (Figs. 2.43 and 2.44, curve 3) supports the above conclusions and demonstrates changes in the fine structure of the ChPC spectrum induced by the ionizing radiation more reliably and demonstrably. These changes concern the following extrema of the curve ΔD^{IV}, namely, $\lambda^{\Delta DIV}{}_{max}$: 428.9, 433.0, 442.8, 443.5, 445.9, 449.3, 453.0, 459.0, 461.0, 462.8, 464.6, 466.1, 468.4, 470.7, 476.7, 479.6, 481.6, 485.0, and 488.2 nm, and also $\lambda^{\Delta DIV}{}_{min}$: 427.2, 430.5 435.1, 439.0, 442.7, 444.8, 447.8, 452.0, 454.9, 457.9, 461.0, 464.6, 467.85, 469.3, 471.4, 475.4, 478.0, 483.8, 486.9, and 490.0 nm.

The listed distinctions between the experiment and the control in Figs. 2.43 and 2.44, with the necessary evidential value emphasize the real impact of the radiation on the structural change in the photosynthetic device. Separately, it is necessary to point to the need to take into account and to consider the basic rule of difference spectrophotometry, according to which the $\Delta\lambda_{max}$ of difference spectra, as a rule, does not coincide with λ_{max} absorption spectra and with their orientation; the appearance of new maxima displaced with respect to initial ones is also probable (Figs. 2.43 and 2.44, curve 3).

However, to draw a conclusion about which elements of the ChPC (the chlorophyll or carotenoids) undergo the greatest damage under γ-irradiation, it is necessary to be careful.

Earlier, the lability of bands of a native spectrum of barley mutants under the influence of inhibitors of photosystems was shown in nanometer areas 428–430, 450, 469–471, and 500 nm (Saakov et al. 1971a, b; Saakov and Nazarova 1972; Saakov 1971a, b, c, 1973; Saakov and Hoffmann 1974), the change of which was explained by the structural damage of ChPC of chlorophyll *a* and carotenoids and also with the suppression of the de-epoxidation reaction of the xanthophyll cycle (Saakov 1971a, b, 1973; Saakov and Hoffmann 1974). The material shown in Fig. 2.43 emphasizes the damage to the ChPC structure in areas of the spectrum 429.6, 432–433, 437–440, and 444–450 nm. Proceeding from the comparison of native spectra of the barley mutant lacking chlorophyll *b*, of spectra of solutions of chlorophyll *a* and pheophytin *a* (Fig. 2.43, curve 4; Fig. 2.44, curve 6), it is possible with a certain degree of confidence to attribute the found changes to structural damage of the native complex of chlorophyll *a*. With sufficient probability, the reason for changes in the bands 428.0, 443.5, 445.9, and 455.0 nm is structural damage to the chlorophyll *b* complex. From the data in Fig. 2.44, a conclusion can be made about the highest lability of the following bands on curve 2: 462.9, 466.5,

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468.1, 471.15, 472.8, 475.6, 478.0, 481.1, 483.1, 486.1, and 489.1 nm. The corresponding band shifts on the curve 3 are found in areas 464.6, 468.3, 469.0, 472.4, 475.3, 476.9, 480.6, and 483.7 nm. Taking into account the data of curves 4 and 5 from our previous works (Saakov and Nazarova 1972; Saakov 1973; Saakov and Hoffmann 1974) and presented in the form of derivative spectra of higher orders than earlier, and also the data in Fig. 2.42, it seems possible that the above-specified bands on curves 2 and 3 in Fig. 2.44 belong to the xanthophyll system.

Earlier, we proved the high lability of Viol under the influence of inhibitors of photosystems and herbicides (Saakov 1971a, b, 1973, 2000a–e). The comparison of curves 4 and 5 in Fig. 2.44 reveals hypochromic effects in the courses of curves of native spectra of a barley leaf after the influence of the uncoupler of photophosphorylation CCCP in a range of wavelengths: 461.0, 463.4, 464.8, 469.8, 471.8, 476.0, 477.3, 478.7, 481.7, 486.25, and 487.8 nm. On the contrary, the hyperchromic effect appears in a number of bands: 462.1, 468.15, 474.6, 480.1, 483.1, and 489.4 nm. In bands 474.5, 482.8, and 489.4 nm, the hypsochromic shift caused by the simplification of polymery of structures is noted, and the bathochromic shift is observed in the band 477.3 nm.

Thus, having confirmed at a qualitatively different level the data of the past years, we can attribute the changes in curves 2 and 3 in Fig. 2.44 to damage to the structures of the pigment complex interrelated to the activity of the xanthophyll cycle (Saakov 1971a, b, 2000a–e).

Summing up the material of this chapter, we can draw the following conclusions. For the first time in a native green leaf, the localization of the damaging influence of γ-radiation in the fine structure of the chlorophyll–protein complex is shown. The influence, first of all, concerns the damage to chlorophyll *a* and links of the xanthophyll cycle. Presumably, there is damage to the structure of pheophytin. More detailed proof of this statement is the subject of a separate investigation. As a result of found changes induced by ionizing radiation in the spectral curves of a native leaf, the conclusion about the disorder of stationary transitions of the step-like pathway of light energy and damage to the activity of the photosynthetic ETC follows. The damage to the fine structure of the photosynthetic device takes place later than the change in the functional activity of phototrophy, as determined with the help of the PAMF method described in the previous sections of this chapter. The derivative spectra of high orders for violaxanthin are presented here for the first time and will undoubtedly be interesting during the performance of analytical works on the chemistry and biochemistry of xanthophylls when studying their physiological activity. The presented materials can serve as a reliable diagnostic tool for the assessment of the state of vegetation in the zone of technogenic accidents both on the ground and during the global positioning satellite survey.

The material of this chapter has brought to the attention of the reader the complex biophysical approach to research on the early changes in membrane

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structures in the whole functionally active live organisms. From this point of view, it is reasonable to apply two methods for the analysis of two different orientations, functional and structural diagnostics, which allow the intravital registration (on live organisms or objects) of the structural state of biomembranes and their supermolecular complexes. There is the unique possibility to obtain information on the state of chloroplast energetic structures connected with the conversion of light energy to the energy of chemical bonds and evolution of molecular oxygen.

The methodological approaches presented in this chapter can help solve practical problems, for example, those posed by the accident at the nuclear power station in Fukushima. Experts have now found substantial radiation pollution with cesium of vegetation, mushrooms (*Lactarius volemus*), and wheat over a distance of 70–80 km from station. According to the Japanese Ministry of Public Health Services, Work and Social Protection, tea plantations in the provinces Tiba and Saitama and sea plankton are polluted with radioactive cesium. To the north and northwest of the Fukushima station, the crops are polluted with strontium-89. Taking into account the strict territorial limits of Japan, the rational localization of farmland will play a large role. The station continues to pollute coastal waters because of leaking of infected water from accumulation tanks of the station (in 2013, 300 L with radioactivity of 80 million Bq/L; at the beginning of 2014, blowouts of radioactive water with radioactivity of 2.7 million Bq/L were registered). The intravital diagnostics of the state of survival rate of plants and seaweed as a result of the radiation aftereffect can be a defining factor.

The experimental material presented in this section and the analysis of literature on the application of the PAM method allow us to be skeptical about the existing theories, hypotheses, points of view, and, unfortunately, ignorant speculations concerning endocellular mechanisms of *Procaryota* and *Eucaryota* cell resistance to the influence of EFEs of the anthropogenous or natural origin.

In the middle of the twentieth century, one of the first scientists to perform research on this subject at a competent level for that time was P.A. Henckel and coworkers (Henckel 1954). Researches of opposite type exsited too (Udovenko 1976).

A review of the state of the problem of studying the resistance of chloroplast membranes with methods of molecular spectroscopy was published by one of authors of this book around the same time (Saakov 1976).

A serious contribution to the formation of biophysical approaches and methods of diagnostics of the vegetative cell state was made by American researchers (Strehler and Arnold 1951). Their studies were developed by the very careful work of B.N. Tarusov, chair of biophysics of Moscow University (Kochetov and Tarusov 1975, 1977; Perelygin and Tarusov 1966; Popov and Tarusov 1964; Tarusov 1966; Tarusov et al. 1962; Dzhanumov et al. 1970; Doskoch et al. 1971; Zakarian and Tarusov 1966). The achievements of the department and its most active scientists are

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consolidated in several editions of the textbook *Biophysics* edited by the corresponding member of the Russian Academy of Sciences, A.B. Rubin (2004).

Thus, in discussing the material in this chapter, the authors have tried to underline the high importance of the problem for Russia and its rather superficial and one-sided level of study.

The authors have not mentioned questions of ecological terrorism, the urgency of which has increased in the last decade. However, we consider that this area and subjects are so wide and many-sided in their ecological, political, and economic aspects that it is better to leave them beyond the scope of this book.

We have brought to the attention of the reader our experimental works, their theoretical interpretation, and also the collection of information from European publications leading in the application of the pulse-amplitude modulated fluorescence method.

It is regrettable to acknowledge, but the amplitude and productivity of the use of this method in Europe, and its achieved successes, got the better of Russian science. Attempts by the Saint Petersburg Electrical and Technical University (LETI) to create a Russian device were successful (Saakov et al. 2010). However, an obstruction to its wide introduction in the practice of scientific laboratories was the absence of Russian optic fibers. Costs associated with creation of the device got stuck on the impossibility of buying optic fibers in Europe.

Nevertheless, the method received recognition in Russia, and, with the help of equipment bought in Europe, experiments are now being carried out.

In cells of plants and animals there are two great energetic systems supplying live cells with ATP, NADPH, and NAD. One of these energetic systems is located in chloroplasts and transforms light energy to the energy of chemical bonds; the second system is placed in mitochondria.

With the enough large amount of factual material presented in this chapter we show the role of stability of the electron transport chain (ETC) in chloroplasts and so the role of life activity of a green cells occurs in dependence of functional activity of ETC at transformation of light energy and ATP formation.

The second energetic mechanism is located in cellular mitochondria. This mechanism determines cell survival by producing energy via another system, namely oxidative phosphorylation. By analogy to the activity of photosynthetic ETC in chloroplasts, mitochondrial energetic processes are responsible for cell resistance (including animal cells).

An additional argument in favor of the statements above is the known lethal action of KCN (an inhibitor of oxidative phosphorylation) on the human body and green cells.

Up to the point that factors of extreme intensity suppress the coupled or separate activity of these two energetic systems of a plant cell, it is possible to speak about the repair reactions of an organism.

The present chapter was written to substantiate a thesis about the primacy of energetic stability. The presented material proves that without competent and purposeful selection of plants using the principle of stability of their energetic

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systems, all trivial selection turns into long-term shamanism of a different orientation, because it is directed towards a search for casually obtained objects.

Some plant breeders do not analyze the biochemical essence of the main reactions of an organism and blindly waste time on obtaining stable grades. All this is a consequence of aimless chaotic selection of material and absence of real collaborative work with biochemists.

On the other hand, the poverty and weakness of the biochemical base in some selection centers does not promote such collaboration. Besides the steady and competent management of inter-disciplinary collaboration is necessary. Otherwise, good intentions are discussed but not realized.

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Chapter 3
Methodological Approaches in Experimental Work

Contents

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3.1 Main Law of Light Absorption by a Substance

Photobiological processes occur under the influence of light and can be registered in ultraviolet (UV), visible, and near-infrared spectral regions. Generally, values of light flux intensity, I, and wavelength, λ, are used in optical measurements. The frequency index $\overline{\nu}$ is also considered to characterize an absorbed light. Frequency is expressed in reciprocal seconds and represents the ratio of a radiation velocity c to a wavelength λ (in centimeters or nanometers):

$$\overline{\nu} = c/\lambda = 3 \times 10^{17}/\lambda_{\text{nm}},$$

where $c = 3 \times 10^{10}$ cm s^{-1} or 3×10^{17} nm s^{-1}, the velocity of light. A frequently used index is the wavenumber $\overline{\nu}$, which is equal to the number of waves in 1 cm. The wavenumber is a reciprocal wavelength expressed in reciprocal centimeters:

$$\left[\text{cm}^{-1}\right] : \overline{\nu} = 1/\lambda_{\text{cm}} = 10^7/\lambda_{\text{nm}}$$

Thus, $\overline{\nu} = 40{,}000$ cm^{-1}, when $\lambda = 250$ nm. A number of spectrophotometers have wavenumbers on scale dials. For example, the wavelength of the cadmium red line, as accepted by international agreement, is equal to 6438.4696 Å (angstroms). So 1 Å is 1/6438.4696 of cadmium red line.

Because only absorbed quanta can have a photochemical impact, the reader should remember that a spectral region of a photobiological process is defined by the absorption spectrum of a substance involved in this process.

The extinction law for actinic monochromatic light absorbed by a substance layer can be written in the exponential or logarithmic form:

$$I = I_0 \times 10^{-\varepsilon C d}; \tag{3.1}$$

$$\lg(I_0/I) = \varepsilon C d, \tag{3.2}$$

where I_0 and I are the light intensity before and after it passes through a layer of substance, solution, or a leaf; ε is a coefficient depending on wavelength and the nature of a substance; C is the concentration of a light-absorbing substance in medium through which light passes; and d is the layer thickness (length of the light path in a cuvette, an optical cell). If the concentration of solution is expressed in moles, then ε is called the molar extinction coefficient (MEC). The value of $\varepsilon\, E^{1\,\%}_{1\,\text{cm}}$ is numerically equal to the optical density of a solution of concentration 1 mol/L at a cuvette thickness of 1 cm (expressed in liters per mole per centimeter). Often,

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solution concentration is expressed as a percentage, and, instead of ε, the so-called specific extinction is used ($\mathbf{E}^{1\%}{}_{1\text{cm}}$), equal numerically to the optical density of a 1 % substance solution at a cuvette thickness of 1 cm. Exactly this value should be taken into account and used during instrument calibration when calculating the absolute concentration of a substance in solution.

The ratio of intensities of passing an incident light is called the transmission coefficient or transmittance and calculated as $T = I/I_0$. The same ratio expressed as a percentage is placed at the left scale of a recording card in types SP-14 and SP-18 Russian spectrophotometers and of some European ones. It is called the "percent transmission" and is given by $T = 100I/I_0$.

More frequently the term "optical density" is used (D), that is, the logarithm of transmission taken with the reversed sign. $D = -1\text{g}T = \varepsilon CD$

From Eqs. (3.1) and (3.2), it follows that, although the absolute quantity of absorbed light energy is directly proportional to an intensity of light flux incident to the object, the percentage (portion) of absorbed light does not depend on it. Therefore, the effect of absorption is assessed not according to the absolute value of light energy absorption, but according to its ratio to the intensity of light incident to the object. The absorption coefficient $(I_0 - I)/I_0$ shows the percentage of absorbed light and the transmission coefficient I/I_0 the percentage of the passed light. However, the absorption coefficient is not proportional to a substance concentration. There is direct proportion between I/I_0 and a substance concentration. The value of is called the optical density of a solution (D). It is used to determine the optical density of a 1-cm-thick layer (Babushkin et al. 1962; Konev and Volotovskii 1974; Lebedeva 1977). Optical density is dimensionless and is used as the absorption characteristic of a substance and to calculate its concentration. The law of proportionality of the degree of light extinction to layer thickness and to the amount of substance through which light passes was formulated by Bouguer–Lambert–Beer, and we call it Bouguer's law, using the name of the first pioneer (Morton 1975; Bershtein and Kaminskii 1975; Lebedeva 1977; Williams 1959; Brandt and Eglinton 1979).

If the thickness of the substance layer or solution is constant, then the dependence $D = f(C)$ is presented as a straight line passing through the origin of coordinates; moreover, the tangent of slope angle α of this straight line is equal to ε.

3.1.1 Reasons for Deviation from Bouguer's Law

If the dependence $D = f(C)$ is broken, that is, the extinction coefficient is not constant but increases or decreases with an increase in C, then apparent deviations from Bouguer's law can arise (Babushkin et al. 1962; Calder 1969; Samsonova and Gak 1971; Rubin 1974; Morton 1975; Bershtein and Kaminskii 1975). The deviations can be caused by physicochemical reasons, conditioned by the discrepancy between the C value used in the calculation and the real value of the concentration of a colored substance in a solution. An operator performing analysis should remember this.

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Another reason for non-Bouguer's law behavior is fluorescence of an analyzed substance. If a dissolved substance can be excited by monochromatic light and emit fluorescence, the fluorescent flux reaching a photodetector causes an increase in transmitted light intensity and a decrease in the experimentally determined optical density. This deviation from Bouguer's law grows with increasing optical density and decrease with a rise in the amount of dissolved substance (the quenching effect) (Braude et al. 1950; Rubin 1974; Konev and Volotovskii 1974; Brandt and Eglinton 1979; Lebedeva 1977).

The reader should bear in mind that a third instrumental reason for apparent non-Bouguer's law behavior can be a defect of the photodetector or of an amplifying circuit in the spectrophotometer, causing nonlinear dependence of instrument readings on light flux intensity. To check a device, an operator should routinely measure the transmission of several neutral light filters or solutions of studied standard substances with known density, usually supplied with spectrophotometers (Cannon and Butterworth 1953; Calder 1969; Sverdlova 1973; Gillam et al. 1970; Morton 1975; Freifelder 1980).

We should also take into account that the most frequent instrumental reason for apparent deviations from Bouguer's law is non-monochromaticity of light flux incident to a sample. In practice, to avoid substantial deformation of a spectral band shape and of the MEC, ε, a spectral width of an output slit of a device, should be much less than the half-width of investigated band $(\Delta\overline{\nu}1/2)$, that is, $S \leq 0.2\Delta\overline{\nu}$ $1/2$ (Sverdlova 1973; Tarasov 1968; Bershtein and Kaminskii 1975).

We should not forget that a significant contribution to light flux heteromonochromaticity is caused by scattered light, that is, by polychromatic emission incident to a tray camera of a spectrophotometer as a result of different reflections and also by scattered light in a dispersive system as a result of a loosely closed cover of an instrument. The influence of scattered light grows with a decrease in I_0, that is, in those spectral regions where emission of a radiation source is small or the optical density of a comparison solution is large. Because of this, spectral measurements must be practically performed in those regions of a spectrum where the absorption of an investigated substance is sufficiently great.

The following group of reasons for apparent deviations from Bouguer's law, often neglected by an operator, is connected with the distribution of an absorbent material in a volume of the analyzed object. First, absorption of the non-polarized light by anisotropic molecules depends on their order strength. The specific ordering of the arrangement of absorbent material molecules can be realized in films (Platonova et al. 1970; Popov and Smirnov 1971). An additional reason for deviations from Bouguer's law is the nonuniform distribution of absorbent material in a light beam (in a cuvette), which appears due to object non-homogeneity. Therefore, working with homogenates of leaves; with suspensions of algae, mitochondria, or chloroplasts; or with turbid solutions, an operator should regularly shake cuvettes to prevent nonuniform particle distribution or the possibility of bubbling (Babushkin et al. 1962; Shibata et al. 1973; Morton 1975; Freifelder 1980).

Research experience shows that there are three effects appearing in measurements of absorption spectra of biological subjects, and they are the most significant

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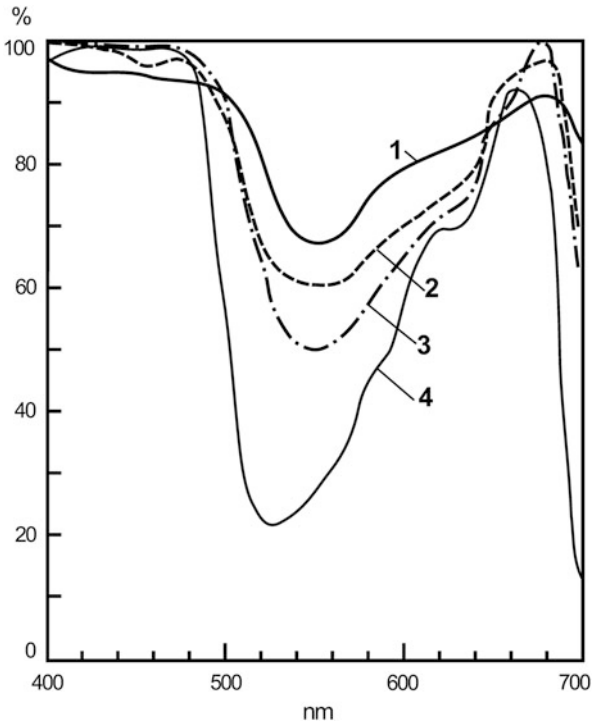

Fig. 3.1 Absorption spectra: (*1*) fresh intact spinach leaf; (*2*) isolated chloroplasts; (*3*) chloroplast fragments; and (*4*) unrefined methanol extract of pigments (Moos and Loomis 1952; Khit 1972)

for errors of spectral determination: nonselective light scattering by uncolored parts of plants, selective light scattering by pigments, and the "skipping" or "sieve" effect (Gurinovich et al. 1968; Moos and Loomis 1952).

We must not forget that biological subjects and structures not only absorb but also scatter light. So only a part of the light passing through the object reaches the instrument photoreceiver, and this creates a false impression of absorption increase. Light scattering also influences the form of spectral absorption curves, causing an overall lift of the spectral curve relative to the density axis. Therefore, corrections for light diffusion in the region of the absorption band should be performed by extrapolation from the region where true absorption is absent, usually from the region located in the long-wave part of the spectrum comparative to the investigated absorption band, for example, from $\lambda = 750$ nm for chlorophyll *a* solutions. Measurements of light-scattering objects should be corrected, and it can be useful when the instrument is supplied with adaptations for light-scattering decrease (Fig. 3.1).

When choosing experimental conditions and an object for study, decreasing the optical heterogeneity of the system (particle sizes in a system and the difference between refractive indices of an incubation medium and the studied particles) is reasonable. In practice this is achieved, on one hand, by using tissue homogenates or suspensions of chloroplasts and mitochondria instead of intact leaves and by decreasing the layer thickness at sufficiently high optical density. On the other hand, addition of substances to the incubation medium that increase the refractive

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index (glycerin, saccharose, etc.) results in a decrease in object light diffusion (Moos and Loomis 1952; Zaidel' et al. 1972).

Therefore, the operator must find the concentration of the added substance at which the optical density of the solution in the region of minimum absorption is lowest. In the case of leaf homogenates, this corresponds to 40–45 % glycerin. This question is described at length in a monograph by Rabinovich (1953) and books by Rubin (1974, 1975).

When performing spectra registration, the reader should remember that the optical density rises because of extensive light scattering and multiple reflection of rays in a heterogeneous biological system. This increase in D is more prevalent in absorption minima, which results in lower absorption selectivity and smoothing of the differences between absorption spectrum maxima and minima. Application of the method of derivative spectrophotometry allows one to substantially decrease the errors caused by light diffusion (see Chap. 3).

Substances absorbing light in biological systems are unevenly distributed, so one part of the ray traversing absorbing particles (chloroplasts, chromatophores) is weakened, while another part "passes" through the sample thickness without absorption. The phenomenon of "skipping," resulting in admixture of actinic (acting) light, causes an optical density decrease that strongly affects the absorption maximum (Morton 1975; Freifelder 1980).

Thus, we should remember that light diffusion and the skipping effect, acting in opposite directions, have several effects, for example, reduction in absorption selectivity, spectra smoothing, and maxima broadening (Popov and Smirnov 1971; Rubin 1975; Williams and Willson 1975; Lebedeva 1977; Williams 1959). It is always necessary to take into consideration that methods of quantitative spectrophotometric analysis of mono- and multicomponent mixtures, when Bouguer's law is valid and when substances do not chemically interact with each other, are based on the principle of additivity of optical densities formulated by Vierordt (1873). In accordance with this principle, the optical density of a complex mixture of substances is equal to the sum of the partial optical densities (see see Figs. 3.7 and 3.8) corresponding to the light absorption of each substance:

$$D = \varepsilon_1 C_1 d_1 + \varepsilon_2 C_2 d_2 + \ldots + \varepsilon_n C_n d_n = d\sum_{i=1}^{n} \varepsilon_i C_i.$$

3.2 Correctness and Accuracy of Spectrophotometric Data

The correctness of measurements is conditioned by the presence or absence of systematic errors of the experiment and by accuracy. Therefore, to assess accuracy, performing a number of repeated measurements is necessary, and to evaluate their correctness, some analogous measurements of standard reference samples should be carried out. The lower the average quadratic (standard deviation) S is, the better will be the reproducibility of results, that is, they are in the narrower interval and are more precise. Correctness is characterized by the difference between the average

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result of measurements and the true value of the measured characterization (i.e., by the measurement error).

Deviations from Bouguer's law, considered in Sect. 3.1.1, cause systematic errors both in MEC values and in the positions of a band's absorption maxima (λ_{max}). An operator should remember that the narrower the absorption maximum, and the shorter the waves in the region in which it is located, the more values of λ_{max} and ε can be misrepresented because of the heteromonochromaticity of emission (Shabalin and Petrova 1969; Litvin et al. 1973b; Gillam et al. 1970; Rubin 1974, 1975).

Special attention should be paid to systematic errors caused by misadjustments of a wavelength scale. For the real-time control of a wavelength scale, using solutions or glass filters with rare-earth elements that have extremely narrow absorption bands is recommended (French 1957b; Dodd and West 1961; Fog and Osnes 1962), for example, filter no. 1 from the complete set of Russian spectrophotometers (SP-10, SP-14, and SP-18), adapted for instrument calibration during their release or repair. Also, a reader could use neodymium filters supplied with some spectrophotometers (from the firms Opton, Shimadzu, and Varian), which are international standards. In the same way, derivatives of holmium (from the firms Hitachi, Opton, PerkinElmer, and Shimadzu) or benzene vapors (Beckman, Jobin Yvon, Varian, etc.) are used in international practice.

The test of a spectrophotometer transmission scale (of optical densities) is hampered by the absence of generally accepted standards of optical density (Bershtein and Kaminskii 1975; Lebedeva 1977; Freifelder 1980).

In practice, as D standards, solutions of stable inorganic compounds characterized by comparatively mildly sloping spectra in UV and visible spectral ranges are most frequently used (Burke et al. 1972; Lebedeva 1977). Of these, a 0.006006 % solution of potassium dichromate in 0.01 N H_2SO_4 had the widest hold and was recommended (Burke et al. 1972; Lebedeva 1977). The optical density of this solution was repeatedly measured by different types of instruments (Bückert and Raffaele 1963; Calder 1969). Values reported by different authors varied considerably: $D = 0.7464–0.7670$ for $\lambda = 235$ nm; $D = 0.8660–0.8793$ for $\lambda = 257$ nm; $D = 0.2865–0.2955$ for $\lambda = 313$ nm; and $D = 0.6408–0.6475$ for $\lambda = 350$ nm (Bershtein and Kaminskii 1975). It now seems that the most reliable MEC is one of potassium dichromate in $HClO_4$, accepted by the National Institute of Standards (previously known as the National Bureau of Standards) in the USA (Burke et al. 1972; Talsky 1994) (Tables 3.1 and 3.2).

It is necessary to remember that, for the same objects, results of D measurements are different for different spectrophotometers (including instruments of one type and when they are previously reliably adjusted) and registered figures are usually distinguished by 0.02–0.05 units of optical density (Brode et al. 1953; Shabalin and Petrova 1969; Gillam et al. 1970; Freifelder 1980). At the same time, λ_{max} values published by different authors vary by not more than 2 nm in the majority of cases (Phyllips 1962; McWilliam 1969). It is possible to use neutral light filters from standard sets for SP-16 and SP-26, produced by the Optical-Mechanical Association of Saint Petersburg (Russia), as ready standards for determining optical density values.

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Table 3.1 Molar extinction coefficient (MEC) values for solutions of $K_2Cr_2O_7$ in $HClO_4$, pH = 2.92 ± 0.02, temperature 25 °C (Bungard et al. 1999; Burke et al. 1972)

	Spectral region (nm)			
	350	313	257	235
$K_2Cr_2O_7$(mg/L)	MEC values			
20.22	3,155.2	1,427.0	4,227.2	3,629.4
40.09	3,159.8	1,426.6	4,241.2	3,640.4
60.12	3,161.8	1,427.6	4,254.4	3,654.2
80.17	3,168.0	1,430.0	4,275.2	3,671.6
99.92	3,171.0	1,431.2	4,288.0	3,683.0

Table 3.2 The set of types of substances for the characterization of standards of various shapes of spectra (solutions in bidistillate)

Type	Substance[a]	Conc g/L	Spectral region	λ_{max}
I	$CuSO_4 \cdot 5H_2O$	20	550–890	779
	$NiCl_2 \cdot 6H_2O$	40	300–480	394
	$Ni(NO_3)_2 \cdot 6H_2O$	40	340–450	391
	$Co(NOj)_2 \cdot 6H_2O$	40	270–350	294
	KNO3	10	260–350	301
	Congo Red	0.01	280–400	341
II	$La(NO_3)_3 \cdot 6H_2O$	20	260–350	301
	$Ce(NO_3)_3 \cdot 6H_2O$	0.01	190–350	252
	KI	0.01	210–260	226
	Amido Black B	0.01	480–720	618
	Congo Red	0.01	400–620	497
III	$NiCl_2 \cdot 6H_2O$	40	550–800	715
	$Ni(NO_3)_2 \cdot 6H_2O$	80	550–850	714
	$Ho(NO_3)j \cdot 5H_2O$	40	520–560	537
	$Nd\ (NO_3)_3 \cdot 5\ H_2O$	40	490–540	523
	$CoC1_2 \cdot 6H_2O$	40	370–600	510
	$Co(N0_3)_2 \cdot 6H_2O$	40	400–600	509
	Methylene blue	0.01	520–740	664
IV	$KMnO_4$	0.05	430–610	525
	$CeCI_3 \cdot 7\ H_2O$	1.0	190–300	253 (240) (223) (212)
	BSA*	1.0	240–320	277
	CH	0.5	240–320	280
	CHG	0.5	240–320	280
	RNase	1.0	240–320	277

[a]*CH* chymotrypsin, *CHG* chymotrypsinogen, *RNase* ribonuclease
* BSA - bovine serum albumin

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3.2.1 Main Factors Influencing the Accuracy of Spectrophotometric Measurements

The possible totality of measurement errors should be considered when performing spectrophotometric analysis. This totality is composed of smaller errors from separate operations:

1. Random errors of preparation of the analyzed solution, influence of turbidity, and fluorescence of the analyzed substance or admixtures contained in the solution.
2. Cuvette error as a result of different light absorption values of cuvettes uncompensated because of different thickness containing absorbing solvent and muliple light reflections (0.05–0.1 % of transmission) (Burnett 1973). Non-reproducibility of the state of optical faces of cuvettes and of their position in a cuvette holder is especially important. This error limits the general accuracy of analysis at low density values (Tereshin 1959a, b; Challise and Williams 1964; Ishii and Satoh 1982a).
3. Error of blank experiment as in point 1.
4. Error in the setting of an analytical wavelength, including a reading error of a wavelength scale and discrepancy between the position of the dispersive element (prism, lattice) and the indicator on a wavelength scale. When working in the region of a gently sloping absorption maximum of analyzed substance or biological structure, inaccurately setting the wavelength does not affect measurement accuracy practically. At the same time, it is necessary to remember that for sharp regions of spectral curves, this error grows to 0.7–1.5 % of the measured value (Ismail and Glenn 1964; Komar' and Samoilov 1969; Bershtein and Kaminskii 1975; Ishii and Satoh 1982a) or substantially more.
5. Direct error of the spectrophotometric measurement, including errors of instrument tuning at 0 and 100 % of transmission (Ismail and Glenn 1964; Komar' and Samoilov 1969; Burnett 1973; Korablev 1967; Litvin et al. 1973a; Konev and Volotovskii 1974; Lebedeva 1977).

The reader should not forget that the relative contribution of the listed factors depends on the nature of an absorption spectrum of an analyzed substance, the special features of an instrument, and analysis conditions. The extent to which a number of requirements are fulfilled should be checked by an operator before starting the experimental work, with repeated control measurements of the light filter sent with the instrument and comparison of this result with a firm result for this standard (Figs. 3.2 and 3.3).

If necessary, namely, in the case of the absence of control light filters and records, it is possible to use light filters from the catalog of colored glass (e.g., ZS-7) published by the St. Petersburg Optical-Mechanical Association. It is necessary to take into account that the problem of the accuracy of spectrophotometric measurements is closely related to the question of optimum optical density (D_{opt}) and the operating range of densities permissible in the analysis. This interval should

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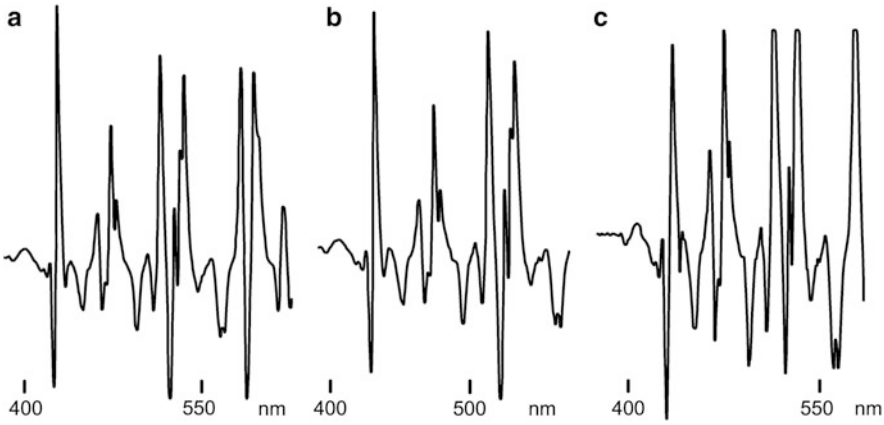

Fig. 3.2 Stability of record reproducibility of the second derivative of the absorption spectrum of the control filter of didymium light filter (absorber) at different mu-factors: (a) the initial spectrum; (**b**) amplification 1.3; and (**c**) amplification 1.0 (Marenko et al. 1972; Marenko and Saakov 1973; Rutman and Saakov 1978; Saakov and Spotakovskii 1973; Rutman et al. 1978)

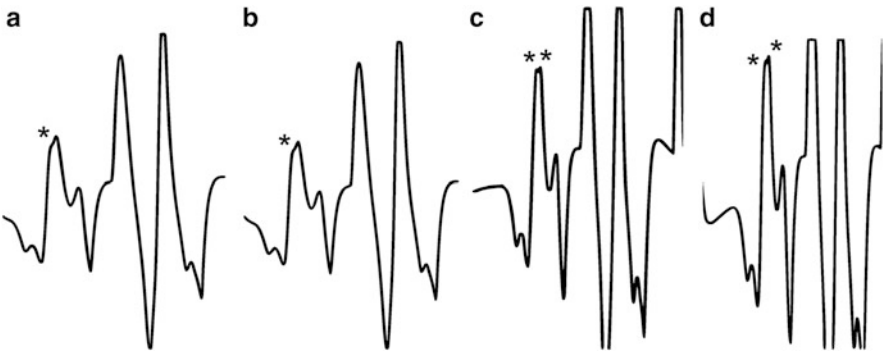

Fig. 3.3 Stability of the resolution reproducibility of the second derivative of the absorption spectrum of the control filter ZS-7 from a filter set produced by the Leningrad optical–mechanical association (LOMO): (a) the spectrum of initial filter; mu-factor (MU) is equal to: (**b**) 1.2; (**c**) 1.0; and (**d**) 1.5 (Rutman and Saakov 1978; Saakov and Spotakovskii 1973)

be selected in such a way that, over the entire range, the standard deviation does not exceed twice the standard deviation at D_{opt}:

$$S_D/D \leq 2(S_D/D)_{\min}.$$

In the paper by Twymann and Lothian (1933), the relative standard deviation of D takes the form

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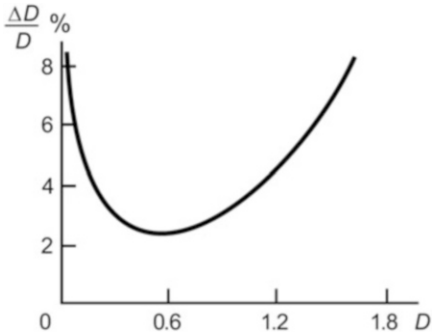

Fig. 3.4 The graph of $\Delta D/D$ error dependence as function of D in the direct spectroscopy method (Rubin 1974)

$$S_D/D = 0.4343 \cdot S_T/D \cdot 10^{-D}. \tag{3.3}$$

Equation 3.3 is deduced by supposing that the analyzed substance complies with Bouguer's law and the standard deviation of transmission S_T does not depend on the T value. Differentiating Eq. (3.3) and setting the derivative to zero, it is possible to obtain the value $D_{\text{opt}} = 0.4343$ ($T_{\text{opt}} = 36.8$ %). From Eq. (3.3), it follows that the value of relative error substantially depends on the optical density of the investigated substance:

$$\Delta D/D = \Delta C/C = 0.434 \Delta T/D \cdot 10^{-D}.$$

In Fig. 3.4, $\Delta D/D$ error is shown as a function of measured optical density D, at an accuracy of a spectrophotometer scale $\Delta(I/I_0)$ equal to 1 %. As can be seen in the graph, the transmission band 20–65 % should be chosen as the optimum region for absolute method measurements. In this case, the minimum relative error corresponds to 0.434 D.

As a result, the operating range of optical densities satisfying Eq. (2.3) is 0.2–0.7, which corresponds to the transmission interval 0.2–0.65. However, Komar' and Samoilov (1963, 1967) experimentally found some discrepancy in the S_D dependence on D. This discrepancy is maximal in the region of large D values. For a large number of spectrophotometric measurements in off-the-shelf devices performed with the usual methods, a reader should choose the value of D_{opt} within the range 0.4–0.8 D (Freifelder 1980).

It is necessary to emphasize that, for a particular instrument, its real measurement error at D_{opt} and the operating density range should be determined only on the basis of an experimental study performed by an operator before the routine analysis and taking into account the recommendations made above (Nagibina and Prokof'ev 1961; Shabalin and Petrova 1969; Sverdlova 1973; Gillam et al. 1970; Morton 1975; Lebedeva 1977).

The influence of many factors on an error of spectrophotometric analysis can be compensated for if analyses of unknown solutions or homogenates are carried out under the same conditions (i.e., the same spectrophotometer, cuvettes, volumetric

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glassware, and weights) and with the same error characteristic of the work of a particular operator (Tarasov 1968; Rubin 1974).

When analyzing complex mixtures or homogenates of biological tissues, the researcher should use a combination of spectral approaches and methods of analysis of registered spectra. The purpose of such combinations is to determine not only one main substance in a mixture, but several components or changes in their states (Brice and Swain 1945; Nagibina and Prokof'ev 1961; Clayton and Thiers 1966; Calder 1969; Shlyk 1971; Moskvin et al. 1973; Perfilev et al. 1985; Spitsyn and L'vov 1985).

The accuracy and correctness of the quantitative and qualitative spectrophotometric analysis of any particular mixture of main compound and admixtures, as well as of the analysis of heterogeneous biological structure, depend on the combination of many factors. They include, first, strictness in fulfilling the conditions and limitations basic for the method used. Furthermore, the accuracy and correctness of an analysis depend on the impurity content and trend of the spectral curve, the correct choice of analytical wavelengths, errors of spectrophotometric measurements, and the accuracy of the preparation of biological and parallel samples. Therefore, the accuracy and correctness of data analysis of a particular system or structure with a particular spectral method can and must be found in a special preliminary study (Gonopolskii 1969; Litvin et al. 1973b; Bershtein and Kaminskii 1975).

Because of the high sensitivity of the method, the above-mentioned conditions become especially important in the registration of derived spectra. "Sensitivity" is tightly bound to the "accuracy" and "correctness" of spectral analysis. For example, the sensitivity of a color reaction is expressed as a number of micrograms of compound with an optical density equal to 0.001 in a layer of solution with a 1-cm cross section. In the next section, we describe one of techniques of derivative spectrophotometry for increasing the sensitivity of spectrophotometers.

3.2.2 Difference (Differential) Spectrophotometry

In described methods of analysis, the optical density of an analyzed solution is measured relative to a comparison solution (according to an accompanying manual), which is a pure solvent (a so-called blank solution). Therefore, it is obvious that direct spectrophotometry is a borderline case of a difference (differential) method when C_0 and $D_0 = 0$. If, instead of the cuvette with the solvent, we use the solution of any second substance and then register the absorption spectrum with a direct method ("usual spectroscopy"), the difference in optical densities of the two objects will be directly measured, that is, the difference absorption spectrum.

We emphasize that the term "differential" is frequently found in the scientific literature, especially in the English language, and it expresses the essence of the Russian term "difference," but not "differential" or, more precisely, "differentiated."

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If I_0 is the intensity of light incident to the cuvette (solution) and I_1 and I_2 are the intensities of light passed through two solutions (objects), then $\lg I_0 - \lg I_1 = D_1$; $\lg I_1 - \lg I_2 = D_2$, and so $\lg(I_1/I_2) = D_2 - D_1 = \Delta D$.

After direct measurement of intensities of light passed through compared solutions, it is possible to find the difference between optical densities of these solutions (Figs. 3.5 and 3.6).

Registering changes in difference spectra substantially increases the accuracy of measurements and sometimes allows one to register such absorption spectra changes reliably, even when the difference is not revealed with the usual method.

With the help of the difference spectrophotometry (an absorption spectrophotometry technique), it is possible to investigate small changes in the optical density of a studied system and at a large extinction value. The difference spectrum is found by subtracting one absolute absorption spectrum from another. In this way, the response to changes in the system under the influence of an external factor (of a chemical or physiological nature) is obtained. This is extremely important in research on pathological changes of the state of blood, energetics of mitochondria and chloroplasts, and for comparison of algae mutants differing visually only slightly.

In studies of biological samples, registering the difference in absorption between two samples as function of wavelength is frequently required when the initial states of samples are very similar. The reader should remember that in this case a higher optical density for each solution (sample) can be used than is applied in the usual

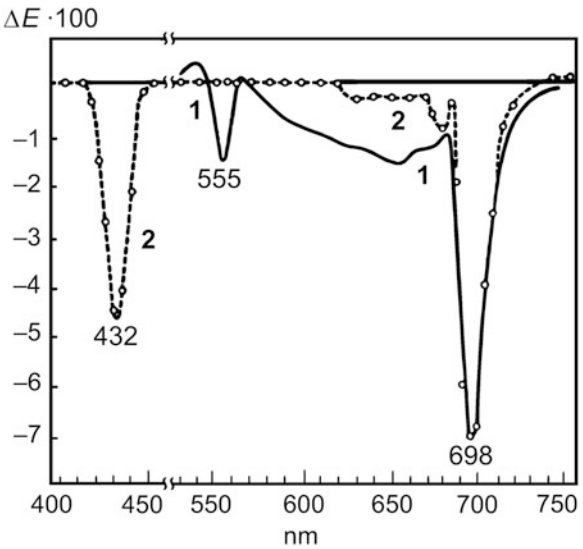

Fig. 3.5 Influence of the exciting light and darkness and also of redox agents on the pigment P700 absorption in the suspension of chloroplast fragments processed by acetone (Kok 1961; Khit 1972): (*1*) the difference spectrum "oxidized form minus restored form"; (*2*) the difference spectrum "light variant minus dark variant"

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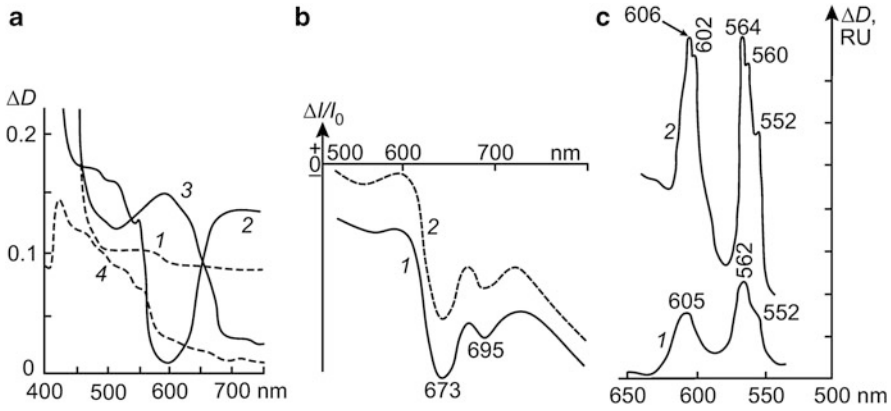

Fig. 3.6 Variants of differential (difference) spectra application: (a) The difference absorption spectra for the unpurified solution of the mixture of water-soluble proteins containing ferredoxin, flavoprotein, plastocyanin, and some others: (*1*) the zero line; (*2*) addition of ferricyanide to the sample; (*3*) addition of ascorbate excess to the sample; (*4*) additional introduction of hydrosulfite (Einor 1970, p. 130). (**b**) Differential spectra "oxidized variant minus restored one" of pigment–protein complexes from intergranular thylakoid fragments of pea chloroplasts containing photosystem 1 (*1*) and from the corn parenchyma (*2*) (Ostrovskaja 1975, p. 162). (**c**) The differential spectrum "restored sample minus oxidized one" of the suspension of mitochondria isolated from rat liver, recorded at room temperature (*1*) and at temperature 120 °C (*2*) (Rubin p. 47)

spectroscopy method. In this case, the optical density of one of the solutions is conditionally set equal to zero. In the case of identical samples, the zero line passes through the ordinate of their density. Transmission of the second solution (or the sample undergoing test) may be (or may become) either less or more than the transmission of the first solution; with respect to this, the optical density at different wavelengths can have both positive and negative values in the diagram of the difference spectrum, that is, be bipolar (Figs. 3.5 and 3.6).

In practice, such a difference spectrum is recorded if one places one substance (e.g., ubiquinol, cytochrome oxidized by NAD^+ or $NADP^+$) into the cuvette of comparison and places ubiquinone or cytochrome restored by $NADPH_2$ into the cuvette for samples. Using the example of the difference in spectra of ubiquinol and ubiquinone it is possible to illustrate the basic properties of difference spectra:

1. Extinction values can be negative and positive (bipolarity of signal).
2. Absorption maxima and minima are shifted, and extinction values are different from their values in absolute spectra.
3. Zero absorption points (isosbestic points) correspond to wavelengths, where oxidized (initial) and restored (final) forms of substance absorb equally.

Measurements of difference spectra have advantages in the case of small differences in optical densities of two compared samples, and this is taken into account when performing this form of analysis. Thus, the accuracy of measurements in this case is proportional to ΔD. So the possibility of comparing two substances or two

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states of the same substance arises; therefore, the important point of analysis is the fact that in both cuvettes the initial concentrations of components are identical. During work with native tissues or solutions of pigments, it is necessary to equalize densities in one absorption maxima. For plant tissues or their homogenates and for algae, the densities are usually equalized in the red maximum of chlorophyll absorption ($\lambda = 683$–685 nm).

The difference in the absorption spectrum of native structures in photobiological studies corresponds to changes occurring in the object under the influence of light, temperature, or chemical agents. A decrease in optical density (negative maximum) indicates a decrease in substance concentration in the final state compared with the initial state, that is, a decrease (increase) in product concentration at the considered wavelength in the experimental cuvette compared with the control; similarly, the optical density increases because of an increase in the concentration of the substance absorbing in the considered region of the spectrum.

It is sufficient to stress that a number of great achievements, for example, in the fields of biochemistry of photosynthesis, study of redox reactions of cytochromes, and energetics of photobiological processes, were achieved with the help of this technique (Chance 1951; Duysens 1954, 1956, Kok 1959, 1969; Witt 1971, 1979; Witt et al. 1961; Einor 1973). A detailed discussion of the successful application of difference spectrophotometry in the 1950s and 1960s is presented in work by Einor (1973). Einor profoundly describes different problems of chloroplast energetics. Regretfully, this book has been unfairly forgotten, but remains until now (it was published in 1973) one of the best books on the problems of reconstruction of photosynthesis energetics and on analysis of the progress made with application of differential spectrophotometry.

3.2.3 *Measurement Errors in Difference Spectrophotometry*

If D_0 is the optical density of a comparison solution and D_X the density of the studied solution, the density of a second solution measured comparatively to the first (D_0) is equal to the difference in absolute optical densities (D_{diff}):

$$\begin{aligned} D_{diff} = D_X - D_0 = \varepsilon d(C_X - C_0) &= \varepsilon d \Delta C; \\ D_{diff} = -1g\, T_{diff} &= -\varepsilon d(C_X - C_0) \end{aligned} \quad . \tag{3.4}$$

To estimate the measurement accuracy of this method, it is necessary to compute the value of $\Delta C_X/C_X$, which in the case of compliance with Bouguer's law is equal to $\Delta D_X/D_X$. After elementary calculations as presented by Rubin (1974, 1975), the optimal optical density for D_{diff} corresponding to a minimal measurement error is

$$D_{diff} = 0.434 - D_0. \tag{3.5}$$

As D_0 increases, the measurement error decreases from 0.434 (at $D_0 = 0$) to zero (at $D_0 = 0.434$) and then remains equal to zero with a further D_0 increase. At $D_0 = 0$,

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an operating range of optimum densities should be chosen equal to 0.2–0.7 D, and as D increases, this interval increases. We emphasize that to increase the measurement accuracy of difference spectra, the optical densities of investigated samples should be high enough and with maximally close values chosen by an experimenter for the selected absorption maximum. In practice, this is realized by carefully equalizing densities in one of the maxima of the investigated absorption spectrum or of native biological material. For example, depending on the purpose of study, the homogenate density of leaves or algae suspensions should be equalized at 480, 515, 650, and 683.5 nm (in absorption maxima of chlorophylls *a* and *b*); for erythrocytes at 560 nm; and for cytochromes at 550–559 and 560–563 nm (Einor 1970, 1973).

In the case $D_0 = 0$ and $D_X = D_{diff}$, Eq. (3.5) automatically becomes the formula for measurement errors from the method of direct spectrophotometry. This emphasizes once more that the method of direct spectrophotometry is a particular case of difference spectrophotometry. After setting the derivative of the first part of Eq. (3.5) equal to zero, we find the optimum optical density for D_{diff} corresponding to a minimum measurement error.

The formal analysis of Eqs. (3.4) and (3.5) can result in the conclusion that the highest accuracy can be obtained after comparing two practically opaque solutions. This paradox occurs because only a measurement error of the analyzed solution D_X was taken into account in the equations. If we add into the reasoning a measurement error of a comparison solution D_0 and a tray error, then dependence of a measurement error D_{diff} does not become equal to zero with an increase in D_0 but has minima (Korablev 1967; Lebedeva 1977). Thus, it is possible to calculate the optimum D_0 value for a given D_X or the optimum D_X value for a given D_0.

Research experience shows that, as a result of the accumulation of errors characteristic of both direct and difference spectrophotometry, the gain in accuracy can only be 5–7 times, instead of the theoretical 10–12 times (Barkovskii and Ganopol'skii 1969). Nevertheless, it is necessary to emphasize that the great achievements of the last 30 years in biophysics and biochemistry of photosynthesis and of chloroplast function occurred because of the application of this analysis technique (Einor 1973; Witt 1971; Saakov 1987).

In each specific case, an assessment of the reasonability of application of direct and difference methods, as well as determination of optimum values D_0 and D_{diff}, can be performed only after a preliminary detailed experimental study of the properties of an analyzed substance or a biological system and of the instrument used for analysis (Willis and Miller 1959; Gonopolskii 1969; Platonova et al. 1970; Blyum et al. 1972; Blank 1973; Golovachev 1976).

As noted earlier, the difference spectrum is found by subtracting one absolute absorption spectrum from another. This gives qualitatively new information on changes in the system under the influence of an external factor. This is especially important in studies of pathologic changes of the blood state, energetics of mitochondria and chloroplasts, and when comparing algae mutants, which are visually almost similar (Kvitko et al. 1977b).

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In a monograph by Barkovskii and Ganopolskii (1969), the existing methods and approaches of difference spectrophotometry are described fully and critically, so we only briefly describe the basic questions of this technique, which are useful for practical work and necessary for understanding the material in Chaps. 4 and 5.

Unfortunately, high-performance difference spectrophotometers are not produced by any Russian companies. We do not think that it is necessary to discuss details of the modification of Russian commercial recording spectrophotometers for high sensitivity and high speed. Interested researchers could look through the book edited by Rubin (1974), a substantial portion of which is dedicated to the practical construction of high-performance difference spectrophotometers. Also, Borisov and colleagues effectively worked on modifying Russian spectrophotometers for the purpose of difference spectrophotometry (Borisov and Mokhova 1964; Borisov et al. 1970).

3.3 Basis of Derivative Spectrophotometry

In spite of the successful development of spectrophotometric analysis techniques, the study of biological objects in their native state, and of multicomponent bio-organic and chemical systems, encounters a number of difficulties.

On the one hand, this is caused by the high optical density, photosensitivity, and strong light diffusion of a biological system and, on the other hand, by its complexity and heterogeneity. Difficulties of the first kind can be excluded by application of more advanced spectrophotometers with high sensitivity and with compensation for light diffusion. Difficulties of the second kind are caused by the fact that the absorption spectrum of a biological object is the superposition of several rather wide and closely placed absorption bands of different substances, each of which can have several modifications characterized by their own physical and chemical properties (chlorophyll–protein and hemoglobin complexes, chelate compounds, etc.). Because of this, the total spectral curve has rather a complex form, and some components either do not manifest at all or are displayed in the form of only weakly expressed bends, inflections, shoulders, or protrusions.

Thus, a researcher has to struggle with the difficulties of ascertaining the number and positions of extrema, precise identification of substances, and their quantitative ratio.

It is significant that for a biological object the resolution limit is mainly dictated not by the technical capabilities of spectroscopic instrumentation, because there are spectrophotometers with high resolution of angstroms and fractions of angstroms, but by an object's structure revealed as a mixture of wide and closely placed absorption bands. Because some maxima of a total spectral curve can be manifested as weakly expressed protrusions, bends, and band asymmetry, and because a spectrum's curvature is visually assessed, this visual assessment introduces additional errors and uncertainty into the question of the existence or position of various absorption bands composing the spectral outline.

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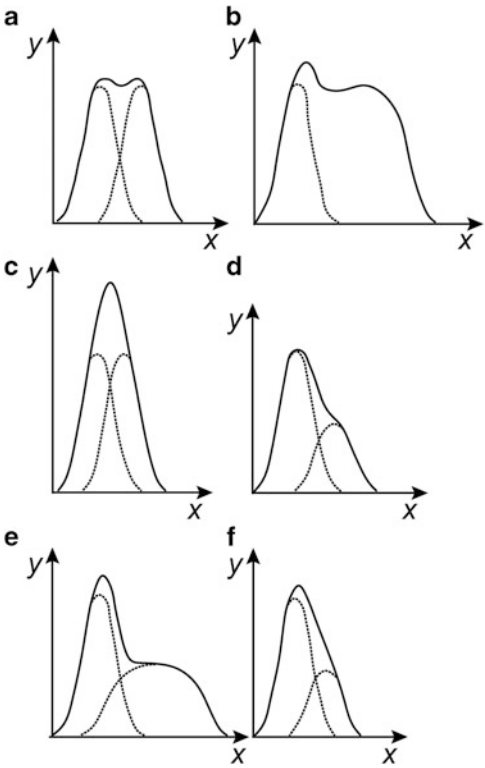

Fig. 3.7 (**a**–**f**) Influence of hidden absorption bands on the change in the total absorption curve outline

If we suppose that an absorption spectrum consists only of Gaussian-type bands differing from each other only in width, intensity, and position relative to the wavelength axis, then even under such ideal conditions the precise interpretation of the spectrum is a very difficult problem. Absorption spectra of many samples have not just one but several maxima, and their forms substantially vary from the Gaussian and from any other curve described with a known mathematical formula (curves of Lorenz, Rayleigh, etc.) (Martin 1957; Rutman et al. 1976b; Dubrovkin and Belikov 1988).

These described difficulties require considerable skill from researchers in making the most precise possible identification of separate absorption bands in the total spectrum (Fig. 3.7). This part of the problem is extensively described in a review by Giese and French (1955). Using a large quantity of model figures obtained by machine analysis, the authors of that review illustrated many variants of overlapping bands and their possible separation in the case of differentiation of models.

The criteria of separation of two symmetrical, closely placed absorption bands were also discussed by Meister (1966a) and Litvin and coauthors (Litvin and Gulyaev 1969; Gulyaev and Litvin 1970).

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In the work of Meister, the resolution limit of two identical absorption bands having a Lorenz curve form (a dispersion outline) is described. The Sparrow criterion for separation of two symmetrical bands is sufficient to reveal a notch between them. The borderline case is a total curve with a flat top; in other cases, two initial curves are not resolvable (see Figs. 3.10b and 3.13).

In the publications of Litvin and coauthors, the Rayleigh criterion is considered in addition to the Sparrow criterion, but in contrast to Meister, the authors apply both criteria to the Gaussian curve. According to the Rayleigh criterion, two symmetrical bands are resolved if the value of the notch between them is not less than half of the maximum value of the ω-band. From the Sparrow criterion, the resolution limit of two Gaussian bands is equal to 0.849ω and the Lorenz bands to 0.557ω. From the Rayleigh criterion, the resolution limit reaches the value of $(1.5–2.0)\omega$ for Gaussian curves (Lester 1970; Dubrovkin and Belikov 1988).

Thus, the Rayleigh criterion is stricter than the Sparrow criterion and is more reliable. However, due to the high level of quality of spectroscopic instrumentation, in practice the Sparrow criterion is more frequently used. The latter could be written as the ratio of the minimum distance between the tops of two symmetrical bands $\Delta\lambda_0$ to the width of these bands at an ordinate equal to 0.5 from the maximum of the ω-value (i.e., $\Delta\lambda_0/\omega$).

If it is necessary to resolve two asymmetrical bands varying in width and in amplitude, then the resolution limit should change. In particular, if the amplitudes of two Gaussian bands are in the ratio $A_1/A_2 = 1/5$ and their half-widths are equal, then the resolution limit will be equal to 1.0ω (Dubrovkin and Sobolev 1976). If with the same ratio of amplitudes the ratio of half-widths is 1/2, then the resolution limit is equal to 0.835ω. This question is described in detail in several papers (Lester 1970; Skujins 1986a, b; Dubrovkin and Belikov 1988), and interested readers could investigate this problem from the original sources.

To overcome previously mentioned difficulties, in recent decades, some methods increasing the analytic–informational capabilities of spectral methods have appeared. One of them is the method of low-temperature spectrophotometry (Frei 1960; Litvin and Gulyaev 1969; Rubin 1974, 1975).

The method is based on the fact that, with a decrease in object temperature to the temperature of liquid nitrogen, absorption bands become narrower by 20–30 %. This narrowing is mainly due to the decrease of the widths of molecular energy levels, change in degrees of freedom of molecules, and the forces of intermolecular interaction.

However, this technique has a number of disadvantages and limitations, causing complication of the experimental procedure. Furthermore, this method cannot be applied to direct studies of processes taking place in a living cell under physiological conditions.

An experimenter reading graphs of spectral curves knows that some maxima of the total absorption curve and of transmission or luminescence spectra are manifested as ill-defined protrusions, inflections, or band asymmetry. Incomparably better results for identification and interpretation of the bands in a total spectrum of a biological subject are obtained by a method that has been given the name

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"derivative spectrophotometry." This technique began slowly, but persistently, to win places in analytical laboratories from the middle of the twentieth century (Hammond and Price 1953; French et al. 1954; French 1957a; Pemsler 1957; Martin 1957, 1959; Habermann 1960a, b; Aramu and Rucci 1966; Meister 1966a, b; Stauffer and Sakai 1968; Gulyaev and Litvin 1970; Gaudillere 1974; Matsushima et al. 1975; Dubrovkin and Sobolev 1976; Abdel-Hamid et al. 1984; Spitsyn and Korepanov 1980; Skujins 1986a, b; Dubrovkin and Belikov 1988; Ojeda Bosch et al. 1995; Ojeda Bosch and Rojas Sanchez 2004; Hagris et al. 1996; Rojas and Ojeda 2009; Saakov et al. 2010; Karpinska 2012).

In due course, the discussed approach was proposed by Sir Prof. E. Rutherford as the method of identification of ill-resolved peaks of mass spectra (Dymond 1924; Dubrovkin and Belikov 1988). The author of the first publication (Dymond 1924) illustrates the use of the differentiation method for first-order derivatives and expresses appreciation to Prof. E. Rutherford for the proposed study method and for permanent interest in the work. Revival of this approach and a new impulse for its development took place at the beginning of the 1950s; this was covered in papers of both chemists and biologists (Hammond and Price 1953; French and Church 1955; Giese and French 1955; Singleton and Collier 1956; French 1957a; Martin 1957, 1959; Butler and Hopkins 1970).

It should be immediately specified that differentiation of spectral curves substantially increases the contrast of detailed spectrum presentation due to the revealing of high Fourier frequencies (Dubrovkin and Belikov 1988). This means that changes induced in the spectrum become more visible after differentiation of spectral curves.

The method won acknowledgment in laboratories of different scientific specializations in Europe and America, and some of the achievements and prospects of its application in the analytical practice of chemists and biochemists are summarized in reviews (Williams and Hager 1970; Saakov 1971a, b, c; Saakov et al. 1971a, b; Rutman et al. 1976a, b; Fell 1980; Miller et al. 1982; O'Haver 1982; Fell and Smith 1982; Dubrovkin 1983, 1989; Perfilev et al. 1985; Spitsyn and L'vov 1985; Skujins 1986a, b; Saakov et al. 1987a, b; Talsky 1994; Ojeda Bosch et al. 1995; Ojeda Bosch and Rojas Sanchez 2004; Rojas and Ojeda 2009; Karpinska 2012) and in the monograph by G. Talsky (1994). The considered approach does not have the limitations characteristic of the method of low-temperature spectrophotometry and allows a substantial increase in the selectivity and informativity of the spectral method of investigation of complex biological structures (French et al. 1954; French and Church 1955; French 1957a, b; Frei 1960; Meister 1966b; Kaler et al. 1967; Litvin and Gulyaev 1969; Saakov 1971a, b, c; Navarro et al. 1972; Litvin et al. 1973a, b; Baranov et al. 1975; Rutman et al. 1976a, b; Dubrovkin and Belikov 1988). It virtually increases the resolution of normal spectrophotometers and excludes errors and uncertainties during visual resolution of a question about the presence of various absorption or luminescence bands.

The method of derivative spectrophotometry enables detection of small differences in spectral outlines, because small abnormalities of the regularity of the initial curve are clearly found in the harmonics of derived spectra.

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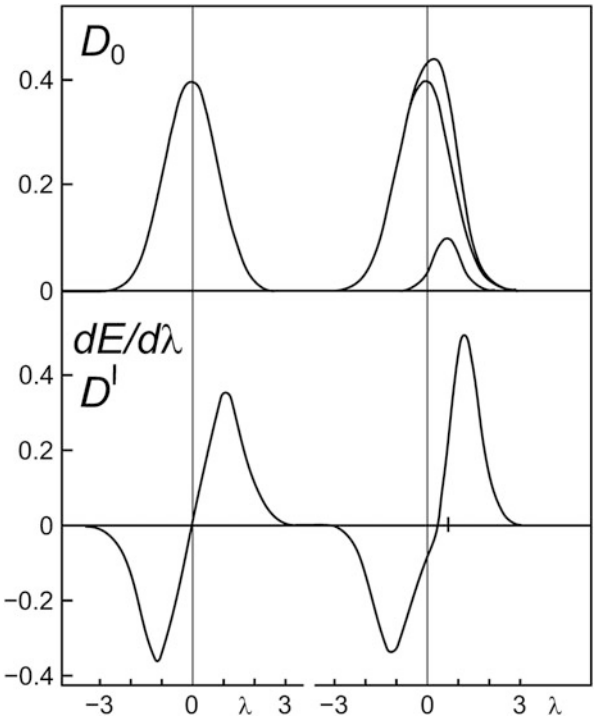

Fig. 3.8 Two curves as the model of the absorption spectrum and their calculated conversions to derived spectra. The *top left* curve shows the symmetrical D^I spectrum. The *right* plot of the same curve shows the addition of another small absorption band, resulting in a hardly noticeable asymmetry of the total outline. This is more clearly revealed on the derived curve below (French 1957b)

The theoretical mathematical validation of the method is extensively presented in a number of articles and reviews (Martin 1957; Bonfiglioli and Brovetto 1964a, b; Gunders and Kaplan, 1965; Goldstein 1970; Hager and Anderson 1970; Williams and Hager 1970; Fell 1980; Dubrovkin 1983; Perfilev et al. 1985; Spitsyn and L'vov 1985; Skujins 1986a, b; Dubrovkin and Belikov 1988; Talsky, 1994; Karpinska 2012).

To facilitate the reader's familiarization with the methodological basis and with areas of its possible application, without particular consideration of the mathematical description of derivative spectrophotometry, in Figs. 3.8, 3.9, 3.10, 3.11, 3.12, 3.13, and 3.14, we illustrate the special features of this method, whose numerous corresponding graphics were published earlier (Litvin and Gulyaev 1969; Butler and Hopkins 1970; Epel and Butler 1972; Hager 1973; Marenko and Saakov 1973; Bershtein and Kaminskii 1975; Leclerc et al. 1975; Rutman et al. 1976a, b; O'Haver 1978; Talsky et al. 1978a, b; Ioffe et al. 1984; Perfilev et al. 1985; Skujins 1986a, b; Dubrovkin and Belikov 1988; Talsky 1994; Saakov et al. 2012).

The derivative of the absorption spectrum of a substance or solution is the result of the differentiation of optical density of the spectral curve D with respect to wavelength λ, to wavenumber ν, or to time t of spectrum scanning, when the latter is strictly synchronized with wavelength scanning.

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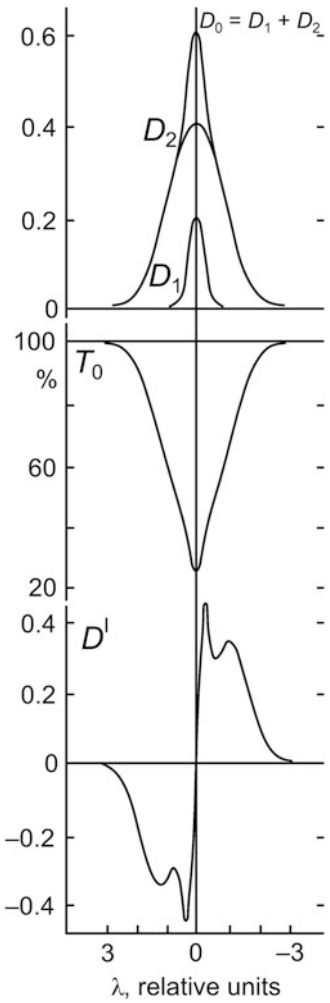

Fig. 3.9 *Top*: Model of the spectral outline consisting of two bands with different width, but with the identical position of maxima. C*enter:* Curve (T_0) is the calculated transmission curve. *Bottom*: Calculated D^I curve of transmission, on which the peak corresponding to a minor component is visible. This means that the derived spectrum can give two peaks identical on absorption, if their band widths are different enough (French 1957b; Giese and French 1955)

It can be seen from Figs. 3.8 and 3.9 that in the region of maximum of the initial curve, the first-order derivative has two peaks: positive, corresponding to the *maximum speed of optical density increase*, and negative, corresponding to the *maximum speed of optical density decrease* of the initial curve. The spectral curve becomes bipolar. At λ_{max} of initial spectrum, its first (D^I) and also all odd derivatives (D^{III}, D^V, D^{VII}, etc.) are equal to zero (see Fig. 3.11a–c). Thus, the precise position of the maximum of the initial spectral curve corresponds to the zero point on the *x*-axis. Because of this, the form of these functions is dramatically different from the form of the initial spectrum (Fig. 3.11). Thus, the first-order derivative spectrum characterizes a curve slope to the *x*-axis or, which is the same, the graph of function change rate (i.e., of the initial curve of spectrum). Possible protrusions,

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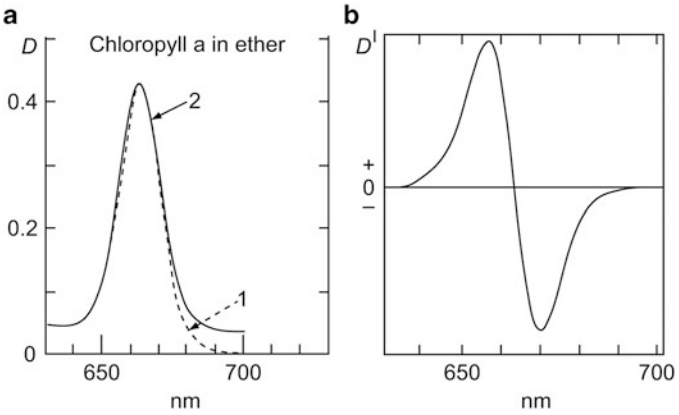

Fig. 3.10 (**a**) The absorption spectrum of the purified solution of chlorophyll *a* in the sulfur ether measured with the instrument Beckman DK2 (curve *2*) and (**b**) its D^I. Integration of the curve *b* is shown by curve *1* (**a**) (French 1957b)

bends, and shoulders, presenting on the initial curve, will be more protuberantly manifested as outline changes of the derivative graph (Figs. 3.8, 3.9, 3.10, and 3.11c).

A second derivative (D^{II}) of the symmetrical absorption curve has a one intensive negative peak and two positive satellites of approximately a quarter the intensity. The half-width of a main peak is approximately one third of the half-width of an initial absorption band. A number of even derivatives (D^{II}, D^{VI}, D^{X}, and D^{XIV}) have negative peaks at λ_{max} of the initial spectrum and, therefore, with reversal of the sign (of the *y*-axis direction) are similar to it in some ways (Martin 1957; Talsky 1994, Skujins 1986a, b, Dubrovkin and Belikov 1988). After conversion into D^{IV}, the half-width of the main peak decreases to approximately a fifth, theoretically, in comparison with the initial spectrum. However, an operator should clearly know that in this case the ratio of intensities of the main peak and positive satellites also reduces (Figs. 3.11 and 3.12) (Dymond 1924; Giese and French 1955; Butler and Hopkins 1970; Bershtein and Kaminskii 1975; Talsky et al. 1978a, b; Skujins 1986a, b). Points of inflection manifest as extrema and for an even derivative as intersection of the zero line. At the same time, the center of the shoulder bend is revealed in even derivatives in the form of acute maxima (D^{II}, D^{IV}) (Talsky et al. 1978a, b; Talsky 1994).

However, registration of the first-order derivative (from now on we will use the introduced designations instead of words) has a number of disadvantages. First, the form of curve is complicated, because the derivative possesses both positive and negative values (i.e., the signal is bipolar); moreover, not one but both maxima (positive and negative) on the graph of derivative (Fig. 3.11) correspond to each maximum of the initial curve.

We would like to accent afresh that at λ_{max} of the initial spectrum, its D^I and also all odd derivatives of higher orders are equal to zero (i.e., the position of the

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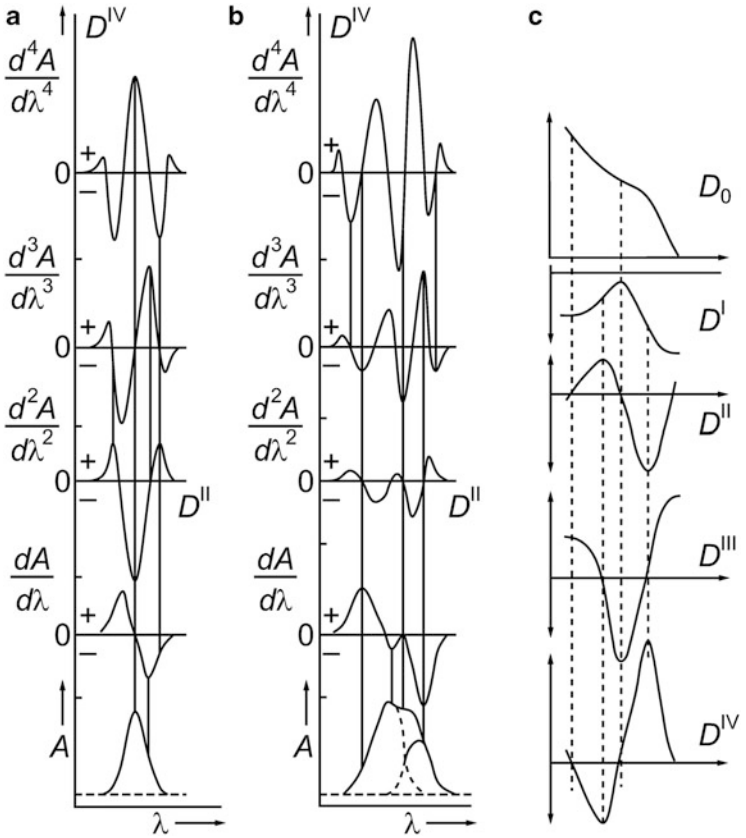

Fig. 3.11 Differentiation of model analytical Gaussian curves: (**a**) the initial curve and its D^I–D^{IV}; (**b**) the initial curve of overlapped Gaussian curves and their D^I–D^{IV}; and (**c**) manifestation and intensification of signal after differentiation of bend shoulders (inflections); the initial curve (D_0) and its D^I–D^{IV} (Talsky 1994; Talsky et al. 1978a, b)

absorption maximum corresponds to the zero point on the *x*-axis). Because of this, the graphic representation of this function is sharply different from the form of the initial spectrum. The half-width of the D^I absorption band remains practically the same as that of the initial absorption or transmission spectrum.

From all the evidence, we can conclude that application of D^I does not increase the resolution of the method, but only specifies the positions of absorption bands. Therefore, it is more promising to use D^{II} for the analysis of spectra and, with the improvement in electronics, D^{IV} (Dymond 1924; Martin 1957, 1959; Butler and Hopkins 1970; Kvaratskheli and Demin 1983).

Conversion of D^I into D^{II} is similar to conversion of the original function D into D^I. The second derivative physically corresponds to acceleration of change in the initial curve D. Finding of extreme points for any function with the help of D^{II} allows the exact determination of the quantity and position of maxima and minima

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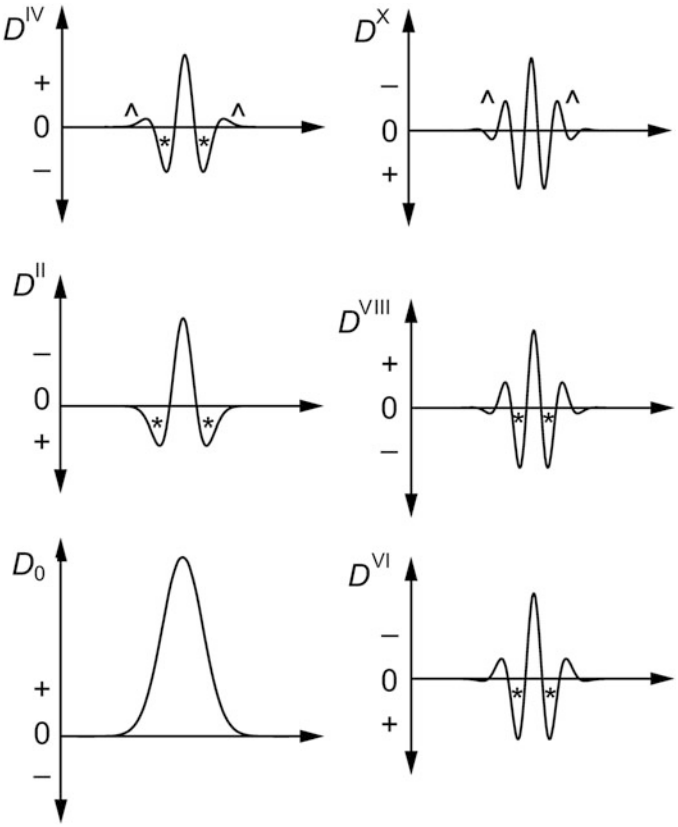

Fig. 3.12 Change character of the ratio of main peaks and satellite intensities; compare D^{II} (*asterisk*) and D^{VI}, D^{X} (*asterisk*), and also D^{IV} (*asterisk* and *caret symbol*) and D^{VIII}, D^{X} (*asterisk* and *caret symbol*)

of the initial curve. Inflections, protrusions, and shoulders in the initial spectrum manifest themselves on the D^{II} curve as separate maxima. The form of the D^{II} curve as curves D^{VI}, D^{X}, D^{XIV}, etc., is close to the form of the original function, but with the opposite sign. Therefore, some even derivatives (D^{II}, D^{VI}, D^{X}, D^{XIV}) have *negative* peaks at λ_{max} of the initial spectrum, and, consequently, with change in the sign (change in the *y*-axis direction), they have some similarity to the initial spectrum, whereas the *form* and the *polarity* of curve outlines D^{IV}, D^{VIII}, D^{XII}, D^{XVI}, etc., correspond to the original function (Fig. 3.11). This allows comparison of the initial and derived spectral outlines quickly and makes the solution of problem of experimental result interpretation easier. Theoretical assessment of the preferential usage of optical density derivatives of a particular order for the increase in determination selectivity of elements with strongly overlapped bands is described in the literature (Kvaratskheli et al. 1981, 1983; Kvaratskheli and Demin 1983; Dubrovkin 1989). However, conclusions from these papers are ambiguous,

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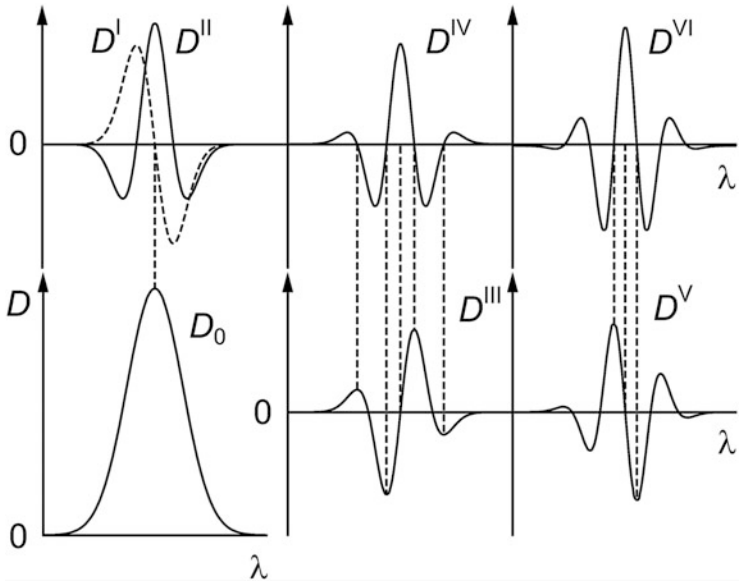

Fig. 3.13 The Gaussian curve D_0 and its analytical derivatives of the first to the sixth (Ioffe et al. 1984)

and there is possibility for further discussion. Briefly, and in an accessible form, the theoretical basis and special features of the method are presented in a monograph by Ioffe and colleagues (1984, Sect. 4.1).

Registration of higher derivatives requires a significant increase in sensitivity, selectivity, and complication of equipment (Dymond 1924; Butler and Hopkins 1970; Leclerc et al. 1975; Rutman et al. 1976a; Cuellar et al. 1978), but, on the other hand, the values of main and parasitic maxima become commensurable, and it is very difficult to identify them in the process of spectra interpretation. It is quite understandable that with the first usages of derivative spectrophotometry, the developed methods of first derivative registration were more widely used (French and Church 1955; Hammond and Price 1953; Habermann 1960a, b; Kaler et al. 1967; Inoue et al. 1975; Skujins 1986a, b) than of the second one (Meister 1966a, b; Drews 1967; McWilliam 1969; Gulyaev et al. 1971; Saakov 1971a, b, c; Hager 1971; Marenko et al. 1972; Navarro et al. 1972; Hager 1973; Marenko and Saakov 1973; Saakov et al. 1973; Saakov and Spotakovskii 1973; Baranov et al. 1974; Udovenko et al. 1974; Saakov et al. 1976; Ishii and Satoh 1982a; Almela et al. 1983). It was only a few laboratories that invented methods of registration of higher derivative spectra (Dymond, 1924; Butler and Hopkins 1970; Rutman and Saakov 1978; Cuellar et al. 1978) that found their application in scientific studies.

Development of the computer technology and corresponding software promoted the solution of problems on the calculation of derived spectra of high orders if only standard spectrophotometric equipment was available in the laboratory (Challise

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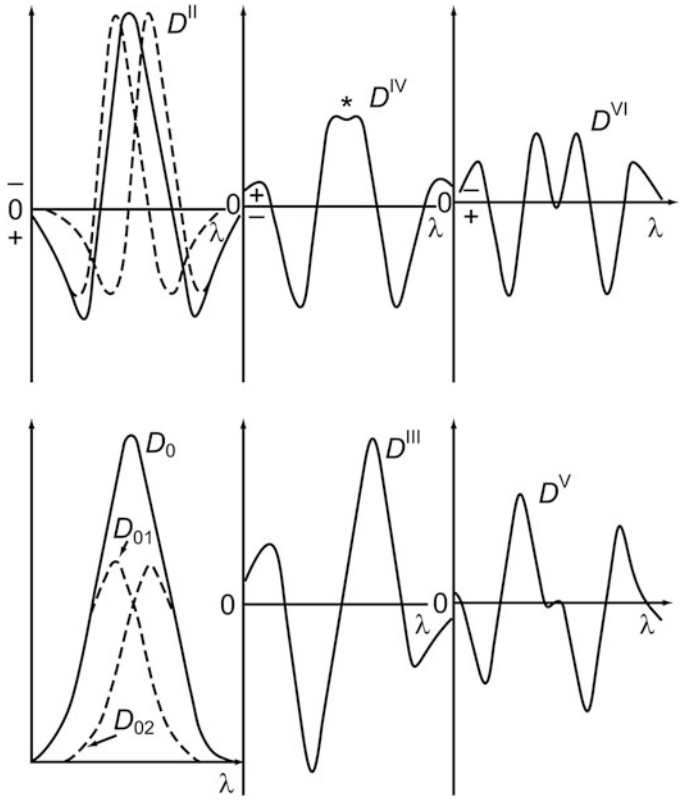

Fig. 3.14 Two symmetrical curves (spectral Gaussian curves) and analytical derivatives of the total curve. *Asterisk* shows the separation of two peaks located from each other at a distance of the half-width of each of them in the D^{IV} spectrum (Ioffe et al. 1984)

and Williams 1964; Savitzky and Golay 1964; Goldstein 1970; Cuellar et al. 1978; Skujins 1986a, b; Talsky 1994; Eliseev et al. 2000).

In Fig. 3.15, an example of derived spectra application for the analysis of prodigiosin (pyrrolyl pyrrylmethylene) is shown. This substance is from the group of natural red pigments, isolated from strains of *Streptomyces* and *Serratia marcescens* (*Bacillus prodigiosus*) (Hubbard and Rimington 1950; Rapoport and Holden 1962; Hearn et al. 1968; Boger and Patel 1988) and used for the fight against leukemia and melanoma cancerous cells (Yamamoto et al. 1999, 2000; Diaz-Riuz et al. 2001; Montaner and Perez-Tomas 2001).

Prodigiosins are a new group of H^+/Cl^- co-transporters that break proton transfer (Hearn et al. 1968; Kataoka et al. 1995; Sato et al. 1998; Melvin et al. 2002). As shown in Fig. 3.15, in acidic ethanol, the main maximum of pigment absorption is decomposed into five or six absorption bands not detected earlier and unknown in the literature (Saakov and Petrova 1996; Saakov 1996b; Saakov et al. 1998). The shoulder bend at 500–510 nm is revealed on the D^{VIII} spectrum

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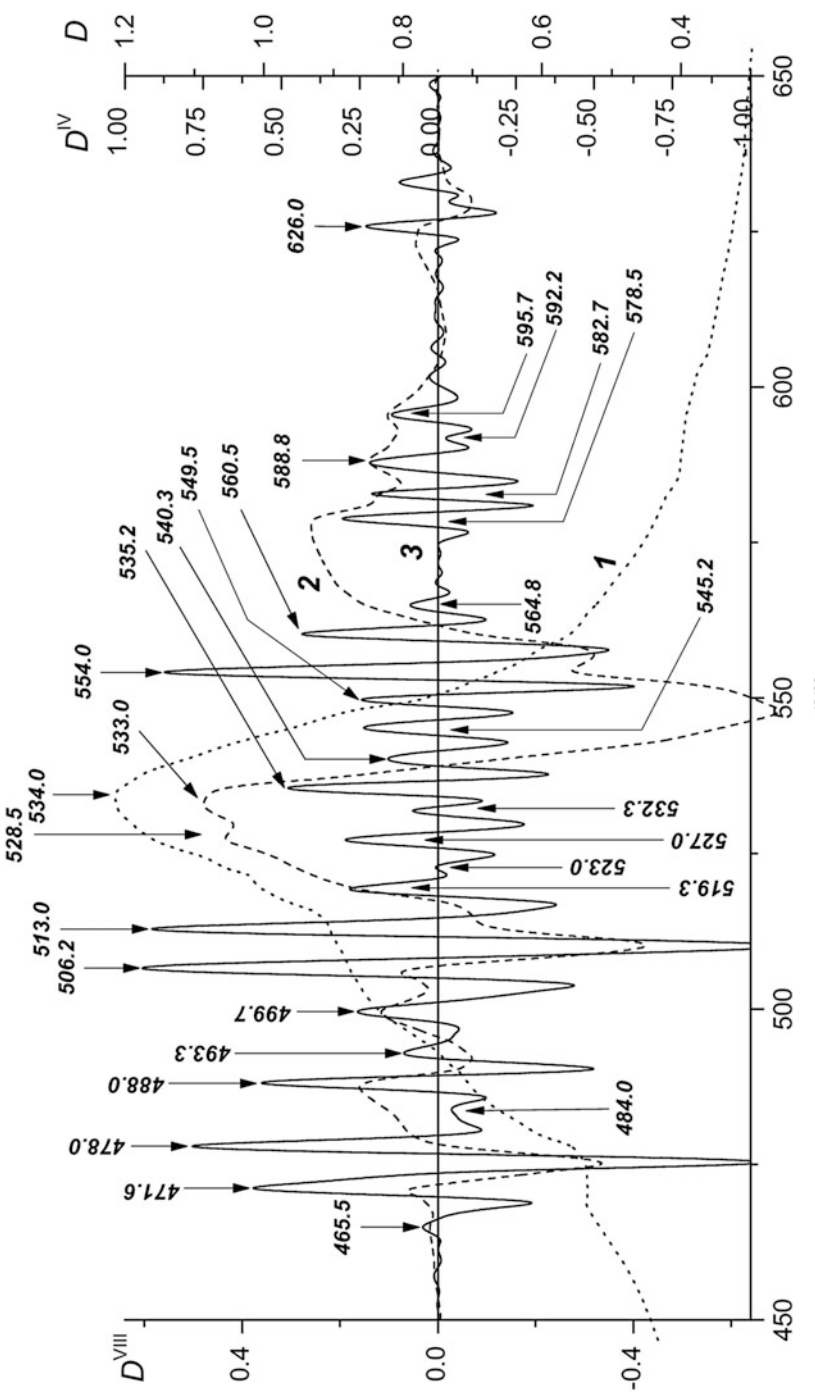

Fig. 3.15 The absorption spectrum of prodigiosin (*1*) and its fourth (*2*) and eighth (*3*) derivatives

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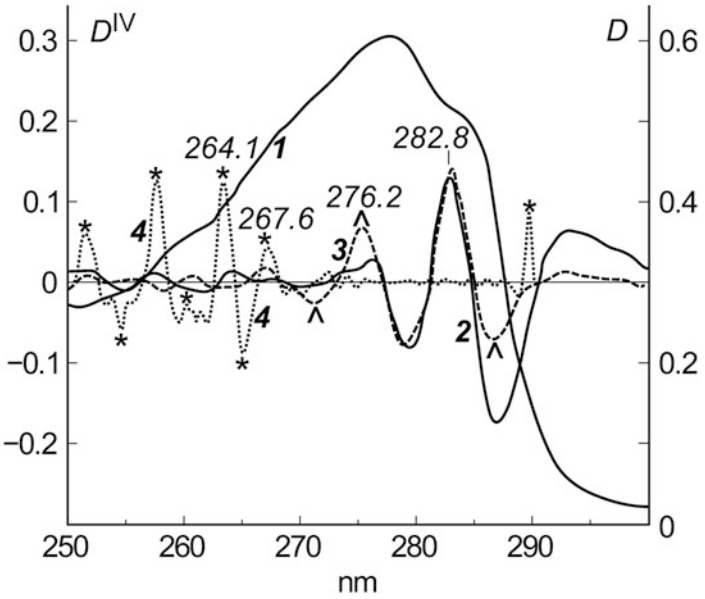

Fig. 3.16 The absorption spectrum of insulin (*1*) synthesized by E. Lilly and its fourth (*2*) derivative (* and ^ denote minimum and maxima for different curves)

as three bands at 499.7, 506.2, and 513.0 nm (Hubbard and Rimington 1950; Rapoport and Holden 1962). The known region of the spectrum, 470 nm, is decomposed into five bands at 471.6, 478.0, 484.0, 488.0, and 493.3 nm. Furthermore, special features of the D^{VIII} spectrum allow assessment of the purification efficiency of isolated prodigiosin preparations or its protein complexes with the necessary certainty, in accordance with manifestation of the above-mentioned spectrum bands and also according to changes in extrema in the region 578.5–595.7 nm (not considered earlier in the literature) under the influence of stress or chemical factors (Saakov and Petrova 1996; Saakov 1996a; Saakov et al. 1998; Saakov and Moshkov 2003; Ryasantseva et al. 2012).

The example of D^{II} analysis of an insulin preparation for diabetics is shown in Fig. 3.16. Molecules of gelatin and polymyxin do not contain tyrosine and tryptophan, but have a phenylalanine residue. Insulin includes both phenylalanine and tyrosine. This is confirmed in Fig. 3.16, which shows the smooth outline of the insulin absorption spectrum (curve 1) and its (non-smooth) fourth derivative (curve 2). Positive extremum of the derivative is at 281.8 nm, which is equal to the main extremum of D^{IV} of tyrosine (curve 3) (Saakov et al. 1998).

In contrast to the low-temperature method, the method of derived spectra registration is actively used for studies of processes taking place in living (native) cells under physiological conditions and with unimpaired biochemical processes, including investigations of different mutants (Frei 1960; Navarro et al. 1972; Inoue

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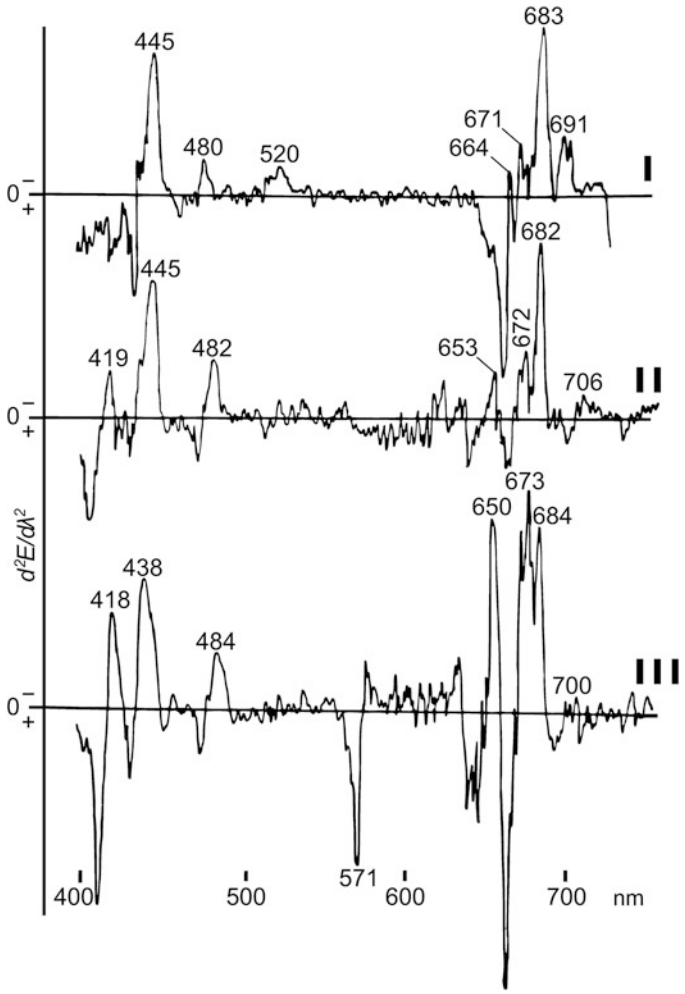

Fig. 3.17 D^{II} spectra of *Chlamydomonas* photosensitive mutant r. N-154 grown heterotrophically (*I*) and its revertant P3-154 grown under heterotrophic (*II*) and mixotrophic (*III*) conditions. From the collection of the Biological Institute of the St. Petersburg State University

et al. 1973; Saakov and Hoffmann 1974; Baranov et al. 1975; Kvitko et al. 1976, 1977a; Saakov et al. 1978a, b) (Fig. 3.17, 3.18, 3.19, 3.20, and 3.21).

It is necessary to take into account an additional advantage of the derived spectrophotometry method, that is, that D^{I} registration substantially decreases the constant component of side absorptions, such as light diffusion, shift, and, in plant studies, changes in a leaf, excessive moistening, or drying (Meister 1966a, b; Saakov 1971a, b, c; Saakov et al. 1973, 1976; Udovenko and Saakov 1976; Skujins 1986a, b). Usage of D^{II} allows one to eliminate an effect of the mentioned side

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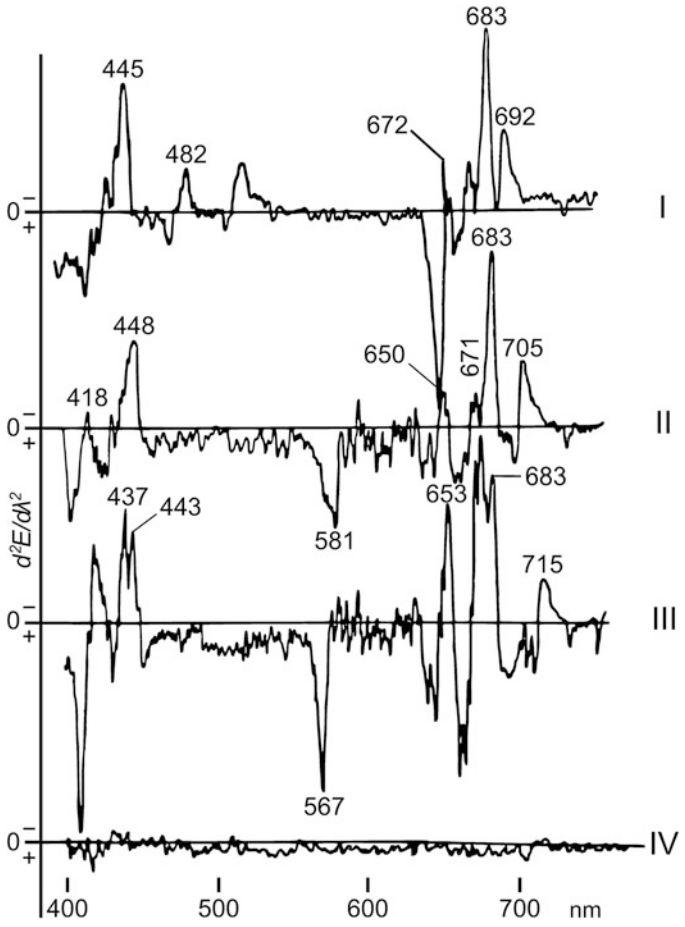

Fig. 3.18 D^{II} absorption spectra of *Chlamydomonas* photosensitive mutant r. (lts_4 zh yellow 13) (*I*) and its revertant $P3_{nem}$-13 (*II*) grown heterotrophically and after light exposure (2,000 lx for 96 h) of the revertant (*III*) and the mutant (*IV*). Compare (*I*) and (*IV*). From the collection of the Biological institute of the St. Petersburg State University

absorptions linearly depending on λ. Only if the listed components have quadratic dependence on l does this influence manifest on the D^{II} graph as a constant component (Rutman et al. 1976b). The presented considerations allow one to limit the number of a priori statements or objections about spurious signals of light scattering in boundary regions of a spectrum.

Derivative spectrophotometry is often used for the resolution of almost or completely overlapped spectral lines (French 1957a, b; Gulyaev et al. 1971; Saakov 1971a, b, c; Udovenko and Saakov 1976; Skujins 1986a, b). Using a computer, Gulyaev et al. (1971) calculated the model curves (Fig. 3.21) illustrating the appearance of two overlapped spectral bands in the second derivative, with different intensity and half-width and located at different distances from each other.

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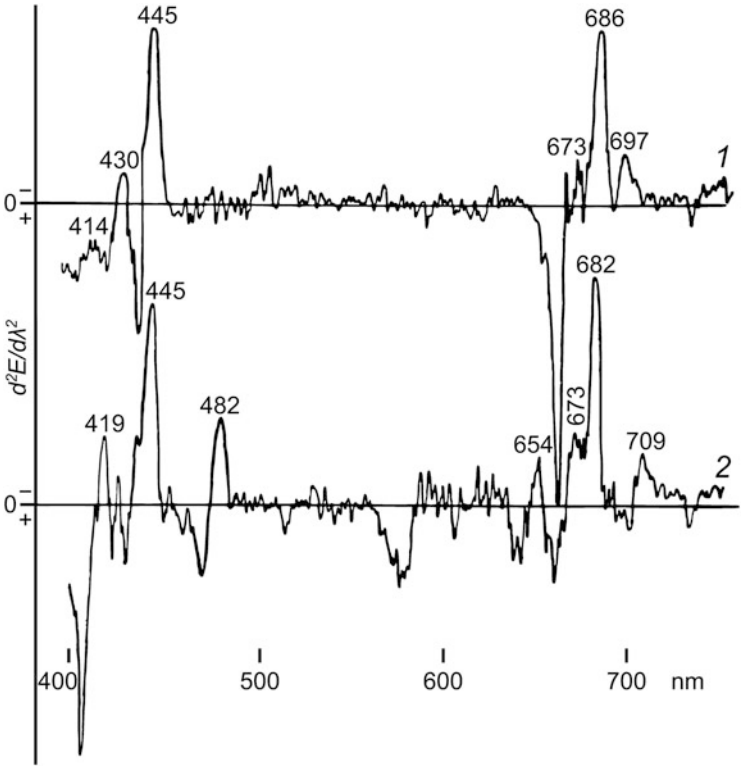

Fig. 3.19 Change in the state of the pigment complex of the light green revertant P6-149 in comparison with the initial photosensitive mutant N-149 of *Chlamydomonas*. (*1*) D^{II} spectrum of heterotrophic culture of the mutant N-149, and (*2*) its revertant P6-149 grown heterotrophically. From the collection of the Biological institute of the St. Petersburg State University

Analysis of the curves in Figs. 3.11, 3.12, 3.13, and 3.14 shows that, even if the overlapping of two bands is not revealed in the initial spectrum, for the D^{II} curve the two bands can be found using the appearance of two negative maxima or the asymmetry of positive satellites. This special feature of the method should be always recalled by an experimenter during spectra analysis (Fig. 3.11b).

The method of derivative spectrophotometry, as one of the special approaches (techniques) of absorption spectrophotometry, allows one to find and to sharpen details of spectral curves hidden within wide spectrum bands or obscured by medium turbidity in the usual method of direct spectrophotometry so that the correct number and improved positions of maxima of blurred or overlapped bands can be found (Figs. 3.22, 3.23, and 3.24) (Talsky 1994).

Therefore, the method can be recommended for the identification of components of complex reaction mixtures (Singleton and Collier 1956; Challise and Williams 1964; Samsonova and Gak 1971; Shibata et al. 1976; Kitamura and Majima 1983; Ivanovic' et al. 1995). The considered method helps in the detection and

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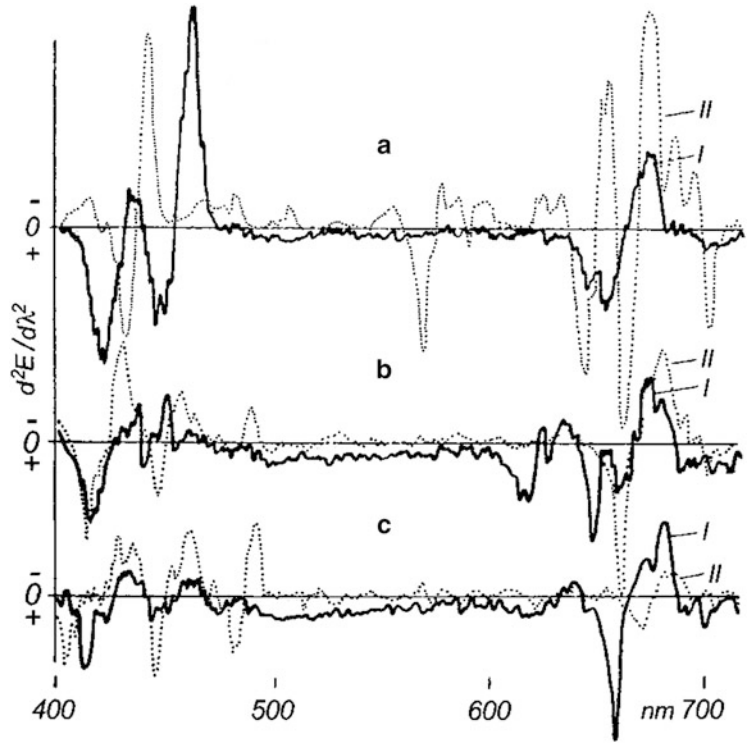

Fig. 3.20 The influence of light on the D^{II} change in photo-indifferent and photosensitive mutants of *Scenedesmus* alga from the collection of the Biological institute of the St. Petersburg State University. (**a**) Photo-indifferent mutant № 43; (**b**) photosensitive mutant № 123; (**c**) photosensitive mutant № 74; (*I*) spectra of mutants grown heterotrophically in the dark; (*II*) the change in spectral structure under mixotrophic conditions

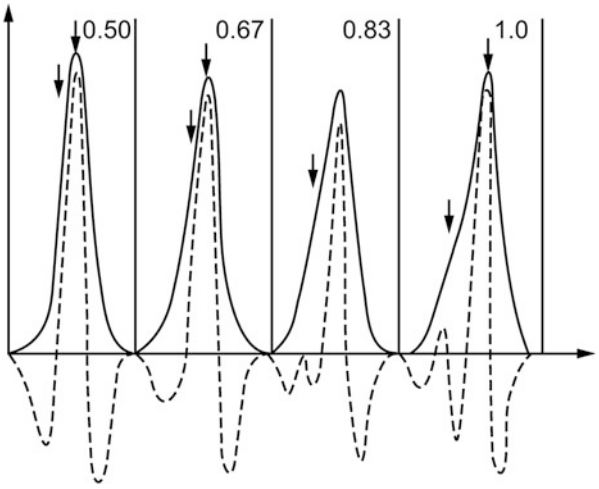

Fig. 3.21 Resolution of two overlapped bands from second derivatives of the absorption spectrum (Gulyaev et al. 1971; Bershtein and Kaminskii 1975). *Figures* on curves correspond to the distances between peaks, in units of band half-widths

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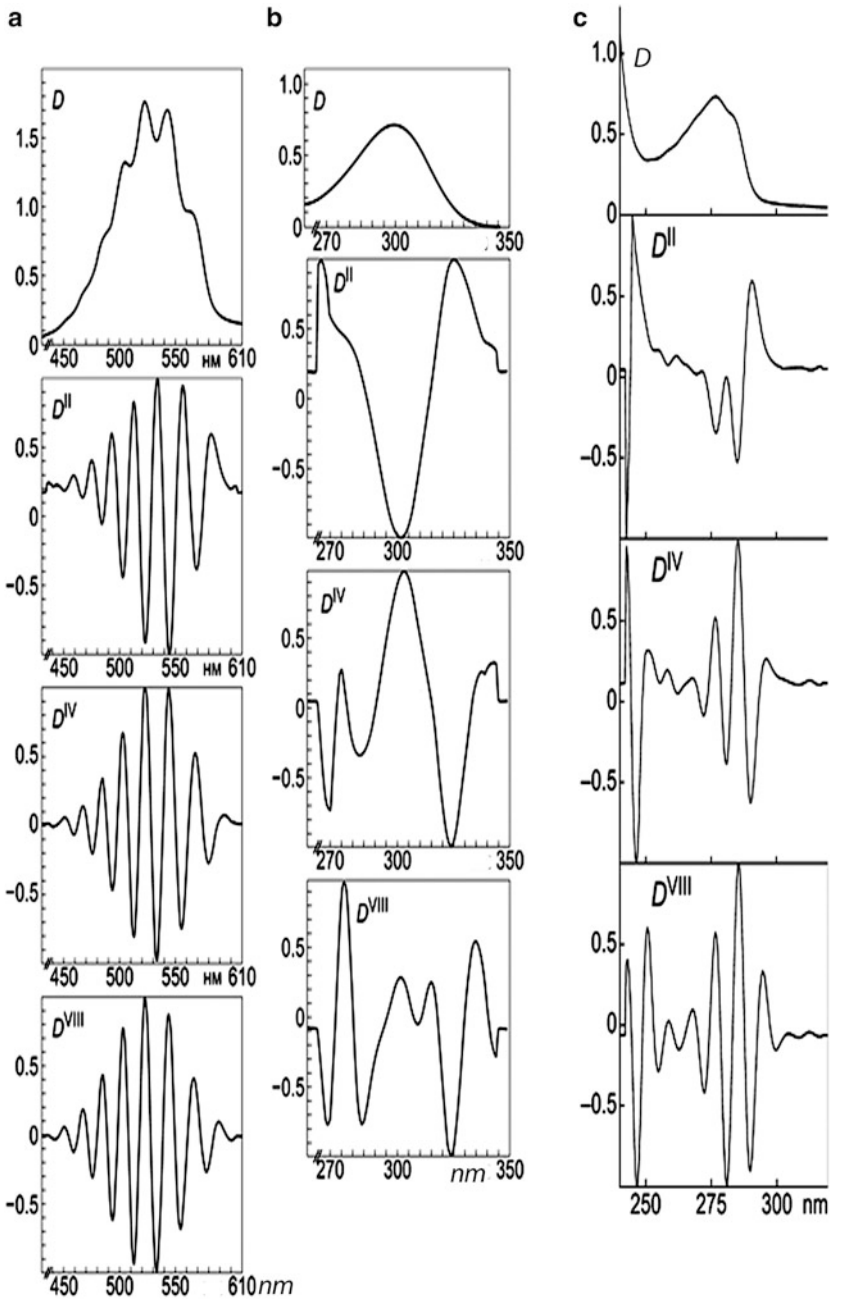

Fig. 3.22 Dynamics of absorption spectra change for solution of: (**a**) $KMnO_4$ and (**b**) KNO_3 (**c**) ribonuclease in dependence on the order of derivative

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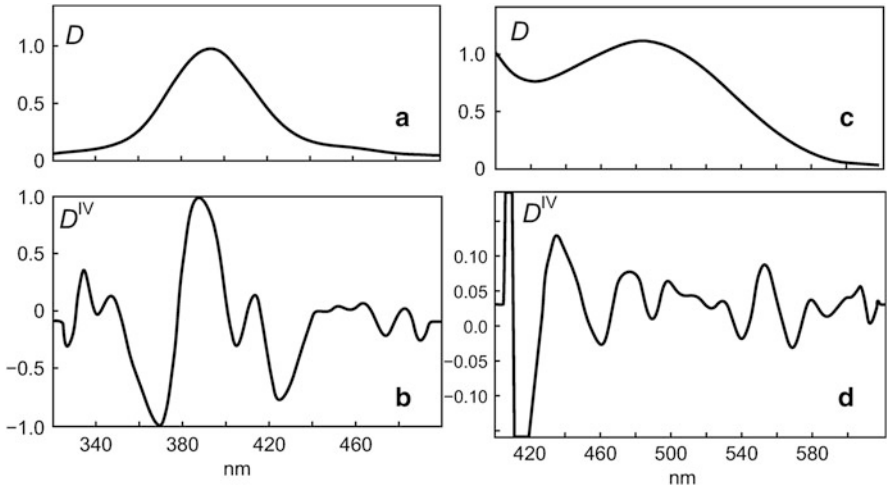

Fig. 3.23 Spectra of aqueous solutions of (**a**, **b**) 40 g/L $NiCl_2$ and (**c**, **d**) 20 mg/L Congo Red

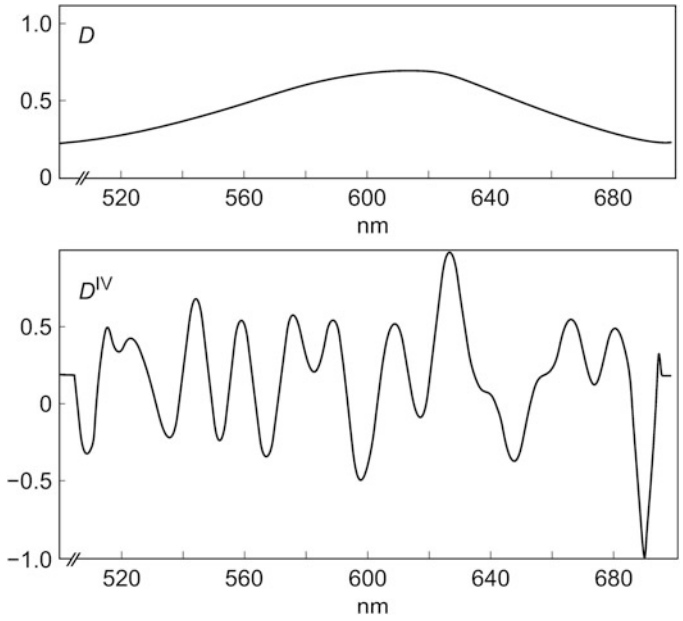

Fig. 3.24 The absorption spectrum of methylene blue (D) and its fourth derivative (D^{IV})

quantitative determination of pollutions, impurities, and undesirable minor components in multicomponent media because the detection of low-intensity peaks of admixtures, overlapped by intensive absorption or transmission bands of base material, is possible (see Fig. 3.11b) (Singleton and Collier 1956; Morton 1975;

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Inoue et al. 1975; Baranov et al. 1976; Such et al. 1980; Spitsyn and L'vov 1985; Skujins 1986a, b). This method of analysis is also used for the assessment of the structure of polymeric compounds (Dodd and West 1961; Calder 1969) and sterols (Olson and Alway 1960), the analysis of electron transitions in organic and inorganic compounds (Ismail and Glenn 1964; Lewis et al. 1970; Hager 1971; Aleksandrova et al. 1982; Nazarenko et al. 1982; Perfilev et al. 1983a, b, 1985; Spitsyn and L'vov 1985), the study of steric (conformational) changes in chelate complexes (Rozengart and Saakov 2002, 2003; Saakov and Rozengart 2005), and the characterization of the native state of biological structures and their special features revealed after mutations of the plastid apparatus or changes to the hemoglobin and plasma of the blood (Fog and Osnes 1962; Navarro et al. 1972; Litvin et al. 1973a, b; Baranov et al. 1974; Saakov and Hoffmann 1974; Kvitko et al. 1976, 1977a, b; Saakov et al. 1978a; Siek and Rieders 1984; Saakov 2005a, b).

Advances in the application of derivative spectrophotometry in analytical chemistry and biochemistry (including by a number of Russian scientists) are presented in large reviews (Talsky 1994; Hagris et al. 1996; Ojeda Bosch et al. 1995; Ojeda Bosch and Rojas Sanchez 2004; Rojas and Ojeda 2009; Karpinska 2012; Saakov et al. 2013).

The material in Fig. 3.22a, b illustrates the expediency of derivative spectra application in the analytical practice of such known compounds as potassium permanganate and potassium nitrate and also of biochemically and physiologically important organic compounds (Fig. 3.22c) for revelation of spectral details imperceptible in the usual absorption spectra.

Congo Red, the disodium salt of 4,4′-bis(1-amino-4-sulfo-2-naphthyazo) biphenyl, is an azo dye and an acid–base indicator. The absorption spectrum has $\lambda_{max} = 505$ nm. In weak-acid medium with pH 5.2, this dye has a red color; in acid medium with pH 3.0, it is blue. The indicator is used for photochemical detection of nitrates.

Methylene blue (*N*,*N*,*N*′,*N*′-tetramethyl thionine chloride hydrate, 3,7-bis (dimethylamino)-phenothiazin-5-ium chloride) is poorly soluble in water and ethanol, soluble in hot water, and easily reduced ($E_0 = +0.53$ V). It possesses oxidization–reduction properties and can play the role of a hydrogen ion acceptor. In aqueous solutions, the monomer form absorbs light of $\lambda_{max} = 668$ nm and the dimeric form $\lambda_{max} = 613$ nm. It is an important organic thiazine dye. In analytical chemistry, it is used for determination of chlorates, perchlorates, cobalt, cadmium, calcium, and magnesium. In medicine, it is applied as an antiseptic and antidote against poisoning with cyanides, carbon monoxide, hydrogen sulfide, and aniline and its derivatives. In microbiology, it is used for the staining of live preparations and also of histological preparations. Dyes of this class are capable of intercalating into nucleic acid structure and tightly binding to the guanosine residues of DNA and RNA. After irradiation with the light of 590 nm, the photoactivated dye oxidizes oxygen to its singlet state. Singlet oxygen damages the genetic material of viruses and thus prevents infection of blood plasma during its application for blood

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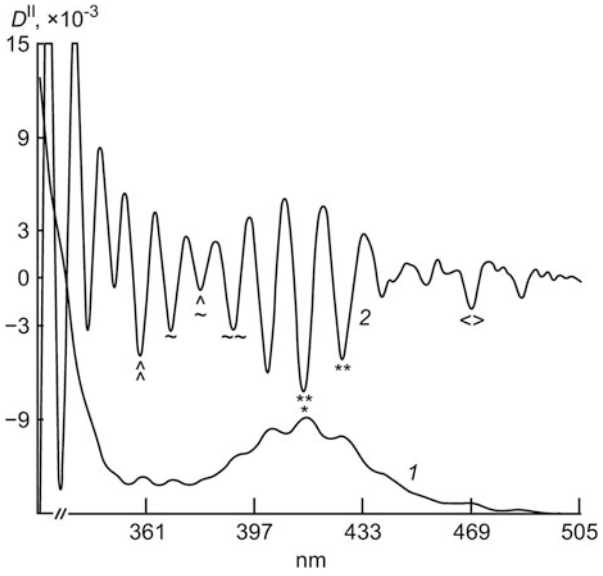

Fig. 3.25 The absorption spectrum of uranyl nitrate in water (*1*) and its D^{II} (*2*). The extrema (maxima) suitable for the rapid analysis of this compound on D^{II} are indicated by *symbols*

transfusion (Fog and Osnes 1962; Navarro et al., 1972; Litvin et al. 1973a, b; Baranov et al. 1974; Saakov and Hoffmann 1974; Kvitko et al. 1976, 1977a; Saakov et al. 1978a; Siek and Rieders 1984; Saakov 2005a, b).

Material in the article by Skujins (1986a, b) prompted us towards the interesting and technologically promising possibility of determination of the detection limits of uranyl nitrate in the region 330–505 nm. During uranium extraction from spent fuel elements or other objects, the easily water-soluble uranyl nitrate $UO_2(NO_3)_2$ is obtained after treatment with nitric acid. In flow injection cuvettes (optical cells) of the spectrophotometer, the qualitative and quantitative analysis of uranyl nitrate can be performed using the characteristics of derived spectra of the second (Fig. 3.25) or fourth derivatives, taking as the basis, for example, spectral bands in the region 397–433 nm. The use of registration of derivative spectra of the second and, better, of the fourth–eighth orders significantly increases the possibility of decreasing the threshold of detection for uranyl nitrate during flow injection spectral determination for a wide variation of concentration range. The flow injection analysis results in the continuous recording of the state of derivative spectra of uranyl nitrate during the solution flow through the standardized cuvette. In the case of darkening of the glass of cuvette walls caused by radiation, it can be replaced. Darkened glass does not distort the picture of spectrum configuration because it works as a neutral light filter. This is also valid for the control of primary information on the trace content and continuous sampling of heptyl and dimethylhydrazone, for example, in air.

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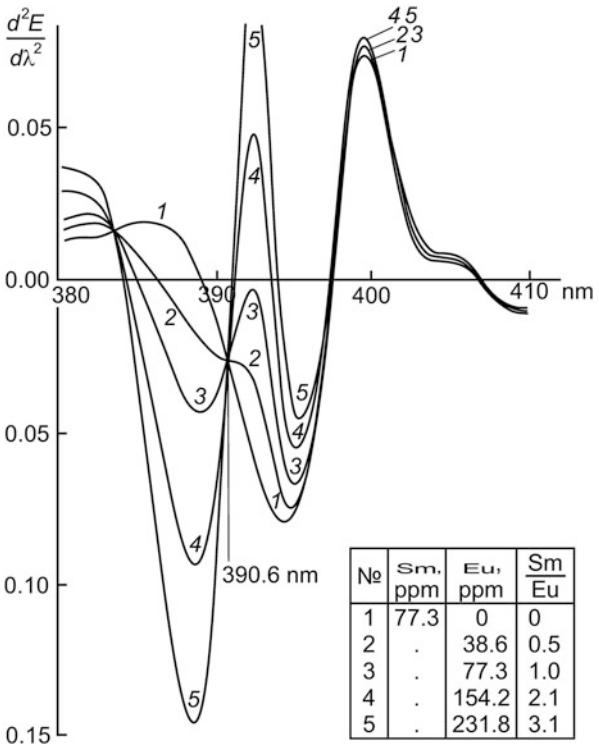

№	Sm, ppm	Eu, ppm	Sm/Eu
1	77.3	0	0
2	.	38.6	0.5
3	.	77.3	1.0
4	.	154.2	2.1
5	.	231.8	3.1

Fig. 3.26 Change character of the D^{II} spectrum of aqueous Sm (samarium) solution with increase in Eu (europium) concentration in it (Ishii and Satoh 1982a)

When checking the accumulation and presence of primary products in air, the proposed approaches can be realized if control of the primary information on trace content is performed at continuous sampling of heptyl and dimethylhydrazone.

Using derived spectra, positive results were obtained in the determination of nanoquantities of alkyl- and alicyclic ketones (Meal 1983). Performing D^{II} registration using the SP Hitachi 556 revealed nanoquantities of nickel and other elements and compounds (Shibata et al. 1972; Ishii et al. 1982a, b; Talsky 1994). Interesting data were registered in the case of selective finding of separate 4f-elements of the lanthanide group (neodymium, holmium, erbium, and thulium) in mixtures with the help of the D^{III} absorption spectrum (Ren et al. 1985); the registration of D^{I} and D^{II} spectra for the combined analysis of elements of group 8 (ruthenium and palladium) in complexes with thiobarbituric acid (Morelli 1983); the analysis of microquantities of lanthanides and 4f-elements samarium and europium with D^{II} and D^{IV} registration (Figs. 3.26 and 3.27) (Ishii and Satoh 1982a); determination of elements of the fourth group (zirconium) in the presence of hafnium with picrin amine E using the D^{I} method; and determination of

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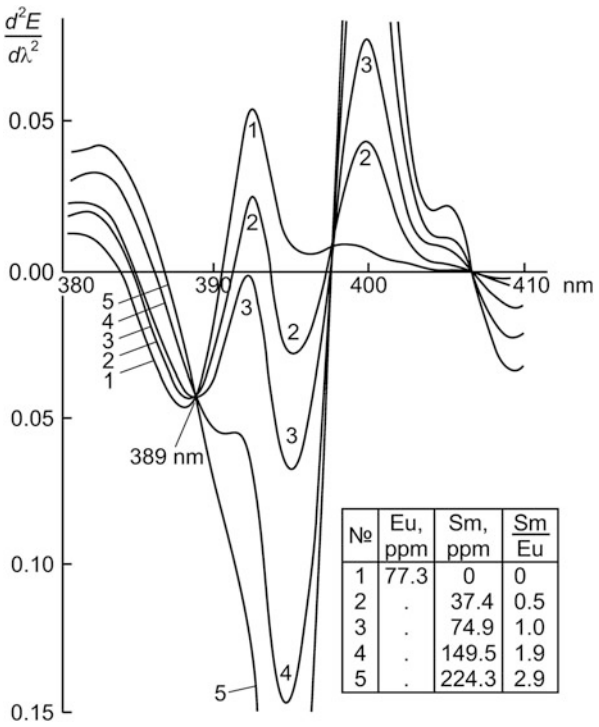

Fig. 3.27 Change character of the D^{II} spectrum of aqueous Eu solution with increase in Sm concentration in it (Ishii and Satoh 1982a)

5f-elements of the actinoid group (uranium) in the presence of 20-fold quantities of thorium and zirconium (Kvaratskheli et al. 1981; Semenov and Perfilova 2000).

To obtain a qualitative picture of derived spectra, it is necessary to find conditions in accordance with the stated study problem and the outline of the absorption spectrum (Spitsyn and L'vov 1985). Intensification or weakening of the manifestation of recorded bands of the derived spectrum depends on values of the band's half-widths in the initial spectrum and on the gradient and symmetry of its bands. The outline of the derived spectrum is more intensively revealed in proportion to narrowing of the band of the initial spectrum. Manifestation of derived spectrum is less for a more mildly sloping peak. The spectrum scanning rate and selection of the time constant (τ; see Sect. 4.1.2, Fig. 4.4) of spectra record regimes play a significant role. This means that *it is necessary to individually study methods of derived signal measurement for every substance or mixture of substances*.

We would like to note that the most complete reviews concerned with description of an application range of the derivative spectrophotometry method are in the following articles from the physicochemical field of studies: Bershtein and Kaminskii (1975), Gans (1982), O'Haver (1982), Miller et al. (1982), Sneddon et al. (1982), Perfilev et al. (1985), Spitsyn and L'vov (1985), Dubrovkin and

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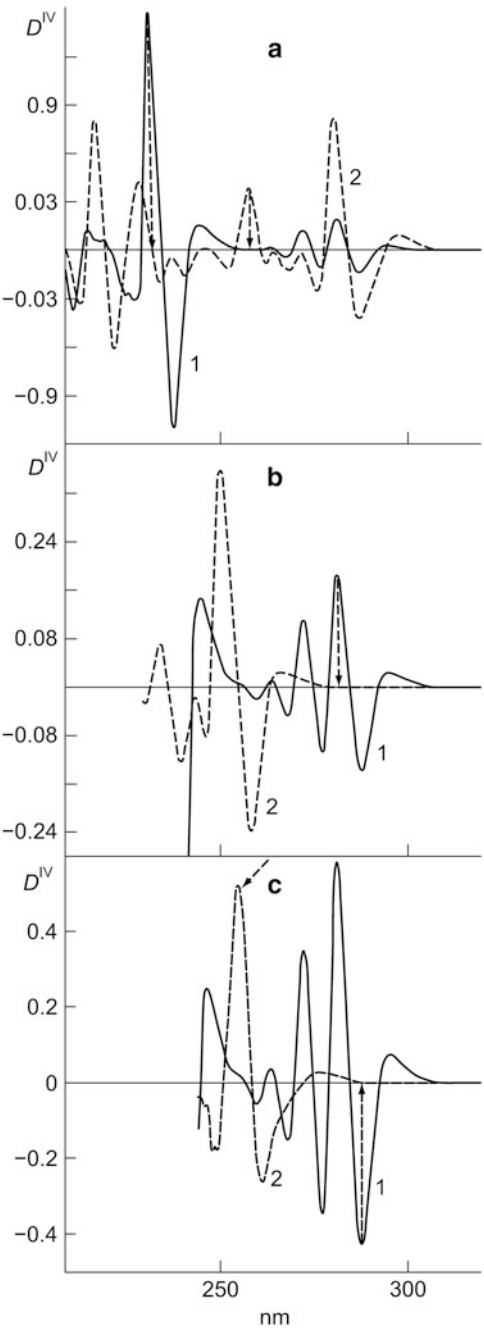

Fig. 3.28 Possibility of determination, with the help of D^{IV} spectra, of binary systems: (**a**) acetylsalicylic acid (*1*) and caffeine (*2*); (**b**) acetylsalicylic acid (*1*) and promethazine (*2*); and (**c**) acetylsalicylic acid (*1*) and phenobarbital (*2*)

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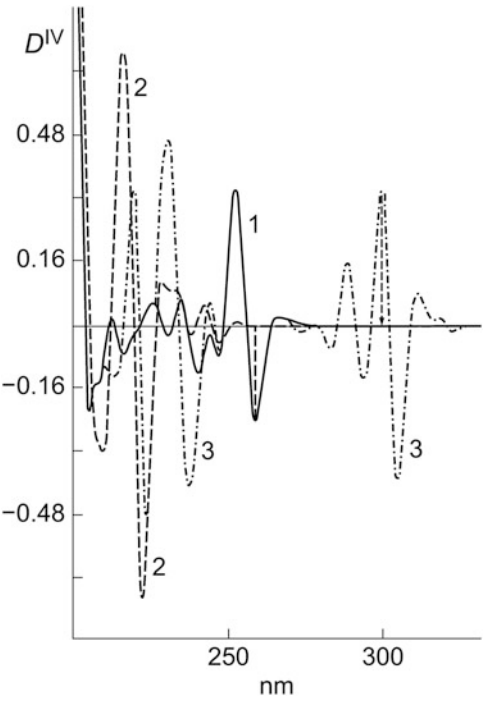

Fig. 3.29 Possibility of determination, with the help of D^{IV} spectra, of tri-component systems: (*1*) promethazine, (*2*) secobarbital, and (*3*) butylated hydroxyanizole

Belikov (1981, 1988), Howell and Hargis (1986), Ojeda Bosch et al. (1995); Ojeda Bosch and Rojas Sanchez (2004), Belikov (2002), and Karpinska (2012). In a number of other papers, different approaches and techniques of derivative application in the chemistry of tranquilizers (Abdel-Hamid et al. 1984), alkaloids (Hassan and Davidson 1984), and phenols (Shibata et al. 1976) are described.

With the methodological support of the biochemical laboratory of the Raymond Poincare hospital (Paris), Dr. D. Ivanovic' and colleagues (1995–2012) used the spectra D^{IV} of binary mixtures to determine promethazine and phenobarbital, and also caffeine and acetylsalicylic acid (Fig. 3.28a–c) in the PerkinElmer UV–VIS device with the double-beam Lambda 5. The same authors carried out the analysis of a ternary mix of promethazine ($\lambda_{max} = 257$ nm), secobarbital ($\lambda_{max} = 217$ nm), and butylated hydroxyanizole ($\lambda_{max} = 232$, 287, 300 nm) (Fig. 3.29).

The success of application of derived spectra accompanied experiments on the determination of indomethacin, ibuprofen, and salicylic acid (Such et al. 1980; Kitamura and Majima 1983; Mahrous et al. 1985); caffeine and amidopyrine, ephedrine and pseudo-ephedrine (with the instrument PerkinElmer 552) (Davidson and Elsheikh 1982; Korany et al. 1984); heroin and morphine (Davidson and Elsheikh 1982; Lawrence and Kovar 1984; Taulier et al. 1986); and in toxicology (Melvin et al. 2002). This method was successfully used for the analysis of

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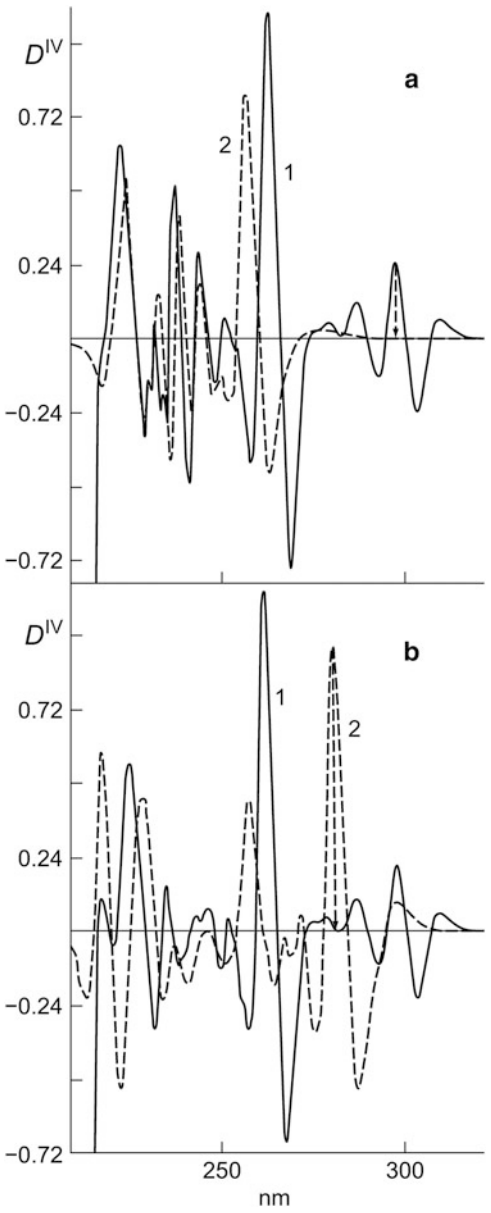

Fig. 3.30 Possibility of determination, with the help of D^{IV} spectra, of binary systems: (**a**) paracetamol (*2*) and L-ascorbic acid (*1*); (**b**) paracetamol (*1*) and caffeine (*2*)

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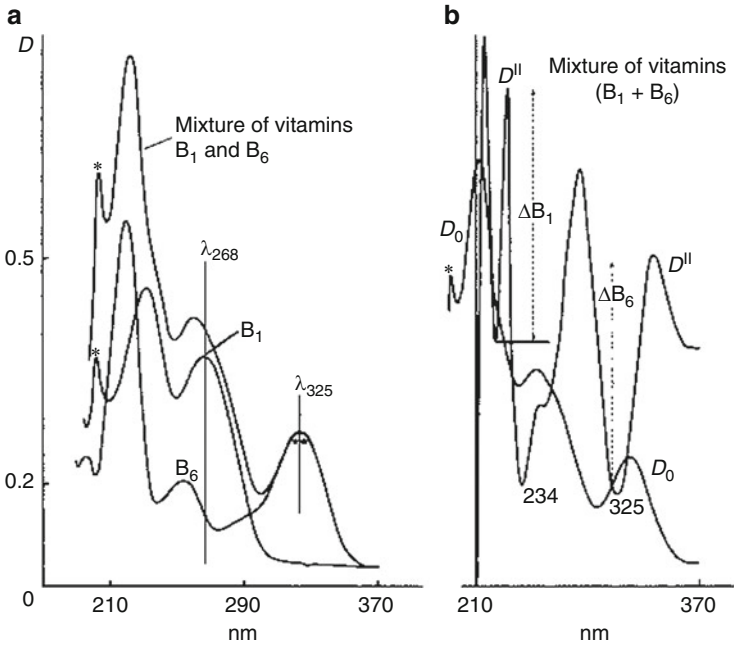

Fig. 3.31 (**a**) UV absorption spectra of vitamins B_1 (20 μg/mL) and B_6 (10 μg/mL) and of their mixture for concentrations 20 and 10 mkg/mL, respectively, in phosphate buffer (pH 7). (**b**) The absorption spectrum of the $B_1 + B_6$ mixture and D^{II} of the spectrum of this mixture

fungicides of the tetramethyldithiocarbamate type (thiram) (Sharma et al. 2003) used for protection of fruits from fungus (with the instrument Shimadzu UV–VIS 160); different hemoglobin derivatives (Saakov et al. 1973; Saakov and Spotakovskii 1973; Saakov et al. 1978b; Siek and Rieders 1984; Parks and Worth 1985; Melvin et al. 2002; Sharma et al. 2003); chlorophyll forms from plants of diverse ecological groups and bacteria (Magomedov and Saakov 1973, 1978; Magomedov et al. 1974; Whitten et al. 1978); and mixture of vitamins, for example, of the B group. Registration of UV spectra for B-group vitamins was performed with the instrument UV–VIS PerkinElmer-200 and derived spectra with the instrument PerkinElmer-200-0628 (Such et al. 1980; Kitamura and Majima 1983) (Figs. 3.30 and 3.31).

The analysis of different admixtures in the flowing water of water pipelines and in river and lake water proved to be very promising (Figs. 3.32 and 3.33) (Hellmann 1994).

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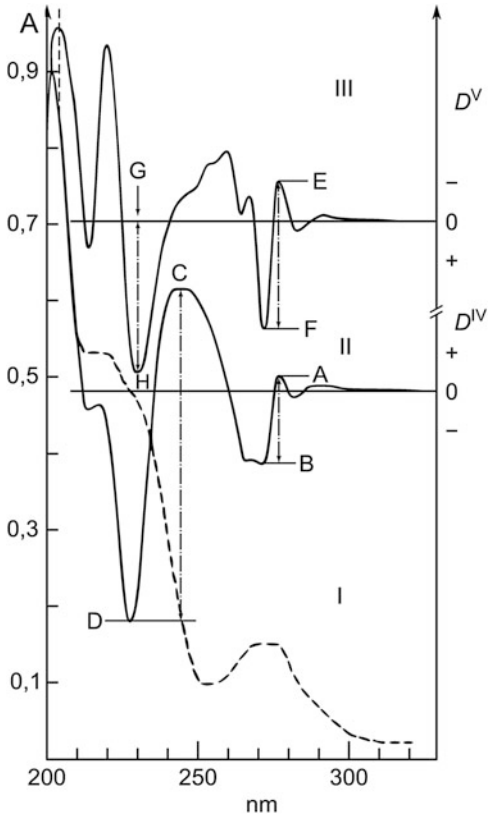

Fig. 3.32 Derivative spectrophotometry of high orders for the analysis of water contamination rate with aniline and phenol (Hellmann 1994). Presence in water of aniline (5 ppm) and phenol (5 ppm). (*I*) the initial spectrum of absorption; (*II*) its fourth derivative (see the vector **A**B); (*III*) the fifth derivative of the water absorption spectrum (compare the vector **A**B on the D^{IV} spectrum with the vector E**F** on the D^V spectrum). Vectors **A**B and **EF** are proportional to the concentration of phenol in water; vectors C**D** and **GH** are proportional to concentration of aniline

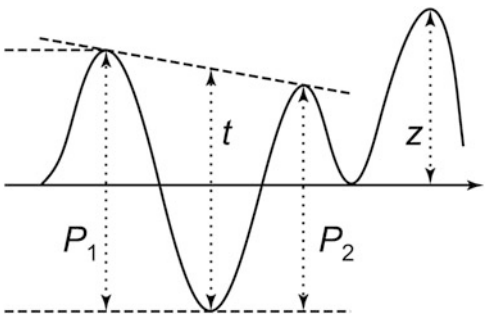

Fig. 3.33 A number of equal variants of calculation of quantitative characteristics of derived spectra, when it is possible to expect linear concentration dependence with retention of values of the band half-width of the substance spectrum. More frequently, the value of amplitudes P_1 and P_2 or of peak amplitude relative to the base line *t* is used. The possibility of using the absolute value of peak from the zero line (*z*) is not excluded

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3.3.1 The Possibility of Quantitative Analysis with the Help of Derived Spectra

For purposes of quantitative analysis, the approaches of derivative spectrophotometry are applied unreasonably little even though the potential capabilities of the method are very promising (McWilliam 1959; Savitzky and Golay 1964; McKay and Scargill 1968; Porro 1972; Ishii and Satoh 1982a; Dubrovkin 1983; Perfilev et al. 1985; Skujins 1986a, b; Saakov et al. 1987a, b; Dubrovkin and Belikov 1988; Semenova and Saakov 1989; Talsky 1994; Saakov et al. 2010). The value of the method can be seen from Figs. 3.34, 3.35, 3.36, and 3.37, for example, for inorganic ions and for the quantitative analysis of amino acids (see Fig. 4.7).

In such determinations, a value for the difference in amplitudes of two neighboring extrema (P_1, P_2) or a minimum amplitude value relative to the tangent line

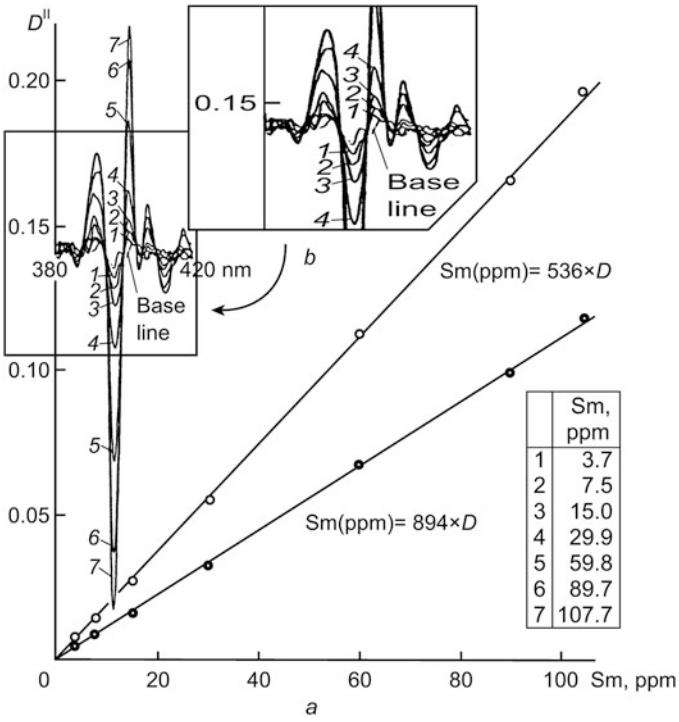

Fig. 3.34 Calibration curves for the determination of samarium concentration using D^{II} spectra with the help of two calculation methods (Ishii and Satoh 1982a): (**a**) original record; (**b**) expanded scale

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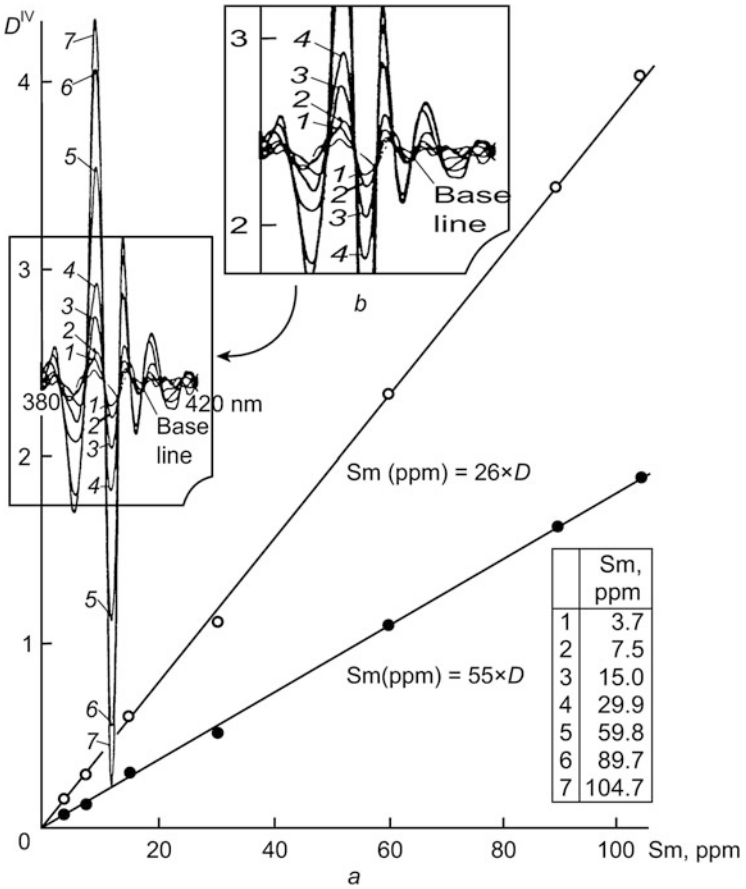

Fig. 3.35 Calibration curves for the determination of Sm concentration from D^{IV} spectra with the help of two calculation methods (Ishii and Satoh 1982a): (**a**) original record; (**b**) expanded scale. *Graphs* in Figs. 3.34 and 3.35 are constructed according to points of changes of peak amplitudes of D^{II} spectra or according to measurement of the peak amplitude from the base line

drawn to two vicinal maxima (Fig. 3.33) is used more frequently Hellmann (1994). When analyzing D^{I} or D^{II} spectra, and in turbid media, the use of the absolute peak value from the zero line is recommended (Talsky et al. 1978a, b; Ioffe et al. 1984; Skujins 1986a, b; Saakov 1987; Talsky 1994).

The techniques of quantitative assessment with derived spectra and analysis of possible measurement errors are described in a review (O'Haver and Green 1976) and in later papers (Perfilev et al. 1985; Dubrovkin and Belikov 1988). The possibility of

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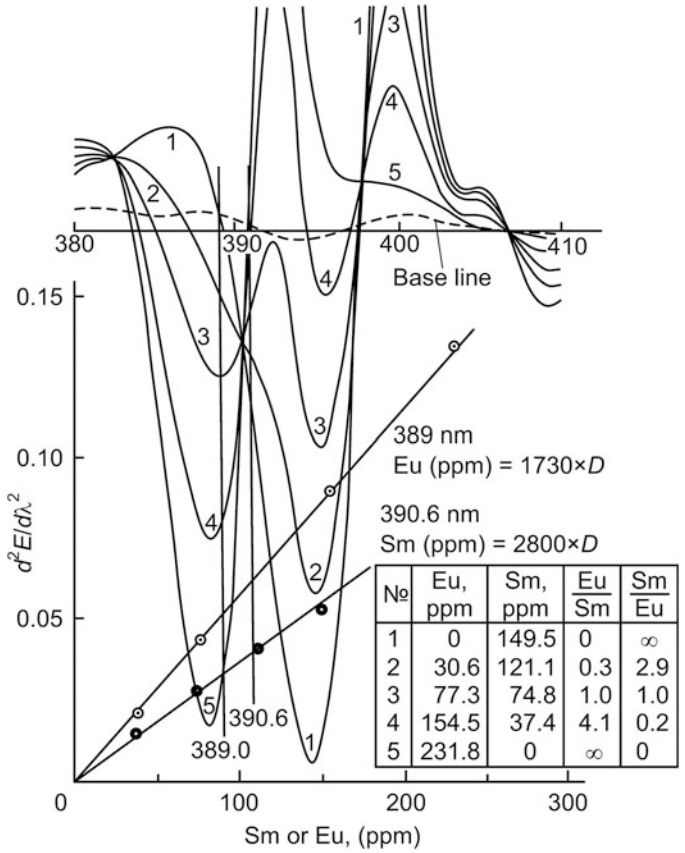

№	Eu, ppm	Sm, ppm	Eu/Sm	Sm/Eu
1	0	149.5	0	∞
2	30.6	121.1	0.3	2.9
3	77.3	74.8	1.0	1.0
4	154.5	37.4	4.1	0.2
5	231.8	0	∞	0

Fig. 3.36 Calibration curves for simultaneous detection of Sm and Eu in aqueous solutions from absorption D^{II} spectra

the quantitative determination of substances in turbid media is accented. For its realization, it is recommended to apply a method based on finding of the distance (t).

If the half-width λ_{max} is constant, the height of the derived spectrum peak is proportional to the substance concentration, which ensures high accuracy together with informativeness and selectivity of the analysis, realization of which by other means is difficult or even impossible. The above determines the fields of rational application of the method, including cases of the aerial or satellite monitoring of terrestrial and aqueous surfaces.

Thus, the figures presented in this section show various approaches for the use of derived spectra for quantitative and qualitative determinations in analytical and practical work.

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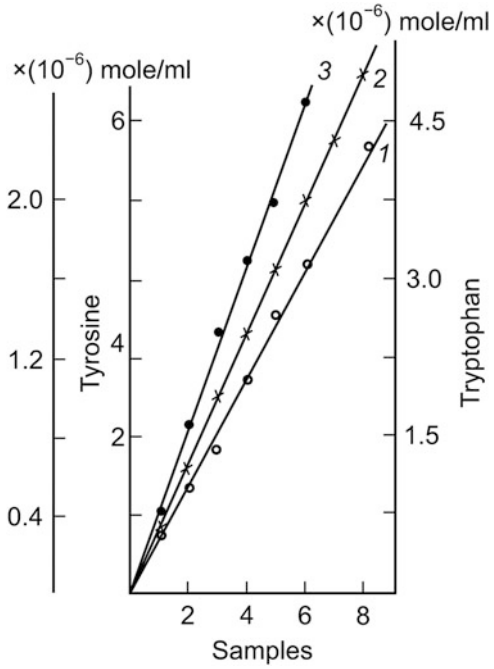

Fig. 3.37 Quantitative determination of aromatic amino acids with the help of D^{II} spectra

3.3.2 Methods of Derivative Signal Registration and Schemes of Differential Analyzers

Since derivative spectroscopy found wide application in qualitative and quantitative analysis (Hammond and Price 1953; Giese and French 1955; French 1957a; Hager 1971; Skujins 1986a, b; Dubrovkin and Belikov 1988; Talsky 1994), the interest in this method has constantly grown. The quantity of literature on the theoretical aspects of the method continuously increases (Giese and French 1955; Bonfiglioli and Brovetto 1964a, b; Bonfiglioli et al. 1967; Hager and Anderson 1970; Porro 1972; Wahbi and Ebel 1974; O'Haver and Green 1976; Dubrovkin 1983; Ioffe et al. 1984), although many communications are too general for practical use or excessively overloaded with a large number of mathematical formulae.

Different approaches to practical application of the method have been developed simultaneously with analysis of the theory. In general, there are three main directions: optical–mechanical, numerical, and electronic-analog (Gunders and Kaplan 1965; Perfilev et al. 1985; Spitsyn and L'vov 1985; Dubrovkin and Belikov 1981, 1988; Ojeda Bosch et al. 1995; Ojeda Bosch and Rojas Sanchez 2004; Rojas and Ojeda 2009).

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3.3.2.1 Optical–Mechanical Methods

The first group of methods for derived spectra registration includes two-wave spectrophotometry (French 1957a, b; Pemsler 1957; Bonfiglioli and Brovetto 1964a, b; Bonfiglioli et al. 1967; Porro 1972; Shibata et al. 1969, 1973; Wahbi and Ebel 1974; O'Haver and Green 1976; Skujins 1986a, b). This method is based on the direct differentiation of optical density with respect to wavelength. It is characterized by the high cost of instruments and also by the capability to register only a first derivative of a studied spectrum without additional expensive alterations to equipment. The method shows advantages over derivative spectrophotometry and, with the development of computer technology, offers new possibilities for researchers of different specializations (Pemsler 1957; Bonfiglioli and Brovetto 1964a; Talsky et al. 1978a; Dubrovkin and Belikov 1988). The same group includes methods of derived spectra registration with wave modulation (Shibata et al. 1969; Hager and Anderson 1970; Hager 1973, 1971; Wahbi and Ebel 1974; O'Haver and Green 1976; Talsky et al. 1978b; O'Haver 1979).

Elements modulating the wavelength could be oscillating or rotating mirrors (Brode et al. 1953; Hammond and Price 1953; Collier and Panting 1959; Willis and Miller 1959; Olson and Always 1960; Meister 1966a, b; Perregaux and Ascarelli 1968; Stauffer and Sakai 1968; Snellman 1968; Snellman et al. 1970; Shaklee and Rowe 1970; Burke et al. 1972), quartz plates or interference filters (Vierordt 1873; McWilliam 1959; Williams 1959; Drews 1967; Gilgore et al. 1967; Zucca and Shen 1973), the oscillatory entrance of either the output slit of monochromator or the sector shutter (Vierordt 1873; French and Church 1955; Baslev 1966; Williams and Hager 1970; Brandts and Kaplan 1973; Fowler et al. 1974; Mukhtarov and Nikolaev 1979), and light source modulation (Fowler et al. 1974). The positive aspect of derived spectra registration with the method of wave modulation is a decrease in signal-to-noise ratio; however, simultaneously, the intensity of light incident to the object is substantially weakened. Realization of this approach requires complex hardware equipment for optical–mechanical and electronic systems (French and Church 1955; Giese and French 1955; French 1957a, b; Bonfiglioli et al. 1967; Goldstein 1970; O'Haver 1976, 1978, 1979).

Using emission modulation in laser gas analyzers, Mukhtarov and Nikolaev (1979) found that application of the first derivative allows one to decrease the lower limit of concentration determination of SO and NO by three to five orders in comparison with the method of differential absorption. Thus, the determination of nanogram quantities of these gases becomes possible, and this is a direct junction with nanotechnology.

The design complexity of equipment is a common feature of these methods of derivative signal realization. A detailed example of the instrument diagram is presented in published work (Goldstein 1970).

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3.3.2.2 Numerical Methods

The second group of methods of derived spectra registration is based on the method of numerical (digital) differentiation (Challise and Williams 1964; Lewis et al. 1970; Lester 1970; Grum et al. 1972; Brandts and Kaplan 1973; O'Haver and Green 1976; Cuellar et al. 1978; Gans 1982), easily realized since the appearance of spectrophotometers with built-in microprocessors or operating with a computer. In recent years, this group of methods has attracted increasing attention (Mikhailyuk 2003).

3.3.2.3 Electronic-Analog Methods

In Russia (former USSR), the third (electronic-analog) method of differentiation of spectral curves is more widely applied (Kaler et al. 1967; Litvin and Gulyaev 1969; Saakov 1971a, b, c; Marenko et al. 1972; Marenko and Saakov 1973; Saakov and Spotakovskii 1973; Spitsyn and Korepanov 1980; Dubrovkin 1989; etc). Combination of this method with further digitalization of data and further data processing with programs has been described (e.g., Magomedov et al. 1974; Magomedov and Saakov 1973, 1978). This method allows one to achieve the desired result with simple hardware.

It is noteworthy that Russian recording spectrophotometers of SP-2 to SP-18 series are constructed in such way that the recorded electrical signal is proportional to the rate of absorption change, that is, to the first derivative of an absorption or transmission spectrum. According to this principle, different devices and attachments to spectrophotometers were designed for the generation and registration of signals of the first and second derivatives of absorption and fluorescence spectra (Kaler et al. 1967; Litvin and Gulyaev 1969; Marenko et al. 1972; Marenko and Saakov 1973; Saakov and Spotakovskii 1973; Zeinalov 1974; Dubrovkin and Sobolev 1976; Rutman et al. 1976a, b; Dubrovkin et al. 1978; Rutman and Saakov 1978; Kucher et al. 1983; Saakov et al. 1987a, b).

Note that the third method is based on the measurement of the rate of spectrum amplitude change, with maintenance of its scanning speed as continuous and uniform (Kaler et al. 1967; Litvin and Gulyaev 1969; Marenko et al. 1972; Marenko and Saakov 1973; Zeinalov 1974).

In recording spectrophotometers, the spectrum scanning speed is strictly dependent on the scanning time of the wavelength range. If the scanning speed of the spectrum is constant (i.e., $d\lambda/dt$ is constant), then for the first derivative $dD/dt = dD/d\lambda(d\lambda/dt)$ (I), and for the second $d^2D/dt^2 = d^2D/d\lambda^2(d\lambda/dt)^2$ (II). However, $d\lambda/dt$ is constant and, consequently, $d^2\lambda/dt^2 = 0$ and $d^2D/dt^2 = d^2D/d\lambda^2(d\lambda/dt)^2$ (III).

Thus, the first and second derivatives with respect to wavelength and to time are equal to each other within an accuracy of constant coefficients, determined by the spectrum scanning speed.

The material described above is not only concerned with derived absorption spectra registration, but also relates to modern requirements and the development

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trends of derived luminescence spectra registration (Navarro et al. 1972; Green 1974; O'Haver 1976; Almela et al. 1983).

We emphasize that one of the first Russian articles in which authors described (in general) the registration method for the first derivative of an absorption spectrum was the publication by Kaler et al. (1967). A similar approach to the technical realization of creation of signal proportional to the first and second derivatives of an absorption spectrum was also described (Martin 1957; Meister 1966a, b; Litvin and Gulyaev 1969; Gulyaev et al. 1971; Saakov 1971a, b, c; Shabalin and Petrova 1969; Litvin et al. 1973a, b; Matsushima et al. 1975; Demchenko et al. 1978; Dubrovkin et al. 1978; Ishii and Satoh 1982a).

The essence of the work of the above-discussed Russian schemes for obtaining the derivative signal is that the electronic signal arriving at the control winding of the synchronous motor of the cam moving the central slit in registering spectrophotometers (series SP 2–18) is proportional to the first derivative. This technical realization has a number of disadvantages, the main one being the presence of a harmonic of 100 Hz, which defines the increased noise level and distorts the formed signal and, therefore, complicates the correct interpretation of a derived spectrum graph (Kaler et al. 1967; Litvin and Gulyaev 1969, 1973; Gulyaev et al. 1971).

Contrary to the recommendations of Kaler and then of Litvin et al., we connected a differentiator directly to the entrance of the final power amplifier stage of a spectrophotometer (Marenko et al. 1972; Marenko and Saakov 1973; Rutman et al. 1976a, b, Saakov et al. 1977; Rutman and Saakov 1978), which allowed us to exclude significant outside signals presented in above-mentioned experiments (Kaler et al. 1967; Litvin et al. 1969, 1971, Litvin et al. 1973a, b).

We–the authors of this book have not yet given a detailed description of the development stages and circuit improvements caused by the appearance of new hardware and the accumulation of research experience. Features of improvements performed by the authors of this book are described in our previous publications (Marenko et al. 1972; Marenko and Saakov 1973; Saakov and Spotakovskii 1973; Rutman et al. 1976a, b; Saakov et al. 1976, 1987a, b; Baranov et al. 1976; Rutman and Saakov 1978) and in papers of other authors (Zeinalov 1974; Dubrovkin and Sobolev 1976; Dubrovkin et al. 1978; Kucher et al. 1983). Practically all our developments have passed many years of testing in laboratory practice, and the results based on them were published in Russia and abroad. The elaborated methods of D^{I} and D^{II} signal registration developed together with the department of physics of SPbETU "LETI" were studied by chemists and biochemists from different laboratories (Kvitko et al. 1976, Kvitko et al. 1977a, b), including many scientists from the school of the well-known physicist, chemist, and academician N.S. Poluektov (Aleksandrova et al. 1982; Nazarenko et al. 1982; Kucher et al. 1983; Perfilev et al. 1983a, b, 1985; Spitsyn and L'vov 1985; Mishchenko et al. 1987), and the results were positively characterized at different scientific forums. However, because of feedback from readers about our previous books (Saakov et al. 2010; Saakov et al. 2013), we have made some text additions and present the scheme solutions used earlier, despite the great developments in the elemental base.

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The active interest in derivative spectrophotometry and the absence of Russian industrial designs meant that researchers started to find their own approaches and solutions (Kaler et al. 1967; Litvin and Gulyaev 1969; Saakov 1971a, b, c).

The differentiating attachment created by us was presented for the first time at a seminar in the Institute of Physiology and Biochemistry of Plants Sib Branch of Acad. Sci. USSR (Irkutsk 1971) in front of academician A.L. Kursanov, Prof. Dr. Kurt Mothes (president of the German National Academy of Sciences, Leopoldina), and also academician V.A. Engelgardt, his wife Prof. M.N. Lyubimova (biochemistry), and corresponding member of Acad. Sci. of USSR F.E. Reimers.

The performed work and the presented experimental material were highly appreciated and recommended for prompt publication. Prof. Mothes suggested publishing the article in his magazine in German. At that time, at the beginning of the 1970s, it was very tempting and prestigious for Russian scientists. But times were different to now, and before publication in Europe, the manuscript had to pass a number of stages. Thereupon, we decided to send the material on circuits to the domestic magazine *Biophysics* (*Biofizika*). The article was accepted by the deputy chief editor, Prof. V.B. Evstigneev, who deeply understood the problems discussed in this manuscript. However, his tragic death meant that the manuscript lay for several months in an editorial staff portfolio and was returned to the authors with a mark about its local interest. It was quite clear that this state of affairs was caused by reasons of completely nonscientific nature and not connected with the quality of the material. The authors quickly published priority schemes of the differentiating attachment in the *News of Academy of Sciences of the Moldavian SSR* (Marenko et al. 1972) and then in a more detailed variant (Marenko and Saakov 1973; Rutman et al. 1976a, b). Due to reasons of a nonscientific nature, V.L. Kaler did not publish the way of obtaining the derivative signal. Scientific material was published in the magazine of Prof. Mothes *Biochemie und Physiologie der Pflanzen* (Saakov 1973a, b) and in the magazine of the Humboldt University in Berlin (Saakov and Hoffmann 1974). Competitors had to be satisfied with such a state of affairs. After all, Kaler et al. (1967) did not describe the scheme of obtaining the derivative signal and published only a few words on the general description of the device, for sufficiently strong reasons. For the same reasons, we published variants of the schemes for obtaining the derivative signal in various journals.

One of the first accessible modifications of the variant of the differentiating unit is presented in Fig. 3.38. This system is characterized by its high reliability and by the absence of parasitic pickups. Its characteristic feature was the presence of a specially developed stabilized constant-voltage source for the electric supply of anodes of tubes. The stabilization coefficient was approximately 500 (Rutman et al. 1976a). The level of noise and pulsation at the output was not higher than 0.5 mV. The application of the special stabilizer for the power supply to tube anodes significantly decreased the noise level and enabled clear records of D^I and D^{II} signals to be obtained.

Moreover, a parametric stabilizer of the constant voltage was used, at the output of which was a bridge circuit for regulation of the output voltage level, providing the compensation of voltage of both polarities when the recording pen was at the

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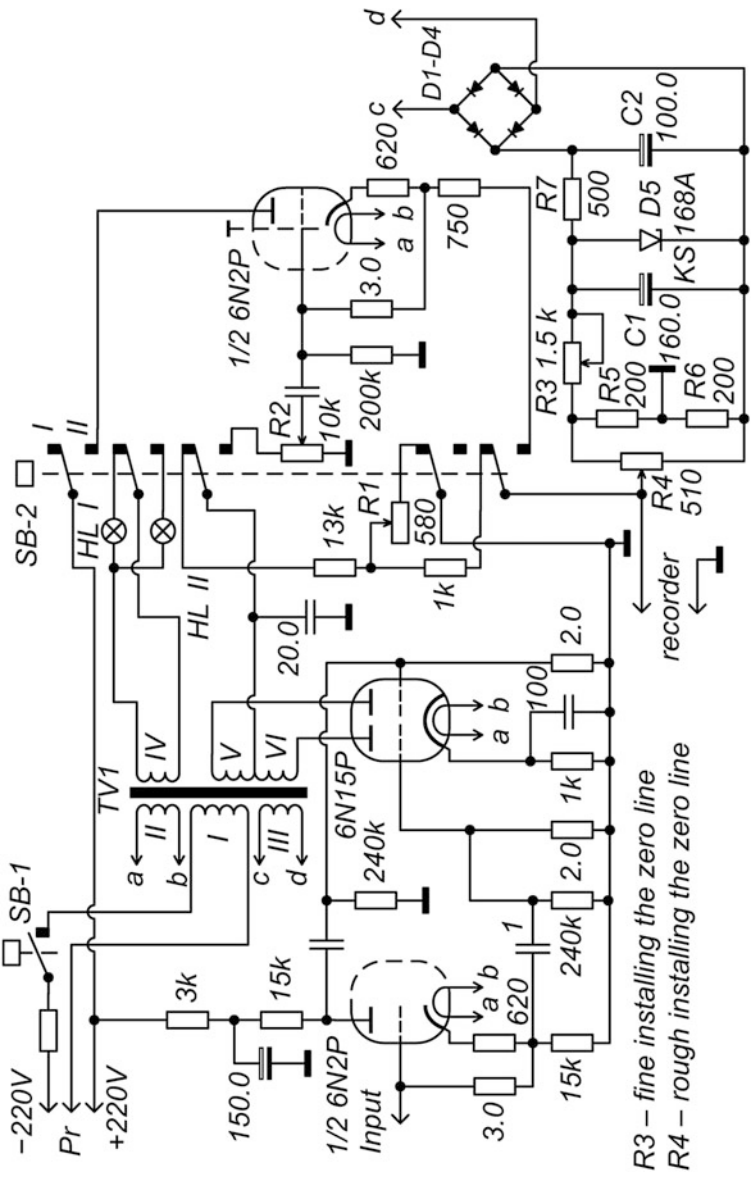

Fig. 3.38 The variant of the electric scheme of the differentiating attachment based on tubes (Marenko et al. 1972; Marenko and Saakov 1973; Saakov and Spotakovskii 1973; Rutman et al. 1976a, b; Saakov et al. 1976)

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zero line (Marenko et al. 1972; Rutman et al. 1976a, b). More details about the work of this scheme and its modification have been published (Marenko et al. 1972; Marenko and Saakov 1973; Saakov and Spotakovskii 1973; Rutman et al. 1976a, b; Saakov et al. 1976).

The next variant of the differentiating unit based on transistors is presented in Fig. 3.39. The scheme works in such a way that the signal from the power amplifier cascade of the spectrophotometer (SP-18, SP-14, SP-18) goes to the emitter follower according to the scheme of the composite transistor (T1, T2), with the aim of eliminating the influence of the differentiating unit on the spectrophotometer operating mode.

The differentiating phase discriminator is based on transistors T3–T5 and serves for the conversion of the alternating current signal coming to its entrance into a direct current signal, the value and polarity of which depend on the input signal phase. The main signal is applied at the base of the transistor T3, and the reference signal (50 Hz) from the transformer winding is applied at the base of T4. When the input signal is absent, the scheme should be balanced in such a way that at the set value (U_{ref}) of voltage at the cascade output (collectors T3 and T5), U_{output} is equal to zero. Then, signals from collectors T3 and T5 through the coordinating emitter followers T6 and T7 and through the switch SA2 arrive at the resistor R9, from which a signal proportional to the value of the first derivative arrives at the entrance of the registering potentiometer. The source of compensating voltage performs the same functions and has the same scheme as that in the tube variant.

The characteristic feature of this differentiating unit is the presence of the scheme of regulation of the differentiation time constant τ and the presence of differentiating amplifiers based on transistors T1, T2 and T3, T4 and serving as amplifying and matching cascades. The resistor R10 serves for regulation of the symmetry of the signal at the amplifier output; the resistor R19 and the condenser C7 serve for smoothing the high-frequency pulsations at the output of the differentiating unit. The resistor R21 and the switch SA2 regulate the level of incoming and output signals, resistors R21 and R23 and, in some variants of this unit, the switch SA3 and the battery are intended for the regulation of the zero line position (Rutman et al. 1976a, b; Saakov et al. 1976, 1987a, b; Rutman and Saakov 1978).

The obtaining of the signal proportional to the second derivative is performed with the help of differentiation of the signal of the first derivative, performed by the differentiating circuit R22C6. The time constant value τ is equal to RC (tau const. of time). For most of a slightly bent spectrum, an increase in the τ value is recommended. The matching of the high output resistance of the differentiating circuit and the low input resistance of the curve recorder is performed by the emitter followers T8 and T9. The transistor stabilizer is used at the power supply source of the unit. The detailed description of the scheme and its work has been published (Rutman et al. 1976a, b).

With modernization of the components of electrical network, the circuits for different devices for shaping and registration of D^{I} and D^{II} absorption spectra with Russian serial equipment were published (Saakov and Spotakovskii 1973, Saakov et al. 1976, 1987a, b; Zeinalov 1974; Dubrovkin and Sobolev 1976; Rutman et al. 1976a, b; Rutman and Saakov 1978; Dubrovkin et al. 1978; Kucher et al. 1983).

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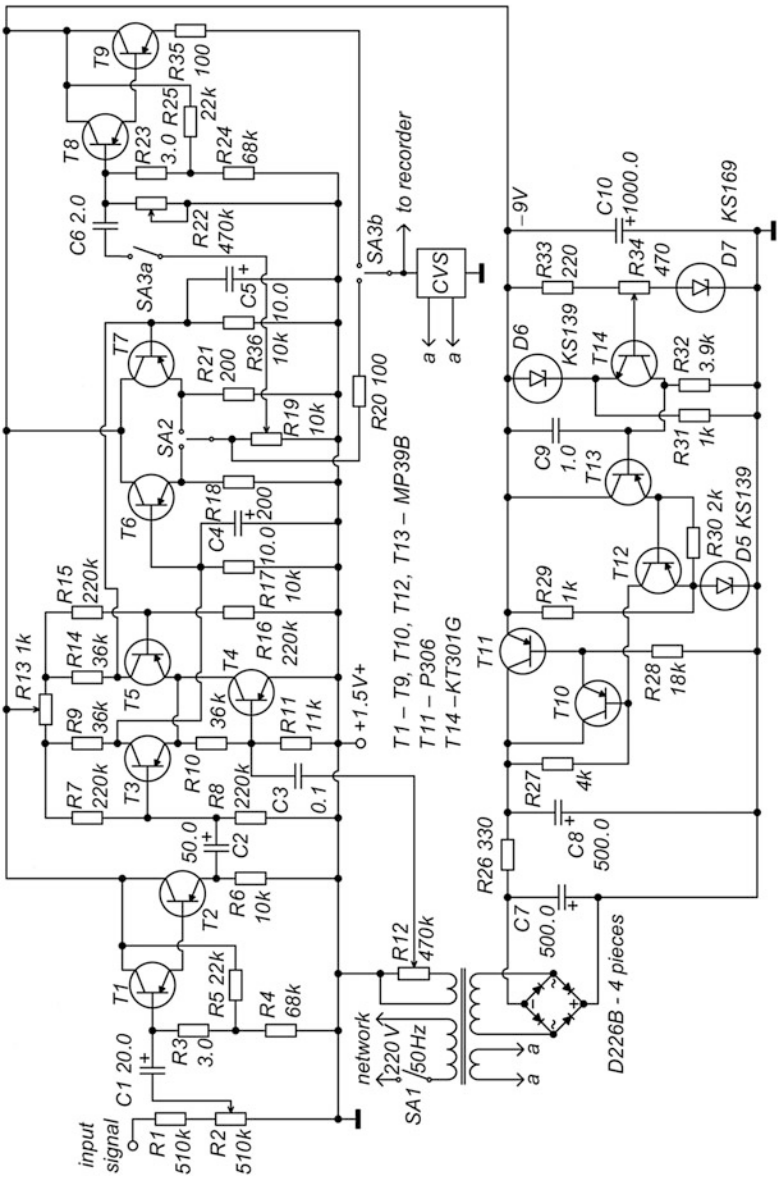

Fig. 3.39 The variant of the differentiating unit based on transistors (Rutman et al. 1976a, b; Saakov et al. 1976, Saakov et al. 1987a, b)

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The principal scheme for a differentiating unit based on field transistors (T1, T5, T6, T9) possessing high input resistance and low noise level is shown in Fig. 3.40. Note that the source of compensating voltage is excluded, and its function is performed by resistors R9 (for D^{I}) and R19 (for D^{II}). This became possible as a result of sending the signal symmetrically with respect to the earth at the input of the recorder of type KSP-4 or EPP-09. For the same reason, the D^{II} signal was obtained by symmetric differentiation of the D^{I} signal with the help of the differentiating circuits C3 and C4 and from the input resistances of transistors T6 and T9. The electric supply of the attachment was provided by the stabilizer of the constant voltage described above. A more detailed description of the work of this scheme has been published (Rutman et al. 1976a, b).

Further, the scheme for obtaining the derivative of infrared spectra has been published (Dubrovkin and Sobolev 1976). The first and second derivations of the signal from the spectrophotometer are carried out by the signal transmitting through the differentiating resistor–capacitor (RC) circuit. Differentiation is performed at the direct current. RC circuits are connected to inputs of cathode amplifiers that provide the matching of the input and output resistance of differentiating cascades. The output follower is based on the balance scheme that reduces the drift of the differentiator. This drift is the basic lack of schemes on the direct current (Dubrovkin and Sobolev 1976). In works of Bulgarian colleagues, the potential of differentiating devices constructed according to the scheme of Zeinalov (1974) was used (Fig. 3.41).

The further variants of our differentiating attachments for spectrophotometers differ from those described above in the way of formation, elemental base, or method of registration of D^{I} and D^{II} signals. However, before describing these, we will consider the problem of optimization of parameters of the differentiating circuit.

Russian and West European authors refer to a number of developments executed together with the chair of physics of SPbSETU "LETI" (Bershtein and Kaminskii 1975; Aleksandrova et al. 1982; Nazarenko et al. 1982; Kucher et al. 1983; Perfilev et al. 1983a, b, 1985; Skujins 1986a, b; Dubrovkin and Belikov 1988; Talsky 1994; Ojeda et al. 1995; Rojas Sanchez and Ojeda 2009). Regrettably, because our publications are cited by Western researchers often only 15–20 years after their appearance, their priority is reduced.

In this respect, it is necessary to pay tribute to the correctness of Prof. G. Talsky (1994) concerning the citing of Soviet author's publications. In his monograph, Talsky quoted about 30 Soviet researchers of chemical and biochemical directions of research. All this is shown in 56 tables and 5 chapters of the monograph and in 828 articles of quoted literature.

It is necessary to especially emphasize the high awareness of European researchers of the published base of Russian works. In the libraries of European scientific centers, there are special employees who, in response to the requests of scientists, search information and make complete databases on the state of world (including Russian) research on a required problem.

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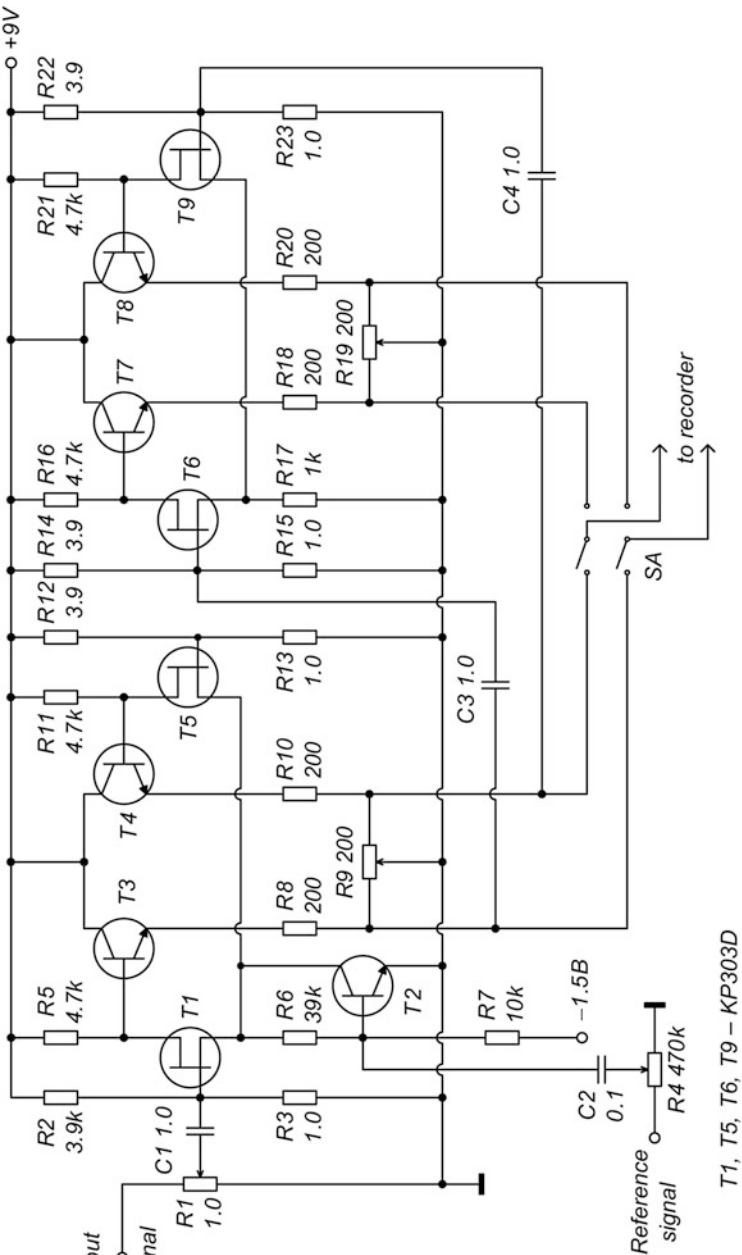

Fig. 3.40 The principal scheme of the differentiating unit based on field transistors

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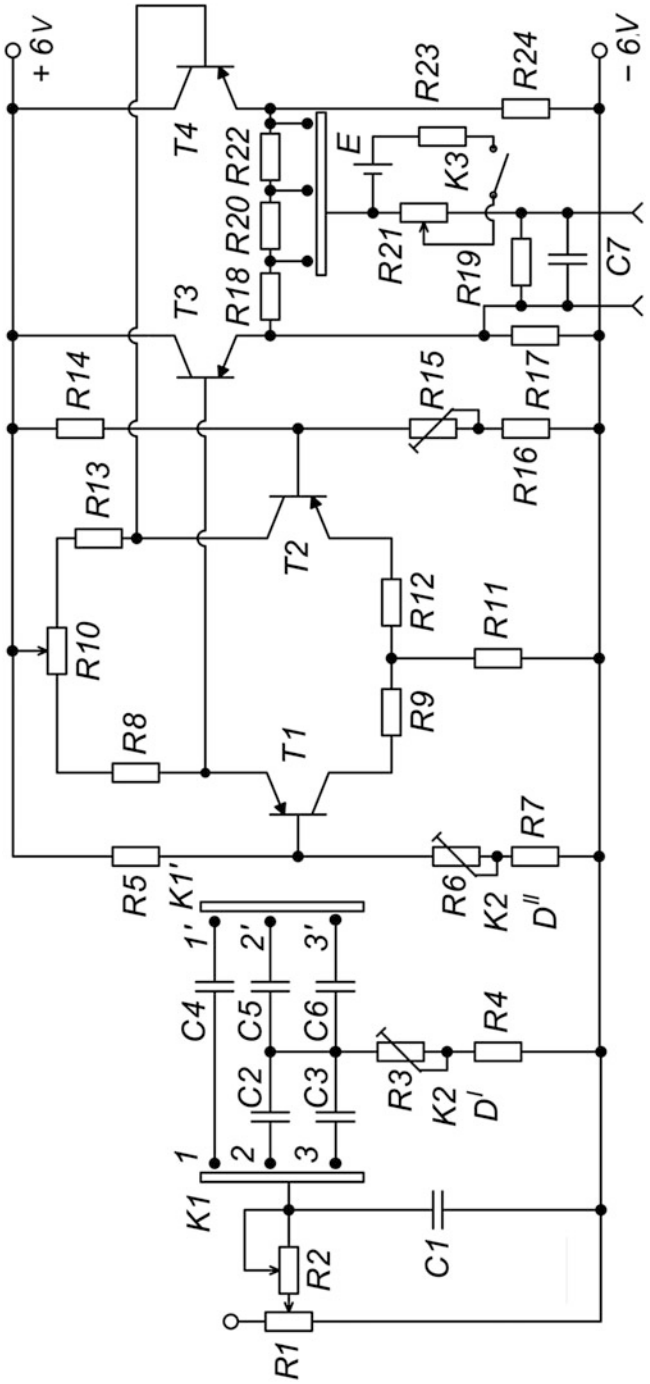

Fig. 3.41 The differentiating unit according to Yu Zeinalov (1974)

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Nevertheless, it does not mean that our works will be cited even if published in English or other European language. On the other hand, when it is necessary for foreign researchers, they quote our publications in journals, republican magazines, papers of institutes, and conferences not translated into English.

By contrast, Russian researchers, who are not even experts in the field of biochemistry, very quickly understood the value of our developments and actively cite them soon after publication (Aleksandrova et al. 1982; Kucher et al. 1983; Perfilev et al. 1983a, b). Except for, correct works of Talsky, follows accent (point) high information value of reviews: Ojeda C.B. et al (1995; 2004; 2009) with several hundreds references of different analytical application of derivative spectra.

3.3.3 *Optimization of Parameters of the Differentiating Circuit*

Let us consider the calculation of optimum parameters of the differentiating circuit and specific methodological solutions leading to an increase in differentiation accuracy and reliability of circuit operation during the analysis of plant and animal tissues or suspensions and solutions of bioorganic substances.

We emphasize that the work of the above-mentioned differentiators is based on the principle of spectrum differentiation not with respect to wavelength, but to time. This principle is realized when spectrum scanning on wavelengths is strictly synchronized with the time of the whole wavelength range transit and, consequently, derivatives with respect to wavelength and to time are equal to each other, with an accuracy of constant coefficients defined by the spectrum scanning speed.

Practical realization of this method, as can be seen from the above-mentioned diagrams, is simple; however, it requires the correct calculation of the differentiating circuit, taking into account the nature of the spectral outline.

An example of this approach is described by Martin (1957, 1959). On the basis of these recommendations, we calculated parameters of the differentiating circuit used in studies with Russian and some European recording spectrophotometers. Not specifically adapting the mathematical formulae for description of a signal at the entrance and output of the differentiating circuit, we will give an instance of basic formulae.

If the signal enters the entrance of the differentiating circuit (Fig. 3.42a) and this signal has the form of a Lorenz curve (Fig. 3.42b), the maximum value of the derivative of this curve at the output of differentiating circuit will be equal to

$$U'_{\max} = \frac{\pm 3\sqrt{3} U_0 r\tau}{4\omega} = \frac{10}{3000},$$

where $U'_{\max}$ is the maximum value of the derivative at the output of the differentiating circuit; U_0 the maximum value of signal amplitude at the entrance of the

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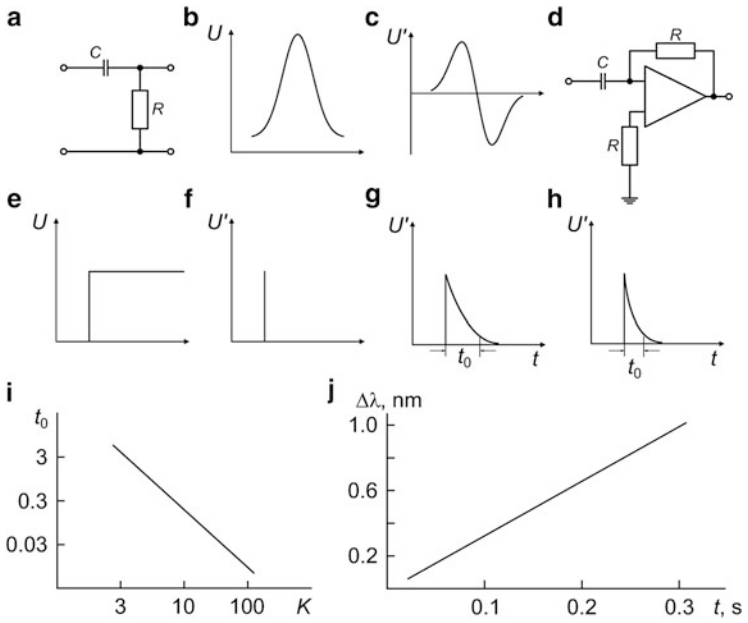

Fig. 3.42 Diagrams and performance records of differentiating circuit and of differentiating amplifier: (**a**) differentiating RC circuit; (**b**) initial signal at the entrance of RC circuit; (**c**) first-order derivative of signal *b*; (**d**) differentiating amplifier; (**e**) signal at the entrance of differentiator; (**f**) signal at the output of ideal differentiator; (**g**) differentiation of signal *d* with the help of RC circuit; (**h**) differentiation of signal *d* with the help of the differentiating amplifier; (**i**) dependence of t_0 on the mu-factor of the differentiating amplifier (t_0 is the time during which signal at the output of differentiator decreases to the value of $1/e$); (**j**) dependence of the average length $\Delta\lambda$ of the differentiation region on the time constant τ of the RC circuit

differentiating circuit; r the spectrum scanning speed (nanometers per second); $\tau = RC$ the time constant of circuit differentiation; and ω the half-width of a Lorenz band (the width of a spectral band at a height of 1/2 the maximum value of spectrum amplitude).

In transistor circuits (Rutman et al. 1976a, b; Saakov et al. 1976, 1987a, b), the maximum signal U_0 (about 3 V) enters the entrance of the differentiating circuit; at the output of circuit, it is necessary to have a signal of not less than 20 mV. Because the first derivative of a Lorenz curve has two maxima (it is bipolar) of equal amplitude (Fig. 3.42c), then $U'_{\max} = 10$ mV; consequently,

$$\frac{U'_{\max}}{U_0} = \frac{3\sqrt{3}}{4\omega} r\tau = \frac{10}{3000}; \tag{3.6}$$

$$\frac{r\tau}{\omega} = \frac{1}{225\sqrt{3}}. \tag{3.7}$$

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If for the spectra record we use a spectrophotometer with fixed spectral scanning speeds (e.g., SP-18 or SP-14), then for the most convenient speed (e.g., № 4), the value of r is equal to 2.9 nm s^{-1}. Because the half-width of the red absorption band, for example, of photosynthesizing models (tissue homogenate, suspension of algae or chloroplasts, etc.) is usually in the range of 30–40 nm, from Eqs. (3.6) and (3.7), it is possible to find the optimum value of the differentiation time constant:

$$\tau = \frac{\omega}{r \cdot 225\sqrt{3}} = \frac{35}{2.9 \cdot 225\sqrt{3}} = 0.03 \text{ s}.$$

At $r = 2.9$ nm s^{-1}, the wavelength range of the visible spectral region (400–750 nm) is scanned in 2 min, that is, in this time the spectrum is divided into 4,000 parts of differentiation. The average length of each part is equal to $\Delta\lambda = 0.0875$ nm ($\Delta\lambda = 350$ nm/4,000 = 0.0875 nm). Thus, the average length of the differentiation part linearly depends on the value of τ. The dependence $\Delta\lambda = f(\tau)$ is shown in Fig. 3.42j. However, taking into account that the accuracy of the wavelength setting in the spectrophotometer is ±1 nm, the time constant of differentiation can be increased practically to 0.1–0.2 s. In this case, $\Delta\lambda = 0.3$–0.4 nm, and at $\tau = 0.3$ s, we have $\Delta\lambda = 0.87$ nm (i.e., its value is close to spectrophotometer precision).

Depending on the parameters of a specific spectral band (amplitude and half-width), selection of optimum conditions for differentiation is performed through choice of the scanning speed and of nominal values R and C of the differentiating circuit. A reader should proceed from the condition that the capacity resistance must be much more than R (Saakov 2000b). For $\tau = 0.1$–0.2 s, the rated capacity (C) must be 4–10 μF; therefore, R values can be in the range of 10–25 kΩ. Having a package with the above-mentioned R and C, we can change τ from 0.04 to 0.25 s, depending on the parameters of the spectral band (its amplitude and half-width). It is also necessary to *change the τ value with a change in the spectral scanning speed*. At the choice of R and C nominals, a reader should proceed from the conditions described (Saakov 2000b). Optimization of τ value influences the manifestation of the fine structure of derived spectra (see Sect. 4.1–4.1.1).

Experience of using of serial spectrophotometers has shown that optimal differentiation conditions can be found if the differentiator ensures a change in the time constant in the range 0.05–0.5 s and if nominal values of R are 5–10 kΩ and C is 2–10 μF (Saakov et al. 1987a, b).

The application of any method always raises the question of its accuracy, in this case, about the accuracy of differentiation. As explained earlier, the differentiating amplifier (DA) (Fig. 3.42d) has a number of advantages over the usual RC circuit (Maiorov 1956; Rutman et al. 1976a, b; Taulier et al. 1986). First of all, there is a significant increase in the level of output signal; second, exclusion of influence of mu-factor instability on the output signal; and third, a significant decrease in the differentiation error (i.e., $t_0 \to 0$) (Fig. 3.42g).

Figure 3.42e–h shows work diagrams of the differentiating circuit (Fig. 3.42 g) and of the differentiator amplifier (Fig. 3.42h) for conditions when a single voltage jump enters (Fig. 3.42e). In Fig. 3.42f the diagram of the output voltage of the ideal

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differentiator is shown. The dependence of t_0 on the amplifier gain (K) with constant R and C is presented in Fig. 3.42i; it is of linear character in logarithmic coordinates. Even if the transfer constant of the differentiating amplifier is equal to 1.0, then t_0 decreases from 3.0 to 0.3 s in comparison with the RC circuit. Thus, the advantages of the differentiating amplifier compared with the RC circuit are obvious.

Specialists have tried to assess the differentiation errors (Smirnov and Badu 1967). Meister (1966b) showed the presence of such errors, but did not give recommendations on how to decrease them. Korobkov (1975) attempted not very successfully to compensate for these errors. Previously, in articles on derivative spectrophotometry for biological native structures, the accuracy level of differentiation was not determined.

The issue of differentiation errors and the value of application of differentiating amplifiers instead of the usual RC circuits are discussed in detail elsewhere (Rutman et al. 1976b). Analysis of literature data allows us to conclude that the use of an RC circuit results in the differentiation error becoming insignificant (2–5 %) after 1–3 s, depending on the value of $\tau = RC$ and that the use of a differentiating amplifier reduces the error to practically zero after only 0.1–0.3 s at the mu-factor $K = 10$ (Fig. 3.42). With an increase in K ($K = 100$), $\Delta \cong 0$ after only 0.01–0.03 s. Thus, correctly selected parameters R, C, and K reduce the differentiation error to such a low value that it is negligible. Details of the calculation and graphs of the differentiation error change when using the differentiating amplifier have ben published (Rutman et al. 1976b).

Concerning the shift in the value of the maximum of the second derivative (Korobkov 1975), because the D^{II} spectrum is formed and recorded in parallel with the initial absorption spectrum and the time constant of the spectrophotometer is more than the time constant of the differentiating attachment (otherwise differentiation would not be possible), it is possible to consider all these shifts as existing only relatively to the true absorption band, but not to the band recorded by the spectrophotometer, i.e., comparison of the initial spectrum with its D^{II} registered with the help of differentiating amplifiers does not reveal shifts in band maxima.

Even in the initial stages of use of the considered method, we performed checking of the differentiation accuracy by applying the procedure proposed by Smirnov and Badu (1967). The checking technique is the comparison of transfer functions of an ideal differentiator (ID) and of a real differentiator (RD), whose block diagram is shown in Fig. 3.43a (Rutman and Saakov 1978). In this case, the RD parameters and their absolute and relative errors are calculated, and also a method to decrease these errors through changing the nominals of separate circuit elements can be found.

RD consists of an adder, an inverter, and an integrator connected to a feedback loop. Without rewriting the mathematical calculations described (Smirnov and Badu 1967), we would like to note that the accuracy of differentiation of the arbitrary time function (on the supposition of equality of $R1$ and $R2$ and the transfer constant of the circuit $K = 1.0$) depends on the integrator parameters, that is, on the

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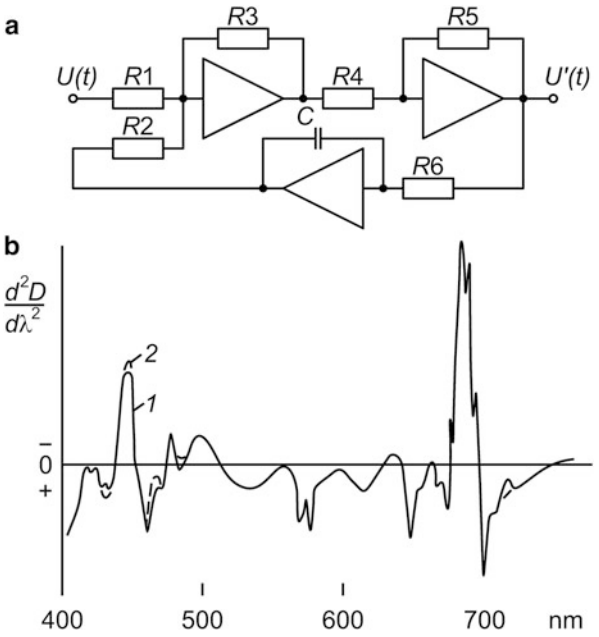

Fig. 3.43 (**a**) Diagram of the real differentiator (RD). (**b**) D^{II} of the absorption spectrum of homogenate of spiderwort leaves

integration time constant τ_0. To have a constant accuracy level when differentiating the arbitrary time function, it is necessary to find τ_0 from the prevailing frequency (Rutman et al. 1976a, b; Saakov et al. 1976, 1987a, b).

For example, if the half-width of the red absorption band of spectrum is equal to 30 nm and the scanning speed of the spectrum is 2.9 nm s^{-1}, then the sinusoid frequency approximating this band will be approximately 0.1 Hz. The time constant of integration is chosen on the basis of this value.

We performed comparison of RD (Fig. 3.43) and of other differentiators (Rutman et al. 1976a, b, 1978; Saakov et al. 1976, 1987a, b) and concluded that the differentiation accuracy of these devices was satisfactory enough as the relative error did not exceed 10 %. Figure 3.43b shows the D^{II} absorption spectrum for a homogenate of spiderwort (*Tradescantia* sp.) leaves. The spectrum was registered with the help of RD and differentiator, manufactured according to the diagram of the differentiating amplifier. The stability of reproduction of spectra recorded in equipment developed by us can be assessed from Figs. 3.2 and 3.3. Similar checking of the stability of derived spectra registration is described (Talsky 1994) (see also Figs. 3.44, 3.2, and 3.3).

Assessment techniques for the degree of deformation of the form and position of the spectrophotometer absorption band are sufficiently well presented in the literature (Tereshin 1959a, b; Babushkin et al. 1962; Tarasov 1968; Rubin 1974, 1975; Bershtein and Kaminskii 1975; Talsky 1994), so we will not describe them. If spectroscopic instrumentation does not change the form of the absorption band by itself and does not shift the band maximum, then a differentiator with correctly

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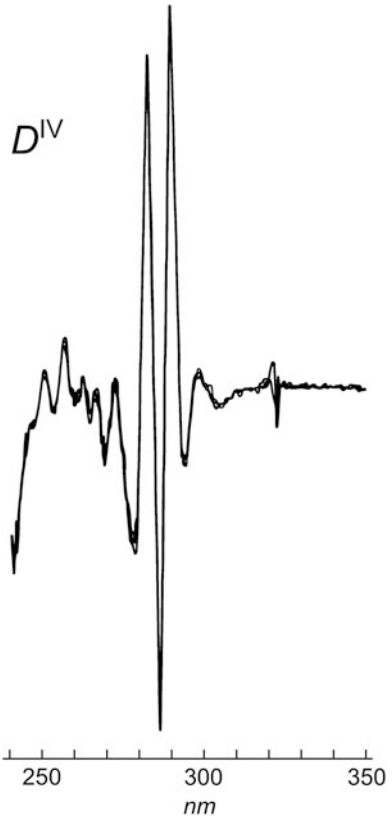

Fig. 3.44 The consecutive sixfold record of curves D^{IV} of absorption spectra of bovine trypsin, concentration 0.5 g/L

selected parameters of differentiation will not add an error to the position of absorption maximum at the wavelength scale.

The analysis described by Rutman et al. (1976a, b) was one of the first attempts at the study and compensation of differentiation errors of the first equipment by shaping and registration of D^{I} and D^{II} signals, although this analysis was probably not ideal and had some imperfections. Examples of the usage of differentiating amplifiers in an apparatus recording D^{I} and D^{II} signals are described in other articles (Rutman et al. 1976a, b, 1978; Saakov et al. 1976, 1987a, b).

3.3.4 Differentiators with New Locating Features

Because the differentiation accuracy and reproducibility of results depend on the stability of parameters of the elements composing a differentiating unit, a differentiator was developed (Rutman et al. 1976a, b; Saakov and Spotakovskii 1973) that was based on integrated microcircuits and according to the diagram of a

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differentiating amplifier (Saakov et al. 1987a, b). We pay attention to the fact that the DA substantially amplifies the level of output signal, noticeably decreases the error of differentiation, and excludes the influence of mu-factor instability on the value of the output signal.

In the presented schematic diagram (Fig. 3.45), the signal is registered from the entrance of the final stage of the power amplifier of the spectrophotometer and comes into the left half of MS1(A1) (assembly of field-effect transistors KPS 104B), which is the matching cascade. The right half of MS1 is the phase-inverting cascade, to the entrance of which the voltage of the reference signal will be applied. The reference signal preliminarily passes through a phase inverter based on MS2 (A2) (the operational amplifier K1UT531A or MS40 ensuring the precise matching of phases of fundamental and reference signals). In the considered differentiator, the principle of synchronous signal detection is used. The signal is modulated by the frequency of 50 Hz, and its envelope is proportional to D^{I}; therefore, after the synchronous detector based on MS3(A3), we register the signal of the first-order derivative of the absorption spectrum. After the active filter based on microcircuit MS4(A4), performing the transformation of symmetrical signal into an asymmetrical signal and also realizing additional filtration of variable components of the signal (cutoff frequency not more than 20 Hz), the signal enters either the entrance of the graph plotter or the entrance of the differentiating amplifier based on the microcircuit MS5(A5). From the output of MS5, the signal proportional to D^{II} is recorded. Supply to the differentiating unit is maintained with the stabilized voltage source described earlier (Rutman et al. 1976a, b). The registration of signal can be performed with recording instruments of types KSP-4 and EPP-09 (with the scale up to 2.5 s) and with a cathode-ray indicator (of type I-10) or with an oscillograph face (S1-18). An electron-beam record is inertia-free and allows registration of more rapid and smaller changes of the spectrum structure. It also ensures the selective large-scale record of individual regions of spectrum and can be useful for an operator performing a number of special experiments (Rutman and Saakov 1978; Saakov et al. 1987a, b).

The considered DA (Differential amplifier) is characterized by high accuracy of differentiation, reliability in operation, and small overall dimensions. The accuracy of DA differentiation is 1–3 % (depending on the phase ratios in the synchronous detector) and was checked with an analog computer MN-10 M, using the procedure described by Smirnov and Badu (1967).

A dedicated analog processor (DAP) was created for processing of electronic data recorded by spectroscopic instrumentation (spectrometers of optical range, spectrofluorometers, microwave spectrometers) (Saakov et al. 1987a, b). DAP in the general case performs the following: search and isolation of informative region of the spectrum, processing (single or double differentiation, measurement of extremum value, integration of spectral curve, etc.), storage of the processing result, and comparison with the normal value of the measured parameter of the spectrum

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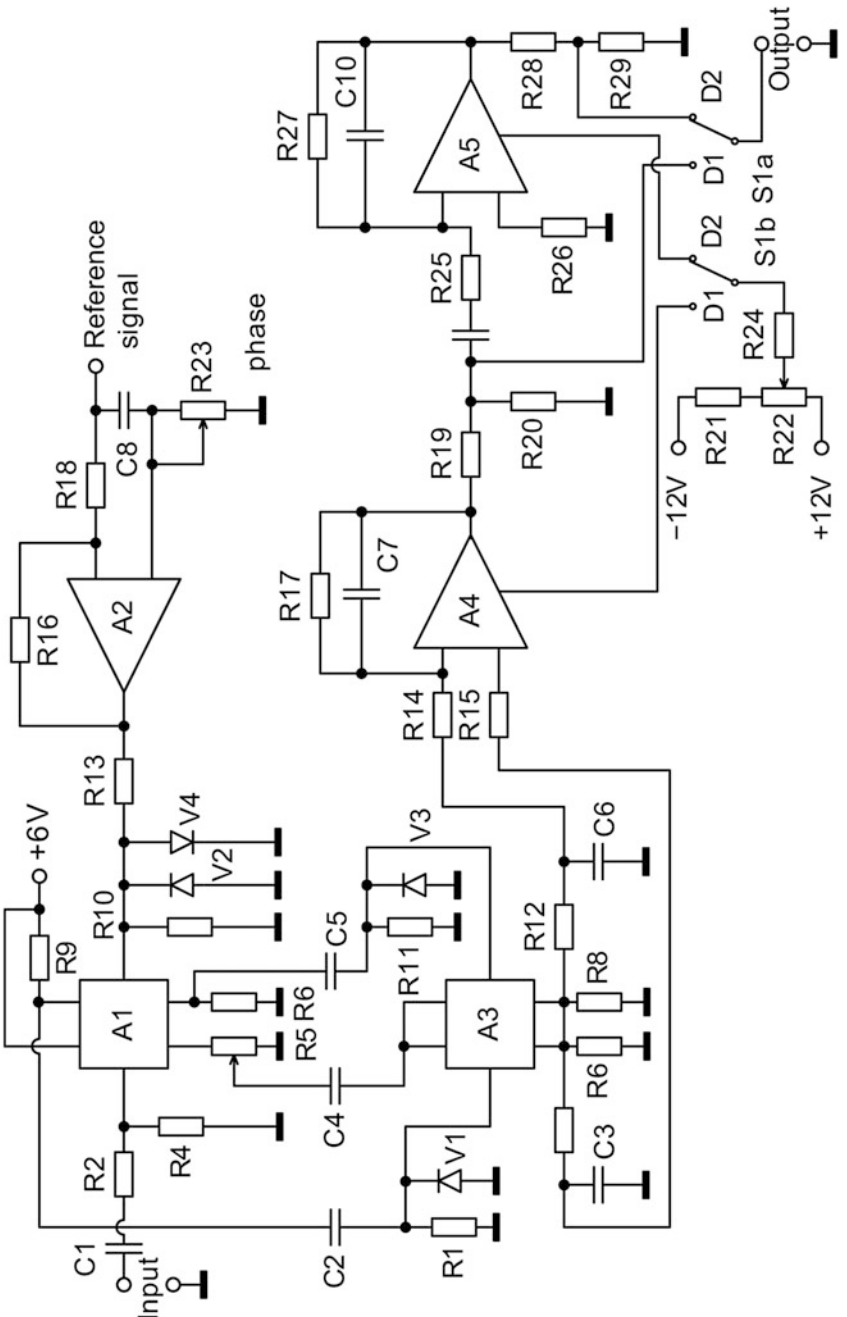

Fig. 3.45 Principal electric diagram of the differentiating attachment on microschemes (Rutman and Saakov 1978)

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(amplitude, slope, area, line width). Information can be sent from DAP to a digital instrument or a computer.

One of the DAP variants was used for the optimum coupling of the electron paramagnetic resonance (EPR) sensor with the computer ASU TP for preparation of the metallo-organic catalyst for polymerization of isoprene caoutchouc. This ASU TP, including EPR sensor and DAP, is successfully operating in production by Nizhnekamskneftekhim (petrochemical industry in Nizhnekamsk) and allows continuous automated monitoring and control of the preparation of the optimum composition catalyst. The use of integrated microcircuits and semiconductor devices in DAP guarantees high stability, reliability of operation, and operational simplicity.

DAP was successfully used in combination with modifications of the instrument Specord UV–VIS (Carl Zeiss, Jena). The graph plotter of this spectrophotometer has two interconnected slide wires of the channel of vertical pen deflection of the instrument automatic recorder. One of slide wires is destined for the remote registration of spectra. Connections of this potentiometer are at the slot located on the back of the spectrophotometer panel (contacts 22, 23, 24). By applying a voltage of 9 V at contacts 22 and 24 from a stabilized source of voltage (SSV) based on transistors V1 and V3 and the stabilitron V2, it is possible to obtain voltage proportional to the optical density of the sample at connections of the slide wire (see Fig. 3.46).

The load of the SSV is the slide wire R1 of Specord and the compensation potentiometer R2. The signal proportional to position of the carriage of the spectrophotometer goes through integrating circuits R4 and C1, decreasing high-frequency noise and interference, and enters at the entrance of the compensating amplifier based on the microcircuit A1, at another entrance of which there is compensation voltage from the R2 resistor slider. With resistors R3, R5, R6, and R7, the mu-factor is assigned; switch S changes the mu-factor in a stepwise manner. With the switch S closed, the input amplifier A1 works in the regime of the voltage repeater and expands the dynamic range of the recorded voltage signal. Potentiometer R2 is set in such a way as to compensate possible voltage shifts in the source of signal and also to select the differentiated region of the spectrum. If amplification is large, then it is possible to exceed the operating range of the compensating amplifier, but with the corresponding position of potentiometer R2, a more detailed representation of necessary spectral region will be recorded.

From the output of the compensating amplifier, the signal can be recorded by a recording instrument or can go to the entrance of the differentiator deriving the first-order derivative. The differentiator consists of amplifiers A2 (A5) and A4 (A7) and elements R8, R9, R10, R14, R15, C3, R12, and R11 (R16, R17, R18, R22, C5, R23, R19, R20); the elements of the second differentiator are listed in parentheses.

The amplifier A2 is connected with elements R9 and R10 of the circuit of negative feedback through the differentiating circuit; moreover, the integrator is

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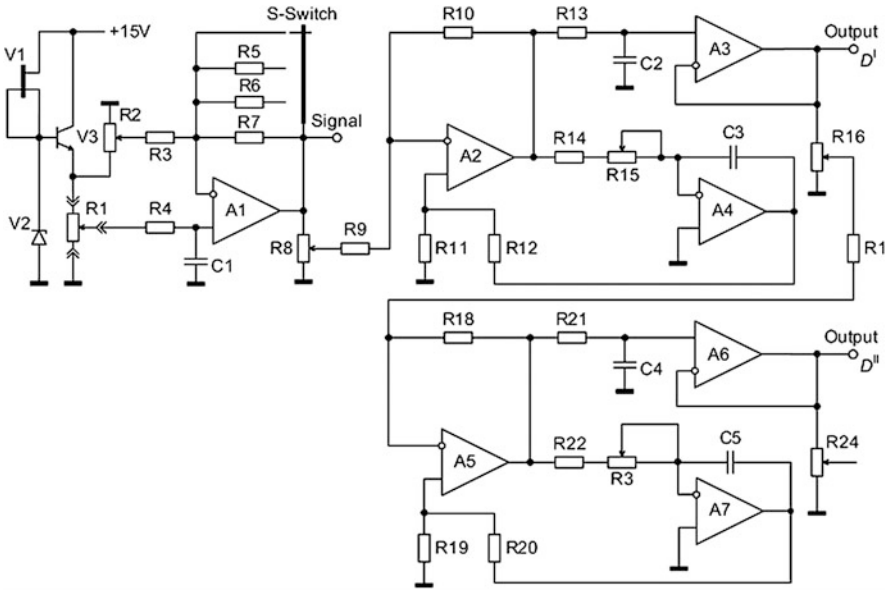

Fig. 3.46 The use of DAP in combination with modifications of *Specord* UV–VIS

connected to the output. The integrator is based on the microcircuit A4 and elements R14, R15, and C3. With the help of the divider R11 ÷ R12, the output of the integrator is connected to the non-inverting entrance of the amplifier A2. This allows the voltage at the output of the amplifier A2 to be proportional to the derivative of the input signal.

Through the integrating circuits R13 and C2, serving for noise decreasing, the D^{I} signal goes to the entrance of the second differentiator. Resistors R8 and 16 regulate the time constant (τ) of differentiation. The amplifiers matching integrating RC circuits with the next loads are assembled in microcircuits A3 and A6.

A reader should remember that the operation of differentiation is combined with some worsening of the signal-to-noise ratio. Therefore, the optimization of this ratio after differentiation can be performed if, instead of integrating chains R13, C2 and R21, R4 shown in Fig. 3.46, we use filters of higher orders.

For better processing of registered information, the functional capabilities of the described device could be enhanced by introduction into its circuit of logarithmic amplifiers or additional differentiating cascades.

Several years later, a diagram of the differentiating attachment on the base of two differentiating amplifiers for the spectrophotometer Specord from the laboratory of the academician N.S. Poluektov (Odessa) was published and used for the analysis of mixtures of samarium and europium (Kucher et al. 1983). Its disadvantages include the obsolete locating features and impossibility of optimizing the differentiation time constant, which decreases sensitivity and the real functional capabilities of the device.

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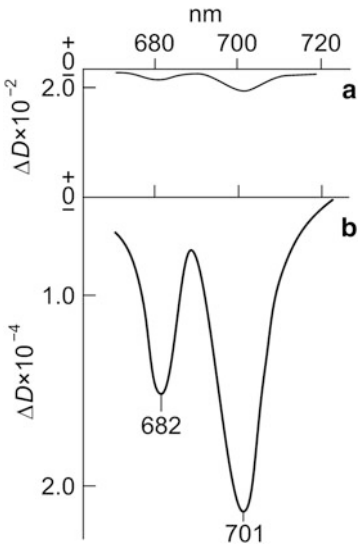

Fig. 3.47 Change in the dynamic range of registration of the difference absorption spectrum ("light minus darkness") for spinach chloroplasts: (**a**) record of the serial spectrophotometer SP-18; (**b**) the same spectrum registered when using a differentiator (Saakov et al. 1987a, b) with turned-off programmed motion

One additional possibility in DA usage, important for the experimenter, should be noted. If we register with the spectrophotometer the signal when playback of the programmed motion is turned off and a differentiator is used in the D^{I} regime, then, when registering an absorption spectrum, it becomes possible to increase the dynamic range by approximately two orders of magnitude (Fig. 3.47). This suggestion can be found in the work of Kaler et al. (1967), but it was not accented enough. Later, this concept was mentioned in another paper (Golovachev 1976), and we reported it in a more final form at several symposia: "Molecular and applied biophysics" in Krasnodar in 1974 (Udovenko et al. 1974); "Plastid apparatus and the resistance of plants" at Leningrad, N.I. Vavilov Research Institute of Plant Industry in 1975; and the "Second all-USSR conference on spectroscopy" in Moscow in 1977 (Saakov et al. 1977). The concept is also described in the collection "Spectroscopic methods in physiology and biochemistry" (published in Leningrad in 1987) and other publications (Saakov et al. 1976, 1987a, b; Saakov and Spotakovskii 1973, Rutman and Saakov 1978).

From the variants of circuits described in literature, it follows that the absence of Russian industrial devices able to record derived spectra forced scientists to find their own equipment designs, improved with the development of the research practice and with achievements of electronic engineering.

This can be compared with each experimenter inventing a bicycle for his own studies, because he does not know all world discoveries in this field and can use only his own financial possibilities. The exceptional separation of researchers in Russia did not allow coordinated development of optimal technical diagrams or the direction of finances for device manufacture at appropriate specialized enterprises.

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3.3.5 *Derivative Spectrophotometry of Difference Spectra*

The considered methods of derived spectra registration using Russian and analogous European instruments have extended the analytical abilities of absorption spectrophotometry and opened up new systematic techniques (Klein and Dratz 1968; Williams and Hager 1970; Whitten et al. 1978; Fell and Smith 1982; Chadburn 1982; Cottrell 1982; Melvin et al. 2002).

The combination of methods of difference and derivative spectrophotometry has proved to be very effective. We proposed a method using Russian recording spectrophotometers and also foreign double-beam instruments with automatic spectrum recording for registration of ΔD^{II} and, if necessary, of higher difference spectra derivatives (Saakov et al. 1973, 1975b, 1987a, b; Mikhailyuk 2003). This method of analysis successfully combines the advantages of difference (see Sect. 3.2.2) and derivative spectrophotometry and allows the recording of the difference spectrum on spectrophotometers with sensitivity below 10^{-3}–10^{-4} optical density units and thus enables the solving of analytical problems that are unsolvable with other devices (Saakov 1971a, b, c, 1987, 2000b, c, d, 2003a, b, c, d Saakov and Hoffmann 1974; Saakov et al. 1976, 2003, 2004).

When using Russian recording spectrophotometers, for the optimum shift of the zero line, it is possible to use a technique of imbalance of the ray flux incident to the photo cell of the instrument. For this purpose, an optical wedge or a set of neutral light filters (e.g., for instruments SP-16, SP-26) or a vertical diaphragm allowing the light flux area to be changed is used. Then, a fixed signal of light flux imbalance will be present at the entrance of an amplifier system of the spectrophotometer and will specify the position of the zero line on the curve tracer of the spectrophotometer.

As the differentiating unit, one of the presented (Rutman and Saakov 1978; Saakov et al. 1987a, b) schemes can be used. The technology of derived difference spectra registration does not differ from the record of derived absorption spectra; however, it requires the strict observance of conditions for difference spectra recording (see Sect. 4.2.2).

The described technique of registration of the second derivative of difference spectra was successfully applied by Japanese researchers for registration of the first derivative of difference spectra of some proteins and aromatic amino acids (Matsushima et al. 1975; Inoue et al. 1975). We draw the reader's attention to this fact because foreign researchers quite often use the methods of Russian scientists without citing their original sources. In practical work, the method of derived spectra registration can be used for very diverse spectral studies (Figs. 3.48 and 3.49) (Bershtein and Kaminskii 1978; Saakov et al. 1978b; Nazarenko et al. 1982; Perfilev et al. 1983a; Skujins 1986a, b; Mishchenko et al. 1987; Talsky 1994).

We used this method for investigation of the spectral discreteness of chlorophyll and hemoglobin molecules, which are almost chemically identical. The discreteness

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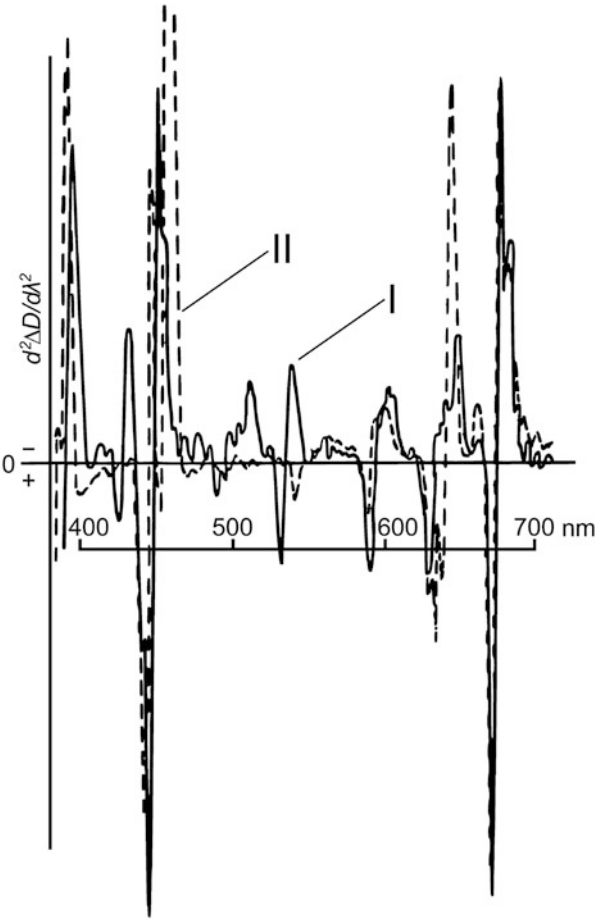

Fig. 3.48 The second derivative of the differential absorption spectrum of solutions: (*I*) pheophytin *a* minus chlorophyll *a* in acetone; (*II*) chlorophyll *b* minus chlorophyll *a* in dioxane

is caused, probably, by a difference in the character of the interaction of pigment molecules with the protein or lipoid complex (Saakov et al. 1973, Saakov et al. 1978a, b). With the help of this method, we discovered in the spectrum of blood the presence of a number of components indicating the spectral heterogeneity of hemoglobin, unnoticeable with registration of usual difference spectra (see Chap. 5).

The registration of the derivative of difference spectrum increases the volume of information about the registered process and allows the detection of insignificant changes in absorption lines unnoticeable with the usual techniques of absorption spectral analysis (Udovenko and Saakov 1976). Examples of the use of the method of derived difference spectra registration is presented in Chaps. 4 and 5.

Summarizing the material discussed in Sects. 3.1–3.3, we hope that the respected reader has gained an insight into the features of derivative spectrophotometry, ways

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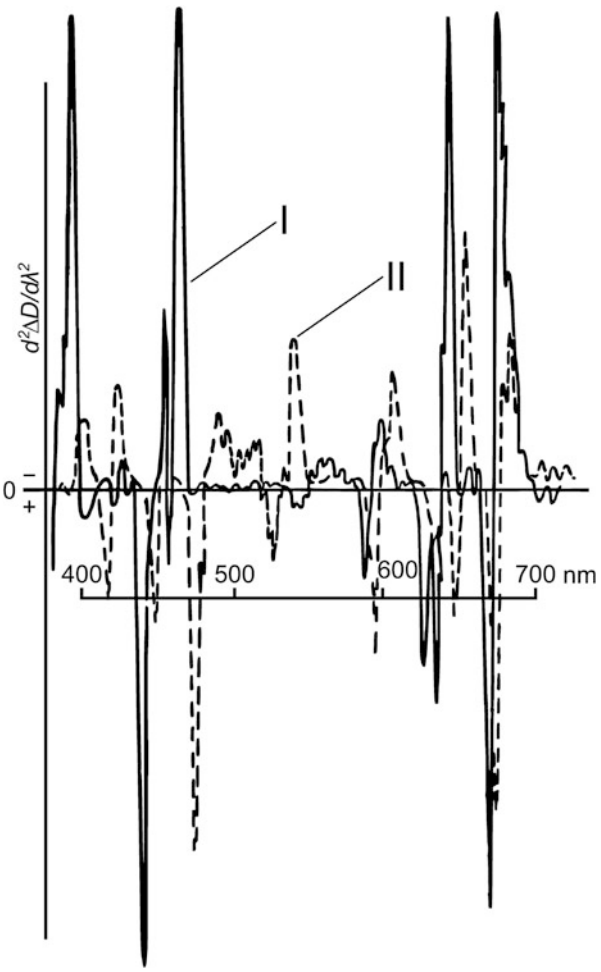

Fig. 3.49 The second derivative of the differential absorption spectrum of solutions: (*I*) chlorophyll *b* minus chlorophyll *a* in acetone; (*II*) pheophytinized total extract of pigments minus green extract in acetone

of obtaining of the derivative signal, possible ranges of application, and the analytical potential of the method in various areas of physical and chemical biology and analytical biochemistry.

However, although obtaining an electronic signal showing the realization of differentiation of the original spectrum is the necessary condition for the realization of the method, it is not enough for the visual assessment of registered processes.

In the following section, we will familiarize the reader with the application of the program package from OriginLab Corporation intended for the visualization of digital data in graphic form.

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3.4 Application of the *Origin* Package for Processing of Numerical Spectra of Biologically Active Substances and Native Structures

General Information on the Origin Package For the last 15 years, *Origin* has been widely used because of the convenience of its application in a scientific experiment. This package represents a tool for processing of mathematical and statistical functions and for creation of plots of these functions, and is also the instrument of data visualization. It integrates with systems of data collection such as LabView, DasyLab, and LabWindows and is compatible with MathCad, MathLab, etc. The detailed description of *Origin* is very large and can be found in the Help section of this package. Here we especially emphasize that we will not attempt to review the full potential possibilities of the *Origin* package, but deem it necessary to speak about the terms and approaches applied by us in using package *OriginPro 8* SRO v.8.0724(B724) for processing and visualization of the numerical data presented in this book. We will try to explain clearly the basic principles and work stages of this convenient program for processing of digital tabular material and its visualization in the form of graphic spectral and chart material. Without detracting from the merits of *Origin* developers, it can be useful to a wide range of readers, especially those who treat arrays of numerical data for analyzing spectral curves. Our recommendations will serve as the necessary basis for the user, who, if desired, can study and apply *Origin* in more depth after reading the corresponding Help section.

Experience of scientific work has shown that disputes and contradictory interpretation in the scientific environment often arise because of terminological disagreements and the ambiguity of their interpretation. Therefore, we will begin by explaining terms used by us. To save space, the empty areas of some windows were cut out; therefore, the proportions of provided menus can differ from the menus opened in the *Origin* package.

3.4.1 Used Terms

The following terms are commonly used:

Interface	A set of tools helping the user to work with various programs and devices.
Project or project file	The file that stores all data and graphs created by the user and logically interrelates them in a whole.
Window	The basic element of a Windows system; a rectangle, the size of which can sometimes be changed and sometimes not (it depends on the type of window). In Windows, all actions are carried out within a window.
Window size management buttons	With the help of these buttons, a window can be minimized, restored or maximized, or closed.

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Scroll bar	The scroll bar appears on the right-hand side or bottom of a window in the case when information is higher or wider than the window size and cannot be viewed completely in a window. With the help of this bar, it is possible to scroll the window content vertically or horizontally.
Toolbar	In each program, there is a toolbar containing buttons for management of the program. Unless specified otherwise, the term "panel" will refer to a toolbar.
Menu	A menu is an element of the graphic interface that allows the user to choose one from several listed actions.
Menu panel	This is a special panel containing the menu providing access to *Origin* features. Unless specified otherwise, the term "menu" is used for a corresponding drop-down menu.
Data window	The data window is an area of the screen performing data visualization. It can differ in appearance (e.g., table, graph, etc.). Unless specified otherwise, the term "window" refers to a data window.
Object	Whole projects and their separate components are known as objects. Almost every object has properties (i.e., parameters), changes to which influence the appearance of an object.
Shot	This is an area of graph bounded by coordinate axes.

Note that the *Origin* program allows work to be carried out not only with the help of a mouse but also by means of a keyboard. Thus, some functions are assigned to hotkeys (combinations of keys on a keyboard that allow various actions to be performed). In the text, any hotkeys corresponding to considered actions will be presented in square brackets.

3.4.2 Interface Description

In Fig. 3.50 it is possible to see the main *Origin* screen showing the basic elements of the program interface. These elements are considered in more detail below:

Window title: The name of an open window and additional information, such as the name of a document opened in this window, are specified here.

Menu panel: Menus providing access to *Origin* program functions are located here.

Toolbar: The main tools applied at work are shown here. Developers of *Origin* chose these tools on the assumption of some universal use of the program. In our work, the default toolbars were sufficient. Thus, the content configuration of these panels is beyond the scope of this description. The same tools can be found in the corresponding menus because icons are identical both for buttons and for menu items.

Working area: The main working area is located under the toolbars. It is divided into various functional zones. Considering these zones from the left of the window to

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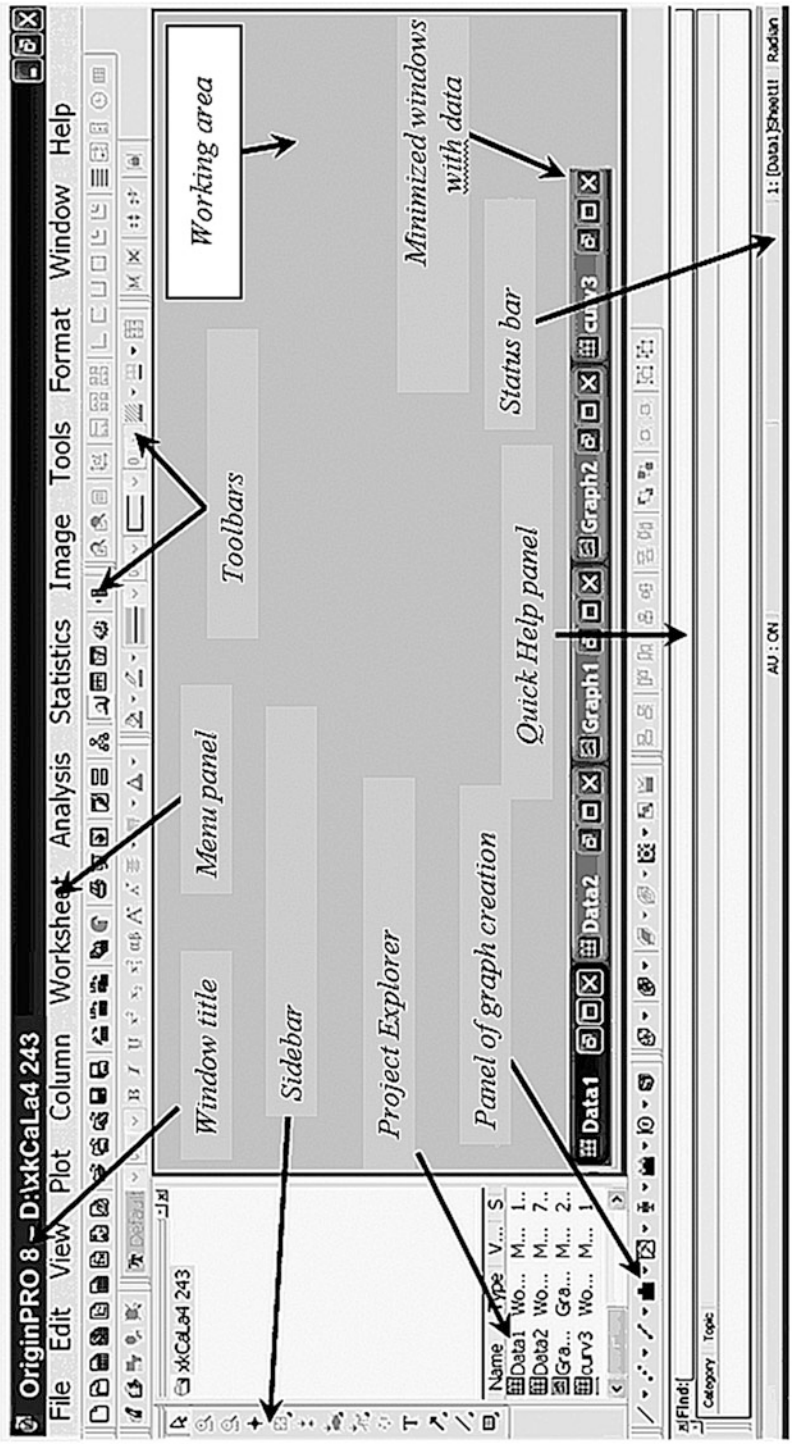

Fig. 3.50 The main *Origin* screen

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the right, it is possible to see the following areas: the *vertical sidebar*, which serves for change of functions with the help of the cursor and is used when working with graphs (its orientation can be changed from vertical to horizontal); the *project review window* is divided into two parts (at the top part of the window is the tree of project folders and at the bottom is the list of objects included in the project, such as tables and graphs); and the *main working area* is a little to the right of these two specified windows, where the content of windows with data and plots can be displayed. Several more windows can be viewed if required, but they were not used by us.

Graph creation panel: This panel is located below the main working area. We will not describe the panel here, because the creation and editing of graphs by means of tools from the *Plot* menu (*Graph creation*) will be discussed in Sect. 3.4.7.1. Under the plot creation panel, it is possible to open the *fast search panel* in the help system (having simultaneously pressed keys Alt and 5 [Alt + 5] or after clicking the right mouse button on working area and choosing (*Quick Help*) from the pop-up menu).

Status panel: The status bar is located at the lower part of the program working window, where auxiliary information is shown (document parameters, the help to menu points; see Fig. 3.50).

3.4.3 Structure of the Menu Panel and Toolbars

It is necessary to emphasize that the structure of the menu panel dynamically changes depending on the current active object. More specifically, when working with a table or a graph, the view of the menu panel is different.

We will first consider the general structure of the panel for a specific operating mode of the program (first, for work with tables, then with graphs), and then we will explain some elements. We emphasize that in this section we only describe the structure of the menu panel. The description of usage of tools suggested for consideration will be presented later. In this context, we will not characterize all possibilities for displaying the program interface toolbars or the corresponding toolbar buttons. However, one possible combination, with the main buttons often necessary in the work, is shown in Fig. 3.50. Some button meanings are presented in Figs. 3.51 and 3.52. The sidebar (see Fig. 3.50) is oriented horizontally in Fig. 3.52.

The choice of content for the toolbar that will appear in the project window can be made in the *Toolbars* tab of the pop-up menu *View* → *Toolbars* [Ctrl + T]. It is necessary to tick the desirable panels (Fig. 3.53a) and to close the menu (*Close*). In another tab of the same pop-up menu, *View* → *Toolbars* → *Button Groups*, it is possible to choose the toolbar buttons. To add a button in the project window, it is necessary to click it with the left mouse button and, without releasing the mouse button, to move the icon to an empty place on the toolbar in the project window (Fig. 3.53b). Then, the mouse button is released.

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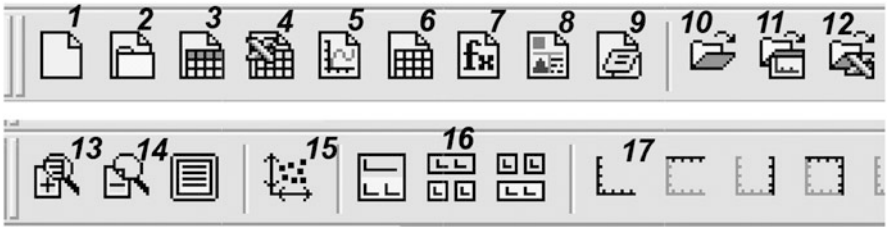

Fig. 3.51 Main icons of toolbars

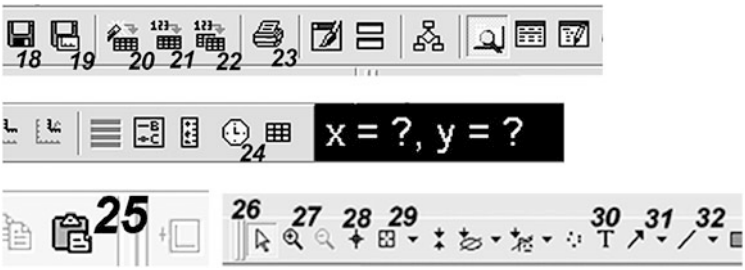

Fig. 3.52 Main icons of toolbars (continuation)

Main Icons
icon N1 *New Project*: creates a new project
icon N2 *New Folder*: creates a new folder in the existing project
icon N3 *New Workbook*: creates a new table for data input
icon N4 *New Excel*: creates a new Excel document
icon N5 *New Graph*: creates a new graph
icon N6 *New Matrix*: creates a new matrix (table)
icon N7 *New Function*: creates a new function
icon N8 *New Layout*: creates the layout where it is possible to group plots and data tables of the project
icon N9 *New Notes*: creates a new explanation or the remark
icon N10 *Open*: opens available documents (projects)
icon N11 *Open Template*: opens existing templates
icon N12 *Open Excel*: opens an existing Excel document
icon N13 *Zoom In*: performs scaling-up when viewing graphs
icon N14 *Zoom Out*: performs scaling-down when viewing graphs
icon N15 *Rescale*: changes the scale of a graph
icon N16 Work with layers and graphs
icon N17 *Add BottomX LeftY Layer*: adds a layer, where the X-axis will be at the bottom and the Y-axis at left
icon N18 *Save Project*: saves the current project
icon N19 *Save Template*: saves the current template
icon N20 *Import Wizard*: importation assistant that helps in data loading

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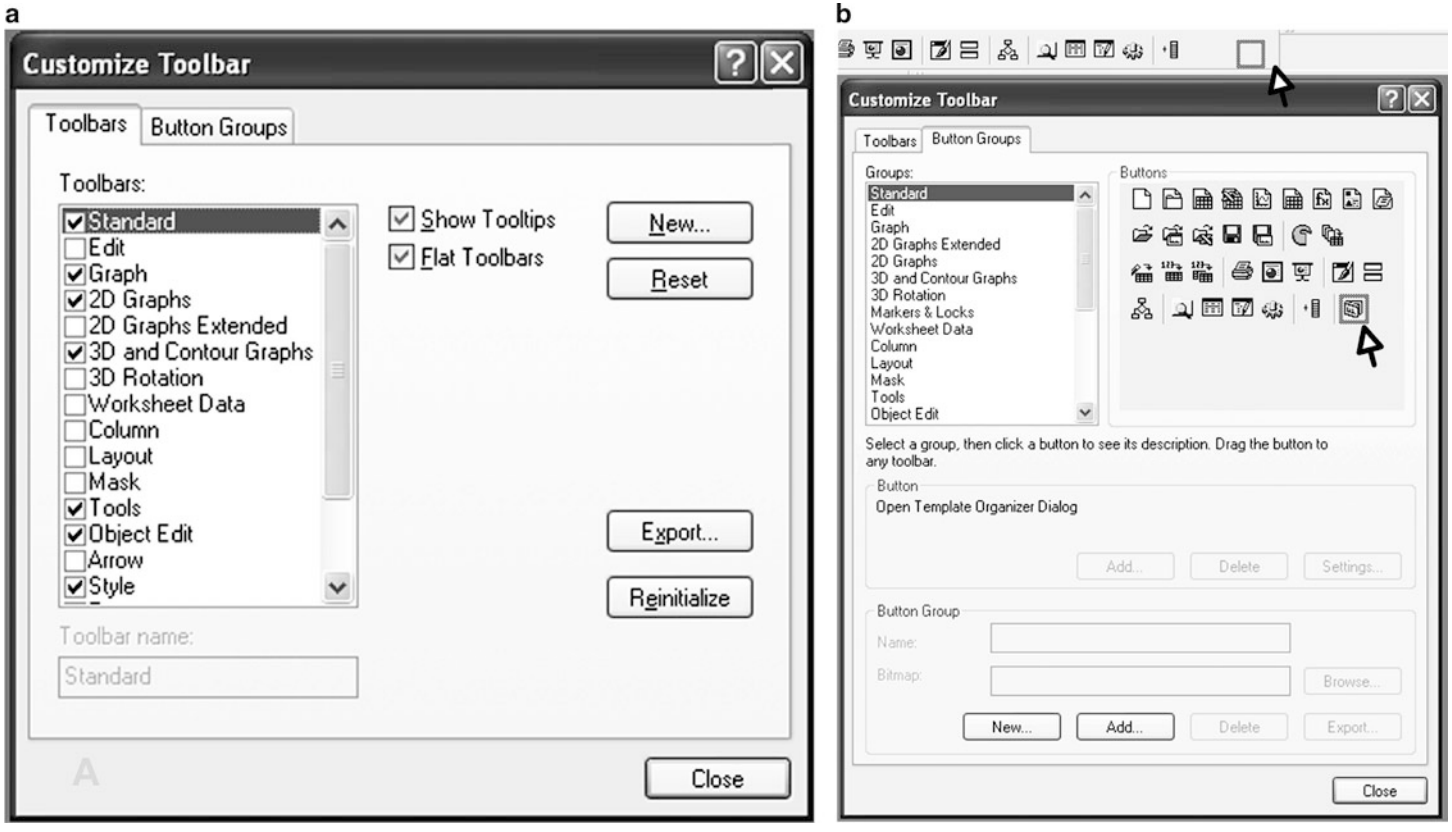

Fig. 3.53 (**a**) The choice of toolbars; (**b**) addition of buttons to the toolbar

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icon N21 *Import Single ASCII*: performs the import of one ASCII file
icon N22 *Import Multiple ASCII*: performs the import of several ASCII files
icon N23 *Print*: prints the project at the printer
icon N24 *Date_Time*: inserts date and time
icon N25 *Paste*: inserts data from the clipboard (an exchange buffer)
icon N26 *Pointer*: switches the cursor into the index mode
icon N27 *Scale In*: switches the cursor into the mode of scale increase, with highlighting of the drawn-around fragment of the axis
icon N28 *Screen Reader*: switches the cursor into the mode of data reading from the graph
icon N29 *Data Reader*: switches the cursor into the mode of view of tabular information associated with a graph point
icon N30 *Text Tool*: inserts a text field on the graph
icon N31 *Arrow Tool*: inserts an arrow on the graph
icon N32 *Line Tool*: inserts a line on the graph

3.4.4 General Description of the Menu Panel

When working with tables, the menu panel consists of the following menus: *File*, *Edit*, *View*, *Plot* (i.e., graph creation), *Column*, *Worksheet* (i.e., data table), *Analysis*, *Statistics*, *Image* (for editing of pictures inserted into *Origin* from files), *Tools*, *Format*, *Window*, and *Help*.

The *File* menu lists the main actions connected with the specific project. For example, saving, loading, printing, and data import/export. The *File* menu contains the following main submenus:

- *New* (i.e., to create a new object) [Ctrl + N]: creates new projects or elements of projects, such as graphs, tables, etc.
- *Open* (i.e., to open an object) [Ctrl + O]: opens the project or its separate components, such as graphs, tables, etc.
- *Open Excel* (i.e., to open Excel tables) [Ctrl + E]: opens the Excel document
- *Close* (i.e., to close the project): closes the current project
- *Save project* [Ctrl + S]: saves the project as a whole. Saving can be done in *Origin* program formats
- *Save project as*: saves the project in *Origin* program formats with additional possibility of its renaming
- *Print:* sends the current object to a printer
- *Print preview*: previews how the object will look after printing
- *Page setup*: opens the printer settings dialogue
- *Import/Export* (i.e., instruments for Import/Export): performs import or export of separate components of the project from documents of other software products (import) or into other documents (export), allowing *Origin* to cooperate with other software (Fig. 3.54)

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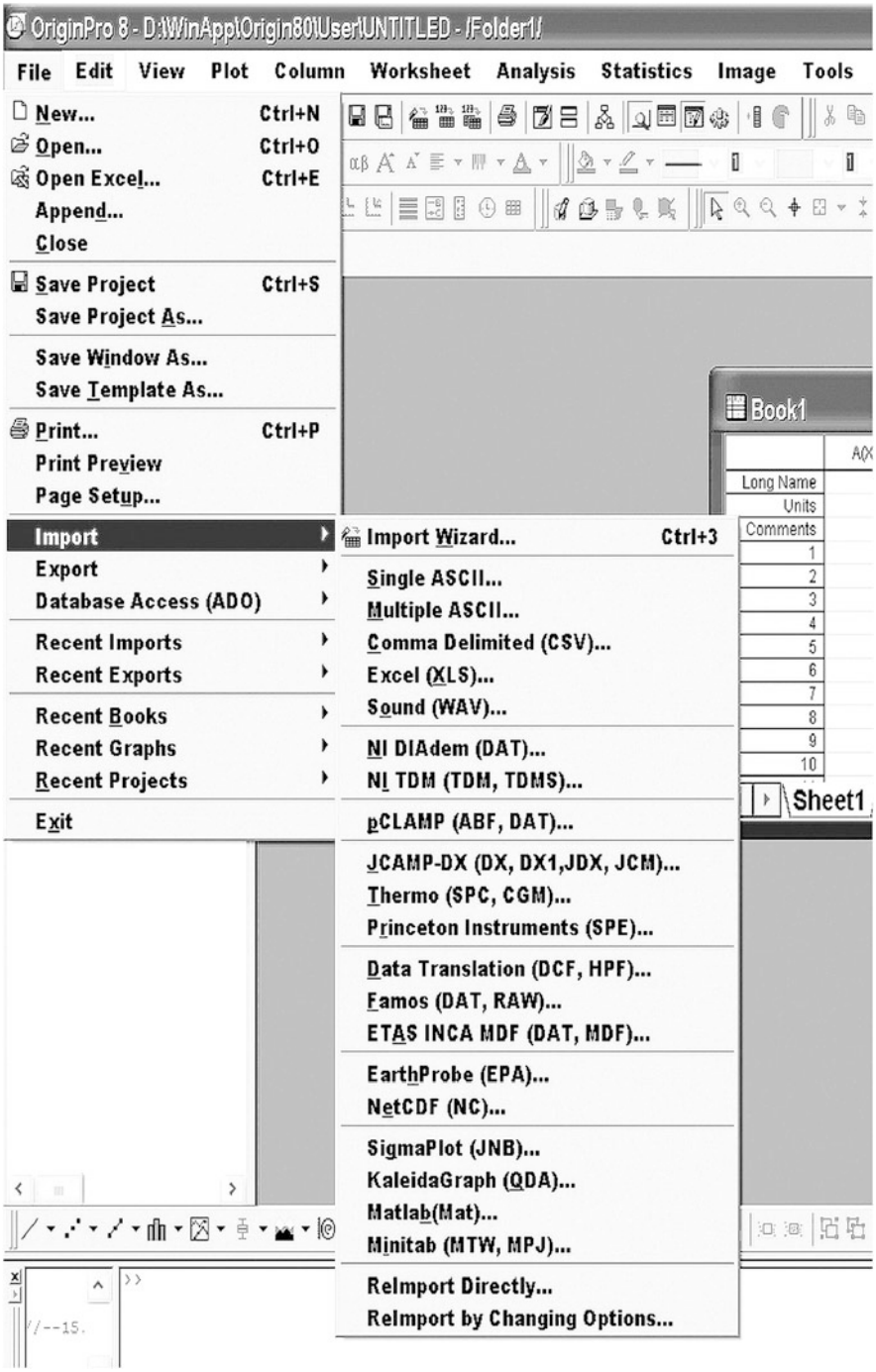

Fig. 3.54 The window demonstrates possibilities of integration of the *Origin* package with other systems of data capture

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- *Recent*... (i.e., recently used objects): contains links to several last projects or objects used in previous work

The *Edit* menu is intended for document editing. In particular, for copying, inserting, cutting, and searching the document. Also, the tabular content visualized as text is meant by "document." The *Edit* menu contains the following main submenus:

- *Undo* (i.e., cancellation) [Ctrl + Z]: cancels the last action; note that this function does not always work correctly; therefore, we recommend saving results (the file) more often.
- *Cut* [Ctrl + X]: cuts the selected text from the document and saves it into the clipboard.
- *Copy* [Ctrl + V]: copies the selected text into the clipboard.
- *Paste*: inserts text from the clipboard.
- *Insert*: adds an empty line (if the line was highlighted in the table), column (when the column was highlighted), or area (for the highlighted area) to the table.
- *Delete*: removes a selected line, column, or highlighted area from the table.
- *Find:* searches for symbols in the table corresponding to the template.
- *Replace*: replaces a number in the table
- *Go to*: jumps to the table cell set by the line and column numbers.

The menu *View*, when working with tables, contains a set of submenus allowing control of the layout of the working window. In the menu are submenus for opening the toolbar control window (*Toolbars*) [Ctrl + T] and submenus for visualization of separate panels: *Status Bar*, *Command window* [Alt + 3], *Quick help* [Alt + 5], *Project explorer* (i.e., the project overview) [Alt + 1], and *Results log* (i.e., a journal of results) [Alt + 2].

The menu *Plot*, when working with tables, gives the possibility to draw graphs according to the selected table columns and contains the following submenus:

- *Line* (i.e., linear): creates a graph, points of which are connected by the line. A user can choose the type of line (continuous, dotted, etc.). Graph points will be not visible.
- *Symbol* (i.e., symbolical): creates a graph, points of which are visible and are not connected by the line. A user can choose which elementary symbol will present points (circle, square, rhombus, etc.).
- *Line + Symbol*: creates a graph in which symbols connected by a line are visible. Technically these three items do not differ from each other; the difference is only how the constructed graph will look.
- *Columns/Bars* (i.e., charts): creates diagrams in the form of columns of identical width, which is inconvenient if the width of columns should be various.
- *Multi Curve:* serves for simultaneous creation of several curves. Includes a number of additional drop-down submenus, some of which help to build several

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coordinate axes (e.g., *Double Y*). *Waterfall* is intended for simultaneous plotting of curves in the same panel (i.e., with common coordinate axes). *Vertical 2* is for the vertical placement of two graph panels in the one picture; *Horizontal 2* is for the horizontal placement of two graph panels in the one picture; *4* is for the horizontal and vertical placements of four graph panels; *9* is for the 3×3 horizontal and vertical placement of nine graph panels; and *Stack* will arrange graphs in different panels, but they will have one common axis.

The menu *Plot* also contains other submenus, apart from the above-specified ones. Examples are the submenus for construction of $3\times$ measured graphs (*3D*), for statistical diagrams (*Statistics*), and for coloring of the area under the graph line (*Area*). A user can explore them if necessary. The majority of these submenus have their own tool possibilities providing the performance of tasks arising before the researcher.

The menu *Column* allows control of the table columns and contains the following main submenus:

- *To establish as X*, *Y*, *Z*: assigns the X, Y, or Z coordinates, correspondingly, to the previously highlighted table column. This option is for the user's convenience when working with the table, because for graph creation in the *graph constructor window* (see Sect. 3.4.7.1), it is possible to specify any columns as any coordinates. If the user previously highlights columns before graph drawing, then the coordinates specified in column names will be chosen as the corresponding values of coordinates on the graph.
- *Set column values:* sets a formula for calculation of column values. This formula can include values in other columns at corresponding lines and/or numbers and functions (see Sect. 3.4.6.1).
- *Add new columns* [Ctrl + D]: provides addition of new columns into the table.
- *Move columns:* carries out automatic moving of columns depending on the chosen command, for example, move a column more to the left, or make it the first column.
- The menu *Worksheet* (i.e., the table) allows operations to be performed on table cells and, among others, contains the following main tools:
- *Sort:* a group of three submenus that carry out sorting/ordering in the highlighted area according to the chosen criterion
- *Clear worksheet:* clears the table completely

The menu *Analysis* groups various tools of the mathematical (e.g., differentiation, integration, smoothing, interpolation, data extrapolation) and the spectral (e.g., Fourier transformation for the data filtration) analyses.

The menu *Statistics* contains tools for statistical analysis.

The menu *Tools* includes various settings, which should not be changed except in case of emergency and a good knowledge of the *Origin* program.

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3.4.4.1 The Menu Panel for Working with Graphs

Above we considered specifics of the toolbar menu for working with *tables*. Now we will underline some features of the toolbar menu change for working with *graphs*.

It is characteristic that for working with graphs (after clicking with the mouse on the graph window), the content of menu options changes. We will briefly consider these changes. As some points were already considered earlier, in this section, only the existence of changes is marked and attention is given only to essentially changed options. The structure of the menu panel for the object "graph" consists of the menus *File*, *Edit*, *View*, *Data*, *Format*, and *Window*.

The menu *File* does not undergo considerable changes.

The menu *Edit* contains the following new submenus:

- *Copy page*: copies the page with a graph from the clipboard. We consider it necessary to warn the reader against the use of this function because when copying a graph (for use in any graphic editor), there is degeneration in the quality of the final image. Only when using *insert* instead of *import*.
- *Copy format*/*Paste format*: two submenus that give the chance to copy/insert style (settings) for decoration of objects in a graph. In our work we did not use these tools and we describe them later only to demonstrate possibilities of the *Origin* program.

The menu *View* changes its content when working with graphs, and the following important new submenus connected with scaling of an image are added:

- *Zoom in* [Crtl + I]: increases an image scale
- *Zoom out* [Crtl + M]: decreases an image scale
- *Whole page* [Crtl + W]: scales object of a current page

The menu *Graph* (when working with a graph material, the menu *Plot* changes to the menu *Graph*) is a new menu that contains instruments for management of, and settings connected with, the creation of graphs. The most important of these are:

- *Plot setup*: opens the window responsible for overall graph control
- *Add plot to layer*: a group of tools for adding the graph or various elements to the layer already available
- *Layer management:* a tool containing settings for layers for the graph
- *Merge graph windows:* creates a picture that is a result of the arrangement on the page of several graphs from combined windows

The menu *Data* contains seven tools, two of which allow correction of points composing the graph, namely:

- Move data points
- Remove bad data points

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The menu *Analysis* includes five submenus for mathematical processing, and each submenu accordingly contains an additional set of submenus.

The menu *Tools* includes 13 positions, and, if necessary, the user can explore these.

The menu *Format* has tabs for editing the properties of the selected (marked) curve on the graph (the tab *Plot*) and of the axis marks (see Figs. 3.68 and 3.75).

The menu *Window* incorporates nine submenus that provide functional management of windows of documents included in the project. The most important of these are:

- *Cascade*: arranges windows as a cascade
- *Tile horizontally*: arranges windows horizontally
- *Tile vertically*: arranges windows vertically
- *Refresh*: updates the window
- *Duplicate*: duplicates the window
- *Properties*: displays the window responsible for settings of the current window

It is necessary to underline that, when working with graphs, the menus specific to tables, such as *Plot*, *Column*, *Worksheet*, *Image*, and *Statistics*, are not displayed on the toolbar.

3.4.5 Techniques of Working with the Software Package Origin for Presentation of Tabular Data

The work in the program *Origin* begins with data input. This can be the manual input of data into table cells, import from a file generated by another software package, or opening of an already available *Origin* file. Because the dialogue window for file opening in the *Origin* project does not differ from the standard dialogue window for file opening in the operating system Windows, we will not discuss it.

It is possible to consider the creation of a new project or a part of a project (e.g., a table) as the work beginning. For creation of a new project, it is necessary to open the menu *File* and to choose the submenu *New*. In the window that appears, the type of created object is chosen, that is, *Project* or *Workbook* (the table). After that, it is necessary to click on the button *OK*. We did not change other options and kept the default settings. It is necessary to note that when *Origin* starts, it creates a new project by default, that is, there is an empty table with $A(X)$ and $B(Y)$ as the titles of columns.

3.4.5.1 Data Import

The data input can be realized using the dialogue window of the *Import* submenu. To bring up this window, you should open the menu *File* and choose the submenu *Import*. After that, in an additional opened menu, it is necessary to choose the format of the file from which the import will be performed or to use the assistant for file

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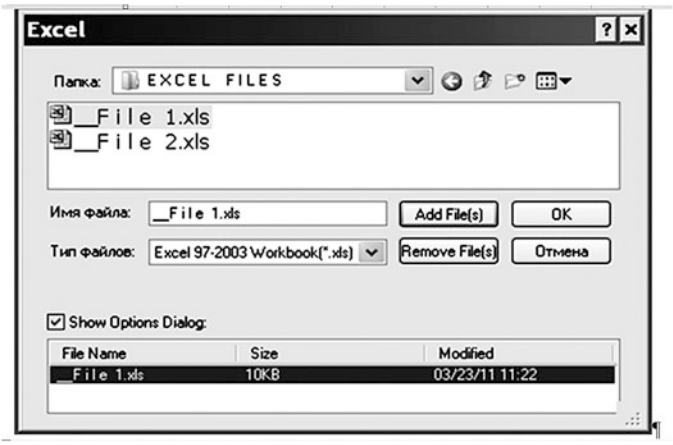

Fig. 3.55 Dialogue window for data import operation

importation. For example, the most widespread formats for import are (the program using the format is specified in brackets) MTW (*Minitab*), xls (*Excel*), mat (*Matlab*), jnb (*SigmaPlot*), and prn (*SpectraCalc*), Single ASCII, and Multiple ASCII (the last two have data in a simple text format, without a link to a specific program) (Fig. 3.55). Note: the abbreviation ASCII refers to American Standard Code for Information Interchange. For the program *Origin*, it is a data format accessible for importation.

Technically, an import is carried out identically for any format. We will consider the import of an Excel file, an example of which is presented in Fig. 3.55.

The field *File Type* allows additional filtration of files displayed in the dialogue panel in accordance not only with a used package but also with their format (some software packages support several associated file formats). The process of import is realized by choosing a file on the top area of the window and pressing the button *Add File*(*s*). After that, the file appears in the bottom part of the window, in the list of files for import. To remove a file from this list, the button *Remove File*(*s*) serves. After the choice of interesting files, it is necessary to press the button *OK*. Differences in the dialogue windows for import of *Single ASCII* and *Multiple ASCII* files are that for the *Single* variant, it is only possible to import one file, and for *Multiple*, several files can be imported simultaneously.

Data highlighting is the main and, probably, the most important working technique. In working with different objects, the process of highlighting can be very different. For example, when working with tables, the highlighting is different to that when editing graph element parameters. Below, how to work with various objects is considered step by step.

We emphasize that many actions are accessible only if a part of an object is highlighted. If marking actions are absent or inaccessible, it is shown by inactive (gray) options in the menu or applied to the whole object.

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3.4.5.2 Working with Tables

Highlighting of Table Elements

The main unit of data storage in *Origin* is the table. Work with a table consists of working with columns, lines, and separate cells or their sets. First, we will consider ways of highlighting table elements.

The simplest way is by mouse click on a table cell. After the click, it is possible to notice that the cell frame is visually displayed as a dark border. If one clicks on another cell, the dark frame goes to it.

For highlighting of more than one cell, it is necessary to mark the cell that will be the beginning of the highlighted area and then continue highlighting keeping the mouse left key pressed. It is also possible to press the *Shift* key and to continue highlighting using the arrows on the keyboard. The most active cell will be white and shows the end of highlighted area. To highlight a whole line or a column, it is necessary to point the cursor to the inside part of the border of the cell defining the line/column and to press the mouse.

To highlight columns that are not adjacent, the key *Ctrl* should be kept pressed, and then columns can be highlighted by clicking the left key of the mouse under their names (when the cursor is on the column name, it changes to an arrow ↓).

To change the width/height of the column/line, it is necessary to move the mouse cursor to the border of two columns/cells and, when the usual arrow changes to double-headed arrow (↕ or ↔), keep the left mouse button pressed and move the cursor in the desired direction.

Addition of Columns to the Table

To add column(s), it is necessary to open the menu *Column* and to choose the submenu *Add New Columns*. In this case, there will appear a window with a field for input of quantity of columns. This window is also reached by simple pressing [Crtl + D]. Note that the column/columns will be added strictly to the right of the current right-hand column in the table. The procedure of addition of columns in the table cannot be cancelled through the menu *Cancellation* [Crtl + Z], but the operation *Removal of columns* serves for this.

To add a column between other columns, it is necessary to highlight one of columns and to choose *Insert* from the drop-down menu by pressing the right key of the mouse. The new column will be inserted left of the highlighted one.

Assignment or Change of a Column Name

In *Origin* the colums are the main associations of cells having the name, and lines have only a numerical identifier their number.

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To change a column name, it is necessary to first click twice on the top cell of the desired column, where the title is set by default as A(X). In the opened window, it is possible to define various options, but we are only interested in the field *Short Name*, since it is used in addressing table elements. In the window *Plot Designation*, it is possible to change the intent of a column (i.e., "X or Y"); then it is necessary to press the button *OK*. The same effect can be achieved by clicking the right mouse button on the column heading and choosing *Properties* in the window that appears.

Column Removal

For column removal, it is necessary to click on its heading using the left button of the mouse (to make sure that the column is highlighted), to press on the highlighted area using the right button of the mouse, and to choose the option *Delete*. In the same way (clicking on the highlighted area with the right mouse button and choosing the option *Delete*), it is also possible to remove any highlighted area in the table. If key *Delete* on the keyboard is pressed, the values entered in highlighted cells will disappear.

Moving of Columns

For column moving, it is necessary to click on a column heading using the left mouse button (to make sure that the column is highlighted), to click on the highlighted area using the right mouse button, to choose the option *Move Column*, and in the next menu to select how and where to move: *Move to first*, *Move to last*, *Move left*, or *Move right*.

Copying and Insertion of Data

For data copying, it is necessary at least to highlight the copied block of data. After highlighting, it is necessary to directly perform the action of copying. This action can be done in several ways:

- Open the menu *Edit* and choose the option *Copy*.
- Click using the right mouse button on the highlighted data block and choose *Copy*.
- Having the already highlighted data block, press the combination [Crtl + C].

For the data insert, the actions are similar to copying, but instead of highlighting the data block, it is necessary to place the frame of the active cell on that cell where the insert should be made. Accordingly, in the menu it is necessary to choose the option *Insert* and in case of hotkeys [Crtl + V].

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Adjustment of the Text Format in Cells

For the adjustment of the text format/view in table cells, it is necessary to execute the following actions:

1. Highlight the necessary block of cells.
2. Click on it using the right mouse button.
3. Choose the option *Format Cells* in the drop-down menu.

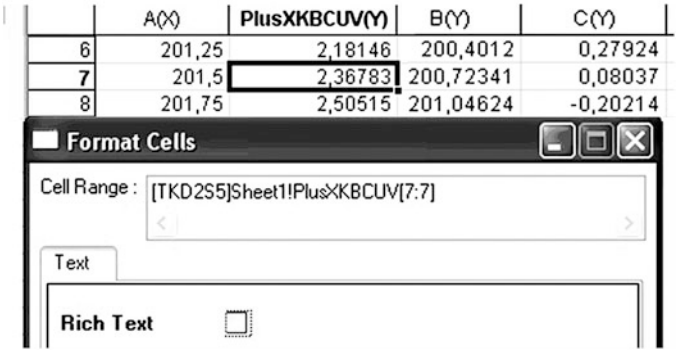

Fig. 3.56 Dialogue window for cell addressing

As a result of these actions, a window containing the following main fields should open:

- *Cell Range:* the information field (without editing possibility) serving for the display of cells that will undergo formatting. The format of cell addressing is presented in the corresponding item (see Sect. 3.4.5.3 below).
- *Rich Text:* activates the possibility of expanded formatting of a text in a cell (e.g., changing font). We did not use this parameter, although the expanded formatting of a text can give better presentation.
- *Format*: this parameter defines how *Origin* should consider and process data in a cell, e.g., text, number, time, date. The reader can choose the needed representation, since it is intuitively clear from the list of variants.
- *Display:* directly defines the way that the data is displayed, according to various standards.
- *Font* and *Font size:* changes the font and font size.

3.4.5.3 The Format of the Address of a Cell or Cell Range

First we will consider the format of one cell address and then of a range of cells

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The Format of the Cell Address

The cell address is given by [Table name]Sheet*!X1 [Y1:Y2] where:

Table name is the table name included in the project. It is desirable to use only Latin letters and figures.

The asterisk symbol (*) designates the sheet number in the table. Most often it is "1."

X1 is the name of the column containing the cell.

Y1 is the number of the line corresponding to the range beginning.

Y2 is the number of the line corresponding to the range end.

In the case of one cell, obviously, Y1 always equals Y2.

Note that the "Sheet" address and the exclamation mark (!) are special identifiers and should not be manually modified. For the example shown in Fig. 3.56, the cell address is:

[TKD2S5]Sheet1!PlusXKBCUV[7:7]

where TKD2S5 is the table name; PlusXKBCUV is the column name; and [7:7] means that the beginning and end of the range are equal to the same value of 7. Thus, in the present example, the cell № 7 of the column PlusXKBCUV of the first sheet of the table TKD2S5 is addressed (Fig. 3.56).

The Format of the Range Address

In this case, we will consider a variant with three highlighted areas (highlighting of additional areas is realized by using the key *Ctrl*; otherwise the previous highlighting will be cancelled each time!). For increased clarity, we will describe a concrete example:

[TKD2S5]Sheet1!(A[2]:PlusXKBCUV[5],A[10:14],B[19]:D[19])

As we see, some elements remain the same: The table name is still in square brackets, and the sheet number (1) is still placed after the word "Sheet." Now we will note the changes. The first is the appearance of parentheses after the exclamation mark and at the end of the record. They specify that everything within them is part of one sheet of one table. Otherwise, the area marked with brackets would be in another place of the line. The second important difference from the previous example of a single interval (in particular, the cell) is that the highlighted areas (or separate cells) are separated by commas. Thus, we have the following ranges:

- A[2]:PlusXKBCUV[5]: this record says that we have the range beginning from the cell A[2] (top left corner) and ending at the cell PlusXKBCUV[5] (right lower corner). As written above, it corresponds to the second cell in column A and the fifth cell in column PlusXKBCUV. In this case we have a rectangular area indicated by the limiting cells on its diagonal.
- A[10:14]: this record specifies that we have the range beginning with the cell A [10] and ending at the cell A[14]. Note that in this case the range represents a fragment of column A.
- B[19]:D[19]: the record shows that we have the range beginning with the cell B [19] and ending at the cell D[19]. In this case we have a fragment of line №19.

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3.4.6 Some Allowed Mathematical Operations

Differentiation
For differentiation, it is necessary to carry out the following actions:

1. Highlight the table fragment. For this, it is necessary to highlight a minimum of two ranges with respect to which differentiation will be performed.
2. Open the menu *Analysis*, change to the submenu *Mathematics*, and then click on the submenu *Differentiate*. The window showing the parameters of operation of differentiation should open.
3. In this window, make sure that the correct ranges are indicated in the field *Input* and that the field *Output* designates the column in which the result of differentiation should be written. To choose the column (or the other interval) for the result output, click on the square gray button with the black right arrow located to the right of the field *Output*. In the drop-down menu, choose the column and press the button *OK* to perform the operation.

The differentiation order is set in the field *Derivative order*. Mostly, we did not use the *Smooth* options.

It is possible in the window of options of differentiation to set the intervals manually; however, these actions require very good knowledge of how to address table elements and the risk of an error is great.

Figure 3.57 presents an example of the differentiation. Clearly visible are the columns highlighted and selected for differentiation and all described fields of parameters of differentiation. Note that in this case the result of the performed operation will be written in the right-hand column B(Y).

Performance of Mathematical Operations on Columns
It is often necessary to perform mathematical transformations on data in columns. For this purpose, it is necessary to execute the following actions:

1. Highlight the column where the result will be written.
2. Place the mouse on the dark area and click the right button. In the menu that appears, choose *Set column values*. As a result, the formula designer window should open (Fig. 3.58).

For example, for conversion of wavenumbers in reciprocal centimeters to wavelengths in nanometers, it is necessary to enter the formula 1/Col (A)*10^7, where Col(A) is the column containing values of wavenumbers. In this case, the window of the formula designer will open as shown in Fig. 3.59.

Further, for example, to calculate the square root [sqrt()] of the sum of cells of columns A(X) and C(Y) at each line and write the result in column B (Y), the entry is sqrt(Col(A) + Col(C)), as presented in Fig. 3.60.

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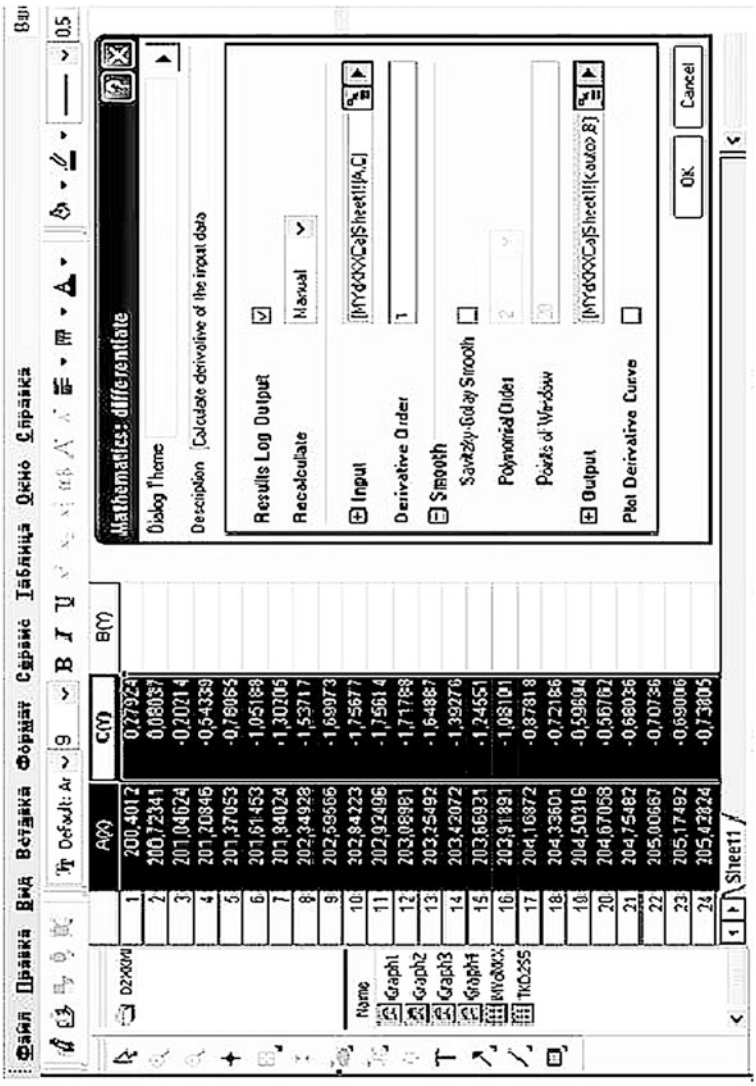

Fig. 3.57 Dialogue window for the differentiation operation

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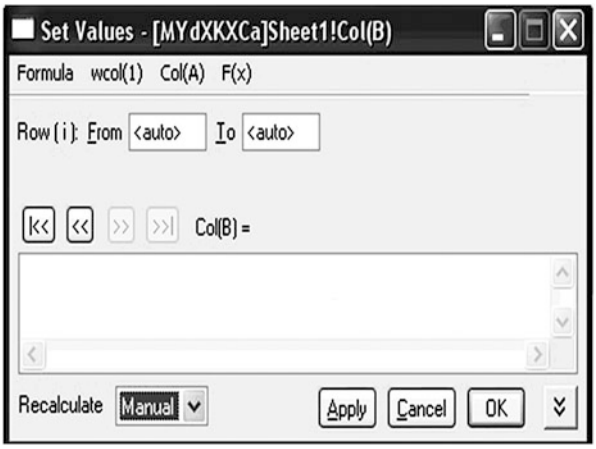

Fig. 3.58 General view of the formula designer window

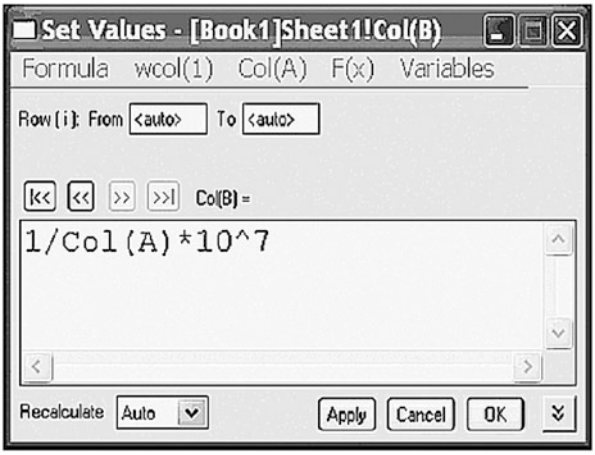

Fig. 3.59 Example of the formula for wavenumber conversion to wavelengths in nanometers

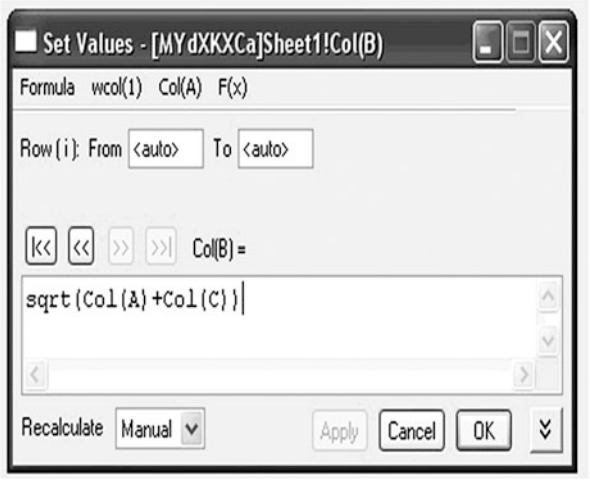

Fig. 3.60 Dialogue window for the general view of a formula example in the formula designer

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Integration

For integration, it is necessary to execute the following actions:

1. As for differentiation, first choose the columns on which the operation will be performed.
2. Open the menu *Analysis* and change to the submenu *Mathematics* and then to the submenu *Integrate*. The window with integration operation parameters should open, as in Fig. 3.61.
3. In this window, make sure that the correct ranges are indicated in the field *Input* and that the field *Output* designates the column in which the result of differentiation should be written. To choose the column (or the other interval) for the

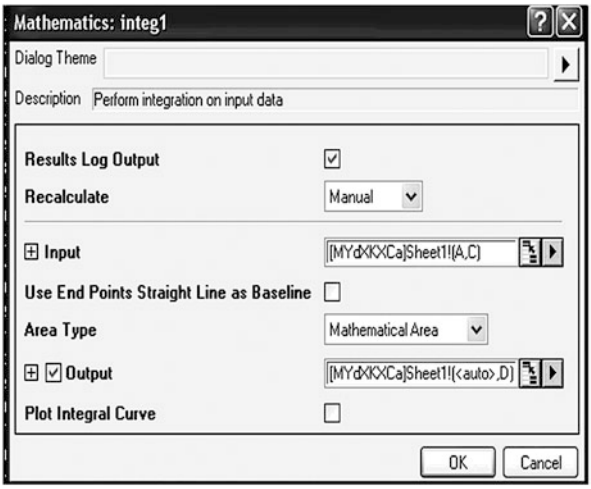

Fig. 3.61 Example of the dialogue window for the integration operation

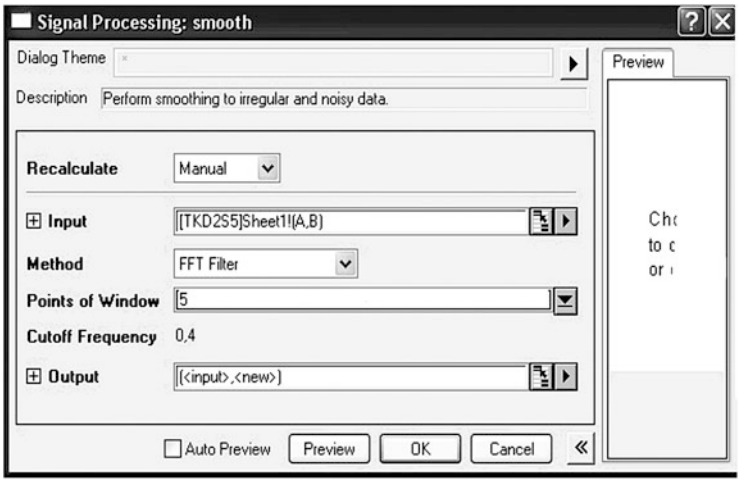

Fig. 3.62 Dialogue window for smoothing the numerical data of graph curves

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result output, click on the square gray button with the black right arrow located to the right of the field *Output*. In the drop-down menu, choose the column and press the button *OK* to perform the operation. We did not change additional options set by default.

Smoothing
To perform the smoothing, it is necessary to open the menu *Analysis* and to choose the submenu *Signal processing*. In the menu that appears, it is necessary to choose the submenu *Smooth*. It is important to pay attention to the number of points used for smoothing. Five points are set by default, but sometimes it is expedient to increase their number 2–3 times according to the contour of a graph curve. The dialogue window of smoothing is presented in Fig. 3.62.

Consider the fields of this dialogue window: *Input* is the address of the range of cells for smoothing; *Method* is the smoothing algorithm. In our case, the algorithm of fast Fourier transformation (FFT) is chosen; *Points of window* is the quantity of points for smoothing; *Output* is the range in which the result will be written; the button *Preview* will show the preliminary result of the operation in a window on the right.

3.4.6.1 Construction and Work with Graphs of the Type "Waterfall"

Graphs constructed in the mode *Waterfall* are very convenient for the comparison of several data sets collected in similar or identical conditions. In *Waterfall* it is possible to change the viewing angle and color of curves and to set for convenience the horizontal and vertical displacement of curves.

In the project table, it is necessary to highlight columns containing numerical data, one column for (X) and several columns for (Y). Next, in the menu *Plot*, choose the submenu *MultiCurve* and then the submenu *Waterfall*. On the display, there will appear the graph *Waterfall* (Fig. 3.63), on which the displacement of curves will be set automatically.

If necessary, to change a step of an axial displacement of graph curves along axis (X) or (Y), it is required to open the window *Plot Details* by a double click of the left mouse button on any curve and to choose *Layer* on the top left corner of the *Plot Details* window. As a result, a new *Plot Details* window will appear (Fig. 3.64), in the right field of which are the tabs *Background*, *Size/Speed*, *Display*, and *Waterfall*. After pressing the tab *Waterfall*, the windows *X Offset* and *Y Offset* will be opened, where the necessary step of the curve displacement along (X) or (Y) can be defined. The entered numbers are not the absolute step of displacement, although the *Origin* description is silent on how these numbers are recalculated in the displacement step.

If the displacement is not required, that is, if curves have to correspond to the table data, it is necessary to specify X Offset = 0 and Y Offset = 0.

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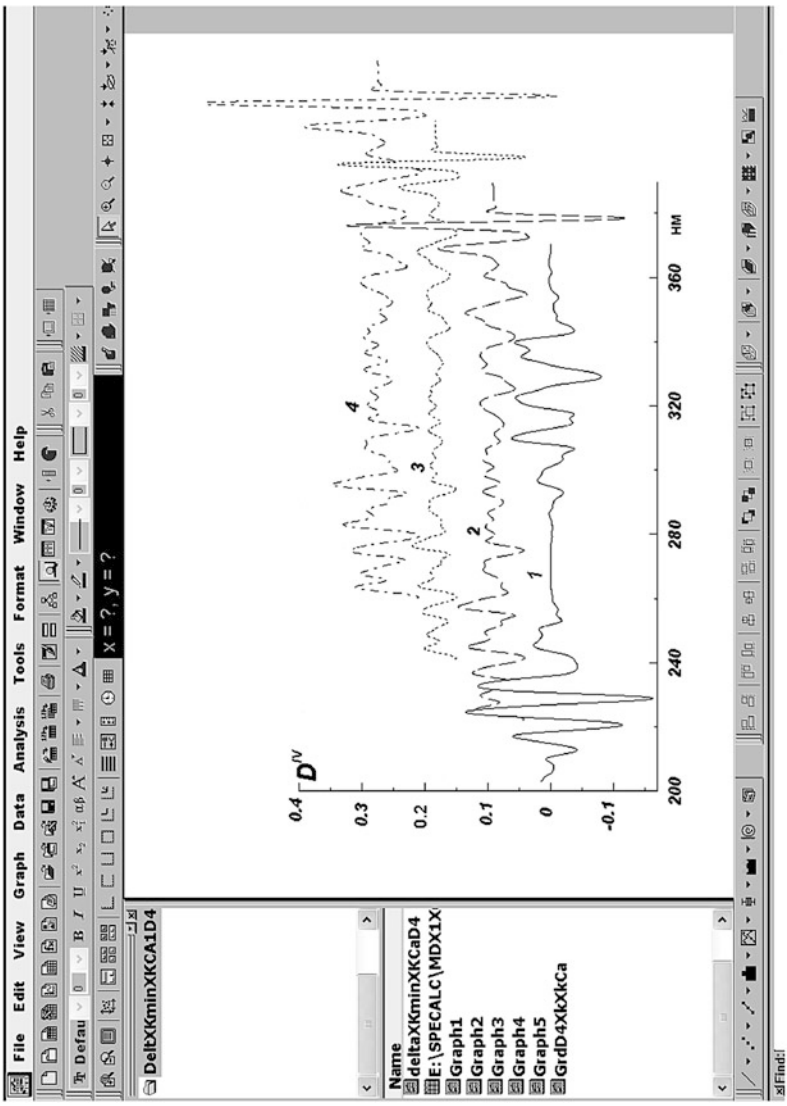

Fig. 3.63 Example of a *Waterfall* graph

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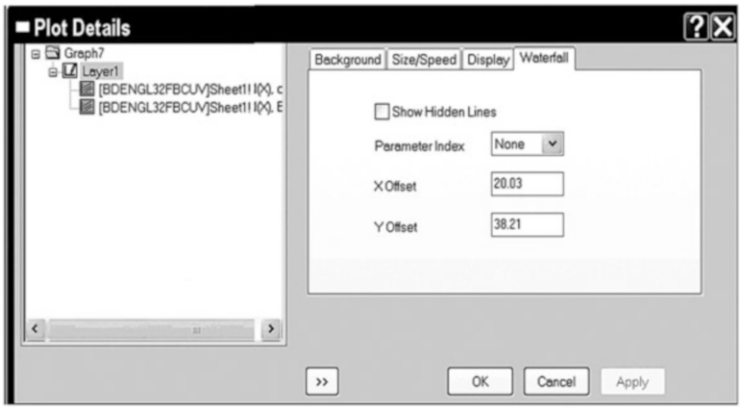

Fig. 3.64 The adjustment of properties for the displacement step for curves of the *Waterfall* graph

It is possible to look at the changed graph without closing the window, having pressed the button *Apply*. To make changes and simultaneously to close the window, it is necessary to choose *OK*.

In order to change the viewing angle, which will correspond to the change in a displacement step, it is necessary to click twice on the graph. After the first click, diamonds will appear that allow one to compress or stretch the graph by pulling them. After the second click, there will appear triangles, with which it is possible to change the viewing angle by pulling them.

To reverse the order of curves, it is necessary to click twice on any axis with the left mouse button and to choose on the left part of the opened menu the axis *Z*, *Z Axes*, and to interchange values in lines *From* and *To*, and then to press *OK*.

Note that if one does not choose *Layer* in the left part of the *Plot Details* window, but instead places the cursor lower (i.e., on the name of any curve), then in the tab *Group* in the section *Edit Mode*, there will be available two circles with the legends *Independent* and *Dependent*. This means that, depending on the choice of option, the properties of the curves of the graph will change, either for one curve individually or for all presented curves simultaneously.

The properties of lines, symbols, and colors of curves are set in the window *Plot Details* in tabs *Line*, *Symbol*, and *Group* (Fig. 3.65 and Fig. 3.66), and their adjustment coincides with that for curves of usual graphs (Fig. 3.67).

In order to change the graph, for example, from linear to linear–symbolic, it is sufficient to place the cursor on the necessary curve and to choose the desirable type from the drop-down menu *Change Plot to* of the right mouse button. The window for adjustment of graph properties will appear as in Fig. 3.65. If the graph is linear, the window will be as shown in Fig. 3.66.

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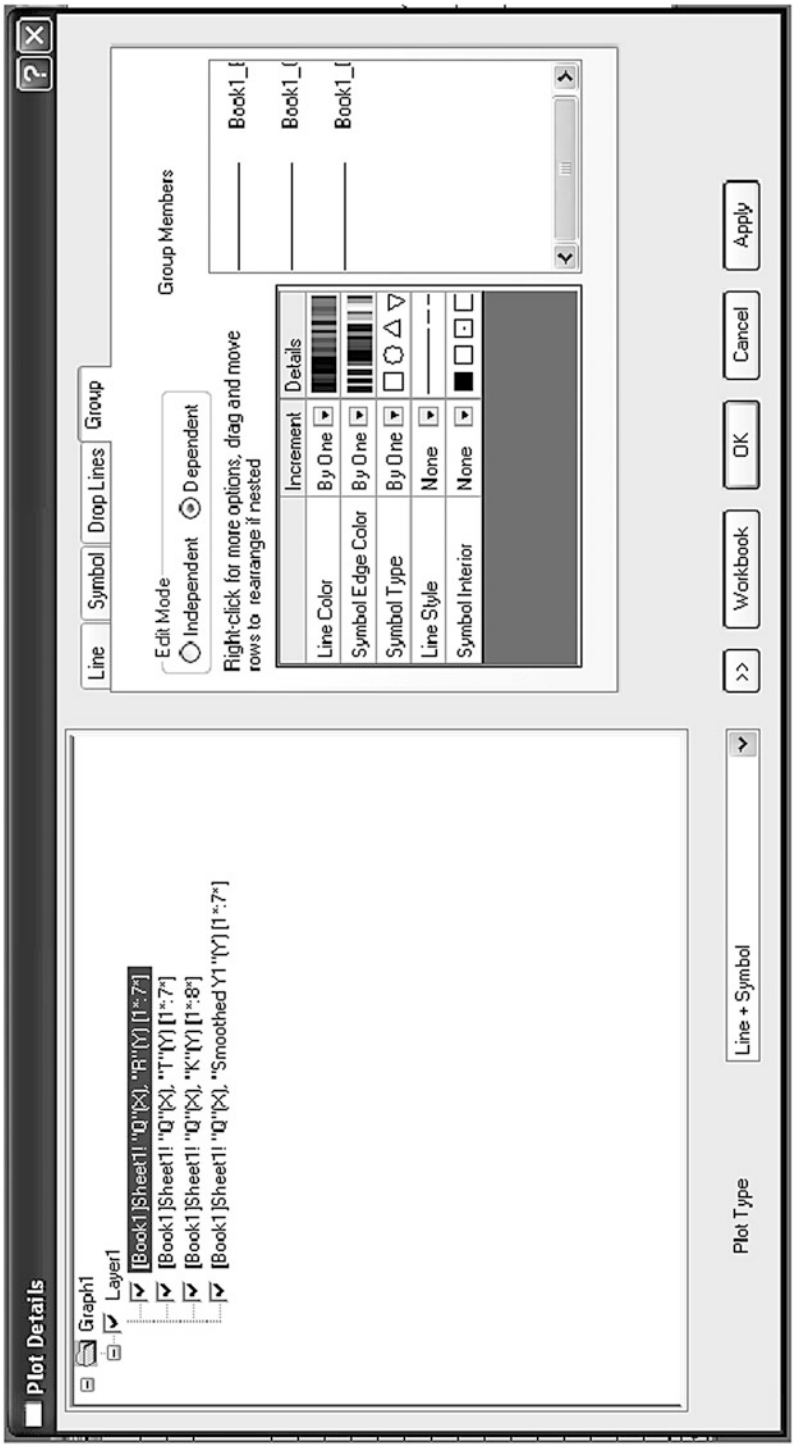

Fig. 3.65 Dialogue window for the adjustment of properties for the *Waterfall* graph

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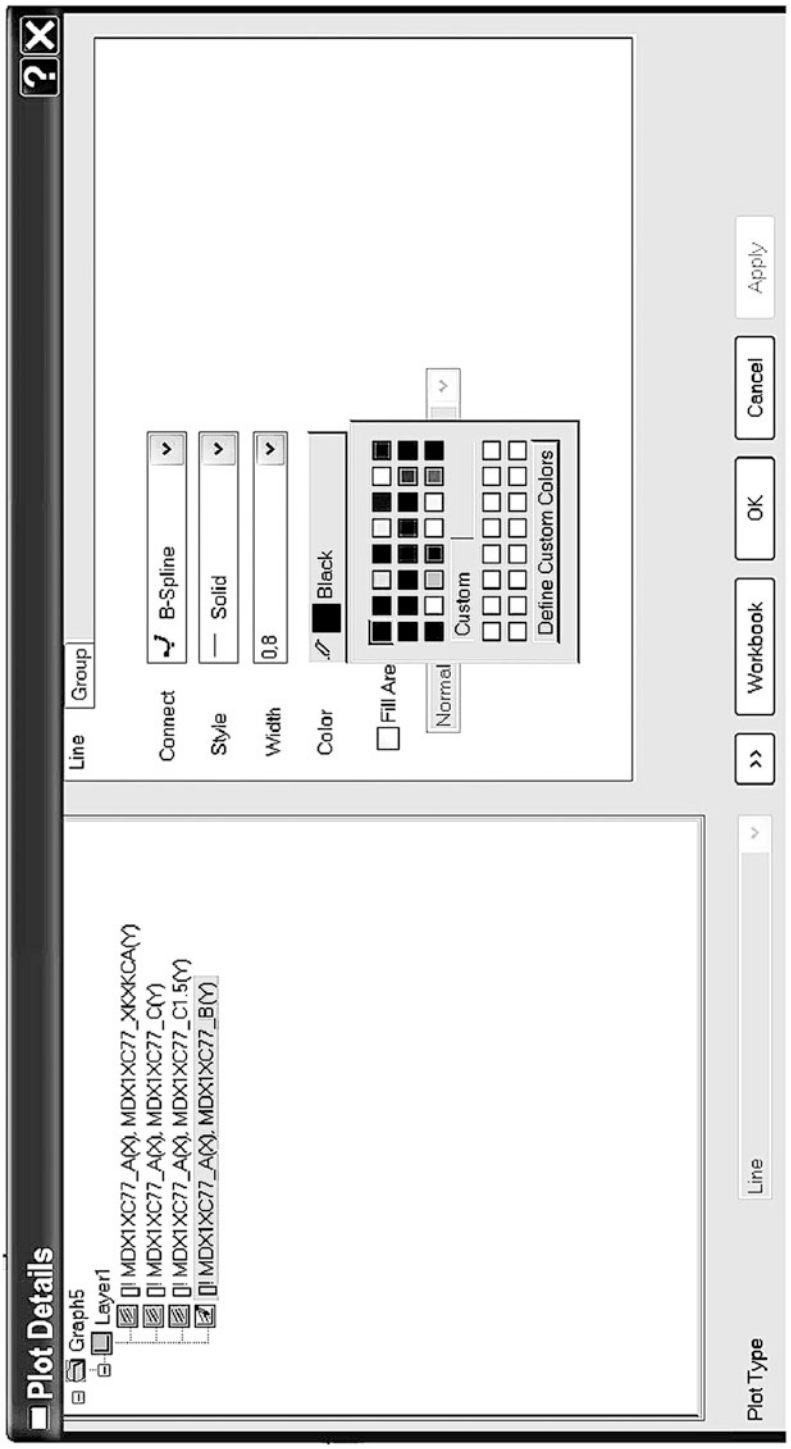

Fig. 3.66 Window for the adjustment of properties for curves of the *Waterfall* graph

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3.4.7 Working with Linear Graphs

3.4.7.1 Graph Construction and Curve Editing

Graph Construction

To create a graph, it is necessary to open the menu *Plot* and choose the necessary type of graph from available submenus. In principle, the way of construction of different graphs is the same; therefore, we describe the universal way. If columns X and Y were not highlighted in the data table, then after the choice of graph

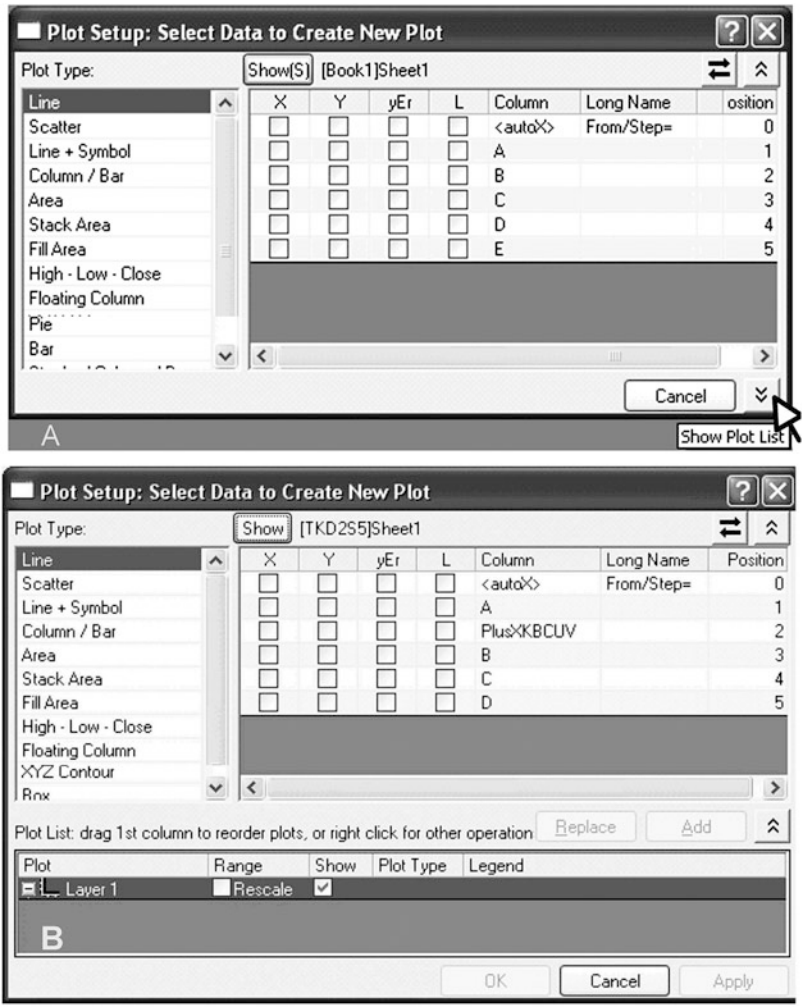

Fig. 3.67 Dialogue window for the graph designer: (**a**) before opening of the additional panel and (**b**) after

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type, the graph designer window will open, as in Fig. 3.67a. (It is necessary to press the bottom right button with the picture of double-down arrows *Show Plot List* to open the additional information panel!). In versions *OriginPro* 8 and *OriginPro* 8.1 (and newer versions), the windows shown in Fig. 3.67 can differ in details.

In the left part of the figure, there is a vertical panel defining the type of created graph. In the table located on the top part of the window, it is necessary to set which column will be the coordinate of the graph. The choice is carried out by ticking the proper boxes; then, the button *Add* will become active. Pressing *Add* will add the graph to the current layer (at graph creation, there will be a single layer). Then it is necessary to press the button *OK*, and the new graph will be created as a new object and at once displayed on the screen.

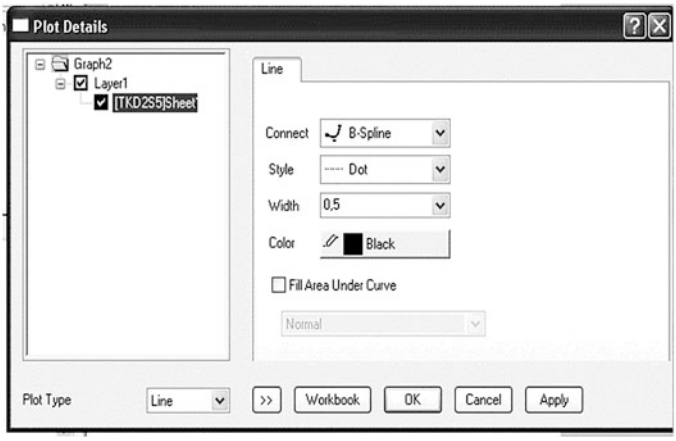

Fig. 3.68 Dialogue window for setting the properties of the graph curves

Adjustment of Graph Properties

As for almost any object of the project, the graph possesses a set of properties that influence its layout. Properties can be grouped in their belonging to various objects of the graph. The general way to view the *Plot Details* window is by double clicking the left mouse button on the graph curve interesting for you. These options allow choice of color, symbol, and thickness of a line (Figs. 3.68 and 3.69).

Adjustment of Plot Line Properties

In Fig. 3.68 it is possible to see the window for adjusting the properties of the line connecting points of a linear graph. The parameter *Connect* defines the way of connecting graph points. Most often we used "B-spline," which is more convenient for curves and allows a smoothed line of the graph. If the parameter *Straight* is chosen, the curve will have corners and look like a zigzag. The parameter *Style* defines the line type (points, a dotted line, a continuous line, etc.). *Width* sets the thickness of the graph line; *Color* sets the color of lines.

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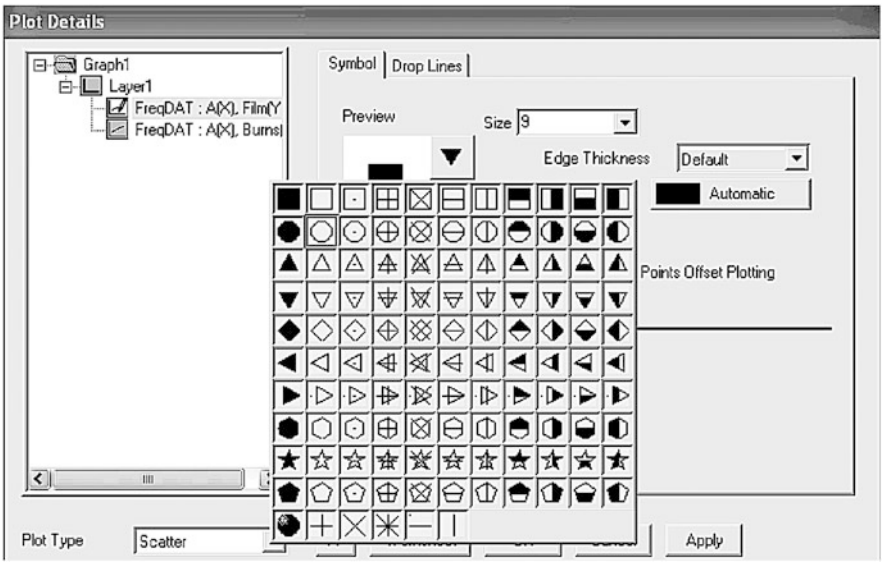

Fig. 3.69 Dialogue window for the adjustment of symbol elements for the graph

The parameter *Fill Area Under Curve* paints the area under the graph in a definite way (at activation of this adjustment, it is possible to see the sample of filling on the right).

For the symbolic and linear–symbolic graphs, the parameter *Symbol* opens the table of symbols and allows choice of the type of points on the graph (Fig. 3.69).

To see the changed graph without closing the window, it is necessary to press the button *Apply*. For graph modification with simultaneous closing of the window, it is necessary to choose *OK*.

Creation of Labels (Legends) on the Graph

For this purpose, the *Text* tool located on the left sidebar (Fig. 3.50) is used. The button looks like the capital letter "T" (Fig. 3.52, icon 30). To create a label, it is necessary to press this button (the cursor should change to the stylized letter *T*) and to press on a place in the graph. The input for the label text will appear in that the area.

Setting of Legend Properties

The legend properties window can be opened by a single click on the legend (if it already is present) with the left mouse button and the next click by the right mouse button above the highlighted area. In the menu that appears, it is necessary to choose the option *Properties*; then the *Object Properties* window will appear (Fig. 3.70). In this window, it is possible to set the typeface, the text of the legend, its background, and its inclination. For an angle of slope, the parameter *Rotate degree* can be used. It is set in degrees.

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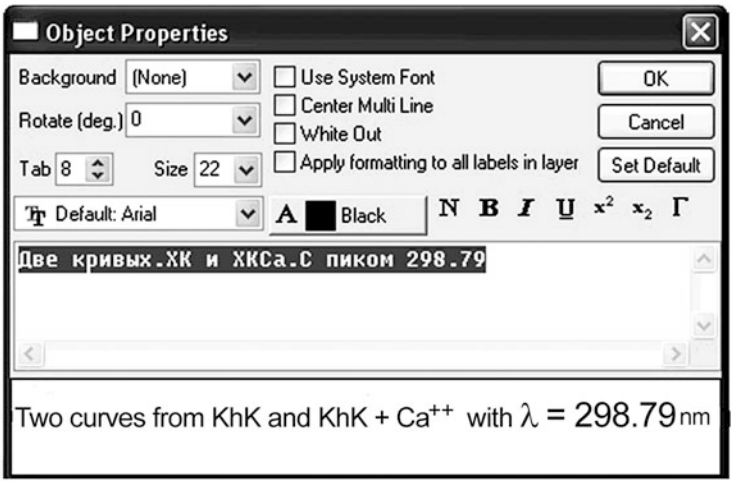

Fig. 3.70 Dialogue window for editing legend properties

The parameter *Apply formatting to all labels in layer* sets the formatting to all labels on the graph, and the button *Set default* will make the defined formatting as default for all new legends.

3.4.7.2 Setting of Properties and Titles of Graph Axes

The window for adjustment of axes properties is obtained by a double click on an axis (any) and consists of the following tabs: *Scale*, *Title and Format*, *Grid Lines*, *Break* (of the axis), *Tick Labels*, *Minor Tick Labels*, and *Custom Tick Labels* (additional options of labels). Note that the change in some properties of the graph, including ordinates (of axes), ticks, and legends, can be opened in submenus *Axes*, *Axis Tick Labels*, and *Axis Titles* in the menu *Format*.

The tab *Scale* (Fig. 3.71) is for setting the scale properties. There are the following main options: The choice of axis is made from the list shown in *Selection*; *From* and *To* give the initial and final values of the scale range; *Increment*; and *Type* (linear or logarithmic scale).

The tab *Title & Format* (Fig. 3.72) shows in the left sidebar, as in the previous tab, the type of the axis for which options are open for correction. The parameter *Title* is responsible for the name displayed near to the axis. Sometimes it is necessary to put ticks inside or outside of the graph shot. The orientation of *Major Ticks* and, accordingly, of *Minor Ticks* in or out of axes is set in the corresponding fields. *Major Tick Length* defines the length of long ticks. The length of small ones is strictly connected with the length of major ticks. The color of the chosen axis is set by *Color*. Other parameters on this tab we left unchanged. The appearance of the window for definition of axes parameters is presented in Fig. 3.72.

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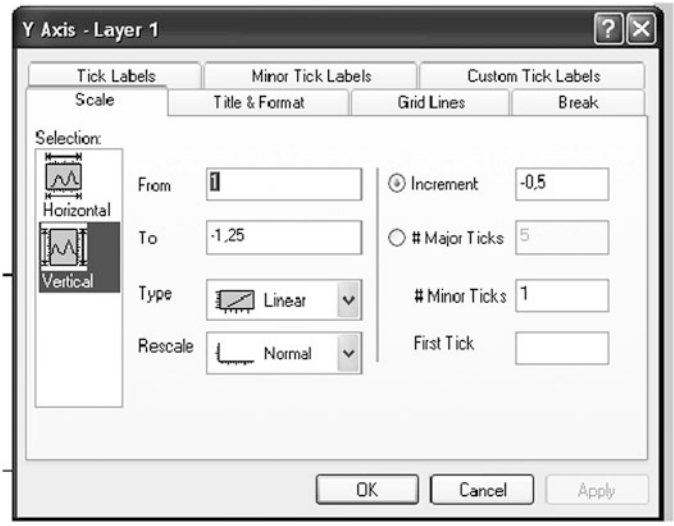

Fig. 3.71 Tab for setting scale properties

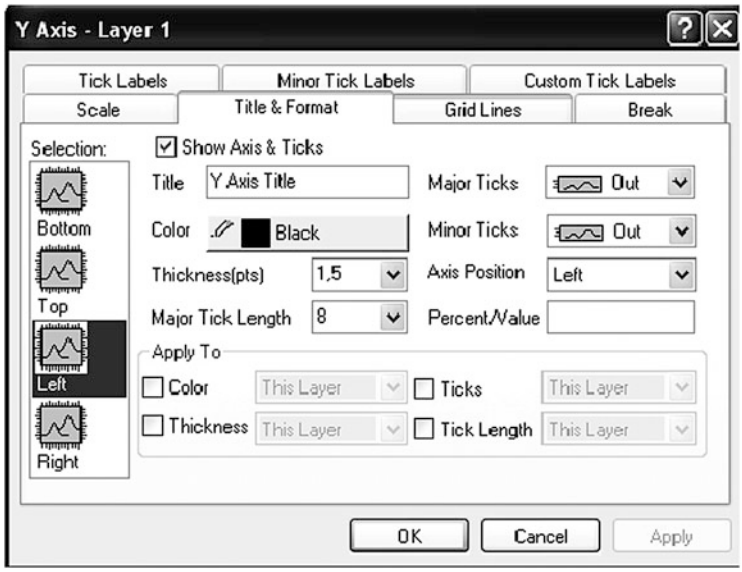

Fig. 3.72 Tab for adjustment of the axes and ticks format

3.4.7.3 Grid Lines

The tab *Grid Lines* is presented in Fig 3.73. On the left, it is possible to choose whether options are to be changed for the vertical or horizontal lines. Tags *Major Grids* and *Minor Grids* define the parameters for lines of the long or short ticks on

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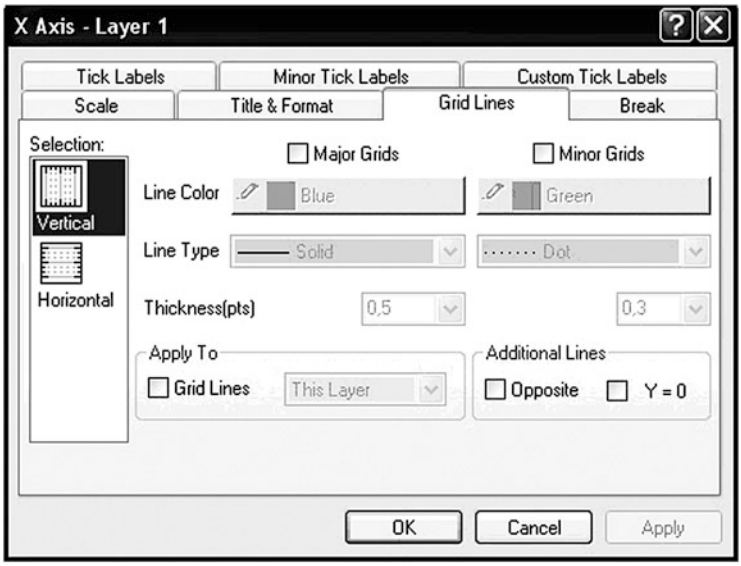

Fig. 3.73 Tab for adjustment of grid lines

axes. Parameters *Line Color*, *Line Type*, and *Thickness* are responsible for the color of lines, their type (continuous, dotted, etc.), and the thickness, respectively. The parameter for vertical lines, $Y = 0$, and for horizontal ones, $X = 0$, is of particular interest because it draws the corresponding zero line.

3.4.7.4 Tick Labels

On the tab *Tick Labels* (Fig. 3.74), it is possible to set the parameters of the test labels near to ticks on axes. The parameter *Type* defines the type of label (numerical, date, time, etc.). Parameters *Font*, *Color*, *Bold*, and *Point* (the primer) set the style and size of the labels. To change the type style, it is necessary to choose one from the drop-down list in the field *Font*. *Display* sets the view of the label representation. For example, in case of a numerical label, it is possible to set its display as decimal (1000 or 1,000) or scientific (1e3). The value of the field *Divide by Factor* sets the multiplier of scaling for values on axes. The view of this tab is presented in Fig. 3.74.

The tab *Custom Tick Labels* (other options of labels) allows adjustment of such parameters as the rotation of labels through any angle set in degrees (*Rotation*), the horizontal and vertical shift of labels (*Offset*), etc. (Fig. 3.75).

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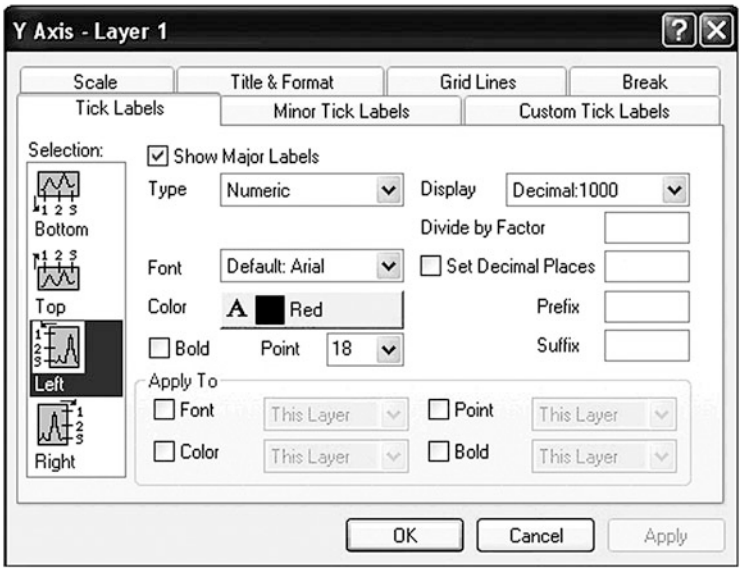

Fig. 3.74 Tab for settings axes labels

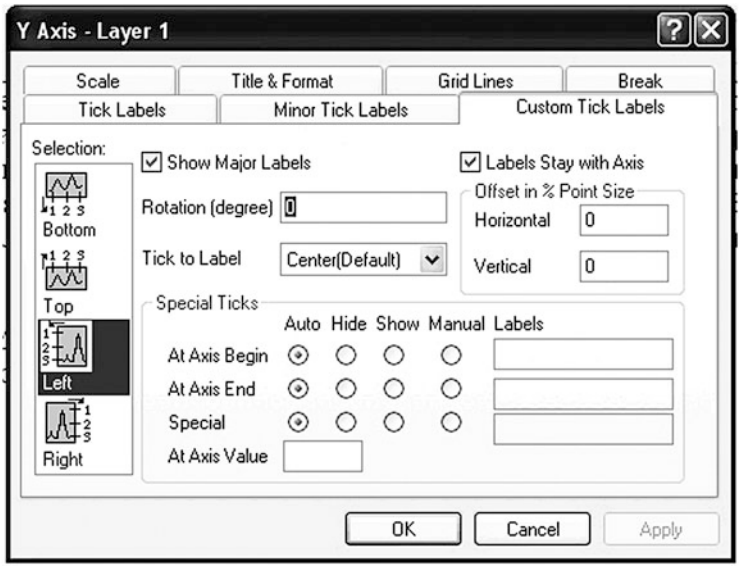

Fig. 3.75 Tab for other settings of axes labels

3.4.7.5 Layers of the Picture

Using a simple example, it is possible to explain what a layer in the program *Origin* is. Let the reader imagine a transparent film on which the graph is drawn. Such a film is a layer. With a layer, it is possible to do the following basic operations: to add

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a new layer, to remove an available layer, to add the graph on a layer, etc. Certainly, when a graph is created, the layer on which this graph is located is automatically created. Also, it is impossible to remove the last layer and simultaneously to see the graph on it. In this case, the object "graph window" will be removed from the project. Note that in this case the function *Undo* (cancellation) does not work. A similar effect is observed with an attempt to cut the graph from the layer.

Addition, Removal, and Editing of a Graph on a Layer

The *layer number* is specified on the button on the top left corner of the graph window. To add a graph to the layer, it is necessary to have the object graph active. After that, it is necessary to click the left mouse button twice on the layer number or to open the menu *Graph* and to choose the submenu *Plot Setup*. As a result, there should appear (as shown in Fig. 3.76) the variant of the dialogue window in which it is possible to add a curve to the graph, similarly to Sect. 3.4.7.1 on graph construction. From the top part of the window, it is possible to choose the table containing the data for graph construction. According to the chosen table, the list of columns for graph construction will be displayed in the central part of the window. After the choice of the desired columns, it is necessary to press the button *Add* (Fig. 3.76). In the bottom part of the window are displayed the graphs already present in the shot.

To remove a graph, it is necessary to choose the target graph by a single click of the left mouse button in the list below the corresponding line and to click on it with the right mouse button. In the drop-down menu, it is necessary to choose the option *Remove*.

At the change of an already existing graph, it should be chosen for removal, and then the parameters in other areas of the window should be changed. After that, it is necessary to press the button *Replace*. This action will replace the current highlighted graph with the new one. Do not forget to place ticks in the column *Show*.

Note that in the field of visualization of the graph list, there are some other points to which it is necessary to pay attention. In our example, above the list of graphs, it is possible to see the legend "Layer 1." This specifies that these graphs are "linked" to layer 1. In other words, they belong to this layer. Thus, it is possible to specify in which layer to add the graph.

Addition, Removal, and Renaming of Layers

For the management of layers, it is necessary to open the menu *Graph* and to choose the option *Layer Management* (Fig. 3.77). In this dialogue window, we only used in our work the tab *Add*. Options for other tabs we left set to default. On the tab *Add*, the following parameters can be adjusted: *Type*, *X Scale*, and *Y Scale*. Then the user can add the graph on a new layer as described above for adding or removing a graph on a layer.

The parameter *Type* defines how the coordinate axes (at the left, right, top, and bottom) will be located on a layer. The parameters connected with axes are responsible for the scale type (linear, logarithmic, etc.). For layer addition, it is necessary to set its parameters by the choice of corresponding options in drop-down lists and then to press the button *Apply*.

Note that in the dialogue window in the left part of Fig. 3.77, there is a list of layers. To remove a layer, it is necessary to choose it in this list, to press the right mouse button, and to choose *Delete Layer*.

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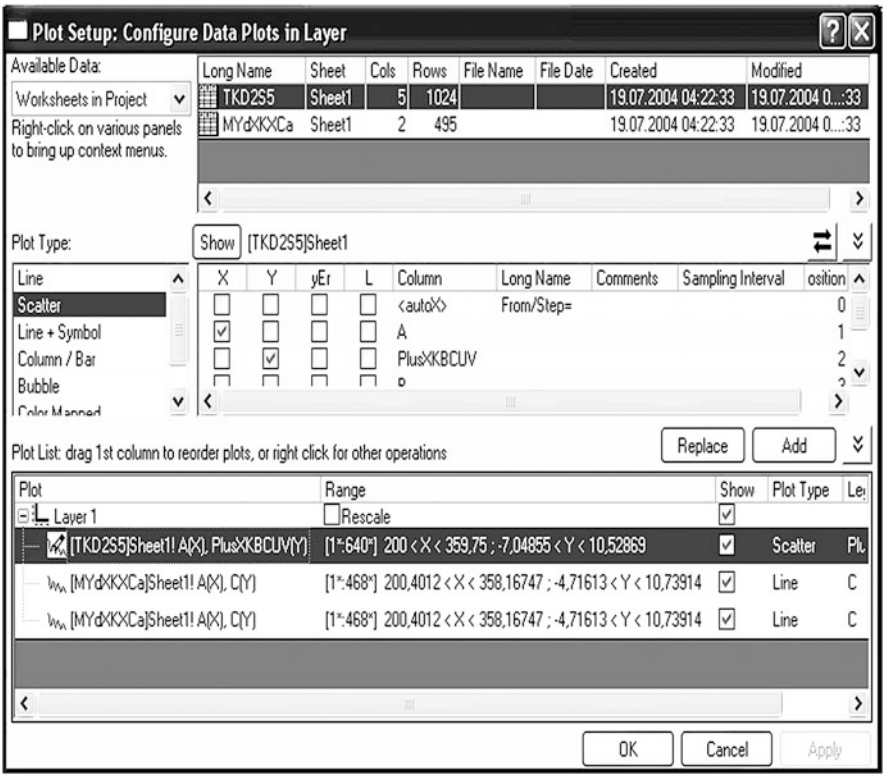

Fig. 3.76 Window for graph adjustment and for graph management on a layer (for *Origin* version 8.1)

In full conformity with an example of layers as transparent films, layers in *Origin* can be reshuffled. For this purpose, it is necessary to choose the necessary layer in the dialogue window of layer options and, having grasped it by the number, to drag it with the mouse upward or downward. We emphasize that, depending on the source of the package *Origin* 8.0, this way can be a little different (Figs. 3.77 and 3.78).

3.4.7.6 Some Essential Operations

Copying, Inserting, and Cutting of Graphs

To copy a graph, it is necessary to click on its shot and to execute copying or cutting. Both copying and cutting are carried out in the same way as for any object supporting these actions, e.g., [Crtl + C] and [Crtl + V].

At graph insertion, the new layer is added. However, note that, during these operations with graphs, there is cutting/copying/insertion of a whole layer; therefore, if there is only one layer, the program will give a warning at any attempt to cut it out [Crtl + X], because the graph would disappear without the possibility to insert it and would be lost.

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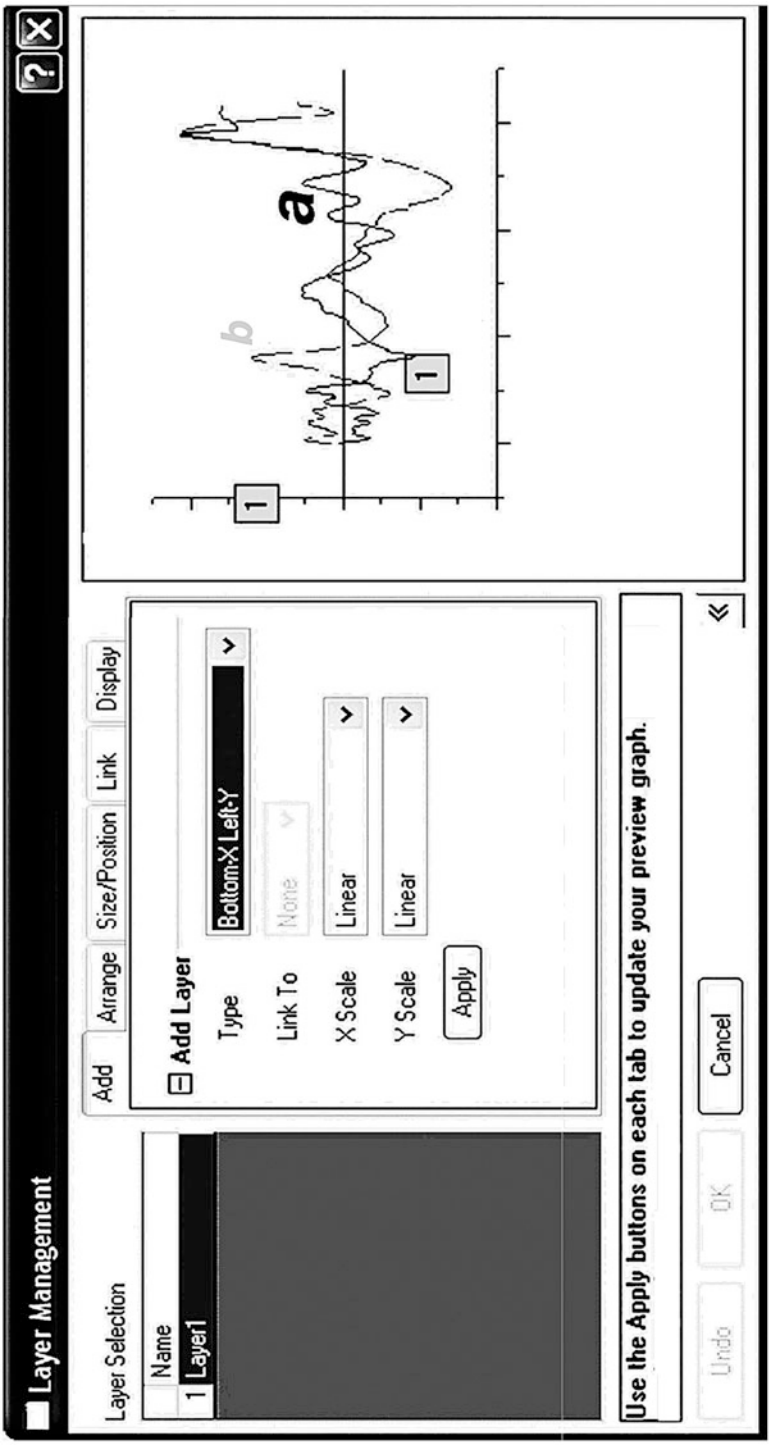

Fig. 3.77 Dialogue window for layer management

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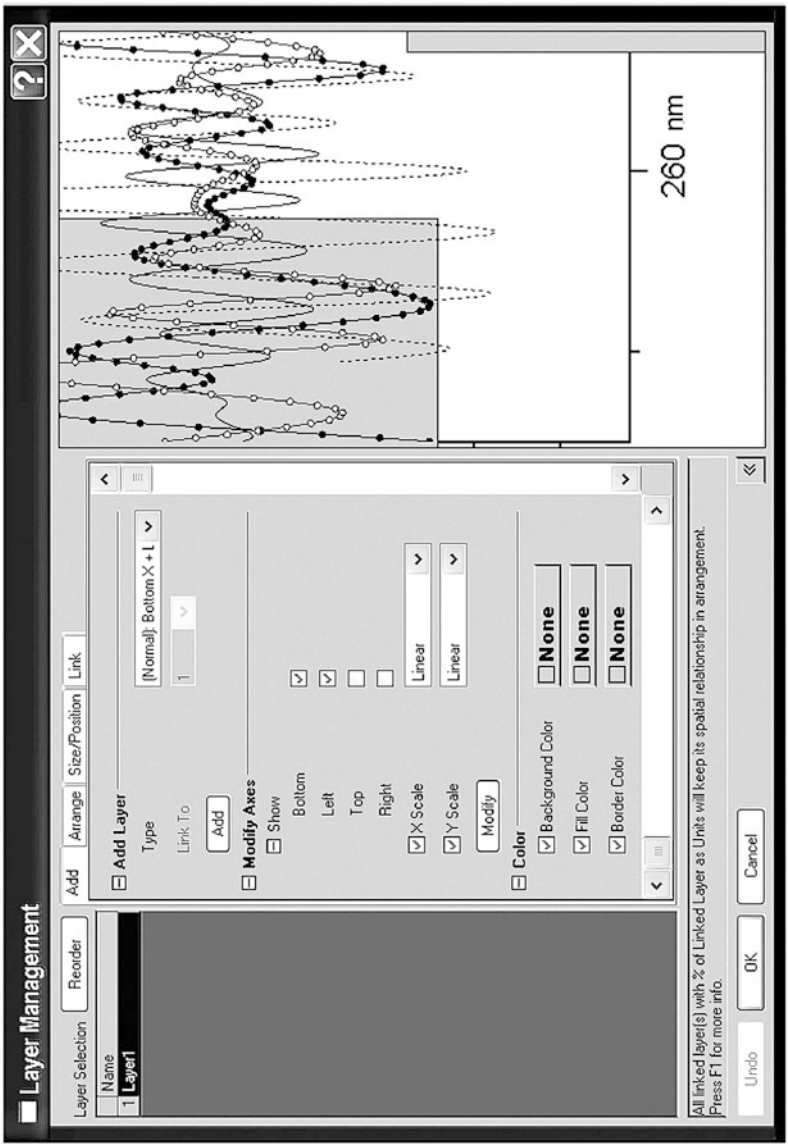

Fig. 3.78 Tab *Add* of the dialogue window for layer management

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Change in the Scale of Graph Display

This operation is called scaling. It is possible to use two ways to change the scale of a graph. The first consists in opening the menu *View* and choosing the option *Zoom in* [Crtl + I] or *Zoom out* [Crtl + M]. The second way is to use the tool *Magnifier* on the sidebar (if it is located at the left, and in our case it is in position 27 in Fig. 3.52). To increase the magnification, it is necessary to choose the button with the plus and for the reduction that with the minus sign (Fig. 3.52). However, there are distinctions between these ways, not only in actions for realization but also in the results of their application. The second way allows the user not only to increase the display scale but also to display at once the interesting area of the graph. This is achieved by highlighting the interesting area of the graph with a frame. Similarly, text labels behave in different ways with different methods of scaling.

The difference between these tools is shown in Fig. 3.79. Note that at the top part of the figure (Fig. 3.79a), the graph is without scaling; in the middle, the scaling was performed by means of the menu (Fig. 3.79b); and at the bottom, by means of the *Magnifier* (Fig. 3.79c).

In the version *OriginPro* 8.1, there is no difference between *Zoom In* and the *Magnifier*; after their choice, it is necessary to click once on the desired area.

To Obtain Coordinates of the Point Position on the Graph; The Linkage of Spectral Bands to the Scale of Wavelengths

For this purpose, it is necessary to use the tool of the sidebar *Data reader* (Fig. 3.52, positions 28, 29).

By clicking on this button, a small window will open that displays the coordinates of the point chosen by the placement and pressing of the pointer on the graph. Moving and pressing the pointer link it to the nearest point of the graph. In the window *X Y* (Fig. 3.52), there will appear the values of coordinates of the point, and on the graph, the point will be highlighted by a red cross. The linkage to the point can be moved by the mouse (click on another point of the graph) or by arrows on the keyboard (to the left or to the right).

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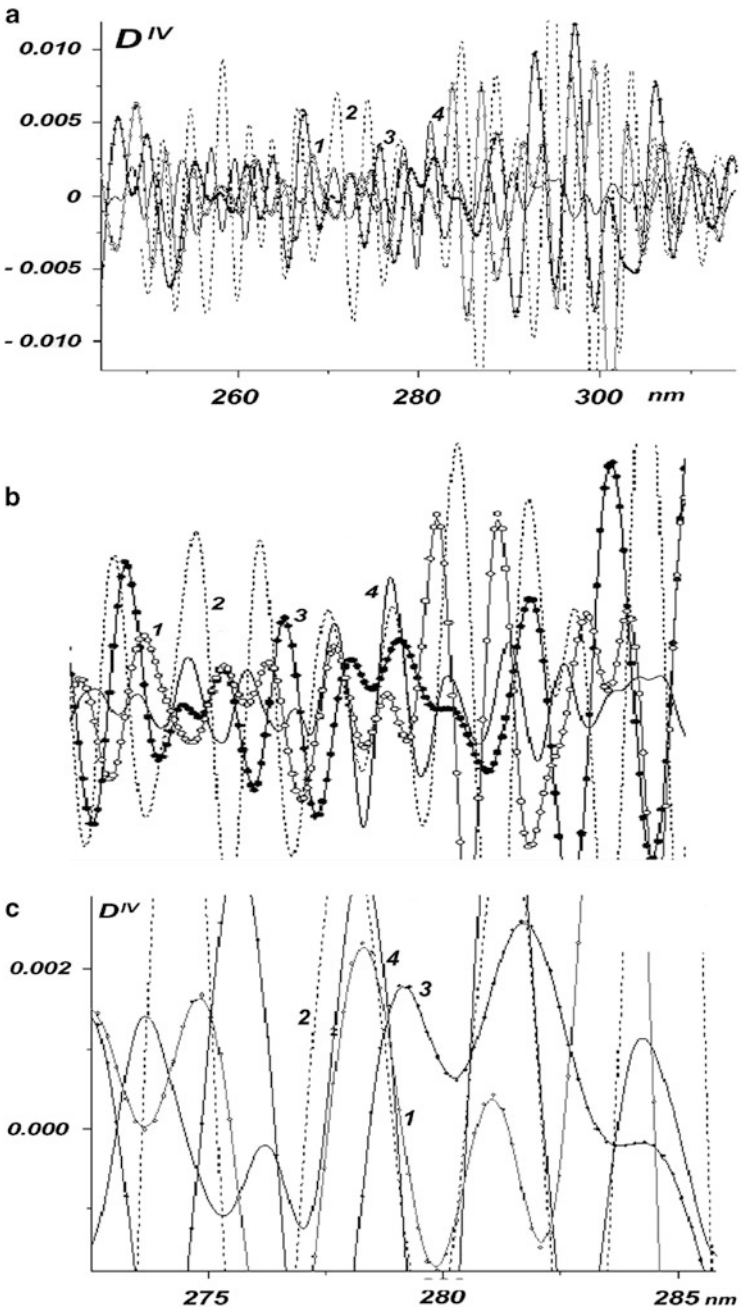

Fig. 3.79 Demonstration of various views of scaling: (**a**) not scaled, (**b**) scaled using the menu option *Zoom in*, and (**c**) scaled using the *Magnifier* tool

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Manipulation of Points on the Graph

Point manipulation refers to correction of the position of a point or removal of a point from an already constructed graph. For these purposes, the corresponding options of the *Data* menu are used.

For removal of a point from the graph, it is necessary to choose in this menu the submenu *Remove bad data points*. The cursor will change its view to a frame with ticks. It is necessary to click on the target point for its removal. Before the removal of points, it is convenient to perform the scaling of the interesting part of the graph.

For point moving, it is necessary to choose in the *Data* menu the submenu *Move data points*. As in the case of removal, the cursor will change, but a point can be moved by placing the mouse cursor on it and "moving" the captured point. To return to the usual view, it is enough to choose the button first from the top on the tool sidebar or to press Esc on the keyboard.

3.4.7.7 Drawing of Arrows on the Graph

To create arrows, a special tool is used. It is the button № 31 on the left sidebar (Fig. 3.52), which looks like an arrow. After pressing this button, the cursor will change to a cross. To draw an arrow, it is necessary to press the left mouse button at the point of the arrow beginning and, keeping it pressed, to move the mouse to the final point of the arrow. Then it is necessary to release the mouse button. After that, the arrow should appear on the figure, and the cursor will have the usual appearance.

3.4.7.8 Editing of Arrow Properties

After drawing an arrow, markers will highlight its beginning and the end point. It is possible to move the terminal points of these markers. In this way it is possible to change the arrow's length, the slope, etc. However, there is also another, expanded, way of editing the arrow parameters. For this purpose, it is necessary to double click on it. A window allowing adjustment of its properties should open, as presented in Fig. 3.80. For convenience, we show the two main tabs *Line* and *Arrow* in one figure. Considering some options for arrows in the program *Origin*:

1. The tab *Line* adjusts the type of the line forming the arrow body and contains the parameters *Color*, *Width*, and *Type*, which define the color, width, and type (continuous, dotted, etc.) of the arrow line.
2. The tab *Arrow* gives the chance to adjust the view of arrowheads. On this tab, it is possible to see two identical groups of options. The left set is responsible for the beginning of the arrow and the right for its end. The parameters for the tails and heads are contained on this tab (Fig. 3.79):

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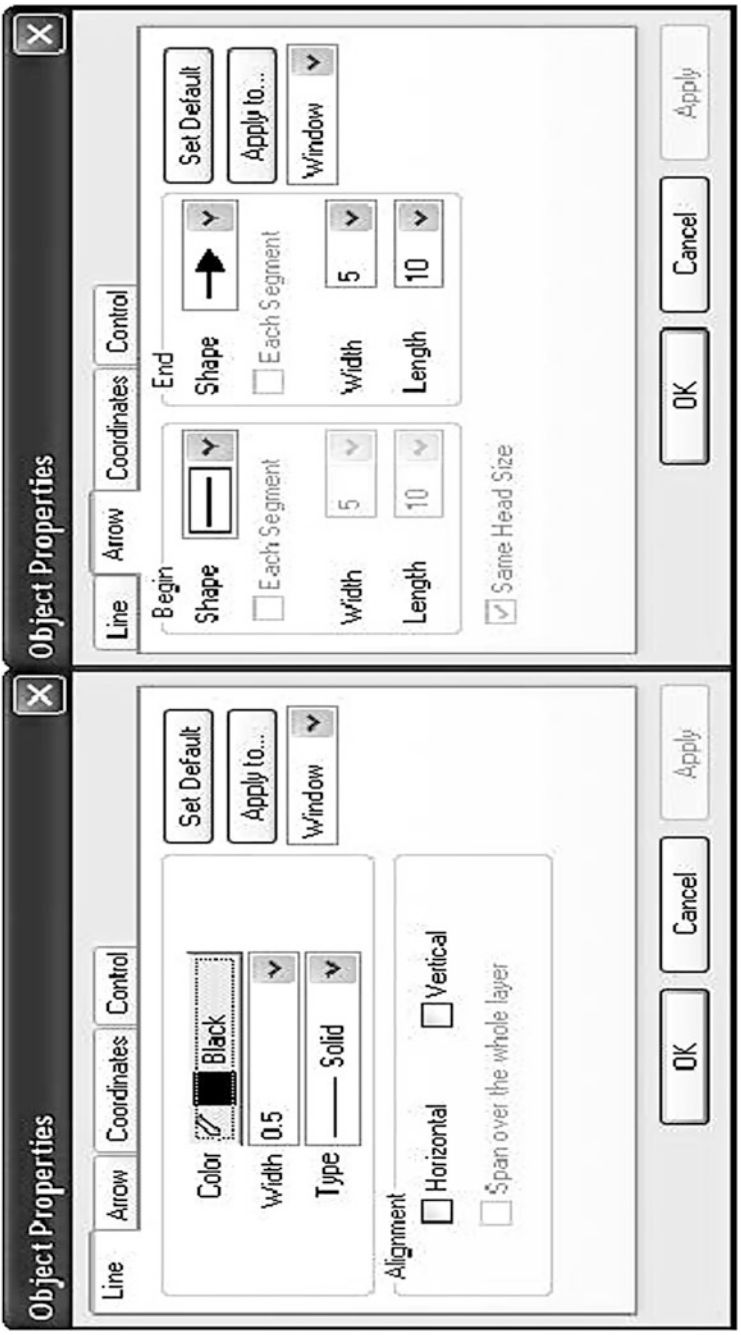

Fig. 3.80 Main properties of arrows

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- *Shape*: the form of head elements of the arrow (actually an arrow, swallow tail, etc.). The reader can choose according to his requirements from the drop-down list showing the options.
- *Width*: the width of arrow heads (not available if the line shape is the final element).
- *Length*: the length of arrow heads (not available if the line shape is the final element).
- *Window*: there is a simultaneous change in the parameters of all arrows in the graph window according to that set. Instead of *Window*, it is possible to choose *Object* (then formatting will be applied only to the highlighted arrow) or *Layer* (for simultaneous formatting of all arrows of one layer). Any of these choices must be followed by pressing the button *Apply to*.
- *Set Default:* sets the entered parameters of an arrow to be used as default, that is, they will automatically be used for drawing of all subsequent arrows.

3.4.8 Saving the Graph as a Picture

The necessity of saving the graph as the picture arises in connection with the requirement to present figures in formats such as jpg, psd, or others accepted by publishing houses.

First it is necessary to open the desired graph. For this it is enough to display its figure. Then the *File* menu should be opened and the option *Export graphs* chosen. As a result, the *Import and Export* dialogue window should appear, as shown in Fig. 3.81

In this window, there are export adjustments. In the field *Image Type*, the format of the final picture can be chosen. The most convenient formats for further work are jpg and psd. In the field *File name*(*s*), it is necessary to enter a file name for the picture file. The field *Path* specifies where the file will be saved (a disk, a folder). The field *Overwrite existing* is responsible for how the program will react if there is already a file with the same name in the specified folder. We recommend that the parameter *Ask* is set for this field, which will lead to a question on the necessity of rewriting an available file. The fields *Export Settings* and *Image Size* allow the margins and size of the picture to be set.

3.4.8.1 Merging of Graphs

To merge graphs, it is necessary to open the menu *Graph* and to choose the option *Merge graph windows*. As a result, the window shown in Fig. 3.82 a should appear.

Let us consider the main options of this dialogue window. For *Keep source graphs*, the presence of a tick does not allow the program to delete initial graphs

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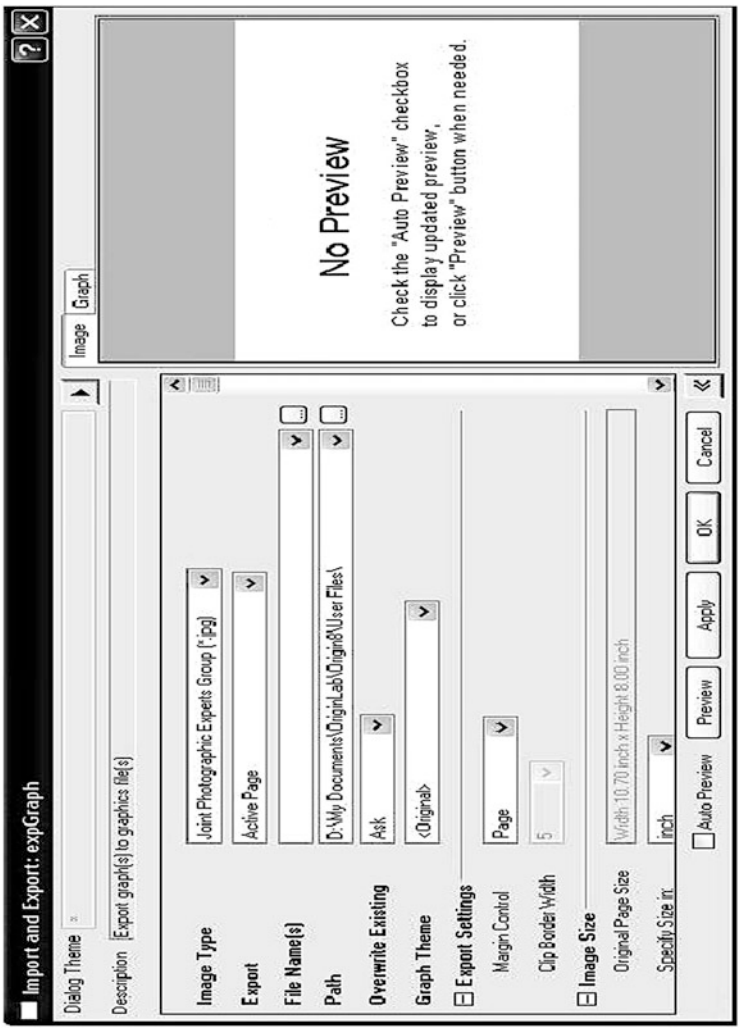

Fig. 3.81 Dialogue window for saving a graph in various formats

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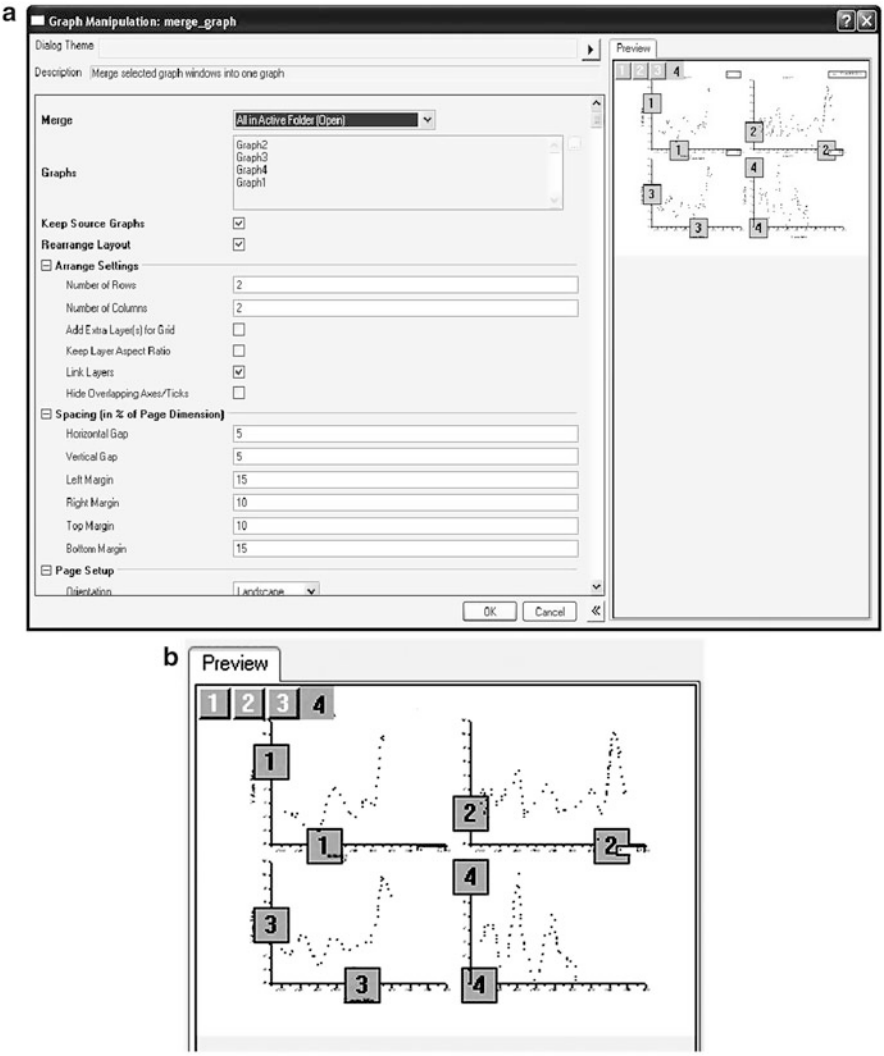

Fig. 3.82 (**a**) Dialogue window for merging of graphs. (**b**) Preview window for merging of graphs

after the merge (Fig. 3.82a). *Number of rows/columns* in the subpoint *Arrange settings* shows the number of rows/columns of the virtual table, each cell of which contains the graph. For example, if the number of rows is set equal to 4 and of columns equal to 2, in our case the graphs would be placed from the top to the bottom, one under another.

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Vertical/horizontal gap in the subpoint *Spacing* (*in % of Page Dimension*) is for setting the intervals between graphs. The fields *Left/right/top/bottom Margin* are for adjustment of the left, right, top, bottom margins. The group of options under *Page setup* allows the page orientation (portrait or album), size, and units of size measure to be set.

In the tab *Preview*, the final result of the graph combination is displayed, and the numbers of layers (Fig. 3.82b) are specified on the graphs.

3.4.9 Approximation of Curves

For an approximation of experimental points on the graph by function (see the example in Fig. 3.83), it is necessary to activate the graph window or to choose the necessary columns directly from the data table (to click with the mouse on the names of the necessary columns without releasing pressed Ctrl) and to choose from the menu *Analysis* the tab *Fitting*. Options in this tab, among others, allow the choice of *Linear Fit* (approximation by a straight line), *Polynomial Fit* (approximation by a multinomial), *Non Linear Curve Fit* (approximation by one of the

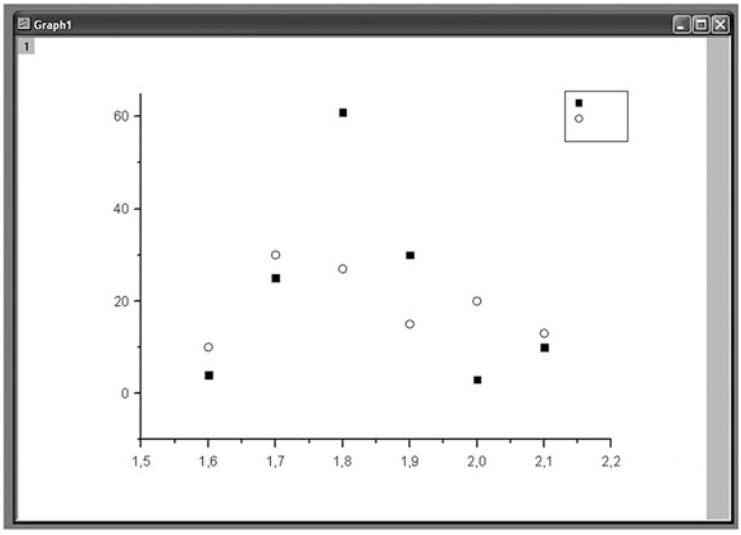

Fig. 3.83 Two graphs, the points of which were approximated by functions

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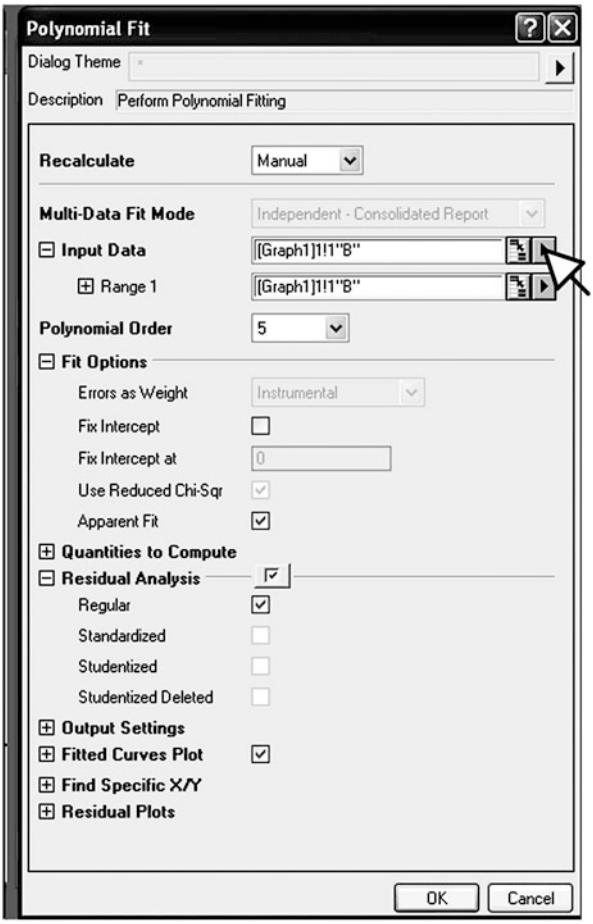

Fig. 3.84 Dialogue window for approximation by a multinomial

functions built into *Origin*), and *Exponential Fit* (approximation by one of the *Origin* built-in exponential functions). In the example shown in Fig. 3.83, the *Polynomial Fit* was chosen. The user can explore the possibilities of other less-used options if necessary.

Having decided upon an option for approximation, it is necessary to click on it using the left mouse button or, if the approximation had already been used earlier, to choose from the submenu *Open Dialog Window*. In Fig. 3.84 an example of the dialogue window for approximation by a multinomial is presented. Features of *Origin* allow the analysis of one chosen graph or several simultaneously. In the line

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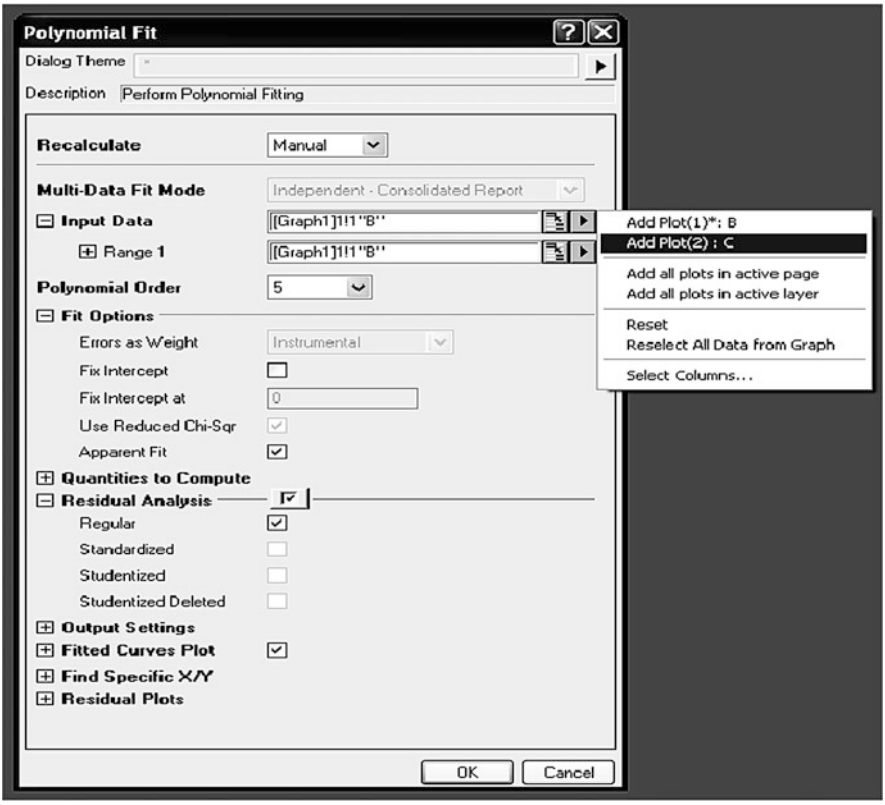

Fig. 3.85 Submenu of the field *Input Data*

Input Data, it is possible to specify the column of ordinates and also to add other columns from the data table. For this purpose, it is necessary to click on the gray button with an arrow that is located to the right of the field *Input Data* and from the opened submenu (Fig. 3.85) to choose the name of one necessary column with the help of *Select Columns*, or to add the names of other columns (*Add Plot*(*2*)*:C* for our example). Note that all graphs will be approximated by multinomials of the order specified in the line *Polynomial Order*. If the orders of polynomials are different, then it is necessary to approximate each graph in turn, without using *Add Plot*. The other parameters used can be those set by default. A tick in the line *Fitted Curves Plot* sets the output of approximating curves to the graph. After the button *OK* is pressed, curves will appear on the graph (Fig. 3.86) as well as a table containing the

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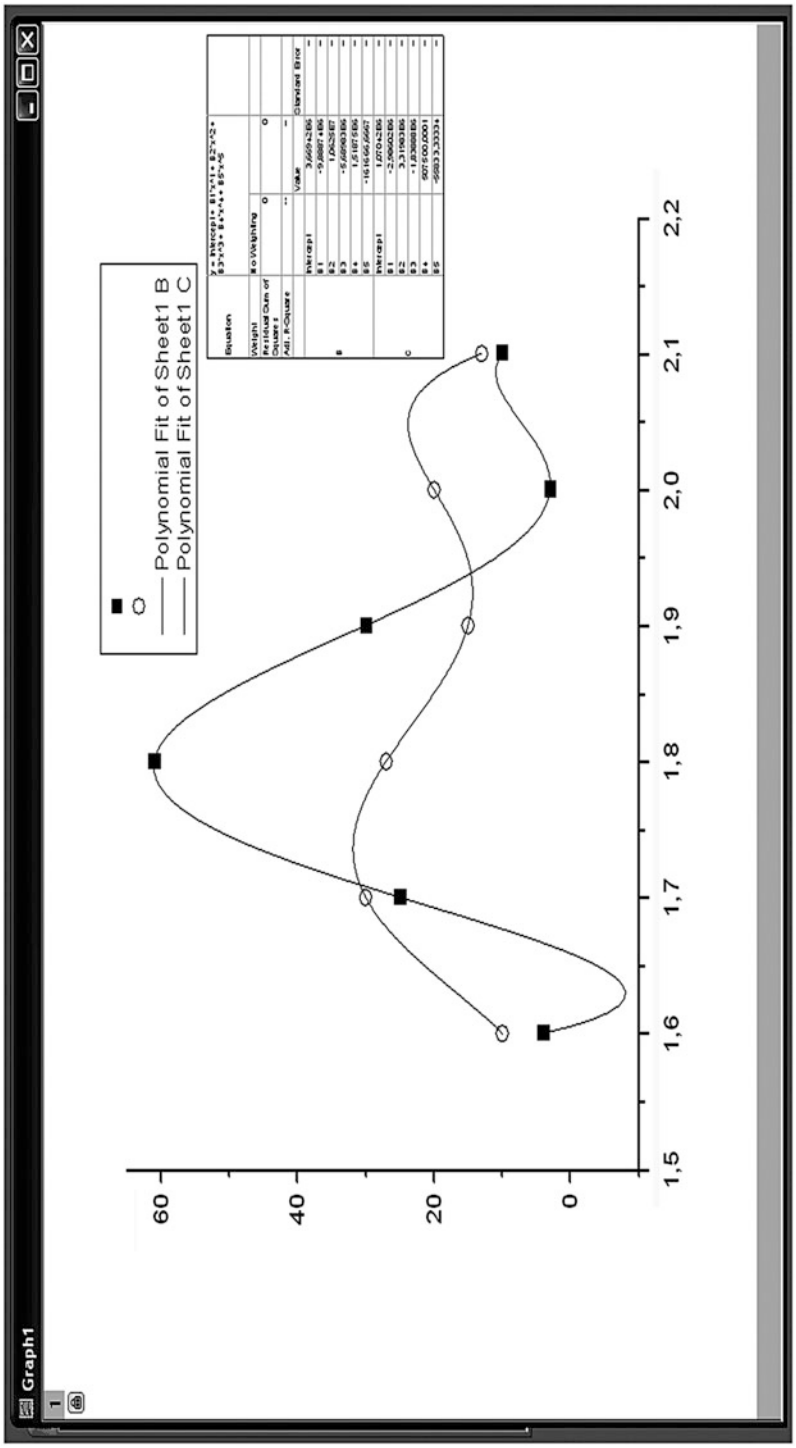

Fig. 3.86 Result of approximation of graph points by fifth-order polynomials

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Table 3.3 Measured polynomial coefficients for the considered example

Equation	$y = \text{Intercept} + \text{B1} * \times \hat{}1 + \text{B2} * \times \hat{}2 + \text{B3} * \times \hat{}3 + \text{B4} * \times \hat{}4 + \text{B5} * \times \hat{}5$		
Weight	No weighting		
Residual sum of squares	0	0	
Adj. R-square	–	–	
		Value	Standard error
B	Intercept	3.66942E6	–
	B1	−9.88374E6	–
	B2	1.0625E7	–
	B3	−5.68983E6	–
	B4	1.51875E6	–
	B5	−161666,6667	–
C	Intercept	1.07042E6	–
	B1	−2.98602E6	–
	B2	3.31983E6	–
	B3	−1.83888E6	–
	B4	507500,0001	–
	B5	−55833,33334	–

coefficients of multinomials or constants of functions (shown more clearly in Table 3.3).

Note also that at the bottom of the data table *Book* are tabs for additional sheets, similar to Excel tabs (Fig. 3.87). These sheets contain the information on parameters of approximating curves and also pictures of their graphs. It is possible to display the graph by clicking on the tab with the mouse.

Dialogue windows for the approximations *Non Linear Curve Fit* and *Exponential Fit* are divided into parts, and their view differs from the window for approximation by multinomials, but the essence remains the same (Fig. 3.88). From the list in the left part of the window, it is necessary to choose first *Function Selection*; in the right part of the window will appear the menu for the choice of function. If the option *Data Selection* is selected on the right, it is necessary to specify one or several necessary columns with ordinates; other points are similar to those in Fig. 3.84.

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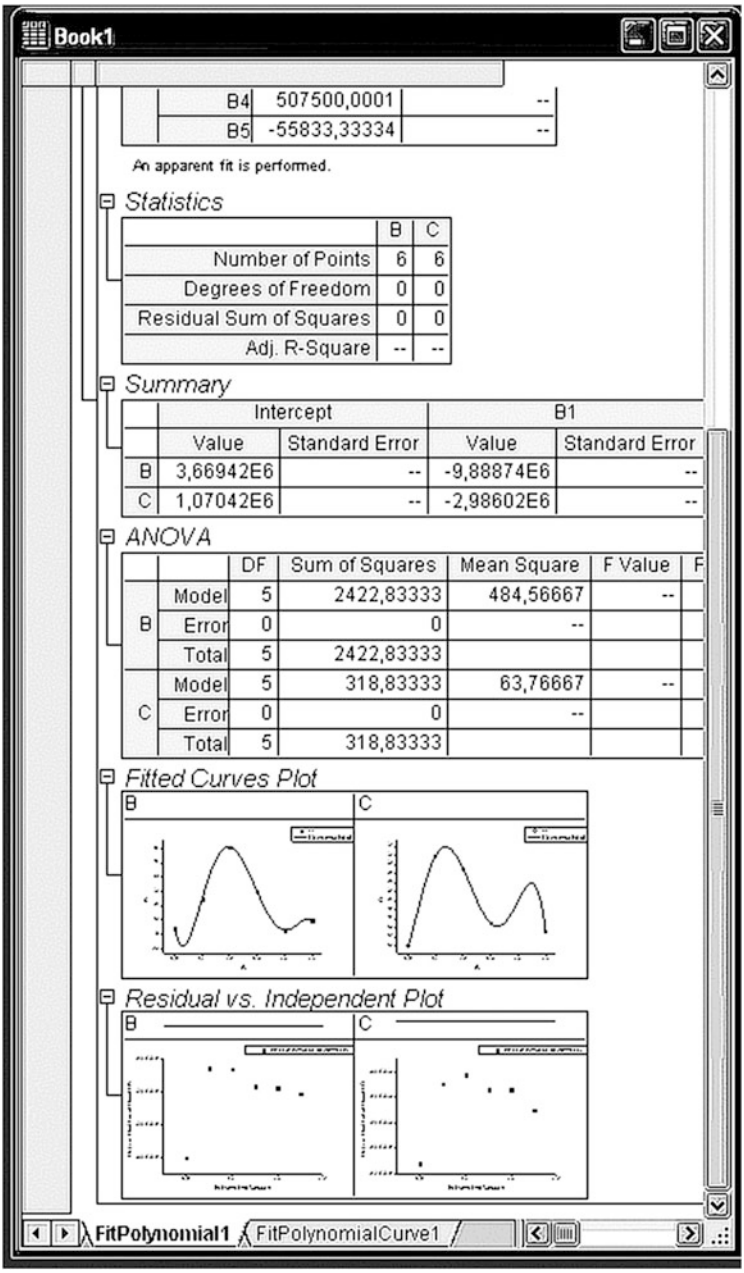

Fig. 3.87 Tab appearing in the data table after approximation

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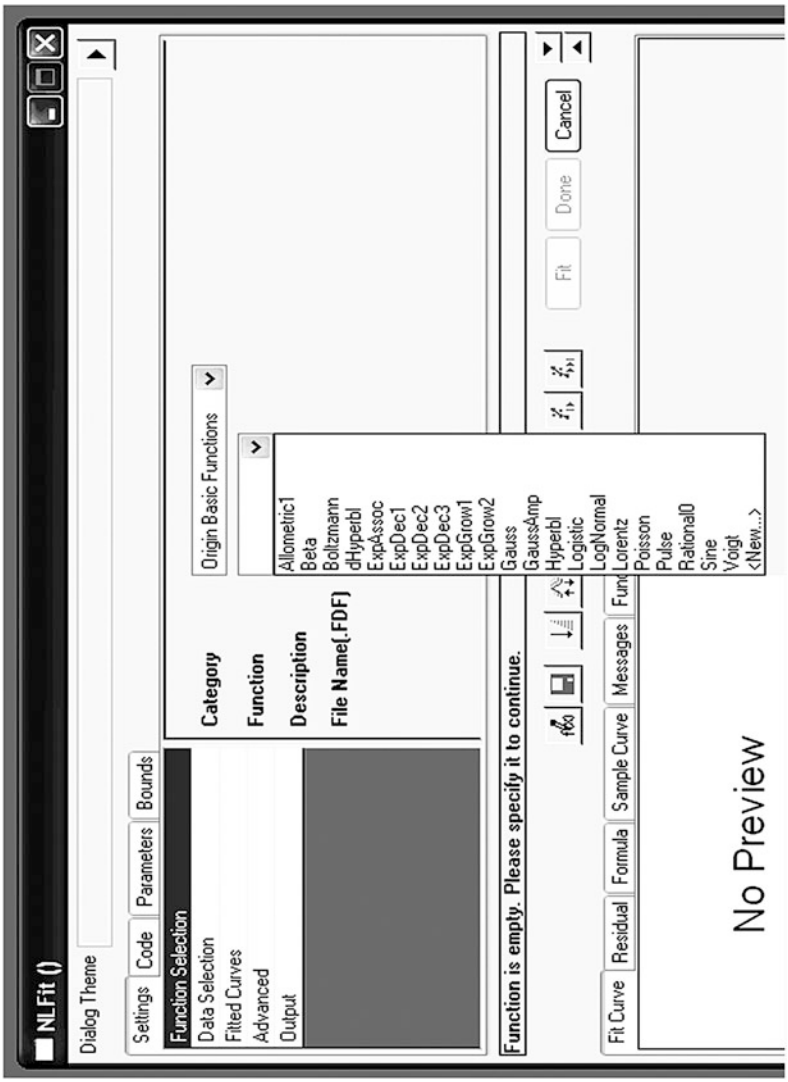

Fig. 3.88 Example of the dialogue window for *Non Linear Curve Fit*

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3.5 New Approaches for Determination of Oxygen Isotopes in Biochemical Works

The importance of oxygen as an element of organic nature defines the high interest in studies of its metabolism and oxidative transformation to molecular oxygen of air.

Stable isotopes O^{17} and O^{18} are frequently applied for research purposes in biological experiments. The natural content of the first isotope is 0.039 % and that of the second is 0.204 %.

The approaches of mass spectroscopic and densitometric methods of analysis, their advantages and disadvantages, and the specificity of preparation of biological samples for analysis are carefully discussed in various reports (Vartapetian 1970; Vinogradov et al. 1962; Anderson et al. 1960; Kutyurin et al. 1969; Trofimov 1953; Budzikiewicz et al. 1969; Dorough and Calvin 1951; Holt and French 1948a, b; and some others).

In Chap. 1, we very briefly discussed the radioactivation analysis method. Because of the dispersion of data in separate publications on the application of this kind of analysis in biological experiment and an absence of details on the methodological orientation, the authors of this book considered it necessary to describe, for the first time, the methodological approaches in more detail. We will consider the possible difficulties and errors of application of the method and also analyze the nuclear reactions that are its basis.

The material obtained on the basis of activation of the isotope O^{18} by nuclear particles is also presented. This review of methodological approaches will promote understanding of the experiments described in Chap. 5.

The specificity of some biochemical research consists in the use of a rather small quantity of substance and of an oxygen isotope in an experiment. It leads to difficulty in the use of mass spectrometry methods, because its sensitivity limits the quantity of substance to the range 10^{-10}–10^{-12} g.

A much more sensitive method of determination of the isotope structure is the radioactivation analysis method (Bauen and Gibons 1968; Kuznetsov 1967; Teilor 1965; Bardes and Owen 1960; Fourcy et al. 1967). This method is based on the determination of the content of an element with the help of the artificial formation of its radioactive isotopes in an experimental sample as a result of the bombardment of the sample by neutrons or charged particles (p, d, α, etc.) accelerated to high energies. Registration of the induced radioactivity of obtained isotopes shows their presence and allows the quantitative content of elements to be found. The sensitivity of the method is 10^{-20} g. This analytical technique is applied in biochemical (the analysis of the oxygen exchange in ATP), chemical, metallurgic, physical, and other research (Andreev et al. 1967; Vartapetian et al. 1966, 1967; Gun-Aazhav and Chultem 1972; Erokhina et al. 1960; Lbov and Naumova 1959; Maslov 1964; Aumann and Born 1964; Fleckenstein et al. 1959, 1960; Fogelstrom-Fineman et al. 1957; Hunt and Miller 1965; Temmer and Haydenburg 1956).

Analysis of the induced radioactivity was suggested for the first time by Hevesy and Levi (1936, 1938), who irradiated various rare-earth elements with the neutrons

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obtained from a radium–beryllium source. Considerable sensitivity of this method was reached after creation of nuclear reactors and accelerators, being sources of particles of high energy (Lawrence and Livingston 1934). The highest sensitivity of the method was reached in accelerators of the charged particles (Lemberg et al. 1966; Boyd 1949; Fogelstrom-Fineman et al. 1957; Meinke 1955).

In work on the analysis of oxygen in silicon (Nozaki et al. 1961), the reaction $O^{16}(\alpha,pn)F^{18}$ was successfully used. In the same experiments, the insignificant value of radioactivity from reactions $O^{18}(\alpha,p3n)F^{18}$ and $O^{17}(\alpha,p2n)F^{18}$ was determined. The sensitivity of the technique was 10^{-7} %. To find small quantities of oxygen, Lbov and Naumova (1959) used neutrons with $E_n = 14$ meV, obtained in the low-voltage accelerating tube through the reaction D(T,n)He^4.

For oxygen determination, the β-radiation arising in the reaction $O^{16}(n,p)N^{16}$ was identified (Andreev et al. 1967). The short half-life period of N^{16} ($T_{½} \approx 7.3$ s) almost excludes the possibility of radiochemical secretion of N^{16}, but thanks to the high energy of β-particles (up to 10 meV), it is possible to provide effective measurement of the radioactivity of the irradiated sample. The works mentioned above studied the application of the reaction O^{16}(T,n)F^{18} for determination of the oxygen presence. In this case, the sample containing the introduced Li^6 was irradiated in the reactor with a particle flux of 1.3×10^{11} neutrons/cm^2 s. Tritons obtained in the reaction Li(n,α)T cause the reaction O^{16}(T,n)F^{18}.

Because the artificial radioactive isotopes of oxygen have small half-life periods ($T_{½}$), the application of these isotopes as the label in comparative biochemical and chemical experiments is connected with great complications of the experimental technique and, consequently, is inexpedient. The corresponding values of $T_{½}$ for O^{14}, O^{15}, O^{19}, and O^{20} are 74 ± 2, 121 ± 2.3, 2.13 ± 1.1, and $2{,}13 \pm 1$ and less than < 14 seconds (Scharff-Goldhaber et al. 1960; Strominger et al. 1958). The use in laboratory practice of the most long living of radioactive isotopes, oxygen O^{15}, as a label was characteristic (Dyson et al. 1958; Evans and Ebert 1961; Ter-Pogossian and Powers 1957).

Before the development of modern methods of γ-spectrometry, the determination of the content of induced isotopes was performed, as a rule, by registration of a radioactive decay curve and of an absorption curve for the β-radiation of isotopes formed as a result of the influence of neutrons or of other charged particles on the investigated sample. We did not avoid this stage of work when carrying out our initial research. We were able to make conclusions about the presence of those or other isotopes using values of half-life periods of isotopes or values of the maximum energy of their β-radiation. The quantitative content of an isotope was determined by comparison of the activities of the investigated sample with the standard. This approach was used in our and in other first works on the application of activation analysis in biochemical research (Saakov 1963a, b, c: 1964, 1965a, b; Sapozhnikov et al. 1961, 1964; Fleckenstein et al. 1960, 1964; Fogelstrom-Fineman et al. 1957; Shneour 1961, 1962a, b).

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3.5.1 *Use of the Reaction* $O^{18}(p,n)F^{18}$ *for the Detection of the* O^{18} *Oxygen Isotope in Biological Objects*

From the end of the 1950s, the nuclear reaction of activation $O^{18}(p,n)F^{18}$ has been applied for detection of the isotope O^{18} in biological and biochemical research. The main advantages of the method are defined by the high sensitivity of the indication of a formed isotope (Fogelstrom-Fineman et al. 1957).

In Chap. 1, we briefly mentioned the discovery of the isotope F^{18} (DuBridge et al. 1937, 1938). The conditions of realization of the specified reaction have now been well studied (Barnes et al. 1937a, b; Blaser et al. 1949, 1951, 1952; DuBridge et al. 1937; Mark and Goodman 1955).

Radioactive fluorine F^{18} spontaneously turns into O^{18} with a half-period of $T_{½} = 122 \pm 8$ min (Blaser et al. 1949, 1951; Ajzenberg-Selove and Lauritsen 1952, 1955, 1959). As a result of positron annihilation, the emission of γ-quanta of energy $E_{\gamma} = 0.51$ meV each takes place. Because the spontaneous radioactive decay $F^{18} \rightarrow O^{18}$ is an exoergic process, the reaction $O^{18}(p,n)F^{18}$ should be endoergic and, hence, possess a threshold (Shpolskii 1950; Burcham 1958). For the considered reaction, the threshold is approximately 2.6 meV (Ajzenberg-Selove and Lauritsen 1952, 1955, 1959; Blaser et al. 1951; Hill and Blair 1956; Hornyak et al. 1950; Richards et al. 1950). The maximum cross section of the reaction $O^{18}(p,n)F^{18}$ (i.e., the probability of the initiation of the nuclear reaction) takes place when the energy of protons is approximately 5 meV (Blaser et al. 1951). However, the experiment shows that it is desirable to perform the irradiation of samples at proton energies not higher than 4.2 meV, because at higher proton energies, the other nuclear reaction, $N^{14}(p,\alpha)B^{11}$, can be induced in biological preparations containing nitrogen. In the course of this reaction, the carbon isotope C^{11} is also formed, according to the reaction $N^{14}(p,\alpha)C^{11}{}_{\beta}+$. Its positron activity ($T_{½} = 20.4$ min, $E_{\beta} + = 0.98$ meV) can mask for a long time the half-life period of F^{18} (Ajzenberg-Selove and Lauritsen 1952; Blaser et al. 1949, 1951).

A newcomer working with an organic substance should remember the possibility of the occurrence of the following nuclear reactions at its bombardment by protons: (p,γ), (p,n), (p,α), (p,d), (p,He^3), and (p,T). These reactions can take place at bombardment by protons of the following stable isotopes of biologically important substances: C^{12}, C^{13}, N^{14}, N^{13}, O^{16}, O^{17}, O^{18}, and P^{31} (Barnes et al. 1937a, b; DuBridge et al. 1938; Hornyak and Lauritsen 1948, Hornyak et al. 1950). As a result of the above-listed reactions, stable isotopes that are not registered by detectors of radioactive radiation are formed. For example, F^{19}, N^{15}, and C^{12} are formed in reactions $O^{18}(p,\gamma)F^{19}$, $O^{18}(p,\alpha)N^{15}$, and $N^{14}(p,He^3)C^{12}$ (Ajzenberg and Lauritsen 1952; Hornyak and Lauritsen 1948, 1950). In other reactions, isotopes with a small half-life period appear, for example, O^{14} and S^{31} in the reactions $N^{14}(p,n)O^{14}$ ($T_{½} = 72$ s) and $P^{31}(p,n)S^{31}$ ($T_{½} = 3.4$ s) (Blaser et al. 1952). In some cases, the thresholds of reactions leading to the formation of radioactive isotopes are high (16–17 meV), and the formation of these isotopes is impossible, for example, thresholds of the reaction $C^{12}(p,d)C^{11}$ or $C^{13}(p,\mathrm{T})C^{11}$ (Richards et al. 1950).

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All radioactive isotopes formed in reactions of protons with stable isotopes *C, N, O*, and *P* and having a half-life period of 2.5 min will have insignificant activity by the time of the possible beginning of measurements of F^{18} radiation, which cannot be taken into consideration. This means that if measurements of the dynamics of the F^{18} half-decay curve are started 75 min after the end of the bombardment by protons, then the activity of all isotopes with $T_{½} < 2.5$ min will have decreased at least 2^{30} times, whereas the activity of F^{18} will not have decreased by half.

From this analysis of possible collateral nuclear reactions, it follows that in reactions of protons with *C, N, O*, and *P*, together with F^{18}, only two radioactive isotopes with a half-life period of more than 2.5 min can be formed, namely, N^{13} ($T_{½} = 10$ min) and C^{11} ($T_{½} = 20$ min). These isotopes are formed in the reactions $N^{13}(p,\alpha)C^{11}$, $C^{12}(p,\gamma)N^{13}$, $C^{12}(p,d)C^{11}$, $C^{13}(p,n)N^{13}$, $C^{13}(p,\mathrm{T})C^{11}$, $O^{17}(p,He^{3})N^{13}$, and $O^{16}(p,\alpha)N^{13}$ (Blaser et al. 1952; Ajzenberg-Selove and Lauritsen 1952, 1955, 1959).

We emphasize that the high threshold of reactions $C^{13}(p,\mathrm{T})C^{11}$ and $C^{12}(p,d)C^{11}$ prevents them occurring at proton energies used by us and other researchers for the indication of isotope O^{18}. The reaction $N^{14}(p,\alpha)C^{11}$ has a threshold of 3.1 meV; however, at neutron beam energies $E_p < 4.2$ meV, the effective cross section of the reaction is small (Blaser et al. 1952).

Of particular interest is the reaction $O^{17}(p,\gamma)F^{18}$. As a result of this reaction, F^{18} is formed, the radioactivity of which can affect the results of an experiment. However, in an assumption that the cross sections of reactions $O^{18}(p,n)F^{18}$ and $O^{17}(p,\gamma)F^{18}$ are approximately identical, it is possible to consider that, when using water enriched with the isotope O^{18}, the main activity of F^{18} will be defined by the contribution of the reaction $O^{18}(p,n)F^{18}$. If H_2O^{18} enriched with O^{18} to 40 % is used, then the content of O^{18} will exceed the content of O^{17} approximately 1000 times (Hunt and Miller 1965). This calculation points to the insignificance of the contribution of the radioactive radiation from the reaction $O^{17}(p,\gamma)F^{18}$ (Blanchard 1955).

Thus, from the analysis of possible reactions of protons with *C, N, O*, and *P* elements, it follows that during work with organic compounds, the revelation of the isotope O^{18} will not be masked by extraneous activity if protons are used with E_p *not higher than 4.2 meV*.

The yield of nuclei F^{18} depends on the quantity of O^{16} isotope in a target and on the number of transferred nuclear particles. This means that, in the case of comparative biochemical research of several samples, it is necessary to strictly consider both the quantity of a preparation put on a substrate taken for an irradiation and the quantity of particles bombarding a target during each exposition. Only in this case it is possible to speak about the *correctness* of the technique and the *accuracy* of experimental results.

There are several possibilities for determination of the number of particles bombarding a target. One method (Fleckenstein et al. 1959, 1960; Fleckenstein and Janke 1964; Snell 1937; Vartapetian et al. 1967) consists in placing a very thin standard target of known quantity of oxygen atoms (N) in the path of the proton beam (before it falls on an investigated target, T). Thus, the irradiation of the target

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and of the standard occurs under identical conditions. Hence, the relative value of the radioactivity arising at the target after the irradiation can be expressed through the ratio T/N, *irrespective of the proton energy*, *the current, the duration of irradiation, the time, and the moment of starting the measurement*.

The number of particles bombarding a target can be also estimated by placing the target in a glass Faraday cylinder and measuring the quantity of electricity transferred by particles bombarding the target by means of an electronic integrator. This was the new approach applied *for the first time* in our experiments.

It is necessary to note that considerable energy is released at the proton bombardment of a target. To avoid the thermal decomposition of a target in the course of irradiation, in our experiments, the Faraday cylinder was cooled by flowing water (Sapozhnikov et al. 1961, 1964; Vartapetian et al. 1965; Saakov 1963a, b, c, 1965a, b). Because of the large leak of electricity due to conductivity through water, measurements of the current with the help of an electronic integrator became impossible.

In this regard, in a number of experiments (Sapozhnikov et al. 1961; Saakov 1963a, b, c), measurements of the current of the proton beam were performed by periodically placing the special Faraday glass in the path of the ionic beam for a period of 1 min. This approach had the disadvantage that during the period between measurements, the intensity of the current could change uncontrollably.

We always paid attention to the importance of controlling the line current (see Chap. 1), because the absence of control leads to *inexact* and, even worse, *inconsistent results*. The author of various works (Shneour 1961, 1962a, 1962b; Shneour and Calvin, 1962) paid attention to the absence of homogeneity of the proton beam and of its necessary control. More often, authors do not mention the control elements in their works, which is especially true for biochemists.

In the process of perfection of the experimental technique, simultaneously with the measurement of the current with the help of an electronic integrator, a scintillation γ-spectrometer was used to measure the yield of γ-rays emitted by a tantalic substrate (on which the target was put) in response to irradiation by protons as a result of the reaction of Coulomb excitation (Saakov 1963a, b, c, 1964, 1965a, b; Saakov et al. 1969, 1970a, b). The yield of γ-rays in this case is proportional to the quantity of protons falling on a target.

To check the stability of the irradiation mode, the same target with the violaxanthine preparation was repeatedly irradiated with protons. The interval between irradiations was 28 h. Results of such irradiation are presented in Fig. 3.89. From the plotted data (Fig. 3.89b), it follows that the difference between registered repeatedly induced activities is not significant; the proton beam is homogenous and is operating stably.

Geometrical positioning of β-particle counters was adjusted within ±0.5 % accuracy using the special standard radiating β-particles. This allowed us to obtain comparable absolute values of the radioactivity of targets using different equipment.

The curve of radioactive decay consists of two parts. One part is more abrupt and shows the presence of nuclei of short-lived isotopes in the target (Fig. 3.89a). The second part is flatter and is characteristic of the manifestation of decay of isotopes

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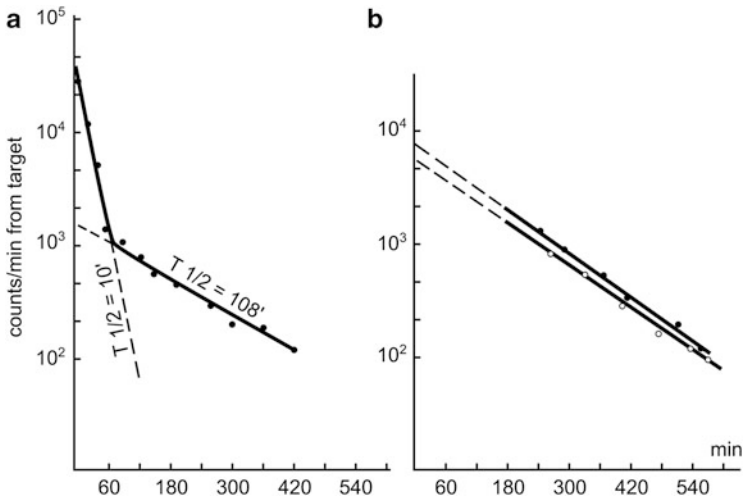

Fig. 3.89 Dynamics of radioactive decay of targets irradiated with protons: (**a**) the curve of radioactive decay; (**b**) dynamics of radioactive decay at a consecutive double irradiation of the target with protons (the interval between irradiations was 24 h)

with longer half-life periods (108 ± 12 min). The measurement of γ-quanta determined a maximum energy of 0.511 meV, testifying to the positron nature of radioactive decay.

From studying the character of curves of the half-life period, we came to a conclusion about the necessity and expediency of the measurement of induced radioactivity of preparations within 18–24 h. Determination of the F^{18} half-period should be made not earlier than 2–2.5 h after irradiation of a target by protons. It is recommended that measurements of the induced radioactivity in targets are performed within 18–24 h.

3.5.2 *Application of the Reaction* $O^{18}(\alpha,n\gamma)Ne^{21}$ *for Determination of the Oxygen Content in Biological Objects*

In the late 1950s, Serdyukova et al. (1957) carried out profound research on the reaction (α,n) for oxygen, which was described for the first time (Hornyak et al. 1950; Bonner et al. 1956). Serdyukova et al. (1957) found that the reaction (α,n) proceeds, mainly, for the isotope O^{18}. The top limit of the cross section of the reaction (α,n) for O^{17} was about 10 % of the cross section of the reaction for O^{18}. The found energy for the first excited level Ne^{21} is equal to 350 keV. With the development of γ-spectrometry, the difficulties connected with the determination of

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half-life periods of isotopes were eliminated by the use of scintillation or semiconductor γ-spectrometers.

The discrete character of the γ-spectrum allows identification of radioactive isotopes and the determination of their content by comparison with a standard, even in the case of the analysis of a mixture containing a considerable quantity of radioactive isotopes. This work was an incitement for the improvement of approaches for the analysis of the O^{18} isotope.

When carrying out our biochemical experiments, Lemberg et al. (1966) suggested an original approach for the determination of the content of isotope O^{18}, which was based on the use of the activation reaction $O^{18}(\alpha,n\gamma)Ne^{21}$. Approximately 70 % of the Ne^{21} nuclei formed in the specified reaction are in the excited state. At their transition in the ground state, γ-quanta of the energy of 350 keV are emitted. In another study (Temmer and Haydenburg 1956), the corresponding background line was observed in the spectrum of γ-rays in research on the Coulomb excitation (CE) of substances containing oxygen impurities, with the help of α-particles.

The described technique was used in a number of biochemical studies (Vartapetyan et al. 1966; Saakov et al. 1969, 1970a, b; Vartapetian et al. 1967). The successful application of this method was reported by one of the authors of this book at the sixth international symposium on the use of stable isotopes, at the Institute of Stable Isotopes (GDR Leipzig) in October 1969.

The main feature of the discussed method was that γ-quanta were detected, not neutrons, and that α-particles accelerated in a cyclotron were applied as the bombarding particles.

The collimating circular diaphragm in the ion guide ensured that the bombarding particles only hit on that surface of the target that was covered with the investigated substance. The substrate with the target was in the center of the Faraday cylinder.

In special experiments, we studied the optimum energy of α-particles, at which the yield of γ-rays is great ($E_\gamma = 350$ keV) and the value of background effects is insignificant. This energy was found to be equal to 4.6 meV. In experiments, beams of helium ions of 10^{-8} A were used. Duration of the irradiation of the target by the beam of particles was 5–10 min. During target irradiation, the spectrum of γ-rays was registered with the help of a multichannel pulse-amplitude analyzer.

The corresponding spectrum is shown in Fig. 3.90. From this figure, it can be seen that at the 80 % reduction in O^{18} content comparatively to control, the spectral peak inherent is nevertheless sufficiently expressed, with corresponding 80 % smaller amplitude. The presented figure concerns the assessment of determination of the absolute sensitivity of the method.

The target used was 50 μg of violaxanthin. The relative content of oxygen in it was approximately 5.3 μg, and the content of the O^{18} isotope was on the order of 2×10^{-9} g. This was the absolute sensitivity of the method.

Unlike the analysis of O^{18} described earlier, the advantage of this method consists in the low power of the particle beam (0.05 W). Therefore, targets were

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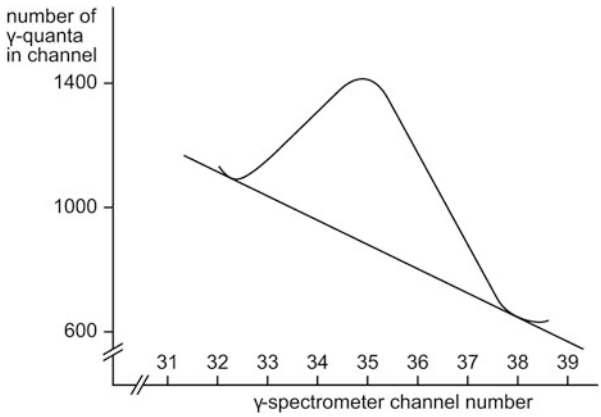

Fig. 3.90 The spectrum of γ-rays in the pulse-amplitude analyzer during the reaction $O^{18}(\alpha,n\,\gamma)Ne^{21}$

not destroyed because of heating, and a unit for cooling of the Faraday cylinder was not required. The additional benefit was the possibility of oxygen determination right after irradiation, instead of the many-hour measurement of decreasing F^{18} radioactivity, with the help of the end-window counter of β-particles.

When analyzing O^{18} in this way, it is also important to know the weight of substance and the number of particles bombarding it. The latter is determined from measurement of the quantity of electricity transferred by bombarding particles, by means of the Faraday cylinder and the electronic current integrator. For the comparative assessment of the O^{18} content in various samples, results of measurement should be normalized to the weight of the sample and to the number of α-particles bombarding the target. Questions of the accuracy of normalization to the number of bombarding particles with the help of the current integrator are described in detail in a master's thesis (Chibirova 1971).

The normalization of the quantity of particles irradiating the target is of vital importance and defines the correctness and the reliability of discussed scientific results. In the publications of European and overseas authors, scientists have not paid attention to this problem at all. This has resulted in disagreements over the interpretation of results of the performed experiments.

In the process of the further improvement of the activation analysis technique, it was suggested to use for normalization the spectra of γ-lines appearing as the result of Coulomb excitation of nuclei, the quantity of which is strictly constant during the irradiation of biochemical preparations. In this case, the area of peaks of the γ-spectra is proportional to the number of α-particles used for the irradiation of the biochemical preparation and can serve for normalization at each exposition (Fig. 3.90).

The Coulomb excitation of nuclei is caused by the long-range electromagnetic influence of charges of nuclei of the target and of the attacking particle. The nucleus excitation is de-energized by emission of the γ-quantum or by conversion of the electron. If the attacking particle is not elementary, the collision can lead to its excitation. The character of electromagnetic forces is well studied, which

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allowed the strict theory of the Coulomb excitation (CE) to be developed (Lemberg 1963).

The used substance should possess the ability to be well sprayed, as a thin layer is necessary. At the same time, this substance should have a high fusion temperature to avoid its evaporation during irradiation by bombarding particles. *Vanadium-51* satisfies these conditions. At CE of the first level of vanadium, γ-quanta of $E_\gamma = 320$ keV are emitted (Fig. 3.91). Thus, for simplification of calculations, it is possible to calculate the triangle area instead of the function integral.

Vanadium was sprayed on a nickel grid, which was placed near to a target such that the beam of α-particles irradiating the target was partially blocked by the grid (Fig. 3.92). The necessary thickness of vanadium sprayed onto the grid was calculated to fulfill the condition that the yield of γ-quanta at CE was comparable to the yield of the reaction $O^{18}(\alpha,n\gamma)Ne^{21}$. The detailed calculation of the vanadium quantity and the technique of its spraying are presented in the work by Chibirova (1971).

For our experiments (Chap. 5), the quantity of vanadium that it was necessary to spray onto a grid to obtain a yield of γ-quanta of vanadium CE comparable to the yield of γ-quanta of the reaction $O^{18}(\alpha,n\gamma)Ne^{21}$ corresponded to a vanadium layer of 29.2 μg/cm^2. Taking into account the porosity of the grid (5:1), the obtained quantity of vanadium required for dusting was equal to 146 μg/cm^2. In experiments, we used a grid with a thickness of vanadium of 250 μg/cm^2.

To observe the lines of O^{18} vanadium in the γ-spectrum, the coaxial detector *p-i-n* of good resolution was used. The photoefficiency of this detector was found with the help of calibrated sources placed in the target location. The energy resolution of the used detector was 40 keV for a photocurrent of γ-quanta of energy 662 keV (Chibirova 1971).

Impulses arriving from the preamplifier were carried by wire to the measurement room. After amplification by a spectroscopic amplifier (such as Enterprise), the impulses entered a multichannel analyzer of "Intertechnique" type, where the amplitude analysis was performed. For control of the necessary intensity of the beam, impulses from the amplifier were output through the single-channel discriminator at the intensitometer, the portable device of which was placed on the control panel of the cyclotron. Data from the multichannel analyzer were transferred to the digital printer and to the recorder.

Measurements showed that the relative content of oxygen in the substrate was on average equal to 0.027 or 0.03 relative units. This value was considered to be the background defining the background of the substrate. The background from O^{18} being present in the substrate did not exceed 10 % of signal of O^{18} contained in the sample. Subtraction of the average value of the background did not cause a large error in the results of measurements. Figure 3.93 presents an example of the analysis of the impulse amplitude for γ-spectra of the background of the substrate and of the vanadium-51 grid.

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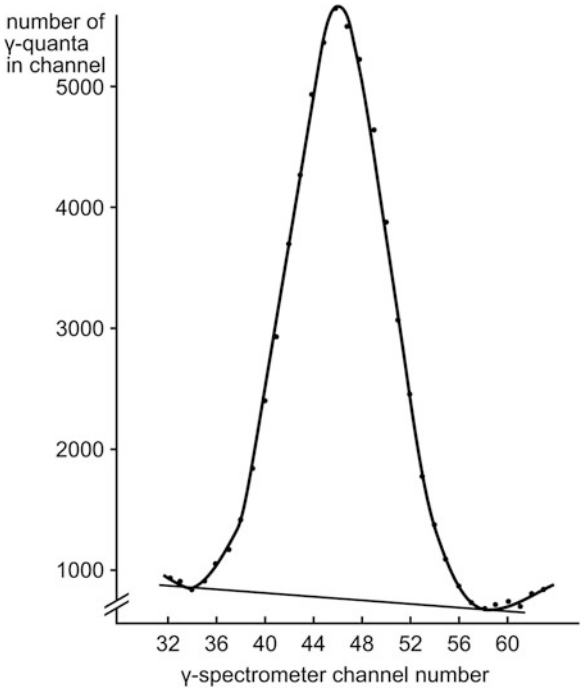

Fig. 3.91 The spectrum of γ-rays in the pulse-amplitude analyzer during the reaction $O^{18}(\alpha,n\,\gamma)Ne^{21}$ when normalized to PbO^{18}

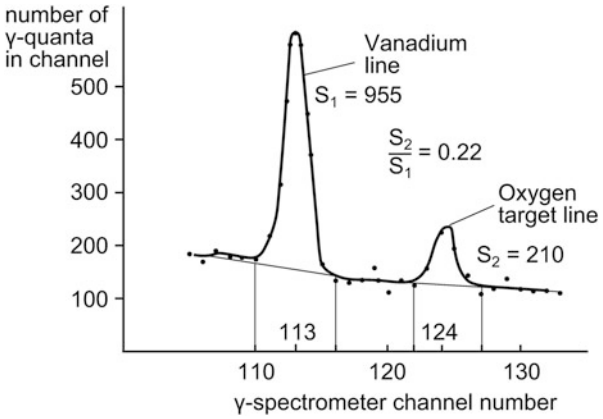

Fig. 3.92 The spectrum of γ-rays in the pulse-amplitude analyzer during the irradiation of oxygen O^{18} and vanadium by α-particles

Thus, by satisfying all requirements of the analysis, we achieved the correctness of the application of the radioactivation method for isotope O^{18} detection in biochemistry.

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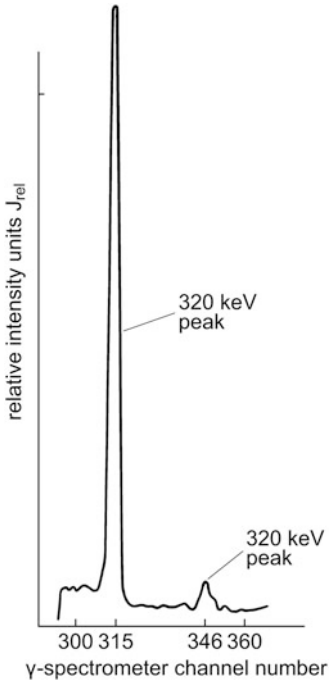

Fig. 3.93 The amplitude of impulses of γ-spectra of the background of the substrate and of the *vanadium-51* grid

3.5.3 *Manufacturing of Targets and Choice and Preparation of Substrates for Radioactivation Analysis*

When choosing the material of the substrate, the first condition is the necessity of taking into account that during the target irradiation the induced radioactivity should not be induced in the underlying surface or matrix substrate or should be characterized by a small half-life period.

The substrate material should be very pure, because the small content of the contaminating element can result in intensive background radioactivity. In pioneer works of this direction, tantalum or platinum matrix were used (Fleckenstein et al. 1959, 1960, 1964; Fleckenstein 1960; Fogelstrom-Fineman et al. 1957). In our first experiments tantalum substrates were also used (Sapozhnikov et al. 1961, 1964; Saakov 1963a, b, 1964, 1965a, b).

We carried out preliminary experiments to compare the radioactivity induced by tantalum and aluminum under experimental conditions. Table 3.4 shows the comparative activity of tantalum and aluminum substrates at various times after their irradiation by protons of $E_p = 4$ meV and current of 10^{-7} A for 10 min.

From the data in Table 3.4, it follows that the use of tantalum matrix substrates is more preferable as it enables working at a much lower level of induced radioactivity.

Tantalum, matrix of diameter of 28 mm served as the bottom of the Faraday cylinder. With the help of a willemite screen, focusing lenses, and diaphragms, the

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Table 3.4 Comparative activity of tantalum and aluminum underlying matrix substrates at various times after irradiation by protons

Material of substrate	Activity after irradiation by protons, counts/min		
	5 (min)	10 (min)	15 (min)
Tantalum	368	128	49
Aluminum	1,072	800	448

cross section of a beam of protons falling onto a target was set to be such that it coincided in form and size with a target deposited in the center of the tantalum substrate (diameter 10 mm).

When we detected O^{18} on the basis of the nuclear reaction $O^{18}(\alpha, n\gamma)Ne^{21}$, we changed the substance of the substrate because of the insignificance of power obtained on targets. In this case, substrates were copper disks of 28 mm diameter, on which nickel was electrolytically applied.

The thickness of the underlying surface, matrix was equal to 4 mm. The weight of the material was chosen by calculation in such way that at real expositions the heating of a target did not exceed 40 °C. For convenience, a special cartridge of a drum type was used, in which were inserted 11 targets; the willemite screen served for beam adjustment. On the beam path, before the target, there was a type-setting collimator and a frame with the vanadium grid. The necessary target of the chosen series was placed under the beam by turning the cartridge axis.

Application of the preparation of the investigated substance on the substrate requires the observance of some conditions:

1. The matrix material should not contain atoms of oxygen.
2. It is necessary to know exactly the quantity of the applied substance.
3. The area occupied by the investigated substance should not exceed the area of the cross section of the charged particle beam.
4. The thickness of the applied layer of the substance should be much less than the proton path. Losses of energy of protons of $E_p = 4$ meV in the organic environment are approximately 14 meV/mg/cm^2.
5. The uniform application of the substance by a glass capillary (instead of a syringe needle) within the appropriate area of the wafer (diameter 10 mm).
6. The substrate material should be an element with a large-enough atomic number that, at the chosen energy of α-particles, nuclear reactions leading to an increase in the induced background radiation do not take place.
7. There should be permanent control of the energy of charged particles.

In biochemical research, small quantities of substance are used (<100 μg); therefore, the targets are thin (their thickness is small in comparison with the path of α-particles). The yield of γ-quanta in a thin target depends on its thickness. If the target is nonuniform, then in the same target, a nonuniform beam of particles will result in a different yield of γ-quanta for the thicker and thinner parts of the target. For the exact determination of O^{18}, it is necessary to have a homogeneous thin target and a homogeneous beam of particles.

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To produce homogeneous targets of biochemical preparations is difficult. Therefore, we used uniform irradiation of a target by applying beam scanning. The focusing of charged particles emitted from a cyclotron was carried out with two quadrupole lenses on the willemite screen. A lens focusing a beam in a vertical plane was connected to a scanning sawtooth voltage. As a result, a scanning beam in the form of a vertical strip was obtained. Application of beam scanning allowed us to achieve uniform irradiation of a target and, thus, exclude the errors appearing because of heterogeneity of a target.

A radiochemically pure fraction of pigments (see Sect. 3.5.4) was applied in the center of copper–nickel substrates. The accuracy of the application of the investigated substance on the substrate was assessed in preliminary experiments. For this purpose, the quantitative content of the pigment in the solution before and after application on a substrate was determined. The change in pigment concentration in the course of application was judged by the change in the optical density in the absorption maximum normalized to a certain volume (see Table 3.5). As a rule, the difference between parallel targets was within ± 8–10 %. An example of the experiment is presented in Table 3.5.

It is necessary to emphasize that in the course of application and elution of the substance from a substrate, its partial loss occurs. On the basis of experience, we came to the conclusion that the value of this loss sometimes reaches 18 % of the amount of substance applied. This loss in the process of application exceeded the difference between the data from parallel targets.

In actual practice of the cyclotron work, the energy of accelerated particles can change by 1–1.5 %. At such change in the energy of α-particles, the yield of γ-quanta emitted during the Coulomb excitation changed only slightly. However, in some cases, the resonant character of the reaction $O^{18}(\alpha, n\gamma)Ne^{21}$ can lead to a considerable change in the yield of γ-quanta of energy of 350 keV.

Investigation of this question (Chibirova 1971) showed that the relative yield of γ-quanta of $E_\gamma = 320$ keV and $E_\gamma = 350$ keV does not significantly change if the energy of α-particles changes by 1–2 % after the fourfold irradiation of the thin inorganic target CuO. This means that the oxygen content in the target practically does not change:

$350/320 = 165 \pm 0.8$ (I); 169 ± 0.18 (II); 172 ± 0.18 (III); 154 ± 0.18 (IV).

Unlike the works connected with the use of a proton beam and the simultaneous cooling of a substrate with flowing water, the application of α-particles forces the researcher to turn greater attention to the *possible burning out of the organic target* in the course of irradiation. This circumstance becomes important in the case of repeated irradiation of the same preparation.

Preliminary experiments demonstrated the good convergence of the yield of induced radioactivity of parallel samples and also showed the influence of repeated irradiation on the yield of the activation reaction per 100 μg of pigment and 1 μC of a transferred charge. At primary irradiation, for example, of light variants of antheraxanthin (with the isotope O^{18}), the yield was equal to 214, 260, and 264 relative units; at the repeated irradiation of the same targets, it was 120, 162, and 137 relative units (Saakov et al. 1970b). By experiment, it was determined that

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Table 3.5 The change in optical density of antheraxanthin at elution from substrates

Initial OD (relative units)	Target number	OD of substance after elution from the matrix	Deviation from the average OD value	Deviation from the average value (%)
0.482	1	0.265	−0.032	−11
	2	0.290	−0.007	−3
	3	0.336	+0.039	+13
	4	0.324	+0.027	+9
	5	0.289	−0.008	−4
	6	0.343	+0.046	+15
	7	0.308	+0.011	+4
	8	0.291	−0.006	−2
	9	0.260	−0.037	−12
	10	0.264	−0.033	−11
Average value		0.297	+0.024	±8

Table 3.6 Dynamics of the change in specific activity of diadinoxanthin isolated from *Euglena* in the process of re-chromatography (imp/100 s μg of carbon)

	Solvent systems in experiments[a]				Specific activity in experiments (imp/100 s μg carbon)			
No.	Experiment I	Experiment II	Experiment III	Experiment IV	I	II	III	IV
1	P:A (6:1)	P:A (6:1)	P:A (6:1)	P:A (6:1)	485	261	236	1,028
2	P	P	P	P	455	185	219	987
3	P:B:M (22:5:1)	P:B:M (22:5:1)	P:B:M (22:5:1)	P:B:M (22:5:1)	450	168	187	813
4	P:M (30:1)	P:Et (30:1)	P:B:M (22:5:1)	P:A:Et (6:1:0.5)	408	155	175	783
5	B:P (3:1)	P:A:Et (6:1:0.5)	P	P:A (5:1)	380	168	168	753
6	P:Et (30:1)	P:A (6:1)	P	P:Et (3:1)	360	166	155	746
7	Det:Et	P	B:P (3:1)	P:A (6:1)	355	160	157	755
8	P:B:M (22:5:1)		P:A (6:1)		367		163	738

[a]Numbers in brackets indicate the proportion of components
P Petroleum ether, *P:A* petroleum ether–acetone, *P:B:M* petroleum ether–benzol–methanol, *P:M* petroleum ether–methanol, *P:Et* petroleum ether–ethanol, *B:P* benzol–petroleum ether, *Det:Et* diethyl ether–ethanol, *P:A:Et* petroleum ether–acetone–ethanol

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repeated irradiation of the target resulted in an average decrease of 38–44 % in the yield of induced radioactivity.

The method of introduction of the isotope O^{18} in investigated compounds or biological objects is defined by goals of the experiment and by the chosen object for each study (Saakov 1963a, b, 1964, 1965a, b; Saakov et al. 1969, 1970a; Sapozhnikov et al. 1961, 1964, 1967; Fogelstrom-Fineman et al. 1957). This isotope can be introduced by contact of the leaf surface with H_2O^{18}, by suspending seaweed or chloroplasts in medium containing H_2O^{18}, or by infiltration of heavy water into leaves.

The radioactivation analysis of the isotope O^{18} allowed *for the first time* information to be obtained on the inclusion of the oxygen of water molecules into the phosphate groups of ATP (Fleckenstein 1960; Fleckenstein et al. 1959, 1960; Fleckenstein and Janke 1964) and into the primary products of photosynthesis (Fogelstrom-Fineman et al. 1957). Oxygen inclusion into stores of vitamins A and K (Vartapetian 1970; Vartapetian et al. 1966, 1967) and of a number of carotenoids (Saakov 1963a, b, 1964, 1965; Saakov et al. 1969, 1970a, etc.) has been studied.

3.5.4 *Purification of Pigment Preparations and Their Radiochemical Purification*

In the period from the middle to the end of the twentieth century, there appeared an indescribable variety of works devoted to various approaches to chromatographic methods of isolation of plastid pigments, and all of them were based on the method suggested by Tswett (1906). From this heritage of several hundreds, we will refer to some publications concerning the radiochemical purification of pigments (Hager 1955, 1957; Jeffrey 1961, 1965, 1968; Sestak 1958, 1959, 1965, 1967; Shlyk 1955, 1959).

The considerable divergence in methods of pigment isolation noted in the literature, the unequal interpretation of results of chromatographic analysis, and the appearance on the chromatogram of unidentified isomers from the same fraction of the pigment have induced us to give particular attention to methodological questions of pigment isolation. The main push for carrying out this analytical work was the total absence of attention to purification of pigments in articles of European researchers working with isotope material, and also our detection on chromatograms of a great number of colorless impurities in zones painted by pigment stains. In isotope experiments, these impurities altered the specific activity of fractions of pigments in the processes of their biosynthesis and preparative isolation. They could lead to artifacts in the course of research on the metabolism and interconversion of pigments.

Any one chromatographic system does not result in the simultaneous complete separation of all plastid pigments. Results of researchers demonstrated the

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unlimited possibilities in the selection of favorable mixtures of organic solvents and in choosing their ratio in a mix (Stanishevskaya 1962, 1964; Sestak 1965, 1967). Any system of solvents for the chromatographic separation of pigments should possess the following properties: A short space of time (1 h) should be needed to differentially separate the main fractions of pigments. The development should not exceed 20–30 cm. The mixture of solvents should not promote destruction of pigments.

Another important factor is the high toxicity of the solvent mixture, because often works with pigments are performed in dark rooms with bad ventilation. Mixtures containing a high percentage of benzene are the most commonly advertised and used systems of solvents for separation of pigments. Let's start with last item (point). The mixes containing benzene, acetone appear in the majority promoted systems of solvents for division of pigments, petrol ether, ethanol and metanol.

Before the characterization of these mixes, we will discuss the toxicological properties of benzene. We will quote a paragraph from the known monograph *Organic solvents* edited by Weissberger (1955):"Benzene is the accumulated poison; as a result of the repeated influence of its small quantities the severe anemia and even death take place in many cases."

Poisoning with benzene occurs almost exclusively through the respiratory tract. Although only small amounts of benzene can penetrate through the skin, Patty (1949) is sure that systematic poisoning is also possible as a result of steeping hands in benzene. At that, such phenomena as skin degreasing, erythema, peeling, and secondary infection can be observed.

It was established that the maximum permissible concentration of benzene was 0.002–0.01 % (Brooks 1937; Drinker and Cook 1949). At the present time, this value has been set to 0.0035 % (American Conference of Governmental Industrial Hygienists 1953 8:246; 1954 9:530). Thus, the class of toxicity of benzene has been increased.

Besides, benzene is very explosive. If half a sheet of A4 format paper is wet with benzene and touched with half a cigarette, it will immediately burst into flames with an explosive clap.

We write all this in order to warn experimenters to be very cautious of benzene. Unfortunately, former students forget university instructions on the safety precautions for working with organic solvents, but in the laboratory, employees should take the studying of safety precautions indulgently and formally.

We write about being careful when working with benzene because no instructions had made an impression until one of the authors performed the experiment with a sheet of paper and a lit cigarette.

Despite the widely advertised technique of carotenoid separation (Popova 1965; Bazhanova et al. 1964; Sapozhnikov et al. 1955, 1956) with the use of mixtures containing a high percentage of benzene, the application of these methods in isotope research became completely unsuitable because of the indistinct fractionation of pigments and also due to the presence of radioactive colorless compounds. The problem arose to decrease quantities of benzene used in mixture of solvents for

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chromatographical analysis at accumulation of pigments preparations and to limit its negative influence on personnel. This problem was caused by the poorly functioning ventilation system which didn't perform air changes of the working room and for a long time benzene vapours accumulated in the laboratory and influenced on reserachers health.

At the early stages of our work, we used recommended procedures (Popova 1965; Bazhanova et al. 1964; Sapozhnikov et al. 1955, 1956), but quickly rejected them after assuring ourselves that those techniques and approaches were absolutely not suitable for reliable radioisotope research.

The main approaches of the technique used by us are stated in various publications (Saakov 1971a, b, c; Saakov and Shiryaeva 1967; Saakov et al. 1969; Lutsenko and Saakov 1969, 1973; Nazarova et al. 1971). The primary system of solvents used for separation of pigments was the mixture of petroleum ether (boiling temperature, T_{boil}, 40–60°) and acetone (P:A, 6:1). This mixture was used as the first system for one-dimensional or two-dimensional chromatography. Good separation of pigments from saponified extracts was realized using the mixture petroleum ether–benzene–ethanol–methanol (22:5:0.5:0.5). This mixture was rarely used, applying it during the second direction of two-dimensional chromatography. Usually the mixture petroleum ether–chloroform (3:1) was used. The range of application of the discussed systems of solvents was checked by long-term work with various vegetative objects (deciduous, grassy, seaweeds).

Verification of the expediency of application of chromatographic paper of European brands such as Whatman № 1, 2, 3; Schleicher ET-81, AE-81, 3-81; and Schuell 2043, 2071, 2247 revealed their sufficient analytical ability. However, for system of solvents P:A (6:1), the quality of pigment separation on these types of paper was not better than on the paper "Leningrad fast" (Nazarova et al. 1969, 1971; Saakov 1963a, b, c; Saakov et al. 1969; Saakov and Shiryaeva 1967; Saakov and Saidov 1965).

Comparative research of systems of solvents for thin-layer chromatography (Hager and Bertenrath 1966, 1967) and suggested by us (Saakov and Shiryaeva 1967; Nazarova et al. 1969, 1971) was performed with the assistance of Prof. Dr. H. Sagromsky (IPK, Gatersleben, Germany) and Dr. Prof. Paul Hoffmann (Humboldt Universität zu Berlin, Germany). This comparison showed that with variations in the polarity of the system, application of P:A (6:1, 5:1, 4.5:1, 4:1, and 3:1) leads to good separation of pigment fractions from saponified and non-saponified extracts.

The comparison of techniques of thin-layer chromatography (Bollinger 1962; Egger 1962; Egger and Voigt 1965; Hiyama et al. 1969; Jeffrey and Allen 1967; Randerath 1965; Sestak 1967; Sherma and Zweig 1967a, b; Stahl et al. 1963) led us to prefer the approach and method of Hager and Bertenrath (1966, 1967). The technique of Kornyushenko and coworkers (Kornyushenko and Popova 1970; Kornyushenko and Sapozhnikov 1969), recommended and advertised in Russia, is a trivial bad and rough (coarse) compilation of that technique (Hager and Bertenrath 1966, 1967).

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3.5.4.1 Radiochemical Purification of Carotenoids

Unlike the extensive literature material on the application of radioisotope techniques in studying of chlorophyll metabolism and its radiochemical purification (Shlyk 1955,1965; Gaponenko 1976; Shlyk et al. 1958), there are a very small number of publications on approaches based on the radiochemical purification of carotenoids (Akhramovich 1972; Godnev and Lipskaya 1965; Godnev, Rotfarb 1962a, b; Saakov 1963a, b, c; Saakov and Shiryaeva 1967; Losev 1964).

Introduction of the method of high-pressure liquid chromatography (HPLC) did not remove the problem of radiochemical purification of preparations when carrying out fine isotope research.

Such circumstances are obviously connected with the high lability of yellow pigments, which defines the complexity of preparative isolation of pure carotenoids free from the colorless compounds accompanying them. These last compounds, being adsorbed together with zones of carotenoids on a paper or in a thin layer, strongly alter the specific activities of pigments and lead to artifacts in the work. This point of view is supported by known experts in the isolation of pigments (Shlyk 1955; Kutyurin and Artamkina 1962; Kutyurin et al. 1962; Dzhawrshyan 1967; Rotfarb 1970; Khodasevich et al. 1966; Anderson et al. 1960; Blass et al. 1959; Costes 1965a, b, c; Fischer et al. 1962). The specified authors underline the necessity of repeated acts of chromatography of pigments fractions in order to achieve the necessary level of radiochemical purity. Application of the HPLC does not remove this requirement. It is exactly in this connection that we criticized the works of Goodwin et al. (Williams et al. 1967; Goodwin and Williams 1965a, b; Goodwin 1969, 1971) in Chap. 1.

The absence of a uniform technique for carotenoid purification resulted in the necessity to develop reliable approaches for the purification of pigments according to problems faced in our research (Saakov 1963a, b, c; Lutsenko and Saakov 1969, 1971, 1972, 1973; Nazarova et al. 1969, 1970, 1971; Saakov et al. 1971; Saakov and Saidov 1965; Saakov 1970; Saakov and Nazarova 1970). The most complete work in this direction was the publication by Saakov and Shiryaeva (1967).

The necessary stage prior to the beginning of chromatographic separation of pigment fractions is the saponification of extracts, which leads to the removal of chlorophylls and of some colorless impurities. The details of operations of saponification are described (Saakov 1963a; Saakov and Shiryaeva 1967). Depending on the object of research, the saponification causes the loss of 18–24 % of the radioactivity of every unsaponifiable fractions of carotenoids. This result showed that it is possible and necessary to consider saponification as the primary link in the radiochemical purification of carotenoids.

After isolation of separate fractions of the main carotenoids, we carried out the repeated sequential re-chromatography of each fraction in replaced systems of solvents until we achieved a constant specific radioactivity of the fraction. Carotenoid preparations applied on a substrate (wafer) were thin enough not to need correction for self-absorption of β-particles in a preparation layer (Shlyk

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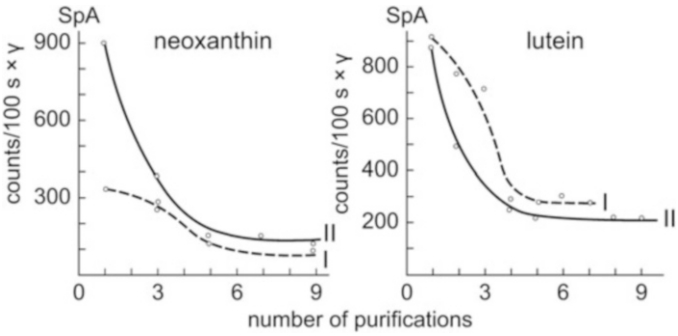

Fig. 3.94 The influence of a number of acts of sequential chromatography on the change in specific activity of preparations of neoxanthin and lutein isolated from leaves of peas (*I*) and corn (*II*). Systems of solvents used were (1) P/A (6:1); (2) P/A (5:1); (3) P/benzene/methanol (22:5:1); (4) P/chloroform (3:1); (5) P/diethyl ether/methanol (30:30:1); (6) P/methanol (3:1); (7) Benzene/P/A (10:2,5:2); (8) pure P; (9) P/A (6:1). The fraction of petroleum ether of $T_{boil} = 40–60$ °C was used

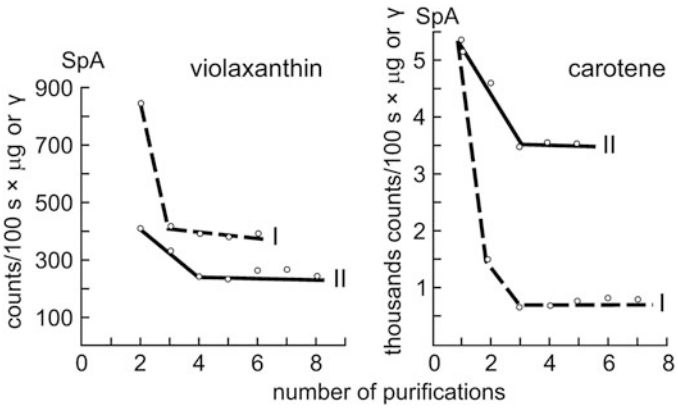

Fig. 3.95 The influence of a number of acts of sequential chromatography on the change in the specific activity of preparations of violaxanthin and carotene isolated from leaves of peas (*I*) and corn (*II*). The systems of solvents used were (1) P/A (6:1); (2) P/A (5:1); (3) P/benzene/methanol (22:5:1); (4) P/chloroform (3:1); (5) P/diethyl ether/methanol (30:30:1); (6) P/methanol (3:1); (7) benzene/P/A (10:2,5:2); (8) pure P; (9) P/A (6:1). The fraction of petroleum ether of $T_{boil} = 40–60$ °C was used

et al. 1959). The specific radioactivity of carotenoids was expressed in impulses per minute per microgram of carbon or of a pigment, depending on the experimental purpose. Examples of purification are shown in Figs. 3.94, 3.95 and in Table 3.7. However, to state that this criterion, namely, the presence of a constant specific radioactivity of the preparation, is enough for high radiochemical purification would be premature and extremely hasty.

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Table 3.7 Changes in the specific activity of carotene fraction with multiple chromatography

No of purifications	Solvent systems[a]		Specific activity (imp/100 s μg pigment)	
	Preparations from *Vicia faba*, Experiment I	Preparations from *Euglena gracilis*, Experiment II	Experiment I	Experiment II
1	P:A (6:1)	P	5,640	4,654
2	Column with Al_2O_3, solvent P	P:A (6:1)	1,500	1,005
3	P:Det:M (30:30:1)	P:B:M (22:5:1)	694	1,190
4	P:Et (14:1)	P:A:Et (6:1:0.5)	702	979
5	P	P:A (6:1)	712	920
6	Repeat, column with Al_2O_3, solvent P	B:P (3:1)	701	945
7	P:B:M (22:5:1)	B:P:A (10:2.5:2)	735	987
8	P:A (6:1)	P:A (6:1)	720	879
9	P	P	738	896

[a]Numbers in brackets indicate the proportion of components
Abbreviations as given in Table 3.6

Work experience has shown that the researcher faces the most considerable difficulties during carotene purification. In this case, in our research, we repeatedly used the combination of column and paper chromatographies. A column with the filler Al_2O_3 can be recommended for (one–two) initial stages of radiochemical purification of pigments (Shukolyukov and Saakov 2001). The column with the applied pigments is washed out using the following sequence of solvents: 100 mL petroleum ether, 100 mL petroleum ether with 10 % diethyl ether, 100 mL diethyl ether with 10 % acetone, and lastly 100 mL acetone. The sum of carotenoids consists of four fractions: carotene, lutein + zeaxanthin, neoxanthin + X-xanthophyll, violaxanthin. The latter is imposed on the violaxanthin fraction. This can be avoided by extension of the adsorbent column height (Shukolyukov and Saakov 2001).

After the fifth chromatography, the preparations of carotene isolated from *Euglena* exposed to H_2O^{18} did not contain impurities in molecules that could contain oxygen. Irradiation of these preparations by a beam of α-particles accelerated in a cyclotron (see Sect. 3.5.2) showed the absence of oxygen contamination of the fraction (Lutsenko and Saakov 1969; Saakov et al. 1969, 1970a). This criterion was already essential, because in earlier works (Sapozhnikov et al. 1961), the fraction of carotene (molecules of which do not contain atoms of oxygen) manifested the radioactivity detected for oxygen.

Our data on the change in the specific radioactivity of carotene isolated from *Euglena* or from the higher plants suggests that separate vegetative objects possess a specific set of colorless compounds inherent in them and accompanying pigments. It means that, probably, there is no necessity to follow the scheme of multiple re-chromatography of pigment fractions for all objects and in all experiments (without a radioactive label). This conclusion points to the specificity of analytical approaches for obtaining radiochemically pure pigments from different objects.

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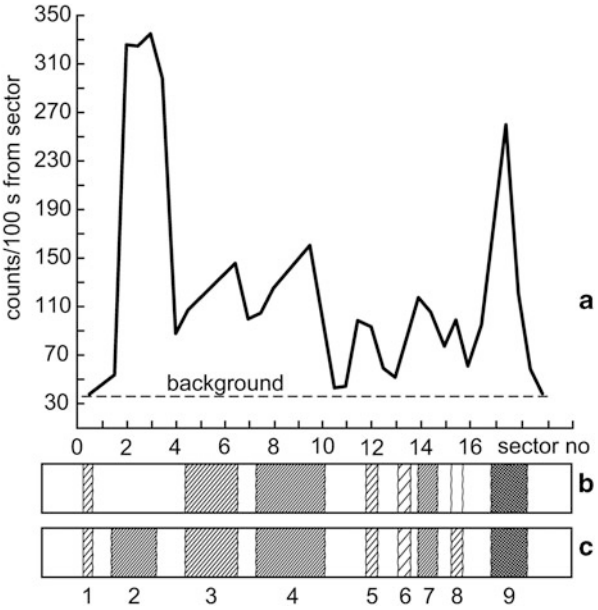

Fig. 3.96 The scheme of radioactivity distribution according to the chromatogram (**a**) for the chromatography of carbon-labeled green extract of pigments (bean, *Vicia faba*; corn, *Zea mays*). The system of solvents was P:A (6:1). (**b**) Visually noted zones of pigments. (**c**) Scheme of the radioautograph from the chromatogram. On this and further figures, the density of hatching defines the dimness degree of zones of the radioautograph. (*1*) the start spot; (*2*) colorless compound; (*3*) the zone of chlorophyll *b* and neoxanthin; (*4*) chlorophyll *a*; (*5*) violaxanthin; (*6*) lutein epoxide; (*7*) lutein; (*8*) pheophytin; (*9*) carotene

The coincidence of radioautograph stains with visually noted zones of pigments on a chromatogram, and the graph of distribution of radioactivity on it after subsequent chromatography of pigment fractions, is one of the indicators of the radiochemical purity of a substance (Saakov 1963a, b, c; Shlyk 1965). Such coincidence demonstrates the degree of correspondence between the distribution of C^{14} radioactivity and the staining of zones of pigments.

Results on the change in the specific radioactivity of carotenoids depending on the number of re-chromatography steps suggest that vegetative objects possess a set of colorless compounds specific for the species and accompanying pigments (Saakov and Nazarova 1970) (Figs. 3.96, 3.97, 3.98, 3.99, 3.100, 3.101, 3.102, 3.103, 3.104, 3.105, 3.106, 3.107, 3.108, and 3.109).

There is no necessity to use many words to describe what the reader will see at once in the presented figures (Figs. 3.96, 3.97, 3.98, 3.99, 3.100, 3.101, 3.102, 3.103, 3.104, 3.105, 3.106, 3.107, 3.108, and 3.109): (1) the discrepancy between the number of zones of colored spots on the chromatograms and the peaks of radioactivity counts and (2) the coincidence of zones of the colorless spots, invisible on chromatograms but revealed in radioautographs, and the curves of chromatogram radioactivity. The manifestation of colorless spots on radioautographs,

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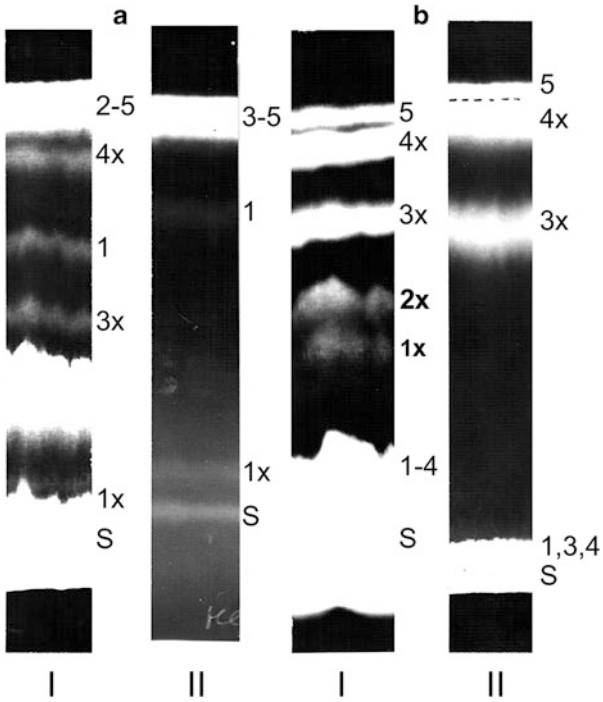

Fig. 3.97 Prints of radioautographs with chromatograms at isolation of neoxanthin (**a**) and of carotene (**b**). (*I*) chromatography of non-saponified extract; (*II*) chromatography of saponified extract; *1–4* numbers of zones of colorless compounds; *S* the starting sport; *1* neoxanthin; *2* chlorophyll; *3–4* xanthophylls; 5 carotene. Systems of solvents: (*I*) benzene/P/ethanol (18:6:1); (*II*) petroleum ether

which sometimes bordered on pigment zones, required additional operations of re-chromatography with changing systems of solvents. Thus, we made sure once more that the criterion of specific radioactivity constancy is not valid.

Figure 3.97 presents material demonstrating the degree of conformity between the radioactivity distribution and stained zones of pigments along the whole length of the chromatogram. Considerable differences in the densities of radioautographs lead to the complexity of their qualitative photographic reproduction. The quantitative dimness of radioautographs is shown in the figures by hatching of various densities.

The fact of the large change in the general content of the pigment compared with the quantity before chromatography and after elution of a pigment can testify to the possible decomposition of the main fraction of the pigment to colorless compounds in the process of repeated purification. The appearance of insignificant radioactivity on the start spot at acts of repeated re-chromatography is noted.

That fact that for some pigments the constant specific activity is only found after three re-chromatography sessions suggests the occurrence of zones of derivatives and of separate colorless compounds from the core chromatographed pigment. This

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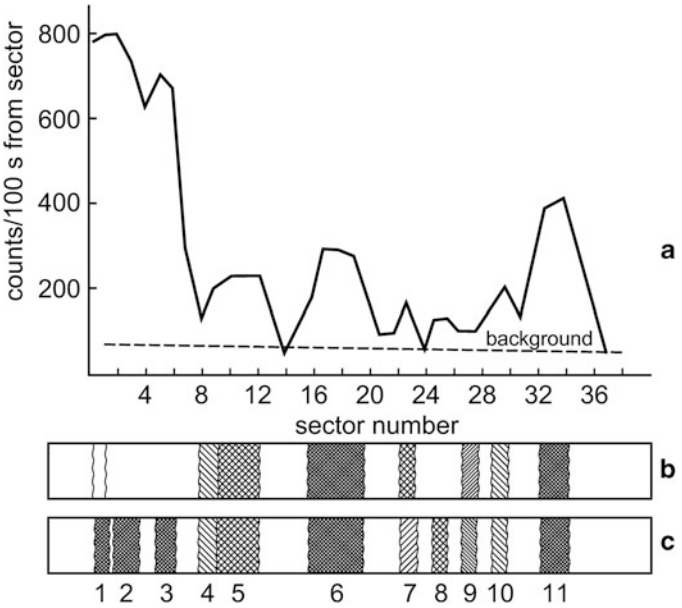

Fig. 3.98 The scheme of radioactivity distribution according to the chromatogram (**a**) at the chromatography of carbon-labeled green extract of pigments from *Euglena gracilis*. The system of solvents was P:A (6:1). (**b**) zones of pigments visually noted on the chromatogram. (**c**) Scheme of the radioautograph from the chromatogram. (*1*) the start line (spot); (*2*, *3*, *8*) zones of colorless compounds; (*4*, *5*) the zone of neoxanthin and of chlorophyll *b*; (*6*) chlorophyll *a* and antheraxanthin (diadinoxanthin); (*7–9*) unidentified compounds; (*10*) pheophytin; (*11*) carotene

remark especially concerns the appearance of derivatives at the third–seventh acts of re-chromatography.

The selection of the solvent system of certain type has a certain value. According to our data, solvents systems containing benzene and chloroform (freshly distilled and kept with the alkali liquor) promote the appearance of stained derivatives and zones of colorless impurities. At the same time, in the process of multiple re-chromatography, the systems of solvents of petroleum ether and methanol, ethanol, or acetone form well-outlined zones of pigments and produce zones of colorless compounds less often. That promotes an appearance of new coloured derivative substances.

To summarize, in this section for the first time in the biochemical literature, an analysis of possible approaches to the optimization of radioactivation methods for the analysis of oxygen isotopes has been made. Conditions and recommendations on the application of reactions of activation of the isotope O^{18} by protons and α-particles in comparative biochemical researches were considered. Problems involved in the preparation of targets and substrates for radioactivation analysis were debated. For the first time, methodological solutions on the preparation of radiochemically pure samples of carotenoids, for application in isotope research of various problems and directions, were suggested and discussed in detail.

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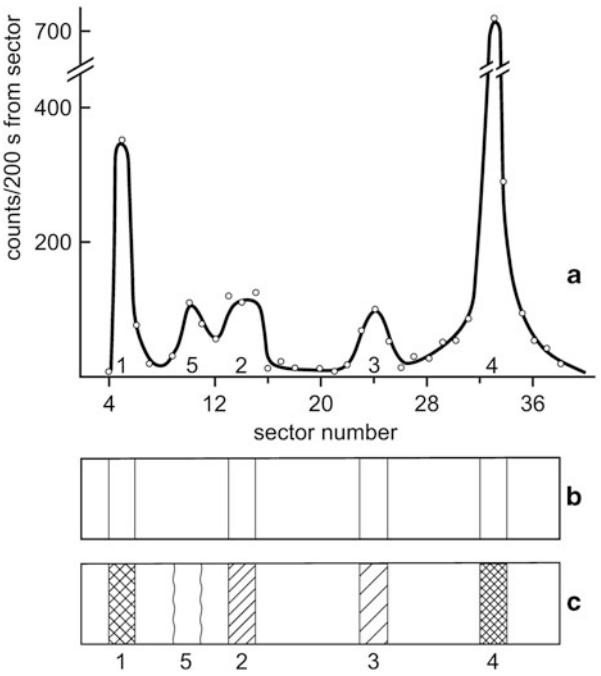

Fig. 3.99 One of the variants of the radioactivity distribution on the chromatogram at separation of carbon-labeled pigments from *saponified* extract from corn sprouts. The system of solvents was P:A (4:1). (*1*) the start line; (*2*) neoxanthin and lutein; (*3*) violaxanthin; (*4*) fraction of carotene; (*5*) colorless compounds

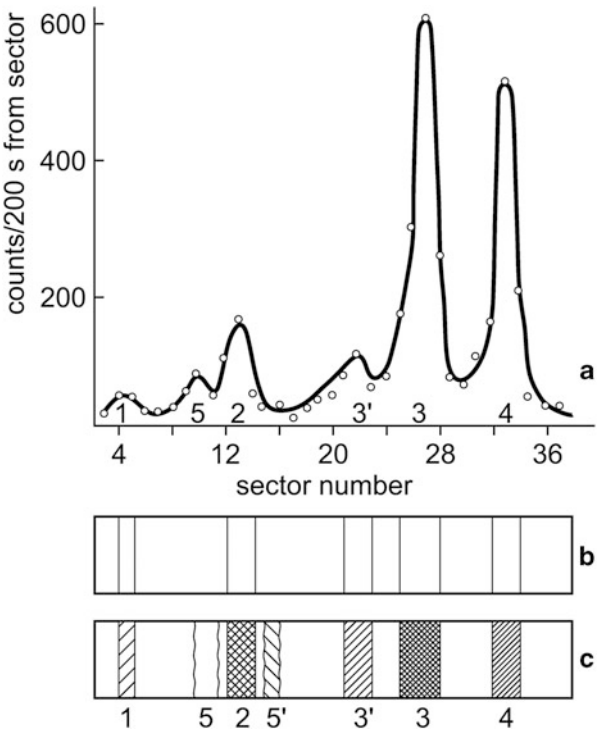

Fig. 3.100 The radioactivity distribution on the chromatogram at separation of carbon-labeled pigments of saponified extract from *Euglena gracilis*. The system of solvents was P:A (6:1). (*1*) the start spot; (*2*) neoxanthin; (*3′*) antheraxanthin + zeaxanthin; (*3*) antheraxanthin (diadinoxanthin); (*4*) carotene; (*5*) and (*5′*) the colorless compounds invisible on the chromatogram

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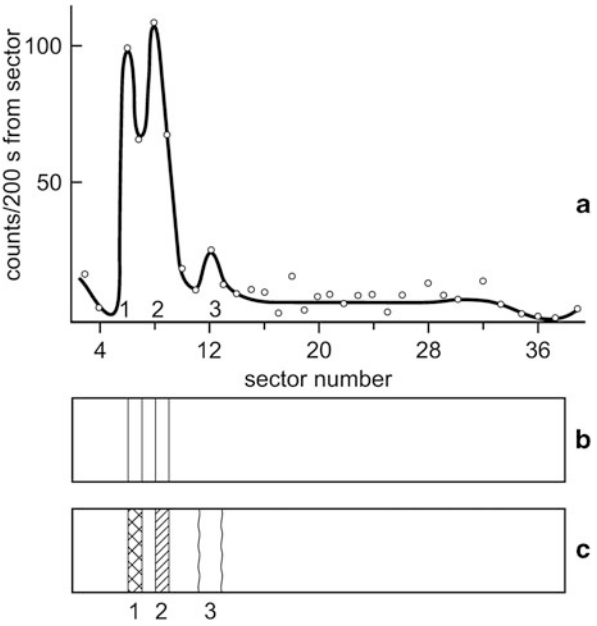

Fig. 3.101 The radioactivity distribution on the chromatogram at the third sequential chromatography of the fraction neoxanthin-^{14}C isolated from cells of *Euglena gracilis*. The system of solvents was P: benzene:methanol (22:5:1). (*1*) the start line; (*2*) neoxanthin; (*3*) colorless compounds

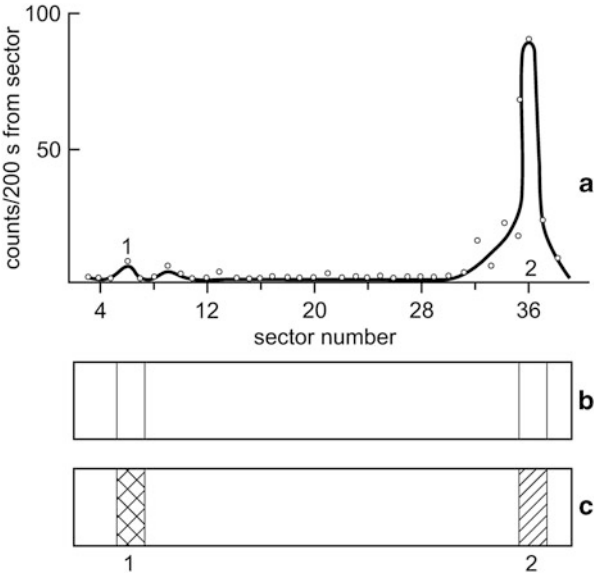

Fig. 3.102 The radioactivity distribution on the chromatogram at the fourth sequential chromatography of the fraction neoxanthin-^{14}C isolated from cells of *Euglena gracilis*. The system of solvents was P:A: ethanol (6:1:0.5). (*1*) the start line; (*2*) neoxanthin

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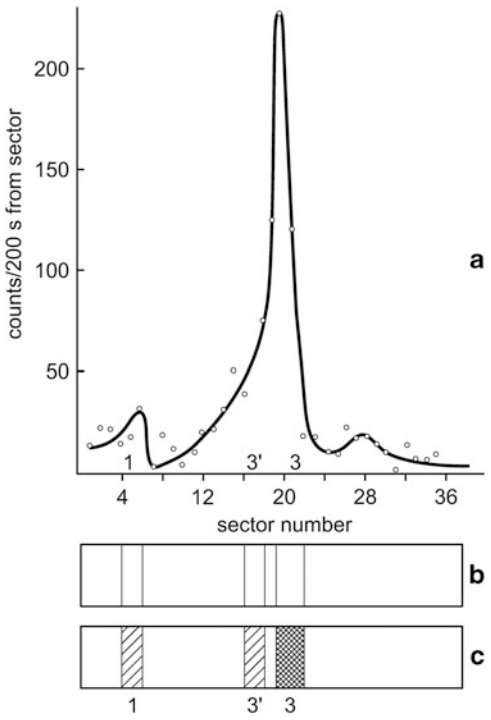

Fig. 3.103 The radioactivity distribution on the chromatogram at the sixth sequential chromatography of the fraction antheraxanthin-^{14}C isolated from cells of *Euglena gracilis*. The system of solvents was P:A (6:1). (*1*) the start line; (*2*) antheraxanthin (diadinoxanthin); (*3′*) the isomer of antheraxanthin

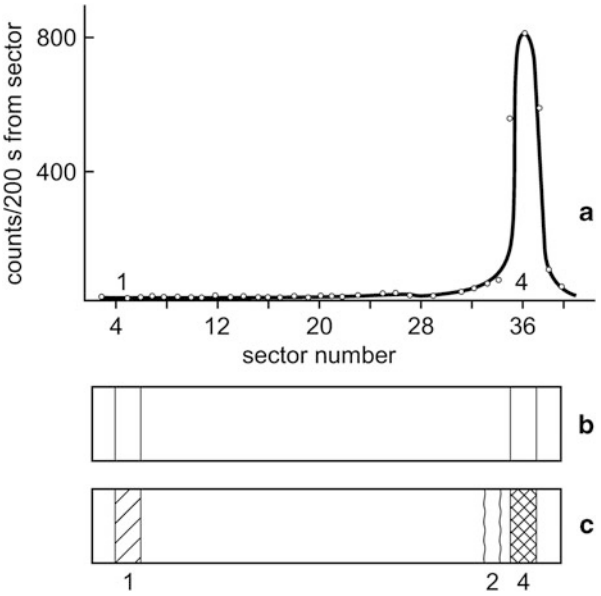

Fig. 3.104 The radioactivity distribution on the chromatogram at the fourth sequential chromatography of the fraction carotene-^{14}C isolated from cells of *Euglena gracilis*. The system of solvents was P:A: ethanol (6:1:0.5). (*1*) the start line; (*4*) carotene; (*2*) colorless compound

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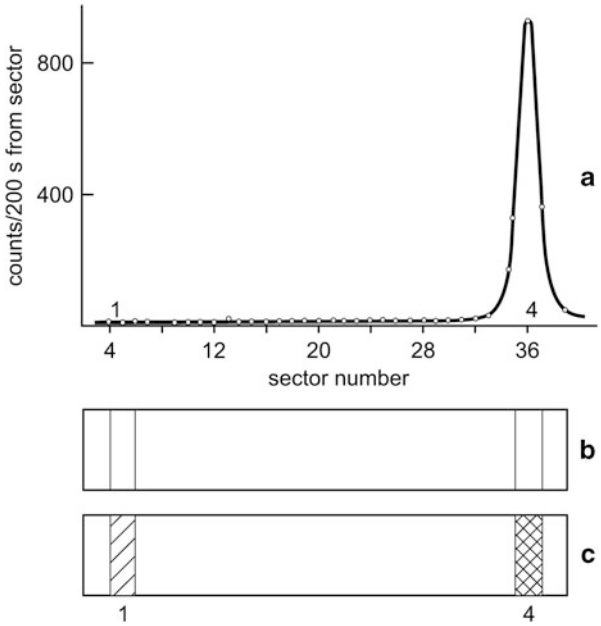

Fig. 3.105 The radioactivity distribution on the chromatogram at the fifth sequential act of purification of the fraction carotene-^{14}C isolated from cells of *Euglena gracilis*. The system of solvents was P:A (6:1). (*1*) the start line; (*4*) carotene

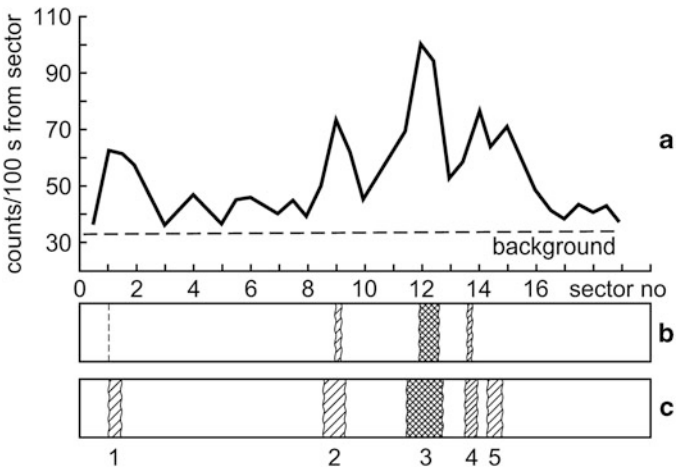

Fig. 3.106 The scheme of the radioactivity distribution on the chromatogram at the fourth act of the sequential purification of the fraction violaxanthin-^{14}C isolated from pea leaves. The system of solvents was P:chloroform (3:1). (*1*) the start line; (*2*) the isomer of violaxanthin; (*3*) violaxanthin; (*4*) the isomer of violaxanthin; (*5*) colorless compound

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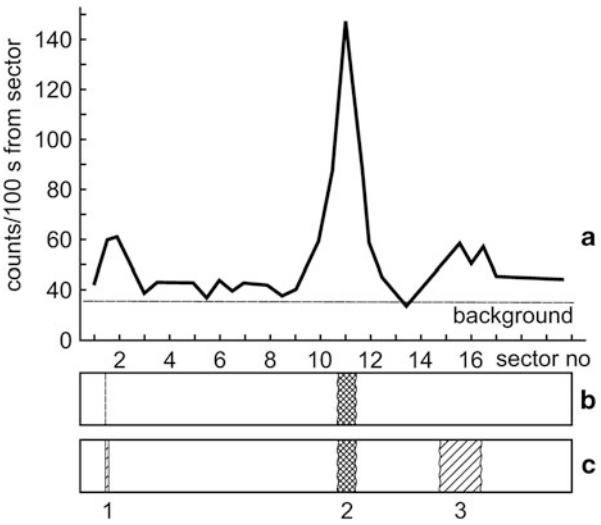

Fig. 3.107 The scheme of the radioactivity distribution on the chromatogram at the seventh act of the sequential chromatography of the fraction violaxanthin-^{14}C isolated from pea leaves. The system of solvents was P:ethanol (7:1). (*1*) the start line; (*2*) violaxanthin; (*3*) colorless compound

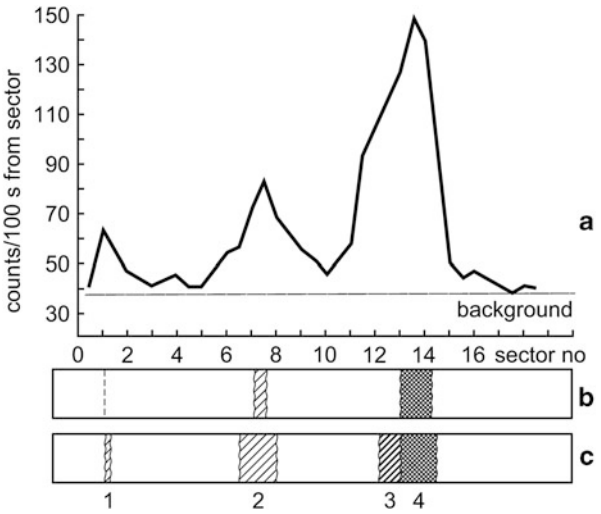

Fig. 3.108 The scheme of the radioactivity distribution on the chromatogram at the sixth act of sequential chromatography of the fraction lutein-^{14}C isolated from pea leaves. The system of solvents was P: chloroform (3:1). (*1*) the start line; (*2*) the isomer of lutein; (*3*) colorless compound; (*4*) lutein

The methodological approaches and solutions suggested by us in Chap. 3, at the very least, serve to acquaint the reader with the material to be discussed in Chaps. 4 and 5 and help in the assessment of critical remarks made in Chaps. 1, 3, and 5. The authors of this book would not tire the reader with methodological excursions into the past and present and would not put effort into additional explanations, if new ignorant publications with the application of poor-quality techniques did not continue to appear. In this chapter, we discussed methods, but the reader should always

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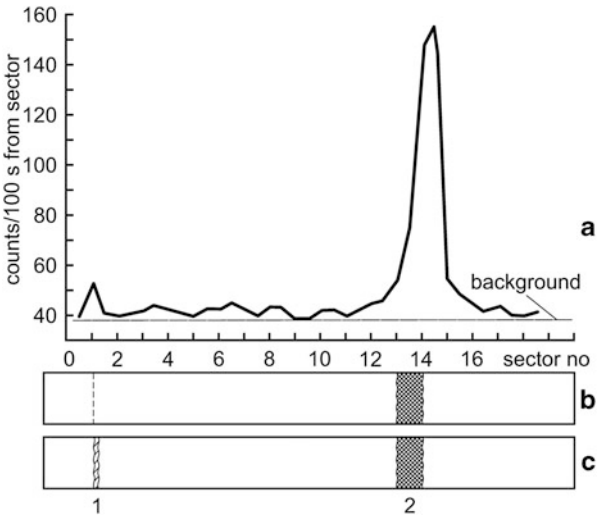

Fig. 3.109 The scheme of the radioactivity distribution on the chromatogram at the eight act of sequential chromatography of the fraction lutein-^{14}C from pea leaves. The system of solvents was P:ethylene (7:1). (*1*) the start line; (*2*) lutein

remember that the methods define the results of work and that behind results there are researchers of this or that degree of methodological and theoretical qualification.

Application of the discussed techniques formed the basis for the experimental work, which is discussed in Chaps. 4 and 5.

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Chapter 4
Application of Derivative Spectrophotometry in Comparative Biochemical Studies

Contents

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In Chap. 3, a number of modern methodological approaches were considered, the application of which in physical–chemical biology ensures that new and sometimes unusual experimental data can be obtained.

In this chapter, we bring to the reader's attention issues of the interaction of biologically active substances with ions of various valences and ways of detecting formed ligands by spectral methods. Evidence of the multicomponent nature of antitumor preparations, and the expediency of using derivative spectra for analysis of muscle relaxants and for qualitative assessment of the state of vitamins and hormones, is presented. This chapter considers features of the derivative spectra of a number of aromatic amino acids (AAAs) and the character of their change under the influence of γ-radiation. The natural continuation of this research direction was the investigation of derivative spectra of proteins and the character of their change under the influence of high temperature and γ-radiation.

4.1 Analytical Significance of Derivative Spectrophotometry in Comparative Studies of Amino Acids and Proteins

In the following material, we focus on the rational application of derivative spectrophotometry for studies of the biochemistry of proteins and the production of superpure preparations.

Protein preparations absorb in the ultraviolet (UV) range of 230–300 nm, with a peak at approximately 280 nm; moreover, the integral absorption of the spectral outline is mainly conditioned by the contribution of residues of AAAs, that is, phenylalanine, tyrosine, and tryptophan. The contribution of sulfur-containing amino acids cystine, cysteine, and methionine to this part of the general UV spectrum is negligible.

As known from the literature, the outlines of spectra of AAAs change depending on the pH of the medium, nature of the solvent, microenvironment of the chromatophore, the chosen spectrophotometer, and experimental conditions (Amiragova et al. 1964; Burshtein 1977; Talsky and Mayring 1978; Demchenko et al. 1978; Demchenko AP 1986; Fraifelder 1980; White et al. 1978; Grigorieva et al. 1985; Semenova and Saakov 1989; Saakov et al. 1990). Figure 4.1 presents the spectra of tyrosine (Tyr) and tryptophan (Trp), and Figs. 4.2a and 4.3b show the spectra of solutions of β-phenyl-β-alanine (β-Phe) and β-phenyl-α-alanine (α-Phe).

In this section, we illustrate the general analytical possibilities and properties of the derivative spectrophotometry method with the help of examples of derived absorption spectra of high orders of AAAs and of a number of proteins.

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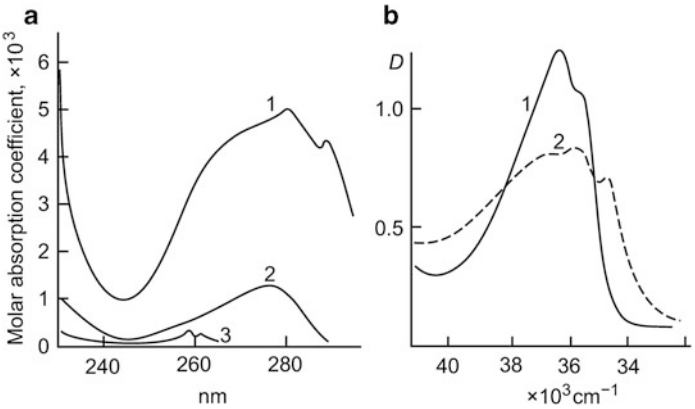

Fig. 4.1 Absorption spectra of tryptophan (*1*), tyrosine (*2*), and phenylalanine (*3*) in aqueous solutions: (**a**) pH 8.0 (Saakov 1987; Semenova and Saakov 1989; Saakov et al. 1990); (**b**) pH 5.15 (*1*) and pH 5.3 (*2*)

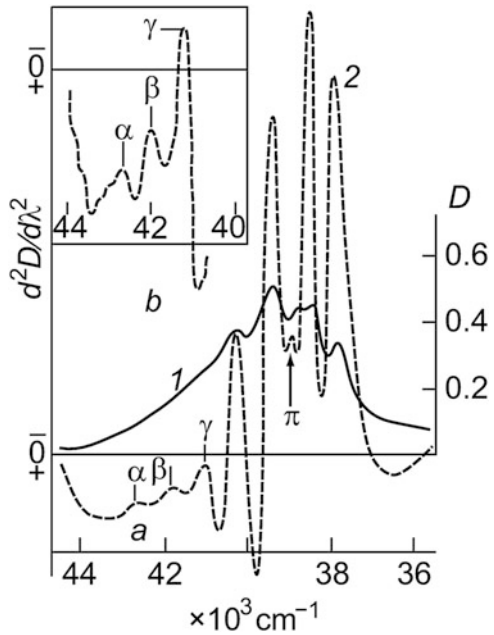

Fig. 4.2 (**a**) The absorption spectrum of 5×10^{-4} M β-phenyl-β-alanine (*1*) and its second derivative (*2*), pH 5.63; (**b**) magnification of the part of D^{II} spectrum

Recall that the basic parameters measured in absorption spectroscopy are the wavelength (λ) corresponding to the absorption maximum (λ_{max}), width of the absorption band, and specific extinction coefficient (ε). Moreover, more frequently the band half-width is analyzed (i.e., bandwidth at an ordinate of half the absorption peak height). Values of λ and ε undergo noticeable changes under the influence of

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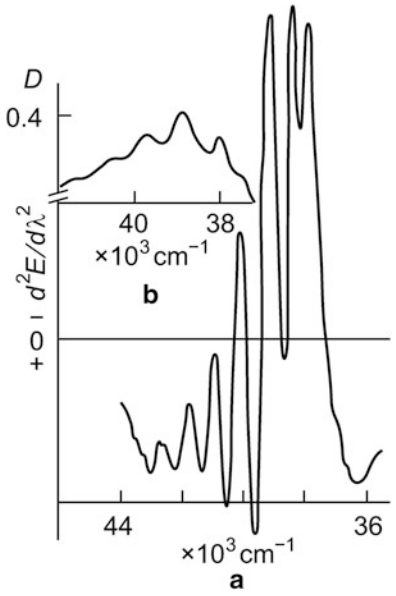

Fig. 4.3 (**a**) The second derivative of the absorption spectrum and (**b**) the absorption spectrum of 5×10^{-4} M β-phenyl-β-alanine solution in water, pH 5.68

the pH of the medium, polarity of the solvent, neighboring molecules, and orientation of vicinal chromatophores (Timasheff and Townend 1970; Burshtein 1977; Fraifelder 1980; White et al. 1978).

4.1.1 Special Features of Derived Spectra of Phenylalanine, Tyrosine, and Tryptophan

Figure 4.2 presents the absorption spectrum of β-Phe and the second derivative of the absorption spectrum. It is evident that curve 2 contains more information about the number and location of spectral bands (SBs) because unobtrusive or practically imperceptible bands in a monotonic change in the initial spectral outline are reliably revealed in the D^{II} curve. Furthermore, on the derived spectrum, it is possible to detect the main bands and to find their positions more precisely on the wavelength scale.

We remind the reader that for the first-order derivative of the absorption spectrum, the position of the maximum of the initial band corresponds to the zero abscissa point, that is, to the point of change in the derivative sign. This property is correct for odd derivatives (D^{III}–D^{V}) and was widely used in experimental papers (Giese and French 1955; Brandts and Kaplan 1973; Cuellar et al. 1978). For even derivatives D^{II} and D^{VI}, the position of a band maximum is located in the negative region, whereas for D^{IV} and D^{VIII}, it is in the positive region (Giese and French 1955; Talsky and Mayring 1978).

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Table 4.1 Absorption spectra D of aromatic amino acids and their D^{II}

Amino acid	Spectrum	Number of the spectral band							
		I	II	III	IV	V	VI	VII	VIII
β-Phenyl-α-alanine, solution, pH 5.68	D, cm^{-1}	...	...	41,440	40,520	39,760	38,920	38,000	37,440
	nm	...	...	Shoulder 241.3	Shoulder 246.8	251.5	257.0	263.1	Shoulder 267.1
	D^{II}, cm^{-1}	42,600	41,660	40,800	39,950	...	...	38,820	37,760
	nm	234.7	240.0	245.1	250.3	256.4	...	261.2	264.8
β-Phenyl-β-alanine, solution, pH 5.63	D, cm^{-1}	...	...	40,700	40,000	39,100	38,500	38,200	37,600
	nm	...	...	Shoulder 245.5	250.0	255.6	Shoulder 259.6	261.8	265.9
	D^{II}, cm^{-1}	42,850	41,830	41,100	40,200	39,330	38,900	38,460	37,790
	nm	233.4	239.0	243.3	248.7	254.2	257.0 π-peak	260.0	264.6
Tyrosine, solution, pH 5.15	D, cm^{-1}	...	...	...	...	...	...	36,350	35,590
	nm	...	...	...	...	...	...	275.1	281.0
	D^{II}, cm^{-1}	...	...	...	...	...	37,300	36,370	35,460
	nm	...	...	...	...	...	268.1	275.0	282.0
Tryptophan, solution, pH 5.30	D, cm^{-1}	...	...	...	...	...	36,840	35,830	34,820
	nm	...	...	...	...	...	271.5	279.1	287.1
	D^{II}, cm^{-1}	...	...	...	...	37,070	36,480	35,870	34,898
	nm	...	...	...	...	269.7	274.1	279.0	287.0

(continued)

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Table 4.1 (continued)

Amino acid	Spectrum	Number of the spectral band							
		I	II	III	IV	V	VI	VII	VIII
β-Phenyl-β-alanine minus β-phenyl-α-alanine	D, cm^{-1}	...	...	40,880	40,000	39,180	...	38,180	37,500
	nm	...	...	Shoulder 244.6	250.0	255.0	...	262.0	266.6
	D^{II}, cm^{-1}	43,140	42,270	41,380	40,580	39,700	38,760	38,070	37,500
	nm	231.8	236.5	241.6	246.4	252.0	258.0	262.6	266.6
β-Phenyl-α-alanine minus β-phenyl-β-alanine	D, cm^{-1}	...	...	41,360	40,400	39,640	38,720	...	37,800
	nm	...	...	Shoulder 241.8	247.5	252.2	258.0	...	264.5
	D^{II}, cm^{-1}	44,040	42,430	41,460	40,600	39,710	38,880	38,070	37,040
	nm	227.0	235.6	241.2	246.3	251.8	257.2	262.6	270.0

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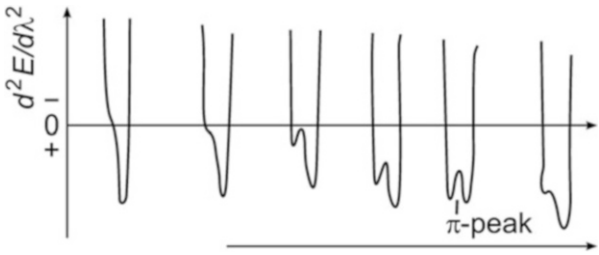

Fig. 4.4 The change in the π-peak intensity in the D^{II} spectrum of β-phenyl-β-alanine in dependence on the change in τ decrease (*arrow*)

Thus, the form of the graph of the second derivative (and other derivatives of even orders) is sufficiently close to the original function (the spectral curve). This allows easy comparison of experimental curves and facilitates tasks (especially in comparative studies) concerned with the interpretation of analysis results; this is, obviously, attractive to researchers.

From data presented in Figs. 4.2 and 4.3, it follows that the D^{II} method allows reliable indication of up to eight bands in the spectrum of β-Phe and up to seven bands in the spectrum of α-Phe. Numerical data on the position of bands for analyzed preparations of β-Phe, α-Phe, Tyr, and Trp, and also the difference spectra of β-Phe against α-Phe and vice versa, are shown in Table 4.1 (Saakov et al. 1987a, b, 1990).

Figures 4.2, 4.3, and 4.5 show that unobtrusive deviations in the symmetry of initial spectral curves of absorption are manifested on the derived spectrum in the form of clearly pronounced peaks; the presence in the initial spectrum of a weak and narrow band overlapped by an intensive and non-characteristic peak is detected on the D^{II} curve.

The dedicated analog processor (DAP; see Chap. 1) used as the differentiator (Saakov et al. 1987a, b; Saakov and Semenova 1989) allows a significant increase in the dynamic range of detection of bands (Figs. 4.2b and 4.5b, c). Changing the mu-factor, it is possible to separate necessary regions of the spectrum; moreover, using a logarithmic chart recorder (e.g., of the type K-201), the amplitudes of main bands become commensurate with the secondary bands, but they conveniently fit in one blank.

Describing the example of analysis of the β-Phe spectral curve, note that special care is required when selecting the time constant of differentiation (τ). The D^{II} curve of the β-Phe spectrum is characterized by the presence of a small, but very specific, β-Phe peak, which we previously designated as the π-peak (Saakov et al. 1987a, b, 1990). Small deviations of τ from the optimum value, particular to this spectral outline, cause deformation of the fine structure of the spectrum, prevent manifestation of small peaks and curve knees on the D^{II} curve, and thus decrease the value and accuracy of this kind of analysis (Fig. 4.4). This detail is extremely important for correct application of the method and was indicated by us earlier in a description of the use of differentiators with RC chains (Rutman et al. 1976a, b); this special feature of the method was also indicated by Demchenko et al. (1978), who used an analog computer for differentiation. Despite the fact that we drew attention to the accuracy of τ selection in our early communications, the

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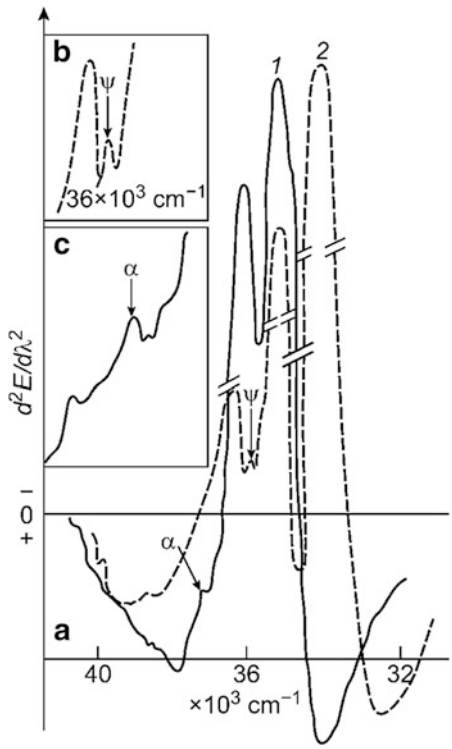

Fig. 4.5 (**a**) D^{II} spectra of aqueous solutions of 5×10^{-4} M tyrosine, pH 5.15 (*1*) and 1×10^{-4} M tryptophan, pH 5.3 (*2*). Magnified parts of the D^{II} spectrum for (**b**) tryptophan with Ψ-peak and (**c**) tyrosine with α-peak

comparative analysis of derived spectra of the same substances from different literature sources forced us to attend again to this issue. The need to control the τ value is manifested in the routine analysis of a wide range of substances or in transfer from the analysis of one class of compounds to another characterized by the smoothness and slope of the spectral outline (Brandts and Kaplan 1973; Litvin et al. 1973; Saakov 1973; Rutman et al. 1976a; Cuellar et al. 1978; Talsky and Mayring 1978).

In connection with the fact that the basic contribution to protein spectra is made by two AAAs (Tyr and Trp) and analyzing the literature, we found some ambiguity in the derived spectra of these amino acids. Therefore, in Fig. 4.5 the second derivatives of Trp and Tyr spectra obtained *in our device* are shown. For Tyr, two maxima are characteristic in region 36,000 cm^{-1}, and a heterogeneous spectrum structure is seen in the region 38,000 cm^{-1} (Fig. 4.5a, b). The spectrum of the second derivative of Trp is characterized by three clearly pronounced maxima, and in the region 36,000 cm^{-1}, there is a small peak Ψ (Fig. 4.5b), which is very similar to the π-peak that is peculiar to β-Phe. Analyzing the structuring of the derived spectra of Tyr and Trp, the reader should also remember that the optimum value of the differentiation time constant does not allow its fine structure to be depicted with sufficient reliability because the shift of its value from the optimum causes larger

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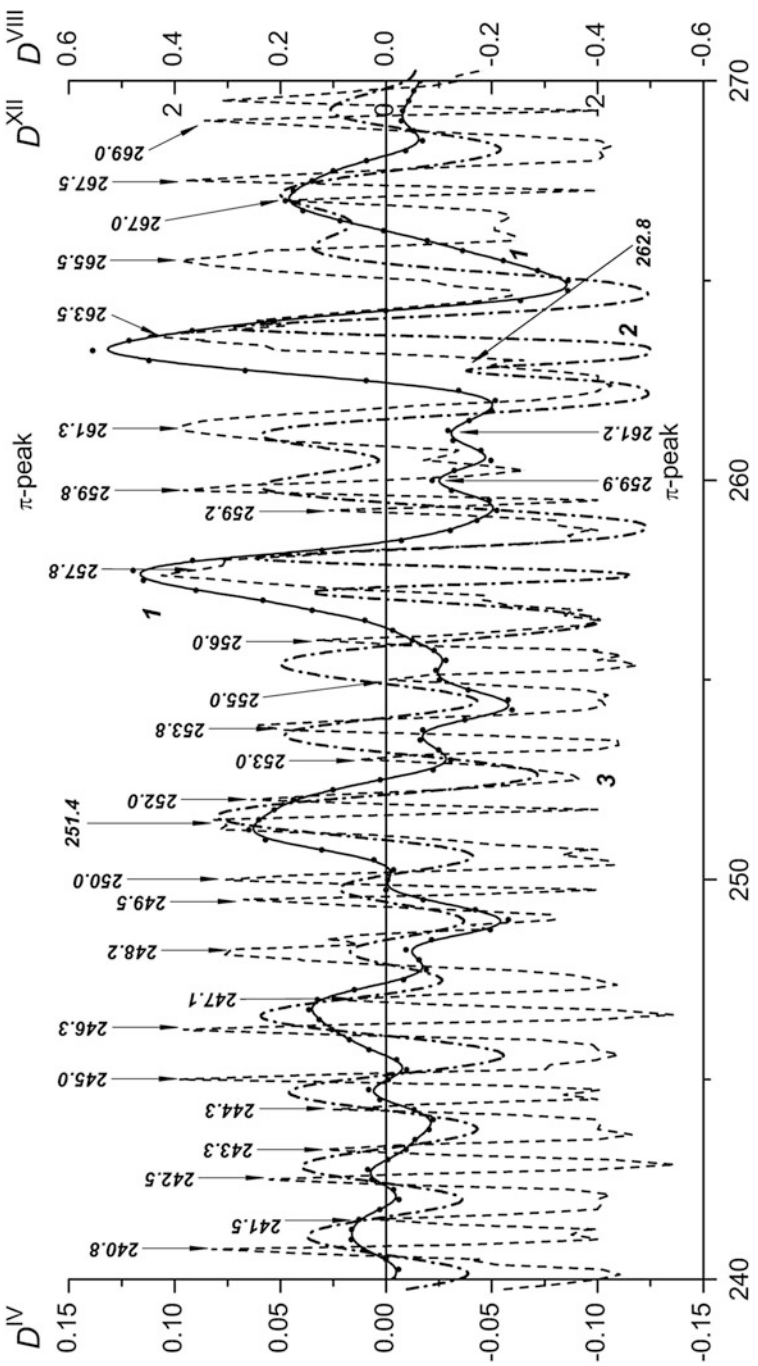

Fig. 4.6 Derivative spectra of high orders for 5×10^{-4} M solution of β-Phe, pH 5.5: (*1*) the fourth derivative of the absorption spectrum; (*2*) the eighth derivative; (*3*) the 12th derivative

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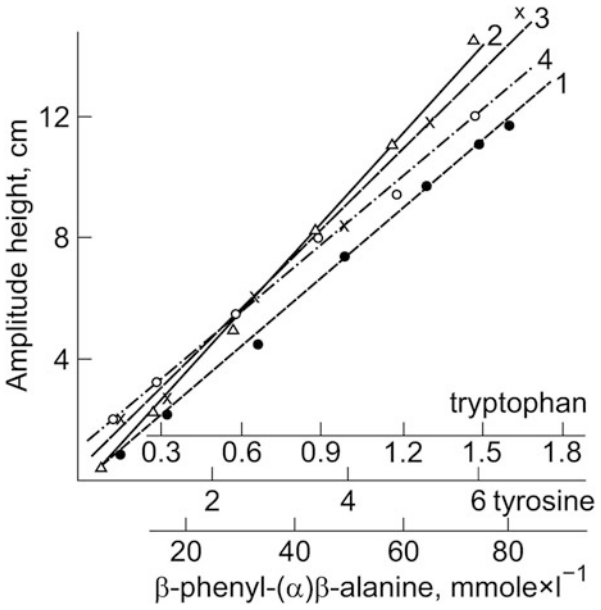

Fig. 4.7 Quantitative determination of tyrosine (*1*), tryptophan (*2*), 3-β-phenyl-β-alanine (*3*), and 3-β-phenyl-α-alanine (*4*) using the second derivatives of absorption

monotony of the spectral curve of the second derivative. We called the attention of the reader to these questions in Chap. 1.

When applying any new method, the question of accuracy, reproducibility, and convergence of obtained results arises, that is, a question about the quality of measurements, which consists of the proximity of results of repeated registrations of spectral curves to each other for experiments performed under same or different conditions. One part of Chap. 3 (3.2-3.3) dealt with the accuracy of differentiation. Our experience with derivative spectrophotometry in different devices (mainly the Russian recording spectrophotometers SP-8, SP-10, SP-14, and SP-18 and German Specord) and with diverse analog converters on lamps, transistors, microcircuits, analog computer MN-10, and DAP (Marenko et al. 1972; Litvin et al. 1973; Saakov 1973; Marenko and Saakov 1973; Rutman et al. 1976a, b; Saakov et al. 1976; Demchenko et al. 1978) showed that the convergence of results is within the limits of 0.3–1.0 % and is largely conditioned by the level of signal mu-factor. We obtained analogous data using instruments from Dr. Armin Meister or the firm SLM-Aminco (DW-2000 FA-220).

The D^{II} spectra of AAAs clearly illustrate a theoretical property of derivative spectrophotometry connected with a decrease in the half-width of main bands in even derivatives, which in turn improves the resolution of overlapped bands. This is illustrated by the DSHO for β-Phe, presented in Fig. 4.6. For analysis, it is best to scan the figure and transfer it to a monitor as a colored image for convenience in finding spectrum components. Earlier criteria of separation of two symmetrical, closely located absorption bands were considered (Meister 1966; Gulyaev and Litvin 1970; Gulyaev et al. 1971; Rutman et al. 1976b). Without discussing the

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choice of different criteria, it is enough to indicate that modern spectral equipment allows use of the Sparrow criterion, its essence being that for resolution of two symmetrical bands, it is sufficient to reveal the existence of a downward dip between them (Rutman et al. 1976b, pp. 131−132).

The undoubted advantage of this method is that the application of even a first-order derivative removes the constant component of side absorptions, such as light diffusion, shift of the object, moistening or drying of the object, and background absorption. Use of D^{II} excludes the influence of side absorptions linearly dependent on λ. The aforesaid limits the range of a priori judgments or objections connected with spurious signals of light diffusion in boundary regions of the spectrum (Rutman et al. 1976b). This means that the loss of any information of the initial spectra is compensated for by a substantial increase in the informativeness of derived spectra (especially in comparative experiments) through an increase in their contrast.

An issue that always disturbs a researcher is the possibility of quantitative assessment of results and the value of the linearity interval of graphs for quantitative analysis. The literature describes limited attempts at quantitative assessment of the results of the derivative spectrophotometry of true solutions and mixtures of substances using diverse means (Savitzky and Golay 1964; Hager 1971, 1973; Shibata et al. 1973; Wahbi and Ebel 1974; Morton 1975; Baranov et al. 1975; O'Haver & Green 1976; Rutman et al. 1976a; Saakov et al. 1978a; Saakov 1987). However, our experience (Semenova and Saakov 1989) (Fig. 4.7) allows us to conclude that when working with solutions of amino acids and other pure preparations, performing quantitative determination using derived spectra with a sufficiently wide interval of graph linearity is quite possible. At the same time, the quantitative determination of substances in native biological structures (chloroplasts, mitochondria, erythrocytes, ribosomes, and others) is a serious analytical problem. All changes in the amplitude of the initial SB, when the half-width is constant, are directly proportional to the amplitude of the second derivative. If its half-width changes simultaneously with a change in the initial band amplitude (this is possible during different conformational changes in native structures under the influence of environmental factors), then on the D^{II} graph, the changes in the half-width and amplitude are registered in parallel. Moreover, if the half-width also increases with an intensity increase for the initial band, then on the D^{II} graph, only an increase in the band half-width with almost constant amplitude can be observed. A decrease in the peak amplitude of the second derivative depends either on the decrease in amplitude of the initial band or on an increase in its half-width, or on the simultaneous influence of these two parameters. Moreover, a change in the amplitude of the D^{II} band is directly proportional to a change in the amplitude of the initial band and is inversely proportional to the square of the half-width of the initial curve (Rutman et al. 1976b).

Quantitative assessment of the application of derived spectra can be performed in different ways using the value of a peak relative to the zero line, the peak-to-peak amplitude of a signal swing, or the difference in amplitude between two vicinal extrema of opposite sign (see Figs. 3.18–3.25). In every case, direct dependence of the investigated substance on the concentration can be expected (Savitzky and Golay 1964).

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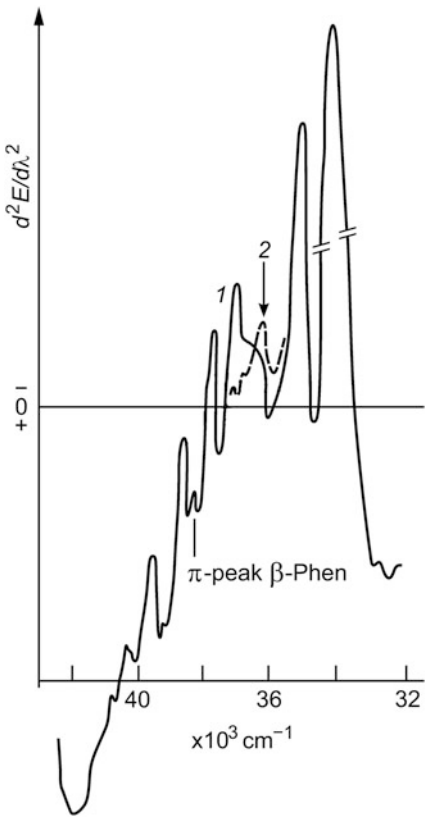

Fig. 4.8 The D^{II} spectrum of a mixture of 1×10^{-4} M tryptophan and 5×10^{-3} M β-Phe solutions (*1*), showing the location of the third maximum of the D^{II} tryptophan spectrum (*2*) (see Fig. 4.5)

A very serious, but in general resolved, problem is the determination of substances in a system consisting of two or more components, the composition of which approaches that of native biological supramolecular structures. In these cases, two ratios are taken into account: (1) the ratio of the intensity of interfering absorption bands to the intensity of the peak or bands of the substance to be determined and (2) the ratio of the half-widths of absorption bands of the interfering substance and of the substance to be determined. Also, the distance between maxima of bands of mixture components is calculated. The graph in Fig. 4.5 illustrates the possibility of jointly determining Tyr and Trp. Figure 4.8 shows that the application of derivative spectrophotometry can solve the complex problem of joint determination of β-Phe and Trp because the value of the molar extinction coefficient of the latter is approximately 30 times higher than that of Phe (Morton 1975).

In the mixture of Trp and β-Phe, features of the fine structure of the D^{II} spectrum specific for β-Phe are revealed, namely, the π-peak. At the same time, the graph in Fig. 4.8 illustrates a known property of derived curves: The superposition of the main peak of one substance with the satellite peak of opposite sign of another substance results in a change in the summary visible spectrum. In Fig. 4.8, curve 2 shows the presence of a third short-wave peak in the D^{II} spectrum of Trp, which disappears in the summary curve. There is also the possible appearance of maxima

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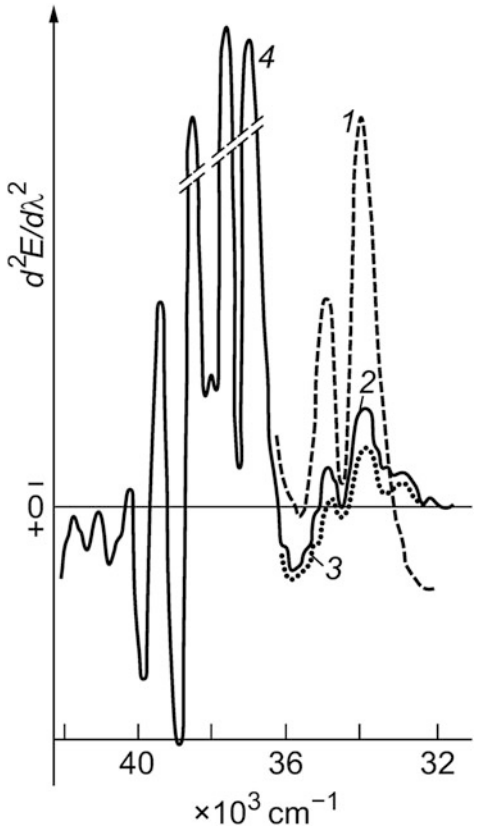

Fig. 4.9 Assessment of detection limit of tryptophan in mixture with β-Phe using second derivative spectra: mixture of solutions of 7.5×10^{-6} M β-Phe plus (*1*) 1×10^{-7} M tryptophan; (*2*) 0.125×10^{-7} M tryptophan; *3* 0.625×10^{-9} M tryptophan. (*4*) The spectrum part corresponding to β-Phe

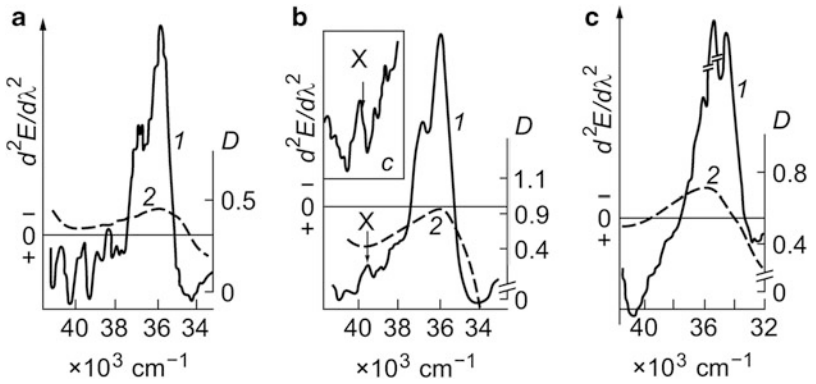

Fig. 4.10 D^{II} absorption spectra of solutions of some proteins: (**a**) human albumin, 5×10^{-5} M, pH 7.2; (**b**) ribonuclease, 2×10^{-5} M, pH 7.3; (**c**) the magnified fragment of spectrum; (**d**) trypsin, 3.1×10^{-6} M, pH 7.2

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of false intensity if, according to the additive law, the addition of bands of identical directivity occurs or addition of false maxima after addition of positive satellites takes place, even when bands are well separated.

Preliminary consideration of the phenomenon of masking some bands with others, which causes apparent disappearance of component bands in the D^{II} spectrum, or, on the contrary, the appearance of false maxima formed as a result of vector addition of positive satellites, is described (Baranov et al. 1975; Rutman et al. 1976a, b; Saakov et al. 1978b). It is extremely important in this case to decide whether the obviousness or non-obviousness of a maximum can be regarded as a sign of the presence or absence of a substance. In this connection, a promising technique for distinguishing maxima and minima of real bands of initial spectra is the comparison of analyzed graphs of derivatives of different orders (e.g., D^{I}, D^{II}, D^{III}, and D^{IV}). This allows a researcher to reject extrema appearing as a result of the above-mentioned special features of derived spectra (see Fig. 4.6).

When studying systems of two or more components, the concept of the detection limit of a minor component characterized by the specific absorption spectrum becomes very important. Usually, the difficult problem of the indication of Trp trace quantities in the presence of Phe is reliably solved with the use of the second derivative method (Fig. 4.9). As shown in Fig. 4.9, the detection of Trp traces of 6.25×10^{-9} M in a 0.1-cm cuvette is not threshold because by increasing the mu-factor with DAP, it is possible to increase the limits of Trp indication in the presence of β-Phe.

The prospect of application of derivative spectrophotometry for the analysis of protein molecules was apparent in the first articles by Inoue et al. (1975) and Matsushima et al. (1975). Figure 4.10 shows the absorption spectra and their second derivatives for ribonuclease lacking Trp chromophore groups, human albumin, and trypsin. The sensitivity of the method to the quantitative and qualitative state of components of the system indicates that this method is very promising for investigating the conformational state of chromophore groups in protein molecules, as shown in the article by Yu. V. Natochin (1985) on research into the influence of EDTA and calcium ions.

4.1.2 *Special Features of Changes in Phenylalanine Spectra Caused by the Influence of γ-Radiation*

In previous studies, we showed the high stability of the chlorophyll–protein complex (ChlPC) of plants exposed to high doses of γ-radiation (Saakov 1993a, c). To explain the reason for such a resistance of ChlPC to radiation, we investigated the optical spectra of a number of proteins, AAAs, and solutions of pigments for the purpose of identifying the selective resistance of separate components of the complex and to compare them with that of the chlorophyll–protein complex as a whole.

It was also discovered that under extreme conditions of plant existence, namely, during anoxia, the oxidizing transformation of β-Phe into Tyr occurs (Chirkova et al. 1989). Radiation–chemical transformations of Phe are thought to be

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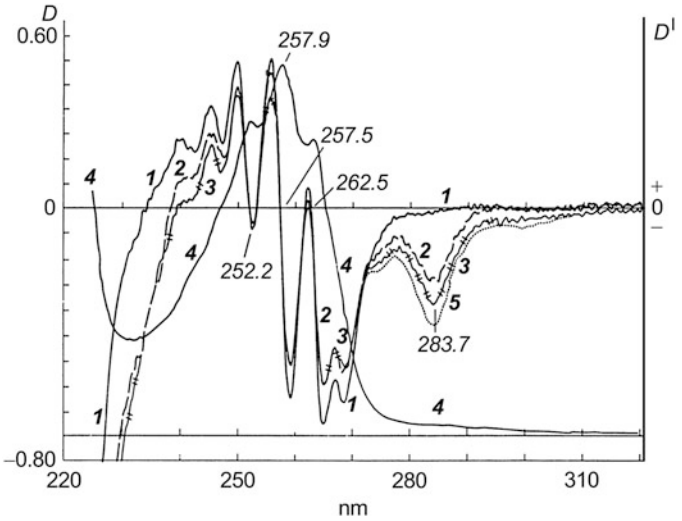

Fig. 4.11 The absorption spectrum of 4×10^{-3} M DL-phenylalanine solution, pH 2.15 (*4*) and the character of its D^{I} change (*1*) under influence of γ-irradiation (*2*, *3*, *5*). *Curves 2*, *3*, *5* show the influence of γ-irradiation in doses of 1.2, 2.7, and 3.6 kGy, respectively

connected with the reactions of deaminization, decarboxylation, and the accumulation of carbonyl derivatives. These reactions are specific for alanine and involve the alanine part of the β-Phe molecule. One of products of β-Phe radiolysis was thought to be Tyr (Amiragova et al. 1964). To explain the generality of the directivity vector of β-Phe reactions under the influence of extreme conditions, the effect of high doses of γ-radiation on the kinetics of the optical density (OD) change and on the spectrum structure of β-Phe solutions was investigated.

The laboriousness of chemical analysis stimulated the authors of this book to use molecular spectrophotometry methods. Despite the apparent good knowledge of optical β-Phe spectra, literature data on this question are rather limited and contradictory (Ziegler 1973; Ichikawa and Terada 1977; Demchenko et al. 1978; Saakov et al. 1987a, b, 1990). We have already described a specific feature of the second derivative of the spectrum of β-Phe solution (Fig. 4.2) connected with the presence of an absorption band characteristic for the analytical work, which we call the π-peak (Saakov et al. 1987a, b, 1990).

In the experimental process, solutions of DL-Phe (4×10^{-3} M) from the firm Serva and solutions of L-Phe were used for the analysis. Absorption spectra and their first (D^{I}), second (D^{II}), fourth (D^{IV}), eighth (D^{VIII}), and 12th (D^{XII}) derivatives were investigated with a DW-2000 spectrophotometer (Aminco, FRG). A capsule containing the ^{57}Co isotope, power about 600 Gy/h, was used as the γ-radiation source.

In Fig. 4.11 (curve 4), the initial absorption spectrum of DL-β-Phe solution (pH 2.15) and its first-order derivative (curve 1) are presented. We chose acid solutions of DL-β-Phe because γ-radiation induces the oxygen effect, which is

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Table 4.2 Absorption bands of DL-phenylalanine solutions, pH 3.6

Type of spectrum	Numbers and positions of the main band of the phenylalanine absorption spectrum (nm)															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
D						252.11		257.54		263.03						
D^{I}_{max}				239.9	245.0	250.16	255.75				266.54					
D^{I}_{0}						252.0	253.7	257.6	261.34	263.0						
D^{I}_{min}					242.0	247.3	252.8	259.1		264.9	268.4					
D^{II}			235.2	240.1		246.5	251.45	257.6	Band asymmetry	263.4	267.3					
			235.33	241.2		246.7	251.7	257.7		263.45	267.6					
D^{IV}	230.8	232.6	235.2	241.2	242.7	246.9	251.4	257.7	260.1	263.3	267.1	272.3	274.0			
			236.4[a]			248.2			260.3		267.2					
			237.4		244.7	248.32		253.8	261.32	269.2						
			239.1		244.8	250.0		255.34	261.35							
D^{VIII}	230.8	232.7	235.2	241.2		247.0	251.2	257.8	261.1	263.1	267.0	269.3	273.9	284.6	286.0	289.4
					242.7	248.4	252.2	259.1		264.2	267.8	270.9	275.3		287.3	290.8
			236.4[a]			249.9	253.8		261.4	265.7		272.3		276.4	288.1	294.0
			237.4		246.0		255.2		π-peak					277.6		295.3
			239.0											278.3		297.9
			239.6				256.2							279.5		299.0
														280.9		
														282.2		
														283.4		

[a]Values of intermediate absorption bands of phenylalanine

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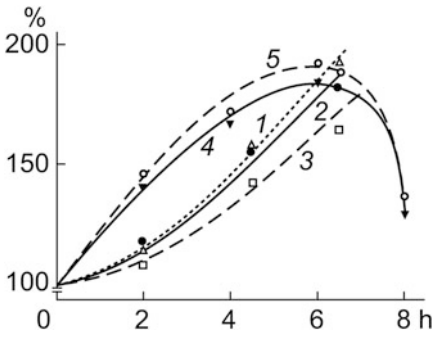

Fig. 4.12 Dynamics of OD change in the principal maximum ($\lambda_{max} = 257.6$ nm) of absorption spectrum of DL-Phe (*4*) and of L-Phe (*5*) under influence of γ-irradiation (600 Gy/h). *Curves 1*, *2*, *3* show corresponding changes in maxima $\lambda_{max} = 252.1$ nm (*1* and *2*) and $\lambda_{max} = 263.08$ nm (*3*)

accompanied by an increase in the output of carbonyl and phenol derivatives. In Fig. 4.14 (curve 1), the spectrum of the fourth derivative is shown, and in Figs. 4.6 and 4.13, the eighth derivatives of optical spectra of β-Phe solutions of different acidity are presented. The data in Table 4.2 summarize our knowledge about SBs of β-Phe and give clear evidence of the advantage of application of high-order derivative spectra for reliable identification of separate SBs for β-Phe. The registration of D^{XII} of the absorption spectrum of β-Phe did not add substantial information in comparison with the D^{VIII} derivative. Only the theoretically expected narrowing of SBs was revealed.

Earlier we indicated that it is possible to reveal the π-peak in D^{II} spectra, depending on the quality of selection of the differentiation time constant (Rutman et al. 1976b; Saakov et al. 1987a, b). Sometimes, the masked presence of the π-peak can be found in the spectrum of the second derivative in the form of asymmetry of the SB $\lambda = 257.7$–258.3 nm. However, reliable detection of the π-peak occurs in the D^{IV} spectrum. The π-peak is located between two main maxima with $\lambda = 257.7$ and 263.3 nm (Fig. 4.6). Sometimes on the D^{VIII} spectrum, there is an additional band with $\lambda = 259.1$ nm in the structure of the π-peak, but this depends on the origin of the used preparation (Fig. 4.13). In the fine structure of D^{XII} spectrum (Fig. 4.6), the asymmetry of peaks of the D^{IV} spectrum can be reliably detected and is manifested in the form of narrow bands at 240.8 and 241.5, 242.5 and 243.3, 244.3 and 245.0, 246.3 and 247.1, 249.5 and 250.0, 251.4 and 252, 253.0 and 253.8, and 255.0 and 256.0 nm; in the region of the π-peak at 259.2 and 259.8 nm; and in the region of the small peaks at 260.5 and 261.3, 262.8 and 263.8, 265.5 and 266.4, 267.0 and 267.5, and 269.0 and 269.5 nm. The application of derivative spectrophotometry allows the simultaneous determination of β-Phe and Trp or Tyr, since the value of the molecular extinction coefficient, for example, of Trp, is approximately 30 times higher than that of β-Phe (Saakov et al. 1987a, b). The detection of π-peak bands in the mixture simplifies solution of this problem.

Thus, the data in Table 2.3 and Figs. 4.6, 4.13, and 4.14 reliably prove the localization and significance of the π-peak and of bands of its components for the

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serious qualitative characteristic of β-Phe preparations and can serve as the methodological basis for assessment of the influence of γ-radiation on β-Phe.

In the process of irradiation of β-Phe preparations by γ-rays, the OD of the solution gradually increases, which is peculiar to each of the main maxima of the absorption spectrum of β-Phe (Fig. 4.12). In this case, the general picture of the absorption spectrum is practically the same. During the irradiation process, the changes appear in the curve of the first-order derivative of the absorption spectrum (Fig. 4.11) and are most detectable in the region of the band $\lambda = 283.7$ nm; the degree of these changes is a function of exposure time or, in other words, a function of the received dose of γ-radiation. At the same time, changes in the fine structure of the absorption spectrum are most clearly manifested in the D^{IV} spectrum. These data are shown in Fig. 4.14. It follows from the data in Fig. 4.14 that γ-irradiation significantly changes the contribution of SBs of the π-peak, induces partition of the maximum $\lambda = 251.4$ nm into two bands, and partitions the main maxima with $\lambda =$ 257.7, 263.27, and 267.1 nm into two bands each.

Analysis of spectral changes detected as the appearance of a number of new SBs (namely, 248.7, 251.9, 252.8, 254.2–254.4, 256.7–256.72, 261.3–261.60, 265.7, 266.6, 267.10–268.2, 270.6, 272.2, 275.1, 276.5, 279.1, 283.7, and 288.9 nm) indicates the presence in the irradiated solution of a compound with spectral properties characteristic of Tyr. On the spectrum for Tyr solutions, we obtained data very similar to the above-mentioned bands. The same data correlate with changes in the D^{I} spectrum in Fig. 4.11 (the band $\lambda = 283.7$ nm caused by manifestation of the main absorption maxima region of Tyr with $\lambda = 276.12$–283.12 nm)

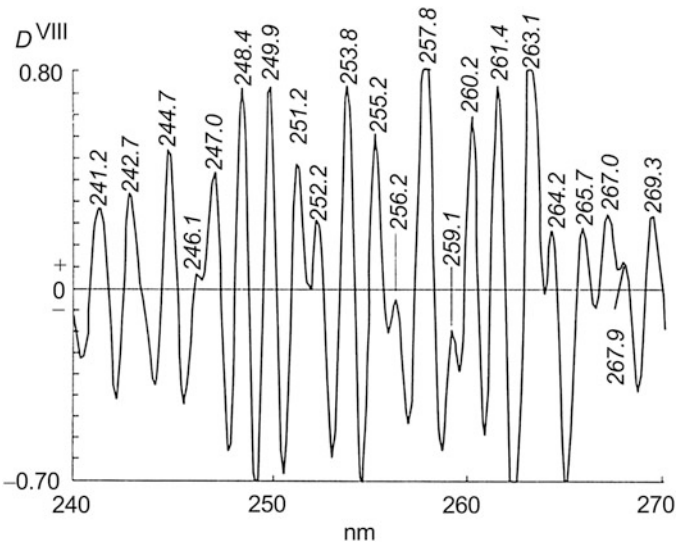

Fig. 4.13 The eighth derivative of the absorption spectrum of DL-phenylalanine solution (pH 2.15). Bands 259.1, 260.2, and 261.4 nm are in the region of the π-peak, detected earlier using D^{II} spectra (Saakov 1987; Semenova and Saakov 1989) (compare with Fig. 4.6)

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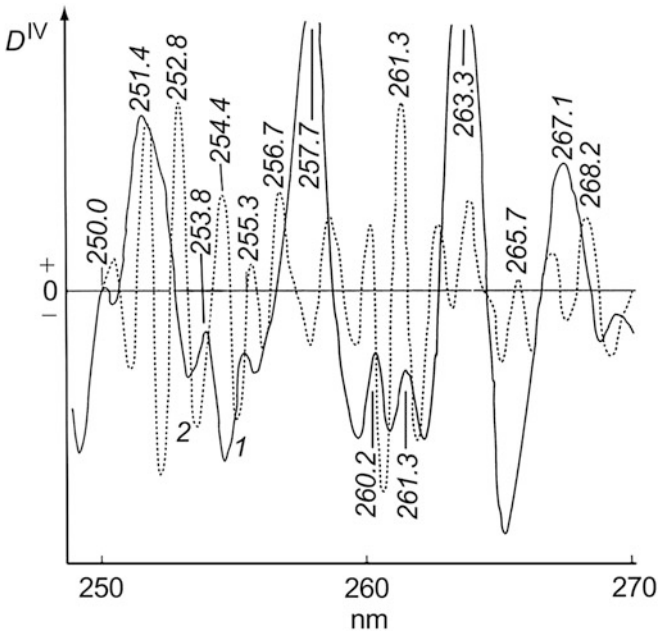

Fig. 4.14 The change character of D^{IV} of the absorption spectrum of DL-phenylalanine solution, not irradiated (*1*) and under the influence of γ-irradiation of 11 kGy (*2*)

and with appearance of the band of the Tyr α-peak in the region $\lambda = 267.0$–269.0 nm, which we described previously for the D^{II} spectrum (Saakov et al. 1987a, b) (Figs. 4.13 and 4.14).

Thus, the data we obtained indicate that during β-Phe radiolysis process, a compound appears that is similar in its spectral properties to Tyr. Taking into account the results of our research on the effect of anoxia on the transformation of β-Phe in mitochondria and of our direct experiments on the transformation of exogenous ^{3}H-β-Phe and ^{14}C-β-Phe in mitochondria (Chirkova et al. 1989), we have reasons to suppose that the substance discovered in the process of β-Phe radiolysis is Tyr (Pronkin and Saakov 1997).

The complex mixture formed during the process of β-Phe radiolysis, in which Tyr is present, could contain elements of the reverse process, that is, loss of the hydroxyl of the Tyr benzene ring with formation of Phe. This explains some nonconformity of mixture spectra with spectra of true Tyr solutions. It is necessary to note that with an increase in the pH value, the oxidizing power of hydroxyl radicals decreases. The presence in the mixture of products of Tyr radiolysis, such as DOPA, dopamine, dopaquinone, melanin, and adrenaline, is not excluded.

According to our results, the effect of identical radiation doses on ChlPC and β-Phe solutions indicates that ChlPC has a stronger resistance to radiation than β-Phe solutions, which is noteworthy. This means that neither solutions of pigments

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(Saakov 1993a) nor solutions of β-Phe individually cause the high resistance of ChlPC to radiation.

Intensification of membrane destruction under extreme influences is accompanied by increased peroxide oxidization of lipids. Free radicals of oxygen occurring in this case, as well as hydroxyls, can participate in the oxidization of bound and free residues of AAAs. It is possible that by inducing β-Phe radiolysis in acid solutions with γ-radiation, we stimulate the oxidization of β-Phe under extreme influences (Chirkova et al. 1989).

Thus, it is possible that our model experiments correspond to the course of an unspecific reaction of β-Phe oxidization caused by the effect of a stress factor on membranes. This was possible to discover using derivative spectrophotometry (Pronkin and Saakov 1997).

4.1.3 *Specific Character of Tyrosine Spectra Changes Under the Influence of γ-Radiation*

To make conclusions about radiation damage to proteins from spectral changes is possible only if the specific character of a change in the optical spectra of separate amino acids is known well (Demchenko 1986; Saakov et al. 1987a, b, 1990). Until recently, there were no AAA spectra registered with the help of modern methods of optical–electronic analysis. This frequently caused arbitrary interpretation of the changes in AAA spectra caused by the influence of extreme factors of the environment (EFEs) (Rowbottom 1955; Brandts and Kaplan 1973; Demchenko 1981, 1986; Demchenko and Zima 1977). Analysis of literature data shows the presence of contradictory information about the picture of tyrosine-derived spectra (Rowbottom 1955; Brandts and Kaplan 1973; Demchenko 1981, 1986).

Our own studies allowed us to find additional bands in the fine structure of the second derivative of the Tyr absorption spectrum (Saakov et al. 1987a, b). The methodological capabilities of past years limited the detailed presentation of spectral outlines of this amino acid; only the first and second derivatives of tyrosine spectra were considered (Ziegler 1973). Using the new analytical possibilities of this equipment, we were able to obtain DSHO of Tyr solutions and studied the character of their change in response to γ-radiation doses that initiated tyrosine radiolysis.

In the process of experimental work, solutions of tyrosine preparations (10^{-3} M, pH 5.0–5.15) from Serva and Sigma were investigated. For registration of absorption spectra (D) and their first (D^{I}), second (D^{II}), third (D^{III}), fourth (D^{IV}), and eighth (D^{VIII}) derivatives, the recording UV–Vis spectrophotometer with built-in computer DW-2000 FA-220 (SLM-Aminco, FRG) was used.

Table 4.3 summarizes the results of the absorption spectra measurements and also lists the main bands isolated with the registration of derived (D^{I}–D^{VIII}) spectra. In previous work, we indicated that in one experiment only the simultaneous

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Table 4.3 Bands of absorption spectra of tyrosine solutions

Type of spectrum	Numbers and positions of the main absorption bands of tyrosine solutions (nm)																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
D									267.04		275.10		283.00				
D^{I}		245.04									275.00						
500 Gy			248.12								275.56						
D^{II}			249.12	252.20	254.16		260.88		268.16		275.28		282.84				
2 kGy			248.15	252.04	254.28	257.92	260.72		267.16		275.28					295.44	
			248.12	252.04	254.00	257.92	260.72	261.12	266.00	270.52	273.00	278.92	281.14	287.88			
D^{III}			249.24[a]														
2 kGy		245.88	247.28	252.04	254.28	258.76	260.72	261.25	266.88	270.52	275.00	278.92	282.56		291.80	296.69	299.17
				253.13[a]				262.12	268.52		276.33				295.26		
4.5 kGy		247.00	248.20	252.32	254.00	256.80	259.26	260.56	266.60	269.12	275.22	277.80	283.68		292.10	294.64	298.60
								261.48[a]			274.16	276.54	279.00			295.90	
								263.80		274.55							
D^{IV}	243.00	245.88	248.12	251.48	254.28	256.52	260.72	263.24	267.16	269.40	275.28	276.30	283.12		292.64	294.32	298.08
	240.80[a]																
2 kGy	242.52	245.60	248.68	251.76	254.28	256.80	260.72	263.52	266.32	268.84	274.40	276.96	283.70	285.00	293.45	295.16	298.60
	240.60[a]									272.20		281.40		288.72			
4.5 kGy	242.24	244.76	249.52	252.32		257.36	260.72	263.94	266.32	269.77	273.90	278.72	283.96		292.92	295.44	298.90
		246.72[a]								271.00		281.72		288.72			
D^{VIII}	243.10	245.90	248.12	251.48	254.28	256.52	260.44	263.52	266.88	269.68	275.28	280.04	283.12	289.00	292.36	294.88	298.08
	240.61[a]									272.20							
2 kGy	242.52	245.60	248.68	251.48	254.28	256.80	260.72	263.52	266.32	269.12	274.44	279.48	282.28	285.10	292.62	295.16	298.52
	240.45[a]										272.20	276.96		288.72			
4.5 kGy	242.34	244.80	247.00	252.32	254.44	257.64	260.72	263.24	266.32	270.80	275.90	278.64	284.24	288.72		295.24	298.95
			249.35[a]							273.90		281.72					

[a]Positions of maxima of intermediate absorption bands of tyrosine solutions

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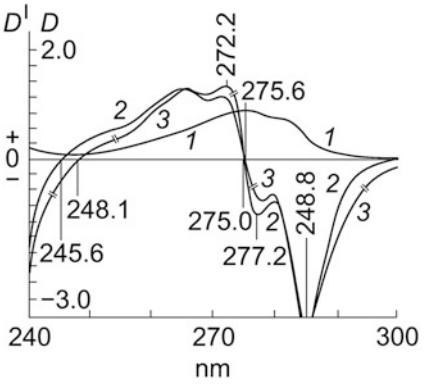

Fig. 4.15 The absorption spectrum of tyrosine solution and the character of its D^{I} change under 600 Gy irradiation: (*1*) the absorption spectrum of tyrosine; (*2*) the control D^{I} spectrum; (*3*) the change in D^{I} after irradiation. *Ordinate*: relative units of absorption (D) and of D^{I}

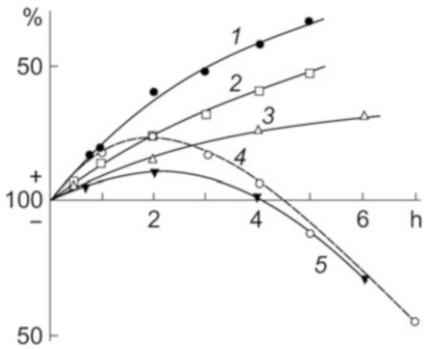

Fig. 4.16 Dynamics of OD change for tyrosine solutions in the principal absorption maximum ($\lambda_{max} = 275.2$ nm) under the influence of γ-irradiation (600 Gy/h). Curves of change for solutions with initial OD of (*1*) 0.977, (*2*) 0.922, (*3*) 0.822, (*4*) 0.303, and (*5*) 0.299. *Ordinate*: percentage change in OD (D)

recording of derivatives of different orders allowed us to solve the problem of the obviousness of one or another band in the fine structure of derived spectra (Rutman et al. 1976b; Saakov et al. 1987a, b).

Comparative analysis of SBs found by registration of different derivatives of the absorption spectrum enabled us to answer whether the obviousness or non-obviousness of a maximum could be considered a sign of the presence or absence of a substance. This situation can arise at the interference of vectors of positive satellites, causing the masking of some bands with others and accompanied by the appearance of false maxima. Differentiation of a monotonic change in the initial spectral outline (Fig. 4.15, curve 1) assists precise determination of positions of the chosen bands on the wavelength scale, which is especially well seen on D^{IV} and D^{VIII} curves (Figs. 4.17 and 4.18, curves 1). Furthermore, the possibility of a considerable increase in the dynamic range of spectra recording created good conditions for the reliable separation of necessary spectrum regions (Fig. 3.34). From Table 4.3 and Fig. 4.18, it follows that the complex structure of the tyrosine spectrum consists of 18–19 bands, whereas previously it was only possible to reliably determine three bands in the D^{II} spectrum (Saakov et al. 1987a, b).

Comparative analysis therefore solves the problem of correct identification of peaks and demonstrates the great advantages of the fourth and eighth derivatives for

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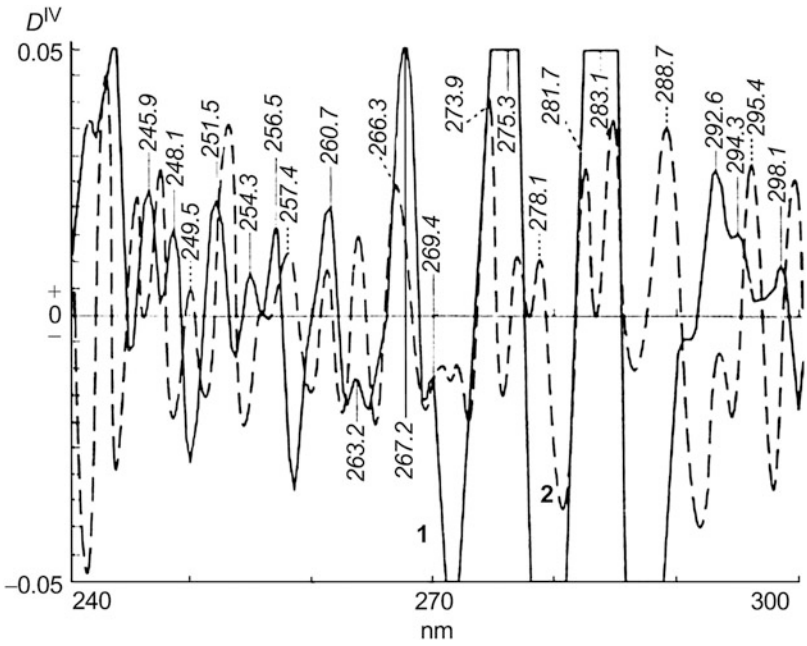

Fig. 4.17 The D^{IV} spectrum of tyrosine solution and the character of its change under irradiation of 2.4 kGy: (*1*) the control D^{IV} spectrum; (*2*) after irradiation. *Ordinate*: D^{IV} of the absorption spectrum

analysis of the real form and structure of the absorption band and of small spectral changes appearing under the effect of EFEs. Thus, a reliable methodological basis was created, and the complex harmonics of derived Tyr spectra was proven.

Figure 4.16 shows the kinetics of absorption change in the main maximum of the Tyr absorption spectrum during radiolysis. The radiation doses used correspond to those accepted (Rowbottom 1955; Amiragova et al. 1964). The increase in OD with increasing radiation dose proves that, in the radiolysis process, compounds are formed with a higher coefficient of molar extinction than that of Tyr. However, with an increase in radiation dose, the curve begins to dip. In this case, the inverse dependence of the decrease in speed of the OD curve on the OD of the initial Tyr solution is revealed. For OD = 0.822 (curve 3), this dip reaches the value of 74 % of the initial density after 29 h and is 4 % after 44 h. For OD = 0.303 (curve 4) and OD = 0.299 (curve 5), the OD decrease reaches 48 and 30 % after 9 h, respectively, and 4 and 10 % after 24 h. The descending branch of the OD curve indicates the appearance in the radiolysis process of compounds with a smaller molar extinction coefficient, for example, β-Phe (Rutman et al. 1976b). Comparison of data in Table 4.3 and Fig. 4.18 with the results in tables from published papers (Amiragova et al. 1954; Vartapetyan et al. 1966) indicates the validity of the conclusion.

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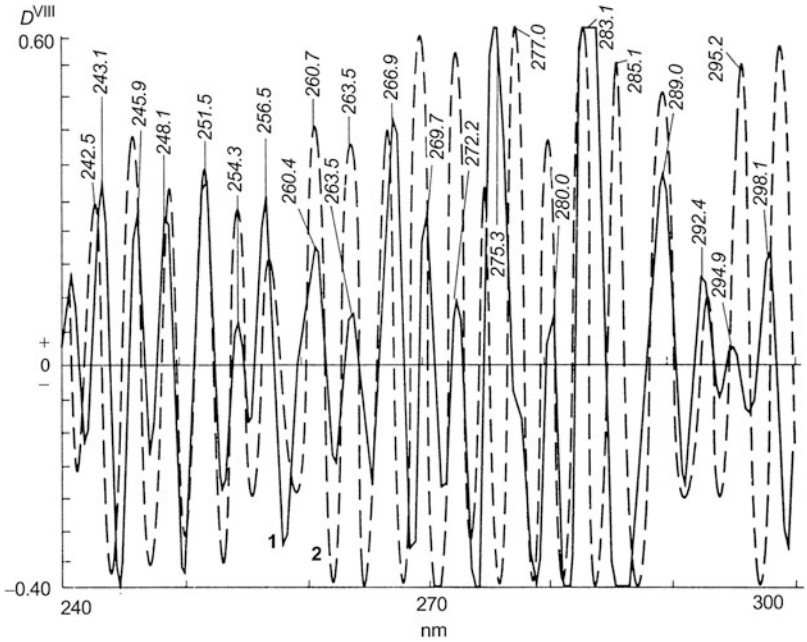

Fig. 4.18 The D^{VIII} spectrum of tyrosine solution and special features of its change under irradiation of 5.4 kGy: (*1*) the control D^{VIII} spectrum; (*2*) after irradiation

Figure 4.15 presents the absorption spectrum of tyrosine, which is characterized by poorly developed vibrational structure and fuzzy maxima. The influence of 600 Gy does not result in a substantial change in the curve of the first-order derivative of the absorption spectrum. Significant changes in the curve are practically not found. These data correspond to the ascending segment of the curves in Fig. 4.16.

A fourfold increase in radiation dose changes the fine structure of D^{IV} spectrum characteristically. Absorption in principal maxima ($\lambda = 275.28$ and 283.12 nm) decreases, their partition into two or three new bands occurs, and new absorption bands appear in the spectrum (Fig. 4.17, curve 2). The data time in the figure corresponds to curves 4 and 5 in Fig. 4.16, when an initial increase in the OD is located on the descending branch and is equal to the initial OD of the solution before irradiation. Following γ-irradiation (5.4 kGy), there is a greater partition of principal absorption maxima (Fig. 4.18, curve 2) and a change in the spectrum picture in the region 260–272 nm. The spectrum analysis allows a judgment to be made about the presence in the complex mixture of radiolysis products of absorption bands peculiar to β-Phe. This agrees with results on β-Phe detection in the mixture using D^{II} spectra (Saakov et al. 1987a, b) and with published data (Saakov 1993e) and Table 4.2. Aerobic conditions of radiolysis promote an increase in the decay rate of tyrosine. In the process of irradiation of water, the hydroxyl radicals

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oxidize the tyrosine aromatic ring through alternative ways (Rowbottom 1955; Amiragova et al. 1964) that results in the formation of 3,4-DOPA and, through it, of dopamine, dopaquinone, and adrenaline.

After acute irradiation of an organism, the total protein quantity changes insignificantly, but the quantity of albumin decreases, which is connected with the disorder of liver function. The destruction of amino acids and, in particular, of AAAs during radiolysis changes the physical and chemical properties of proteins, and this disrupts the osmotic properties of blood plasma. The analogous reaction of blood plasma was met in research on the influence of other EFEs (Sokolova et al. 1991, 1992, 1993).

Thus, the possibility to assess the degree of Tyr radiolysis from DSHO creates, in turn, the basis for a rapid method for assessing radiation damage to protein structures on the basis of spectrophotometric analysis of proteins using the fourth or eighth derivatives of their absorption spectra. The high informativeness (information capability) of derived spectra enables them to be used to assess changes in the value of the albumin-to-globulin ratio, which is used as an indicator of the degree of radiation injury. This question is considered more fully in Sect. 4.1.8.

Thus, this section has presented the reliably measured DSHO for Tyr solutions and shown the directivity of their change under the effect of high doses of γ-irradiation, which serves as the basis for rapid resolution of questions about the depth of destruction of protein structures with γ-irradiation.

4.1.4 Character of Tryptophan Derived Spectra Changes Under the Influence of γ-Irradiation

In assessment of the influence of ecological catastrophes, particularly radiation accidents, on the physiological processes of animals and plants, two questions arise that are connected with assessment of the physiological state of the animal or plant organism in the zone of ecological risk: (1) How can the level of damage to a living cell be instrumentally assessed rapidly and reliably (Saakov 1992; Saakov et al. 1993; Pronkin and Saakov 1997)? (2) What mechanisms of change in the metabolism underlie the formation of adaptation processes, the reparation of functional reactions, or a lethal outcome for the organism? Further, how do these mechanisms influence the physiological activity of plant and animal cells?

In previous work, we considered the specific character of damage to the energy mechanisms of the green cell under the influence of high doses of ionizing radiation, using for this purpose the capabilities of derivative spectrophotometry of high orders (DSHO) for assessment of the depth of chloroplast damage (Saakov 1993c, d).

In this section, we attempt to assess, with the help of DSHO, the products of radiation transformations of Trp and their possible influence on the adaptation mechanism reactions in plants and animals.

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In the array of diverse information about the physiological response of animals and plants to extreme ecological impacts (EEIs), the significant role of the state of protein structures and of AAAs under the influence of ionizing irradiation has been shown (Kuzin et al. 1955; Goncharova and Sheverdov 1993; Saakov 1993e). In this case, a change in β-Phe and Tyr concentrations was combined with the formation of dopamine, thyroxine, noradrenaline, adrenaline, and melanin (Saakov 1993b, e; Pronkin and Saakov 1997).

It is known that, as a result of Trp metabolic transformations, this amino acid can be the precursor of such cell mediators as 5-hydroxytryptamine (serotonin, Srt) and 3-oxyindolyl acetic acid (3-IAA), which actively influence the course of physiological reactions in animals and plants. There is a noted specific similarity between radiation–chemical and biological ways of Trp oxidization (Amiragova et al. 1964). This suggests that the radiation effect on cell processes and on their coordination is determined not only by the toxic action of ammonia liberated after deaminization of amino acids (Barron 1955), but also by the formation of a number of biologically active cell mediators that affect the activity of functional processes and the behavioral reactions of organisms to EEIs.

We studied changes in the optical and absorptive properties of solutions of DL- and L-tryptophan under the influence of γ-irradiation. It is important to understand the character of Trp spectra change because the intensity of Trp absorption is 30 times greater than that of Phe at equal concentrations and four times greater than that of Tyr. This means that the outlines of optical spectra of proteins containing Trp are formed to a high degree by the totality of contribution of the Trp SBs.

As γ-source, we used a screened capsule containing ^{57}Co isotope with radiated energy $E_\gamma = 122.06$ and 136.47 keV; the half-life $T_{1/2}$ was 271.3 days, and its power was 6.50 Gy/h. The absorption spectra (D) and their D^{I}, D^{II}, D^{III}, D^{IV}, and D^{VIII} derivatives were registered with the help of the recording UV–Vis spectrophotometer DW-2000 FA-220 (SLM-Aminco, FRG). Separate recordings were carried out in the instrument UV–Vis-Specord (Carl Zeiss, Jena, FRG) in analog or numerical form with subsequent computer processing of spectra using an appropriate program (Saakov et al. 1990; Saakov 1992). The application of numerical DSHO recording confirmed the reliability of registration of the harmonics of Trp bands in analog form, including a new band for Trp (named the Ψ-peak) visible in D^{II}–D^{VIII} spectra and characteristic for tryptophan indication (Saakov et al. 1987a, b).

Table 4.4 summarizes the results of the measurement of Trp absorption spectra and presents the broad range of bands isolated using registration of its DSHO. We emphasize the need in the experiment for registration of derived spectra of both even and odd orders in order to make a conclusion with high reliability about the presence of an absorption band of the investigated substance and to exclude the influence of false satellites.

Registration of D^{IV}–D^{VIII} spectra (Fig. 4.19) helped to divide the principal absorption maximum (compare with Fig. 4.5) of Trp at $\lambda = 278.6$ nm (see Fig. 4.21, curve 1) into seven bands not previously known in the literature; the second maximum at $\lambda = 286.4$ nm into seven bands; the zone of Ψ-peak at

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Table 4.4 Bands of absorption spectra and their derivatives for tryptophan solutions

Type of spectrum	Numbers and positions of the main absorption bands of tryptophan solutions (nm)																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
D		271.50							278.60					286.40			
D_0^{I}	241.80			273.60					278.20					286.70			
$D_{\min}^{\mathrm{I}}$												283.20					290.80
D^{II}		271.36	272.90		274.20	276.60	277.40	278.30	279.20	280.70	282.80	285.30	286.66[a]	287.14[a]		288.10	289.80
					275.20[a]				280.10[a]		281.90[a]	283.60[a]				288.60[a]	290.70[a]
D^{III}	270.50	271.30	272.52	273.70	274.80	276.70	277.60	278.70	279.80	280.92	282.28	284.50	285.24	286.10		288.24	289.40
		271.95[a]		274.20[a]	275.52[a]	277.35[a]	277.90[a]	279.24[a]	280.10[a]	281.76[a]	283.10[a]						291.10[a]
D^{IV}	269.20	271.32	272.64	273.72	274.92	276.70	277.56	278.64	279.72	280.80	282.36	283.44	284.76	285.84	287.16	288.12	
					275.93[a]												
	270.12	271.32	272.64	273.72	274.92	276.72	277.56	278.64	279.72	280.80	282.36	283.44	284.76	285.84	287.16	288.12	289.51
					275.78[a]												
Bands of near ultraviolet region																	
D^{III}														266.90		268.00	269.00
D^{IV}	250.30	251.15	253.84	255.52	256.60	258.04	258.70	260.30	261.34	262.36	263.32	264.40	265.00	266.56	267.52	268.50	
Bands of far ultraviolet region																	
D^{II}	291.50	292.60		294.25	295.44	296.20	297.40	298.70									
D^{III}	291.88	292.50	293.44	294.20	295.36	296.40	297.70	299.10	299.80								
					295.80[a]	297.16[a]	298.30[a]										
D^{IV}		292.50	293.12	294.20	295.20	296.40	297.68	299.10	300.20	301.14	301.93	302.84	305.10	306.30	307.23	308.18	309.90
			293.96[a]														

[a]Position of maxima of intermediate absorption bands in tryptophan solutions

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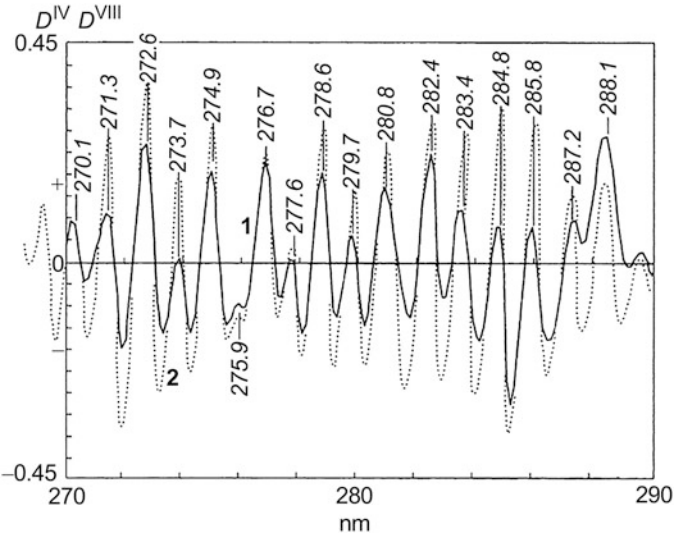

Fig. 4.19 The fourth and eighth derivatives of the absorption spectrum of DL-tryptophan solution (0.5×10^{-4} M, pH 5.3). Bands 272.6, 273.7, 274.9, 275.9, and 276.7 nm are in the region of the Ψ-peak (Saakov et al. 1990; Saakov 1987; Semenova and Saakov 1989)

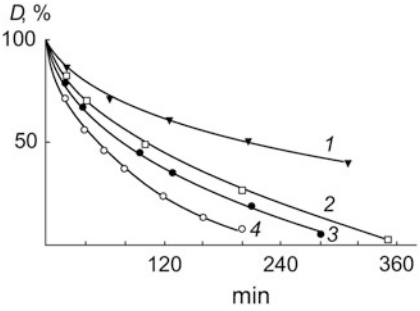

Fig. 4.20 Dynamics of OD change for the principal maximum ($\lambda_{max} = 278.6$ nm) of DL-tryptophan absorption spectrum under influence of γ-irradiation (650 Gy/h). Curves of change for solutions with initial OD of (*1*) 0.889, (*2*) 0.544, (*3*) 0.217, and (*4*) 0.10. *Ordinate*: percentage change in OD (*D*); *abscissa*: time in minutes

$\lambda = 274$ nm into five bands; and the bend shoulder at $\lambda = 271.5$ nm into five bands. The recurrence of bands in spectra of even and odd derivatives stresses the correctness of their detection. As a result of this, a reliable methodological base for the rapid registration of radiation transformations of Trp was created. The method can be recommended for application in analytical works with different proteins, AAAs, or other substances having a spectral outline.

The irradiation of Trp solutions with γ-rays causes an OD decrease in the principal absorption maximum, which can be interpreted as the formation of

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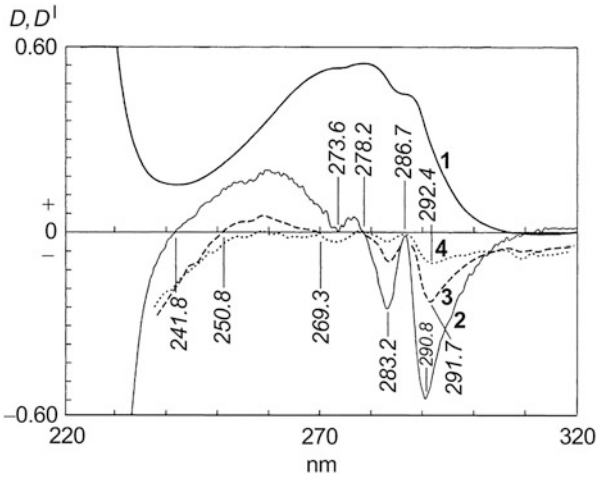

Fig. 4.21 The absorption spectrum of DL-tryptophan and the character of its D^{I} change under γ-irradiation: (*1*) the absorption spectrum of tryptophan; (*2*) the control D^{I} spectrum; (*3*) change in D^{I} after 400 Gy irradiation; (*4*) change in D^{I} after 600 Gy irradiation. *Ordinate*: *D* and D^{I}, relative units

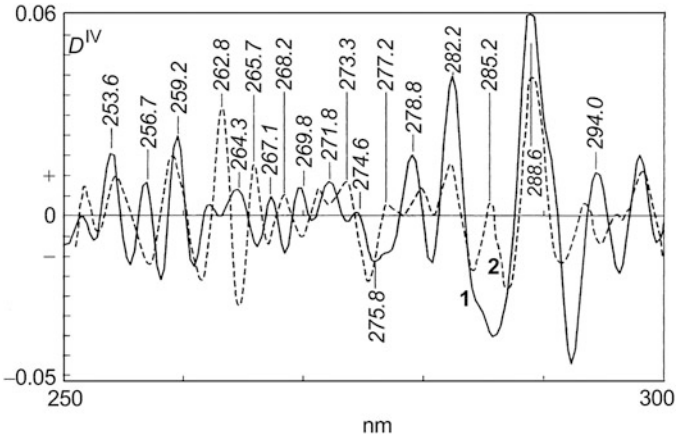

Fig. 4.22 The change character of D^{IV} of the absorption spectrum of L-tryptophan solution (0.3×10^{-4} M, pH 5.0): (*1*) after 400 Gy irradiation; (*2*) after 600 Gy irradiation

intermediate products with a smaller molar absorption coefficient. The curve shape is analogous with that for the pigments of chloroplasts (Saakov 1993c) and is radically different from the curves of radiolysis of β-Phe and Tyr (Saakov 1993b, e). The rate of Trp destruction at identical radiation doses is inversely proportional to the initial OD (Fig. 4.20). In general, the picture of spectrum *D* practically does not change, which accents the high percentage of Trp present. However, on the curve of the D^{I} spectrum, the changes in the far UV range of the Trp spectrum are visible (Fig. 4.21, curves 2–4, $\lambda = 291.7$ and 273.6 nm). The level of these changes is a function of the received dose of γ-irradiation. From the data in Fig. 4.22, it follows that the greatest shifts in the D^{IV} spectrum, caused by influence of

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γ-irradiation, are connected with changes in the bands of the principal maximum ($\lambda = 278.80$ and 282.2 nm) and the second maximum ($\lambda = 285.2$ and 288.60 nm). The picture of bands of the Ψ-peak ($\lambda = 274.46$ and 275.5 nm) is also significantly disrupted. The structure of bands in the near UV range of spectrum seriously changes ($\lambda = 268.20$, 265.70, 262.80, and 256.70 nm).

It is known that serotonin (Srt) is spectrally identified with two absorption maxima at $\lambda = 275.0$ and 293.0 nm (Amiragova et al. 1964). Applying the usual direct spectrophotometric methods, it is difficult to separate the harmonics of bands of one or other substances in the mixture of products of radiolysis. The analytical capabilities of DSHO allow us to conclude with some probability that the bands in the region $\lambda = 274.6$, 276.9, 293.4, and 294.0 nm are connected with the presence of Srt in the mixture. Taking into account the optical properties of IAA (Baranov et al. 1975; Morton 1975), the presence of bands (Fig. 4.22) $\lambda = 262.8$, 273.3, 277.2, and 285.2 nm also corresponds to its presence in the mixture of products of Trp transformation.

In the blood of animals and humans, Trp is characteristically present in the form of Trp bound to protein and also in the form of free amino acid (15–20 %). The high speed of Trp renovation and metabolism is noted. The same is peculiar to plant cells. Data indicates the predominance of Trp deaminization during its radiolysis, with formation of 3-IAA. By this, it is possible to explain the activation of growth processes in plants at the population level in places of radiation catastrophes. At the same time, in solutions containing oxygen and in cellular structures, oxidization of the fifth carbon atom in the Trp benzene ring results in formation of 5-hydroxytryptophan, and decarboxylation of the latter forms Srt. The decarboxylation of Srt causes 5-IAA formation. So it is possible to explain the appearance in the cell of active mediators that condition biochemical mechanisms of change in physiological reactions in animal and plant cells under EEIs.

During irradiation of an organism, the Srt content in the blood, spleen, and gastrointestinal tract decreases as a consequence of Trp metabolic disorder (Kurskii and Baksheev 1974). A decrease in Srt content is thought to increase the aggressiveness of irradiated males and is also accompanied by simultaneous increase in the tissue Srt antagonist dopamine (Morton 1975; Saakov 1993e). At the same time, it is shown that low-frequency vibrations, which decrease the behavioral activity of experimental animals, result in an increase in Srt and histamine contents in the pulmonary tissue (Akhmetzyanov et al. 1994). Furthermore, the data presented in Table 4.4 and Figs. 4.19, 4.20, 4.21, and 4.22 indicate that the previously discovered high stability of the chlorophyll–protein complex and of protein-synthesizing systems (Saakov 1993c; Saakov et al. 1993; Goncharova and Sheverdov 1993) is not caused by radioresistance of tryptophan molecules, because transformations of the latter with the formation of biologically active mediators occurred in response to a smaller dose of γ-irradiation than in irradiation of plant objects (Saakov et al. 1993; Saakov 1993d; Goncharova and Sheverdov 1993). This phenomenon coincides with the data described in previous sections on the stability of ChlPC and β-Phe.

Thus, it is shown that the analytical possibilities of DSHO (of four to eight orders) are applicable for rapid assessment of the appearance of Trp radiolysis

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products. The resultant mediators are combined with the mechanism of adaptive reactions in animals and plants in zones of ecological risk.

4.1.5 Comparative Characteristics of Albumin Denaturation Changes Under Exposure to High Temperatures and Radiation

The presence of denaturation changes in protein molecules after their irradiation by an ionizing radiation source (IRS), in the presence of oxygen in solution, by doses substantially exceeding the lethal dose for human and animals has been discussed and summarized (Kuzin 1962). The view (Kuzin 1962; Khenokh and Lapinskaya 1955, 1956) that the absorption SBs of AAAs, albumin (Alb), and globulin (Glb) during irradiation were invariable was reliably disproved by our articles (Saakov 1993b, e, 2000b, c). This became possible because of the fundamentally new information possibilities of the DSHO method and the thorough research of kinetic curves of OD changes during radiolysis. Changes in the fine structure of absorption spectra of protein and amino acid preparations appeared under the influence of γ-radiation and were detected with the help of the DSHO method. Such changes can be explained as the appearance of damage to the native configuration of protein molecules caused, possibly, by induced denaturation changes (Pronkin and Saakov 1997; Saakov 2000b, c). In this case, acceleration of Alb disintegration under IRS influence is combined with the destruction of membrane Alb–lipid structures, simultaneous oppression of the function of Alb in the transport of Trp and metal ions, and failure of the maintenance of osmotic pressure in the plasma.

For purposes of broadening the fields of use of DSHO, we performed a comparative study of the fine structure changes in the absorption spectra of Alb solutions under thermal and radiation influences, inducing protein denaturation.

In connection with the fact that aqueous Alb solutions correspond better to the protein state in native structures than dry preparations (Kuzin 1962), with the help of the procedure (Saakov 1993b, e, 1998, 2000b, c), we studied the influence of γ-rays of isotope ^{57}Co on preparations of bovine and human serum albumins (from Sigma) and on a number of medical serum preparations. The registration of absorption spectra and of their DSHO was performed using the UV–Vis spectrophotometer DW-2000 FA-220 (SLM-Aminco, FRG). The recording of spectra in analog form was carried out using the instrument UV–Vis-Specord (Carl Zeiss, Jena, FRG) according to the published procedure (Saakov et al. 1987a, b). For the possibility of comparison and unification of data obtained in numerical and analog forms and of recording scales, the spectra were scanned and digitalized with the help of the program *Graph Digitizer* 2.14. Further graphing was carried out with the help of the program *Microcal Origin* 6.1, giving the possibility to calculate errors and to perform operations of differentiation simultaneously. A good agreement of registrations of parallel samples in spectral measurements and reproducibility of

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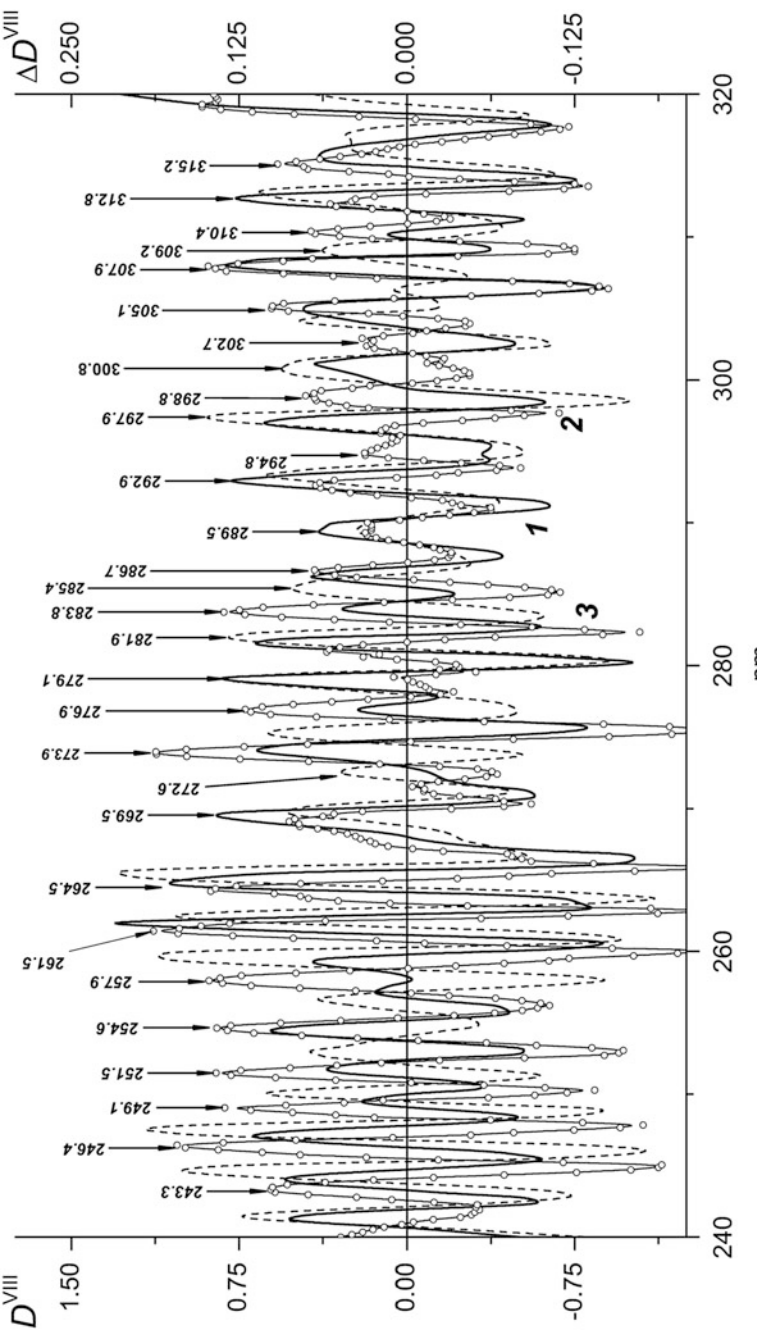

Fig. 4.23 Influence of temperature on the change character of D^{VIII} of the albumin absorption spectrum in the region 240–320 nm: (*1*) D^{VIII} of albumin solution (pH 7.2; control); (*2*) change in D^{VIII} of albumin solution under influence of temperature 75°C for 5 min; (*3*) differential spectrum ΔD^{VIII} "control minus experiment." *Ordinates*: OD, relative units (*left* for *curves 1* and *2* and *right* for *curve 3*); *abscissa*: wavelength, nm

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records for the same sample (±2 %) and from experiment to experiment (±8 %) was very important.

Many years of DSHO experience showed the prospect of using this method for analysis of the smooth outlines of protein spectral curves and of changes in the fine structure of their spectra under different kinds of influence (Saakov et al. 1987a, b, 1990; Saakov 2000b, c). Because of this, taking into account the increased informativeness of derived spectra of the eighth order (D^{VIII}), we investigated and compared the character of their change for Alb solutions under the influence of different temperatures and different doses of γ-radiation. The specific character of DSHO for Alb and their dynamics under the influence of different doses of γ-irradiation has been described (Saakov 2000c).

From the data in Fig. 4.23, it follows that a small shift in the pH of the Alb solution (pH 7.2), in comparison with the data of Saakov (2000c), causes significant movements of the SB set because of the ionization of chromophores (Fraifelder 1980; Saakov et al. 1987a, b). This movement was not detectable using the old spectroscopy technique (Kuzin 1962; Khenokh and Lapinskaya 1955, 1956). For convenience, for comparison of changes in the fine structure, we considered parts of the spectrum in regions 240–320 or 250–300 nm.

An increase in temperature promotes an increase in OD values, that is, there is a *hyperchromic* effect that probably indicates formation of a less-ordered structure of Alb molecules comparative with the initial structure (Fig. 4.23, curve 2). This hyperchromia is usually the criterion for denaturation or for protein transfer into an unfolded state. In this case, it is possible to speak with some confidence about the larger accessibility of internal AAA residues (Tyr and Trp) to the solvent used. The stated conforms both to theoretical positions (Kuzin 1962) and to the kinetics of Alb OD increase under the influence of γ-radiation (Fraifelder 1980; Saakov 2000c). Obtained data confirm and complement previously published results (Saakov et al. 1987a, b; Fraifelder 1980; Saakov 2000c) at a qualitatively new level. It should be immediately emphasized that the new DSHO technology allowed the discovery, for the first time, in a turbid medium and in the complex harmonics of the D^{VIII} Alb spectrum, of OD changes in some SBs produced by the hypochromic effect. The following regions of spectrum could be attributed to this change: 257.9, 262.0, 269.5, 273, 276.9, 283.8, 289.5, 293.0, 305.0, 308.0, 313.0, and 317.0 nm. We focus the reader's attention on this hypochromia of separate SBs, because earlier in the literature, the presence only of an integral hyperchromic effect in the protein spectrum (Kuzin 1962; Khenokh and Lapinskaya 1955) characteristic of denaturation changes was discussed. Specifically, due to new methodological DSHO possibilities, the hyperchromic effect was found in the harmonics of only some separate bands of the D^{VIII} spectrum (λ regions 241.0, 244.0, 247.0, 251.0, 256.0, 252.0, 259.0, 265.7, 281.9, 285.0, 297.3, and 300.0 nm).

From our data shown in Fig. 4.23, it follows that the process of Alb denaturation is accompanied by the shift of a number of maxima of SBs into the long-wave region, a *bathochromic* shift (λ regions 245.0, 248.0, 250.0, 253.0, 259.0, 262.5, 265.5, 275.0, 293.5, 308.9, 313.0, and 317.0 nm). Other SBs are *for the first time* characterized by a *hypsochromic* shift into the short-wave part of the spectrum (λ

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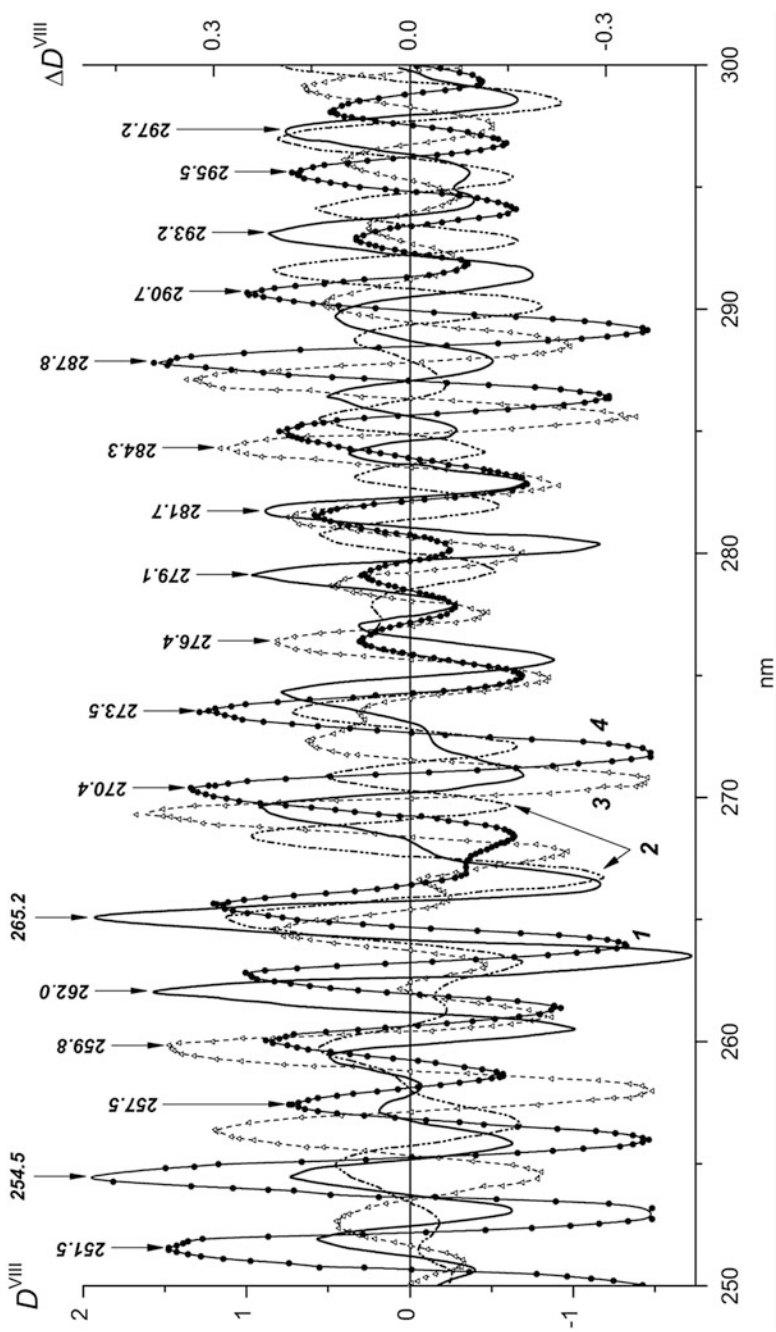

Fig. 4.24 The influence of different doses of γ-radiation on the change in D^{VIII} of albumin solution (analogous with Fig. 4.23): (*1*) control; (*2*) influence of γ-radiation dose 3.78 kGy; (*3*) influence of γ-radiation dose 13.23 kGy; (*4*) difference spectrum ΔD^{VIII} "control minus experiment" 3.15 kGy. *Ordinates*: OD, relative units (*left* for D^{VIII} spectra, *right* for ΔD^{VIII}); *abscissa*: wavelength, nm

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regions 253.0, 256.0, 272.0, 285.4, 300.0, and 304.0 nm). Some SBs do not change their position under the influence of temperature (i.e., 269.5, 279.2, 281.9, 289.5, 297.3, and 300.0 nm). We suggest that their invariability is adequate in the case of radiation influence.

Finally, an OD change opposite to the initial one occurs for an insignificant number of SBs (λ regions 251.5, 257.9, 273.9, 276.9, 283.8, and 306.0 nm). Additional information about the character of temperature-induced disorders of the D^{VIII} spectrum follows from the form of the differential spectrum (ΔD^{VIII}), which was obtained by the subtraction of one absolute absorption spectrum from another, for example, by subtraction from the control D^{VIII} Alb spectrum of the experimental variant under thermal influence (Fig. 4.23, curve 3). In this case, new values of extinction did not coincide with the directivity of initial spectra, maxima and minima of absorption could be displaced, and the appearance of new maxima was possible (λ regions 271.0, 257.9, 294.8, 298.8, 302.7, and 315.2 nm).

From the data shown in Fig. 4.24, it follows that the harmonics of the control D^{VIII} Alb spectrum (curve 1) is identical to that in Fig. 4.23. Comparative assessment of the influence of different doses of γ-radiation is shown by curves 2 and 3 in Fig. 4.24. The effect of a 3.78-kGy dose results in changes in the fine structure of the $\mathrm{D}^{\mathrm{VIII}}$ spectrum that are different to those resulting from high temperature, despite the fact that shifts are present in the spectrum both with a bathochromic shift (regions 255.0, 257.5, 259.8, 291.6, and 294.0 nm) and with a hypsochromic shift (regions 268.4, 273.5, 276.4, 278.0, 280.5, 283.0, 285.5, 289.0, 291.6, and 297.2 nm). Shifts are practically absent in regions 251.5, 254.5, 259.8, 262.0, 265.2, and 297.2 nm. A hypochromic effect with OD decrease is registered for the first time in regions 255.0, 257.5, 261.8, 265.0, 270.4, 279.0, 280.7, 287.0, 288.7, 290.0, 293.8, and 295.5 nm. Similarly, a reliable hyperchromic OD increase with this radiation dose is observed only in SBs 286.0, 292.0, and 294.0 nm.

These data can be interpreted as the existence of protein structures (pools, conformations) with differing resistance to the external influence of radiation. An increase in the radiation dose to 13.23 kGy causes turbidity of the solution and induces *hyperchromic* OD increases in general similar to those in Fig. 4.23, and affects regions 256.0, 259.8, 269.4, 276.4, 284.3, 287.0, and 299.3 nm. Simultaneously, *hypochromic* OD effects appear in regions 252.6, 262.0, 264.6, 272.3, 279.1, 281.4, and 293.3 nm. OD changes in spectral harmonics are accompanied by *bathochromic* shifts in regions 252.6, 256.3, 287.0, and 290.3 nm and by *hypsochromic* effects at 264.6, 269.3, 272.1, 276.4, 278.7, 296.0, and 299.0 nm. The absence of reliable shifts of SBs along the *x*-axis is registered in spectral regions 259.8, 262.0, 281.7, 282.0, and 293.2 nm. Also, as under the thermal influence, OD changes that are differently directed in comparison with the control appear at 254.5, 257.9, 267.8, 272.2, 292.0, and 294.9 nm. The differential spectrum of ΔD^{IV} (Fig. 4.24, curve 4) "control minus experiment" reveals a shift of maxima and minima of absorption, accenting the movements of positions of Alb absorption bands under the influence of γ-irradiation.

Thus, analysis of changes induced by temperature and γ-irradiation, namely, changes in the fine structure of D^{VIII} spectral harmonics, confirms that the DSHO

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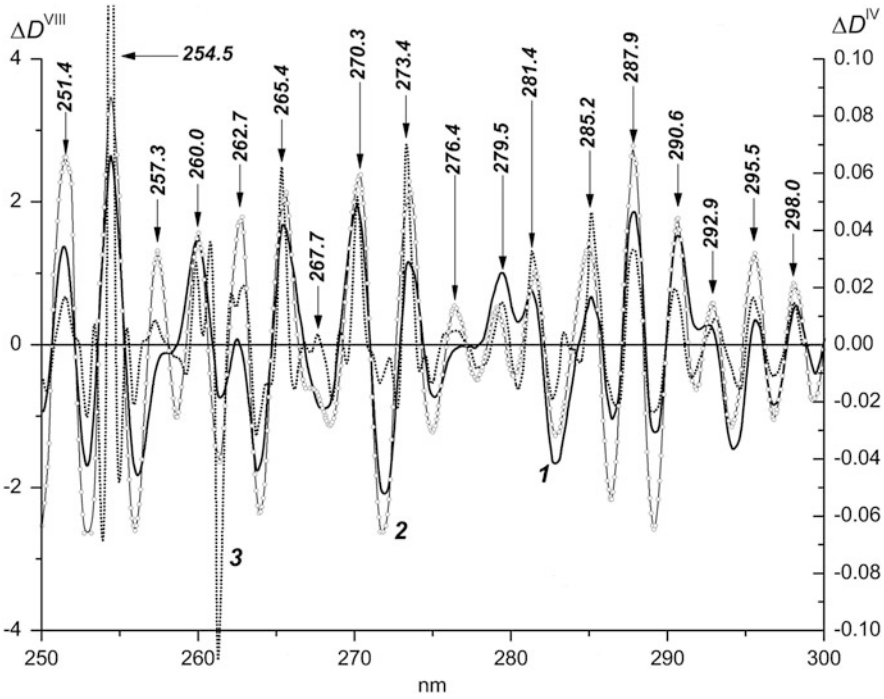

Fig. 4.25 Example of comparison of theoretical and experimental difference spectra ΔD^{IV} "control minus experiment": (*1*) initial curve ΔD^{IV} "control minus experiment" used for theoretical calculation; (*2*) experimental curve of difference spectrum ΔD^{VIII} (3.15 kGy); (*3*) theoretical curve of difference spectrum ΔD^{VIII} calculated from *curve 1*. *Ordinates*: OD, relative units (*left* for *1* ΔD^{IV}, *right* for *2* and *3* ΔD^{IV}); *abscissa*: wavelength, nm

method is a reliable method of assessment of the specific character of denaturation changes, combined both with hypochromic and hyperchromic effects in separate bands of the Alb spectrum and also with changes in the fine structure of spectra caused by bathochromic and hypsochromic shifts, or with the absence of shifts of some SBs.

We digitalized spectra and carried out their further graphic visualization with the program *Microcal Origin* 6.1. The control differentiations of derived spectra were carried out, from the lowest orders to the high ones, with sequential smoothing on seven to ten points of curves before five and after eight differentiations with the help of Fourier transform and also after five to seven differentiations with the help of *Smoothing*: *Adjacent Averaging* on five points. An example of this calculation is presented in Fig. 4.25. Results of differentiation show that in the case of registration of derived spectra of the second to fourth orders, the calculated transfer to the eighth and higher orders does not add substantial errors for the interpretation of experimental results.

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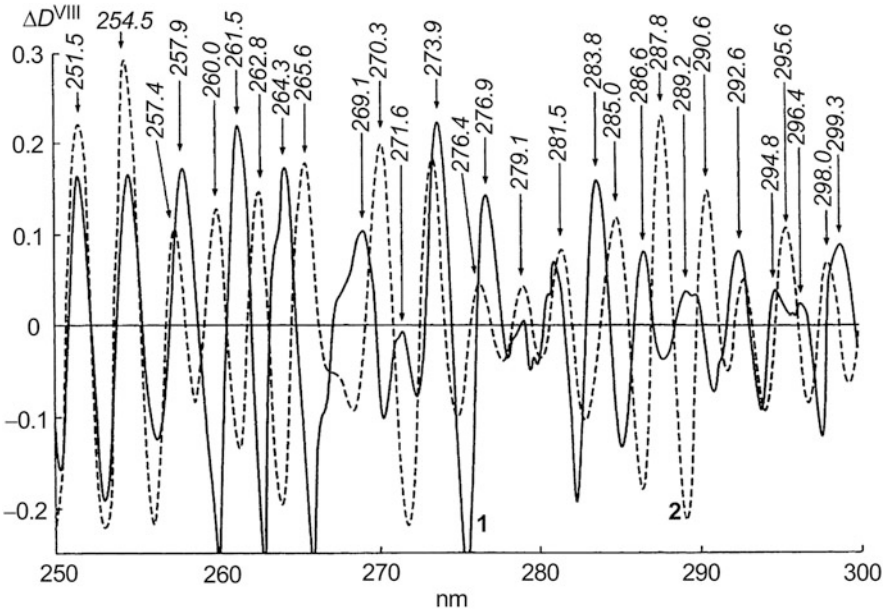

Fig. 4.26 Comparison of difference albumin spectra "control minus experiment": (*1*) under thermal denaturation; (*2*) under denaturation induced by γ-irradiation. *Ordinate* and *abscissa* as in Fig. 4.25

Thus, in the absence of technology for D^{VIII}–D^{XII} spectra registration, it is possible to reproduce DSHO by calculation and to obtain completely comparable and correct data.

In the comparison of different shifts of Alb spectra harmonics induced by temperature and γ-irradiation, only a few regions are revealed as common for the influence of both factors: *bathochromic* shifts (250.0–252.0 and 259.0–259.8 nm); *hypsochromic* shifts (272.0 and 285.0–285.5 nm); the absence of wavelength shifts (281.7–281.9 nm); differently directed changes in absorption (257.5–257.9 nm); and *hypochromic* effects (257.5–257.9 and 287.1–289.5 nm). The listed coincidences are rather exceptional considering the totality of elements of sharp disparateness induced in derived Alb spectra by temperature and γ-irradiation causing Alb denaturation. Nevertheless, this disparateness captures the attention; they are interesting and can be the subject of more thorough and promising studies.

The demonstrable picture of similarity and dissimilarity of DSHO harmonics in the fine structure of spectra during denaturation changes is presented in Fig. 4.26. From these data, it follows that sometimes the SB location of experimental variants coincides in an interval of the wavelength scale (regions 251.5, 254.5, 257.5–257.9, 273.9, 276.4–276.9, 279.0, 281.5, and 292.6 nm). In other cases, new SBs are induced (regions 260.0, 262.8, 265.6, 269.1, 270.3, 283.8, 285.0, 286.6, 287.8, 289.2, 290.6, 294.8, 295.6, 296.4, 298.0, and 299.3 nm), the positions of which are

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bathochromically or hypsochromically shifted with respect to the control. We emphasize that the appearance of these shifts is sometimes combined with the formation of new SBs (e.g., regions 260.0, 262.8, 264.3, 265.6, 271.6, 286.6, 287.8, 289.2, and 290.6 nm).

Thus, it is possible to conclude that changes in the fine structure of D^{VIII} spectral harmonics during denaturation changes of Alb, induced by thermal and radiation exposure, occur due to both unfolding of different quantities of AAA residues and, probably, their different quality. To determine to which AAA the derived spectra of the investigated heterogeneous system correspond is very complicated because of the presence of products of both radiolysis and denaturation in the mixture. However, the data previously described (Saakov 1994a, 1998) indicate that some SBs (Figs. 4.23 and 4.24) correspond to Tyr and Trp and also to products of their radiolysis. The above conforms to the point of view stated in articles (Khenokh and Lapinskaya 1956; Kuzin 1962) about the significant role of the hydroxyphenyl groups of Tyr residues during denaturation changes. In this case, the positions of some SBs indicate the participation of the imidazole and indole groups of Trp in denaturation changes of the spectrum, with different contributions.

The experimental material obtained by us suggests the existence of separate and specific mechanisms of Alb molecule transformation during thermal and radiation-induced denaturation. In this case (Fig. 4.24), the simultaneous damage of a large number of hydrogenous bonds, connected with a hyperchromic OD shift, requires significant doses of γ-irradiation. The aggravation of states induced by γ-irradiation is intensified by the additional influence of temperature or another external factor before or after exposure to γ-irradiation (Kuzin 1962; Saakov 2000d).

It is possible to suppose that the binary influence of external factors causes the joint damage to separate protein structures, which is summarized in the character of the change in the spectral picture according to the principle of additivity, similar to that taking place with photo-inhibition (Saakov 2000f).

Thus, for the first time, we have discovered the differential presence of hypochromic and hyperchromic effects in separate SBs during denaturation induced by temperature and γ-radiation. Simultaneously, a number of SBs are characterized by the differently directed OD change in comparison with control and also by bathochromic or hypsochromic shifts with a change in the fine structure of the spectrum. Only a small number of SBs do not have wavelength and OD shifts, which a reader should take into consideration in calculations of Alb quantity in damaged preparations. Separate regions of SBs were identified as being general for the influence of both factors according to the character of response to denaturation changes. A similar study of the behavior of the totality of separate SBs of Alb during its denaturing has not been performed earlier. Data analysis allows conclusions to be made about the different qualitative and quantitative mechanisms of denaturation changes in Alb solutions under the influence of temperature and γ-irradiation. These mechanisms should be considered in regions of natural disasters and ecological catastrophes when using reserves of blood plasma and other albuminous medical preparations.

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4.1.6 Changes in γ-Globulin Optical Spectra Under γ-Irradiation

Application of γ-globulin preparations (γ-Gl) for the treatment and preventive maintenance of a wide spectrum of infectious diseases is predetermined by the presence of antibodies in the γ-Gl fraction, namely, antibodies to many viruses, bacteria, and their toxins. Simultaneously, γ-Gl increases the general resistance of an organism through unspecific stimulation of immunogenesis, softening the course of infection. The γ-Gl fraction of blood serum is a mixture of the different immunoglobulins realizing an immune or antitoxic function; in this case, antibodies participate in the neutralization of foreign antigens. During irradiation of animal organisms with lethal and sublethal doses of γ-radiation (4–10 Gy), protein hydrophility is disrupted and there is partial loss of their antigenic properties. Examination of the population of inhabitants in a 30-km zone of the Chernobyl accident revealed a mass decrease in immunity, intensification of chronic diseases, and induction of new pathologies (Gesellschaft für Reaktorsicherheit 1987; International Atomic Energy Agency 1986). A decrease in the γ-Gl content during radiation sickness was noted, while the quantity of α- and β-globulins simultaneously increased. Furthermore, a change in the ratio of albumin-to-globulin content (Alb/Glb ratio) is considered to be one of the early signs of pathological changes in the composition of blood plasma and a function of radiation injury depth.

Investigation of the character of changes in the properties of endogenous immunoglobulins under extreme influences of different kinds is promising for discovering the mechanisms of their action and retention of the immune activity of preparations. Key problems during radiation injuries under conditions of ecological catastrophes and other extraordinary situations are a decrease in the effectiveness of active and passive immunization of irradiated organisms and the appearance of pathologic states, which are accompanied by a change in the γ-globulin fraction of blood proteins.

The influence of γ-ionizing radiation on solutions of bovine γ-Gl (25 mg/50 mL in 0.2 % NaCl solution; Sigma) was investigated. Absorption spectra in numerical form and their DSHO were obtained using the UV–Vis spectrophotometer DW-2000 FA-220 (SLM-Aminco, FRG). The recording of spectra in analog form was carried out with the instrument UV–Vis-Specord (Carl Zeiss, Jena, FRG) using the accepted procedure (Saakov et al. 1987a, b, 1990). For comparison and unification of data obtained in numerical and analog forms, the spectra were scanned and then digitalized with the help of the program *Graph Digitizer* 2.1. Further graphing and visualization of graphs, and also calculation of errors and regression coefficients were performed with the help of the program *Microcal Origin* 6.1. In general, the discrepancy between parallel samples in experiments did not exceed the significance level $r < 0.5$ %. As γ-source, we used a screened capsule with the isotope ^{57}Co, with radiation energy $E_\gamma = 122.06$ and 136.47 keV; the half-life period $T_{1/2}$ was 271.3 days and the power of γ-irradiation was 630 Gy/h.

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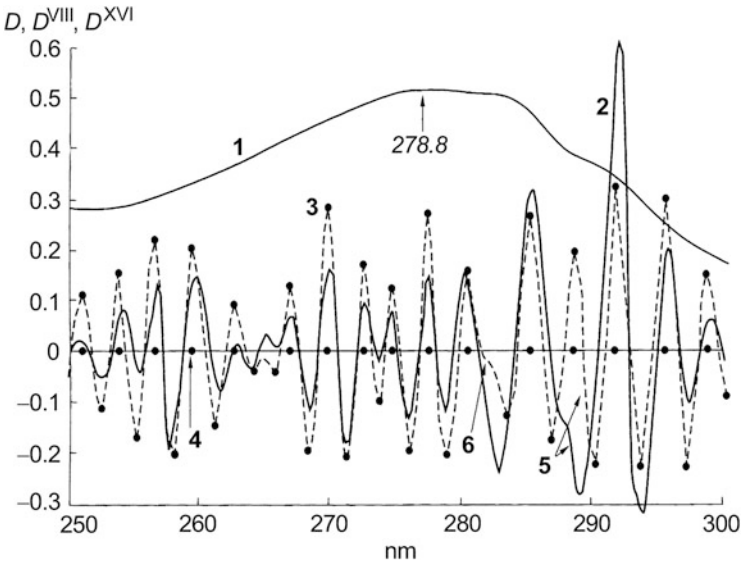

Fig. 4.27 The absorption spectrum (*1*) and its eighth (*2*) and 16th (*3*) derivatives for γ-Gl solution. *Black circles* (*4*) mark intersection of the wavelength axis with curves of third and fifth derivatives of the absorption spectrum. *Arrow 5* indicates resolution of inflection on the D^{VIII} curve as the peak on the D^{XVI} curve. *Arrow 6* indicates manifestation of the bend shoulder (inflection) on the D^{XVI} curve, absent on the D^{VIII} curve. *Ordinates*: OD, relative units; *abscissa*: wavelength, nm

The analytical capabilities of modern molecular spectrophotometry showed that the apparent good knowledge of the optical spectra of AAAs, creating the spectral outline of proteins, was far from complete (Saakov 1993b, e, 1998). In the process of our work, absorption spectra of γ-Gl solutions and their D^{I}–D^{V}, D^{VIII}, D^{XII}, and D^{XVI} derivatives were investigated. The choice of the order of derivatives was conditioned by the fact that, in contrast to D^{II}, D^{VI}, and D^{X}, the positions of true maxima of absorption bands in D^{IV}, D^{VIII}, D^{XII}, and D^{XVI} are located in the positive region (see Chap. 1), which makes it easier to compare experimental curves and to interpret results. For odd derivatives, the position of the maximum of initial absorption bands corresponds to the zero abscissa point (i.e., to the point of derivative sign change). Thus, the coincidence of abscissa intersection points for graphs of odd derivatives indicates the obviousness of maxima of D^{VIII} and D^{XVI} curves (Fig. 4.27).

Differentiation of the monotonic initial spectral γ-Gl outline, characterized by poorly developed vibrational structure and fuzzy maxima, reveals in the range 250–300 nm up to 16–17 absorption bands sufficiently reliably detected by the D^{VIII} registration method. The presence of the same obvious bands in the spectra of even and odd derivatives stresses the correctness of their determination. From the data in Fig. 4.27 and Table 4.5, it follows that application of the DSHO registration method has, for the first time, ensured the possibility of differentiation of the totality of

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Table 4.5 Positions of absorption spectra bands and of their derivatives of different orders for γ-Gl solutions

	Numbers and positions of the main bands of the absorption spectra for γ-Gl solutions (nm)								
Spectra types	1	2	3	4	5	6	7	8	9
D									
D^{I}_{max}	234.3					259.2		264.6	272.1
D^{I}_{0}	230.1	236.3	241.9		250.2	253.3	260.3	266.5	271.2, 273.1
					251.4	256.9	262.9	267.4	274.2
D^{I}_{min}	234.3		243.5		251.9			266.2	
D^{II}				245.5	254.1		260.1	265.7, 266.3	269.6
				249.4				267.4	
D^{III}	229.6	236.1	241.9		250.3	254.1	260.9	266.2	269.0
					253.5	254.9	262.8	267.4	271.0
						257.1			273.0
D^{IV}				244.4	252.8	257.1	260.1, 262.8	265.3	269.9
				245.1	253.5		260.2	267.4	271.1
				249.2	254.2		260.6	269.0, 269.3	273.5
D^{V}	229.8	236.3	242.0		250.3	254.9	260.3	267.4	271.0
					253.5	257.2		269.1	273.0
D^{VIII}	227.0	234.9	240.4	247.6	250.9	255.4, 256.5	260.9, 262.3, 263.0	265.3	271.3
	229.5	237.2	244.1	248.5	251.7, 252.8	258.2, 259.8	263.6, 264.4	266.1, 266.9	272.6
	231.7			249.5	254.3, 254.9			269.0, 269.3	273.0, 273.6
D^{XII}	230.1	236.9	240.3	247.2	251.1	256.7	262.8	267.1	270.6
				249.7	253.9	259.8	264.5	269.9	274.8
D^{XVI}	229.5	236.9	240.3	247.1	251.1	256.7	262.8	265.1, 267.0	272.6
			244.1	249.5	253.9	259.8		269.9	274.8

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Spectra types	Numbers and positions of the main bands of the absorption spectra for γ-Gl (gamma-globulin) solutions								
	10	11	12	13	14	15	16	17	18
D	278.7		289.9						
D^{I}_{max}	279.1		287.0	290.5, 293.4					
D^{I}_{0}	276.2	281.9		290.0	297.0				
	279.3			292.1					
D^{I}_{min}		283.3	287.0	290.6	294.0	301.8	307.3	311.3	315.3
					294.5	304.0		313.3	
D^{II}	274.2	285.0	285.3	292.0		300.3			
	275.7, 276.3	285.3	288.7						
	278.5								
D^{III}	276.2	281.8	288.9	292.0	296.7	300.5	303.6	311.3	315.3
	278.5	284.9				301.7	307.3	313.3	
	279.3								
D^{IV}	275.7	279.9	285.3	291.7	295.2, 295.6		306.3, 308.8		
	277.4	280.7	289.8		297.9				
	278.4				299.0				
D^{V}	276.3	281.6		290.0	296.6	300.4		307.4	311.1
	278.1	284.8		292.0		301.9			313.1
	279.3					303.8			
D^{VIII}	275.9, 276.3	280.2, 280.7	285.3	289.7	295.2	300.3	305.5	308.8	312.7
	277.8	281.8	287.0	291.5	297.7	302.4		310.5	313.1
	278.4	282.8	288.5		298.8				315.8
D^{XII}	277.4	280.5	285.2	291.7	295.6	300.4	305.4	309	311.3
			288.4		298.6				
D^{XVI}	277.4	280.5	285.3	291.7	295.4	300.3	305.5	308.6	314.9
		282.2	288.2		298.7	302.5		310.0	

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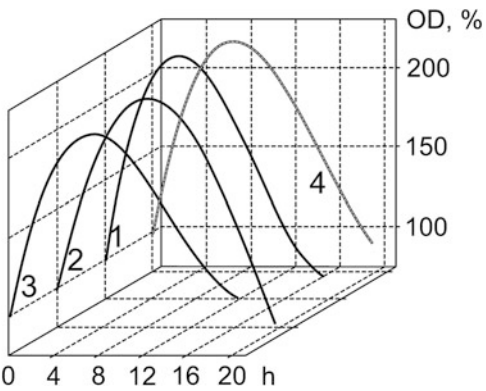

Fig. 4.28 Kinetics of OD change in the principal absorption maximum ($\lambda_{max} = 278.8$ nm) for γ-Gl solution under influence of γ-irradiation. Raw data were processed with the help of the third-order polynomial regression method using the program *Microcal Origin* 3.0. *Curves1–3* show three series of experiments using three parallel probes in the experiment; *curve 4* shows the average of three series of experiments. *Ordinate*: OD change, %; *abscissa*: time of irradiation, hours

absorption bands in the γ-Gl spectrum, revealing bands not described earlier in the literature.

The character of band manifestation in derivatives of the eighth to the 16th order shows that the bend shoulder on D^{VIII} is seen in the form of a peak only on the D^{XII} curve (Fig. 4.27, position 5; Fig. 4.30). Almost imperceptible to the eye is the wave nature of the D^{VIII} curve manifested in the form of the bend shoulder on the D^{XVI} curve (Fig. 4.27, position 6). The manifestation of other bands in D^{VIII}–D^{XVI} spectra is practically identical. It can be concluded that, for routine physiological biochemical studies, the registration of derived spectra of the eighth order is sufficient. Thus, the combination of DSHO with computer analysis of data obtained in numerical and analog form creates a reliable basis for experiments.

For an γ-Gl solution, the kinetics of OD change in the principal absorption maximum can be approximated with a high order of accuracy by a curve of form $y = -ax^2 + bx + c$. As can be seen from Fig. 4.28, OD sharply increases in the first hours of irradiation, indicating formation of new products with a high specific molar extinction coefficient. Calculation of the first-order derivative corresponding to the process rate indicates the maximum accumulation of radiolysis products (RPs) in first 2–4 h. Then, after a 2–3-h period of dynamic equilibrium, a decrease in the kinetic curves begins, which can be explained by domination of the secondary process of radiolysis of products obtained from the γ-Gl preparation. Descending branches of kinetic curves coincide to a certain degree with the trend of kinetic curves of Tyr and Trp radiolysis described earlier (Saakov 1994b, 1998). Hence, it seems possible that products close to Tyr and Trp are formed in the process of γ-Gl irradiation.

Figure 4.29 shows the dynamics of D^{IV} and D^{VIII} differential spectra change after 4 and 8 h of irradiation, emphasizing the increase in specific changes in the

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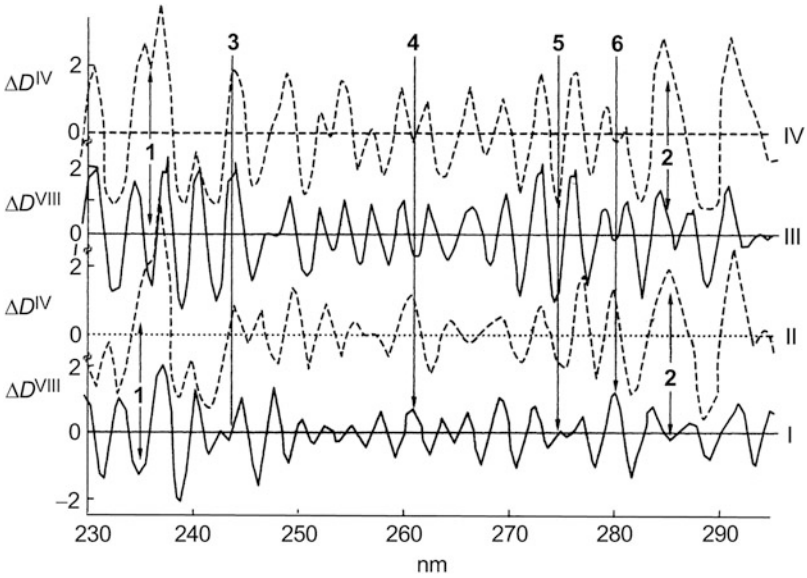

Fig. 4.29 Dynamics of difference spectra ("control minus experiment") change in the fourth derivative (*curves II* and *IV*) and the eighth derivative (*curves I* and *III*) of the γ-Gl absorption spectrum under influence of γ-irradiation: *I* and *II* after 4 h (2.52 kGy); *III* and *IV* after 8 h (5.04 kGy). *Arrows 1–6* indicate zones of difference spectra characterized by maximal difference from control. *Ordinates*: OD, relative units; *abscissa*: wavelength, nm

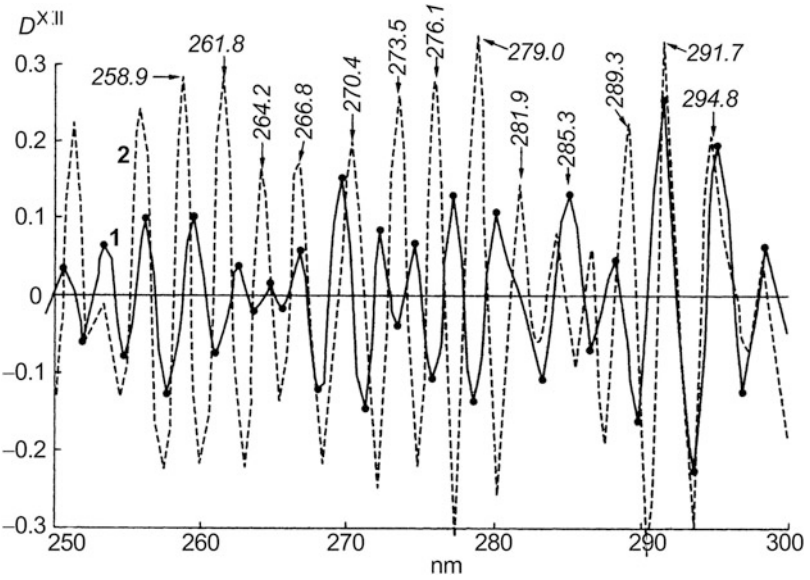

Fig. 4.30 The 12th derivative of the γ-Gl absorption spectrum (*1*) and influence of γ-irradiation (1.89 kGy) on the character of its change (*2*). *Arrows with numbers* show location of absorption maxima of the totality of radiolysis products. *Ordinates*: OD, relative units; *abscissa*: wavelength, nm

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spectral outline with an increase in the radiation dose. The most characteristic changes are noted in regions 243.0, 261.0, 274.0, and 280 nm. Furthermore, the comparison of curves I and II and also of III and IV in Fig. 4.29 (arrows 1 and 2) clearly demonstrates an advantage of D^{VIII} for localization of damaging influences. To investigate the presence of possible RPs of γ-Gl, we will consider the data in Fig. 4.30. Comparison of the spectral D^{XII} outline of RPs of γ-Gl with the data on DSHO of Trp from the paper by Saakov (2000b) shows their obvious similarity. Taking into account the heterogeneity of the reaction medium, including the totality of initial substance and its RPs, it was hardly possible to expect a complete identity between RPs and Trp spectra. Nevertheless, the harmonics of SBs (Fig. 4.30, curve 2) indicates with high probability the presence of Trp in the solution. Descending branches of the kinetics of γ-Gl radiolysis (Fig. 4.28) indicate the formation of products with a low coefficient of molar extinction, one of which could be 5-hydroxytryptamine (serotonin, Srt) (Saakov 1993b).

An increase in the content of Srt in irradiated preparations of γ-Gl leads to reactions of an organism such as damage to motor coordination, weakening of muscular tone, and reduction in motor activity (Saakov 2000b). Irradiated preparations of γ-Gl introduced into an organism can activate damage mechanisms typical for an excess Srt content, functionally influencing, for example, the thermotaxic center. As a biogenic amine, Srt performs the function of chemical neuromediator in the synaptic transfer of nervous activity, simultaneously working as regulator of different steps of cellular metabolism, changing the permeability of plasma membranes of nervous and unstriped-muscle fibers for Ca^{2+} ions and increasing their entry into the cytoplasm (Kurskii and Baksheev 1974). It is possible that an increase in the Srt content as a result of γ-Gl radiolysis is one element of the system of urgent physical chemistry regulation of osmotic pressure (Monin et al. 1985; Natochin et al. 1985) or promotes oppression of the uriniferous function, connected with ureter spasm. On the other hand, the radioprotective effect of Srt is known, caused both by its influence on the bioenergetics of an organism, namely, on oxidative phosphorylation, and by manifestation of its active electron-donor properties in binding free radicals produced during radiation injury (Kurskii and Baksheev 1974).

It was shown that the spectral analysis of DSHO of Tyr and Trp solutions creates a basis for the rapid methodological assessment of the radiation injury of protein structures (Saakov 1993b, 1998). The data in Figs. 4.29 and 4.30 develop this statement and demonstrate that the rapid diagnostics of changes appearing in the spectral outline of immune structures and of their localization is possible with a high degree of reliability.

Thus, the presented materials describe for the first time the DSHO of γ-Gl solutions and allow us to recommend DSHO as a very promising method for characterizing the state of γ-Gl preparations, assessing the presence of admixtures in them, and revealing structural changes occurring in γ-Gl during storage of preparations or under the influence of extraordinary environmental situations. The presence of products of radiolysis can explain the possible nontraditional physiological action of γ-Gl preparations damaged by irradiation. The probability of their

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negative influence could be prevented by introduction of mono-oxidase, with the formation of 5-oxyindolyl acetic acid or *p*-chlorophenylalanine, which causes a drop in the serotonin level.

4.1.7 *Special Features of γ-Globulin Spectra Changes During γ-Globulin Denaturation Caused by Exposure to High Temperature and Radiation*

Investigation of the character of changes in the properties of endogenous and exogenous immunoglobulins under different extreme influences, on the organism and on medical preparations, is promising both for explanation of mechanisms of their action and for assessment of the result of their immune activity retention. A decrease in the effectiveness of active and passive immunization in irradiated organisms and the appearance of pathologic states (accompanied by changes in the γ-Gl fraction of blood proteins) are found in response to radiation or thermal damage under conditions of ecological catastrophes or other extraordinary situations. This was confirmed by the aggravation of organic diseases and induction of new pathologies in populations of inhabitants and animals in the zone of the Chernobyl accident and in neighboring regions (Gesellschaft für Reaktorsicherheit 1987; International Atomic Energy Agency 1986). Therefore, in this section, we make the comparative assessment of changes in the character of the harmonics of SBs of the eighth (D^{VIII}), 12th (D^{XII}), and 16th (D^{XVI}) derivatives of the γ-Gl absorption spectrum under the influence of temperatures of 55 °C (T_{55}) (10 min) and 70 °C (T_{70}) (5 min), and also of γ-irradiation ($D^{\mathrm{VIII}}{}_{\gamma}$, $D^{\mathrm{XII}}{}_{\gamma}$, and $D^{\mathrm{XVI}}{}_{\gamma}$), and investigate the protein denaturation changes coupled with them.

Due to the fact that aqueous γ-Gl solutions correspond better than dry preparations to the protein in vivo state (Kuzin 1962), the influence of different doses of γ-irradiation of ^{57}Co isotope on the special features of changes in γ-Gl (Sigma № g-7516) was investigated (Saakov 2000b). The registration of absorption spectra and of their DSHO was performed with the help of the UV–Vis spectrophotometer DW-2000 FA-220 (SLM-Aminco, FRG). The recording of spectra in analog form was carried out using the instrument UV–Vis-Specord (Carl Zeiss, Jena, FRG) according to the published procedure (Saakov 1993b, e, 2000b). For the purpose of comparison and unification of data obtained in numerical and analog forms and of scales of diagram recording, the spectra were scanned and digitalized with the help of the program *Graph Digitizer* 2.14. Further graphing was carried out with the help of the program *Microcal Origin* 6.1, which gives the opportunity to perform operations of differentiation, to calculate experimental errors, and to find the exact location of extrema on the wavelength scale (Saakov 2000f). The prospect of using this method both for the analysis of smooth outlines of spectral curves of γ-Gl and for studying changes in the fine structure of spectra under different types of influence was shown earlier (Saakov 1993b, e, 1998; 2000b). The coincidence of

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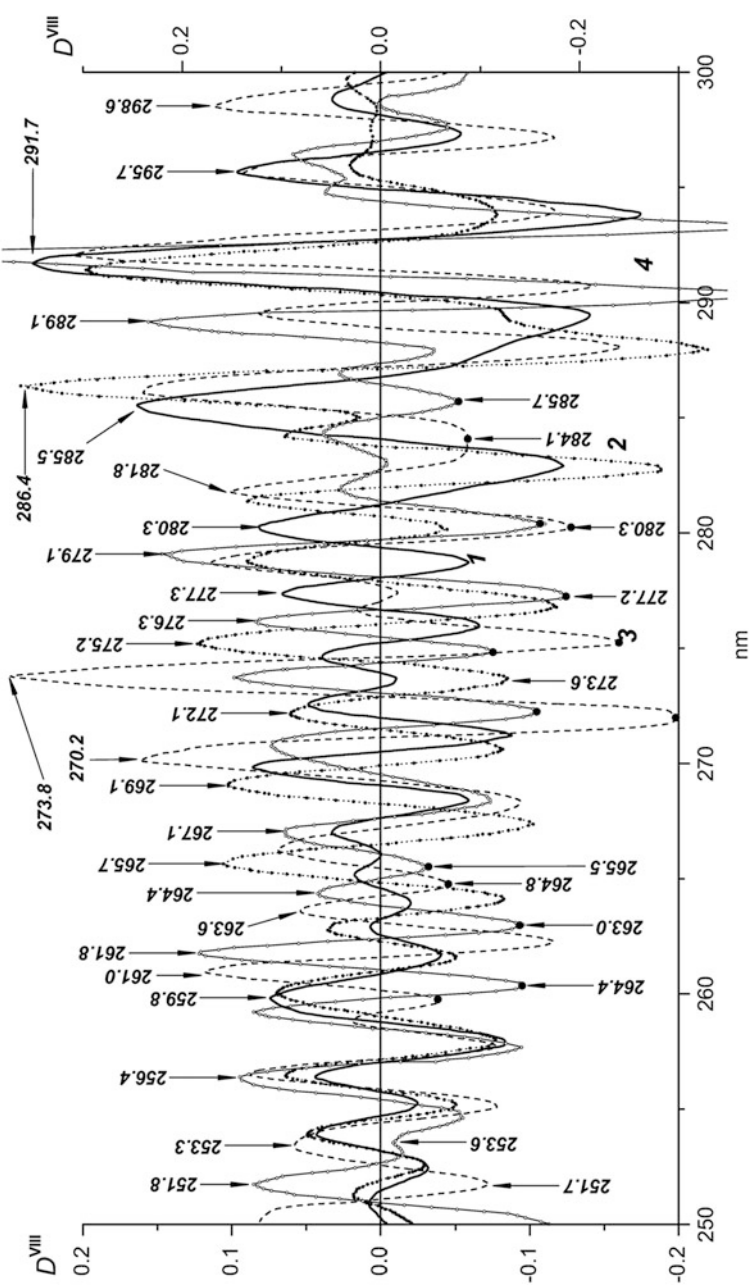

Fig. 4.31 The change character of D^{VIII} of the γ-Gl absorption spectrum under thermal and radiation denaturation: (*1*) control solution as described (Sapozhnikov et al. 1964); (*2*) D^{VIII} induced by temperature of 55°C; (*3*) D^{VIII} induced by temperature of 70°C (4.2×10^9 erg); (*4*) D^{VIII} induced by γ-irradiation (approximately 4.2×10^7 erg). *Black circles* indicate inversely directed changes of amplitudes induced by influencing factor. *Ordinates*: OD, relative units; *left* for *1*, *2*, *3*; *right* for *4*. *Abscissa*: wavelength, nm

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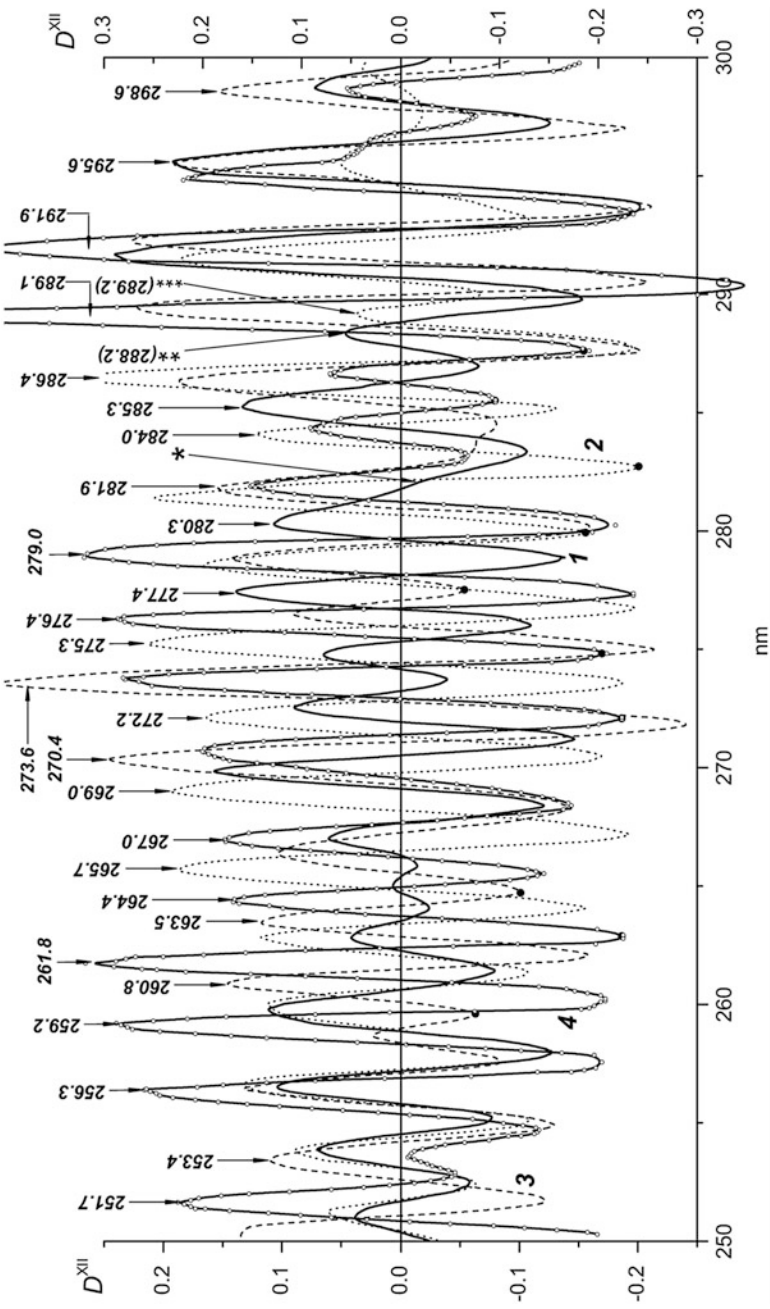

Fig. 4.32 The character of change in D^{XII} of the γ-Gl absorption spectrum under thermal and radiation denaturation: (*1*) D^{XII} of control solution; (*2*) D^{XII} induced by temperature of 55 °C; (*3*) D^{XII} induced by temperature of 70 °C; (*4*) D^{XII} induced by γ-irradiation. *Ordinates*: OD, relative units; *left* for *1* and *2*; *right* for *3* and *4*. *Abscissa*: as in Fig. 4.31

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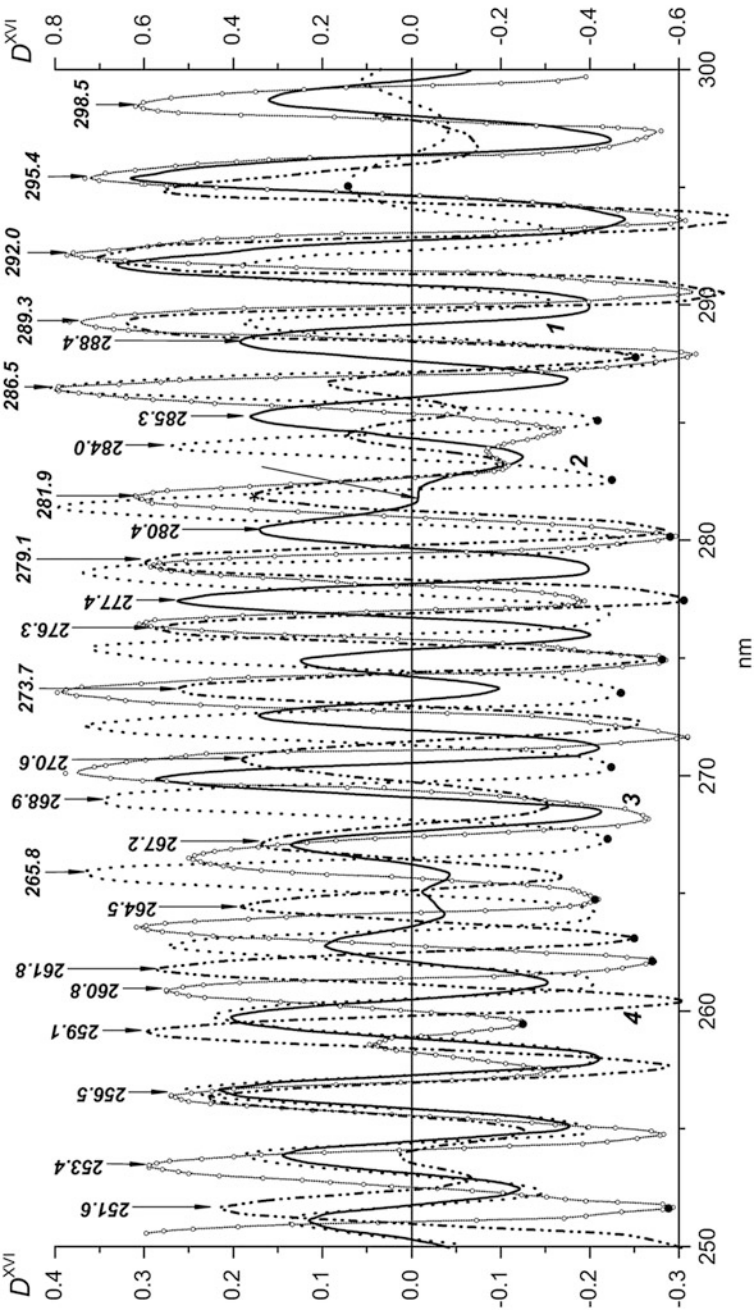

Fig. 4.33 The change character of D^{XVI} of the γ-GI absorption spectrum under thermal and radiation denaturation: (*1*) D^{XVI} of control solution; (*2*) D^{XVI} induced by temperature of 55 °C; (*3*) D^{XVI} induced by temperature 70 °C; (*4*) D^{XVI} induced by γ-irradiation. *Asterisk* indicates the bend shoulder, visually assessed in Fig. 4.32 as inflection. *Ordinates*: OD, relative units; *left* for *1* and *3*; *right* for *4*. *Abscissa*: as in Fig. 4.31

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registrations of parallel samples in spectral measurements and the reproducibility of records for the same sample (±2.5 %) and from experiment to experiment (±9 %) indicate the reliability of the chosen method.

From consideration of the totality of the curves in Figs. 4.31, 4.32, and 4.33, it follows that an increase in temperature often increases OD values, that is, there is a hyperchromic effect, which probably shows the formation of the less-ordered structure of γ-Gl molecules in comparison with initial organization. This hyperchromia is the criterion for the denaturation degree or of protein transfer to an unfolded state. In the case of protein, we can speak with sufficient confidence about the larger accessibility of internal AAA residues to the solvent (seen from Tyr and Trp spectra). This agrees both with theoretical concepts (Kuzin 1962) and with the kinetics of OD change for γ-Gl under the effect of γ-irradiation (Saakov 2000b). From obtained data, separate hyperchromic spectral bands -SBs induced by the influence of temperature were isolated: $D^{VIII}T_{55}$: 251.0, 256.5, 262.8, 265.7, 269.1, 272.1, 275.2, 286.4 nm; $D^{XII}T_{55}$: 251.7, 256.3, 259.2, 261.8, 264.4, 267.0, 270.7, 273.8, 276.4, 279.0, 281.9, 284.4, 289.1, 294.8 nm; $D^{XVI}T_{55}$: 251.0, 253.8, 256.7, 259.7, 262.7, 265.8, 268.9, 272.1, 275.4, 276.0, 281.4, 284.0, 286.5 nm (1); and $D^{VIII}T_{70}$: 250.1, 253.4, 256.6, 261.0, 263.6, 266.3, 270.2, 278.0, 278.7, 281.9, 286.2, 289.5, 298.6 nm; $D^{XII}T_{70}$: 250.1, 253.4, 256.5, 260.8, 263.5, 266.4, 270.4, 273.6, 276.5, 278.9, 281.9, 286.3, 289.5, 292.3, 295.6, 298.6 nm; $D^{XVI}T_{70}$: 250.3, 253.4, 256.5, 260.8, 263.6, 266.4, 270.2, 273.4, 276.5, 278.9, 281.9, 283.7, 286.5, 292.0, 295.0, 295.4, 298.5 nm (2). The hyperchromic effect was also discovered during induction of denaturation changes by γ-irradiation: $D^{VIII}{}_{\gamma}$: 251.8, 256.3, 259.2, 261.8, 264.4, 267.0, 270.7, 273.8, 276.4, 279.0, 281.9, 284.4, 289.1, 294.8 nm; $D^{XII}{}_{\gamma}$: 251.7, 256.3, 259.2, 261.8, 264.4, 267.0, 270.7, 273.8, 276.4, 279.0, 281.9, 284.4, 289.1, 294.8 nm; $D^{XVI}{}_{\gamma}$: 251.6, 256.2, 259.1, 261.8, 264.5, 267.2, 273.7, 276.3, 279.1, 289.2, 292.0 nm (3). Special features of DSHO ensured the registration of the hyperchromic effect in the absorption spectrum, not integrally as stated earlier (Kuzin 1962), but in separately derived SBs in the harmonics of D^{VIII}–D^{XVI}. For SBs (1) and (2), a good coincidence of the shift of SBs, as seen for (3), is noted. Small discrepancies are explained by the manifestation of properties of derivative orders, which follows from the comparison of curve 1 in Figs. 4.31, 4.32, and 4.33, showing a resolution increase with an increase in the derivative order (Fig. 4.32, $\lambda = 281.9$, 288.2, and 289.2 nm; Fig. 4.33, $\lambda = 281.9$, 288.4, and 289.3 nm). At the same time, the differences between SBs (1), (2), and (3) are sufficiently significant to indicate a difference both in the quantitative and qualitative participation of different AAAs during the formation of the less-ordered structures and different accessibility of inner Tyr and Trp residues to the solvent.

The new DSHO technology assisted us to reveal for the first time, in turbid medium and in the complex D^{VIII}–D^{XVI} harmonics, in some SBs, the OD change manifested as the *hypochromic* effect. The following SBs can be attributed to this: D^{VIII} T_{55}: 281.3, 284.2, 295.8, 299.7 nm; $D^{XII}T_{55}$: 289.2, 291.5, 295.6, 299.6 nm; $D^{XVI}T_{55}$: 291.7, 299.5 nm (4); and $D^{VIII}T_{70}$: 258.9, 276.6, 292.0, 295.7 nm; $D^{XII}T_{70}$: 258.7 nm; $D^{XVI}T_{70}$: 258.6 nm (5). During denaturation changes caused by γ-irradiation, hypochromia is found in SBs $D^{VIII}{}_{\gamma}$: 253.6, 286.9, 294.8, 296.4,

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298.6 nm; $D^{XII}{}_{\gamma}$: 284.4, 286.7, 298.6 nm; and $D^{XVI}{}_{\gamma}$: 253.8, 270.7, 284.5, 286.6 nm (6).

We focus on the hypochromia of some SBs in the fine structure because it was not previously possible to establish hypochromia of SBs accompanying the state of more ordered structures because of methodological complexities. We would like to note that the disappearance of hypochromia during denaturation changes can serve as the measure of α-helicity, which is the most stable form of peptide chains. Also, some SBs (Figs. 4.31, 4.32, and 4.33) are found that do not change OD values under the influence of temperature and γ-irradiation: $D^{VIII}T_{55}$: 253.9, 280.3–281.4 nm; $D^{XII}T_{55}$: 284.0–285.3, 288.2–289.2 nm; $D^{XVI}T_{55}$: 288.4–289.2 nm; $D^{VIII}T_{70}$: 285.5–286.1, 295.7 nm; D^{XII}: 259.2–259.8 nm; $D^{XVI}{}_{\gamma}$: 280.4, 281.9, 286.7 nm (7). The listed SBs (7) are different for thermal and radiation exposure. Under the influence of temperature, the response of SBs in DSHO harmonics insignificantly differs from D^{VIII} to D^{XVI}, which is possibly caused by both the quantitative effect of the influence and by the special feature of DSHO. Finally, in an insignificant number of SBs, the OD changes occur opposite to the initial ones and are shown in Figs. 4.31, 4.32, and 4.33 by dark circles. To assert that these shifts are connected with a hypochromic effect would be imprudent, because it is more probable that they are combined with hypsochromic and bathochromic shifts, as considered below.

Further, from Figs. 4.31, 4.32, and 4.33, it follows that the denaturation process, induced by T_{55} and T_{70}, is accompanied by the shift of a number of SB maxima to the long-wave region (i.e., bathochromic shift): $D^{VIII}T_{55}$: 251.3, 275.2, 278.8, 281.4, 286.4, 299.7 nm; $D^{XII}T_{55}$: 275.3, 278.7, 281.4, 286.4, 289.2, 299.6 nm; $D^{XVI}T_{55}$: 275.4, 278.6, 281.4, 286.5, 289.3, 299.5 nm (8); and $D^{VIII}T_{70}$: 261.0, 263.6, 270.2, 273.8, 278.7, 281.8, 286.2, 289.5, 292.0 nm; $D^{XII}T_{70}$: 260.8, 263.5, 270.4, 273.6, 278.9, 281.9, 286.4, 289.4, 292.3 nm; $D^{XVI}T_{70}$: 260.8, 263.6, 270.2, 273.4, 281.9, 283.7, 286.5, 289.3, 292.0 nm (9). A bathochromic shift during denaturation changes is also induced by γ-irradiation: $D^{VIII}{}_{\gamma}$: 251.8, 261.8, 264.4, 270.8, 273.7, 276.2, 279.2, 281.9, 287.0, 291.9, 296.5 nm; $D^{XII}{}_{\gamma}$: 251.7, 270.7, 273.7, 276.4, 279.0, 281.9, 286.6, 289.1, 291.9 nm; $D^{XVI}{}_{\gamma}$: 270.0, 270.6, 273.7, 286.7, 289.3, 292.0 nm (10). Influences of T_{70} and γ-irradiation increase the number of shifted SBs, although the positions of band shifts rarely coincide. On the one hand, a bathochromic shift could indicate the formation of aggregated "crosslinks" by radical molecules of γ-Gl (Kuzin 1962). On the other hand, the displacement of Tyr, Trp, and β-Phe into a less polar environment is possible, which causes an increase in λ_{max} and ε (resulting in the hyperchromic effect).

Thus, the damage to the native configuration of γ-Gl during its denaturation is manifested as a change in protein optical properties and as a DSHO shift. In the case of a bathochromic shift induced by temperature, DSHO SBs (8 and 9) correspond more to each other than to bands induced by γ-irradiation (10). As for the comparison of (1), (2), and (3) in the case of the hyperchromic effect, consideration of the bathochromic shift shows that there is more similarity between variants (8) and (9) than with variant (10), which highlights differences in the denaturation changes.

A set of separate SBs is characterized by a hypsochromic shift to the short-wave part of the spectrum: $D^{VIII}T_{55}$: 265.7, 269.1, 272.1, 284.2, 291.3 nm; $D^{XII}T_{55}$: 265.7,

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269.0, 272.2 nm; $D^{XVI}T_{55}$: 265.8, 268.9, 272.1 nm (11); and $D^{VIII}T_{70}$: 250.1, 253.4, 258.9, 266.3, 269.0, 273.8, 276.7, 278.8 nm; $D^{XII}T_{70}$: 250.1, 253.4, 258.9, 266.4, 276.5 nm; $D^{XVI}T_{70}$: 250.3, 253.4, 256.5, 258.6, 266.4, 276.5, 278.9 nm (12). A hypsochromic shift is also registered with γ-irradiation: $D^{VIII}{}_{\gamma}$: 259.1, 284.3, 298.1, 294.8 nm; $D^{XII}{}_{\gamma}$: 256.3, 259.3, 261.8, 264.4, 284.4, 294.9 nm; $D^{XVI}{}_{\gamma}$: 256.2, 259.1, 261.8, 264.5, 276.3, 279.1, 284.5, 294.8 nm (13). The small discrepancies between SBs (11) and (12) are more conditioned by properties of the order of the chosen derivative and significantly differ from bands of the hypsochromic shift (13). It is established that some SBs do not change their positions under the influence of temperature: $D^{VIII}T_{55}$: 253.9, 259.8–260.0, 262.8, 295.7–295.6 nm; $D^{XII}T_{55}$: 251.1, 253.8, 256.5, 259.8, 262.8, 274.8–275.3, 291.4–291.6, 295.5–295.6 nm; $D^{XVI}T_{55}$: 251.0, 253.8, 256.7, 259.7, 262.7 nm (14); and $D^{VIII}T_{70}$: 256.6, 295.7, 298.6 nm; $D^{XII}T_{70}$: 256.6, 298.6 nm; $D^{XVI}T_{70}$: 295.4, 298.5 nm (15). The absence of the shift of SBs is also possible to see after γ-irradiation: $D^{VIII}{}_{\gamma}$: 253.6, 256.4, 291.7–292.0, 298.6–298.8 nm; $D^{XII}{}_{\gamma}$: 253.6, 256.3–256.4, 267.0, 298.7 nm; $D^{XVI}{}_{\gamma}$: 253.8, 267.2, 298.7 nm (16). After consideration of (7), (14), and also (15) and (16), common SBs are found that do not change their OD values (D^{VIII}: 280.3, 281.4 and D^{XVI}: 280.4, 281.9 nm) and bands without bathochromic and hypsochromic shifts in the spectra D^{VIII}–D^{XVI} (253.6–253.8, 256.3–253.0, 298.6–298.8 nm). Attention should be paid to these bands in the study of external influences on γ-Gl, and with their use, it is reasonable (and more correct) to perform new quantitative calculations.

After consideration of the specific character of DSHO changes for γ-Gl, induced by thermal and radiation exposure, we can conclude that changes in the fine spectral structure in D^{VIII}–D^{XVI} harmonics during denaturation are specific for the influence of temperature and γ-irradiation. This specificity is manifested both by the unfolding of different quantities of AAAs and, very probably, by their different quality (Saakov 2000f). It is possible that energy, absorbed by γ-Gl, results in the formation of a radical state of the protein molecule, analogous to the state of free nucleotides appearing during DNA hydrolysis, with the corresponding hyperchromia. At the same time, analogous hyperchromic and hypochromic effects were found in a study of the effects of thermal and radiation exposure on Alb (Saakov 2000f). The presence of bathochromic and hypsochromic shifts was also characteristic for Alb preparations.

Thus, the material presented in this section on the existence of specific mechanisms of denaturation changes in molecules of γ-Gl under thermal and radiation exposure coincides with the directivity of Alb denaturation changes (Saakov 2000f). This concept is supported by the data in Fig. 4.34 showing the presence of hyperchromic and hypochromic effects and also of bathochromic and hypsochromic shifts in compared variants of experiments and highlights the dissimilarities in the changes in the fine structure of spectra under exposure to high temperatures and γ-radiation. In accordance with the properties of difference spectra, OD values can be non-coinciding with the directivity of initial spectra (Figs. 4.31, 4.32, and 4.33), and absorption extrema can be shifted (Saakov 2000f).

It should be stressed that the comparison of similarity and dissimilarity of changes in the fine structure of spectra for Alb and γ-Gl indicates the larger manifestation of dissimilarity of denaturation changes for Alb. Taking into account

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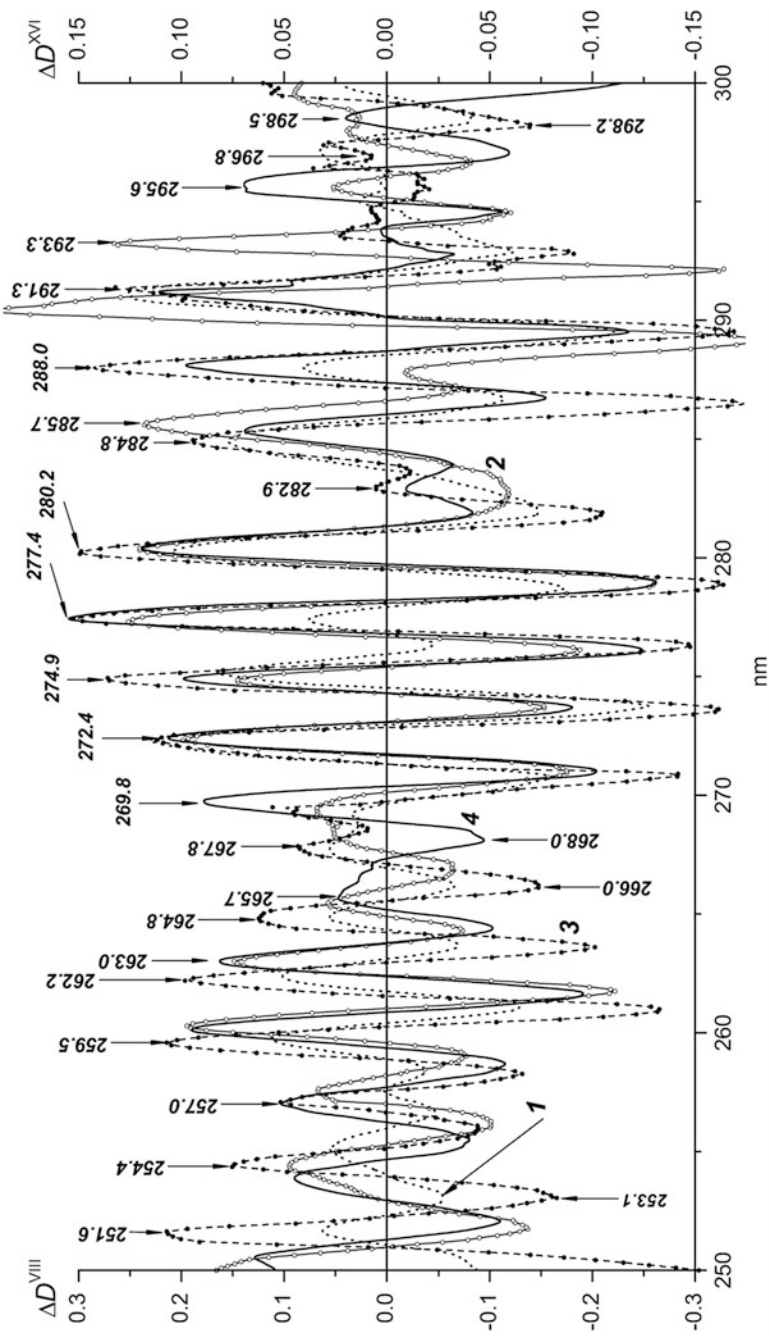

Fig. 4.34 The change character of ΔD^{VIII} and ΔD^{XVI} of spectra "control minus experiment" of γ-GI solutions induced by temperature and γ-irradiation: (*1*) ΔD^{VIII} (control T_{70}); (*2*) ΔD^{VIII} (control γ); (*3*) ΔD^{XVI} (control T_{70}); (*4*) D^{XVI} (control γ). *Ordinates*: as in Fig. 4.32; *left* for *1* and *2*; *right* for *3* and *4*. *Abscissa*: as in Fig. 4.31

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data on the specific character of denaturation changes for γ-Gl and under the influences described above, there are foundations to speak about the specificity of denaturation mechanisms in different proteins under the influence of high temperature and γ-irradiation. Attention should be paid to this when storing reserve funds of physiological preparations in the zone of ecological catastrophes.

Thus, our experiments gave, for the first time, data on the specific character of hypochromic and hyperchromic effects; on bathochromic and hypsochromic shifts of separately considered bands of the eighth to 16th derivatives of the γ-Gl absorption spectrum; and also on the invariability of a number of SBs during denaturation changes in the protein induced by exposure to high temperatures and radiation. It is possible that this stability is caused by specific protein conformations or protein pools. The statement about the exclusiveness of the presence of only the hyperchromic effect (Kuzin 1962; Amiragova et al. 1964) during denaturation of γ-globulin and Alb is withdrawn.

4.1.8 Coupling of Albumin Derived Spectra Changes with the Accuracy of Determination of the Albumin/Globulin Coefficient for Radiation Injuries

Research into the influence of an ionizing radiation source (IRS) on proteins is coupled with the study of the central elements of mechanisms of radiation influence on the organism and its adaptive reactions or death. The state of the fine native protein structure is predetermined by the harmonious course of evolutionarily conditioned physiological biochemical processes in animals and plants. Albumin (Alb), in particular, is the main protein of the blood plasma and realizes a regulatory function in maintaining colloidal-osmotic pressure, the transport of substances, and the deposition of protein reserves of the organism.

In this connection, knowledge of the character of changes in the state of Alb in the organism, in plasma medical preparations, and in food products that have been irradiated in radiation catastrophes or in the process of sterilization and conservation plays a primary role.

In clinical practice, it was found that the course of many pathological processes during infectious diseases, tumors, increased function of the adrenal cortex and thyroid gland, and also under the influence of an IRS is accompanied by a decrease in Alb content (Kukhta et al. 1986; Smolichev 1965). A decrease in the Alb/Glb ratio is considered to be the manifestation of early pathological damage to the blood composition under IRS influence (Volkin and Kohn 1951; Westphal et al. 1953; Bauer et al. 1954; Smolichev 1965). It is also considered that the total protein quantity during radiation sickness changes insignificantly (Smolichev 1965); moreover, a decrease in the serum Alb content is combined with a simultaneous increase in α- and β-globulins (Kuzin 1962; Westphal et al. 1953).

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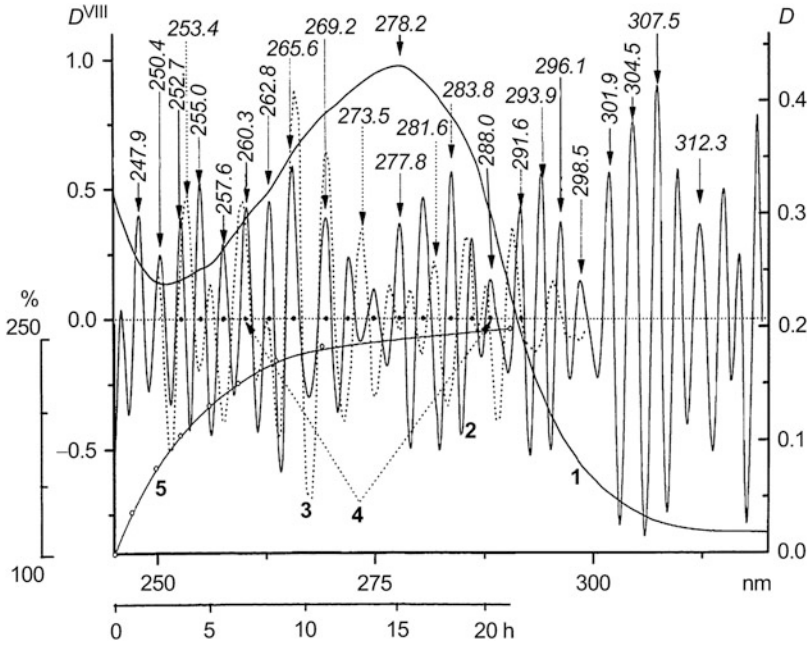

Fig. 4.35 The absorption spectrum of bovine serum albumin (BSA) solution (*curve 1*) and kinetics of its OD change in the principal absorption maxima under the influence of γ-irradiation (power 600 Gy/h) (*curve 5*); D^{VIII} of the absorption spectrum of BSA solution (pH 7.68 for *curve 2*, pH 6.9 for *curve 3*). *Black circles* (*4*) indicate the places of intersection of the zero line with D^{III} and D^{V} curves for BSA solution, pH 7.68. *Ordinates*: OD, relative units or %; *right* (*D*) for *curve 1*; *upper abscissa*: wavelength, nm; *lower abscissa*: time for *curves 4* and *5*

Research into the influence of an IRS on the kinetics of an absorption change in β-Phe, Tyr, Trp, and γ-Gl, performed with the help of the new capabilities of DSHO, showed the need for more detailed study of special features of Alb absorption kinetics and of some bands of its spectrum as functions of the received radiation dose (Saakov 1993b, e, 1998, 2000b, c).

Because aqueous Alb solutions correspond more than dry preparations to the state of protein in native structures (Kuzin 1962), we investigated the influence of γ-rays of ^{57}Co isotope (power 600 Gy/h) on preparations of bovine (BSA) and human serum albumins from the firm Sigma using the published procedure (Saakov 1993b, e, 1998, 2000b, c). The registration of absorption spectra and DSHO in numerical form was performed with the spectrophotometer DW-2000 FA-220 (SLM-Aminco, FRG) (Saakov 1993b, e). The spectra record in analog form was carried out with the help of the instrument UV–Vis-Specord (Carl Zeiss, Jena, FRG) using a previously developed procedure (Saakov et al. 1976, 2004). For comparison and unification of data in numerical and analog forms, the spectra were scanned and saved in the computer and then digitalized with the program *Graph Digitizer* 2.14. Further graphing of spectra was performed with the program *Microcal Origin* 6.1, which allows approximation of kinetics curves of an

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absorption change under IRS influence and the calculation of errors of radiation transformation processes and regression polynomials. The discrepancy of parallel tests in experiments did not exceed the significance level of $\rho < 0.5$ %. Experience of work with DSHO showed the suitability of using this method for the analysis of smooth contours of spectral curves of a number of proteins (Saakov et al. 1987a, b; Saakov 2000c). Therefore, taking into account the good separation of bands in derived spectra of the fourth and eighth orders (Saakov 1993b, e, 1998, 2000b, c), we investigated the character of their change in Alb solutions under the influence of γ-radiation.

The protein spectral outline is formed, mainly, by contributions of SBs of three AAAs: β-Phe, Tyr, and Trp. Specific coefficients of molar extinction of Tyr and Trp are higher than that of Phe by four and 30 times, respectively. Therefore, it is possible to consider with some reliability that the spectral outline of a protein is mainly formed by the joint contribution of Tyr and Trp absorption bands. The initial spectral outline of Alb is characterized by a poorly developed oscillating structure (Fig. 4.35, curve 1) with one fuzzy principal maximum of absorption, whereas in the D^{VIII} spectrum, 29 absorption bands are found for the selected wavelength range (curve 2). A change in pH and density of solution can cause an insignificant shift of a number of SBs and of the quality of their resolution (curve 3). Reproducibility of SBs in the spectra of even and odd derivatives reliably accents the correctness of absorption maxima registration and, furthermore, indicates the sufficiency of D^{VIII} determination for physiological and biochemical studies (Saakov 1993b, e, 1998, 2000b, c).

With exception of the kinetic curve of OD change for γ-Gl, the kinetic curves of Alb, β-Phe, Tyr, and Trp can be approximated by the Boltzmann equation:

$$Y = A2 + \frac{(A1 - A2)}{1 + \exp((x - x0)/dx)}$$

However, for data unification, we used the equation of polynomial regression. In this case, the kinetics of OD change in the solution of Alb in the principal maximum of absorption ($\lambda = 278.2$ nm) under the influence of γ-irradiation can be expressed by an equation of the form

$$Y = 102.736 + 27.753X - 1.885X^2 + 0.043X^3$$

The character of the trend of kinetic curves of Alb OD transformation in response to radiation is indicated by the difference from the trend of the γ-Gl curve (Saakov 2000b, c):

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$$Y = 86.230 + 37.780X - 3.119X^2 + 0.064X^3 + 8.512 \times 10^{-7}X^4 - 1.149 \times 10^{-7}X^5$$

and similarity with the kinetic curve of Tyr OD radiation transformations (Saakov 1993b):

$$Y = 102.435 + 8.246X + 13.622X^2 - 5.695X^3 + 0.912X^4 - 0.053X^5$$

and that of β-Phe (Saakov 1993e):

$$Y = 86.23 + 37.58X - 3.12X^2 + 0.064X^3 + 8.51 \times 10^{-7}X^4 - 1.15 \times 10^{-7}X^5$$

At the same time, in contrast to Tyr, the kinetic curves of Trp OD radiation transformations (Saakov 1998) are approximated by a polynomial regression of the form

$$Y = 99.242 - 58.154X + 29.795X^2 - 9.682X^3 + 1.521X^4 - 0.090X^5$$

The stated suggests the weighty contribution of Tyr to the character of the Alb OD kinetic curve. The Alb OD change kinetics shown in Fig. 4.35, 5 indicates the formation of substances with high specific coefficients of molar extinction in the radiolysis process, possibly Tyr and Trp. The absence of the descending branch on

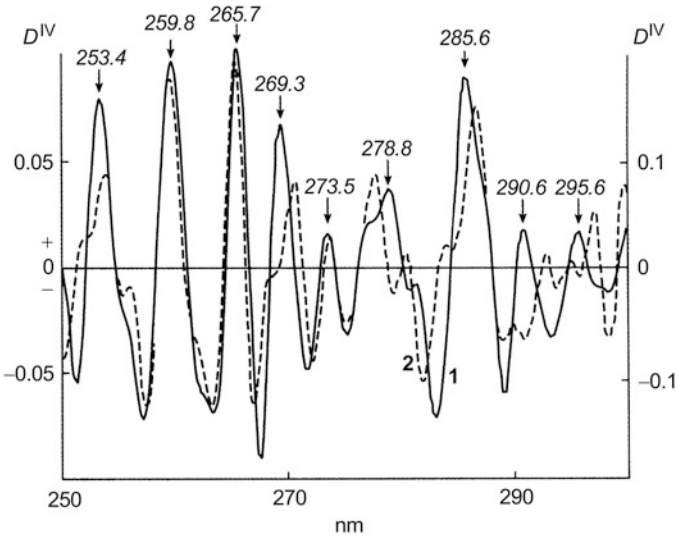

Fig. 4.36 The D^{IV} change in absorption of BSA solution (pH 6.9) under the influence of γ-irradiation of 1.2 kGy: (*1*) control; (*2*) experiment. *Ordinates*: OD, relative units; *right* for *curve 2* in compliance with OD increase on curve 5 in Fig. 4.35 (the same is true for Figs. 4.37 and 4.38). *Abscissa*: wavelength, nm

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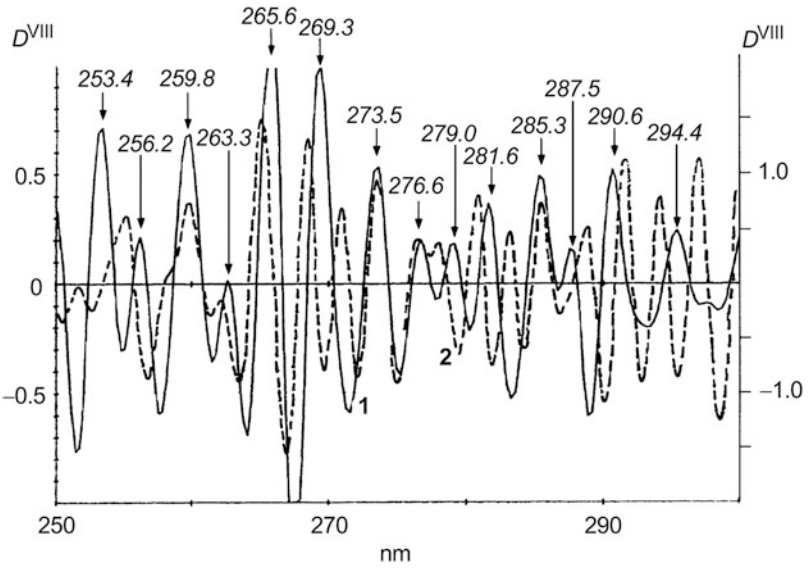

Fig. 4.37 The D^{VIII} change in absorption of BSA solution (pH 6.9) under the influence of γ-irradiation of 3.6 kGy: (*1*) control; (*2*) experiment. *Ordinates*: OD, relative units; *right* for *curve 2*. *Abscissa*: wavelength, nm

the Alb OD kinetic curve in the studied time interval serves as evidence that Trp plays small part in its formation. Previously, the Tyr content increase in blood and intensive oxidization of Tyr under aerobic conditions with the formation of 3,4-DOPA and dopachrome were discovered using different methods (Saakov 1993b, e, 2000c).

One source of newly formed Tyr is β-Phe (Saakov 1993e). Also, γ-irradiation of Tyr and β-Phe causes the formation of thyroxine, adrenaline, and noradrenaline.

The fact that absorption maxima of β-Phe at $\lambda = 252.1$, 257.6, and 263.1 nm have similar but different inclination of the kinetic curves of OD radiation transformations (Saakov 1993e) requires extreme care in making statements about the *invariability* of the spectral outline of absorption spectra of Phe, Tyr, Trp, and proteins under irradiation (Kuzin 1962; Khenokh and Lapinskaya 1956). It is possible to accept the conclusion, as being closer to reality, that the detection of possible changes in the fine spectral outline of the absorption spectrum is conditioned by such features of the chosen measurement method as sensitivity and the SB resolution limit for proteins and amino acids (Saakov et al. 1987a, b; Saakov 1993b, e, 1998). Even small pH changes (Fig. 4.35, curve 3) result in both the shift of some SBs and the resolution of previously invisible SBs of absorption. Data on the influence of γ-radiation on BSA (Figs. 4.36, 4.37, and 4.38) clearly illustrate a change in the fine structure of spectrum at different time points of the kinetic curve (Fig. 4.35, curve 5). After 2 h (Fig. 4.36), besides an increase in OD of the solution, changes in SBs are visible in the region of $\lambda = 254.4$, 269.3, 278.8, 285.6, 290.6, and 295.6 nm. SBs practically without the shift are found at $\lambda = 259.8$, 265.7, 273.5, and

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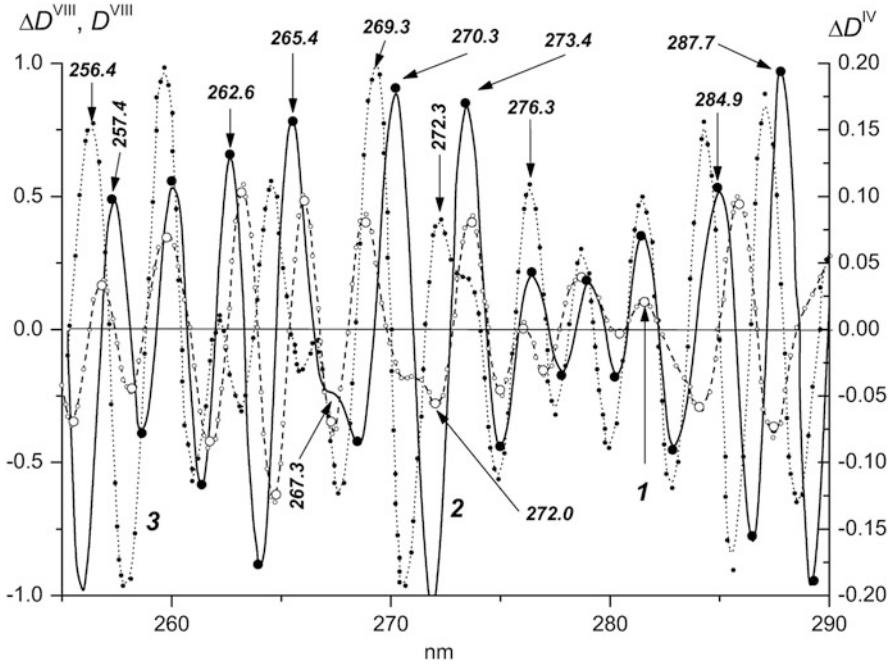

Fig. 4.38 The change character of difference spectra "control minus experiment" ΔD^{IV} (*curve 1*, 600 Gy) and ΔD^{VIII} (*curve 2*, 3 kGy), and the D^{VIII} spectrum for BSA solutions (pH 6.9) (*curve 3*, 12.6 kGy) at different levels of irradiation. *Ordinates*: OD, relative units; *right* for *curve 1*. *Abscissa*: wavelength, nm

285.3 nm. This means that, in limits of the time interval from 1 to 21 h of γ-radiation influence, we should be very careful in making conclusions about a decrease in Alb quantity, especially if quantitative assessment was performed on the basis of a change in the OD value only in the principal maximum of absorption.

In calculation of the coefficient Alb/Glb, the SB stability at $\lambda = 259.8$, 273.5, and 285.3 nm should be taken into account. This position concerns a change in SBs for the consideration of the speed decrease in Glb radiation transformations in the interval 12–18 h (Saakov 2000b, c), because the trends of OD change for BSA and γ-Gl are different. In any case, in the calculation of the Alb/Glb ratio, it is necessary to indicate extremely exactly the time interval of measurement, the influence or aftereffects of γ-radiation, and the method of Alb and Glb determination. If not, distressing variant readings can appear because hours of γ-radiation influence and many days of their aftereffects are characterized by different radiation effects (see Kuzin 1962, p. 204) and trends in the kinetics curves.

The change in D^{VIII} spectral outline at 6 h after the beginning of irradiation (Fig. 4.37) shows, in contrast to the point of view of Khenokh and Lapinskaya (1956), a large number of appeared bands, namely, $\lambda = 255.0$, 264.6, 268.2, 270.7, 278.0, 280.9, 288.3, 291.3, 294.0, and 297.0 nm. The Alb SBs most resistant to the influence of γ-radiation were found, precisely, at $\lambda = 258.8$, 265.6, 269.3, 273.5, and

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285.3 nm. With some probability, several SBs could be assigned to Tyr ($\lambda = 255.0$, 268.2, 270.7, and 280.9 nm) and others to Trp ($\lambda = 264.6$, 278.0, 288.3, and 290.0 nm). However, this supposition should be made carefully because in the heterogeneous medium of RPs, it is unlikely to expect the differentiated revelation of the whole totality of SBs characteristic of Tyr or Trp (Saakov 1994b, 1998; Saakov et al. 1970a, b). From the comparison of data in Figs. 4.36 and 4.37, it is evident that the conclusion about the SB stability at $\lambda = 259.8$, 273.5, and 285 nm is correct for high amounts of radiation. This should be taken into account in calculation of the clinical coefficient Alb/Glb or other indices of γ-radiation injury. Possibly, this is caused by as yet unestablished protein structures having increased radioresistance. The same concerns the stability of SBs for γ-Gl, namely, $\lambda = 256$, 258.0–259.5, 266.8, 269.6–270.5, 285.2, 291.67, and 294.8 nm. The repeated manifestation of this apparently random fact indicates the presence of still-hidden but existing mechanisms.

Thus, in calculation of the Alb/Glb coefficient, we recommend consideration of *only* bands with $\lambda = 259.5$, 269.6, and 285.2 nm, which are stable during the irradiation both of Alb and γ-Gl.

The data in Fig. 4.38 show that the difference between experiment and control, following from trends of kinetic curves (Fig. 4.35, curve 5), finds qualitative expression in the difference spectra ΔD^{IV} and ΔD^{VIII} as a function of radiation dose. At the same time, the influence of doses of 3.6 and 12.6 kGy (Fig. 4.38, curves 2 and 3) does not allow any conclusions to be made about their exceptionally specific influence on the change in Alb spectral outline and demonstrates the low percentage increase in OD kinetics (Fig. 4.35, curve 5).

Thus, this section describes, for the first time, the D^{VIII} spectrum for BSA and its qualitative changes in dependence on the pH of the solution. The kinetics of OD change as a function of dose of γ-radiation is presented. The reliable proof of the qualitative change in the fine structure of Alb spectrum under the influence of γ-radiation is obtained, which contrasts with positions prevailing earlier in the literature (Kuzin 1962; Khenokh and Lapinskaya 1956). The SBs most resistant to γ-radiation influence are established in structures of Alb and γ-Gl and can be used as the basis for theoretical calculations in clinical diagnostics of the radiation exposure coefficient Alb/Glb. Furthermore, the results suggest the interesting possibility of existence of protein structures with increased radioresistance.

Several years after the publication of our data on the Internet, in the journal *PLoS Biology*, 20 March 2007, information about the success of the team of US scientists under the leadership of Dr. Michael J. Daly from the Department of Pathology of the Military Medical University in Maryland appeared, according to which, the reason for survival of the bacterium *Deinococcus radiodurans* under high levels of γ-irradiation is the existence of a powerful mechanism of protein protection from oxidization, because of special fermentative mechanisms involving the bivalent manganese ion. New concepts moved the focus of research on the resistance of bacterium cells from DNA damage to damage of proteins and the existence of fermentative radioprotectors based on manganese. It is possible that the new model of radioresistance can help to bring together the great mass of contradictory results

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existing in the literature and promote experiments searching for natural radioprotectors.

In connection with the possibility of Tyr and Trp presence in RPs, we draw attention to the possibility of appearance of the active mediators 3,4-DOPA, IAA, thyroxine, Srt, adrenaline, and others (Saakov 1993b, e, 1998, 2000b, c) as products of secondary radiolysis. These mediators can condition the activity of biochemical mechanisms of change in physiological reactions. This observation could prove to be essential during the irradiation of blood reserves, serum, medical Alb preparations, or foodstuffs containing Alb in radiation catastrophes, sterilization, or conservation.

Thus, this section summarizes data on the derived spectra of Phe, Tyr, and Trp and on the character of their change under the influence of radiation. The specific character of the existence of RPs of amino acids and the possibility of influence through these products on the physiological state of an organism in a zone of anthropogenic catastrophes are shown. The quantitative determination of the amino acid content is illustrated. The possibility of DSHO use for assessment of the depth of radiation damage to Alb and Glb, which are accompanied both by hyperchromic and hypochromic effects, is proven. The specific character of changes in the spectra of Alb and Glb under radiation and thermal influences is established. On the basis of the obtained data, the existence of protein pools or protein conformations that are little affected by radiation influence is suggested. On the basis of the analysis of the fine structure of derived spectra, a new method for calculation of the Alb/Glb coefficient for clinical diagnostics is proposed.

Thus, due to modern analytical techniques, significant corrections have been made to earlier points of view on the influence of radiation on AAAs and proteins and on the kinetics of radiolysis of these preparations.

In total, the material presented in Sect. 4.1 is an illustrated study guide on the comparative study of the influence of radiation on changes in the spectral characteristics of amino acids and proteins. These features are closely coupled to conformational changes in protein molecules, resulting in changes in their physiological functions.

4.2 Derivative Spectrophotometry for Analysis of Guanidine Preparations

Some guanidine derivatives are biologically active substances and are used, in particular, for the treatment and prophylaxis of protozoal diseases. Of these substances, a special position is occupied by 1,3-bis-[(*p*-chlorobenzylidene)amino] guanidine (Cl-BAG) produced by Russian industry and named not very successfully as “chimcoccid.” This compound is a highly effective anticoccidial preparation against coccidioses and toxoplasmosis of animals (Khovanskikh 1984). Cl-BAG could also be of potential interest for the treatment of human

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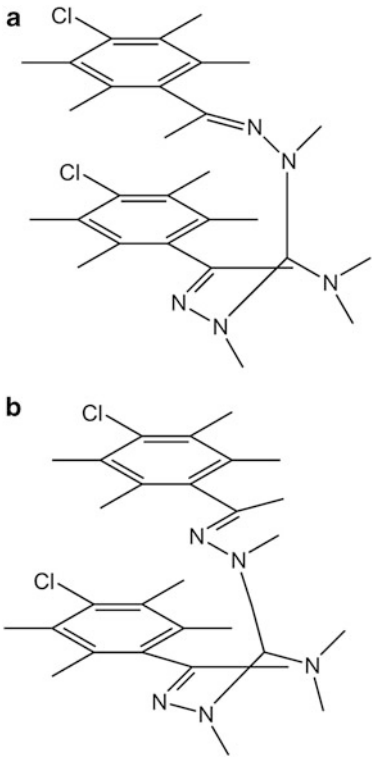

Fig. 4.39 (**a**, **b**) The two most preferable conformations of Cl-BAG (compound 1) according to computer calculations

toxoplasmosis (Khovanskikh et al. 1984). However, the molecular mechanism of Cl-BAG influence remains unexplained. Therefore, attention is drawn to the similarity between the structures of Cl-BAG structure and some adrenergic compounds, the molecules of which contain two benzene rings bound with a flexible alkylamine chain and with electron-donor substituents in *para*- and *meta*-positions. It has been shown that optimum conformations of these adrenergic compounds are stabilized by attraction of parallel benzene rings (Govyrin and Zhorov 1994). Moreover, electron-donor substituents are situated relative to each other at distances allowing chelation of metal ions. On the basis of the analysis of structural–functional and conformational–functional relations of a number of adrenoreceptor agonists (substances exciting adrenoreceptors or increasing noradrenaline concentration in the synaptic chain, their action being similar to that of adrenaline) and adrenoblockers (preventing interaction of mediator with adrenoreceptors), it was supposed that adrenoreceptor agonists participate in metal transfer into the hydrophobic cave of the receptor (Govyrin and Zhorov 1994). Experimental and theoretical data confirm that the probable pretender to this role is calcium (Khovanskikh et al. 1984; Govyrin and Zhorov 1994).

Structural similarity of Cl-BAG to the mentioned adrenergic compounds stimulated us to investigate its conformational possibilities and to assess its potential

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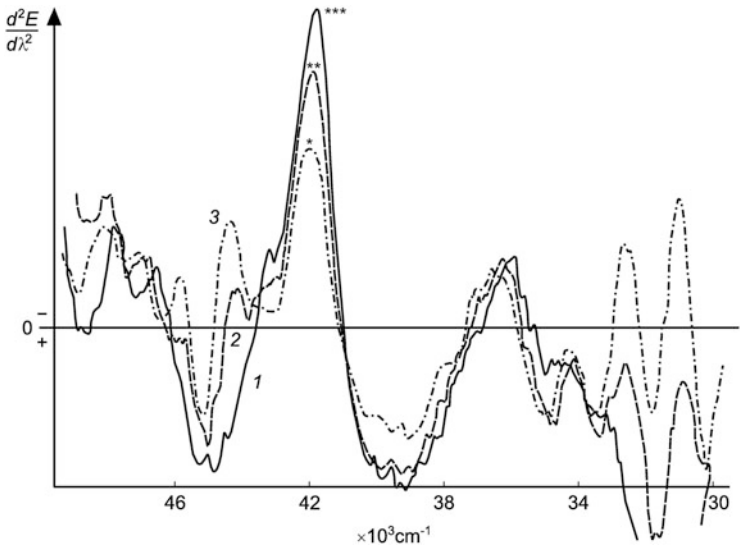

Fig. 4.40 The second derivative of UV absorption spectra of: (*1*) Cl-BAG; (*2*) mixture of Cl-BAG with 0.15 mM Ca^{2+}; (*3*) mixture of Cl-BAG with 0.25 mM Ca^{2+}, obtained in laboratory device

calcium-binding properties (Zhorov et al. 1985; Rozengart et al. 2003). Despite the fact that classical and quantum-chemical calculation methods give slightly different values for the relative energy of two folded Cl-BAG forms, both these methods predict the substantial advantage of these forms in comparison with the planar conformation (Zaionts et al. 1982; Grigorieva et al. 1985; Zhorov et al. 1985; Rozengart et al. 2003).

Calculation of conformational energy and search of steady conformations of the protonated BAG form were performed using an approximation of the method of atom–atom potential functions (approximate computation of force fields) (Zaionts et al. 1982; Grigorieva et al. 1985). The global minimum of energy, found as a result of calculations, corresponds to the conformation shown in Fig. 4.39a. The second preferable conformation, with an energy of 0.5 kcal/mol, is presented in Fig. 4.39b.

In addition to the conformations presented in Fig. 4.39, the preferable forms include two additional enantiomeric (stereochemical) conformations with energy equal to theirs and also found by calculation. The energy of the completely elongated planar stable conformation is equal to 8.0 kcal/mol. The most advantageous of planar Cl-BAG conformations have an energy of more than 6 kcal/mol. Of the folded stable conformations, those with energies nearest to the energy of optimum forms differ from the global minimum by at least 3 kcal/mol. The main reason for preferring folded Cl-BAG conformations is the non-valent attraction of benzene rings. Calculation using the atom coordinates of the stable conformations

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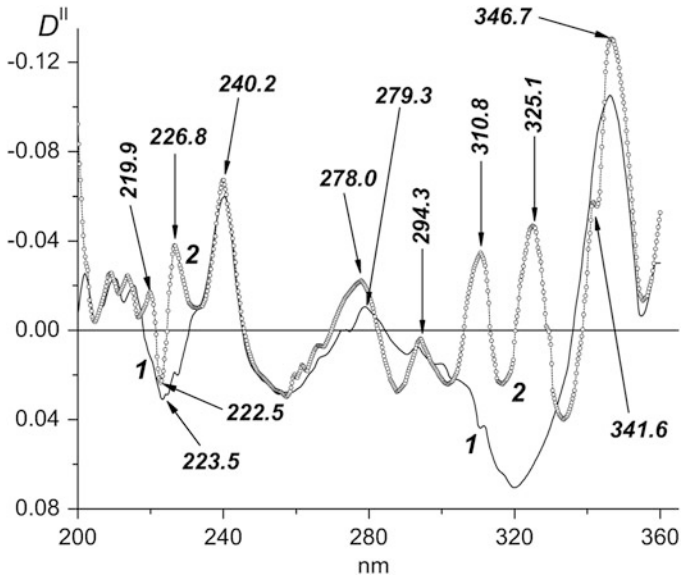

Fig. 4.41 The second derivative of UV absorption spectra of: (*1*) Cl-BAG and (*2*) the mixture of Cl-BAG plus 0.25 mM Ca^{2+}, obtained after data processing with the help of *Spectra Calc* and visualization in *Origin* 6.1

presented in Fig. 4.39 (Zhorov et al. 1985; Zaionts et al. 1982; Grigorieva et al. 1985) and of the completely elongated planar conformation showed that their energies were equal to 0.70 and 6.7 kcal/mole, respectively. In the most preferable Cl-BAG conformations, the distance between chlorine atoms is equal to 3.7 Å (Khovanskikh et al. 1984; Govyrin and Zhorov 1994; Rozengart et al. 2003).

Thus, calculations show that the folded Cl-BAG conformations in which chlorine atoms remote in the valence chain are spatially close are substantially more preferable than other stable conformations. This suggests the predisposition of optimum Cl-BAG conformations to form chelate complexes with metal ions (Khovanskikh et al. 1985; Govyrin and Zhorov 1994; Rozengart et al. 2003), which was confirmed (Zhorov et al. 1985). Further more detailed data expanded our conceptions on the possibility and specificity of complex formation depending on the nature of the chelated ion.

Figure 4.40 (curve 1) shows the second derivative of the absorption spectrum of Cl-BAG solution. In comparison with the usual absorption spectrum, it has clearly manifested and specific Cl-BAG maxima in regions 46,000–44,000; 42,000; 39,000; and 36,000 cm^{-1}, whereas the absorption spectrum of this substance is characterized by a smooth line, with insignificant peaks complicating the precise identification of fuzzy principal absorption maxima and accenting the poorly developed vibrational structure of the spectrum.

After addition of equimolar quantities of Ca^{2+} ions to Cl-BAG solution, the hyperchromic effect can be observed in absorption spectra as an increase in the OD

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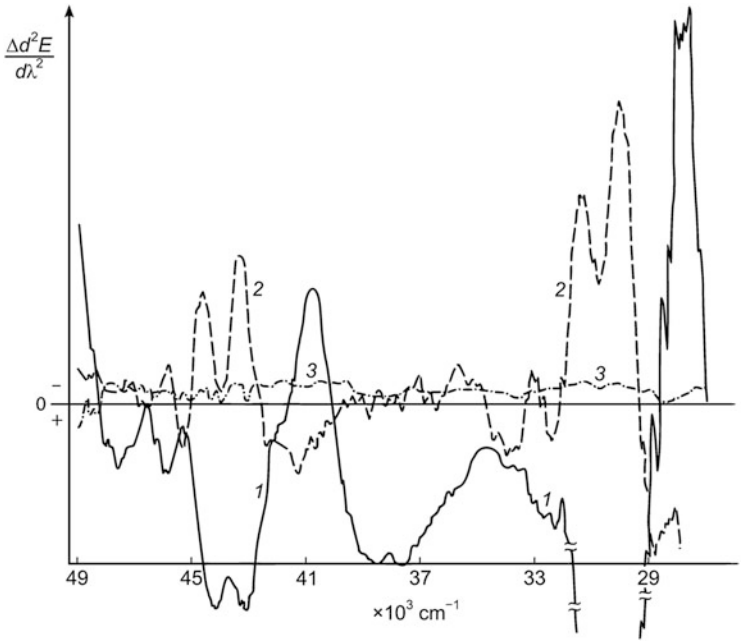

Fig. 4.42 The second derivative of difference absorption spectra of equimolar solutions: (*1*) Cl-BAG against Cl-BAG + Ca^{2+}; (*2*) Cl-BAG + Ca^{2+} against Cl-BAG; (*3*) Cl-BAG + K^{+} against Cl-BAG

in the main absorption maximum. This suggests formation of a less-ordered structure than the initial structure. Twofold dilution of the solution caused by the addition of calcium ions was accompanied by insignificant change in initial OD values. This suggests that the influence of Ca^{2+} results in formation of a complex with significantly higher molar extinction coefficient. Figures 4.40 and 4.41 (curve 2) show the appearance of a complicated spectrum structure of the calcium complex in comparison with the control; an absorption increase in regions close to 46,000 and 44,000 cm^{-1}; and the appearance of two maxima at 31,000 and 33,000 cm^{-1} (the minimum is at 32,000 cm^{-1}). Additional experiments showed that the OD increase was connected to some degree with an increase in Ca^{2+} ion concentration; moreover, in derived spectra, the value of signal frequently went beyond the boundaries of the diagram. Thus, addition to the Cl-BAG solution of equimolar Ca^{2+} quantities results in a hyperchromic effect in D^{II} spectra, combined only with insignificant reduction in OD values during twofold dilution. In Fig. 4.40 (curves 2, 3), an increase in absorption in regions close to 46,000; 45,000; 33,000; and 31,000 cm^{-1} is registered; moreover, the level of OD increase is connected to some degree with an increase in Ca^{2+} concentration.

The OD decrease in the main maximum of D^{II} spectrum of Cl-BAG was inversely proportional to Ca^{2+} concentration and also caused by dilution of solution

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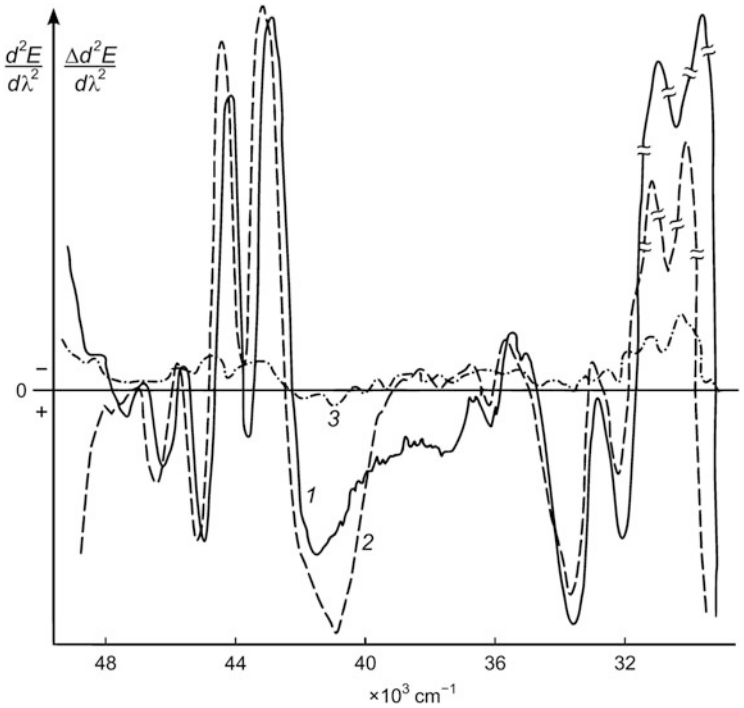

Fig. 4.43 The second derivative of the absorption spectrum of: (*1*) Cl-BAG + La^{3+}; D^{II} of difference absorption spectra; (*2*) equimolar solutions of Cl-BAG + La^{3+} against Cl-BAG, (*3*) equimolar solutions of Cl-BAG + Mg^{2+} against Cl-BAG

after addition of Ca^{2+}. This was confirmed by differential spectra (Fig. 4.42, curves 1, 2). Reflection symmetry of the dynamics of curves 1 and 2 once more stresses the reliability of revealed changes in the D^{II} spectra of the Ca^{2+}–Cl-BAG complex. The insignificant shift of absorption maxima of differential spectra relative to the D^{II} Cl-BAG spectra and to its Ca^{2+}–chelate spectra is theoretically quite permissible. The data in Fig. 4.42 (curve 3) confirm our early hypothesis (Zhorov et al. 1985) about the absence of Cl-BAG interaction with K^+ and Mg^{2+} ions. The differences between the fine structure of the Cl-BAG spectrum and its calcium complex most clearly manifest in the spectrum of the fourth-order derivatives (Fig. 4.44).

The material of Figs. 4.45 and 4.46 also allows us to return to consideration of the important role of chlorine atoms in the Cl-BAG molecule in chelation of metal ions. First of all, there are substantial differences between the D^{II} Cl-BAG spectra and that of its chlorine-free derivative, BAG (Fig. 4.46, curves 1, 2). However, the most important is that the spectra reveal that BAG is not capable of interacting with Ca^{2+} (Figs. 4.45 and 4.46, curves 2 and 3).

The difference spectrum of Cl-BAG in the presence and absence of calcium ions (Figs. 4.40 and 4.41) denotes the significant disturbance caused by the influence of calcium ions on the electron structure of the molecule. The differential spectrum of

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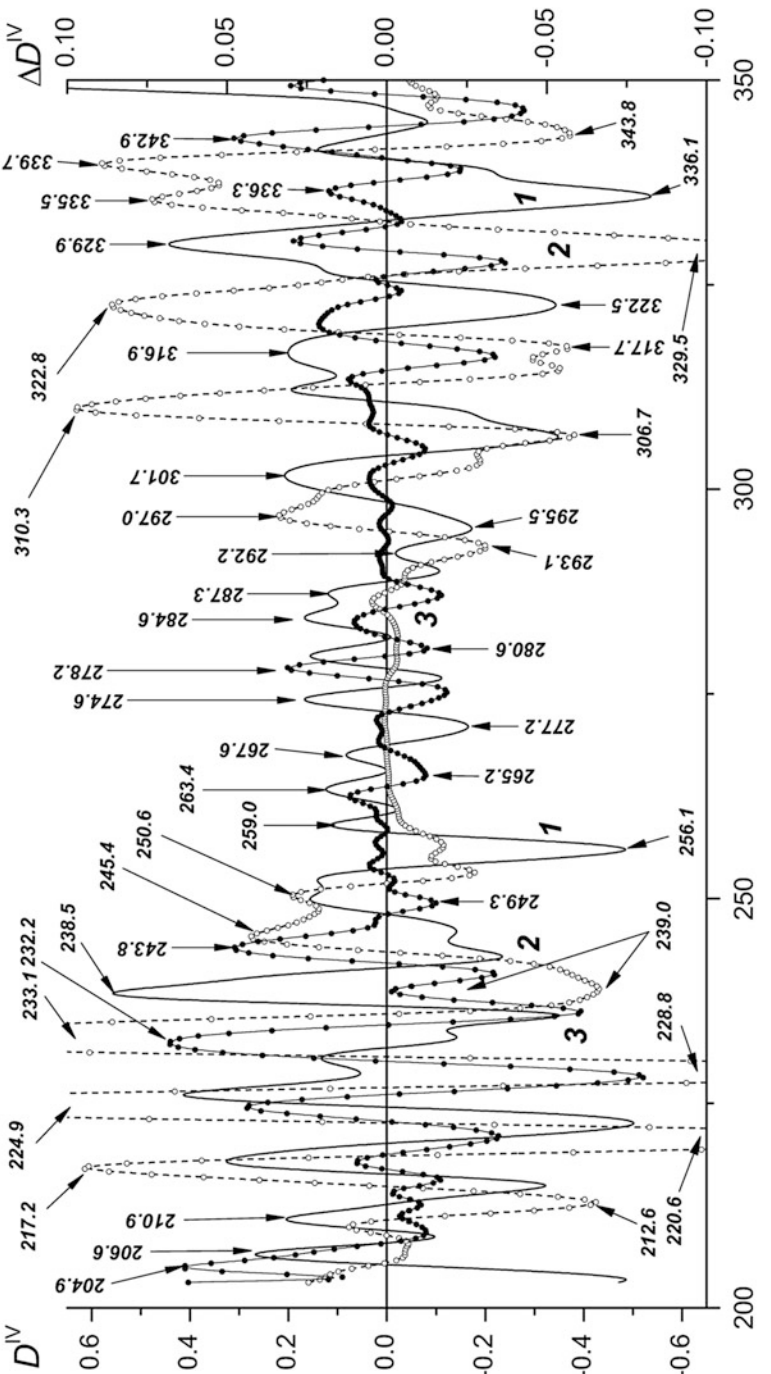

Fig. 4.44 The fourth derivative of the absorption spectrum of: (*1*) Cl-BAG, (2) its complex with Ca^{2+}; (*3*) D^{IV} of the difference absorption spectrum of Cl-BAG against Cl-BAG + Ca^{2+}

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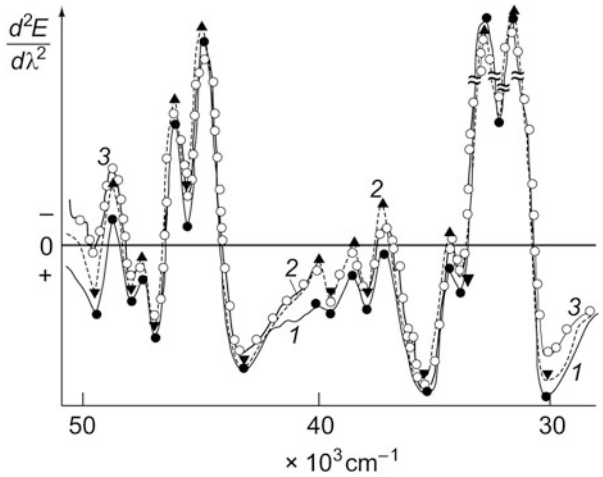

Fig. 4.45 Second derivatives of spectra of: (*1*) 0.25 M BAG solution; (*2*) BAG interaction with Ca^{2+}; (*3*) BAG interaction with La^{3+}

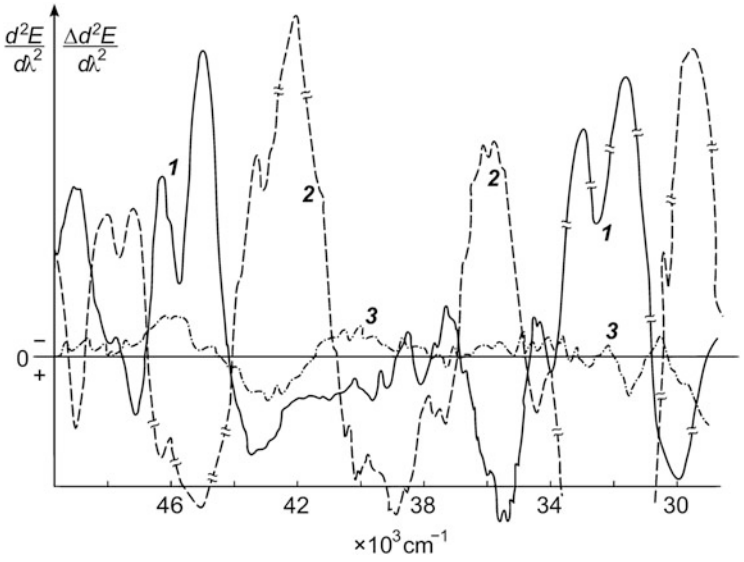

Fig. 4.46 (*1*) D^{II} of the BAG absorption spectrum. D^{II} of difference absorption spectra: (*2*) Cl-BAG against BAG; (*3*) BAG + Ca^{2+} against BAG

chlorine-free derivative BAG in the presence and absence of calcium (Fig. 4.46) indicates the absence of interaction of this compound with calcium. This testifies that chlorine atoms play a determining role in interaction of calcium with Cl-BAG. It is experimentally found that BAG does not show anticoccidial activity (Khovanskikh 1984; Khovanskikh et al. 1984; Zhorov et al. 1985; Govyrin and Zhorov 1994). According to repeated tests, UV spectroscopy shows that BAG

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practically does not interact with ions of potassium and magnesium (Zhorov et al. 1985; Rozengart et al. 2003).

We have described the different ways of demonstrating Ca^{2+} interaction with Cl-BAG and BAG in great length in order to exclude different interpretations of this issue.

The general conclusion, made on the basis of calculation of the spatial BAG structure and from UV spectroscopy data of BAG and its physiologically inactive analogue, is that the presence of anticoccidial activity coincides with the presence of calcium-binding ability, which is determined by the three-dimensional closeness of chlorine atoms.

4.2.1 *Chelating Ability of 1,3-Bis-[(*p*-Chlorobenzylidene) Amino]Guanidine: Complexes with Ca^{2+} and La^{3+} Ions*

In connection with the material presented in Sect. 4.2 on the interaction of Cl-BAG with K^+, Mg^{2+}, and Ca^{2+}, the investigation of chelate complex formation with La^{3+} is of separate interest. For this ion, the value of the ionic radius (Handbook of chemist 1962; Saakov et al. 1987a, b; Govyrin and Zhorov 1994) and the character of its biological effects are comparable with those of Ca^{2+}. Lanthanum ion is an inhibitor of membrane Ca^{2+}-ATPases of cells and of Na^+/Ca^{2+} ion exchangers (Zhorov et al. 1985; Govyrin and Zhorov 1994); furthermore, it is a nonspecific antagonist of Ca^{2+} channels (Marley et al. 2000).

The second derivative of differential (difference, ΔD^{II}) spectra reveals, with a large degree of accuracy, divergences in the dynamics of change in initial spectral curves between control and experiment. Note that according to the theory of differential spectra, the positions of maxima and minima of difference curves can significantly differ from the positions (ordinate and abscissa) of extrema of initial spectra, that is, extrema of absorption can be shifted relative to the initial spectra (Fraifelder 1980; Saakov et al. 1987a, b).

This confirmed by the character of differential spectra for Cl-BAG + Ca^{2+} against Cl-BAG (Figs. 4.43 and 4.44, curve 3). The insignificant shift of absorption maxima of differential spectra relative to the Cl-BAG absorption spectra and to its calcium complex is quite permissible theoretically. Manifestation of the hyperchromic effect for protein structures indicates the formation of a less-ordered structure in comparison with the initial state; it is a criterion for the degree of denaturation and transition of a protein molecule into an unfolded state (Kuzin 1962).

La^{3+} actively interacts with Cl-BAG (Fig. 4.47, curve 3), and complication of the D^{II} spectrum manifests as the appearance of maxima in regions 49,000–46,000; 47,000–45,000; 40,000; 35,000; 33,000; and 31,000–32,000 cm^{-1}. They indicate significant disturbance of the electron structure of the molecule of the La^{3+} complex, manifested in the appearance of new absorption bands close to 48,400; 47,000; 44,300; 42,800; 40,350; 38,300; 36,650; 35,400; 32,800; 31,100; and 29,700 cm^{-1} (225.1, 232.3, 248.95, 263.4, 284.0, 299.3, 307.3, 325.5, and

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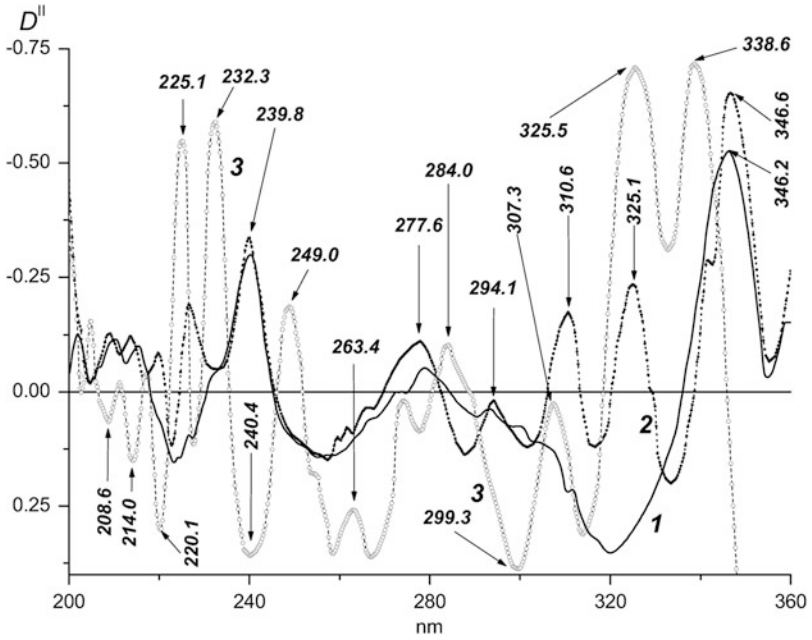

Fig. 4.47 Second derivatives of Cl-BAG spectra and of products of its reaction with Ca^{2+} and La^{3+}, obtained after digitalization and visualization of data with the help of programs *Graph Digitizer* 2.11 and *Origin* 6.1

338.6 nm). Although some conclusions could be made on the basis of absorption spectra, the large fuzziness of their outlines stimulated the application of the second derivative to ensure finding newly formed bands in the spectral outline and to detect their more precise positions (Saakov et al. 1987a). Furthermore, the amplitude of signal of the D^{II} spectrum indicates the exceptionally salient hyperchromic effect with high degree of reliability, and this effect probably corresponds to simplification of the structural order of complexes.

Figure scales do not allow the peak-to-peak amplitude of signals to be shown. This is confirmed by examination of the curve shape of the differential spectrum ΔD^{II} "Cl-BAG + La^{3+} minus Cl-BAG" (Figs. 4.47 and 4.48, curves 2), which enables more precise identification of the location of newly appeared bands and of lanthanum-induced changes to the fine structure of the spectrum. In this case, a small hypsochromic shift to the short-wave region (44,550; 43,000; 31,000 cm^{-1}) relative to the Cl-BAG spectrum is observed for the main absorption maxima of the ΔD^{II} spectrum. This allows, with high degree of reliability, to speak exactly about the role of lanthanum in the shaping of the new structure of the spectrum.

To analyze the role of chlorine atoms in the Cl-BAG molecule during complex formation (Zhorov et al. 1985), unsubstituted 1,3-bis-(benzylideneamino)guanidine (BAG) was investigated. The main maximum of its absorption spectrum is significantly moved to the short-wave spectral region 31,000 cm^{-1} in comparison with Cl-BAG, that is, there is a typical *hypsochromic* shift of spectrum relative to initial

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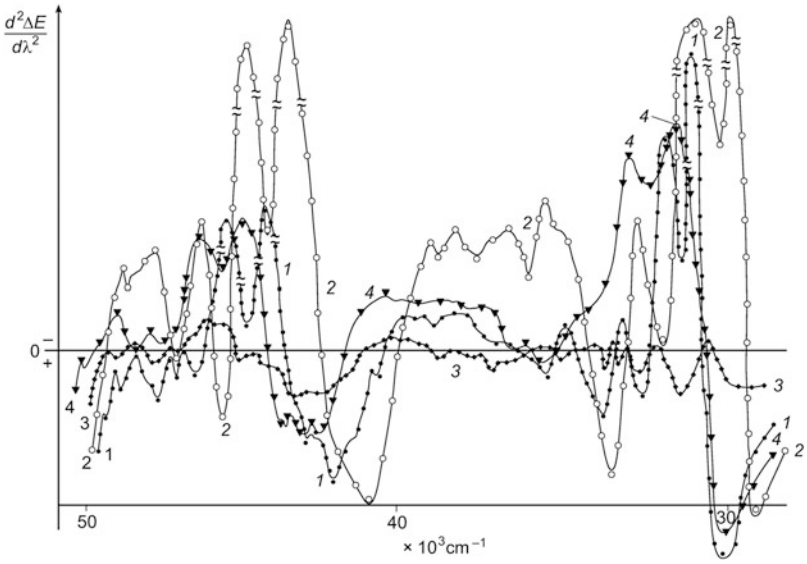

Fig. 4.48 Special features of second derivatives of difference spectra: (*1*) Cl-BAG + Ca^{2+} against Cl-BAG; (*2*) Cl-BAG + La^{3+} against Cl-BAG; (*3*) BAG + Ca^{2+} against BAG; (*4*) BAG + La^{3+} against BAG

compound (Figs. 4.45 and 4.46, curves 1). Simultaneously, substantial differences in the D^{II} Cl-BAG and BAG spectra are revealed, seen from dynamics of curve 1 in Fig. 4.45. In addition to the hypsochromic shift of the main absorption maximum to region 31,000 cm^{-1}, two maxima in region 46,000–44,000 cm^{-1} are formed, which are also hypsochromically moved relative to corresponding maximum of Cl-BAG. Characteristic extrema for BAG are a maximum at 48,000 cm^{-1}, three maxima in the region 40,000–36,000 cm^{-1}, and a maximum at 34,000 cm^{-1}. BAG, in contrast to Cl-BAG, practically does not interact with calcium ions (Fig. 4.45, curve 2), which is also evident from the differential spectrum "BAG + Ca^{2+} against BAG" (Fig 4.46, curve 3).

Absorption spectra of BAG + La^{3+} clearly demonstrate the presence of a significant *hyperchromic* effect in the initial absorption curve that probably corresponds to unfolding of the complex and its transition to a new conformational state. Furthermore, from the D^{II} spectrum (Fig. 4.47, curve 3), the complication of the vibrational structure of the spectrum and the appearance of clearer extrema follow. Changes in the fine structure of the BAG electron spectrum induced by lanthanum are visible on the differential spectrum "BAG + La^{3+} against BAG" (Fig. 4.48, curve 4). In principal absorption maxima, a hypsochromic shift (44,550 and 43,000 cm^{-1}) is observed. This allows, with high degree of reliability, to speak exactly about the role of lanthanum in creation of the new structure of the spectrum.

The specific character of the fine spectral structure of Cl-BAG and its Ca^{2+} and La^{3+} complexes is evidenced by registration of derived spectra of the fourth order. Taking the D^{IV} spectrum of Cl-BAG (Fig. 4.49) as the basis, analysis of the fine

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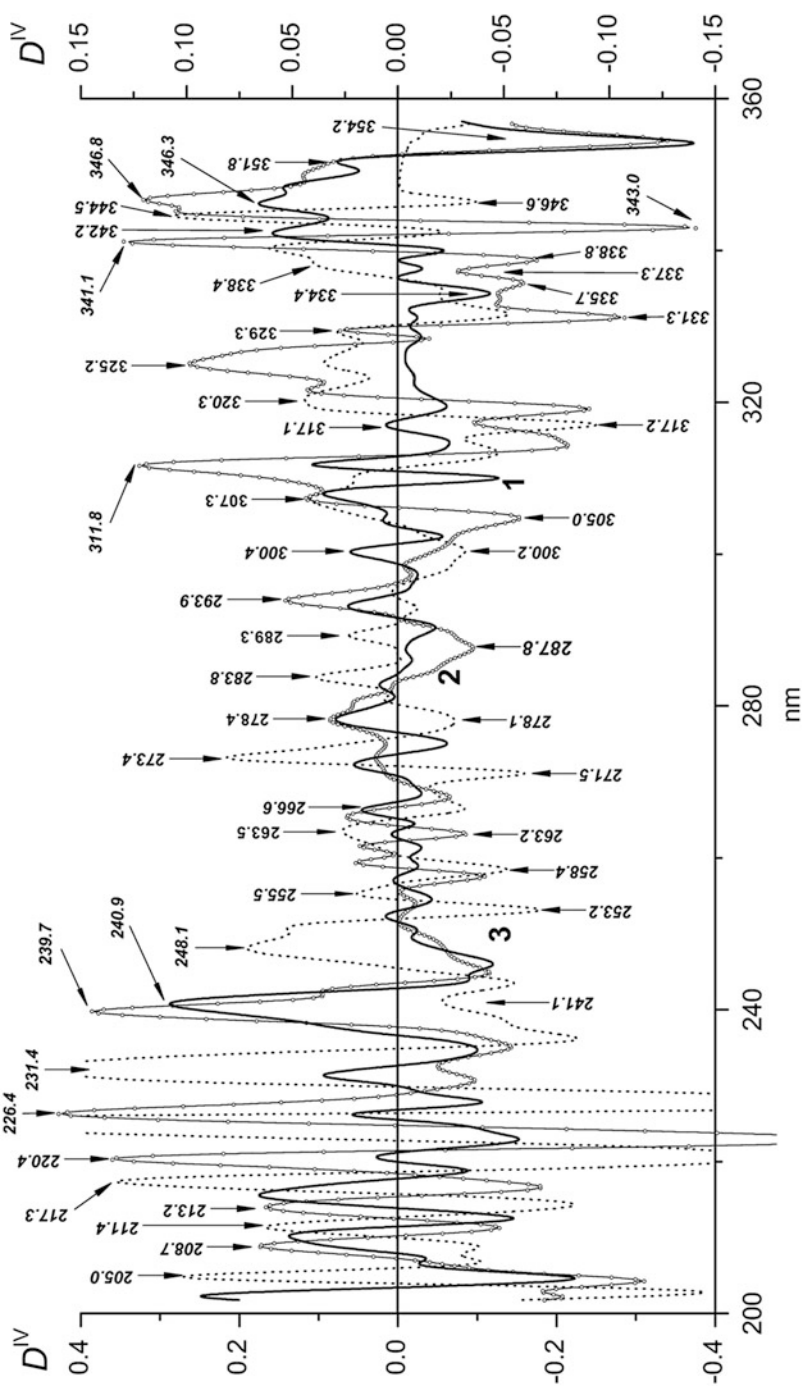

Fig. 4.49 Specific character of derived spectra of fourth order of solutions (0.25 mM): (*1*) Cl-BAG; (*2*) its complex with Ca^{2+}; (*3*) its complex with La^{3+}. The *left ordinate* axis is for *curves 1* and *2*; *right* for *3*

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structure of its spectrum in comparison with its complexes with metals was carried out. For the Ca^{2+} complex, the following bands are common with Cl-BAG for λ_{max}: 208.7, 213.2, 220.35, 226.4, 232.3, 252.35, 255.4, 266.5, 273.4, 278.4, 293.0, 307.3 and for λ_{min}: 204.35, 212.68, 223.02, 234.5, 245.7, 268.0, 275.0, 287.8, 305.0, 314.7 nm. The hyperchromic effect of the Ca^{2+} complex appears in bands 208.7, 213.2, 220.35, 226.4, 232.27, 239.67–240.92, 256.1, 259.63, 265.57–266.55, 272.27, 278.36, 290.1–293.9, 307.3, 311.83, 320.3–329.3, 341.1, 346.8, and 351.8 nm. This means that an integral increase in OD in the absorption spectrum is determined by the contribution of the above-listed bands, which is not seen on the usual absorption spectra. Furthermore, the dominant role of Ca^{2+} in creation of the spectral hyperchromatism looks real enough. Simultaneously, the hypochromic effect in the spectrum of the Ca^{2+} complex was discovered, that is, the decrease in absorption value is opposite to initial SBs of Cl-BAG at $\lambda_{min} = 216.3$, 231.7, 257.0, 263.2, 268.3, 287.8, 305.0, 314.7, 319.4, 331.2, 335.7, and 338.8 nm. The general domination of hyperchromic effect can be explained by the predominance of the additive increase in the OD of the Ca^{2+} complex independently of the presence of the hypochromic effect in the above-listed bands.

The analogous comparative analysis of OD bands of D^{IV} spectrum for Cl-BAG and its La^{3+} complex revealed the following agreements for $\lambda_{max} = 210.1$, 217.2, 224.0, 231.35, 240.9–241.0, 266.55, 272.3, 278.4, 281.3, 290.1–293.9, 307.3, 340.7–342.0, and 346.8 nm. The hyperchromic effect in bands of La^{3+} complex is revealed at 205.0, 211.4, 217.3, 225.1, 231.4, 248.0, 255.5, 263.5, 273.4, 283.8, 289.3, 307.3, 320.3–329.3, and 338.4–344.5 nm. From this, as in the case of the Ca^{2+} complex, it follows that the additive manifestation of the hyperchromic effect is caused by an increase in absorption of separate SBs. However, in the La^{3+} complex, the part in which bands coincide with Cl-BAG manifests a hyperchromic effect. This hyperchromatism is combined with the creation of partly new SBs at 225.25, 248.1, 273.4, 283.8, 289.3, and 320.0–329.3 nm. At the same time, for the La^{3+} complex, the agreement of minimum extrema with bands of Cl-BAG is found for bands 204.4, 211.7, 223.1, 228.0, 234.7, 245.3, 268.0, 275.1, 287.8, 302.0–305.0, 314.3, 331–334, 335.7, 343.0, and 354.2 nm. Simultaneously, bands of hypochromic effect appear for the La^{3+} complex at 202.9, 214.2, 220.4, 230.8, 253.2, 257.6, 263.2, 266.8, 271.5, 287.8, 300.2, 305.0, 317.2, 328.8, 343.0, and 346.6 nm. Thus, in Ca^{2+} and La^{3+} complexes, manifestation of hyperchromatism is conditioned by the difference of additive OD of bands of hyperchromic and hypochromic effects. These data confirm the need for the derivative approach to reveal hyperchromatism in absorption spectra (Saakov 2000a, e).

The results of experiments carried out by the authors allowed us to conclude the following:

1. The precise localization of absorption maxima of Cl-BAG and BAG was ascertained in D^{II} spectra; these maxima are fuzzy on the usual absorption spectra.

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2. A more complex structure of BAG spectra was noted in comparison with Cl-BAG, which indicates lower stability than the structure of Cl-BAG molecules.
3. The presence of Cl-BAG interaction with Ca^{2+} and La^{3+} ions was reliably established, and the specific character of the fine spectra structure of formed complexes was revealed.
4. The exclusiveness of the hyperchromic effect after formation of Ca^{2+} or La^{3+} complexes with Cl-BAG was refuted; a hypochromic effect was found for a number of bands.
5. The absence of interaction of BAG with Ca^{2+} was established.
6. Because of the similarity of D^{II} spectra for BAG + Ca^{2+} and BAG + La^{3+}, it was only possible with the help of difference ΔD^{II} spectra to reveal the differences, which indicated the possibility of interaction of lanthanum ions with BAG.
7. Experimental data indicated selectivity and specificity of the influence of some metal ions on the formation of chelate complexes with Cl-BAG and BAG (Zhorov et al. 1985; Rozengart and Saakov 2002; Rozengart et al. 2003).

Furthermore, the described material serves as additional proof of the advanced application of DHSO for the analysis of biologically active substances forming chelate complexes.

4.2.2 *Special Features of Ca^{2+} Binding by Mono-, Bis-, and Tris-Substituted Guanidine Derivatives*

A study of the structure and molecular mechanisms of action of Cl-BAG (Khovanskikh 1984) indicated that in the most preferable folded conformations of the Cl-BAG molecule, the chlorine atoms remote on the chain were brought to a distance of 3.7 Å as a result of attraction between benzene rings, as shown by calculations of molecular mechanics (Khovanskikh 1984; Zhorov et al. 1985). The possibility of formation of a Cl-BAG + Ca^{2+} chelate complex was confirmed by comparative spectrophotometric studies (Rozengart et al. 2003; Saakov et al. 2003). It was found that the derivative *N*,*N*′-bis-(benzylideneamino)guanidine (BAG) does not manifest anticoccidial activity and is practically lacking the ability to bind Ca^{2+} (apparently as a result of the absence of chlorine atoms) (Rozengart et al. 2003; Rozengart and Saakov 2003). All this gave the basis to suppose that the basis of the anticoccidial activity of Cl-BAG is its capability for Ca^{2+} chelate formation.

There was an interest in developing the above-mentioned experiments using the new methodical basis to study the possibility of Ca^{2+} complex formation for mono- and tris-substituted guanidine (MG and TG, respectively) derivative analogues of Cl-BAG. The structural formulae of the preparations are shown below:

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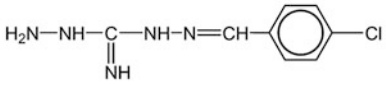
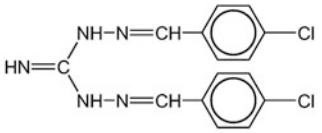
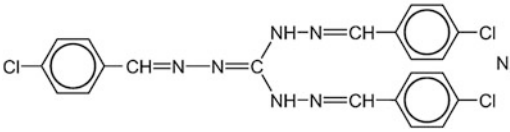


N-(*p*-chlorobenzylidenamino)guanidine
monosubstituted derivative - MG



N,N'-*bis*(*p*-chlorobenzylidenamino)guanidine
bis-substituted derivative - Cl-BAG



N,N',N"-*tris*(*p*-chlorobenzylidenamino)guanidine
tris-substituted derivative - TG

In carrying out experiments on the formation of complex compounds of biologically active substances with Ca^2, we wanted to emphasize the special features of change in spectral curves and to stress the prospects of registration of difference DSHO for the rapid and precise identification of created complexes.

It was possible to register derived spectra of the second to fourth orders simultaneously because the differentiator allowed signals to be recorded in analog form (see Chap. 3). Research experience proved the reasonability of transfer of analog signals into numerical form. For this purpose, the digitalization of spectral curves was performed with the program *Graph Digitizer* 2.14 according to N. Rodionov (http://nick-gr.chat.ru). Digitalized data were interpolated with the program *Origin* 6.0 and then fast Fourier transform (FFT)-filtered and transferred into the program *Spectra Calc*. After transfer, spectra of the second derivative were smoothed on seven points and differentiated for calculation of the fourth derivative of absorption spectra. After the last operation, smoothing on 17 points was carried out, allowing reliable separation of the main signals characteristic of absorption bands and exclusion of signals from outside noise.

To detect fine special features of the spectra structure change after chelate complex formation, difference (differential) spectrophotometry was also used because it reveals the difference between an initial preparation and a complex. This allowed us to find spectral changes in derived spectra with substantially higher accuracy and to characterize the specific character of interaction of the investigated guanidine derivatives with Ca^{2+}. We emphasize that according to the fundamental rule of difference spectrophotometry, the appearing absorption extrema normally do not coincide with the absorption maxima of initial preparations.

Although the absorption spectra of discussed substances are characterized by insignificant smooth changes that often complicate precise identification of fuzzy principal absorption maxima and stress the poorly developed vibrational structure of the spectrum, derived spectra have a pronounced structure. Obtained data are presented in Figs. 4.50, 4.51, 4.52, 4.53, 4.54, 4.55, 4.56, and 4.57. The second derivatives of absorption spectra of solutions of MG, Cl-BAG, and TG are shown in Fig. 4.50 (curves 1–3), each of which in comparison with usual spectral absorption curves has clearly pronounced and specific extrema of absorption. For MG (curve

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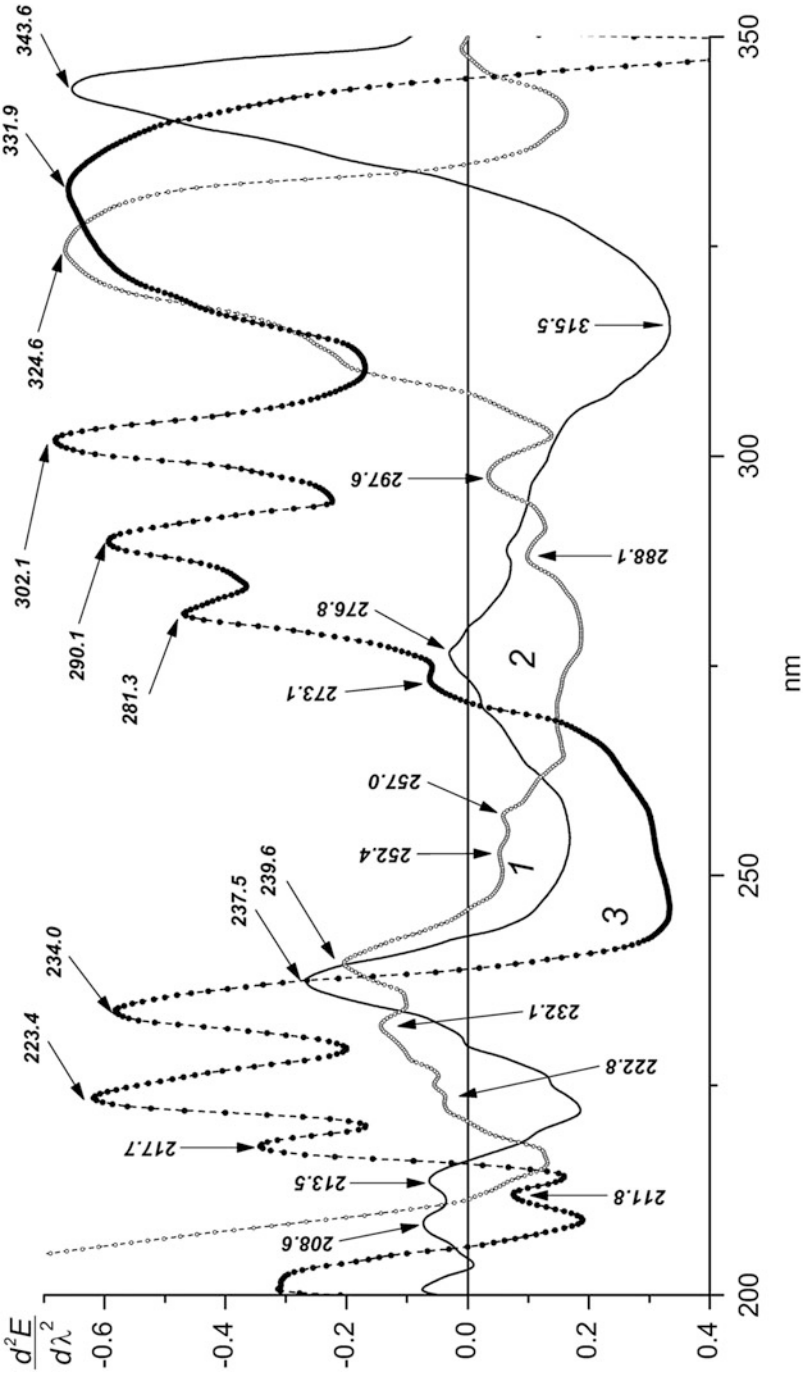

Fig. 4.50 Second derivatives of absorption spectra of solutions (0.25 mM): (*1*) Cl-BAG; (2) TG; (*3*) MG. *Arrows* show positions of the most interesting and specific extrema. *Ordinate:* OD, relative units

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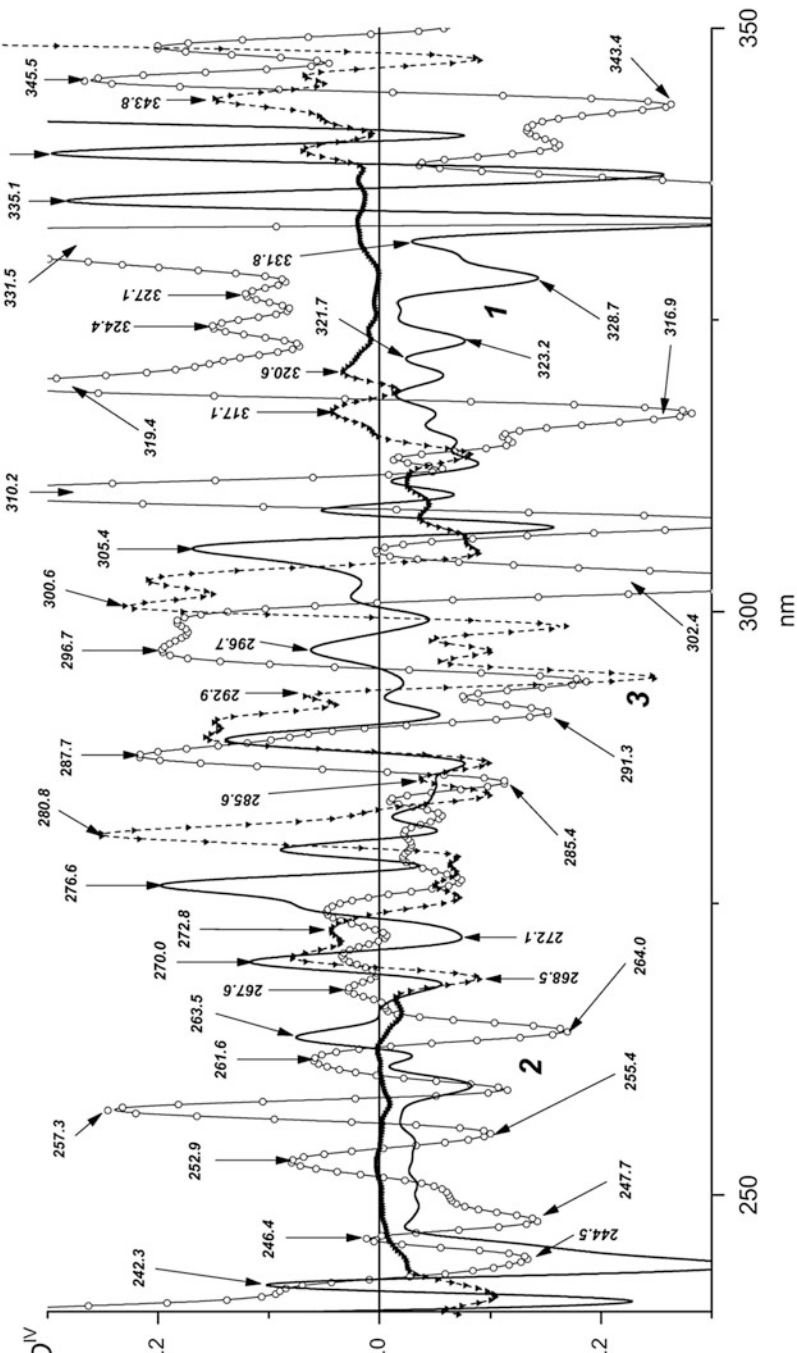

Fig. 4.51 Fourth derivatives of absorption spectra of solutions: (*1*) Cl-BAG; (*2*) TG; (*3*) MG. *Arrows* show positions of the most interesting and specific extrema. *Ordinate:* OD, relative units

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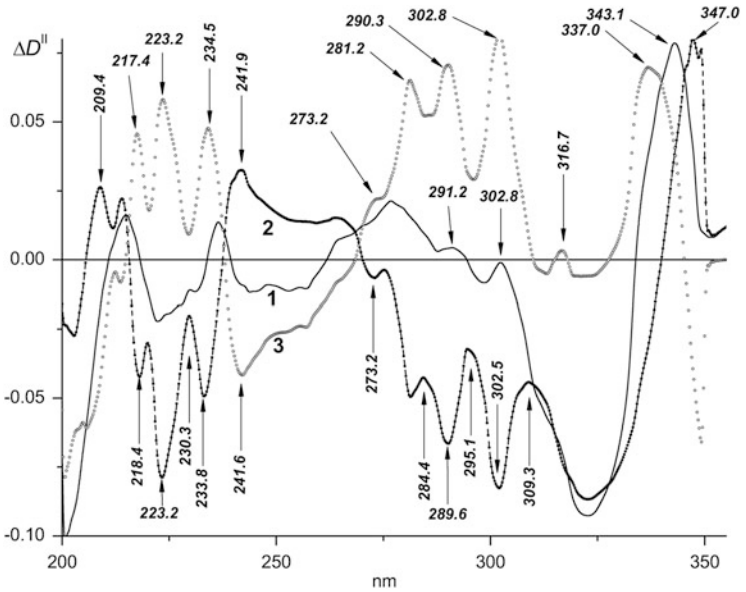

Fig. 4.52 The second derivative of difference spectra: (*1*) TG against Cl-BAG; (*2*) MG against Cl-BAG; (*3*) TG against MG

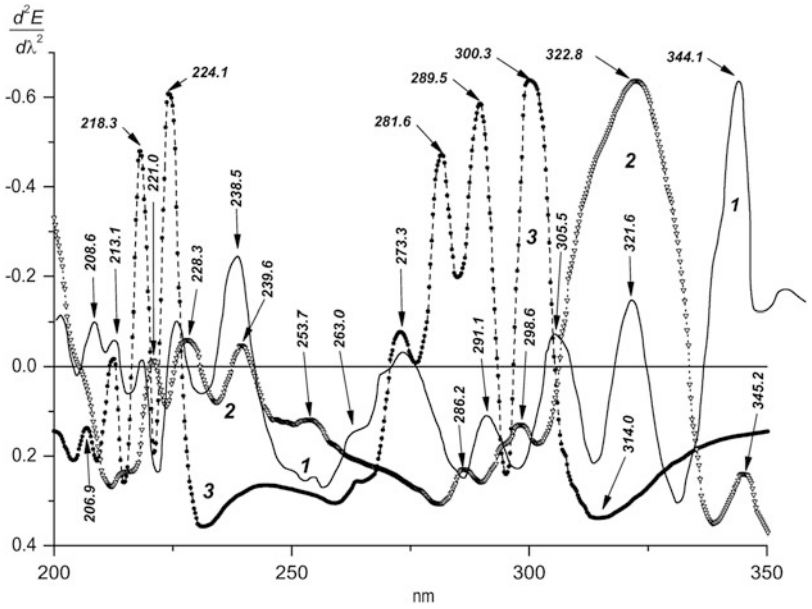

Fig. 4.53 Comparative spectra of second derivatives of solutions of chelate complexes: (*1*) Cl-BAG + Ca^{2+}; (*2*) TG + Ca^{2+}; (*3*) MG + Ca^{2+}. Other designations are as in Fig. 4.50

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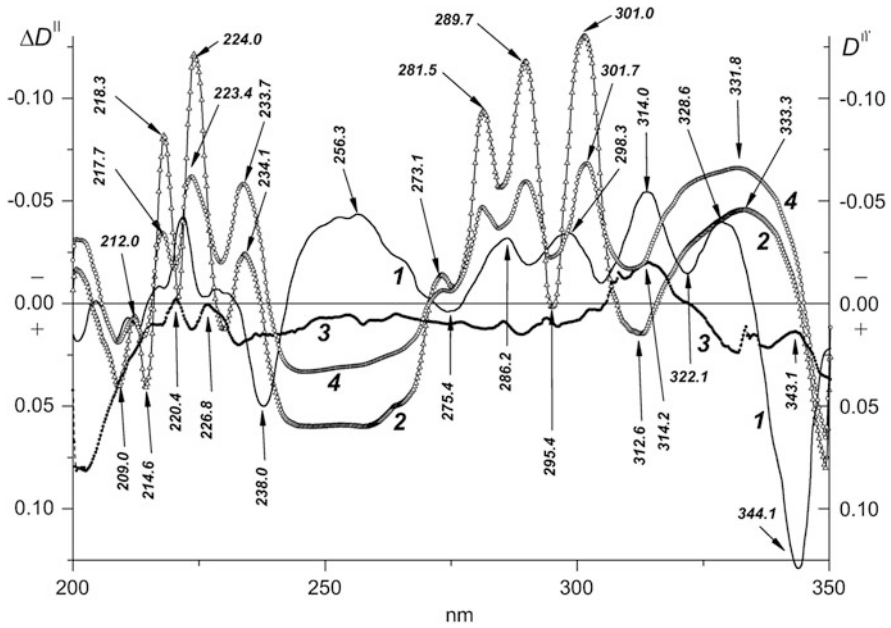

Fig. 4.54 Specific character of second derivatives of difference (differential) spectra: (*1*) Cl-BAG against Cl-BAG + Ca^{2+}; (*2*) MG against MG + Ca^{2+}; (*3*) TG against TG + Ca^{2+}; (*4*) the second derivative of the absorption spectrum of MG solution is shown for comparison with the difference spectrum *2*. Other designations are as in Fig. 4.50

3), four bands are characteristic in the far UV region at $\lambda_{max} = 211.9$, 217.7, 223.4, and 234.0 nm; in the near UV region, five bands are characteristic at $\lambda_{max} = 273.1$, 281.3, 290.1, 302.1, and 331.9 nm. For Cl-BAG (curve 1), characteristic absorption bands have extrema at $\lambda_{max} = 208.6$, 213.5, 237.45, 276.8, 315.5, and 343.6 nm. For TG (curve 2), the structure of the spectrum is weakly pronounced (i.e., significant extrema at $\lambda_{max} = 222.8$, 232.1, 239.7, 288.1, 297.6, and 324.6 nm). Figures 4.51 and 4.52 clearly illustrate the analytical capabilities of DSHO and the special features and abilities of derived difference spectra.

As described earlier (Rozengart and Saakov 2002), after the addition of equimolar quantities of Ca^{2+} to Cl-BAG solution, there was manifestation of the hyperchromic effect in absorption spectra, combined with an increase in OD in the principal maximum. This allowed us to suppose the formation of a less-ordered structure of the complex in comparison with the initial state. Twofold dilution of the solution during addition of calcium is accompanied by an insignificant change in the OD values. This suggested that the Ca^{2+} influence results in formation of a complex with substantially higher molar extinction coefficient. Figure 4.53 (curve 1) illustrates the complicated nature of the spectrum structure of the calcium complex in comparison with the control. An increase in absorption in bands is registered at $\lambda_{max} = 218.4$, 225.8, 238.5, and 273.3, especially at 291.1 and 305.5, and also at 321.6 and 344.1 nm.

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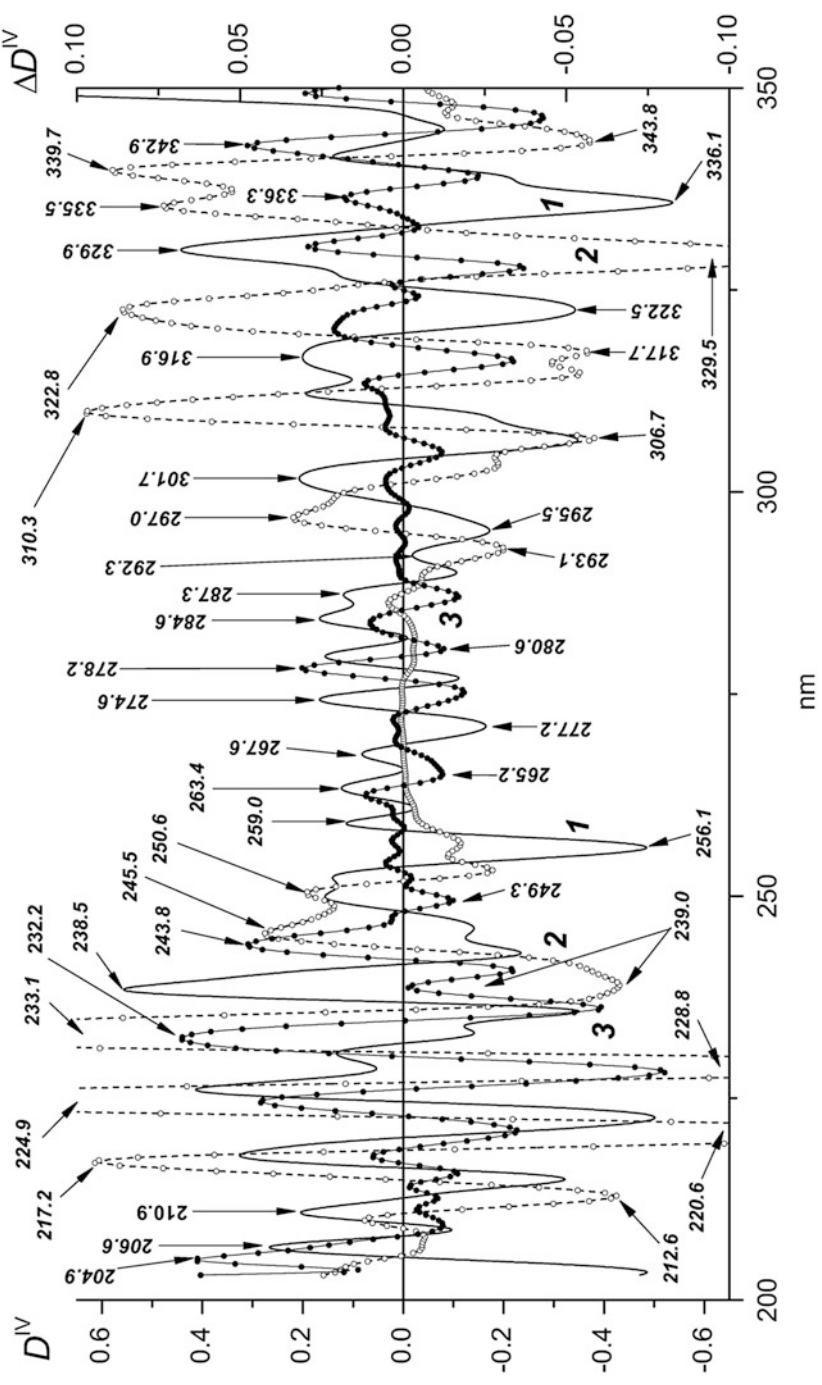

Fig. 4.55 Specific features of difference absorption spectra of fourth order for solutions (0.25 M): (*1*) Cl-BAG against Cl-BAG + Ca^{2+}; (2) MG against MG + Ca^{2+}; (*3*) TG against TG + Ca^{2+}. The *left ordinate* axis is for *curves 1* and *2*; *right* for *3*. Other designations are as in Fig. 4.50

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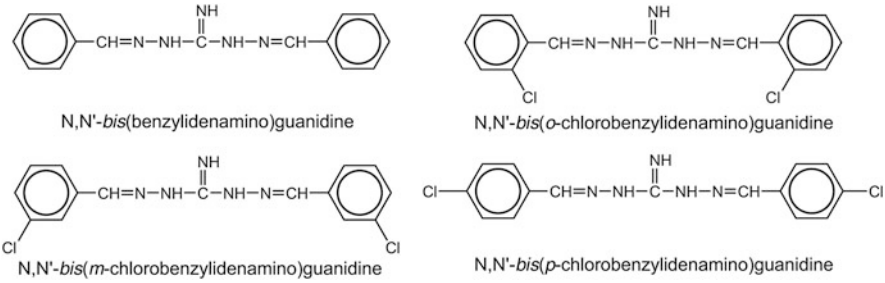

Fig. 4.56 Formulae of BAG derivative analogues with different positions of the chlorine atoms in the molecule

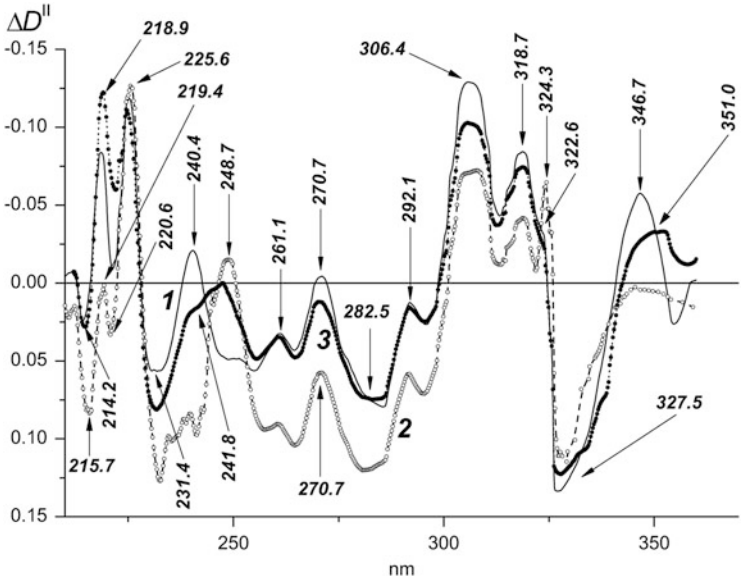

Fig. 4.57 The second derivative of differential (difference) spectra of *para* (*1*)-, *ortho* (*2*)-, and *meta* (*3*)-isomers of *N*,*N*′-bis-(benzylidene amino)guanidine minus BAG. *Ordinate*: relative units of the second derivative of difference spectra

Experiments showed that the level of OD increase depends on an increase in Ca^{2+} concentration; moreover, the value of the signal frequently went off the diagram scale on derived spectra. This is confirmed by the character of the difference spectra "Cl-BAG against Cl-BAG + Ca^{2+}" (Fig. 4.54, curve 1). Theoretically, an insignificant shift of absorption maxima of differential spectra relative to absorption spectra of Cl-BAG and its calcium complex is quite acceptable. Manifestation of the hyperchromic effect, for example, for protein structures, probably indicates the formation of a less-ordered structure in comparison with the initial state; it is a criterion for the degree of denaturation and of protein molecule transfer to an

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unfolded state (Kuzin 1962). Analogous manifestation of the hyperchromic effect during interaction with Ca^{2+} is observed for TG. Discovered hyperchromatism of OD is accompanied by significant *hypsochromic* shift of the principal absorption maximum of TG, combined, mainly, with disappearance of the band at $\lambda_{max} = 331.9$ nm (see Fig. 4.50, curve 3) and with the hypsochromic shift of extrema at $\lambda_{max} = 206.9$, 212.4, 218.3, and 224.1 nm.

Besides the found hypsochromic shift, attention is also directed to the redistribution of ratio of extrema amplitudes (Figs. 4.50 and 4.53, curve 3) in the far UV region at $\lambda_{max} = 211.9$, 217.7, 223.4, and 234.0 and at $\lambda_{max} = 206.9$, 212.4, 218.3, and 224.0 nm. Simultaneously, the presence of the hypochromic effect in the region of 314 nm, accompanied by disappearance of the band 331.9 nm, is notable. Interaction of TG with Ca^{2+} does not result in such significant changes in the spectrum as the reaction of MG and Cl-BAG with calcium ions. At the same time, in the absorption spectrum of TG, the level of OD decrease does not correspond to twice the decrease in concentration. The discovered spectral shifts are more similar to manifestation of probable interaction with Ca^{2+} and to weakly pronounced manifestation of hyperchromatism, with a small hypsochromic shift of the principal absorption maximum. There is noticeable change in the fine structure of D^{II} spectrum (Fig. 4.53, curve 2) at $\lambda = 214.5$, 220.96, 228.3, 239.6, 286.2, and 298.6 nm and hypsochromic shift of the maximum at 322.75 nm relative to the maximum at 324.6 nm (Fig. 4.50, curve 2).

Therefore, there is reason to suppose that interaction of guanidine derivatives with Ca^{2+} causes an increase in the molar extinction coefficients of formed complexes, but the level of induced hyperchromatism and of hypsochromic shifts suggests different degrees of interaction intensity of MG, Cl-BAG, and TG with Ca^{2+}. It was difficult to foresee the presence or absence of interaction with Ca^{2+} and its level for different guanidine derivatives.

However, some hidden complications were hypothesized on the basis of analysis of insignificant changes in the D^{II} spectrum structure of the Ca^{2+} + TG complex (see extrema at $\lambda_{max} = 220.96$, 228.3, 229.6, 298.6, and 322.75 nm). The monotony of curve 3 in Fig. 4.54 demonstrates the absence of significant spectral differences between TG and its probable complex with Ca^{2+}. At the same time, even the presence of small extrema at $\lambda = 220.45$, 226.8, and 314.2 nm suggests the presence of at least a weak interaction of the TG derivative with Ca^{2+}. These data corroborate the conclusion that there are different degrees of interaction of guanidine derivatives with Ca^{2+}.

Even more information about the specific character of changes in the electron structure of absorption spectra of chelate complexes is gained from the fourth derivative of difference spectra "compound minus compound + Ca^{2+}." These data are presented in Fig. 4.55. Comparison of curve 3 in Figs. 4.54 and 4.55 confirms the absence of monotony in the change character of the spectral curve "TG minus (TG + Ca^{2+})." Theoretically, supposing the absence or probability of minimum interaction of TG with Ca^{2+}, we could expect the spectral line to approach the zero line. If we reject insignificant curve fluctuations, then in this case, there are about 23–27 extrema accenting a spectral difference between the initial guanidine

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derivative TG and its supposed complex with Ca^{2+} (bands at $\lambda = 204.9$, 213.9, 217.8, 224.4, 228.7, 232.2, 238.7, 243.8, 249.3, 263.3, 265.2, 275.1, 278.2, 280.6, 283.5, 287.2, 305.0, 313.5, 316.3, 320.1, 327.8, 330.5, 336.3, 339.1, 342.9, 346.4, and 349.3 nm). Even taking into account the large scale of the right-hand axis of the graph in Fig. 4.54 (curve 3), the curve fluctuations actually prove the difference between the spectral TG state and the complex $TG + Ca^{2+}$. Another matter is the level of interaction and the conformational configuration of the formed chelate complex, shown as the difference curve 3 of ΔD^{IV} spectrum in Fig. 4.55.

Thus, on the basis of the data in Figs. 4.54 and 4.55, we *for the first time* gave experimental confirmation of the interaction of TG with Ca^{2+}, based on the sensitivity and informativeness of the high-order derivative spectrophotometry method. The basis of the working hypothesis on TG interaction with Ca^{2+} was established with experiments on the analysis of $TG + Ca^{2+}$ complex using the D^{II} method.

The data in Fig. 4.55 (curve 2) clearly show a difference in spectral states of the initial MG preparation relative to its chelate complex with Ca^{2+}. In this case, for industrial analysis of synthesized complexes, the presence of bands at $\lambda_{max} = 217.2$, 224.9, 233.1, 245.5, 250.6, 293.1, 297.0, 306.7, 310.3, 314.3, 317.7, 322.8, 329.5, 339.7, and 343.8 nm can be considered a standard indication of MG interaction with Ca^{2+}. Thus, the ΔD^{IV} method (see Sect. 3.3.3) reliably and persuasively demonstrates the possibility of the analytical detection of formation of $MG + Ca^{2+}$ chelate complexes and allows obvious separation of the main SBs characteristic of the chelate MG derivative.

Despite the fact that the results of Figs. 4.53 and 4.54 can be explained as interactions of Cl-BAG with Ca^{2+}, the data in Fig. 4.55 (curve 1) present at a qualitatively new level the distinctive special features of the spectral structure of the Cl-BAG molecule from the $Cl\text{-}BAG + Ca^{2+}$ complex. This is manifested as the domination of extrema both in the positive regions (at $\lambda_{max} = 206.6$, 210.9, 217.8, 225.9, 230.4, 238.5, 249.7, 259.0, 263.4, 267.6, 274.5, 279.9, 284.6, 287.3, 292.4, 301.7, 312.0, 316.9, 329.9, and 341.6 nm) and in the negative regions (at $\lambda_{max} = 208.5$, 214.9, 222.5, 235.5, 242.9, 256.1, 277.2, 276.8, 290.0, 295.5, 306.7, 322.5, 336.1, and 345.0 nm). The interpretation of a number of coincidences of SBs of difference spectra goes beyond the scope of this book because it requires thorough physicochemical studies that are still at the stage of experiment.

It follows from difference spectra (ΔD^{II} and ΔD^{IV}) that, as a result of interaction of MG, Cl-BAG, and TG with Ca^{2+}, a set of qualitatively different products appear. These products are characterized by the specific character of the electron spectra structure, stressing the qualitative special features of formed complexes. The initial products MG, Cl-BAG, and TG, different in their conformational states, interact with Ca^{2+} and manifest a specific spectral character, whose special features are revealed in the position of bands of the far and near UV regions, and this can serve as the analytical basis of their diagnostics. For example, in the near UV region (200–250 nm), it is possible to separate a group of bands that are similar for MG, Cl-BAG, and TG in many respects. This group is apparently revealed due to manifestation of the properties of benzene rings. In the region 210–212 nm, hyperchromatism is found for the band 210.9 nm for Cl-BAG and

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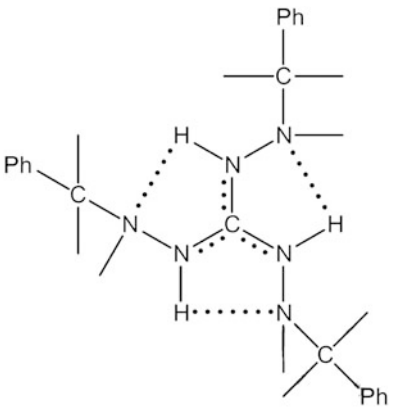
hypochromia at 212.6 nm for MG. TG bands occupy the intermediate position, close to the base line, which can be interpreted as a weak interaction with Ca^{2+} or its absence. Analogous TG bands are observed in spectral regions close to 256.0, 277.0, 295.0, and 310.0 nm. In the regions of 217.5, 224.9, and 233.1 nm, MG, Cl-BAG, and TG show a hyperchromic effect in difference spectra, corresponding to an OD decrease in these spectral regions of calcium complexes. By contrast, in the region of 238.5–239 nm, hyperchromatism of Cl-BAG accompanies hypochromia of MG. In the far UV region, for MG bands, a hypsochromic shift is revealed relative to Cl-BAG bands (using this more studied compound as the standard) in regions 297.0, 310.0, 313.0, 335.0, and 340.0 nm. Furthermore, it is clearly evident that, as a rule, the negative extremum of Cl-BAG corresponds to the positive extremum of MG, for example, in regions 295.0–297.0, 306.7–310.3, 316.9–317.7, 322.5–323.0, 329.5–329.9, 335.5–336.1, and 342–343.8 nm. The clarification of reasons for the observed spectral special features is the object of further deep study.

Differences between MG, Cl-BAG, and TG in their ability to bind Ca^{2+} are probably conditioned by the spatial structures of these guanidine derivatives. As described (Zhorov et al. 1985), the Cl-BAG structure has a biplanar conformation in comparison with guanidine. It is possible to suppose that in TG the configuration of the central guanidine group substantially changes:



The possibility of formation of three penta-cycles due to intramolecular bonds makes this structure probably more planar, which, apparently, complicates chelation with Ca^{2+}. A reader should note that TG is practically lacking anticoccidial activity (Khovanskikh 1984). This can serve as additional evidence in favor of our hypothesis (Zhorov et al. 1985) that the anticoccidial activity of Cl-BAG is connected with the ability to chelate Ca^{2+}.

Thus, in this section, the comparative difference spectra of the fourth derivative for MG, TG, and Cl-BAG are described and, with a high degree of reliability, reveal the interaction of guanidine derivatives with metal ions. It was established that the degree of interaction of mono-, bis-, and tris-derivatives of guanidine with Ca^{2+} is

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different. The analytical approaches proposed by us can be used in wider biochemical practice for detection of purity criteria for biochemical preparations and of their interaction with metal ions.

4.2.3 *Special Features of Interaction of Bis-[(Chlorobenzylidene)Amino]Guanidine Derivatives with Ca^{2+} Depending on the Chlorine Atom Position in the Molecule*

Calculations of the structure of the effective antiprotozoal substance *N*,*N*′-bis-[(*p*-chlorobenzylidene)amino]guanidine (*p*-Cl-BAG) carried out with the help of molecular mechanics (Zhorov et al. 1985) showed that in folded conformations, which are the most preferable, the chlorine atoms remote in the valence chain are separated by a distance of 3.7 Å as a result of attraction between the two benzene rings (Zhorov et al. 1985). This enables Ca^{2+} chelate complexes to form, as experimentally proven by us (Zhorov et al. 1985; Saakov et al. 1987a, b; Rozengart et al. 2003). At the same time, the analysis showed that *N*,*N*′-bis-(benzylideneamino) guanidine (BAG), not containing chlorine atoms, is not predisposed to complexing with Ca^{2+} and does not manifest anticoccidial activity (Zhorov et al. 1985; Rozengart et al. 2003).

A hypothesis was suggested about the specific role of chlorine atoms for manifestation of anticoccidial activity, combined with the ability of *p*-Cl-BAG to form complexes with metal ions (Saakov et al. 2003, 2004; Saakov and Rozengart 2005). This hypothesis was confirmed and developed in further studies (Saakov et al. 2003).

Investigation of the specific character of interaction mechanisms of mono- and tris-substituted guanidine derivative analogues of *p*-Cl-BAG found significant differences in their abilities to bind Ca^{2+} (Saakov et al. 2003). There was conjectured dependence of the found special features of chelate complexes with Ca^{2+} on the spatial structure of the considered guanidine derivatives. In the case of *N*,*N*′,*N*″-tris-[(*p*-chlorobenzylidene)amino]guanidine, the more planar structure is created as a result of a change in the central guanidine group and the formation of three penta-cycles due to intramolecular hydrogen bonds. This structure does not forbid, but substantially complicates, Ca^{2+} chelation (Rozengart and Saakov 2002). At the same time for *N*-((*p*-chlorobenzylidene)amino)- N′-guanidine limitations for Ca^{2+}chelating were not found (Saakov et al. 2003).

It was interesting to perform a comparative spectrophotometric study of chelate formation with Ca^{2+} in dependence on the position of the Cl atoms in the Cl-BAG molecule, that is, to compare Cl-BAG (*p*-Cl-BAG) and its *o*-chlorine (*o*-Cl-BAG) and *m*-chlorine (*m*-Cl-BAG) derivatives. These studies are described in this section. In the process, changes in the derived spectra of three compounds (Fig. 4.56) were

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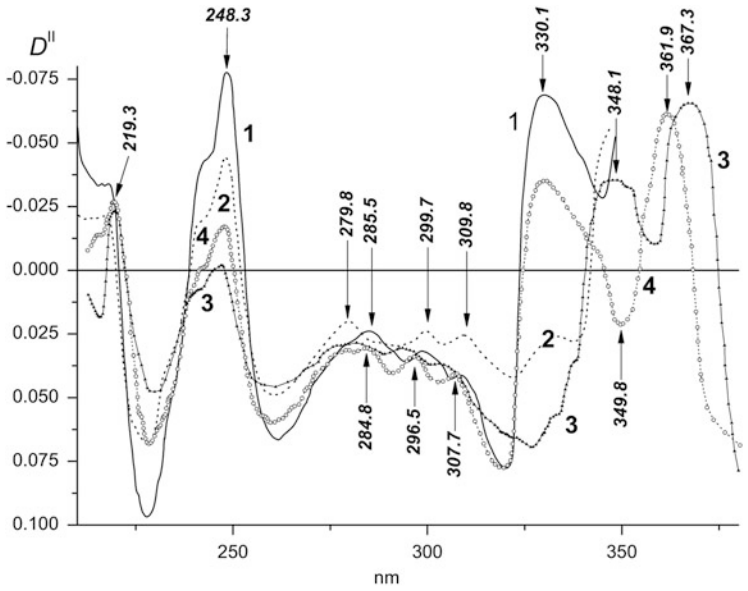

Fig. 4.58 The second derivative of the absorption spectrum of: (*1*) *o*-Cl-BAG and (*3*) *m*-Cl-BAG and of their complexes with the calcium ion (*curve 2* for *o*-Cl-BAG and *curve 4* for *m*-Cl-BAG)

investigated with the arrangement of chlorine atoms in the molecule in *para*-, *meta*-, and *ortho*-positions.

Quality, reliability, and reproducibility of results were checked with three or four consecutive records with three or four repetitions of the experiment (Rozengart and Saakov 2002, 2003; Rozengart et al. 2003). With the help of the program *Origin* 6.1, digitalized data were interpolated with an interval of 0.25 nm, smoothed out by FFT filtration, and then differentiated using the program *Spectra Calc*. Further processing of curves was done as described earlier. Visualization of numerical data was performed with the help of *Origin* 6.1. To obtain data on changes in the fine spectra structure, the procedure of recording differential (difference) derived spectra was used (Rozengart and Saakov 2002, 2003; Saakov et al. 2003). This approach allows, with a high degree of reliability, the detection of spectral changes in curves of derived spectra and reveals bands more precisely. We think it is necessary to emphasize that, in accordance with the rules of difference spectrophotometry, extrema appearing on the curve of difference spectrum do not coincide, as a rule, with the extrema of absorption curves of initial preparations.

Results on the registration of derived spectra of studied compounds and data concerning the specific character of their interaction with Ca^{2+} are presented in Figs. 4.57, 4.58, 4.59, and 4.60. The first task was assessment of the specific character of change in the second derivative of Cl-BAG absorption spectra (D^{II}) in dependence on the chlorine atom position in the BAG molecule. We carried out the registration of difference spectra of the second derivative (ΔD^{II}) "*p*-Cl-BAG minus

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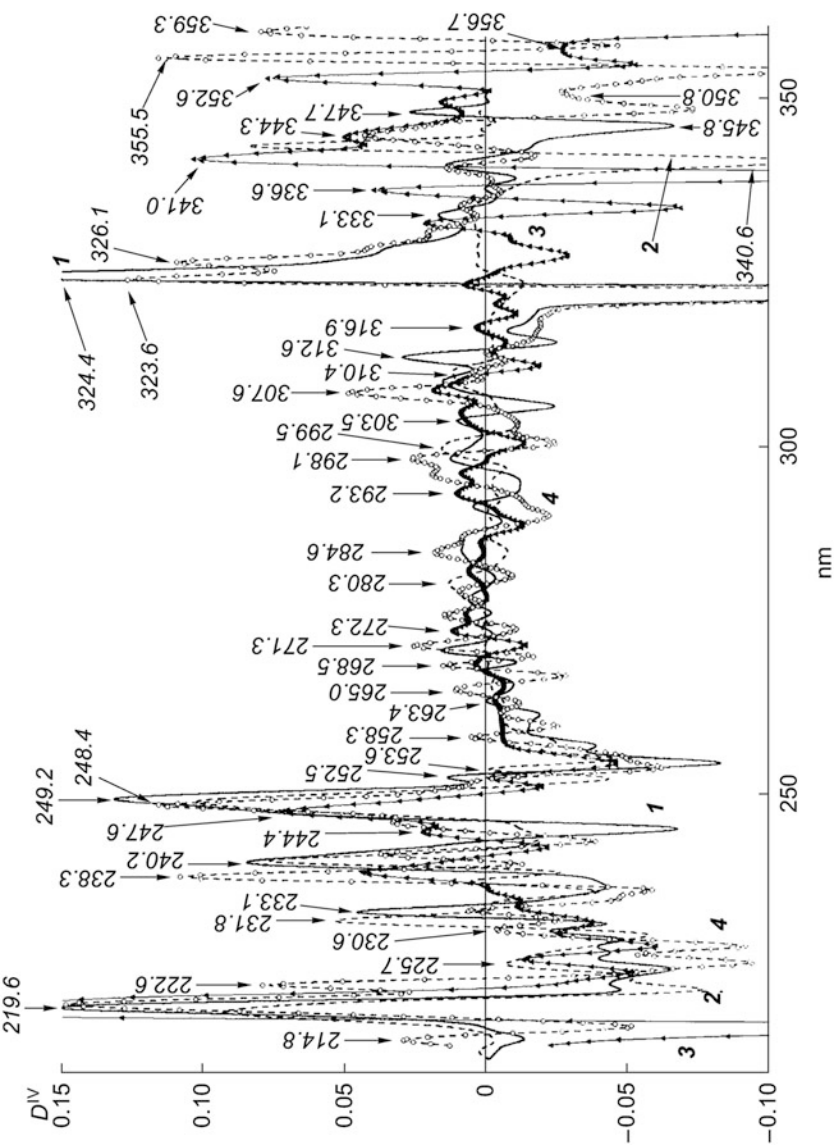

Fig. 4.59 The fourth derivative of the absorption spectrum of: (*1*) *o*-Cl-BAG and (*3*) *m*-Cl-BAG and of their complexes with the calcium ion (*curve 2* for *o*-Cl-BAG + Ca^{2+} and *curve 4* for *m*-Cl-BAG + Ca^{2+})

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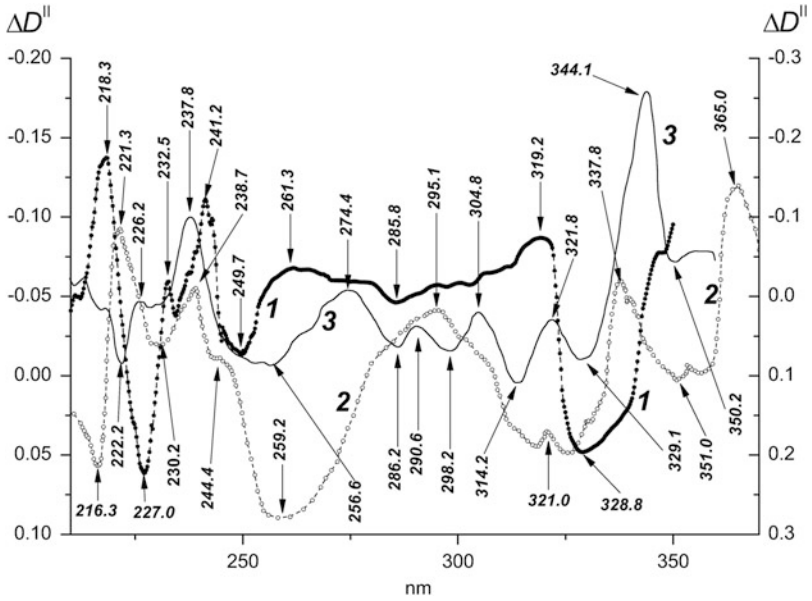

Fig. 4.60 The second derivative of difference spectra: (*1*) *o*-Cl-BAG minus *o*-Cl-BAG + Ca^{2+}, (*2*) *m*-Cl-BAG minus *m*-Cl-BAG + Ca^{2+}, and (*3*) *p*-Cl-BAG minus *p*-Cl-BAG + Ca^{2+}. The *left ordinate* is for *curve 2*; *right* for *curves 1* and *3*

BAG," "*o*-Cl-BAG minus BAG," and "*m*-Cl-BAG minus BAG" (see Fig. 4.57) to find how the chlorine atom position in the molecule influences the D^{II} curve of the absorption spectrum.

The data in Fig. 4.57 indicate that the difference spectra of these three compounds have no great and principal differences. Found differences were mainly concerned with curve 1 (*p*-Cl-BAG minus BAG) and consisted of the appearance of hypsochromic shift at band 240.36 nm (compared with bands of curves 2 and 3 close to the same spectral region). This indicates that the *para*-position of the chlorine atoms in the *p*-Cl-BAG molecule results in the appearance of the characteristic band 240.36 nm, whereas for *ortho*- and *meta*-positions of the Cl atoms, the specific band is at 248.7 nm. A question requiring deeper analysis arises: What is the mechanism of formation of the band at 240.36 nm? In other words, does this band appear de novo, or is it only a manifestation of the hypsochromic shift as a result of the chlorine atom presence in the *para*-position in the *p*-Cl-BAG molecule? It is possible that the fourth-order differentiation of the difference spectrum of curves 2 and 3 causes more pronounced extrema in the region 239.6–241.8 nm (Fig. 4.57). For bands 218.9, 225.6, 261.1, 270.7, 282.5, 292.1, 306.4, 318.7, 327.5, and 346.7 nm, we can confidently speak about their coincidence for *para*-, *meta*-, and *ortho*-substituted compounds. There is a noticeable inflection in the region 324.3 nm for *meta*- and *para*- positions of the band peak for the *ortho*-position of Cl atoms. Simultaneously, the manifestation of band 219.4 nm is decreased for the

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ortho-position. Consideration of difference spectra curves in Fig. 4.57 shows that the position of the chlorine atom in chlorine-substituted BAG derivatives does not generally change the picture of the spectrum. The band 240.4 nm for *p*-Cl-BAG is the exception.

From Fig. 4.58, in which original D^{II} spectra for *m*-Cl-BAG and *o*-Cl-BAG are presented, it follows that the positions of extrema in the far UV (in the region of 219.3 nm) correspond, generally, to bands in Fig. 4.57 in the region 218.9–225.6 nm and that those in the region of 248.3 nm coincide with bands 246.7–248.7 nm of the ΔD^{II} spectrum. The hypochromic effect in the extremum 248.3 nm indicates a decrease in the molar extinction coefficient of *m*-Cl-BAG. In the wide region 279.9–320.2 nm, the differences are insignificant. Furthermore, in the region 330.1 nm, substantial differences between curves 1 and 3 appear, probably because of the Cl atom position (positive extremum at 330.1 nm of curve 1 for *o*-Cl-BAG corresponds to negative extremum at 327.7 nm of curve 3 for *m*-Cl-BAG). For *m*-Cl-BAG in the near UV, a *bathochromic* shift of maxima relative to *o*-Cl-BAG in the region 348.1–367.3 nm is characteristic. Although the picture of interaction of *p*-Cl-BAG with Ca^{2+} was described by us in sufficient detail (Rozengart and Saakov 2002; Rozengart et al. 2003), data on the interaction of *o*-Cl-BAG and *m*-Cl-BAG with Ca^{2+} and on the possibilities of metal complex formation for these compounds are absent in the literature. The material in Fig. 4.58 (curves 2 and 4) fills this gap.

The first conclusion from comparison of curves 1–2 and 3–4 in Fig. 4.58 is the interaction of *o*-Cl-BAG and *m*-Cl-BAG with Ca^{2+} with the formation of complexes. The second conclusion is the specificity of interaction of *ortho*- and *meta*-chlorine-substituted isomers with calcium ions.

This specificity for the *o*-Cl-BAG + Ca^{2+} complex is shown by the hypochromic effect of the band 248.3 nm in absorption spectra and D^{II} spectra; the appearance of the hypsochromic shift of the band 279.85 nm; and also the fine spectral structure in the region 279.9–309.8 nm. It is possible that hypsochromic shift of band 285.5 nm occurred. However, on the basis of indirect data, we can suggest that band 279.9 nm shows spectral properties of the newly formed complex with Ca^{2+}, which is accompanied by disappearance of band 285.5 nm. The negative extremum at 319.7 nm moves bathochromically. The sharp hypochromic effect of the band 330.1 nm and the appearance of a band in the region 348 nm were noticed.

For the *m*-Cl-BAG complex with Ca^{2+}, a hyperchromic effect of the band 248.3 nm in comparison with the corresponding absorption band of *m*-Cl-BAG both in absorption spectra and in D^{II} spectra, and also the increased structuring of bands in the region 279.9–319.7 nm, is characteristic. In contrast to *o*-Cl-BAG, for the spectrum of the *m*-Cl-BAG complex, the hypsochromic shift of maxima 348.1 and 367.3 nm, respectively, to 330.1 and 361.9 nm is specific. Also, there is a hyperchromic effect in the region 248.3 nm, described by us for the reaction of Ca^{2+} with *p*-Cl-BAG. The presence of the latter suggests the formation of a less-ordered structure of complex in comparison with the initial structure. The disappearance of positive maximum at 348.1 nm is accompanied by formation of a negative extremum at 349.8 nm. Consequently, during interaction with Ca^{2+} in *ortho*- and *meta*-chlorine-substituted analogues of Cl-BAG, a significant change in properties of

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spectral curves occurs, and this change coincides to a high degree with the same change for *p*-Cl-BAG.

The specificity of interaction with Ca^{2+} found for *o*-Cl-BAG and *m*-Cl-BAG compounds is also characteristic for *p*-Cl-BAG, for which the differentiation of positive and negative extrema in the spectrum structure is better. There are principal absorption maximum at 344.1 nm and six specific maxima at 226.4, 238.2, 273.3, 291.3, 306.1, and 321.6 nm (Rozengart and Saakov 2002).

Thus, the comparative analysis of spectra of interaction of *p*-Cl-BAG, *o*-Cl-BAG, and *m*-Cl-BAG with Ca^{2+} highlights the individual spectral characteristics of formed complexes reliably detected with the help of the D^{II} method. Even more evident proof of the validity of this conclusion follows from the analysis of curves in Fig. 4.51. In view of the properties of D^{IV} spectra in the region of far UV, 214–253.6 nm, the individual positions of extrema of *ortho*- and *meta*-chlorine-substituted isomers and their Ca^{2+} complexes are reliably detected. In the region 258.3–316.9 nm, from the various totality of bands, the difference between the spectra of *ortho*- and *meta*-chlorine-substituted isomers and the substantial individuality of the bands of their Ca^{2+} complexes are easily seen. Especially pronounced are the differences in the individual positions of D^{IV} SBs in the near UV region, which correspond to the data of D^{II} spectra. These special features are characteristic of the *para*-, *ortho*-, and *meta*-positions of chlorine atoms in phenyl radicals of benzylideneguanidine and are caused by the formation of complexes of these compounds with calcium ions.

The conclusion is confirmed by the differential spectra (Fig. 4.60) "*o*-Cl-BAG minus *o*-Cl-BAG + Ca^{2+}," "*m*-Cl-BAG minus *m*-Cl-BAG + Ca^{2+}," and "*p*-Cl-BAG minus *p*-Cl-BAG + Ca^{2+}." It is known that when the difference spectral curve approaches the zero line, then there is no difference between the original substance and its supposed complex with metal (Rozengart et al. 2003; Rozengart and Saakov 2002), that is, there is no interaction of original substance with the metal ion. Also, existence of the complex *p*-Cl-BAG + Ca^{2+} was reliably proven in earlier studies (Zhorov et al. 1985; Saakov et al. 1987a, b; Rozengart et al. 2003). In Fig. 4.60, curves 1 (*o*-Cl-BAG minus *o*-Cl-BAG + Ca^{2+}) and 2 (*m*-Cl-BAG minus *m*-Cl-BAG + Ca^{2+}) demonstrate the difference in states of *o*-Cl-BAG and *o*-Cl-BAG + Ca^{2+} and in states of *m*-Cl-BAG and *m*-Cl-BAG + Ca^{2+}. Interpretation of spectra allows us to conclude that for both initial isomers, the Ca^{2+} interaction character is specific. There can be seen zones similar for all three curves (218.3–222.6, 232.0–244.4, 249.7–256.6, 328.8–329.1, 337.8–344.1 nm). At the same time, each curve has individual spectral features inherent only for it, which stresses the specificity of interaction of *para*-, *ortho*- and *meta*-chlorine-substituted BAG with Ca^{2+}. Using separate bands, characteristic, for example, of the differential spectrum of the *ortho*-complex (218.3, 227.0, 241.2, 249.7, 319.2, and 328.8 nm), it is possible to define pharmacological purity. The latter also concerns bands of the *meta*-chlorine-substituted complex (221.32, 230.2, 238.7 nm) and the shoulders at 244.4, 259.2, 295.1, 321.75, 337.8, and 365.0 nm. Special consideration should be given to the specific character of bands of the difference spectrum "*p*-Cl-BAG against *p*-Cl-

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BAG + Ca^{2+} at 222.2, 226.2, 237.8, 256.6, 274.4, 290.7, 304.08, 321.8, 329.1, and 344.1 nm for the known anticoccidial preparation (curve 3).

As described earlier (Zhorov et al. 1985; Rozengart and Saakov 2002; Rozengart et al. 2003; Saakov et al. 2003), the three-dimensional *p*-Cl-BAG structure has a folded conformation as a result of the non-valent attraction of benzene rings. The question arises: To what degree does the biplanar conformation corresponds to *ortho*- and *meta*-chlorine-substituted analogues? We could expect that in *o*-Cl-BAG, because of the possibility of rotation, the chlorine atoms are not spatially placed above each other and thus they do not prevent the attraction of benzene rings. In *m*-Cl-BAG, such rotational possibilities of chlorine atoms are somewhat limited, which results in insignificant difficulties in the approach of benzene rings. At the same time, in *p*-Cl-BAG, where chlorine atoms are located above each other (Zhorov et al. 1985; Rozengart and Saakov 2002; Saakov et al. 2003), such interference is more probable.

On the other hand, the presence of chlorine atoms in the Cl-BAG molecule probably results in formation of dipoles. In this case, in *p*-Cl-BAG, the close location of similarly charged chlorine atoms supposes dipole repulsion of similar poles. Because of the possibility of rotation of chlorine atoms, this effect is practically absent in *o*-Cl-BAG and is manifested to a small degree in *m*-Cl-BAG. For these two isomers, we could even suppose opposite dipole–dipole interaction. Unsubstituted BAG lacks both steric and dipole factors; therefore, it must be worse than *o*-Cl-BAG and *m*-Cl-BAG and have an advantage over *p*-Cl-BAG, in the possibility of biplanar structure formation. As our studies showed, for the binding of Ca^{2+}, both chlorine atoms are probably necessary, spatially located in a specific position relative to each other. The refinement of this position is the subject of our further experiments.

Thus, we have described, for the first time, the D^{II} spectra of *m*-Cl-BAG and *o*-Cl-BAG and demonstrated the interaction of these compounds with Ca^{2+} on the basis of the analysis of derived spectra. The characteristics of special spectra features of the metal complexes have been presented. Also for the first time, we have shown the spectra of the fourth derivative of absorption spectra of the considered compounds and of their complexes with calcium and demonstrated the analytical expedience of their application. The original data on the differential spectra of *para*-, *meta*-, and *ortho*-chlorine-substituted compounds in comparison with unsubstituted [(benzylidene)amino]guanidine are convincing. This reliable tool for the analysis of *para*-, *meta*-, and *ortho*-chlorine-substituted [(benzylidene)amino]guanidine derivatives is proposed for use in their chemical production.

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4.2.4 Specific Character of Ca^{2+} Interaction with [(Benzylidene)Amino]Guanidine Derivatives Containing Electron-Donor or Electron-Acceptor Substituents

The similarity of the Cl-BAG structure to a number of adrenergic compounds is noteworthy. In molecules of these compounds, there are two benzene rings connected by a flexible alkylamine chain with electron-donor substituents in *para*- and *meta*-positions. Analysis of the structural–functional and conformational–functional relations of a number of adrenomimetics and adrenoblockers suggested their participation in metal ion transfer into the hydrophobic cave of the receptor (Govyrin and Zhorov 1994). Calcium was considered a probable candidate for such a cation even though experimental and theoretical data indicated that the Ca^{2+} cation does not manifest a high tendency to form complexes. The structural similarity of Cl-BAG to adrenergic compounds stimulated the study of its conformational possibilities and its potential calcium-binding properties (Zhorov et al. 1985). Comparative spectrophotometric experiments on mono-, bis-, and tris-substituted aminoguanidine derivatives, and also on Cl-BAG analogues distinguished by the position of the chlorine atom in the molecule, were performed (Govyrin and Zhorov 1994; Zhorov et al. 1985; Rozengart and Saakov 2002; Rozengart et al. 2003; Saakov et al. 2003). As a consequence of these works, the interaction of Cl-BAG with Ca^{2+} and La^{3+} was found; moreover, the selectivity and specificity of the influence of different metal ions on the formation of chelate complexes with Cl-BAG analogues were shown. The specific character of the fine spectra structure of formed complexes was revealed with a high degree of reliability, and the absence of interaction of the unsubstituted analog BAG with Ca^{2+} was ascertained (Zhorov et al. 1985; Rozengart et al. 2003; Saakov et al. 2003). Analysis of the DSHO of mono-, bis-, and tris-substituted aminoguanidine derivatives interacting with Ca^{2+} revealed the qualitatively varying products of complex formation, which were different in the specific character of the electron spectra structure (Saakov et al. 2003). It was shown that the different location of the chlorine atom in the benzene rings of the Cl-BAG molecule does not generally change the picture of the spectrum; the band 240.36 nm for *p*-Cl-BAG is the exception. Comparative analysis of derived spectra of Ca^{2+} interaction with *p*-Cl-BAG, *o*-Cl-BAG, and *m*-Cl-BAG clarified the individual spectral characteristics of formed complexes (Fraifelder 1980).

In connection with the above, there is special interest in the study of special features of Ca^{2+} interaction with Cl-BAG analogues that have substituents other than Cl in the benzene rings of the molecule.

As objects of investigation, we used bis-[4-(dimethylamino)-(benzylideneamino)]guanidine (Me_2N-BAG) with the substituent having (similarly to Cl) properties of π-donor and of σ-acceptor and bis-[(4-nitrobenzylidene)amino] guanidine (NO_2-BAG) containing a strong π-acceptor substituent in the benzene ring.

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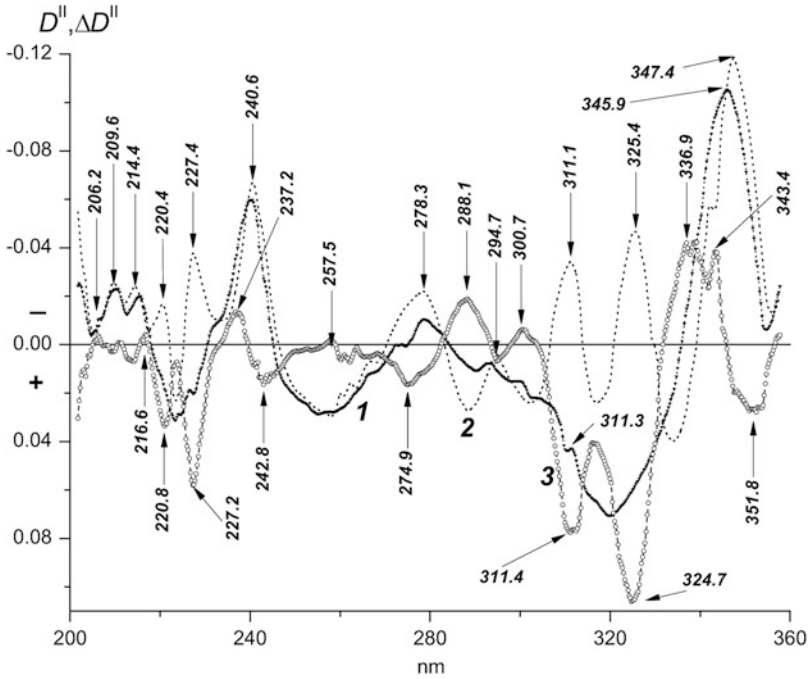

Fig. 4.61 Second derivatives of the absorption spectra of 0.25 M BAG solution (*1*) and its chelate complex with Ca^{2+} (*2*). The second derivative of the difference spectrum "the chelate complex against Cl-BAG" (*3*) was obtained by calculation. *Arrows* show positions of the most interesting and characteristic extrema for the each curve

The possibility of FFT filtering of numerical data and subsequent smoothing of derived curves allowed us to reliably separate main signals and to exclude unwanted noise. The absorption spectra of investigated compounds are characterized by a smooth line and by smoothed outlines of main absorption maxima. In connection with this, the use of derivative spectrophotometry of high orders completely proves its value.

The obtained data are presented in Figs. 4.61, 4.62, 4.63, and 4.64. From Fig. 4.61, it follows that application of the D^{II} registration for the interaction of Cl-BAG with Ca^{2+} detects the appearance of six new peaks at 219.9, 226.8, 278.0, 294.3, 310.8, and 325.1 nm and a shoulder at 341.6 nm. Probably, the complication of the spectral outline indicates the formation of a complex with Ca^{2+}. The comparison of spectral outlines of BAG + Ca^{2+} and Cl-BAG + Ca^{2+} and also of differential spectra "BAG minus BAG + Ca^{2+}" and "Cl-BAG minus Cl-BAG + Ca^{2+}" creates a reliable basis for concluding that Cl-BAG interacts with calcium ions with formation of a chelate complex characterized by specific features of the D^{II} spectrum (Rozengart et al. 2003).

The electronic structure of the molecule is radically different for Cl-BAG and NO_2-BAG. In particular, because of the significant contribution of the structure

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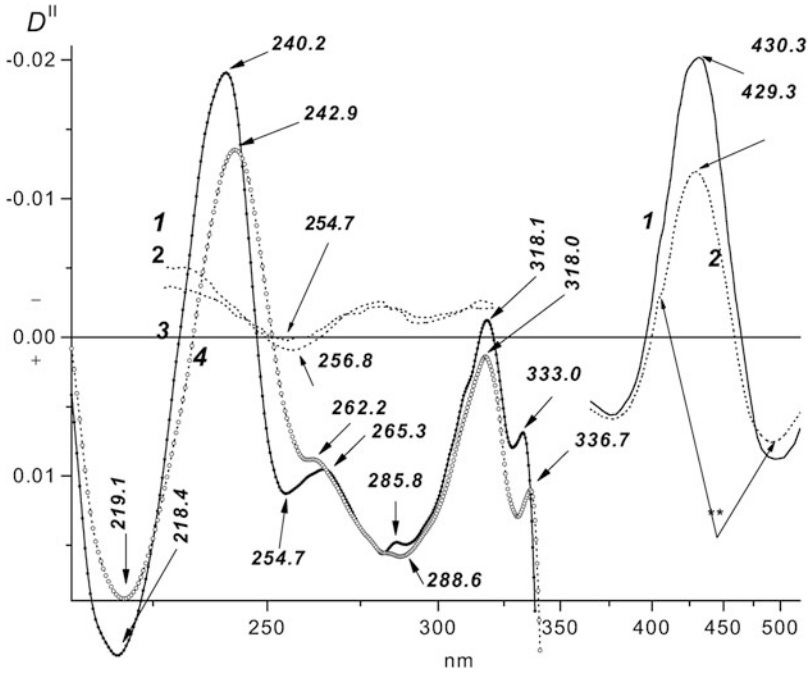

Fig. 4.62 Second derivatives of the absorption spectra of 0.25 M NO_2-BAG solution (*1*) and its interaction with Ca^{2+} (*2*). D^{II} of Me_2N-BAG solution (*3*) and its interaction with Ca^{2+} (*4*)

with charge separation, aromatic substituents of NO_2-BAG are strongly polarized, which causes destabilization of folded conformations of the molecule. Furthermore, because of strong polarization of aromatic substituents, interaction of NO_2-BAG with metal ions does not result in a great change in the electron structure (additional polarization) of the chromophore fragment. This means that a reader should not expect cardinal changes in the absorption spectra of NO_2-BAG in the presence of metal ions. This is confirmed by the similarity of the smoothed absorption spectra of NO_2-BAG and NO_2-BAG + Ca^{2+}. At the same time, even from registration spectra of the second derivative of the absorption spectrum (Fig. 4.62, curves 1 and 2), insignificant differences between curves are revealed (at 254.7 and 256.8 nm and also indicated with asterisks). Decrease in the absorption value for curve 2 is caused by dilution after addition of Ca^{2+} solution.

Another picture is observed for the interaction of Me_2N-BAG containing a dimethylamine substituent, which, as in the case of Cl, has properties of both π-donor and σ-acceptor.

From comparison of the data in Fig. 4.62 (curves 3 and 4), the difference in D^{II} spectra between Me_2N-BAG and Me_2N-BAG + Ca^{2+} is seen as a shift of main extrema of the spectrum ($\lambda = 242.9$, 254.7, 262.2, 285.8, 333.7, and 336.7 nm). There is basis to suppose that the spectral changes are produced by Ca^{2+} interaction with the Me_2N-BAG molecule. It is obvious that there is some similarity in the

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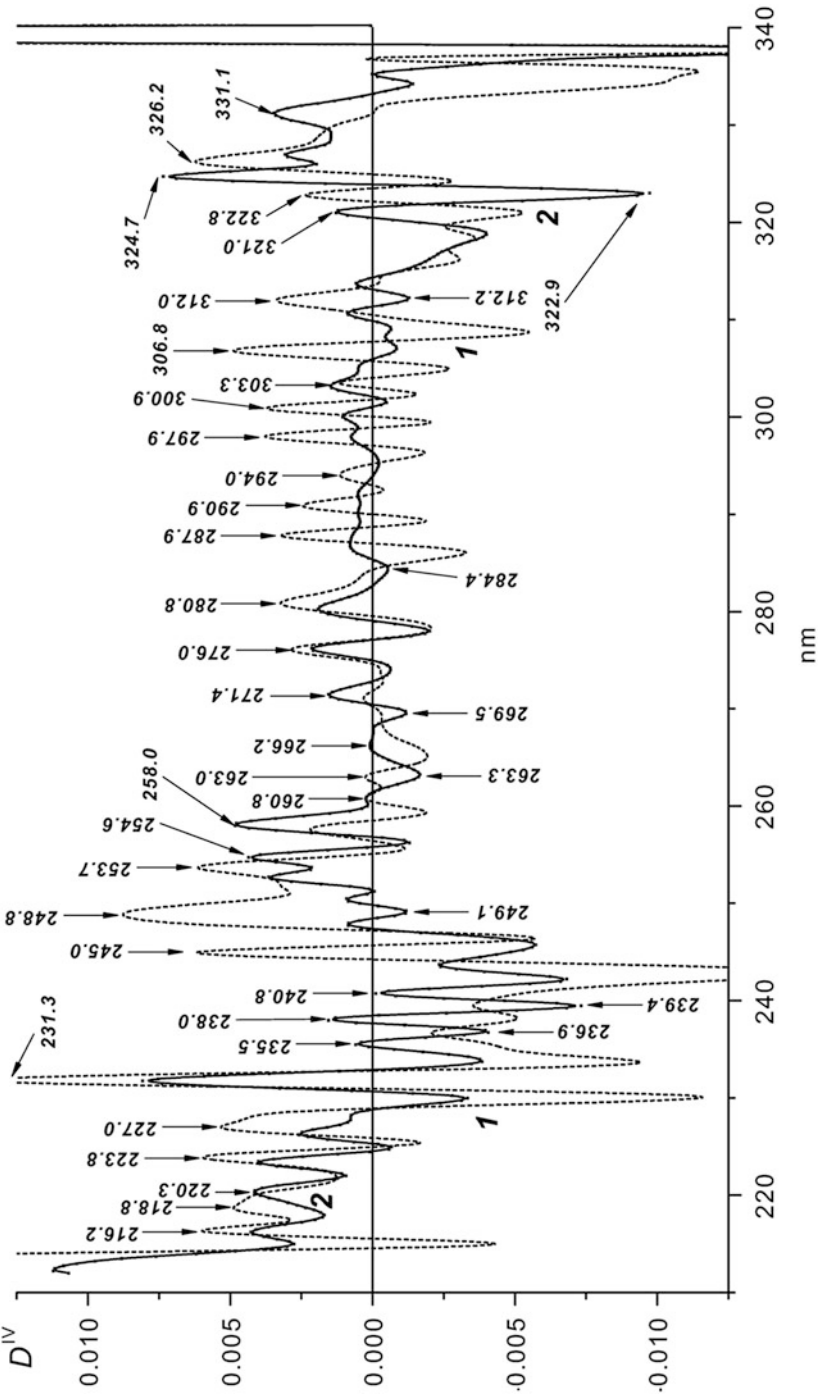

Fig. 4.63 Fourth derivatives of absorption spectra of Me_2N-BAG solution (*1*) and of its possible complex with Ca^{2+} (*2*) in the UV spectral region

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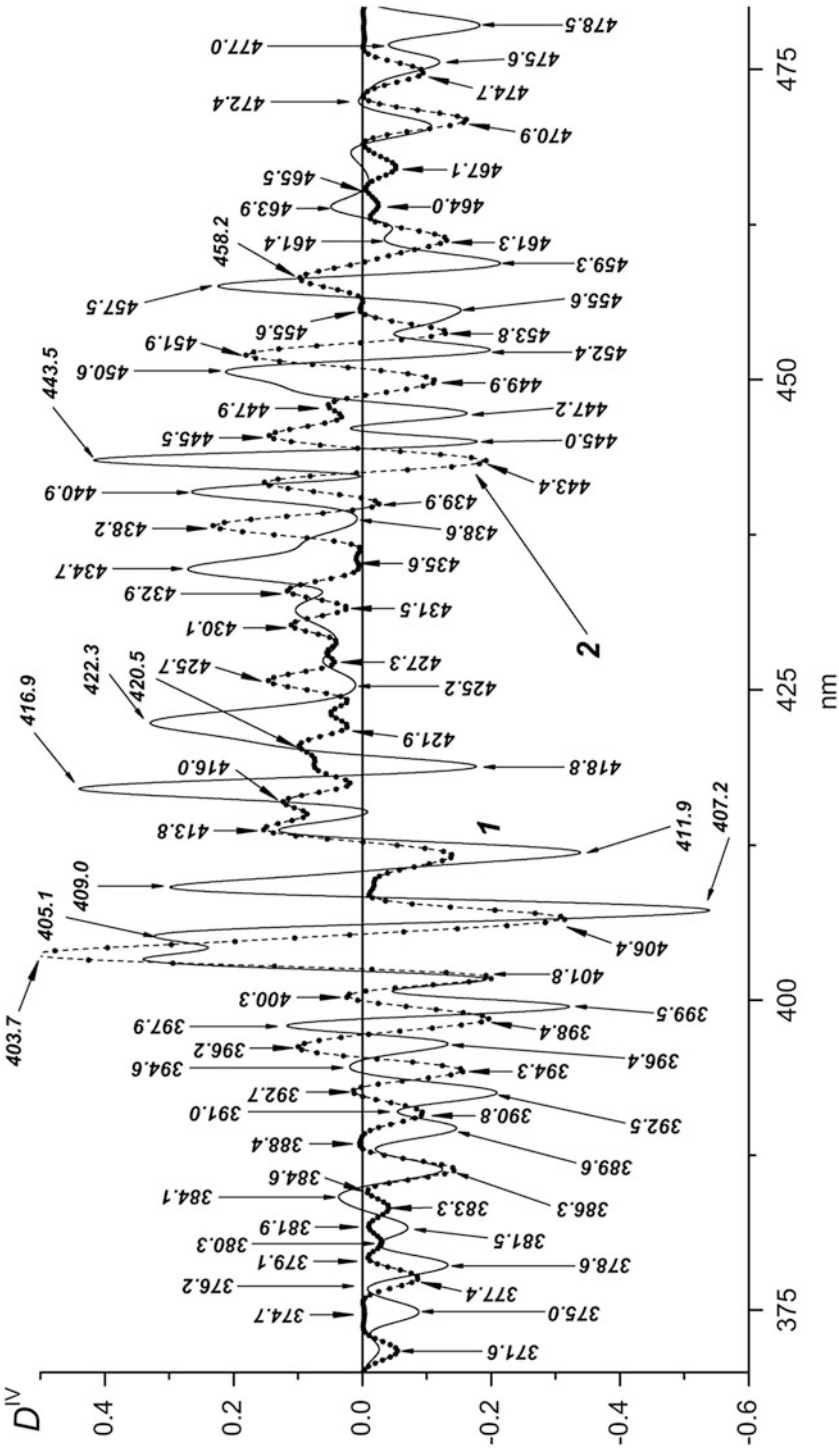

Fig. 4.64 Fourth derivatives of absorption spectra of Me_2N-BAG solution (*1*) and of its possible complex with Ca^{2+} (*2*) in the visible spectral region

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nature of Ca^{2+} interaction for Cl-BAG and Me_2N-BAG. This is confirmed by comparative results of spectra registration of the fourth derivative of Me_2N-BAG and Me_2N-BAG + Ca^{2+} (Fig. 4.63). The most significant differences in the fine structure of D^{IV} spectrum between considered curves are found at $\lambda = 218.8$, 235.5, 238.0, 240.8, 245.0, 248.8 and 249.1, 253.7, 263.0, 266.2, 287.0, 290.9, 297.9, 300.9, 306.8, 312.2, 322.8, 324.7, and 331.1 nm. Changes in extrema orientation of curve 2 with respect to curve 1 highlight a distinction between Me_2N-BAG and Me_2N-BAG + Ca^{2+} and indicate the reality of interaction of Ca^{2+} with Me_2N-BAG with the formation of complex.

A significant difference between control and experimental variants is observed in the visible range of the spectrum (Fig. 4.64). This concerns bands 374.7–375.0, 377.4, 379.1, 380.3, 381.5, 384.1, 391.0, 392.7, 394.6, 396.2, 398.4, 403.7–405.1, 409.0, 416.9, 421.9, 427.7, 431.5, 434.7–435.6, 438.2–438.6, 443.4, 449.9–450.6, 451.9, 453.8, 463.9, 467.1, and 477.0 nm, which clearly reveal differences between the spectra of Me_2N-BAG and its possible calcium complex.

The data in Fig. 4.63 indicate significant changes in the fine spectral structure of Me_2N-BAG under the influence of calcium ions. These changes are, mainly, in bands 252.3, 262.7–263.0, 268.1, 288.5, 292.2, 298.1, 303.1, 308.1, 311.3, 313.8, 326.3, 336.3, and 339.7 nm, and they allow conclusions to be made about the active interaction of Ca^{2+} with Me_2N-BAG.

Thus, on the basis of comparative spectrophotometric studies, the specific character of the interaction of Ca^{2+} with Cl-BAG and Me_2N-BAG is shown. Pronounced interaction was not observed for NO_2-BAG and BAG. Seemingly, for the manifestation of Ca^{2+} complexing capability, the presence of substituents such as chlorine or a dimethylamine group with properties of π-donor and σ-acceptor is necessary in the BAG molecule.

4.2.5 Special Features of Calcium Ion Interaction with Substituents of Bis-[(4-Hydroxy-3-Methoxybenzylidene)amino]Guanidine and Bis-[(4-Cyanobenzylidene)amino]Guanidine

As described above (see Chap. 3.3), earlier application of the registration of derived spectra of second and higher orders promoted the appearance of an array of original data that would have been impossible to obtain using the trivial methods of absorption spectrophotometry (Saakov et al. 1978a; Levillain et al. 1985; Eskondari et al. 2003; Erk 2004). We were convinced of this after examining about 3,000 references in the database of the US National Center for Biotechnology Information (NCBI) on the application of derivative spectrophotometry in biochemistry, pharmaceutical chemistry, physiology, and medicine. New steps in this direction were made by us when researching the specific character of interaction of anticoccidial (benzylideneamino)guanidine derivatives, in particular Cl-BAG, with a number of

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metals (Zhorov et al. 1985; Rozengart and Saakov 2002). Analysis showed that in the presence of Ca^{2+}, the unsubstituted bis-(benzylideneamino)guanidine (BAG), in which there are no chlorine atoms, is not capable of complexing with Ca^{2+} and does not manifest anticoccidial activity (Khovanskikh et al. 1984). A hypothesis about the specific role of Cl atoms for the manifestation of anticoccidial activity, combined with the ability of Cl-BAG to form complexes with metal ions (Zhorov et al. 1985; Rozengart and Saakov 2002), was suggested, confirmed, and developed in our further studies (Rozengart et al. 2003; Saakov et al. 2003).

Mono- (Cl-MG) and tris-substituted (Cl-TG) derivatives of Cl-BAG differ in their conformational states and manifest specific spectral character when interacting with Ca^{2+}. These special features are seen in the position of bands of the far and near UV regions. This was proposed as the analytical basis for their diagnostics. From the examination of difference spectra of the second and fourth derivatives (ΔD^{II} and ΔD^{IV}), it was found that interaction of Cl-MG, Cl-BAG, and Cl-TG with Ca^{2+} results in qualitatively various products that are characterized by the specific character of the electron spectra structure, highlighting the qualitatively special features of the formed complexes. It was suggested that the differences in the ability of Cl-MG, Cl-BAG, and CL-TG to bind Ca^{2+} is conditioned by the spatial structure of these guanidine derivatives. We suggested that in CL-TG the configuration of the central guanidine group significantly changes (Saakov et al. 2003). The possibility of formation of three penta-cycles due to intramolecular hydrogen bonds makes this structure more planar, which, apparently, complicates chelation with Ca^{2+}. Cl-TG practically lacks anticoccidial activity (Khovanskikh et al. 1984), which serves as additional evidence in favor of our hypothesis (Zhorov et al. 1985) about the connection between the anticoccidial activity of Cl-BAG and the ability to chelate Ca^{2+}. Detailed study of the spectral change character as a function of the Cl atom position in *para*-, *ortho*-, or *meta*-locations showed that the position does not principally change the picture of derived spectra, with exception of the band 240.36 nm for *p*-Cl-BAG. However, interaction with Ca^{2+} reveals the individual spectral characteristics of formed complexes (Rozengart and Saakov 2003).

Further investigation was made of the special features of Ca^{2+} interaction with Cl-BAG analogues containing substituents in the benzene rings that were different from Cl and different in their electron-donor and electron-acceptor properties. These studies revealed the specific character of Ca^{2+} interaction with Cl-BAG and bis-[4-(dimethylamino)-(benzylideneamino)]guanidine (Me_2N-BAG), both having properties of π-donor and σ-acceptor. For bis-[(4-nitrobenzylidene)amino] guanidine (NO_2-BAG), possessing a strong π-acceptor substituent in the benzene ring, there was no clearly manifested interaction with Ca^{2+} observed in the second derivative of the absorption spectra.

Works on the application of derivative spectrophotometry of high orders for assessment of the reaction of biologically active organic compounds with complex substituent groups with metal ions were absent from the literature. We decided to fill this gap with the help of the analysis and registration of comparative derivative spectra. We looked for comparative special features of Ca^{2+} interaction with two

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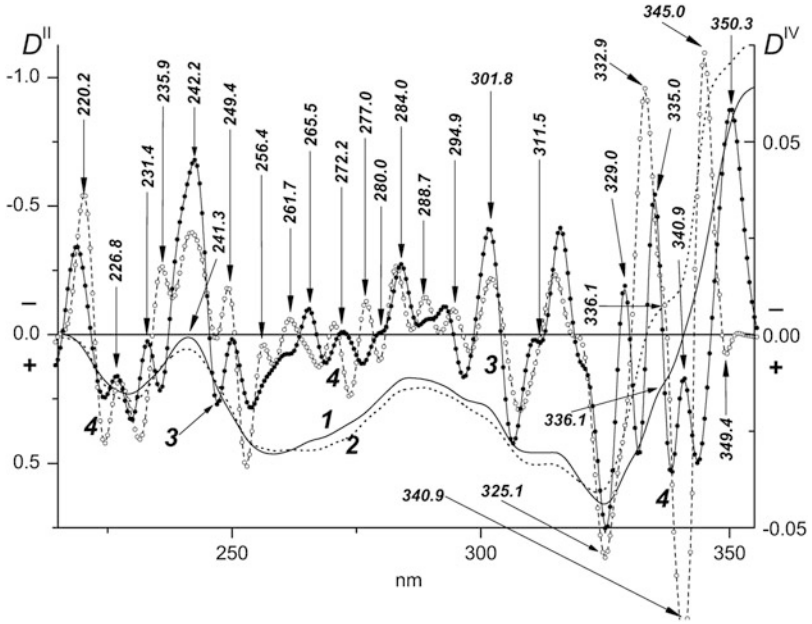

Fig. 4.65 Special features of changes in derivative spectra of solutions of bis-[(4-hydroxy-3-methoxybenzylidene)amino]guanidine (*p*-OH, *m*-OCH_3-BAG) during interaction with Ca^{2+}: (*1*) D^{II} of the absorption spectrum (*p*-OH, *m*-OCH_3-BAG), control; (*2*) D^{II} for the product of interaction *p*-OH, *m*-OCH_3-BAG + Ca^{2+}; (*3*) D^{IV} of the absorption spectrum of the solution *p*-OH, *m*-OCH_3-BAG (control); (*4*) shows the change in D^{IV} during interaction of *p*-OH, *m*-OCH_3-BAG with Ca^{2+} (experiment)

other derivatives of BAG, bis-[(4-hydroxy-3-methoxybenzylidene)amino]guanidine (*p*-OH, *m*-OCH_3-BAG) and bis-[(4-cyanobenzylidene)amino]guanidine (*p*-CN-BAG).

Experiments were performed using the tested and established procedure of registration of derived spectra of second and fourth orders (Chap. 3). Analog signals were transferred into numerical form. Digitalized data were used in the graphic mathematical packet *Microcal Origin* 6.1, the main purpose of which is graph plotting (i.e., visualization of numerical data). The curve plotted on the basis of numerical data was interpolated with an interval of 0.25 nm, smoothed out by FFT filtration, and then, if necessary, further differentiation was carried out using the program *Spectra Calc*. This program offers positive capabilities for analysis and imaging of spectral curves. After differentiation, curves were smoothed out on seven points (FFT smoothing), and numerical data were again used in *Origin* 6.1 for the visualization of total results. The program automatically finds the positions of positive and negative extrema of a spectral curve.

Cl-BAG was used as the standard for comparison of interaction of aminoguanidine derivatives with Ca^{2+} (see Fig. 4.61). Interaction of the latter with Ca^{2+} was checked many times, and the specific character of spectral

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characteristics of formed complexes did not give rise to any doubts (compare curves 1 and 2 in Figs. 4.61 and 4.53, respectively) (Rozengart and Saakov 2002). The accuracy of the new SBs specific for the Cl-BAG + Ca^{2+} complex (Fig. 4.61, curve 2) with $\lambda_{max} = 200.5$, 227.4, 278.3, 294.7, 311.1, and 325.4 nm was reliably confirmed by extrema of the difference spectrum (ΔD^{II}) (curve 3) with $\lambda_{max} = 220.8$, 227.2, 242.8, 257.5, 288.1, 300.7, 311.4, 324.7, 336.9, 343.4, and 351.8 nm.

Results of Ca^{2+} interaction with a preparation of *p*-OH, *m*-OCH_3-BAG are shown in Fig. 4.65. In absorption spectra, the difference between the spectral curves of solutions *p*-OH, *m*-OCH_3-BAG and *p*-OH, *p*-OCH_3-BAG + Ca^{2+} was conditioned only by the degree of dilution after addition of solution with calcium ions. However, the D^{II} spectra revealed discrepancies between spectral curves 1 and 2. For example, we can concede the presence of some specific spectral differences in bands with $\lambda_{max} = 241.3$ nm; in the region 261.7–320.5 nm; especially at 300.5 nm and 312.5 nm; and then at 336.1 nm and 345–350.3 nm. To check this, the registration of D^{IV} spectra (Fig. 4.65, curves 3, 4) was performed. Advantages of the latter method for analysis of the fine structure of specific spectra character do not need repeating and have been described in detail (Saakov et al. 1987a; Rozengart and Saakov 2002, 2003). Comparison of curves 3 and 4 in Fig. 4.65 shows the following features.

In the described spectral region, there are reliably recorded differences in the spectral curves for *p*-OH, *m*-OCH_3-BAG and *p*-OH, *m*-OCH_3-BAG + Ca^{2+}. These differences mainly consist of the formation of new maxima of absorption (absent on curve 3 for *p*-OH, *m*-OCH_3-BAG) at $\lambda_{max} = 256.4$, 261.7, 277.0, 288.7, 294.9, 332.9, and 345.0 nm and the negative extremum at 340.9 nm. It does not really matter whether the maxima at 332.9 nm and 345.0 nm are newly formed or whether they are a result of hypsochromic shift of bands 335.0 nm and 350.0 nm, or, for example, of bathochromic shift of bands 329.0 and 340.9 nm. The important thing is a fundamental change in the fine structure of the D^{IV} spectrum of curve 4 relative to curve 3. In this case, significant changes in the D^{IV} spectrum are shifts of positive SBs with $\lambda_{max} = 220.2$, 235.9, 249.4, 270.5, 282.9, 314.8, and 319.3 nm and shifts of negative extrema 231.3, 252.8, 273.66, 279.9, 286.3, 291.7, 298.1, and 307.9 nm. Changes on the right side of the graph were described earlier.

These facts allow us to conclude that the interaction of *p*-OH, *m*-OCH_3-BAG with calcium ions produces reliably detected changes in the spectral picture. The internal mechanism of these changes requires additional experimental investigation and serves as the subject of further experiments.

Another picture of Ca^{2+} interaction is observed for another Cl-BAG derivative, *p*-CN-BAG (Fig. 4.66). On the basis of absorption spectra registration, it is impossible to find visible interaction of Ca^{2+} with *p*-CN-BAG. Spectral changes are manifested only as a typical absorption decrease in the region of principal maximum of the visible range at 399 nm. Registration of the second derivative of absorption spectrum also reveals an absorption decrease in maxima at 248.3, 296.3, and 398.7 nm. At the same time, close examination of spectral curves reveals differences at 221.5, 268.0, 307.0, and 320.3 nm in the UV region of spectrum and

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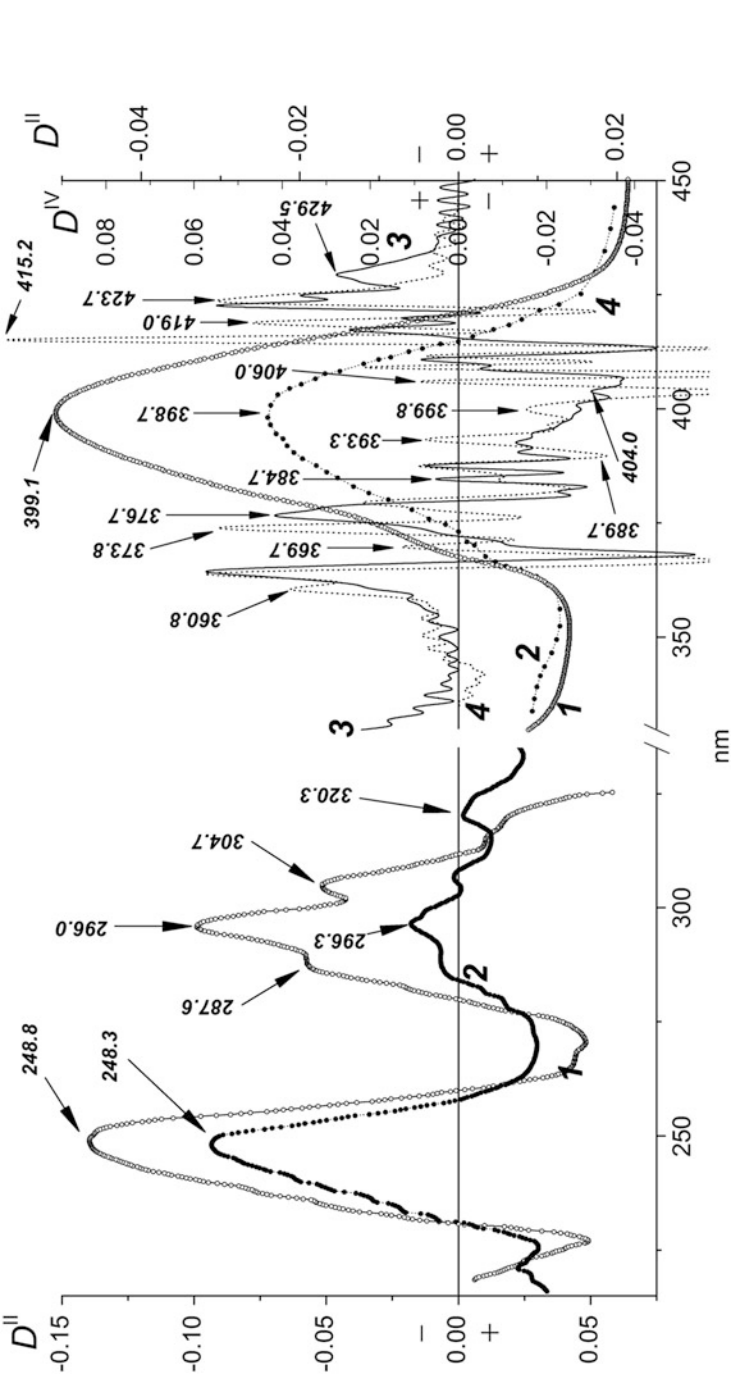

Fig. 4.66 Special features of changes in derived spectra of bis-[(4-cyanobenzylidene)amino]guanidine (*p*-CN-BAG) during interaction with Ca^{2+}: (*1*) D^{II} of the absorption spectrum of *p*-CN-BAG solution in UV and visible regions (control); (*2*) the same, but during interaction of *p*-CN-BAG with Ca^{2+}; (*3*) D^{IV} of the absorption spectrum of *p*-CN-BAG solution (the visible spectral region, control); (*4*) D^{IV} for solution of *p*-CN-BAG + Ca^{2+} in the visible spectral region (experiment)

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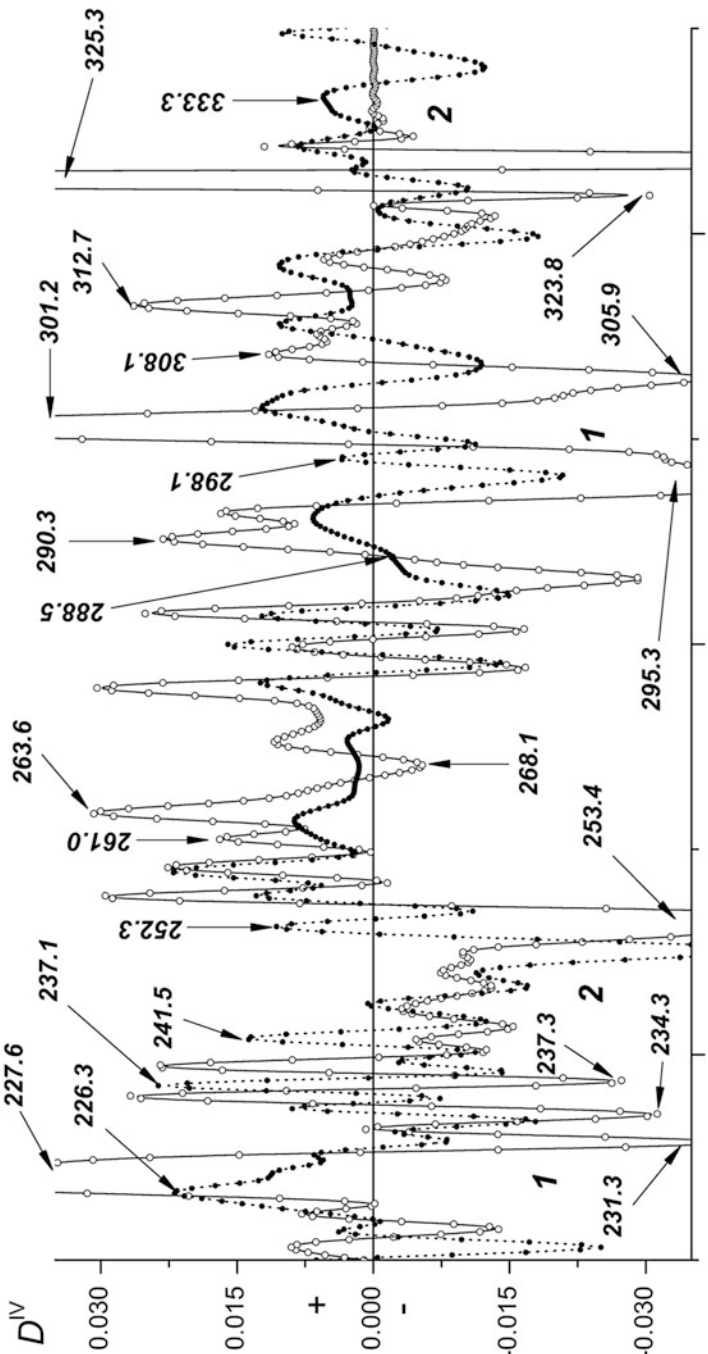

Fig. 4.67 Specific features of change in D^{IV} of the absorption spectrum of *p*-CN-BAG solution during its interaction with Ca^{2+}: (*1*) the control D^{IV} spectrum for *p*-CN-BAG in the UV spectral region; (*2*) the D^{IV} spectrum for the product of interaction of *p*-CN-BAG + Ca^{2+} in the UV spectral region

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at 340.7, 369.5, and 435.3 nm in the visible range. Equalization of concentrations in the principal absorption maximum at $\lambda_{max} = 399.1$ nm (curve 1) and registration of derived spectra of the fourth order (curves 3 and 4) persuasively demonstrate the difference in the spectral pictures of initial preparations of *p*-CN-BAG and *p*-CN-BAG + Ca^{2+}. The most pronounced differences between curve 4 and the initial curve 3 are discovered in SBs with $\lambda_{max} = 337.5$, 342.0, 345.3, 347.5, 350.5, 354.3, 360.8, 369.7, 373.8, 379.5, 384.68, 389.7, 393.33, 396.3, 399.8, 404.0, 406.0, 409.16, 411.7, 415.1, 419.0, 423.7, 429.5, 434.7, 438.3, and 441.7 nm. These distinctions are caused, first, by both hypsochromic and bathochromic shifts of bands relative to the initial control; second, by the appearance of new bands, not differentiated earlier; and third, by the presence of the hyperchromic or hypochromic effect in newly formed bands or in bands corresponding to their position in the control.

Examination of special features of the fourth-order derived spectra in the UV spectral region for *p*-CN-BAG + Ca^{2+} (Fig. 4.67) also reveals very significant differences in the spectral harmonics of *p*-CN-BAG and *p*-CN-BAG + Ca^{2+}. The most pronounced differences are described below. An interested reader could reveal the totality of fine differences by scanning the figure and increasing its size on a monitor, together with coloring of spectral curves. First of all, note extrema characterized by their position in the reversed phase, namely, at $\lambda_{min} = 221.2$, 222.9, 225.4, 235.75, 237.75, 252.3, 261.0, 268.1, 288.5, 298.1, 303.1, 311.2, 312.7, 324.3, 333.3, and 336.3 nm. Note also maxima characterized by a bathochromic or hypsochromic shift relative to curve 1 at $\lambda_{max} = 226.3$, 237.1, 244.8, 276.2, 280.0, 316.9, and 322.9 nm; manifestations of the hypochromic effect relative to curve 1 in bands $\lambda_{max} = 226.3$, 255.5, 262.8, 270.6, 276.2, 282.85, 292.3, 303.1, 319.75, and 336.2 nm; and the presence of the hyperchromic effect relative to curve 1 in bands $\lambda_{max} = 241.5$, 244.9, 252.3, 279.9, 311.18, 317.0, and 333.3 nm. Consideration of the totality of listed differences between curves 1 and 2 allows us to conclude the presence of specific disturbances in the spectral harmonics of the compound *p*-CN-BAG interacting with calcium ions. These disturbances were reliably detected during registration of D^{IV} spectra.

Differences in the spectra of Cl-BAG derivatives containing substituents with different acceptor–donor properties, after their interaction with Ca^{2+}, were noted by us earlier (Saakov et al. 2004). It was reliably established that Ca^{2+} interacts with Me_2N-BAG having a substituent with π-donor and σ-acceptor properties (similarly to Cl). For NO_2-BAG containing a strong π-acceptor substituent in the benzene ring, it was not possible to make a categorical conclusion about interaction with Ca^{2+}, although we indicated the existence of differences in D^{IV} spectra between NO_2-BAG and NO_2-BAG + Ca^{2+}. Despite the fact that we already had data on registration of derived spectra of the fourth order, we supposed that, because of the strong polarization of aromatic substituents during interaction of NO_2-BAG with metal ions, a substantial change in the electron structure (additional polarization) of the chromophore fragment does not occur. However, with improvement in sensitivity and informativeness of the analytical method, our work obtained results contrasting with previously prevailing concepts. We observed such differences from the initial

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preparation on the D^{IV} curve for NO_2-BAG + Ca^{2+} in spectral regions with $\lambda_{max} = 374.7$, 384.1, 392.7, 403.7, 409.0, 416.0, 420.5, 425.7, 431.5, 435.6, 438.2, 443.4, 445.5, 449.9, 451.9, 458.2, 461.3, and 467.1 nm. Changes in the spectral harmonics of the preparation NO_2-BAG + Ca^{2+} consisted both in appearance of new SBs, absent in the initial preparation of NO_2-BAG, and in appearance of batho- and hypsochromic shifts of already existing SBs. Discovered changes were accompanied by hyperchromia and hypsochromia of separate previously existing SBs. Interpretation of these data in the light of presented materials does not exclude questions about the deeper nature of NO_2-BAG interactions with Ca^{2+}.

We have not yet clarified the nature of Ca^{2+} interactions with Cl-BAG analogues possessing different substituents, but we consider it necessary to take notice of the presence of this phenomenon. Explanation and discussion of the mechanisms of Ca^{2+} interaction with NO_2-BAG are the subjects of separate theoretical and experimental investigations.

Thus, this section has presented derived spectra of D^{II} and D^{IV} for *p*-OH, *m*-OCH_3-BAG and *p*-CN-BAG and also of products of their interaction with calcium ions. Interpretation of spectra allowed us to form an opinion about the interaction of these substances with Ca^{2+}, with the formation of chelate complexes (ligands) having specific spectral features that could serve for the analytical identification of reaction products. The reliability of application of fourth-order derived spectra for the interaction analysis of *p*-CN-BAG and *p*-OH, *m*-OCH_3-BAG with metal ions was demonstrated.

We have persuasively shown the great possibilities of derivative spectrophotometry for comparative analysis of the interaction reactions of biologically active materials with metal ions and for characterizing the specific character of compounds containing different acceptor–donor groups.

4.3 Proof of the Polycomponent Composition of the Promising Antitumor Drug Ukrain

Due to increasing environmental pollution with carcinogenic substances and with an increased prevalence of oncologic disease, increasing attention is focused on the search for new, more effective anticancer therapies, which is an important field of study in physiology and biochemistry at the end of the twentieth and beginning of the twenty-first centuries. In recent years, there have been frequent publications in European literature on assessment of the biological activity of the alkaloid preparation Ukrain (UKR). Interest in this preparation is caused by the fact that, according to experimental data, it is characterized by pronounced medicinal properties and can be used in oncologic practice as an antitumor remedy. It was discovered that it is cytostatic for malignant cells in concentrations non-cytostatic for normal cells (Nowicky 1980; Uglyanitsa et al. 2000; Ukrain 2000). Addition of UKR to a culture of malignant cells decreases their oxygen consumption and

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decreases synthesis of DNA, RNA, and proteins (Taborska et al. 1995). This induces the programmed death, apoptosis, of malignant cells (Han et al. 1991). The mechanism of this action remains the subject of discussion.

The original raw source of alkaloids for production of UKR is the plant killwort (*Chelidonium majus* L.), a weed from the papaverous family Papaveraceae (Uglyanitsa et al. 2000; Taborska et al. 1995). The part of this plant richest in alkaloids is the root; total content of alkaloids in the root is 2–3 %, in the aerial part 0.5–1.5 %, and in leaves about 0.25–0.4 %. With the help of classical methods, more than 30 alkaloids were revealed in *Ch. majus* (Taborska et al. 1995). Alkaloids extracted from *Ch. majus* were separated using liquid column chromatography (with gradient elution) into 20 peaks, 13 of which were identified with the help of individual alkaloid markers (Han et al. 1991). The absolute and relative content of alkaloids substantially changes in the process of plant ontogenesis and depends on the conditions of its cultivation (soil, climate, etc.). Individual alkaloids are distinguished by the value and directivity of their biological effect. It is shown that the physiological and therapeutic effectiveness of substances prepared from *Ch. majus* can change, depending on the nature of the alkaloids predominant in the preparation (Taborska et al. 1995). In this connection, some authors consider it reasonable to indicate the territory where the plants used for production of an investigated series of UKR remedies were grown (Han et al. 1991).

In a number of articles by Russian (Nikolskaya et al. 2000; Kuznetsova et al. 1999, 2001) and European (Ciebiada et al. 1996) authors, it is reported that UKR is obtained by interaction of the chosen alkaloids with thiophosphoric acid. However, the technological documentation of the producer (Nowicky Pharma, Vienna, Austria) explains (see Nowicky 1999a, p. 343) that UKR is obtained by interaction of triaziridide of thiophosphoric acid with a mixture of preliminarily extracted alkaloids. The approximate composition of the mixture is chelidonine 49 %, protopine 13 %, stylopine 12 %, allocryptopine 9 %, and berberine 3.2 %. Other components are sanguinarine, chelerythrine, and coptisine, the content of each of which is less than 3 %.

Information in the literature about the chemical composition of UKR preparations is very contradictory. For example, from the paper by Liepins et al. (1996), it seems that UKR is an individual substance, the semisynthetic derivative of thiophosphoric acid and the alkaloid chelidonine. There are even reports on the experimental measurement of inhibition constants for UKR inhibition of cholinesterases and monoamine oxydases in ferments (Kuznetsova et al. 1999, 2001). However, the technological documentation from the pharmaceutical firm Nowicky Pharma (Nowicky 1999b; Uglyanitsa et al. 2000) and instructions on the physiological application of the preparation state that the UKR preparation contains, besides the main chelidonine component with the formula $(C_{66}H_{75}N_6O_{18}PS) \cdot 6HCl$, a mixture of triaziridide derivatives of different alkaloids and also (Uglyanitsa et al. 2000) remains of free triaziridide (about 14 mol%) and of native alkaloids (not entering in the reaction with triaziridide).

In addition, in preliminary experiments, we found another significant feature, namely, the nonstandard nature of UKR preparations. Preparations of different

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series of production differed substantially (by 1.5 times) in their ability to inhibit cholinesterase activity, which ensures the synaptic conductivity of nerve impulses. It is difficult to imagine that such high differences could be caused by differences in the UKR content in ampules of solutions of different series. The most probable reason is the initial technological differences in the composition of these UKR samples, caused by phenotypic special features of the initial plants (*Ch. majus*).

In this connection, we decided to investigate the identity, uniformity, and degree of composition complexity of different UKR samples. To solve this task, we carried out the comparative analysis of solutions of different UKR series with the help of derivative spectrophotometry of high orders (DSHO), using the accepted procedure (see Chap. 3).

For the analysis, UKR samples were taken as dosed solutions with substance concentration of 1,000 μg/mL for four series: № 500873 (I), № 544324 (II), № 544323 (III), and № 590115 (IV). Before spectrophotometric measurement, the original solutions were diluted 80 times to a concentration of 12.5 g/mL (for the mass of dry preparation). For dilution, freshly prepared 0.1 M borate buffer (composition 0.1 M $Na_2B_4O_7 + 0.2$ M HCl, pH 7.8) was used. This freshly prepared buffer (in contrast to phosphate, acetate, and most other buffer solutions) does not manifest noticeable absorption in the investigated spectral region (200–760 nm) when the thickness of the photometrically scanned layer is 20 mm.

The digitalization of spectral curves was performed with the program *Graph Digitizer* 2.14, according to N. Rodionov. Further treatment of data for production of D^{II} and D^{VIII} spectra was carried out with programs *Spectra Calc* and *Origin* 6.1. The functional abilities of the program *Spectra Calc* allow via simple operations the equalization of ODs of investigated UKR series in the necessary extrema, which was especially important in measurements of difference (differential) ΔD^{IV} spectra. In the experiment, we recorded five to seven subsequent spectra of the same sample. Quantitative assessment of derived spectra can be accomplished with different methods, for each of which it is possible to expect a graph showing direct dependence on substance concentration (Savitzky and Golay 1964; Saakov et al. 1987a, b). Methods include using the amplitude value of a peak relative to the zero line, peak-to-peak amplitude of signal swing, and difference in amplitude of two vicinal extrema of opposite sign. Modern software allows the calculation of DSHO and also the calculation of the integral (area) of a peak. On this basis, one can assess

Table 4.6 Values of $[I]_{50}$ in reactions of human acetylcholinesterase with different inhibitors

Inhibitors	Series №	$[I]_{50}$ (10^{-2} g/L) ($n = 6$)
Iodous tetrabutylammonium (TBA)	1	6.6 ± 0.3
	2	6.5 ± 0.3
Iodous tetrapropylammonium (TPA)	1	9.4 ± 0.3
	2	9.4 ± 0.2
Remedy Ukrain (UKR)	1	4.3 ± 0.2
	2	6.4 ± 0.3

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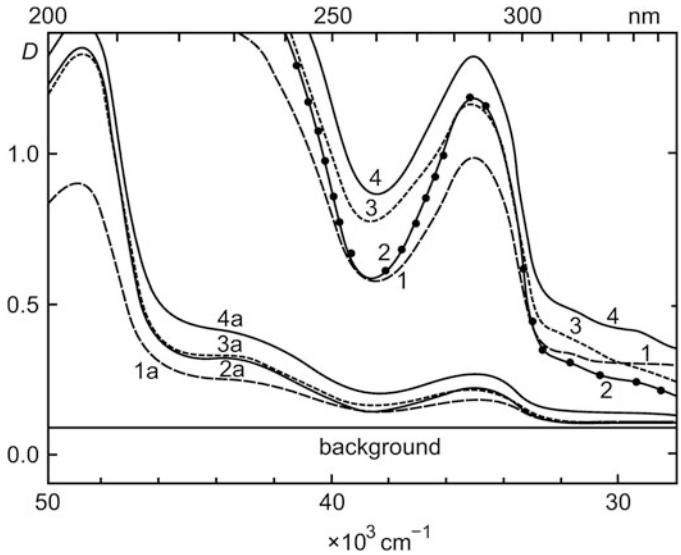

Fig. 4.68 Absorption spectra of buffer solutions of UKR remedy samples for a thickness of the photometrically scanned layer of 20 mm (*curves 1–4*) and 5 mm (*curves 1a–4a*) in 0.1 M borate buffer solution (pH 7.8). Comparison solution was water. Sample I *curves 1* and *1a*; sample II *curves 2* and *2a*; sample III *curves 3* and *3a*; sample IV *curves 4* and *4a*. Background absorption was of freshly made 0.1 M borate buffer solution (pH 7.8) for 20 mm thickness of the photometrically scanned layer. *Abscissa*: wavelength; *ordinate*: relative OD units

changes in the quantitative composition of components (*Peak Explorer* 1.0; *PeakFit* v.4) and perform, if necessary, statistical calculations (*SigmaPlot* 2000).

Catalytic activity during cholinesterase hydrolysis of substrate in the absence and presence of an inhibitor (the UKR preparation) was determined by Yu.G. Zhukovskii and coauthors with the help of the photometric method of Ellman et al. (1961) with modifications (Zhukovskii et al. 1996). Conditions were as follows: 25.0 ± 0.1 °C, pH 7.5 ± 0.05, and 2 mM acetylthiocholine substrate (Sigma, USA) in 0.02 M phosphate buffer solution containing 0.1 M KCl and 1 mM Ellman's reagent, 5,5′-dithio-bis-(2-nitrobenzoic acid) (Sigma, USA). The unit *E* of fermentation activity was taken as the hydrolysis of 1 μmol acetylcholine substrate in 1 min under the following analysis conditions: 2 mM acetylcholine in 0.02 M phosphate buffer, pH 7.5, containing 0.1 M KCl, and temperature 25 °C. The effectiveness of UKR inhibiting activity was evaluated according to the concentration required to cause a 50 % decrease in hydrolysis speed ($[I]_{50}$, g/L). Duration of the inhibition reaction to the moment of addition of substrate solution was 2 min. As cholinesterase, we used acetylcholinesterase (NF 3.1.3.7, hAChE) from erythrocytes of human blood (Sigma, USA); the specific activity of the preparation was 4 *E*-units (AChE per milligram).

In preliminary experiments, Zhukovskii and colleagues discovered that UKR preparations of different production series manifest different inhibiting ability. In

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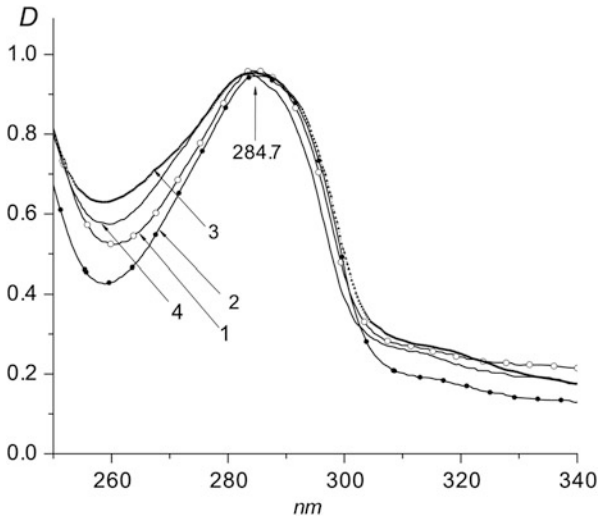

Fig. 4.69 Use of the *Spectra Calc* program for equalization of ODs of Ukrain preparations in the main absorption maximum and for comparison of adjacent spectral areas. *Curves 1–4* are for samples I–IV, respectively

particular, the value of $[I]_{50}$ was equal to 43 ± 2 mg/L for series I and 64 ± 3 mg/L for series II (Table 4.6).

The noticeable difference in the inhibiting ability of selected series of UKR preparations was not caused by divergence in the weight concentrations of preparations in the analyzed solutions. Individual chemical compounds having fixed and constant composition do not manifest discrepancies in values of $[I]_{50}$. The probable reason for the divergence of $[I]_{50}$ values for UKR could be the chemical heterogeneity of original samples of this remedy. To check the uniformity of UKR series, the following experiments were performed.

At the first stage, a comparison of absorption spectra of aqueous buffer solutions of UKR samples from four different series of production was performed. Weight concentrations of the substance in photometrically scanned solutions were identical, and the thickness of the photometrically scanned layer was equal to 20 mm (Fig. 4.68, curves 1–4) or 5 mm (Fig. 4.68, curves 1a–4a).

A common feature of solutions of four UKR samples was the presence of one minimum in region 258–260 nm and two clear absorption maxima in regions 204–206 nm and 282–286 nm. Besides these two maxima, from barely noticeable bends in the absorption curves, it was possible to infer the manifestation of nine more absorption bands (hidden maxima) at 203.0, 206.0, 234.0, 244.0, 263.0, 290.0, 315.0, 339.0, and 370.0 nm. Moreover, each of the four UKR samples had its own distinctive special features of the spectral curve. In particular, it is evident from Fig. 4.68 that the relative values of absorption noticeably changed with a change in light wavelength. For example, in spectral regions 200.0–257.0 nm and 259.0–303.0 nm, absorption of sample solution III (curve 3) was higher than the absorption of sample solution I (curve 1) and almost coincided with that of sample II (curve 2). But in the spectral region 303.0–350.0 nm, the ratio of absorption values became different. Absorption of sample solution III intersected with that of sample I, but

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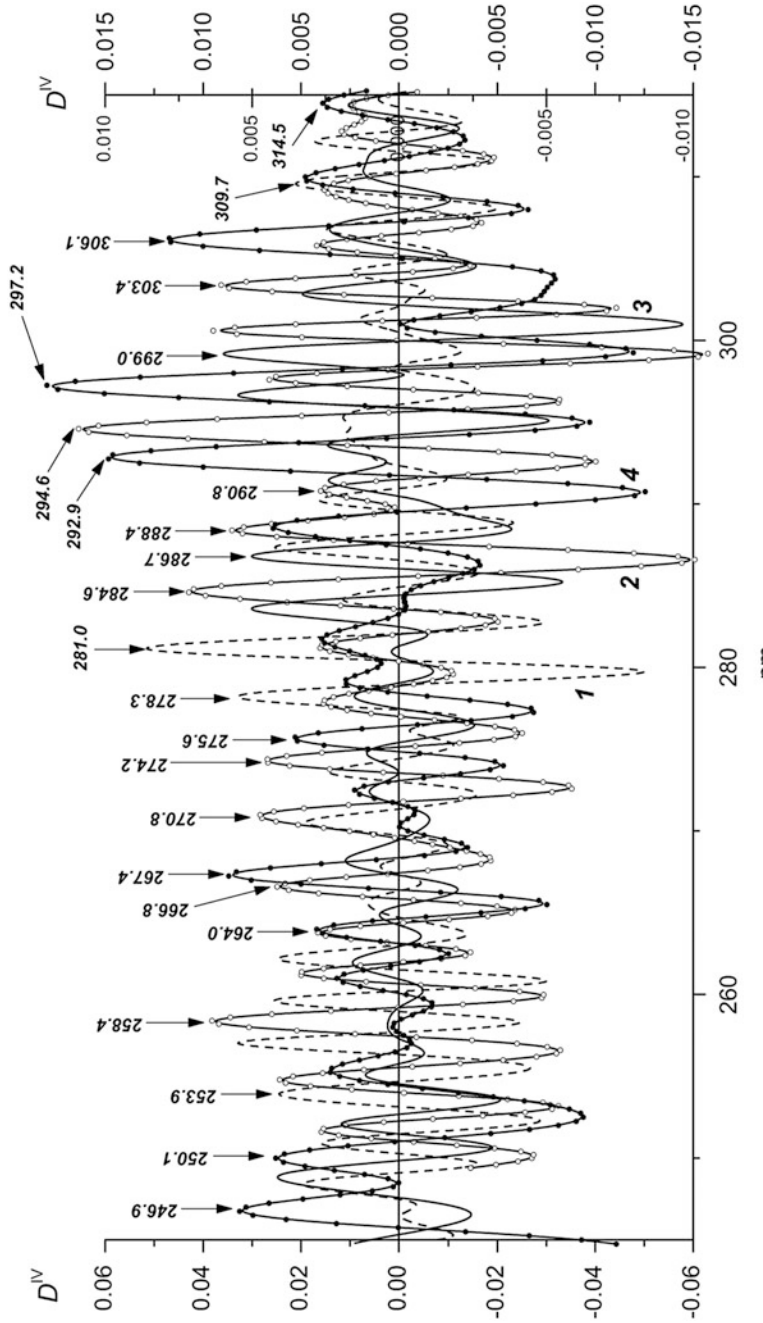

Fig. 4.70 The fourth derivative of absorption spectra of buffer solutions of four Ukrain samples: (*1*) series № 500873 (I); (*2*) series № 544324 (II); (*3*) series № 544323 (III); (*4*) series № 590115 (IV). *Abscissa*: wavelength, nm; *ordinate*: relative OD units; the *left ordinate* is for *curve 1*; the *right outer ordinate* for *curves 2* and *3*; the *right inner ordinate* for *curve 4*

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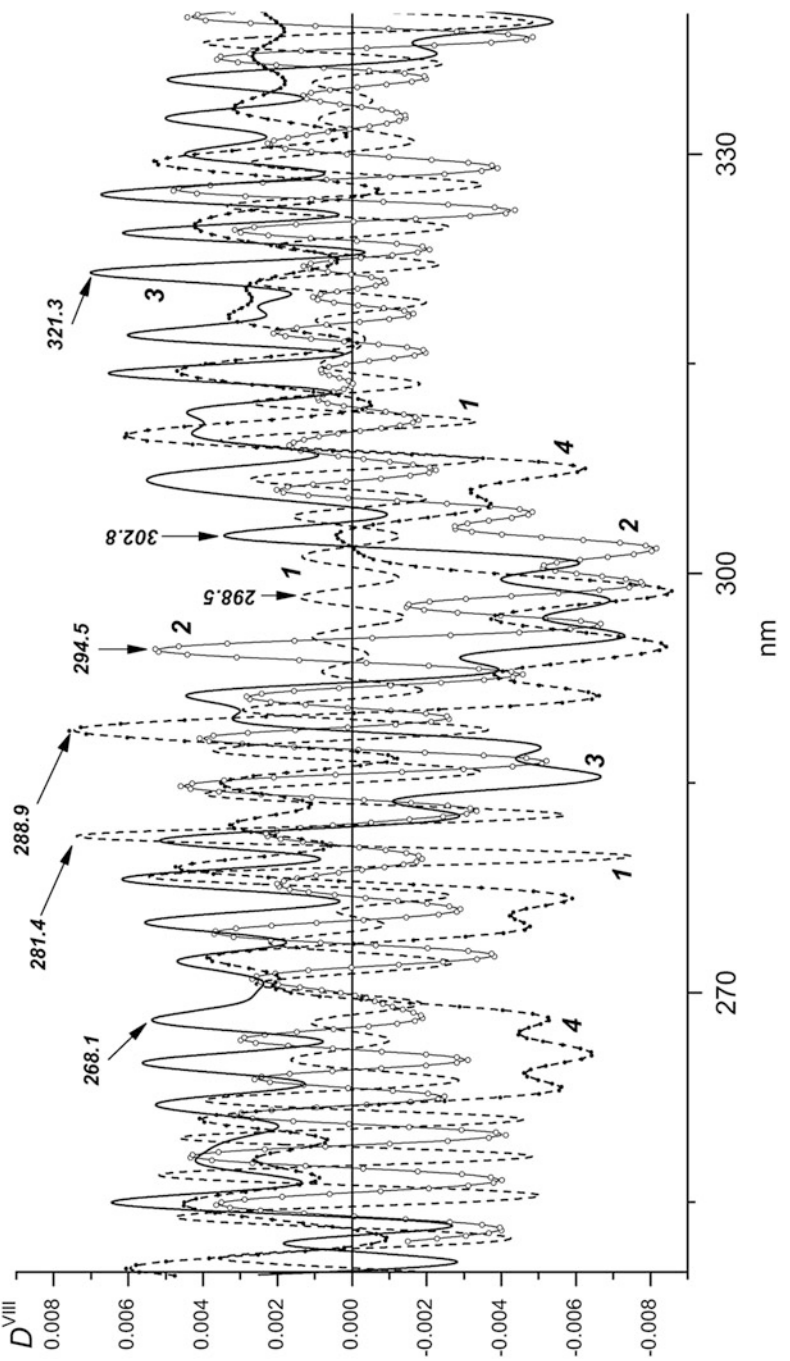

Fig. 4.71 The eighth derivative of absorption spectra of buffer solutions of four Ukrain samples. Curve designations are as in Fig. 4.69. *Abscissa:* OD, relative units

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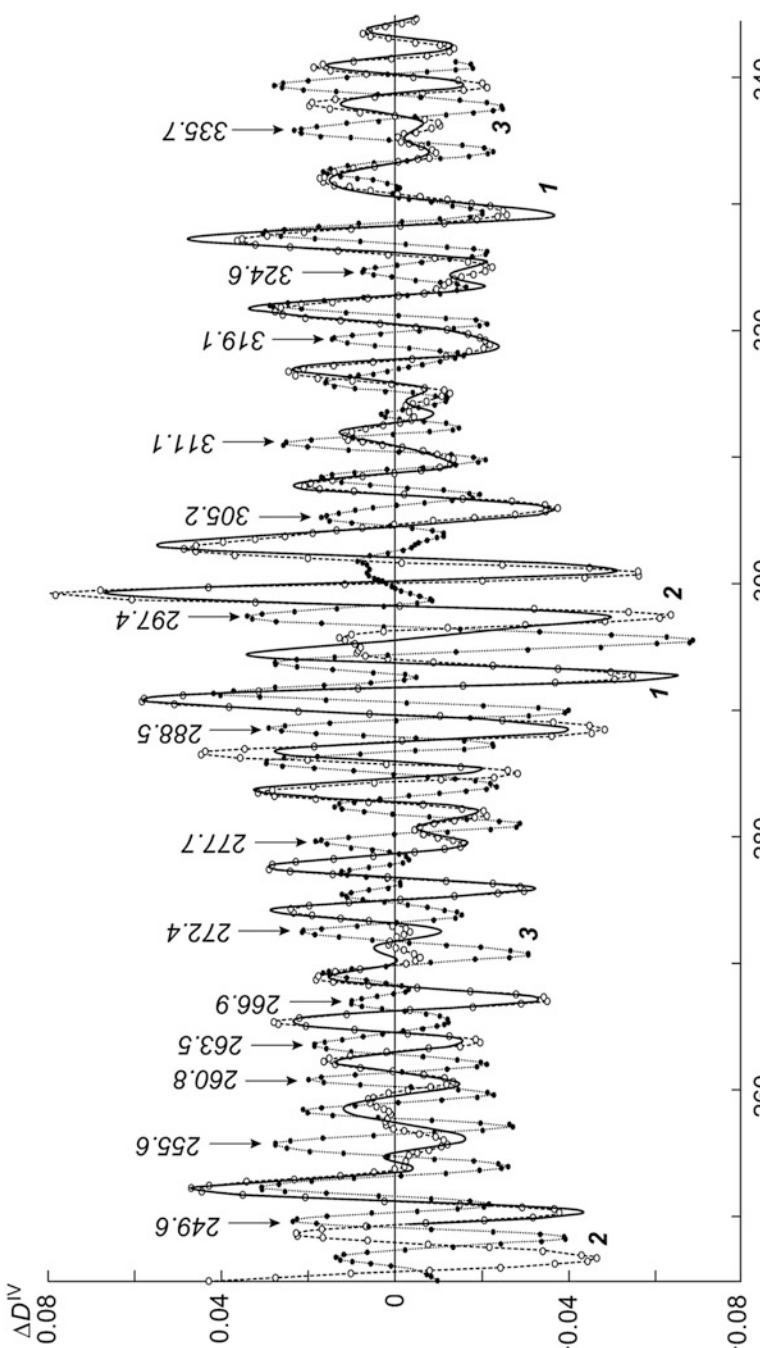

Fig. 4.72 The fourth derivative of difference absorption spectra: (*1*) solution of Ukrain series II minus solution of series IV; (*2*) solution of series II minus solution of series III; (*3*) solution of series II minus solution of series I. *Axis* designations as in Fig. 4.70

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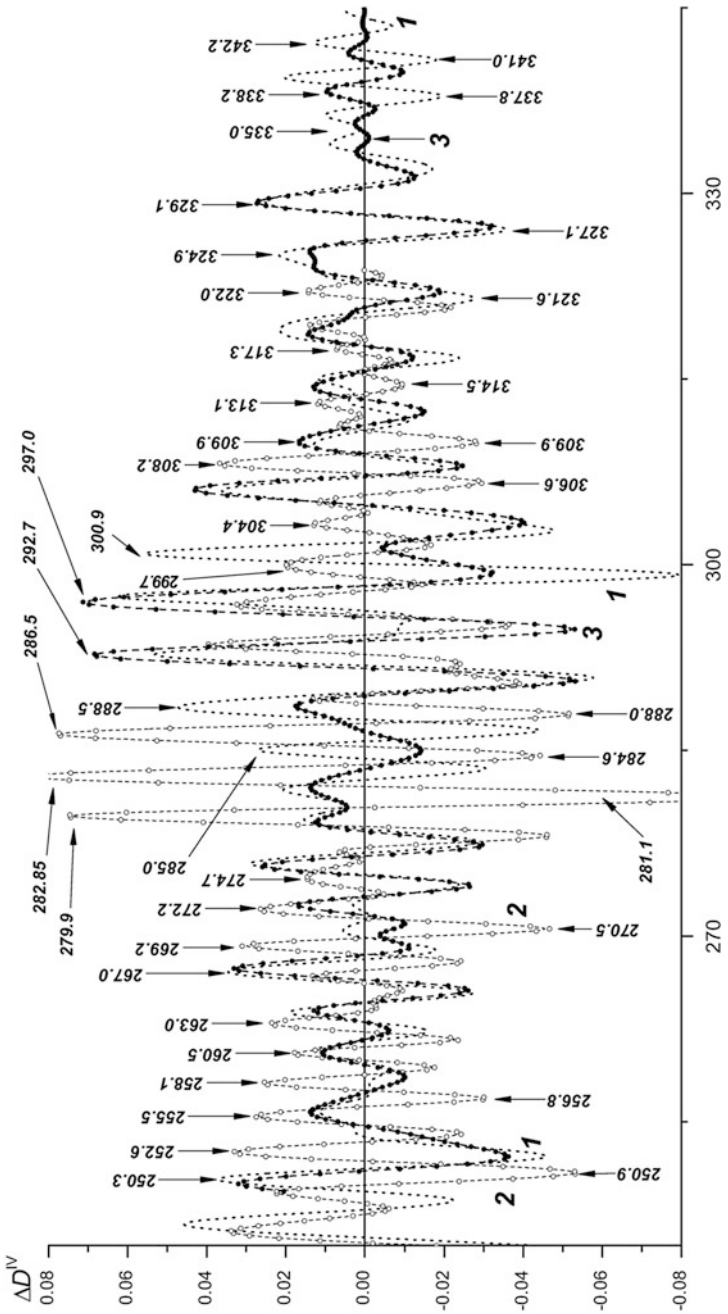

Fig. 4.73 The fourth derivative of difference absorption spectra: (*1*) solution of Ukrain series № 590115 (IV) minus solution of series № 544323 (III); (*2*) solution of series № 590115 (IV) minus solution of series № 500873 (I); (*3*) solution of series № 590115 (IV) minus solution of series № 544324 (II). *Axis* designations as in Fig. 4.70

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both were higher than the absorption of sample solution II (i.e., curves could be located in priority 4, 3, 1, 2). In the region 295.0 nm, curves 2 and 3 were similar. We emphasize that identical weight quantities of substances were taken for the analysis.

The program *Spectra Calc* can reveal differences in spectral curves more clearly. It gives the possibility to displace, directly on the monitor, the contours of spectral curves to the same value of absorption (OD) in the region of principal absorption maxima. We want to highlight this feature of the computer technique because it finds new lines and spectra specificity (Fig. 4.69).

This operation detects additional special features in the specific character of each of the four investigated spectral curves. For example, ascending parts of curves (at 260.0 nm) are located from bottom to top in the order I, II, III, IV; the descending parts of the same curves (at 310.0 nm) are in another order, II, I, III, IV (Fig. 4.69). Furthermore, the capabilities of the program ensured the significant narrowing of spectral curve outlines of samples from checked series and allowed us to establish differences in positions of principal maxima more exactly. The position for series I was 282.3 nm, for II 283.4 nm, for III 285.2 nm, and for IV 284.3 nm. The analysis of listed special spectra features confirms that tested samples of UKR preparation are different in the relative content of components in their composition.

To obtain more reliable information for unambiguous interpretation of the specific character of spectra and for more precise definition of localization of hidden absorption maxima of UKR samples, we used the abilities of derivative spectrophotometry of the fourth to eighth orders. Results are presented in Figs. 4.70, 4.71, 4.72, and 4.73. The illustrative area of Figs. 4.70 (D^{IV}) and 4.71 (D^{VIII}) shows the presence of many absorption bands, whose position is completely specific for each sample from the four studied UKR series (I–IV). For comparative analysis of the location of absorption maxima, we selected the curve of series II as occupying the intermediate position between absorption spectra (Fig. 4.68, curve 3). For convenience of comparison, the spectral region was limited by the narrower wavelength range, as presented in Fig. 4.69. From the data in Fig. 4.69, it follows that series I is characterized by a number of absorption bands, λ_{max} of which either substantially differs from the corresponding maximum for series II or coincides with its minimum extremum. These bands are 250.1, 253.9, 257.0, 259.6, 270.5, 276.2, 290.2, 294.9, 298.5, 301.0, 304.3, and 312.0 nm. Analogously, there were found bands for series IV that did not coincide with the spectral curve of series II (e.g., bands with $\lambda_{max} = 258.4$, 264.0, 266.8, 270.8, 284.6, 288.4, 294.6, 297.8, 300.5, 309.0, and 321.8 nm). Performing further comparison, we obtain for series III specific λ_{max} different from those of series II, namely, 246.9, 250.1, 264.0, 267.4, 270.0, 275.6, 279.2, 288.8, 292.9, 298.0, and 301.1 nm.

From Fig. 4.69, it follows that simple comparison of series I with series III or IV and also of series III with series IV reveals a number of bands that indicate the presence of diverse components in the mixture that are different in their spectral characteristics. This fact is confirmed by the results presented in Fig. 4.71, where specific special features of derived absorption spectra of the eighth order are shown. Careful examination of configuration of SBs in the region of 300 nm reveals

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non-coinciding spectral outlines of the four investigated UKR samples. Thus, derivative spectrophotometry allowed us to discover the totality of many hidden absorption peaks of components in photometrically scanned solutions and to detect specific features of investigated series. To the reader interested in this remedy, we recommend scanning the figure and studying it on a computer monitor using increased size and coloring.

Our results are well-grounded and allow us to conclude that each of the tested UKR preparations contained a mixture of several light-absorbing substances (probably alkaloids or their derivatives) and not just one individual alkaloid. UKR preparations of different series are distinguished not only qualitatively but also by their quantitative composition, which follows from the increase in OD values (hyperchromia) of separate bands (Fig. 4.68, curves 1, 3, 4). In other words, preparations are not only multicomponent in the alkaloid composition but also are nonstandard quantitatively and qualitatively. The nonuniformity of preparations probably influences the effectiveness of their physiological action.

To confirm these conclusions, we performed the registration of derived difference ΔD^{IV} spectra. Results are presented in Figs. 4.72 and 4.73. In Fig. 4.72, series II was again chosen as the basis for comparison. The comparison of series II and series IV (curve 1) revealed a number of prevailing bands in the difference spectrum, with $\Delta\lambda_{max}$ at 252.4, 254.7, 258.5, 262.5, 269.2, 271.2, 274.2, 277.65, 283.6, 286.8, 290.8, 294.3, 299.3, 303.1, 307.6, 312.0, 316.0, 321.7, 327.5, 331.9, 338.0, 340.8, and 343.8 nm. The analogous comparison of series II and series I (curve 3) allowed the detection of the totality of bands in the sign-positive spectrum part, with $\Delta\lambda_{max}$ at 246.8, 249.6, 252.3, 255.6, 258.6, 258,3, 260.8, 263.5, 266.9, 272.4, 275.4, 282.5, 285.7, 288.5, 291.4, 293.8, 297.3, 301.2, 305.2, 308.3, 311.1, 313.3, 315.8, 319.1, 321.8, 332.5, 335.7, and 339.4 nm. Further analysis of the difference spectrum "series II minus series III" (curve 2) enabled us to find $\Delta\lambda_{max}$ for a number of bands: 245.3, 248.7, 252.2, 259.2, 262.3, 265.4, 269.0, 274.3, 277.5, 280.7, 283.6, 286.6, 290.9, 295.6, 299.0, 302.0, 307.8, 311.8, 316.8, 321.5, 327.2, 332.1, 337.7, 340.9, and 343.5 nm. The reader should remember that according to the fundamental rule of difference spectrophotometry, the directivity of difference spectra can be non-coinciding with the directivity of initial spectra, and values of $\Delta\lambda_{max}$ of difference spectra, as a rule, do not coincide with $\Delta\lambda_{max}$ of the absorption spectra; the appearance of new absorption maxima is possible. The extrema of the positive region of the three compared curves do not coincide with each other, they have hypsochromic or bathochromic shifts relative to curve 1 (chosen as the basis), and the manifestation of a hypochromic or hyperchromic effect is found in a number of bands.

The above-stated agrees with results presented in Fig. 4.73. For this figure as the comparison spectrum, we chose the absorption D^{IV} spectrum of series III, also occupying an intermediate position in Fig. 4.68, and series II. From Fig. 4.73 (curve 1), it follows that in the ΔD^{IV} spectrum "series III minus series II," there is reliably detected a number of absorption maxima having specific positions that do not coincide with maxima of these series in Figs. 4.70 and 4.71, namely, 246.6, 250.3, 254.2, 255.7, 260.6, the small maximum 258.1, 260.5, 263.9, 267.0, 270.4,

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272.6, 275.8, 279.5, 281.7, 285.5, 288.5, 292.7, 297.5, 300.9, 306.1, 309.7, 313.2, 315.1, 318.9, 324.9, 329.1, 334.0, 336.2, 339.4, and 342.2 nm. We emphasize that in the case of identity of component compositions, the difference spectrum of the two series would be expressed by a line with extremely insignificant deviations from the zero line position.

If we conditionally accept curve 1 (Fig. 4.73) as the basis for comparison, then relative to it, the totality of positive maxima of curve 2 ("series III minus series I") has both bathochromic and hypsochromic shifts. More precisely, these differences are as follows: hypsochromic shift with hypochromic effect (246.0, 249.4, 266.7, 274.7, 296.6, 299.7, 304.0, 305.0, 317.3 nm); hypsochromic shift with hyperchromic effect (255.5, 263.0 nm); newly formed absorption bands (252.7, 258.1, 269.2, 308.2, 322.0 nm); curve shoulders in the form of peaks on derived spectra of higher orders (264.8, 291.5 nm); bathochromic shift with hyperchromic effect (279.9, 286.5, 289.2); bathochromic shift with hypochromic effect (276.8, 289.2, 293.7, 311.25, 313.1, 315.0, 317.3, 319.4 nm); and absence of shift with small hyperchromia in band 260.5 nm. Thus, curve 2 substantially differs from curve 1, which indicates their *non-identity*. Thus, the spectrum ΔD^{IV} "series III minus series I" shows differences in the composition of components of the tested series.

Further comparative analysis of curve 3 (Fig. 4.73) relative to curve 1 ("series III minus series IV") reveals hypsochromic shifts with simultaneous hypochromia in bands 249.9, 255.7, 270.0, 318.8, 333.1, 338.2, and 341.3 nm and a hyperchromic effect in bands 272.3, 275.8, 292.7, 297.0, and 314.3 nm. Pronounced hypochromia is noted in bands 260.4, 263.9, 279.3, 281.9, and 288.5 nm. A bathochromic shift with simultaneous hypochromia is found in band 301.3 nm; the hyperchromic effect is discovered for band 309.9 nm; and the bathochromic shift occurs at 267.4 nm. On the spectrum ΔD^{IV}, the negative extremum appears at 284.9 nm, whereas on curve 1, there is a positive absorption band at 285.0 nm. On curve 3 at 320 nm, the shoulder appears manifested on the D^{VIII} spectrum in the form of a maximum. Analogous curve deflection appears in the region 324.9 nm. Consideration of the difference spectrum "series III minus series IV" also indicates the dissimilarity of sample composition. From the data presented in Fig. 4.73, the conclusion follows that investigated series of UKR preparation are distinguished by their qualitative composition and by the numerical ratio of their components.

Thus, the data in Figs. 4.72 and 4.73 stress a difference between investigated series of UKR preparations. The presence of hyperchromic and hypochromic effects in difference curves indicates a quantitative difference in the components of analyzed samples. This conclusion, besides the presented data, is also based on experimental and theoretical developments of other articles (Saakov et al. 1987a, b; Zhukovskii and Saakov 2002). The corresponding manifestation of hypsochromic and bathochromic shifts argues for a difference in the qualitative composition of analyzed preparations.

Results presented in Figs. 4.70, 4.71, 4.72, and 4.73 indicate the individuality of substance combination of each of the four UKR samples. This can be the reason for the differences in biochemical and physiological activity of UKR preparations of

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different series. Our results are in line with concepts described in other articles (Nowicky 1999b; Uglyanitsa et al. 2000), whose authors support the concept of multicomponent UKR, and contrast with statements (Liepins et al. 1996) about monocomponent UKR preparations.

Thus, in spite of the fact that UKR promotes the viability of animals irradiated by γ-radiation, helps to restore hemopoiesis, improves functioning of the immune system (Han et al. 1991), and has antiviral, antimicrobial, and anticancer properties, it is extremely difficult to unambiguously formulate the mechanism of these effects because of unstable heterogeneity of produced UKR preparations. It is possible that inhibition of malignant cell growth or other physiological activities could be the property not of the main UKR components, but of contaminants. One of the ways to increase the composition identity of UKR samples could be cultivation of *Ch. majus* in a phytotron under strictly controlled conditions.

Thus, for the first time in analytical practice, the fourth to eighth derivatives of absorption spectra were used to study samples of the biologically active remedy UKR. The complex, heterogeneous, and specific structure of the sample composition was established for each series of UKR. The totality of the complex structure of many hidden absorption peaks of components in photometrically scanned solutions was revealed and individual differences were found. It was experimentally proven incorrect for some authors to consider the Ukrain preparation as a monocomponent chemical compound and to characterize it by individual formula of chemical composition, molecular weight, and values of constants for irreversible and reversible fermentation inhibition. The latter only makes sense for individual inhibitors of fixed chemical composition. Results described in our joint article with Yu.G. Zhukovskii (Zhukovskii and Saakov 2002) can assist in improving the quality of biochemical and physiological studies in which the anticancer preparation UKR is applied. The method of registration of DSHO can be successfully recommended for the comparative analysis of other biologically active preparations of alkaloids for the purpose of clarification of their homo- or heterogeneity.

4.4 Derived Spectra Application for Analysis of Derived Forms of the Non-depolarizing Muscle Relaxant Tercuronium and of Vitamins and Hormones

4.4.1 Comparative Analysis of Tercuronium Derivatives

Some capabilities of derivative spectrophotometry can be illustrated by the example of D^{II} spectra investigation of a number of derived forms of the non-depolarizing myorelaxant (NM) tercuronium (Saakov et al. 1987a, b).

The pharmacological properties of the compounds investigated by us were studied by Starshinova (1980). Table 4.7 presents data on the neuromuscular blocking activity, obtained in experiments on the rectus abdominis muscle of the

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Table 4.7 Neuromuscular blocking activity of compounds

Preparation	Radical R	Frog Muscle Rectus abdominis $K_{D,p}\cdot(10^{-7}$ M)	Cat Front shin ED_{50} (μmol/kg)	Rabbit Gastrocnemius ED_{50} (μmol/kg)
Tercuronium	$(C_2H_5)_3N^+$—	1.5 ± 0.3 (6)	0.08 ± 0.01 (9)	0.020 ± 0.003 (4)
IEM-840	$(CH_3)_3N^+$—	14 ± 2 (4)	0.30 ± 0.04 (4)	0.25 ± 0.05 (4)
IEM-940		6.0 ± 1.5 (4)	1.00 ± 0.20 (4)	0.80 ± 0.10 (4)
IEM-941		2.5 ± 0.6 (4)	0.50 ± 0.05 (4)	0.35 ± 0.06 (4)
Tercuronium bromide	$(C_2H_5)_3N^+$—	1.5 ± 0.2 (4)	0.08 ± 0.03 (4)	0.020 ± 0.001 (5)
IEM-1080		...	...	...
IEM-1081		...	...	...

K_D is the dissociation constant of substance in M. Acetylcholine was used as the agonist. ED_{50} is the dose causing the partial (50 %) blockade of neuromuscular transfer at intravenous introduction. In brackets are the number of experiments

frog *Rana temporaria* (fRAM) and also in experiments on cats and rabbits (under narcosis).

The most active muscle relaxants are tercuronium (IEM-858, IEM stands for the Institute of Experimental Medicine of the Northwest Branch of the Russian Academy of Medical Sciences, SPb) and tercuronium bromide. Their dissociation constants (K_D) found in experiments on choline receptors of fRAM are equal to 1.5×10^{-1} M, and doses causing the blockade of neuromuscular transfer in experiments on cats and rabbits are equal to 8×10^{-8} and 2×10^{-8} mol/kg, respectively. Consequently, these substances have a high affinity for the choline receptors (CR) of skeletal muscles. The replacement of ethyl radicals in the molecule of tercuronium by methyl radicals (IEM-840) results in a significant decrease in blocking activity, caused by a decrease in the affinity of this compound for CR. Figure 4.74 presents the absorption spectra of separate investigated derivatives of tercuronium. Figures 4.75 and 4.76 show, for tercuronium and some of its derivatives, the second derivatives of absorption spectra or difference spectra of the second derivative, equalized on OD in the region 35,000 cm^{-1}.

Application of the D^{II} method allows us to reliably separate four bands in the far UV range and to register more strictly the position and fine structure of a main maximum in the near UV range (Fig. 4.75b). Each of the preparations is distinguished by a sufficiently specific spectrum, although the general spectral outlines are similar.

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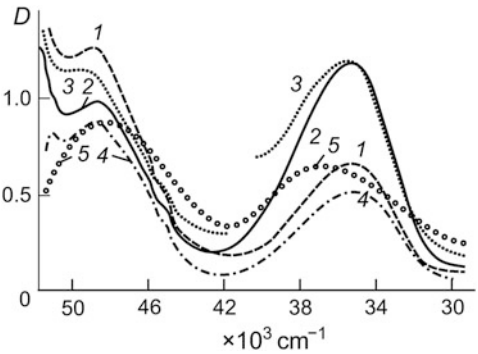

Fig. 4.74 Absorption spectra of tercuronium derivatives: (*1*) IEM-858; (*2*) IEM-841; (*3*) IEM-940; (*4*) IEM-840; (*5*) tercuronium bromide

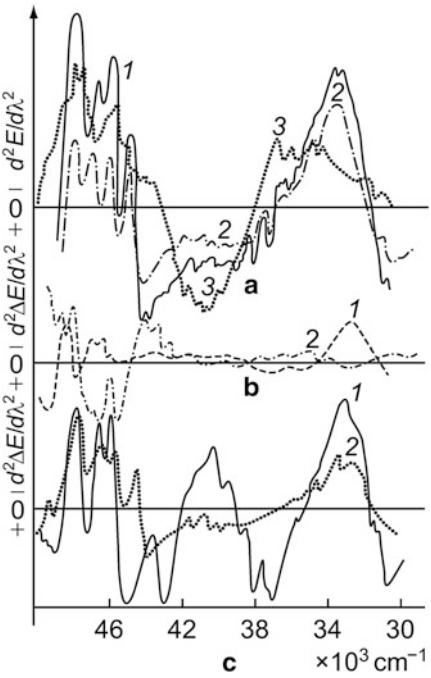

Fig. 4.75 The second derivative of absorption spectra of derivative compounds of tercuronium: (**a**) D^{II} spectrum of tercuronium (IEM-858) (*1*) and of its chloride (*2*) and bromide (*3*); (**b**) D^{II} of difference spectra (ΔD^{II}) "tercuronium minus IEM-840" (*1*) and "tercuronium minus IEM-1080" (*2*); (**c**) ΔD^{II} of spectra "tercuronium minus tercuronium bromide" (*1*) and "tercuronium minus IEM-1081" (*2*)

The dissociation constant of IEM-840 in experiments on fRAM was about ten times greater than the K_D of tercuronium. Decrease in CR affinity of the compound IEM-840 is caused by a decrease in hydrophobicity of the molecule as a result of replacement of six ethyl radicals by six less-hydrophobic methyl radicals. The difference D^{II} spectrum (Fig. 4.75b) shows that the replacement of radicals causes an absorption decrease in the region 34,000 cm^{-1} for the preparation IEM-840. The replacement of six ethyl radicals by two methyl and two cyclohexane radicals changes the difference D^{II} spectrum only in the far UV region.

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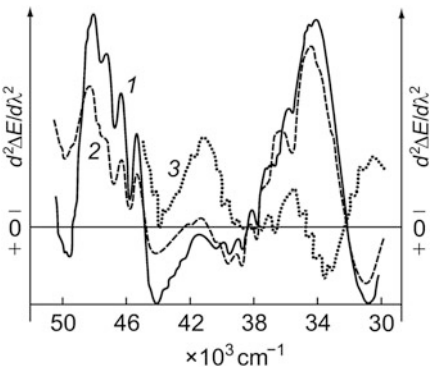

Fig. 4.76 Second derivatives of spectra of tercuronium derivatives IEM-941 (*1*) and IEM-940 (*2*); ΔD^{II} of spectra "IEM-941 minus IEM-940" (*3*)

Tercuronium bromide is characterized by an absorption decrease in the region 40,000 cm^{-1} and by hypsochromic shift of the main maximum (Fig. 4.75a, c). The replacement of four ethyl radicals by two cyclohexane rings changes the character of the difference D^{II} spectrum, especially in the far UV range, promoting formation of a new band in the region 45,000 cm^{-1}.

According to experimental data on fRAM, the affinities of compounds IEM-940 and, especially, IEM-941 for CR are higher than the affinity of IEM-840, but lower than that of tercuronium. This is because the hydrophobicity of radicals of the cation heads of IEM-940 and IEM-941 is higher than the hydrophobicity of methyl radicals of IEM-840, but lower than the hydrophobicity of ethyl radicals of tercuronium. From the data in Fig. 4.76, it is evident that the outlines of D^{II} spectra of preparations IEM-940 and IEM-941 are similar to the outline of the tercuronium spectra. At the same time, the difference D^{II} spectrum "IEM-941 minus IEM-940" indicates that their differences are probably combined with different physiological influences of these substances. On the basis of the K_D values, the dose of IEM-940 and IEM-941 needed to block neuromuscular transfer in experiments on cats and rabbits should be less than the blocking dose of IEM-840. However, the blocking activity of these compounds is proven to be lower than that of IEM-840. This is caused, probably, by different types of blocking action. Compounds IEM-940 and IEM-941 are non-depolarizing myorelaxants (NM), whereas IEM-840 has depolarizing (DM) properties. Under the effect of NM, the blockade is developed when not less than 80 % of CR are occupied (Ciebiada et al. 1996). DM can cause blockade as a result of interaction with a much smaller percentage of CR. For this one reason, the effective DM concentration can be much less than the dose of NM.

In this connection, the difference spectrum is interesting (Fig. 4.75b) because the absence of significant deviations in spectra between tercuronium and IEM or other preparations can be the starting point of diagnostics that could allow, at least approximately, the assessment of their possible physiological effect. Ill-defined differences in the absorption spectra of examined preparations can become obvious when using D^{II} spectra, especially in this case when ethyl radicals are replaced by methyl and cyclohexane radicals. Using the spectral method, we have found

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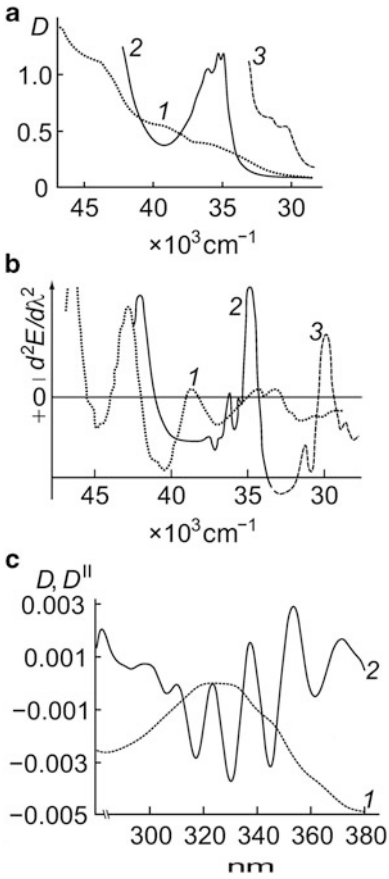

Fig. 4.77 Absorption spectra (**a**) and their second derivatives (**b**) of dihydrotestosterone in ethanol (*1*), vitamin E (*2*), increased concentration of vitamin E in hexane (*3*). (**c**) Absorption spectrum (*1*) and the second derivative (*2*) of testosterone solution (from Fluka, analytically pure) in dioxane (purified for spectroscopy)

specific changeability of biochemical structures of preparations and taken a step towards revealing their physiological properties.

4.4.2 The Reasonability of Using Derived Spectra for the Analysis of Commercial Preparations of Vitamins and Hormones

To conclude this section, we briefly discuss one more field of rational application of the method of D^{II} spectra registration—the solution of analytical problems in work with hormonal preparations and vitamins, especially when determining these substances in two- and three-component commercial mixtures (Saakov et al. 1998; Saakov 2001a, b, 2002, 2003).

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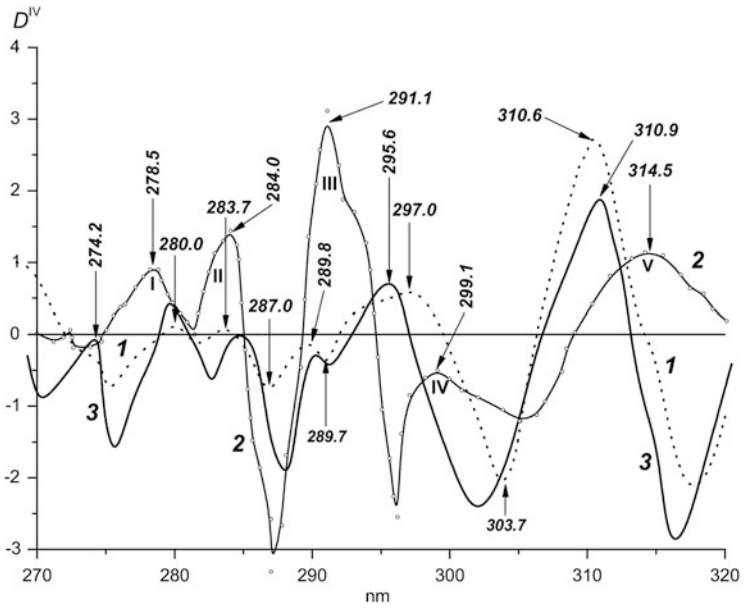

Fig. 4.78 Fourth derivatives of absorption spectra of Russian commercial preparations of vitamins A (*1*) and E (*2*) and of their mixture in isopropyl alcohol (*3*). *Abscissa*: wavelength, nm; *ordinate*: relative units. Concentration of vitamin E was decreased to 10 % of the initial commercial solution

An earlier attempt to apply first-order derivative spectra registration for the characterization of steroid hormones gave encouraging results (Olson and Always 1960). It is completely natural that the D^{II} registration method can help obtain even more information about the structure of spectra of hormones. Comparison of curves in Fig. 4.77a–c, for preparations of dihydroxitestosterone in ethanol and testosterone in dioxane, allows us to isolate two reliably detected peaks on the curve of the second derivative. The amplitudes of these peaks can be used for quantitative determinations in a mixture with other hormones of the same nature.

A similar picture appears during spectrophotometric measurements of vitamins having characteristic absorption bands in the UV range. Curve 2 in Fig. 4.77a corresponds to the absorption spectrum of a vitamin E commercial preparation manufactured in Russia (Saakov et al. 1987a, b). Using the method of addition of the standard solution of preparation to its analyzed solution, it is possible to find components of the curve that are not visible at low concentrations of the substance (Fig. 4.77a, curve 3). On the D^{II} spectrum of the vitamin E solution, five characteristic bands of the curve are seen; moreover, two peaks (35,000 and 30,000 cm^{-1}) can serve as reliable criteria for the presence of the preparation in the mixture, and this can be used in technological operations without preliminary chromatographic purification of the vitamin. Analogously, on the D^{II} spectrum of testosterone in

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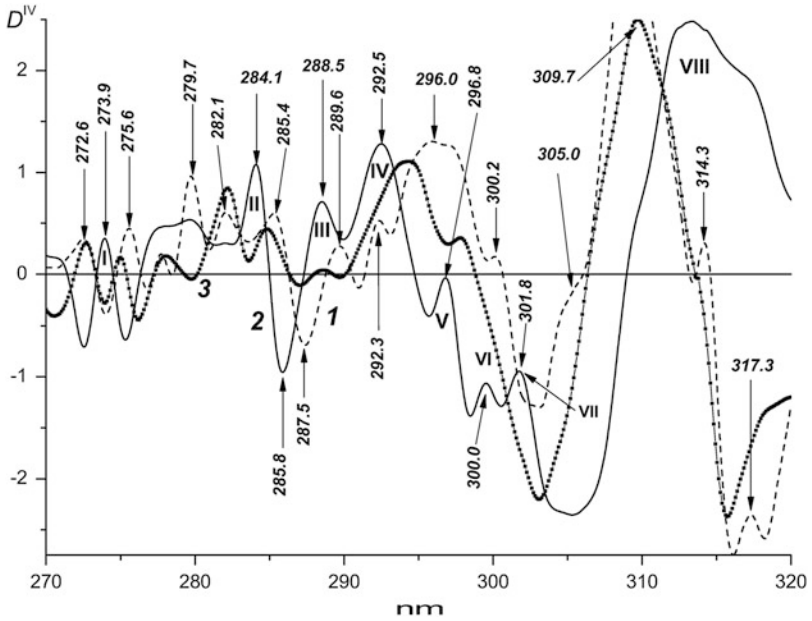

Fig. 4.79 Fourth derivatives of absorption spectra of European commercial preparations of vitamins A (*1*) and E (*2*) and of their mixture in ethanol. *Axes* and dilution of vitamin E as in Fig. 4.78

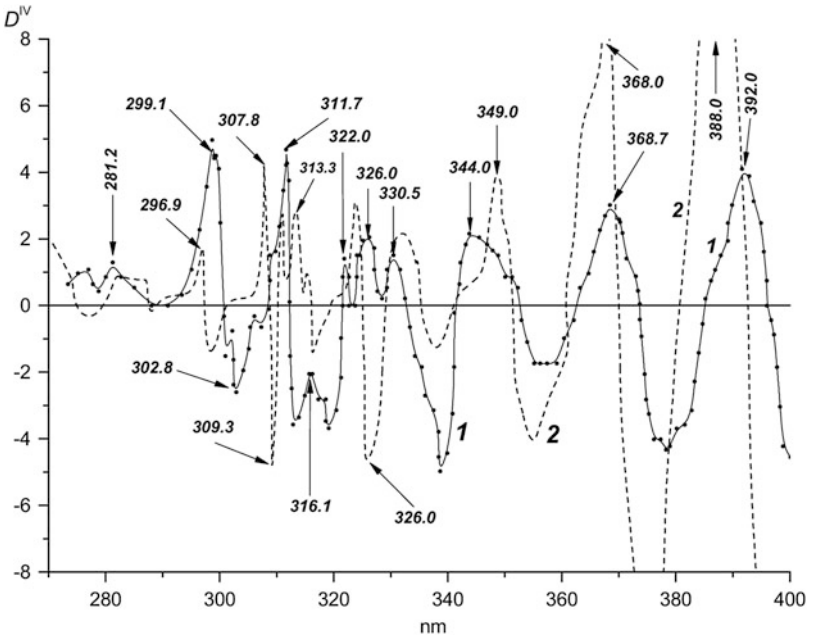

Fig. 4.80 Fourth derivatives of absorption spectra of vitamin A in ethanol (*1*) and of vitamin A acidified with 0.1 N HCL (*2*) (Saakov 2002)

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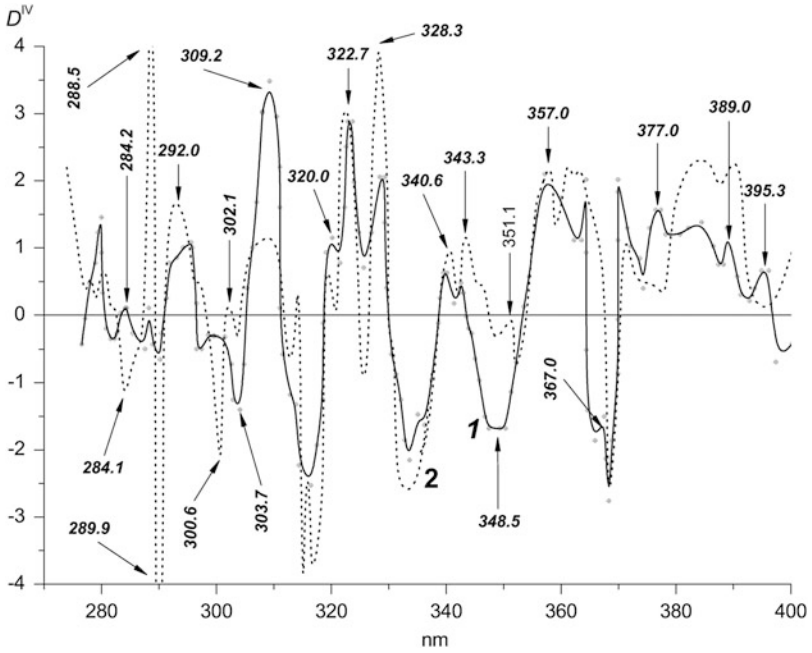

Fig. 4.81 The character of spectrum structure change in the absorption spectra of commercial vitamin A under influence of UV radiation: (*1*) control solution; (*2*) the same solution after irradiation (Saakov 2002)

dioxane (Fig. 4.77c, curve 2), there are six negative peaks that correspond to hidden maxima of the initial curve (curve 1).

In Figs. 4.78, 4.79, 4.80, and 4.81 are shown the fourth derivatives of spectra of vitamins A and E and of their mixtures and also the influence of hydrochloric acid and UV light on spectral changes of these substances. For the solution of a Russian commercial preparation of vitamin A in isopropyl alcohol, 12 positive and negative extrema are observed (Fig. 4.78, curve 1). For the ethanol solution of a European preparation of vitamin A, 25 positive and negative extrema are found (Fig. 4.79, curve 1). The smoothness of contours of curve 1 (Fig. 4.78) can be explained by the large heterogeneity of the preparation and its less-refined purification. This concerns spectral regions 272.0–276.0, 279.7–292.3, 296.0–303.7, and 303.7–318 nm.

In the case of vitamin E measurements, an almost analogous situation takes place (Figs. 4.78 and 4.79, curve 2). For the Russian preparation, 11 extrema in the positive and negative regions of the spectrum are detected. For the European preparation of vitamin E, 21 extrema are found. The spectrum of the mixture of Russian preparations of vitamins A and E has 13 extrema (Fig. 4.78, curve 3), and the spectrum of the mixture of European vitamins A and E includes 19 extreme points. This indicates that the Russian vitamin preparation was more heterogeneous than the European preparation.

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The nature of the response of an ethanol solution of vitamin A (European preparation) to the influence of hydrochloric acid proves the presence in solution, besides vitamin A, of the totality of the outside products reacting with HCl (Fig. 4.80).

Figure 4.81 presents the specific character of changes in the fourth derivative of the absorption spectrum for an ethanol solution of a commercial preparation of vitamin A under the influence of UV irradiation. Complication of the spectral curve contour occurs. Together with a hyperchromic shift at 288.5, 292.0, 302.1, 314.0, 328.3, 349.6, 343.3, 346.2, 351.1, 357.0, 361.2, 363.2, 384.1, and 390.1 nm, which demonstrates simplification of the substance structure, hypochromic shifts occur in regions 284.1, 289.9, 300.6, 316.7, 325.4, 333.2, 377.6, and 395.3 nm, suggesting the formation of more ordered structures.

The considered examples of use of the derivative spectrophotometry method demonstrate only a small area of the rational application of this method. With the presented material, we want to attract the attention of researchers to this method, which is already widely used in Europe in diverse systems of chemical technological control and in analytical work. In Russia, unjustifiably prolonged procrastination occurs. There is no need to list all the possible analytical tasks reliably solved with the help of differentiation of spectral curves (Saakov et al. 1987a, b; Semenova and Saakov 1989; Saakov 1992). These tasks constantly appear in connection with spectral analysis for comparative assessment of biochemical and pharmaceutical preparations (Monin et al. 1985; Natochin et al. 1985; Saakov 1993a–e, 1994a, b, 2002; Saakov and Rozengart 2003); analysis of native supramolecular structures of erythrocytes, chloroplasts, mitochondria, and ribosomes; in chemistry of rare-earth elements and glasses (Kogan et al. 1990a, b); in chemistry of dyes and other organic compounds; and in many other areas (Semenova and Saakov 1989; Saakov 2001a, b).

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Chapter 5
The Range of DSHO Application in Experiments with Pigments of Plants and Animals

Contents

Over a number of years our interests have been concerned with the study of transformations of carbon-labeled or hydrogen-labeled carotene and basic xanthophylls in plant cells. This interest was caused because the hypothesis that light induces the

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direct reduction of violaxanthin (5,6,5′,6′-diepoxy-5,5′,6,6′-tetrahydro-β-carotene-3,3′-diol) into lutein (3,3′-dihydroxy-α-carotene, β,ε-carotene-3,3″-diol) without intermediate products (Sapozhnikov et al. 1957, 1959; Bazhanova and Sapozhnikov 1963) was not methodologically based. It is paradoxical, but this supposition already had opponents before publication (Moster and Quackenbush 1952a, b; Cholnoky et al. 1956, 1957, 1958) and still more afterwards (Anderson et al. 1960; Blass et al. 1959) because the stated theoretical concepts of Sapozhnikov and coworkers were not the only possible alternative. The opinions existing on this question are described in the review by Saakov and Konovalov (1966).

Interest in this problem was stimulated by Cholnoky et al. (1956), who suggested that involvement of the xanthophyll system could explain the functional role of carotenoids in the cellular structures of plants and animals in the process of oxidative metabolism under the influence of ecological stress factors of various origins. Sapozhnikov proposed that the violaxanthin–lutein pair is one of the main links of oxygen transfer from water to the molecular oxygen of air. This suggestion was made without any reliable or unreliable evidence. Having at least one accurate experiment, with non-controversial interpretation, was necessary to convert the idea into scientifically ascertained truth.

Let us quickly recall the basic course of events, discussion of which was extended for a century. Initial definition of the problem arose from papers by known German chemists Willstätter and Stoll (1913, 1918), who showed the possibility of a change in the ratio of carotene to xanthophylls under the influence of external environmental conditions. On the basis of their experiments, the authors suggested the existence of interconversions between carotene and xanthophylls. However, at the level of scientific development of their time and limitations in methodological capabilities, the German chemists did not find coupling of transformation of separate and various components of the carotenoid system. Lubimenko (1916, 1963) paid special attention to the ratio of oxidized and restored carotenoid forms and to their compatibility in ontogenesis of plants. He stressed the urgency for researchers of plastid physiology to investigate pigment conversions and the functions of various pigments connected with these transformations in chloroplasts and chromoplasts. Because of the high lability of components of the carotenoid system, the question of their quantitative ratio is very interesting. The real solution to this question depends not only on the analytical abilities of the researcher but also on how frequently samples are taken during a day, week, month, and vegetative season. Good quality sampling and storage before analysis and the technical training of researchers are vital. There are hundreds of articles on assessment of the dynamics of pigment content, but they are not united by a unified purpose, methodology, or analytical approach. Because of this, there is a rather wide scattering of obtained data and often far-fetched interpretations of results.

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We too devoted attention for a number of years to research on the dynamics of carotenoid content in the vegetation process of studied objects in an effort to reveal laws concerning the ratios of carotene and xanthophyll quantities (Saakov 1968a, b; Saakov et al. 1967). So we know very well the accompanying difficulties of correct performance in such areas of research.

We pay special attention to the research of Lichtenthaler and Becker (1971). These authors established an interesting phenomenon. According to their data, in comparison with the content of chloroplasts, in etioplasts (chloroplasts that have not been exposed to light), the quantities of β-carotene (β-Car), neoxanthin (Neo), and partly violaxanthin (Viol) are lower than the quantities of zeaxanthin (Zea) and antheraxanthin (Ant). In the process of thylakoid formation, the content of β-Car, Neo, and Viol increases, but the content of Ant and Zea decreases. Tracing the content dynamics of the six main carotenoids over 36 h after beginning illumination, the authors found an increase in β-Car quantity with a simultaneous decrease in the contents of Viol, Neo, Ant, and Zea. Lutein (Lut) concentration did not change during this time. The authors reached the conclusion that the change in the ratios of all components of the carotenoid system is at variance with the sum of the yellow pigment fraction. This coincides with the position of Willstätter and Stoll (1913, 1918).

It is necessary to stress that correct investigation of the content dynamics of such a labile and multicomponent system as the carotenoids is difficult and extremely unrewarding work. Maybe for this reason relevant research was undertaken on only three xanthophylls (Viol, Ant, and Zea), the contents of which change under the influence of light, most demonstrably in the first 10 min. Sapozhnikov's proposal was the result of methodologically substandard work taking into account only two xanthophylls, Viol and Lut (Sapozhnikov et al. 1957, 1959; Bazhanova and Sapozhnikov 1963), and not taking into account at least four other main components of the system.

Despite a number of controversial positions and the absence of direct experimental data, publications by Cholnoky et al. (1956, 1957, 1967, 1969) attracted attention. In these articles, there is a rational approach and researchers pay attention to the need for a full study of all components of the carotenoid system, especially when assessing their functional value.

However, the theme begun by the German chemists Willstätter and Stoll and picked up in studies by Lubimenko was brought to a dead end by 1950. In some papers (Sapozhnikov 1937; Sapozhnikov and Lopatkin 1950; Sapozhnikov et al. 1953), the experiments by Willstätter and Stoll were repeated. The authors showed that the ratio of carotene (Car) to xanthophylls increases over 30 min under conditions favorable for photosynthesis. The total quantity of carotenoids changed only slightly, but there was no answer about whether the Car content increases or the xanthophyll quantity decreases.

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But there is no smoke without fire. From where did thoughts about transformation of Viol into Lut appear? We have looked back and read already forgotten works. At that time, two publications by Moster and Quackenbush (1952a, b) had been released; these stimulated Sapozhnikov's imagination, which poured out in his concept about the reversible reaction of Viol and Lut conversion under the influence of light. So far, there was critical consideration of articles of a certain scientific direction, which brought many researchers to a dead end. We describe in more detail good investigations conducted before the cited works but forgotten for various reasons.

These sound investigations were also forgotten.

We consider it necessary to first remind the reader about the works of Moster and Quackenbush (1952a, b). First, Moster and Quackenbush revealed that a temperature increase from +5 to +20 °C promotes a decrease in Zea content with a simultaneous increase in β-Car content. This fact is rather interesting and suggests a connection between the Zea content and β-Car. Second, the temperature increase to +20 °C (illumination of 500-foot candles) was accompanied by an increase in Viol content and a corresponding decrease in Lut content. The effect was reversible when the temperature was further increased to +35 °C. In the second part of this work, the authors established that an increase in object illumination to 2,500-foot candles assisted an accumulation in the object (wheat) of Zea and, accordingly, a decrease in β-Car content. Simultaneously, increased illumination resulted in *Lut accumulation* and was accompanied by *decrease in the Viol content*. From this fact comes the concept of Sapozhnikov et al. (1957, 1959), Sapozhnikov and Bazhanova (1958), Bazhanova and Sapozhnikov (1963), and Bazhanova et al. (1964) about the transformation of Viol into Lut under the influence of light on a plant. Further, the blind acceptance and repetition of this idea are evident in many PhD dissertations and publications. Very sad to say, a number of European and overseas researchers are not aware of the old, intelligent, and worthy European articles and quote publications of a secondary origin, which is doubtful from a scientific point of view. Maybe because Soviet and Russian researchers were always limited in receiving western scientific information, they hunted it down, studied it, and remembered it better than young modern scientists from Europe and America.

A Nobel laureate from the University of California Radiation Laboratory (UCRL), Melvin Calvin, along with others (Anderson et al. 1960; Blass et al. 1959; Shneour and Calvin 1962), did not agree with the point of view of authors of other papers (Sapozhnikov et al. 1957, 1959; Bazhanova and Sapozhnikov 1963). Calvin considered that his data on the identical specific radioactivity of the carbon skeletons of Viol and Lut with incorporation of ^{14}C from carbonic acid dioxide in the process of photosynthesis is a necessary but insufficient condition for asserting the direct

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(without intermediate products of reaction) transformation of Viol into Lut. Simultaneously, by experiments and in Calvin's articles, it was emphasized that the reduction in Viol content in light was not accompanied by a corresponding increase in Lut content. Even the position of Nobel laureate Melvin Calvin is not known by European and American researchers, and they continue to cite erroneous statements on the reaction of plant xanthophylls to light.

Later, contrasting with the position of Sapozhnikov, a team of chemists under the leadership of Yasnikov (Kiev, Ukraine) came to the same conclusion (Bershtein et al. 1969, 1971; Petrenko et al. 1970). At this point, it would be possible to end the discussion, especially because H. Yamamoto and coauthors (Honolulu, HI) (Yamamoto et al. 1962, 1972), Costes (Paris-Grignon, France) (Costes 1963a, b, 1965), and later Hager (Tübingen, Germany) (Hager 1966, 1967b, 1969; Hager and Bertenrath 1962; Hager and Mayer-Bertenrath 1967) obtained identical results on the transformation of Viol through Ant (3,3′-dihydroxy-5,6-epoxy-β-Car) into Zea ((3*R*,3′*R*)-β,β-carotene-3,3′-diol) on the basis of thoroughly developed methodological procedures.

We emphasize that different points of view, methodologically and carefully substantiated by precisely verified methodology, have the right to exist. It is, however, paradoxical, but sometimes the lack of evidences or controversial data don't attract attention and such hypotheses are supported for a long time.

In the case of small particular successes, the effort spent did not correspond to the results obtained, which also led to the oblivion of these works. In the abundant totality of material, the main problems were not solved: What are the pathways of de-epoxidation of Viol under the influence of light on the photosynthetic apparatus? Is the Viol molecule actually converted into Lut without intermediate products of reaction? Given findings could not be expressed in such a categorical way; they could only be considered as the preliminary stage of a study about the functional value of this reaction. Alas, spending more time on these far from objective works is not worthwhile because the theoretical ideas were neither experimentally convincing nor solely possible. It is a pity that for a long time there was no sufficient agreement about this theoretically very important question. However, the responsibility for long-standing unsubstantiated assertions and insistence is divided between the author of this concept and his colleagues.

In USSR, at best, the names of scientists not agreeing with accepted point of view were perservere hushed up for many years. The appearing fault, at that time caused by the fear of being added to the list of ideological enemies, did not allow critical assessment of the situation. For many years, false and antiscientific postulates were drummed into the heads of student audiences. Textbooks by key experts were withdrawn and forbidden. For example, for a number of years in the biological department of Leningrad University, the course of formal genetics and variation statistics was read practically in secret. Years of work were required, and considerable means and numerous facts from different countries for the probative persuasion of an opponent, but nobody was responsible for the

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harm caused by conjunctural assertions. The monopolism of opinion led to the rotting of theory. As an example, T. D. Lysenko, whose position, disregarding any objections and facts, ruinously affected the development of science. Everything that could not be explained with the ideas and postulates of this school was swept aside as nonexistent and inessential. The tame environment wished to forget the known postulate, "the leader, to whom people dare not protest, always say ditto and who is named inerrable, is doomed." (Suvorov 2003).

From the data of Moster and Quackenbush (1952a, b), it was *allowable* to make the following conclusions: There is a correlation between the contents of Viol and Lut. An increase in temperature turns the reaction backwards. Illumination promotes the accumulation of Zea with a corresponding decrease in Car content, which also allows the proposal that there is a correlation with the change in pigment content. Contrary to postulates of Sapozhnikov and coworkers, intensive illumination facilitates the accumulation of Viol and a corresponding decrease in Lut. This very interesting data formed the basis of many years of disputes and lively debates.

One of authors of the present book, as a young specialist already having experience with spectral and radioisotope research methods in the laboratories of O. V. Zalenskii and M. J. Shkolnik, was invited to enter a PhD program under the supervision of D. I. Sapozhnikov. The postgraduate student was set the task of proving the direct transformation of the carbon skeleton of the Viol molecule into the molecule of Lut and, with this experiment, confirming and more reliably substantiating Sapozhnikov's theory at a new methodological level. Illusions disappeared in a year and a half. It was shown that the carbon skeleton of infiltrated or introduced preparations of radiochemically pure ^{14}C-Viol during the process of metabolism in leaves and chloroplasts turns into molecules of Neo, Lut + Zea, and Car. As a consequence of the intransigent insistence of Sapozhnikov, only partial data that supported the scientific position of the supervisor were published (Sapozhnikov and Saakov 1962). However, after discussion of the work with academician A. I. Oparin, Oparin recommended the experimental data for publication in the *Reports of USSR Academy of Sciences* in a more complete form but contradicting D. I. Sapozhnikov's position (Saakov 1963a, b, c, d). Publications that try to reanimate the thesis about the direct conversion of Viol into Lut sometimes appear, the authors do not want to understand that they seem to be serious researchers only for themselves, when they see in a mirror.

Years passed. The scientific result was practically zero. Academician N.M. Sissakian, supervising space cosmos biology and medicine at that time, initially paid attention to the discussed concept and to its possible functional role and practical application. The Viol–Lut system, advertised as the link in the transformation of water oxygen to molecular oxygen, was suggested for use in increasing the generation of oxygen in ecological closed systems. Sissakian approached the theme critically and saw the hopelessness and far-fetched nature of this direction for investigations concerned with life-support systems. This was the opinion of Institute of Medical and biologic problems Acad. of Sci. USSR. It contradicted the discussed thesis of Sapozhnikov concerning the participation of xanthophylls in carriage of water oxygen for space ships supply. However, finally

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and forever, it became complicated to establish the boundary between fact and fiction, as shown by the sentences on the concept of Viol transformation into Lut described in the review by Hager (1980) and in the short, sharp publication in the world-famous journal *Nature* (Haspel-Horvatovicova 1966).

It is necessary to give credit to the author of the position of direct Viol transformation into Lut, who after long years of perseverance and sharp discussions was finally forced under the pressure of facts to reject his point of view (Sapozhnikov 1973). In the end, with this statement he reversed the situation of the lost debut into his apparent benefit. It was made finely and, as always, "wisely."

Scientists (and their coworkers) simply, as if at a command, forgot "the theory" and its functional value. This is wonderful because for a long time it has been known that reaching the truth is always tragic and coupled with dramatic collisions of ideas, characters, and fates. The development of science results from the fact that the researcher, after advancing a hypothesis, has to change it and conform old ideas to new data. This does not always occur, however, nor only by great scientists, and this is the result of doubt. Indeed, it is not easy to judge oneself, and one cannot fail to respect those who have enough civic courage to do this.

Despite the fact that the contrived nature of two concepts of Sapozhnikov and coworkers was rejected by time and further substantiated experiments performed in various countries, there sometimes appear in the Russian press "especially original" communications, in which the balance on conversions of the two xanthophylls is considered.

We recall the history of pigment biochemistry not for renunciation of ourselves at that time, nor for transference of present knowledge onto the past, but to accent all the difficulties of evolution in reaching the truth. Actually, ideas about the transformations of xanthophylls were formed over more than 50 years as a result of efforts of biochemists from different countries studying animals and plants and using dissimilar methodological platforms in the hunt for truth. In this respect, it is difficult to agree with the obstinate rectilinear concepts described in ten and a half defended and forgotten theses.

Over the years, a more thorough and more objective court appeared, the precise experiment. By the way, from one article to the next, Calvin often changed his working hypotheses about the primary product of the inclusion of carbon in the process of photosynthesis, and this did not decrease his great role in science. Nobel laureate D. Arnon acted similarly with his working hypotheses concerning the mechanism of photophosphorylation.

Over a number of years, one of the authors of this book (Saakov 1963a, b, c, d, 1964, 1965a, b, 1966, 1967, 1968a, b, 1990a, b, c, 1970a, Saakov 1989a, b, 2005a, b; Saakov and Shiryaeva 1967; Saakov and Nasarova 1970; Sagromsky and Saakov 1970) investigated the reaction of transformation of Viol, which required using synthesized labeled preparations of Car and highly pure xanthophylls (Saakov 1963a, b, c, d, 1964, 1965a, b, 1966, 1967, 1968a, b, 1970a, 1989a, b, 1990b, 2005a, b; Saakov and Shiryaeva 1967; Saakov and Nasarova 1970; Sagromsky and Saakov 1970). Data about the connection of band 460–470 nm, which is present in the second derivative of the spectrum of native leaves of a number of barley

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mutants lacking chlorophyll *b*, with the manifestation of Viol de-epoxidation, were also obtained (Maslova and Meister 1969; Saakov 1971a; Sagromsky and Saakov 1970).

We refer to a large number of articles because this reflects the long-standing stages of the improvement in procedures for radiochemical purification of carotenoids and because there is a question about the reaction directivity of carotenoid biosynthesis inside and outside chloroplasts. This topic of the directivity of carotenoid biosynthesis reactions and the speed of inclusion of new carbon atoms of photosynthetic origin in carotenoid molecules was not able to be discussed. This question was not subject to consideration for many years because it was completely dominated by the known specialist in carotenoids, T. W. Goodwin (Liverpool, UK) (Rogers et al. 1967; Wieckowski and Goodwin 1967; Goodwin 1971). But the irrefutable data of the French and German researchers M. Rohmer (Strassburg, France) and H.K. Lichtenthaler (Karlsruhe, Germany) (Rohmer et al. 1993; Lichtenthaler, et al. 1997a, b; Rohmer 1999; Lichtenthaler 2000b), were published in independent European journals, and reported at the symposium on prenyl lipids. This is discussed later in Sect. 5.4.

It is useful to discuss the problem of transformation of carotenoids because it is closely associated with the active application of spectral analysis for the characterization of some carotenoids. It is accepted to consider their oxidized forms (xanthophylls) as derivatives of α-Car or β-Car. The characteristics of these pigments are described in books edited by Goodwin (1965) and O. Isler (Basel, Switzerland) (1971). However, for works concerned with radiochemical purification of samples, using only the usual spectral parameters was not sufficient. Call of the times required the additional characterization of substances using derived absorption spectra in addition to existing criteria of radiochemical purification, such as invariance of the specific activity of a substance in the process of its repeated re-chromatography, absence of extraneous colorless admixtures on radioautographs, localization of the substance spot and invariance of its R_f on chromatograms, and spectral characterization of the compound. For the past 35 years we have been using these criteria in our works. Note that when considering the radioactivity of various pigment fractions in our experiments, we are referring to preparations that passed radiochemical purification criteria and control using derived spectra. Unfortunately, in many works by our European colleagues performed with labeled preparations, this part of the experiment was not characterized with sufficient strictness.

Elaboration of a more thorough methodological approach, free from the shortcomings of previous studies of this question, allowed us to establish another sequence of steps of the considered reaction and to show that the loss of epoxy groups in the Viol molecule does not occur simultaneously in both ionone rings and that the phenomena of xanthophyll transformation are not conditioned by extraneous (other) dyed or colorless admixtures (Saakov and Shiryaeva 1967; Saakov and Nasarova 1970; Saakov 1990d).

Because we cite many European and Russian articles in our reviews and because the state of the question is described fully enough (Saakov and Konovalov 1966;

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Saakov et al. 1970a, b, c, 1971b; Saakov 1989a, 1990a, c), we only include references to the most necessary literature.

Our early experiences showed that the process of de-epoxidation does not stop at dihydroxy derivatives, but that the Viol molecule, containing four atoms of oxygen, is restored to Car (Saakov 1963a, b, c, d, 1964, 1965a, b, 1966, 1967, 1968a, b, 1969a, b, 1989a, b, 1990a, b, c, 2005a, b; Saakov and Shiryaeva 1967; Saakov and Nasarova 1970; Sagromsky and Saakov 1970). Soon after our first articles on this subject, publications by C. Costes (1963a, b, 1968) appeared, in which the author referred to our studies and confirmed their main conclusions. These data also indicated the validity of the ideas of Willstätter and Stoll (1913, 1918) and Sapozhnikov (1937) about the possibility of xanthophyll transformation into Car. However, for a long time the scientists of the early twentieth century considered the totality of oxidized carotenoids only as xanthophylls. Later, it was shown that exogenous labeled carotenoids are rapidly drawn into the reaction of interconversion (Saakov and Konovalov 1966; Saakov et al. 1970a, b, c).

Additional evidence for the validity of our conceptions came with the results of experiments assessing the gradation of the specific activities of labeled pigments participating in transformation reactions after the incorporation of exogenous ^{14}C- or ^{3}H-Viol. It was concluded that Car is the end product of the pathway of Viol-reducing transformations, when the basic carbon skeleton of the pigment still remains. High temperatures or addition of the herbicide diuron stopped the reaction. The experiments were carried out with intact leaves and were confirmed by experiments with chloroplasts and algae. Results suggested the existence of a redox cycle of reactions involving the *whole system* of chloroplast carotenoids (Saakov 1964, 1965b, 1970a, b, 1989a, 1990a, b, c). Further work on the migration of ^{14}C- or ^{3}H-carotenoids demonstrated the participation of assumed intermediate components of the cycle in a sequence of reactions of xanthophyll transformation.

5.1 New Data on Derived Spectra of High Orders of Some Carotenoids

To control the purity of introduced and isolated products of carotenoid metabolism, the criteria of high-order derived spectra were used. Performing spectral measurement in two or three solvents with different polarities is necessary to define to what substance a particular spectrum belongs to. Figures 5.1–5.16 show the specific character of derived spectra of high orders (DSHO) for Viol, Lut, Neo, and Car.

Solutions of xanthophylls and Car in polar and nonpolar solvents were spectrophotometrized with a UV–Vis-Specord 40 instrument (Carl Zeiss, Jena, Germany). This instrument prints out positive and negative extrema of spectral curves, which is convenient for initial control of digitalizing operations. The obtained absorption spectra and their derivatives of the fourth order (D^{IV}) were

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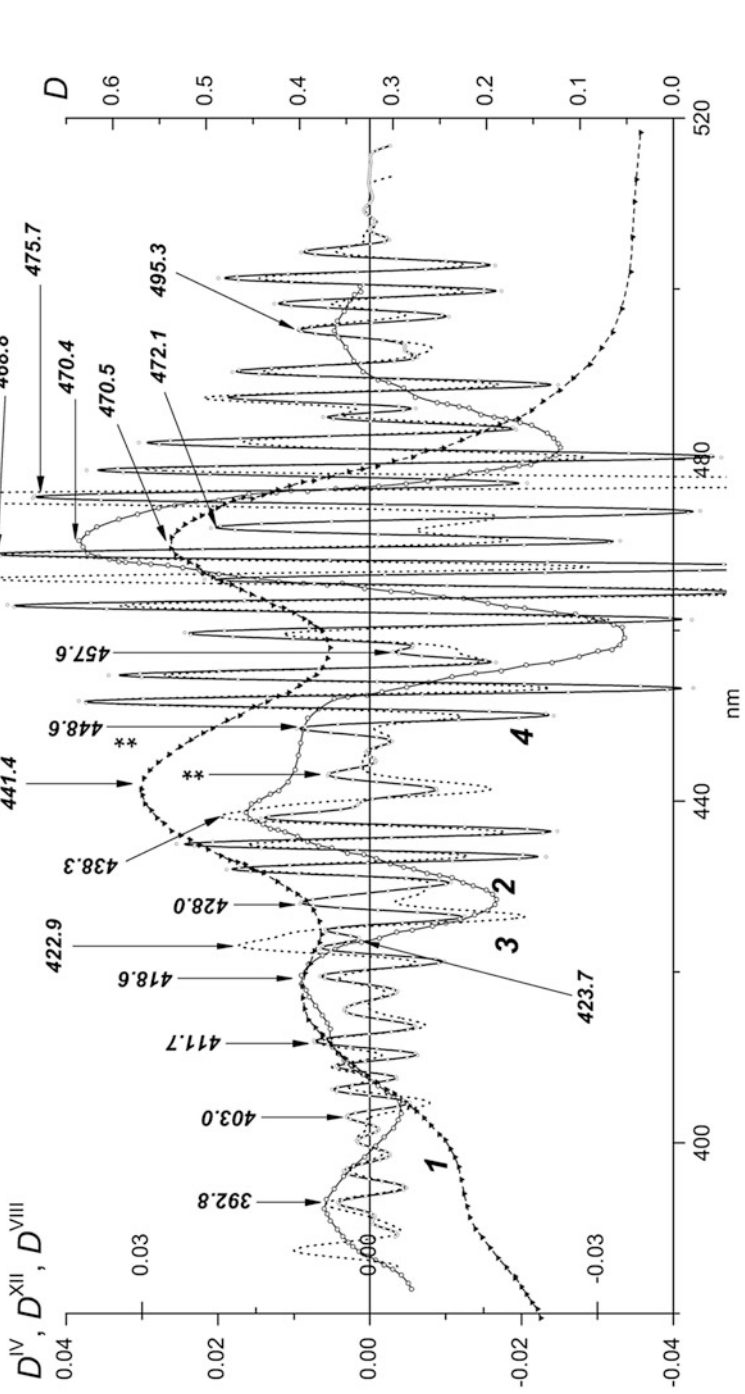

Fig. 5.1 Spectrum of the saponificated and six-time purified Viol solution in ethanol (*1*) and its fourth (*2*), eighth (*3*), and twelfth (*4*) derivatives. *Arrows with two asterisks* indicate characteristic regions of spectra where the use of D^{XII} allows us to isolate the specificity of the fine spectral structure, revelation of which on the D^{VIII} curve is difficult. In further figures, characteristic parts of the spectrum on the D^{XII} curve, where peaks are resolved, are also shown with *asterisks*. *Ordinates* OD, relative units; *abscissa* wavelength, nm

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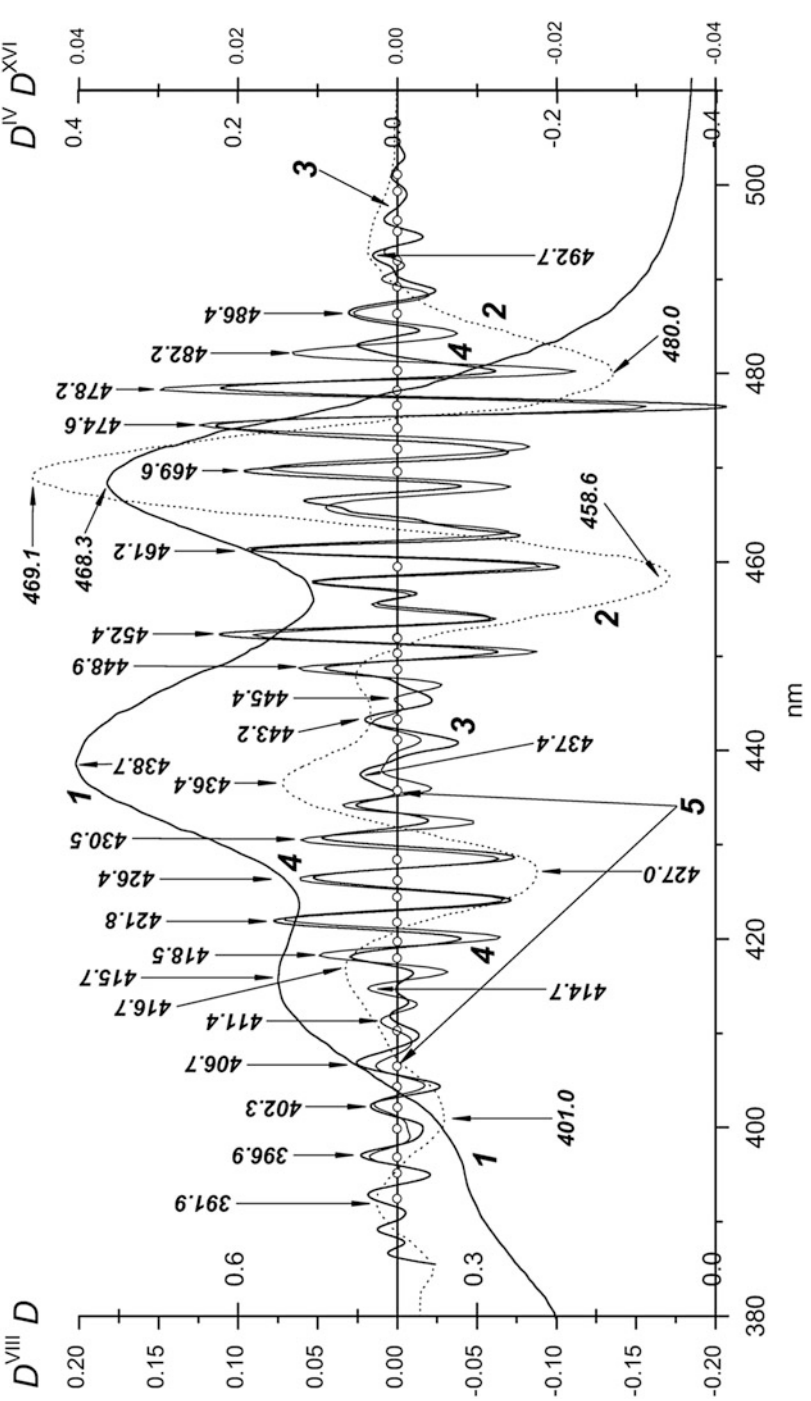

Fig. 5.2 Derived spectra of six-time purified and saponificated Viol solutions in acetone, of the fourth (*2*), eighth (*3*), and sixteenth (*4*) order; (*1*) the absorption spectrum; (*5*) intersection points of the D^{VII} curve of the absorption spectrum with the zero axis. *Ordinates* OD, relative units; *abscissa* wavelength, nm

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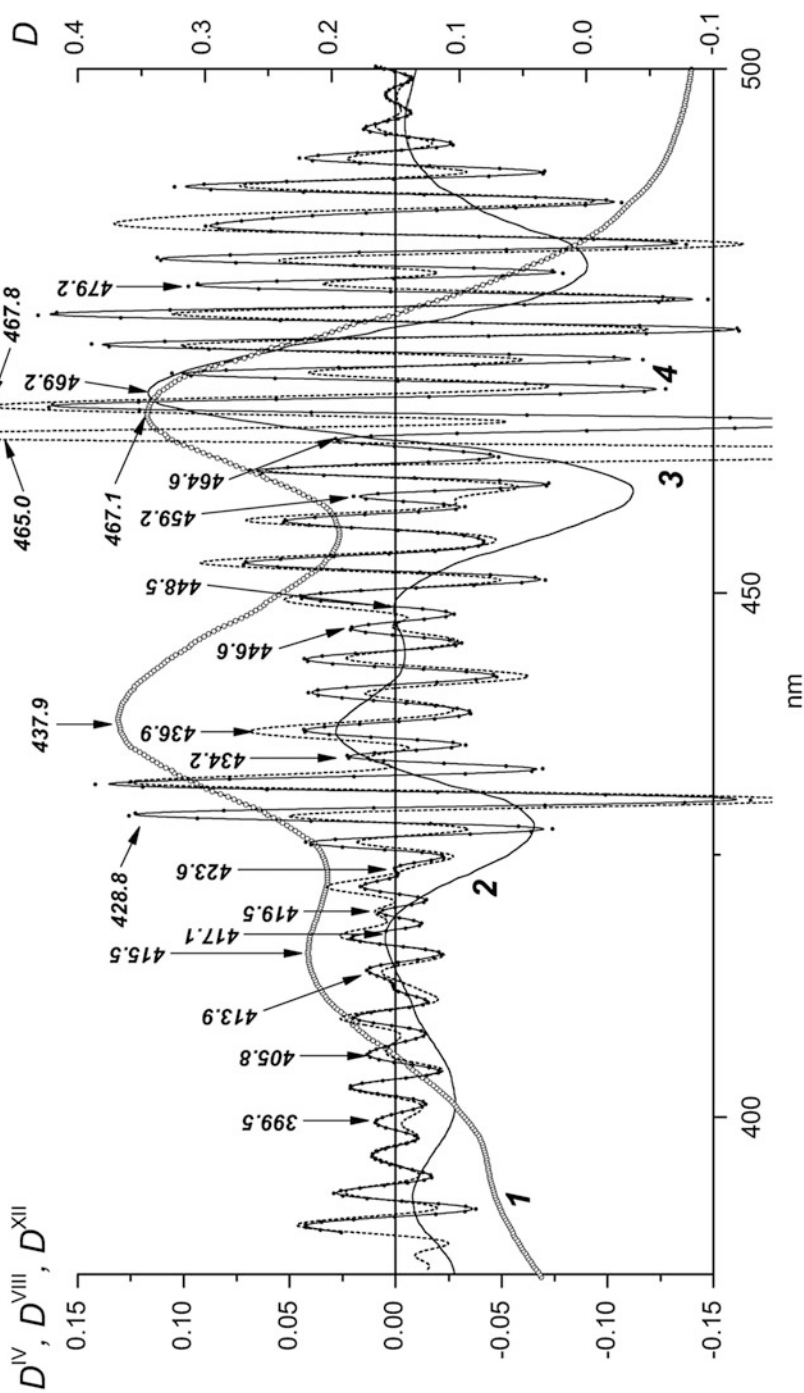

Fig. 5.3 The absorption spectrum of the saponificated and six-time purified Viol solution in methanol (*1*) and its fourth (*2*), eighth (*3*), and twelfth (*4*) derivatives. *Arrows* show absorption maxima for D and D^{IV}. *Arrows* accent regions of difference in D^{VIII} and D^{XII} spectra. *Ordinates* as in Fig. 5.1

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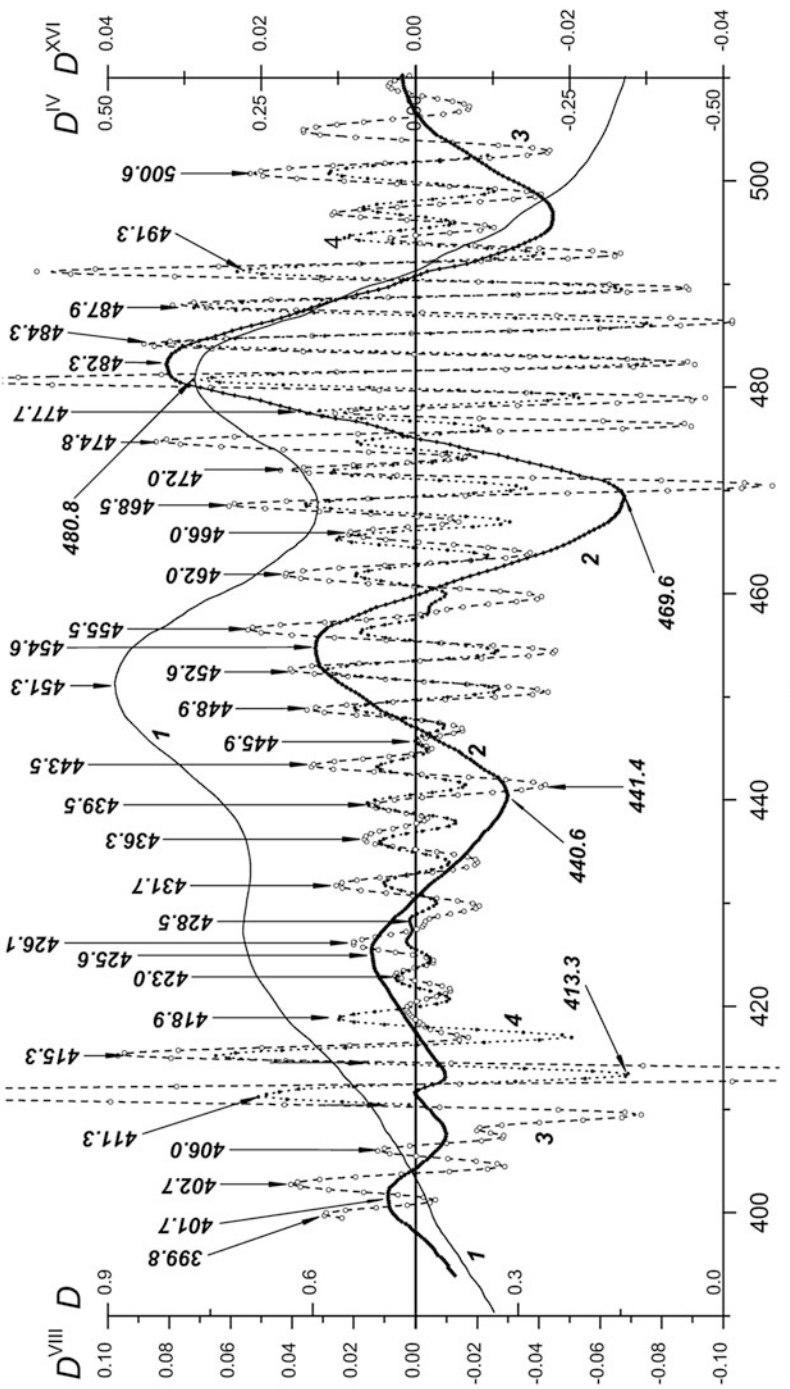

Fig. 5.4 Derived spectra of six-time purified and saponificated Viol solutions in carbon tetrachloride of the fourth (*2*), eighth (*3*), and twelfth (*4*) orders; (*1*) the absorption spectrum. *Ordinates* as in Fig. 5.1

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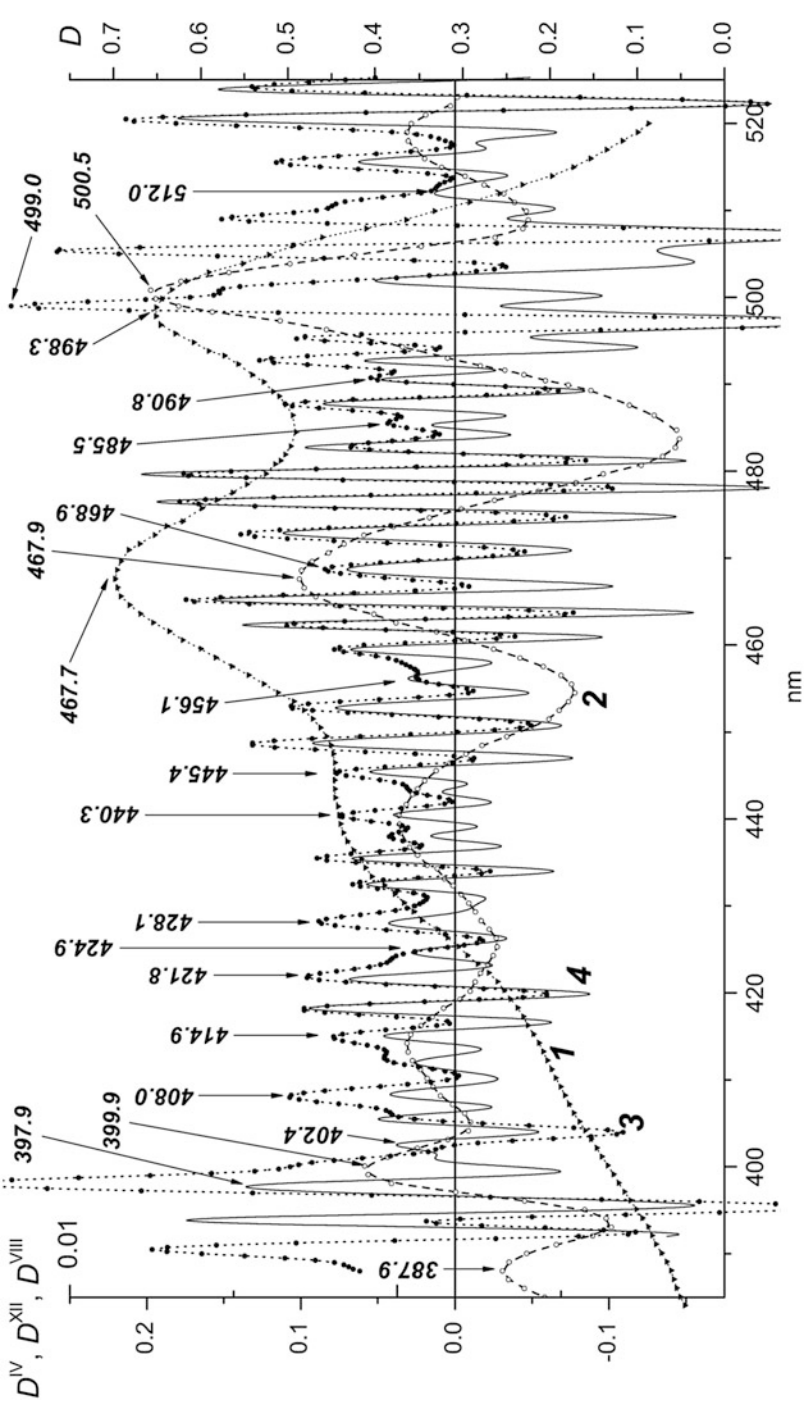

Fig. 5.5 The spectrum of saponificated and six-time purified Viol solution in carbon disulfide (*1*) and its fourth (*2*), eighth (*3*), and twelfth (*4*) derivatives. Designations and *ordinates* as in Fig. 5.1

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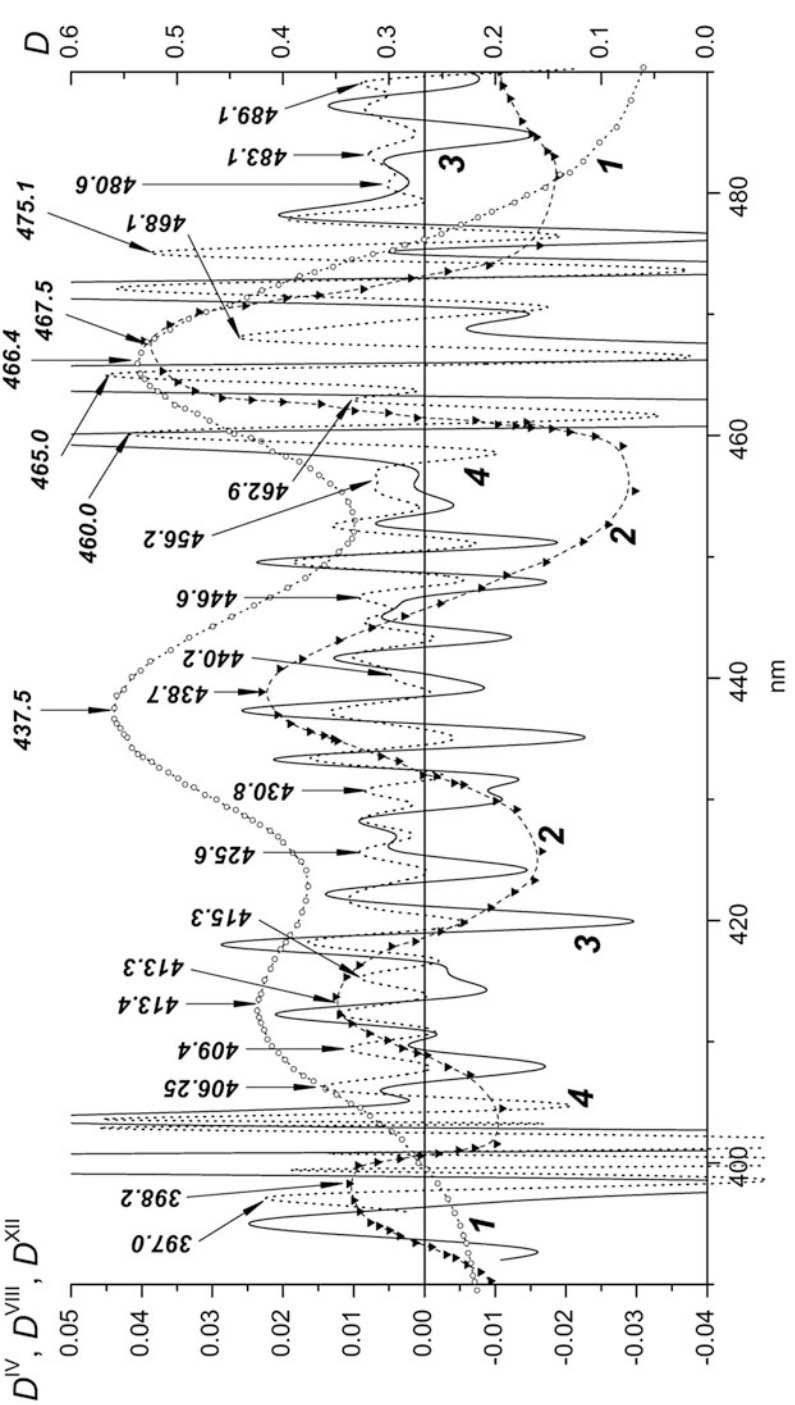

Fig. 5.6 The absorption spectrum of the saponificated and six-time purified Neo solution in ethanol (*1*) and its fourth (*2*), eighth (*3*), and twelfth (*4*) derivatives. Designations and *ordinates* as in Fig. 5.1

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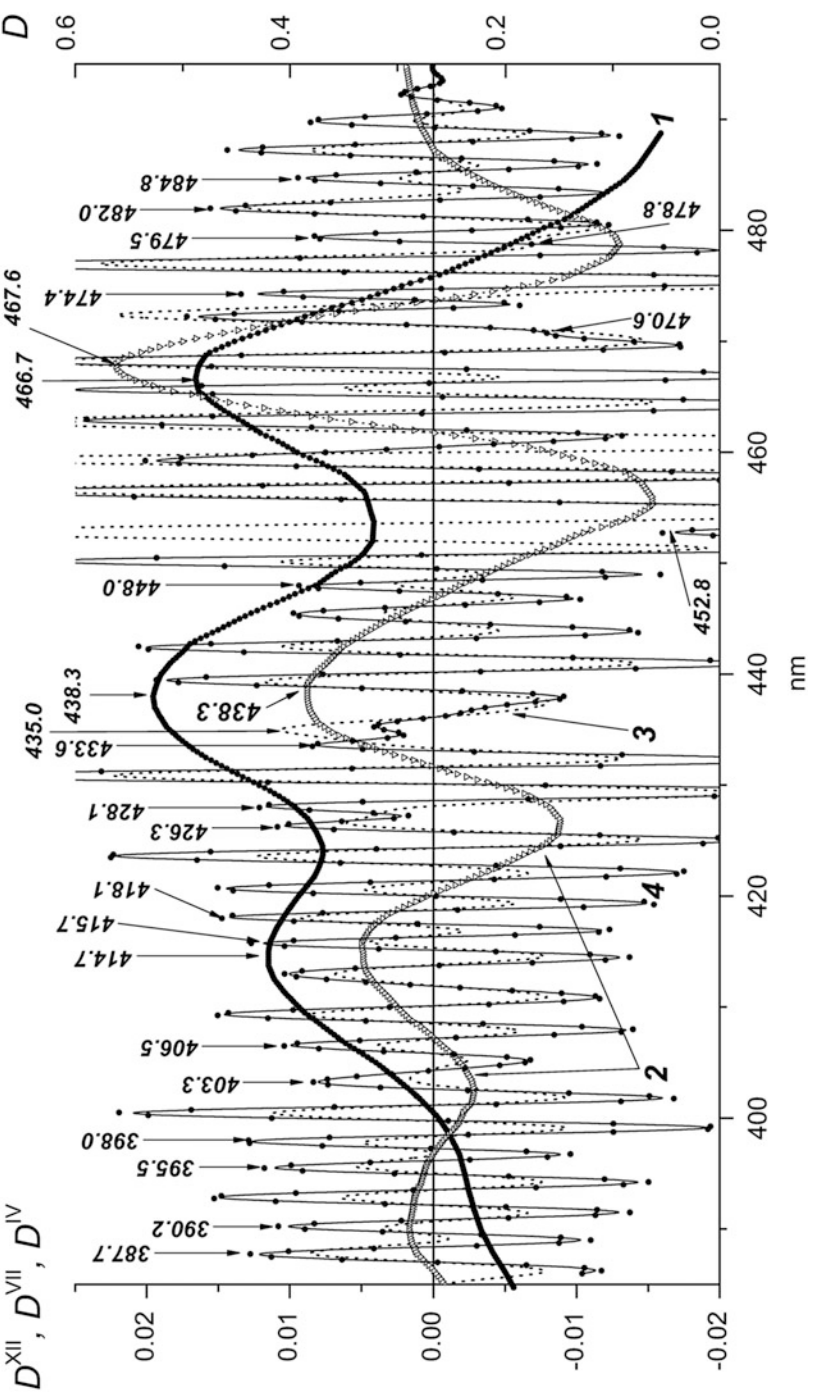

Fig. 5.7 The absorption spectrum of saponificated and five-time purified Neo solution in acetone (*1*) and its fourth (*2*), eighth (*3*), and twelfth (*4*) derivatives. Designations of *ordinates* and *arrows* as in Fig. 5.1

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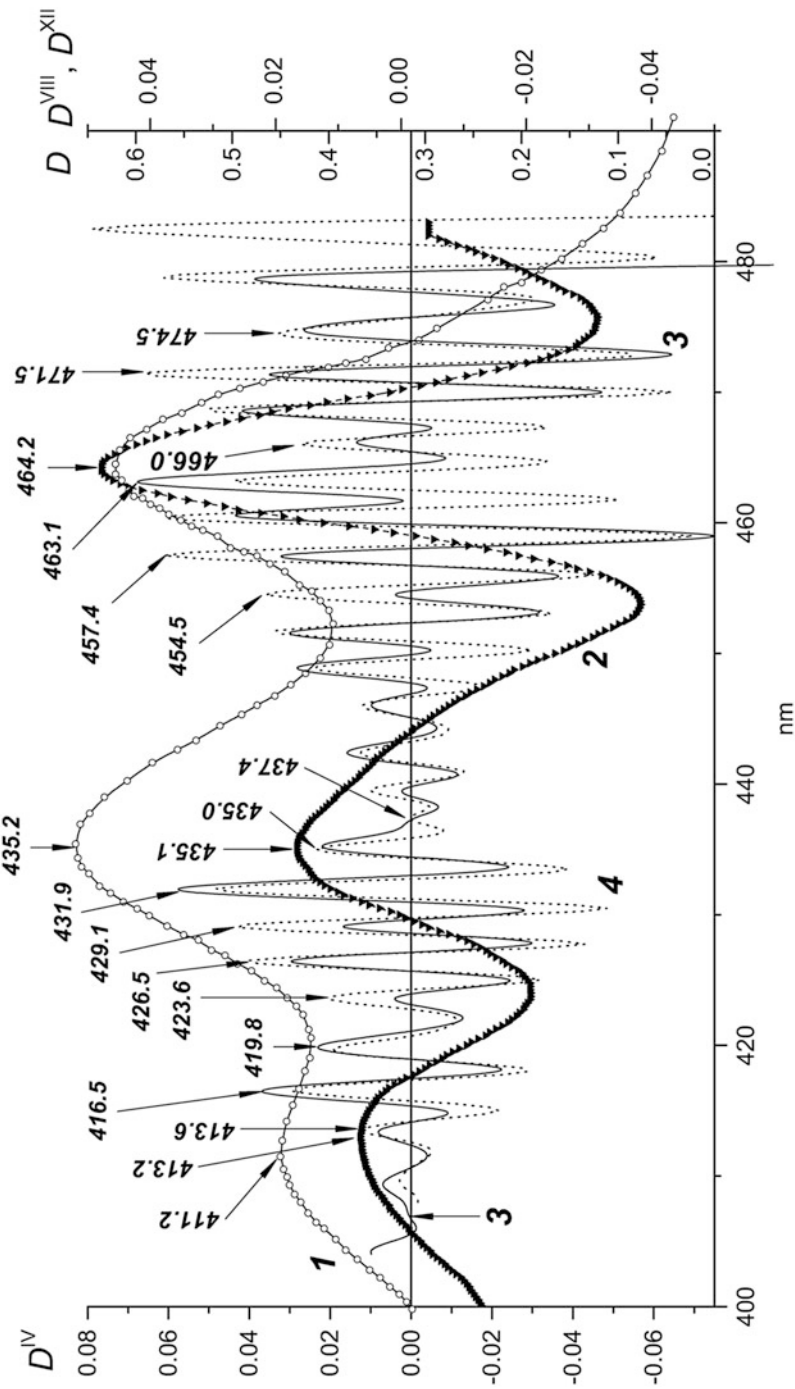

Fig. 5.8 The absorption spectrum of the saponificated and six-time purified Neo solution in methanol (*1*) and its fourth (*2*), eighth (*3*), and twelfth (*4*) derivatives. *Arrows* and *ordinates* as in Fig. 5.1

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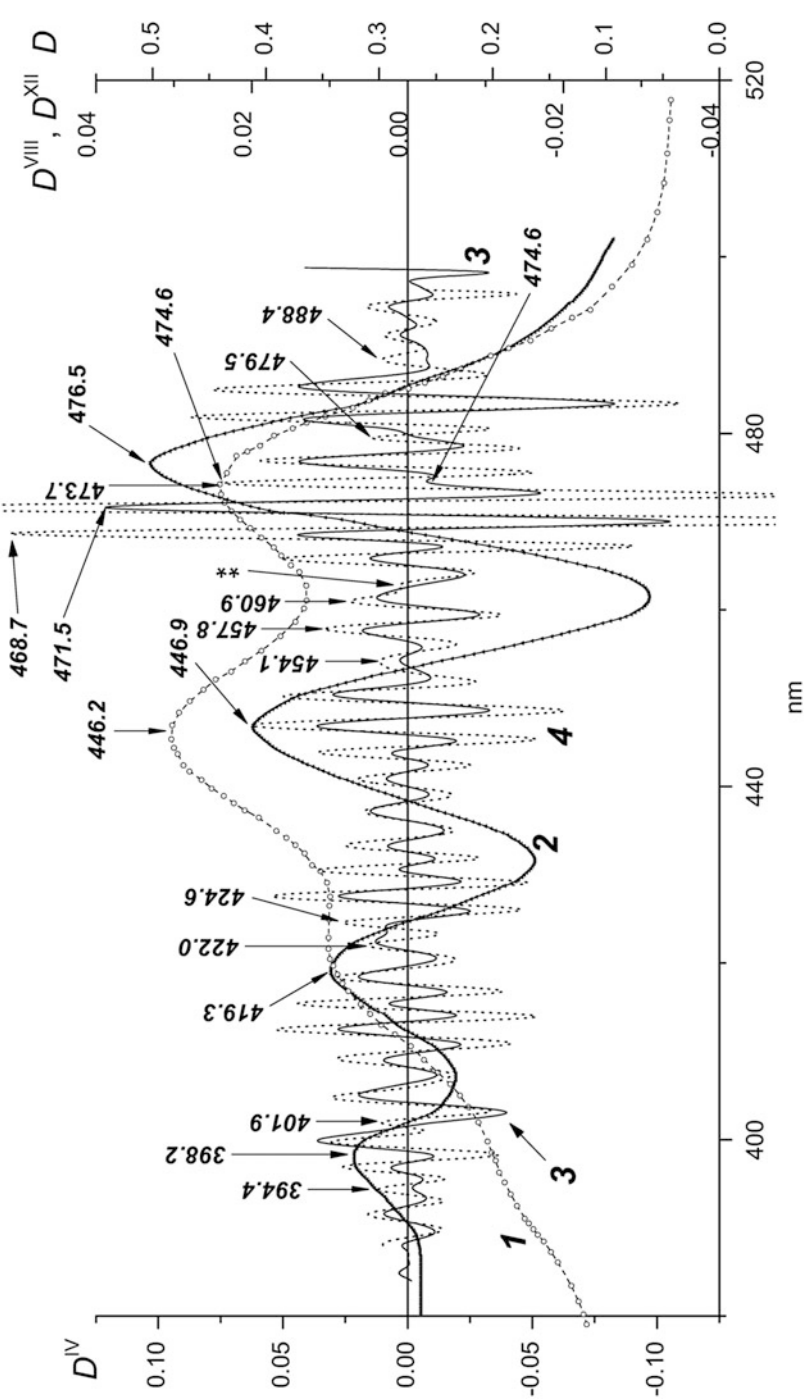

Fig. 5.9 The absorption spectrum of the saponificated and six-time purified Lut solution in ethanol (*1*) and its fourth (*2*), eighth (*3*), and twelfth (*4*) derivatives. Designations and *ordinates* as in Fig. 5.1

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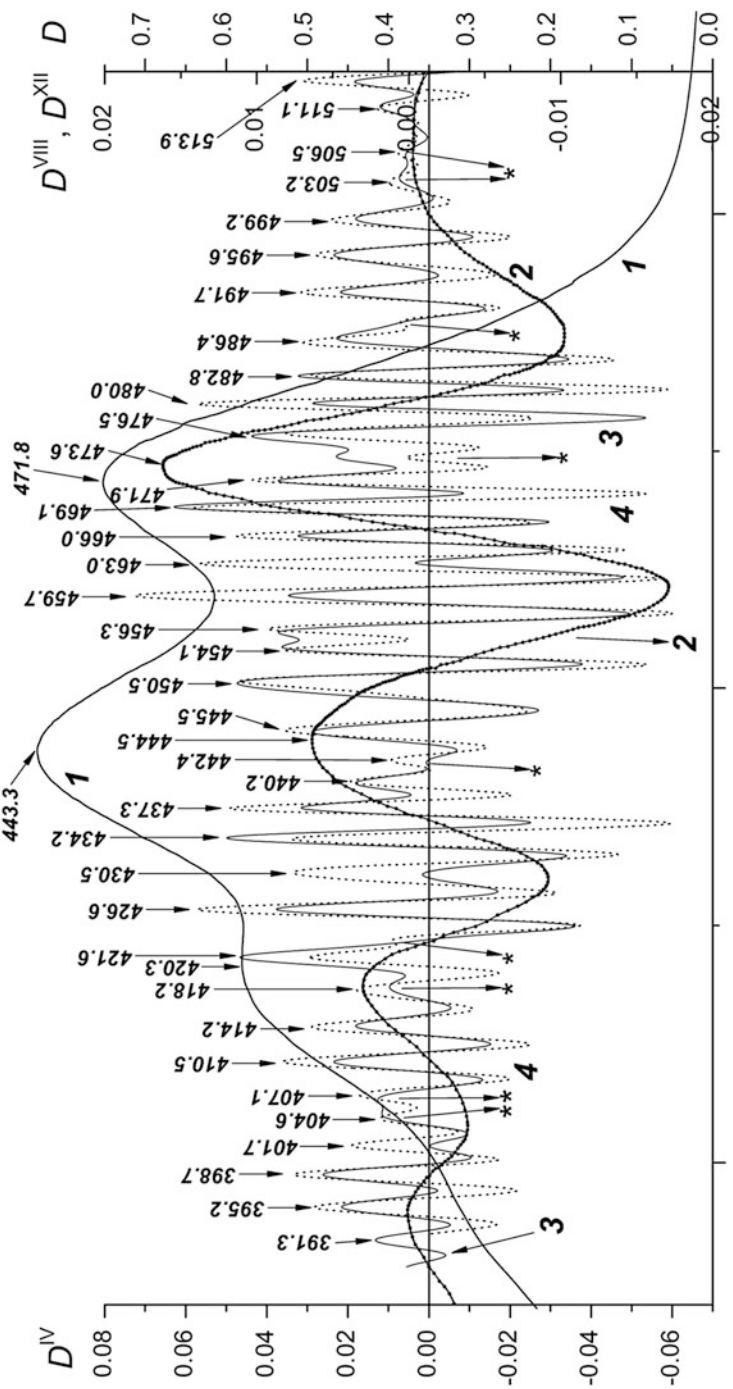

Fig. 5.10 The absorption spectrum of the saponificated and six-time purified Lut solution in methanol (*1*) and its fourth (*2*), eighth (*3*), and twelfth (*4*) derivatives. *Asterisks* and *ordinates* as in Fig. 5.1

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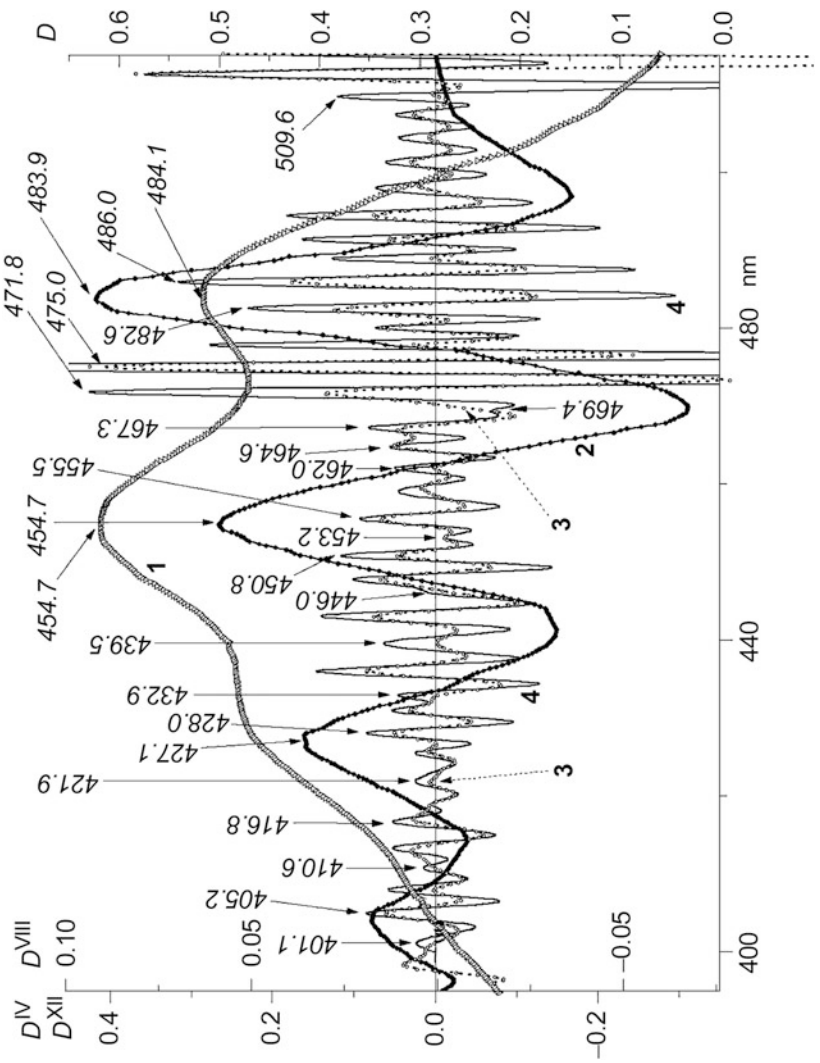

Fig. 5.11 The absorption spectrum of the saponificated and six-time purified Lut solution in carbon tetrachloride (*1*) and its fourth (*2*), eighth (*3*), and twelfth (*4*) derivatives. Designations as in Fig. 5.1

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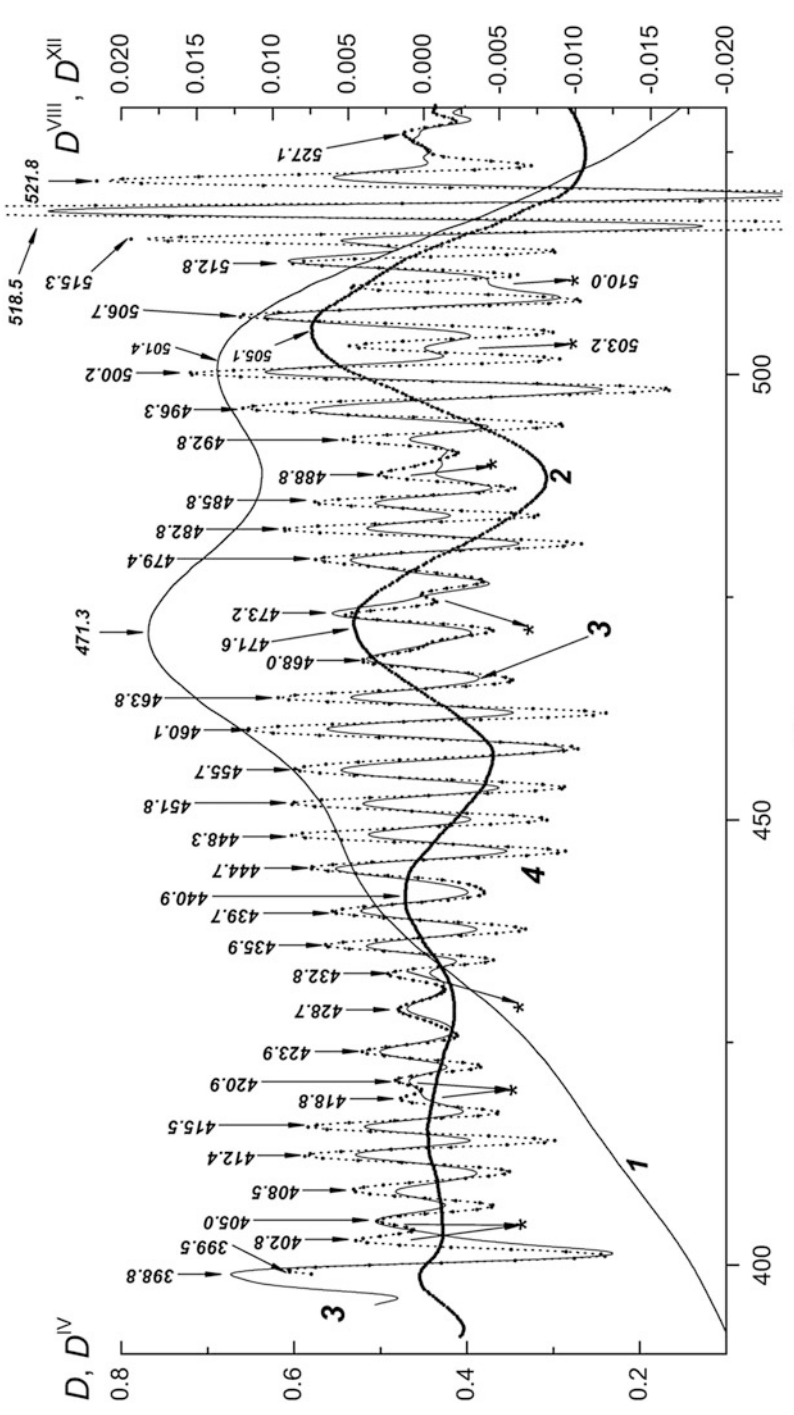

Fig. 5.12 The absorption spectrum of the saponificated and six-time purified radiochemically Lut solution in carbon disulfide (*1*) and its fourth (*2*), eighth (*3*), and twelfth (*4*) derivatives. *Asterisks* and *ordinates* as in Fig. 5.1

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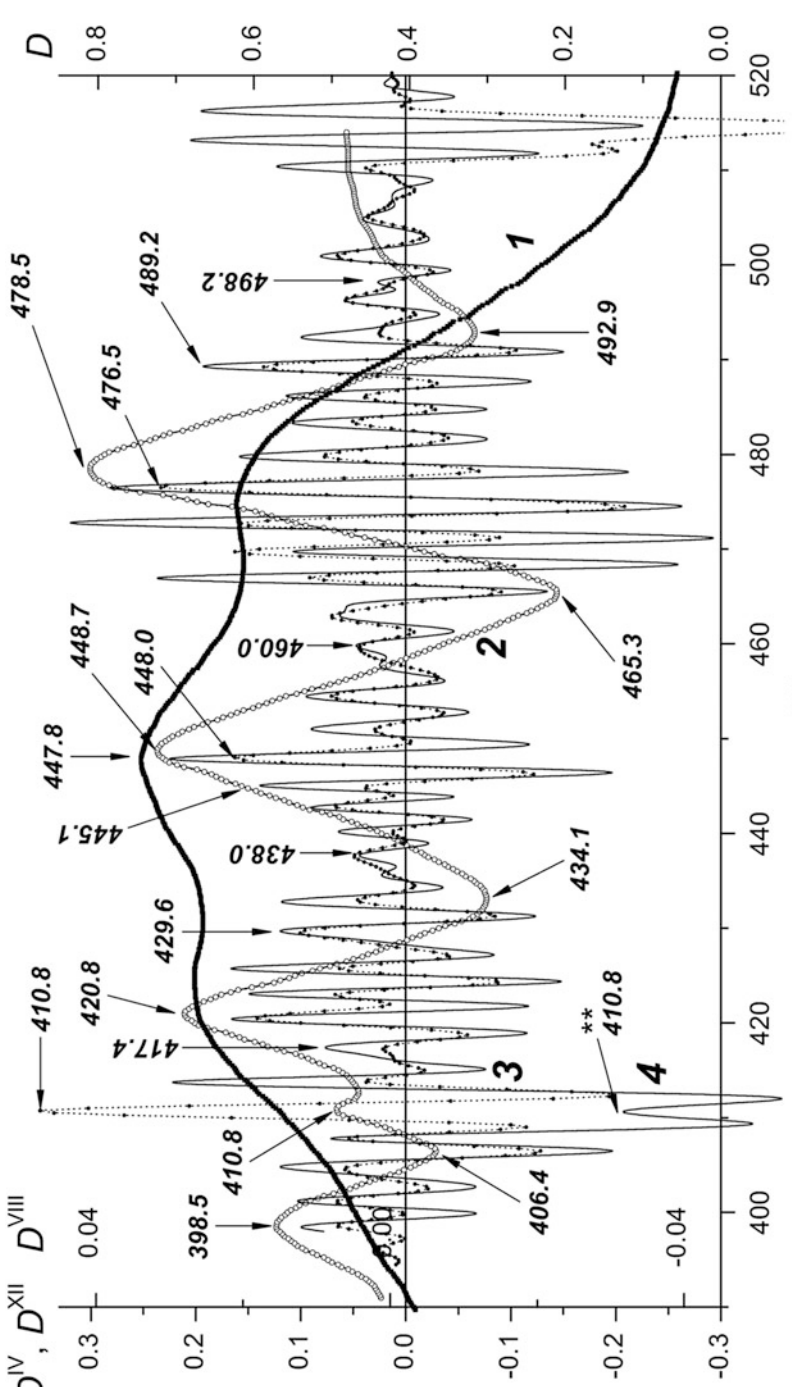

Fig. 5.13 The absorption spectrum of the saponificated and six-time purified Car solution in methanol (*1*) and its fourth (*2*), eighth (*3*), and twelfth (*4*) derivatives. Designations as in Fig. 5.1

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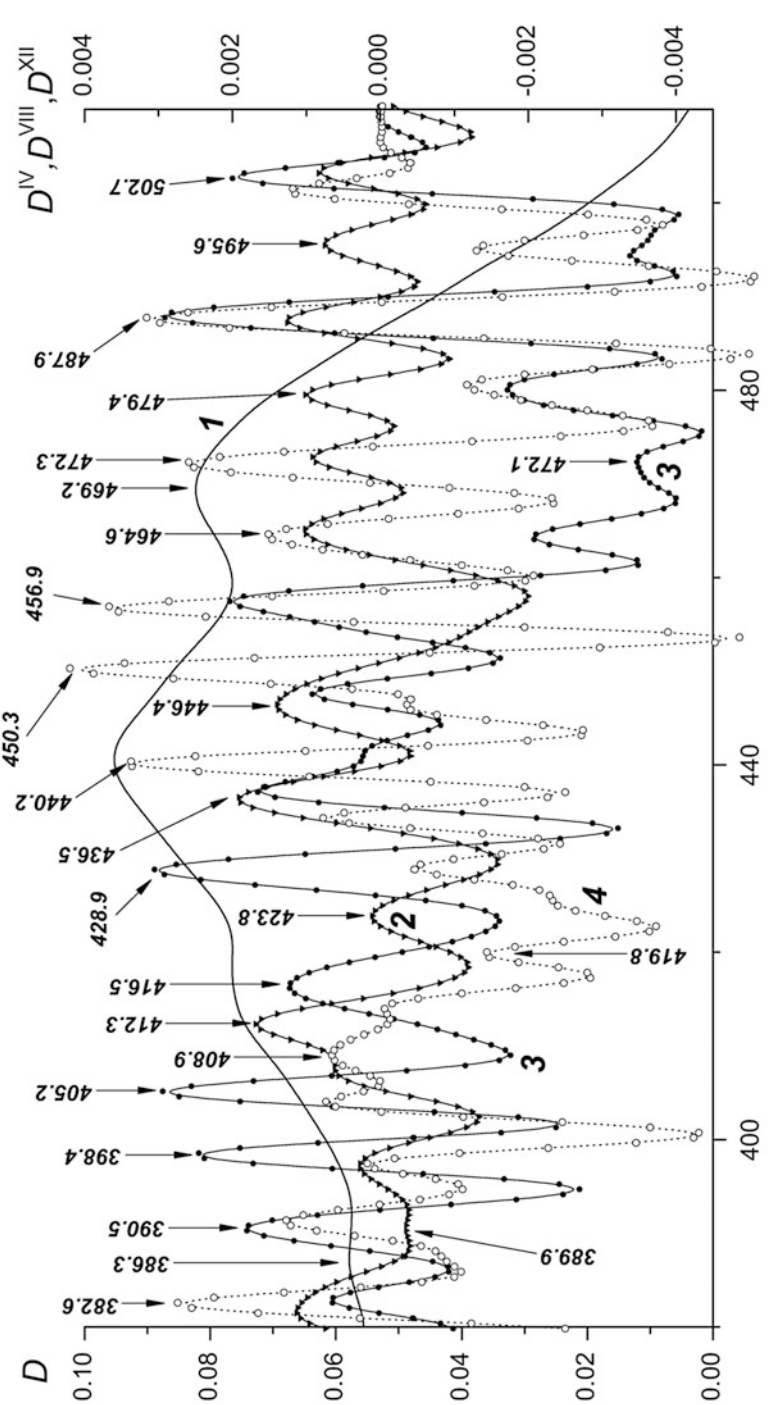

Fig. 5.14 The absorption spectrum of the saponificated and six-time purified Car solution in petroleum ether (30–40 °C) (*1*) and its fourth (*2*), eighth (*3*), and twelfth (*4*) derivatives. The D^{XII} spectrum significantly improves indication of spectral bands absent on the D^{VIII} spectrum. Designations as in Fig. 5.1

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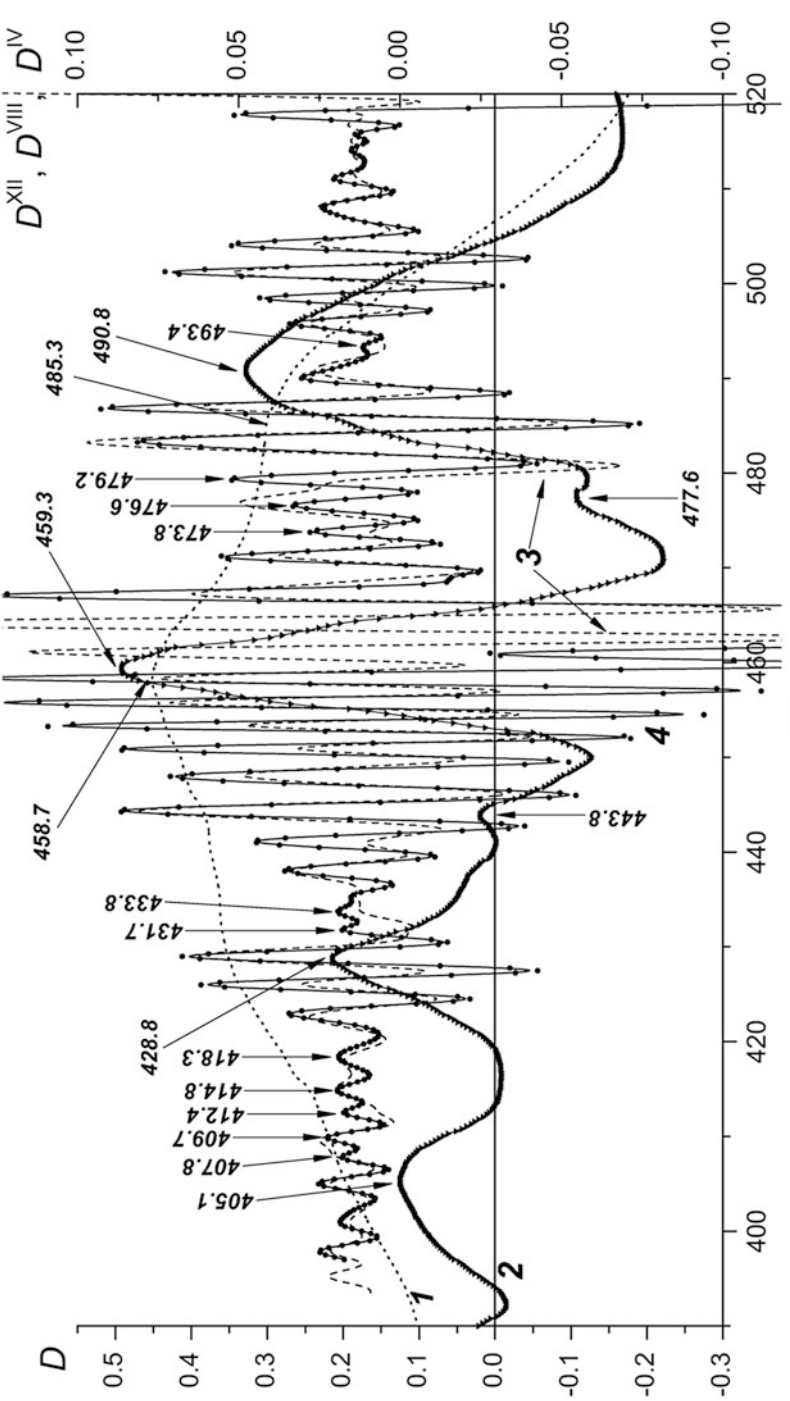

Fig. 5.15 The absorption spectrum of the saponificated and six-time purified Car solution in carbon tetrachloride (*1*) and its fourth (*2*), eighth (*3*), and twelfth (*4*) derivatives. *Arrows* and *axes* as in Fig. 5.1

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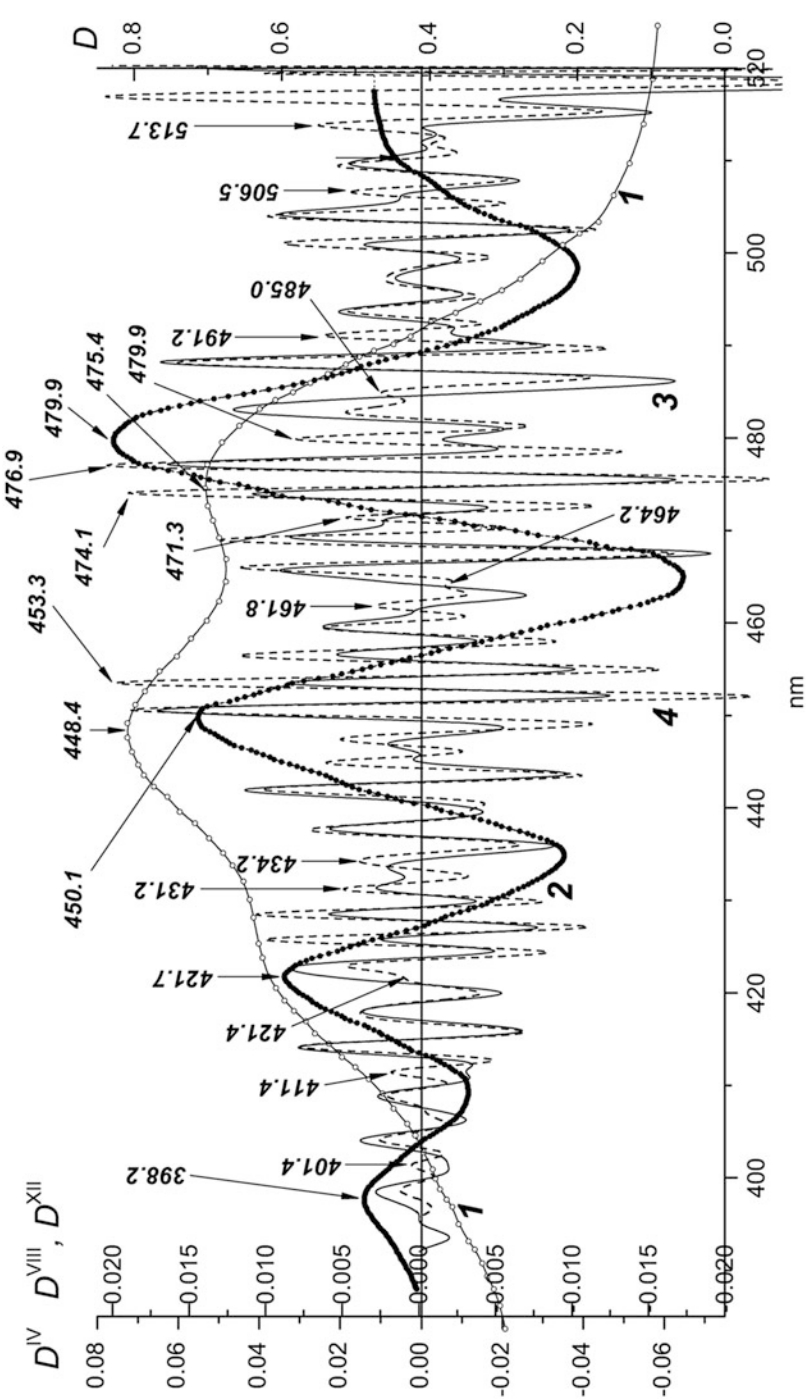

Fig. 5.16 The absorption spectrum of the saponificated and six-time purified Car solution in hexane (*1*) and its fourth (*2*), eighth (*3*), and twelfth (*4*) derivatives. *Arrows* and designations as in Fig. 3.1

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digitalized with the program *Graph Digitizer* version 2.16. Further differentiation of spectra and graphing were carried out with the help of the programs Microcal *Origin* 6.1 and *Spectra Calc*, respectively.

The absorption spectra of carotenoids and their DSHO shown in Figs. 5.1–5.16 were previously absent from the scientific literature. From the data in Fig. 5.1 (curve 2), it follows that application of D^{IV} reliably reveals five absorption maxima in the spectrum of Viol in ethanol, whereas in the curve of spectrum D, only three maxima are reliably found. The advantages of D^{VIII} spectrum do not need to be additionally explained, because the number of bands revealed on the spectrum is practically six times more. The D^{XII} spectrum attracts attention because it allows the determination and localization of band extrema on the wavelength scale. In particular, this concerns those bands that are only weakly expressed in the D^{VIII} spectrum, namely, in λ regions 403.0, 418.6, 428.0, 448.6, 457.6, 472.1, 475.7, 484.9, and 492.9 nm. The properties of the D^{XII} spectrum are especially manifested in the principal absorption maxima at 441.4–443.1 and 448.6 nm (Fig. 5.1, indicated by arrows marked with two asterisks).

For routine analyses, it is necessary and sufficient to register D^{IV} spectra, which reveal specific features of the spectra structure with sufficient obviousness (Figs. 5.1, 5.3, 5.6, and 5.8, curve 2). For better identification, D^{VIII} spectra are recorded. The expediency of D^{XII} spectrum registration can appear in purification of especially pure preparations, which follows from the comparison of curves 3 and 4 in Figs. 5.1, 5.10, and 5.12.

We emphasize that for reliable identification of a pigment, specialists in analytical biochemistry of carotenoids should present its spectral characteristics in polar and nonpolar solvents and sometimes in three or four solvents of different polarity. Therefore, Figs. 5.3–5.5 show the spectra of Viol in three different solvents. Figures 5.5 and 5.12 show the spectra of Viol and Lut in carbon disulfide. In Fig. 5.5 (curve 2), the advantage of D^{IV} spectrum for identification of unobtrusive changes in the D spectrum is visible. Analogously, Fig. 5.1 reveals the advantage of a D^{VIII} spectrum for location of absorption maxima and for identification of bands not manifested on the D^{IV} spectrum. Due to the smaller half-width of spectrum bands on D^{XII} curves, it is possible to specify the location of weakly expressed maxima and bend shoulders of the D^{VIII} spectrum curve. This concerns the identification of spectrum bands in regions with $\lambda = 399.9$, 402.4, 412.0–414.9, 421.8, 424.9, 438.1–439.3–440.3, 443.2–445.4, 456.1–459.7, 485.5–487.7, 498.9–501.9, 512.0–515.4, and 517.7 nm. (Because of the small size of pictures in the book, it is expedient to view scanned figures on a computer monitor using larger scale or color and to observe the specificity of resolution of various bands.)

The same applies to Lut, as can be seen in the absorption spectra in Figs. 5.11 and 5.12 and their D^{IV} to D^{XII} derivatives for Lut in freshly distilled carbon tetrachloride and in carbon disulfide. The finding of four clearly detected peaks on the D^{IV} spectrum illustrates the positive properties of the D^{IV} differentiation method. Curves of the D^{VIII} spectrum have smaller half-width of bands (Fig. 5.11, curve 3), which allows more accurate determination of the location of principal maxima. At the same time, on the D^{XII} spectrum (as in Figs. 5.1 and 5.2), there are

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regions where it is possible to identify hidden spectral bands better or identify those only intended to appear on the D^{VIII} curve. This concerns regions at $\lambda = 401.1$, 408.0, 410.6, 416.8, 418.9, 421.9, 425.5, 428.0, and 432.9 nm. The character of the change in curve contour in transfer from D^{IV} to D^{XII} is noticeable in the region of 446 nm and also at 453.2, 462.0, 464.6, 467.3, 471.7, 488.9, 498.1, 501.3, 504.4, 507.4, and 509.6 nm. For the qualitative identification of pigments, the value of D^{XII} bands is difficult to overestimate (Saakov and Nasarova 1970; Saakov and Konovalov 1966; Saakov et al. 1970a, 1971b). In these figures, the positions of determination for more precise detections are shown with arrows on the D^{XII} spectra.

In Fig. 5.13, the Car spectra in methanol are presented. The specific character of derived spectra of fourth and eighth orders (D^{IV}, D^{VIII}) also applies to Car. Disintegration of the peak band is noticeable at $\lambda = 410.8$ nm, as is the appearance of bands, hidden on the D^{VIII} spectrum, in regions $\lambda = 417.5$, 435.6–437.8, 440.1, 444.9, 450.9, 457.65–459.7, 467.1, 492.3, 498.2, and 507.4 nm.

Thus, together with material previously published by us about derived Viol spectra in acetone, carbon tetrachloride, and methanol (Saakov 1993a, 2004); Lut in methanol and carbon disulfide (Saakov 2003b); Car in petroleum ether and in carbon tetrachloride (Saakov 2003b, 2004); and Neo in acetone (Saakov 2004), the presented material creates a reliable basis for carotenoid identification during their isolation and purification in studies of ways of label inclusion in some fractions of pigments (see Sects. 5.2–5.6 and 5.7.4).

In addition to the spectra of carotenoids (Figs. 5.1–5.16), to create a more complete picture of the spectral features of xanthophylls richly presented in chloroplasts, we invite the readers to note the range of pigments, the isolation and purification of which require meticulous work. The spectral characterization of Ant, mutatoxanthin, ζ-Car, phytoene, lycopene, Phytofluene, and neurosporene is seen extremely rarely. Moreover, the peculiarities of the derived spectra of these pigments *are not described anywhere in professional literature*. It became possible to demonstrate the spectra of these compounds as a result of the technical assistance of our long-time friend and coworker, the engineer Gregory Rutman, now working in the USA, in his Saint-Petersburg's archive material on these preparations are kept (see Figs. 5.17–5.23). The authors express their appreciation to Mr. Gregory Rutman.

In this section, data on the DSHO for xanthophylls and Car, appearing earlier in different works, are united for the first time. This is of essential value for the development of analytical biochemistry of pigments for their use in pharmaceutical chemistry and for an assessment of purification criteria of physiological and vitamin preparations. For example, European firms have recently started to produce vitamin A preparations with the addition of Lut and Zea, and now Russian firms are beginning to manufacture bilberry preparations with the addition of Lut that are useful for eyes.

We did not set out to create a derived spectra bank for various compounds and limit ourselves to presentation of spectra of those substances that were actively used in our research work.

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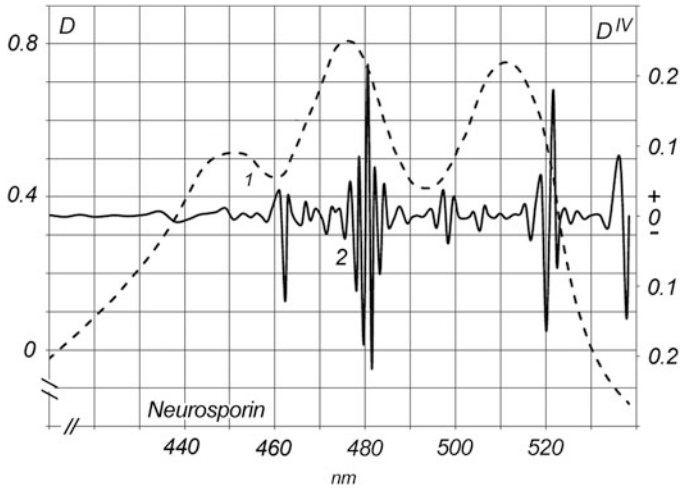

Fig. 5.17 The absorption spectrum of three-time purified neurosporin solution in petroleum ether (40–60 °C) (*1*) and its fourth (*2*) derivative

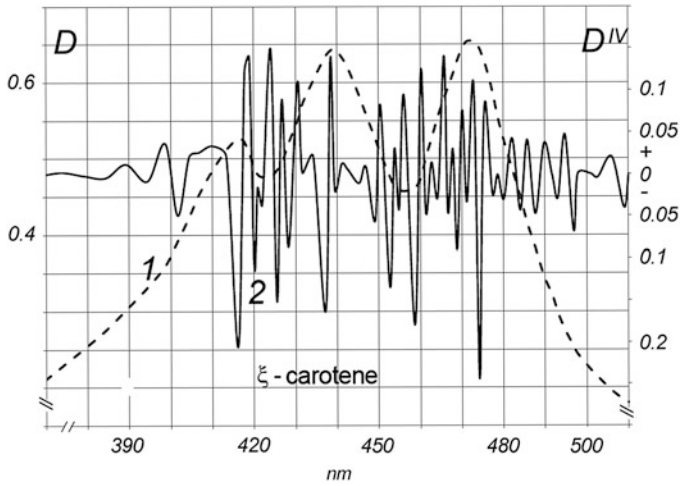

Fig. 5.18 The absorption spectrum of three-time purified ζ-carotene solution in petroleum ether (40–60 °C) (*1*) and its fourth (*2*) derivative

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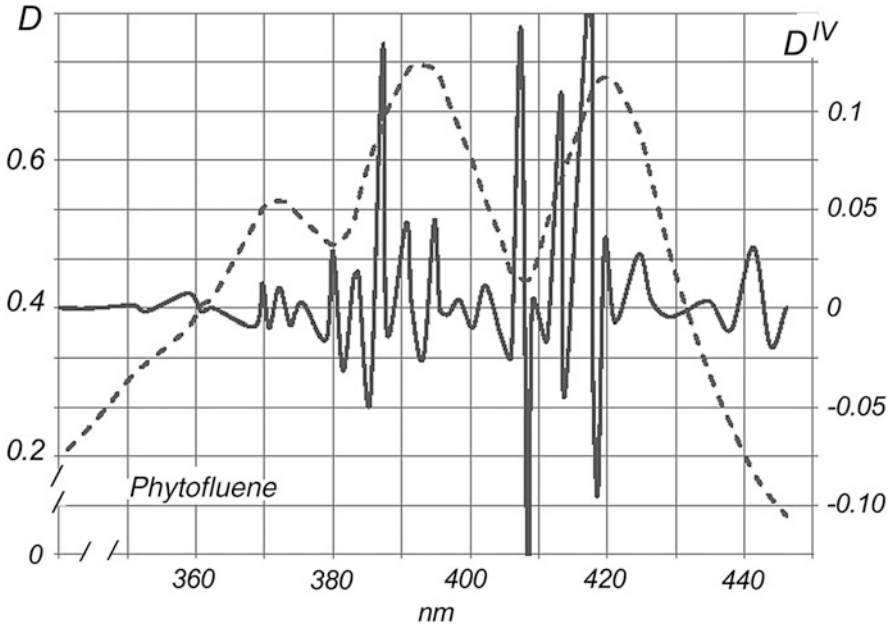

Fig. 5.19 The absorption spectrum of three-time purified Phytofluene solution in petroleum ether (40–60 °C) (*1*) and its fourth (*2*) derivative

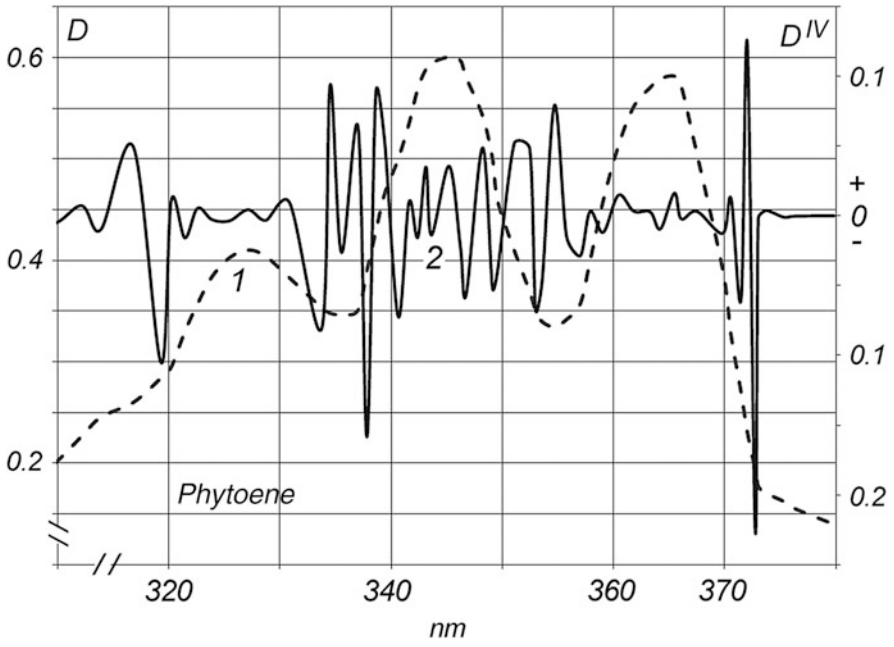

Fig. 5.20 The absorption spectrum of three-time purified Phytoene solution in petroleum ether (40–60 °C) (*1*) and its fourth (*2*) derivative

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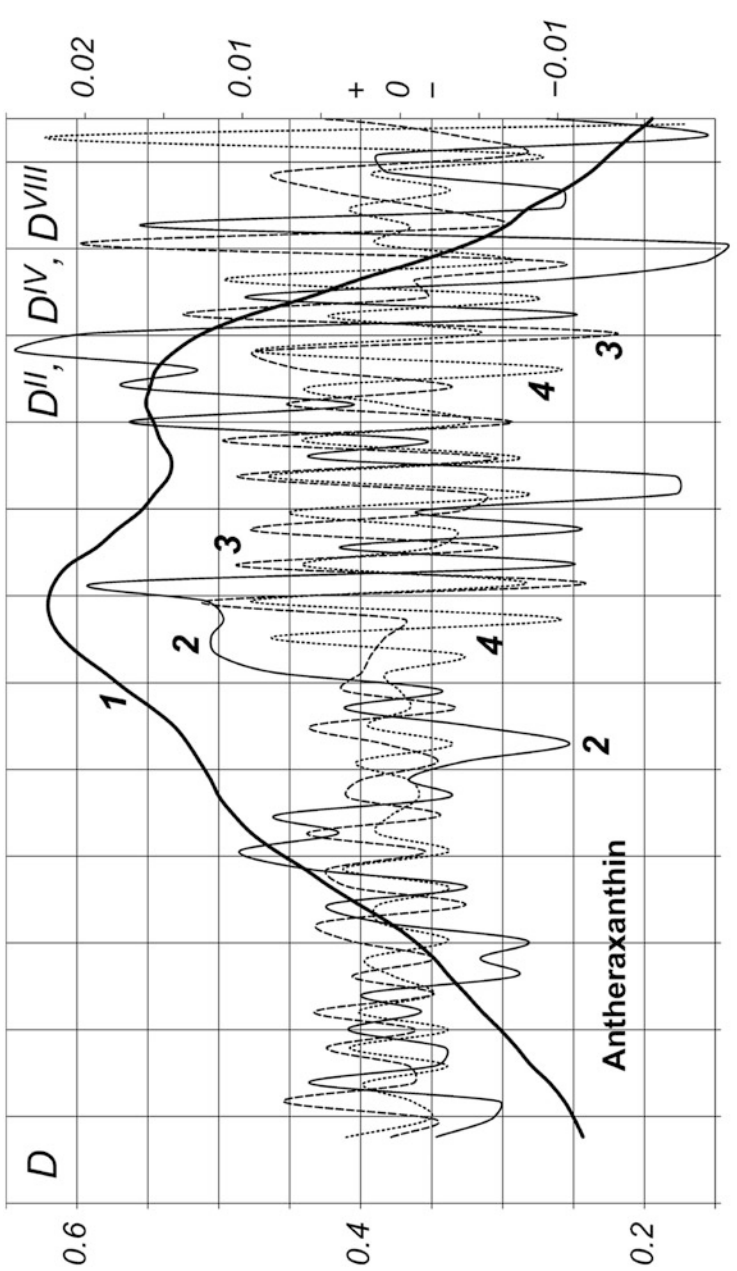

Fig. 5.21 The absorption spectrum of three-time purified antheraxanthin solution in petroleum ether (40–60 °C) (*1*) and its second (*2*), fourth (*3*), and eighth (*4*) derivatives

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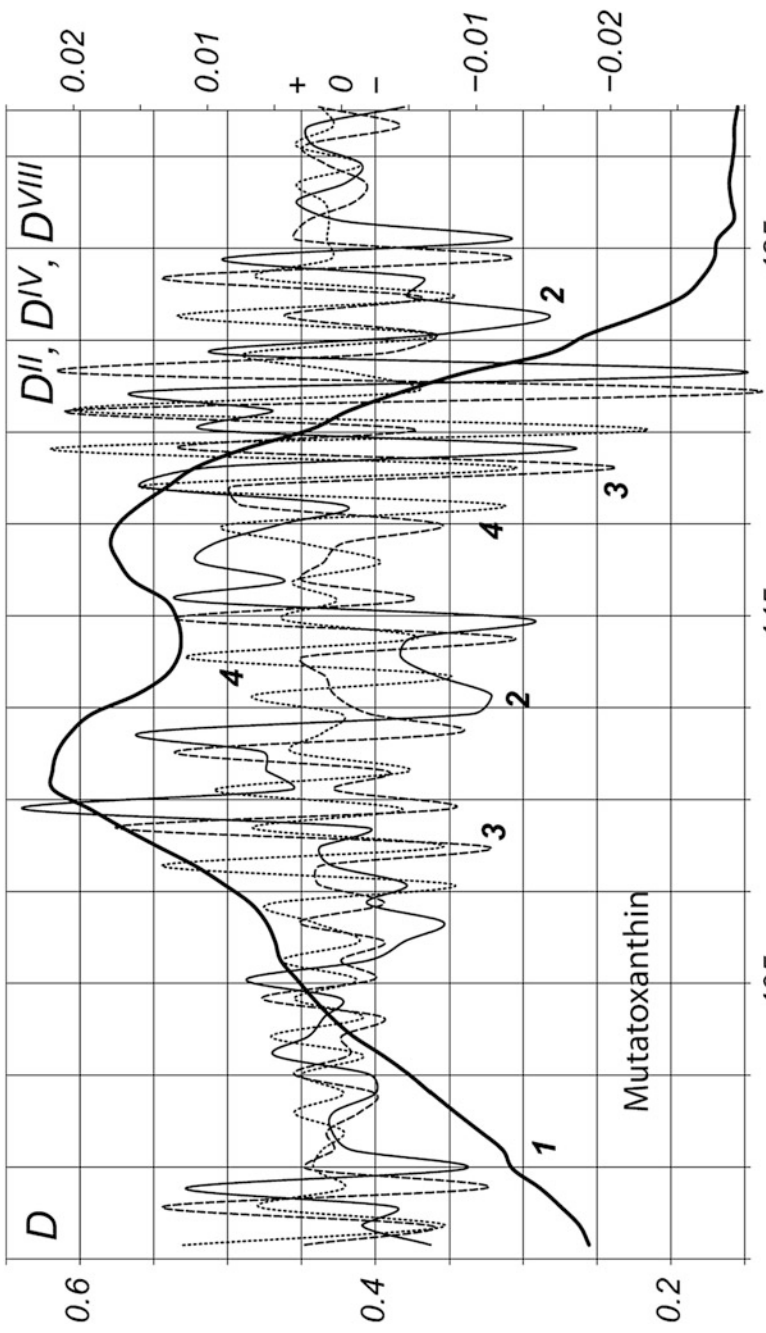

Fig. 5.22 The absorption spectrum of three-time purified mutatoxanthin solution in petroleum ether (40–60 °C) (*1*) and its second (*2*), fourth (*3*), and eighth (*4*) derivatives

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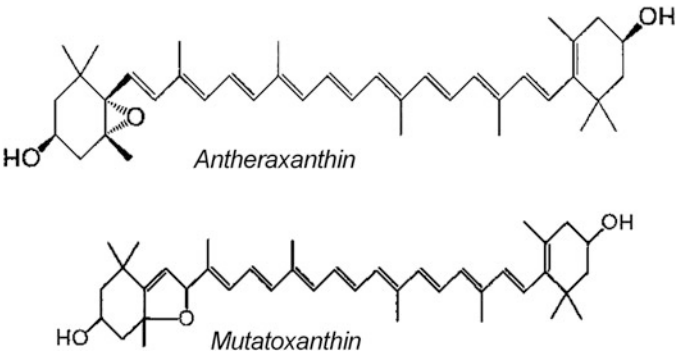

Fig. 5.23 Formulae of two minor xanthophylls, antheraxanthin and mutatoxanthin

The data of Figs. 5.21 and 5.22 reflect features of derivative spectra of two xanthophylls (see formula Fig. 5.23) which differ in their position of oxygen atom in ionone rings. Application of derivative spectra in a more comprehensive sense at an estimation of features of the structural organization and an arrangement of separate nuclear groupings is the perspective analytical way for the characterization of purity of allocated preparations and their structural organization in many experiments in organic chemistry and physical - chemical biochemistry.

5.2 Neoxanthin as a Probable Key Product of Formation of α- and β-Carotenoid Derivatives

During the past 20 years, the role of Zea in processes of protection of living cells from photodynamic effects (Demming-Adams 1990) has been actively discussed. It is supposed that the unused energy of photon flow can be accumulated in photosystems and cause destructive reactions, accompanied both by growth of restored acceptors in reaction centers (RCs) of photosystem 2 (PS-2) and by formation of a triplet state of chlorophyll that promotes the occurrence of singlet oxygen, causing reactions of photo-oxidation in which carotenoids are involved. In a paper by Demming-Adams (1990), Zea transformations in a xanthophyll cycle are not mentioned at all. However, further in this work, it is shown that Zea formation at excess light, when the probability of occurrence of the photodynamic effect is great, is coupled with an increase in the fraction of components of a xanthophyll cycle from 5–15 to 40 %, percentages taken from the sum of chloroplast carotenoids. It is tempting, but doubtful. From a demonstrative field of view, that fact is missing that the quantity of Zea in chloroplasts is extremely insignificant (less than 10 % of Lut fraction). Furthermore, and for an active turnover of its funds, it is necessary for there to be conformity in the speeds of Zea biosynthesis and of the return dark reaction of its oxidization to Viol. The latter is ten times less than the speed of the direct reaction in

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light. So there is a logical disparity in the estimated reaction speeds; more precisely, the following were excluded from consideration: First, during the midday increase in Zea content, its day and 24-h dynamics assume permanent active synthesis and simultaneous removal of Zea funds. Second, the possibility of Zea formation from Lut of other xanthophylls and Car is not taken into account, which results in indistinct and unintelligible interpretation of what occurs with Zea in the process of removal of its excessive funds or during its interaction with singlet oxygen (Demming-Adams 1990). Where are these stores of Zea transformed?

It is important to know the starting and supporting mechanisms of the reaction. During light influence, the Viol quantity decreases, but not to zero, and its pool does not disappear. Zea content increases, but not infinitely. So there is support and updating of Viol pools through some channels and simultaneous degradation of Zea funds. But, as a rule, the least stable compounds are updated. Here, then, there is coupling of biosynthesis reactions (i.e., updatings) and of pigment transformation reactions (degradation of initial pools). Questions of carotenoid biosynthesis are considered in Sect. 5.4.

In spite of the fact that direct evidence for the existence of these transformations was presented a long time ago in the literature, and detailed reviews on the state of the question have been published (Saakov and Konovalov 1966; Saakov 1968a, 1970b, 1989a, 1990a, d Saakov et al. 1970a, 1971b), material attempting to have the last word in the discussion of this uneasy problem continues to appear (Depka et al. 1998). For example, Trebst and coauthors (Bochum, Germany, see Depka et al. 1998) found a decrease in β-Car content in PS-2 in response to intense light and an increase in Zea that was inadequate for the de-epoxidation reaction (DER) (Blass et al. 1959). This result contrasts with the ideas of Calvin, Yamamoto, and Hager about the influence of light on the carotenoid system. This fact was interpreted by Blass et al. (1959) as hydroxylation of Car to Zea in response to light stress. Such an explanation of indirect experiments is not original, because experiments with radiochemically pure ^{14}C-Car, ^{14}C-Lut, ^{14}C-Viol, ^{14}C-Neo, and ^{14}C-Ant (Saakov 1963a, b, d, 1964, 1965a, b, 1966, 1967, 1968a, b, 1970a, b, 1989a, 1990a; Saakov and Shiryaeva 1967; Saakov and Nasarova 1970) and calculation of the corresponding isotope balance reliably proved the possibility of Car transformation into its dihydroxy derivatives at intense light (60,000 lx). With the recommendation of academician A.I. Oparin, these results were published in *Proceedings of the USSR Academy of Sciences* (*Doklady Akad. Nauk.*) (Saakov 1968a), that is, 30 years prior to the publication by Depka et al. (1998). To close this question, it is necessary to remind the reader that in 1989 and 1990 articles were published (Saakov 1989a, 1990a) in a German journal on the oxidizing transformations of labeled Car in chloroplasts and on their inhibition and reactivation. At the same time, articles (Saakov 1989b, 1991) were published in *Reports of USSR Academy of Sciences*, also concerning this topic. However, European researchers did not often notice the obvious (evident proof).

Our data from direct experiments using radioactive labels accented the connection of Car transformations with Lut and Zea and showed the generality of oxidizing transformations of Car in green algae and in animals, found as absence of damage to the initial carbon skeleton of Car. Thus, the mentioned works indicated the presence

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of transformation reactions uniting carotenoids in an integral system (Haspel-Horvatovicova 1966; Saakov 1968a, 1989a) and answered the question about what occurs and where Viol and its dihydroxy derivatives (Zea and Lut) formed in de-epoxidation disappear to. Neither Lut nor Zea can remain infinitely, accumulating in the chloroplast through a DER that is ten times faster than the back epoxidation reaction of Zea or of Lut (Sapozhnikov et al. 1957; Bazhanova and Sapozhnikov 1963; Yamamoto et al. 1962). However, *researchers did not pay attention to this extremely important fact*. We informed foreign colleagues about our works, sending them publications and letters. In 1973, prints of the German articles (Saakov 1973b) were sent to Professor Trebst, in which gratitude was expressed for granted reagents and also our articles were cited. But the colleague did not consider it necessary to mention them. Nevertheless, the priority of research in this field belongs to Soviet researchers (see also Saakov 1968a; Bershtein et al. 1969, 1971, 1970).

In reality, the problems of xanthophyll transformation are significantly wider and deeper than the three components of the xanthophyll system discussed by a number of researchers from Western Europe, the USA, and Russia. For example, Lohr and Wilhelm (1999) experimentally found an important phenomenon in *Phaeodactylum tricornutum*. They proved that Viol is the common precursor for carotenoids with allene and acetylene groups, that is, Viol transforms into diadinoxanthin and then, from it, into fucoxanthin. Peterman et al. (1997) showed the participation of Neo in xanthophyll cycle reactions. Bungard et al. (1999) joined this position when they discovered the appearance of Neo in *Cuscuta reflexa*. Neoxanthin is usually absent in *Cuscuta*, and, instead, lutein-5-6-epoxide accumulation is typical. The appearance of lutein-5-6-epoxide demonstrates the compensatory effect, and the appearance of α-derivatives of xanthophylls can be considered as connected to Neo disappearance in cells. Pogson et al. (1998) made a similar conclusion when working with *Arabidopsis* mutants. The authors supposed that β-Car structurally and functionally compensates Lut in the investigated mutant (Pogson et al. 1996). Sharma and Hall (1996) ascertained that a high Viol content is accompanied by a low quantity of Car in old leaves of sorghum. In the process of photoinhibition, the increase in Zea quantity in new leaves was significantly higher than the decrease in Viol content. The authors concluded that in new leaves the new formation of Zea occurs through an independent pathway that is different to Viol de-epoxidation, that is, they suggested the existence of *a separate pathway of Zea formation not connected with Viol transformations*.

We have illustrated with the presented examples the thesis that, in researching light-induced transformations of xanthophylls, it is absolutely incorrect and inadmissible to limit the sphere of analysis only to consideration of changes in the contents of Viol, Ant, and Zea.

Study of labeled Viol and Lut transformation indicated a role for Neo as a possible key pigment specifying the probable orientation of the reaction during formation of derivative compounds with β- or α-ionone rings (Saakov and Konovalov 1966; Saakov 1970a, b, 1990d, 2005a; Saakov et al. 1970a, 1971b) and also the participation of Zea in these reactions.

In rare works on the light reaction of the xanthophyll cycle, the invariance of Neo content is indicated. Experiments on simple detection of the quantity of xanthophylls

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are not sensitive enough for determination of hardly noticeable changes in Neo content. On the other hand, experiments with radiochemically purified ^{14}C-Neo show the possibility of its transformation into Viol, Lut, and Car (Saakov et al. 1971b). The identification of the Neo allenic structure (Depka et al. 1998) promoted the proof of reality of epoxyxanthophyll transformation and of their furanoid forms into xanthophylls of simpler structure, during treatment with lithium hydride aluminate. In particular, furanoid Neo turned without destruction of its carbon skeleton into Zea, identical to Zea isolated from *Physalis*; Viol into Zea; and epoxy-β-Car into Car (Cholnoky et al. 1969; Bartlett et al. 1969). These experiments ascertained the identity of chemical and biochemical mechanisms of de-epoxidation (Saakov 1990d). In one article (Schnepf and Czygan 1966), the possibility of enzymatic deoxidation of carotenoids of *Arum masculatum* and *Typhonium divaricatum* was investigated, and the authors came to a conclusion similar to that stated above. Thus, experiments of chemists and biochemists created a reliable basis for discussion of the intermediate system (role) of Neo in conversion of Zea metabolism.

Further experiments were carried out on melliferous bees (*Apis mellifera*); dragonflies (*Odonata* sp.); the leaves of pea (*Pisum sativum*), clover (*Trifolium* sp.), and string beans (*Phaseolus vulgaris*); and pea chloroplasts. Methodological questions are discussed in some works (Saakov 1990d, 2005a). ^{14}C-Neo was isolated using the established technique (Saakov et al. 1971b; Saakov 1989a, 1990a), and 25 mCi of $^{14}CO_2$ was added into the biosynthesis chamber to increase the specific activity (SpA) of Neo preparations. An additional control was performed with registration of DSHO, from fourth to twelfth (Saakov 2003b, 2004). The use of D^{XII} derivatives reliably detects specific spectral bands of xanthophylls and Car, invisible or hardly intended on derivative curves of the eighth order (Saakov 2003b, 2004). For qualitative identification of pigments, the value of bands of D^{XII} is difficult to overestimate.

Results of performed experiments are presented in Tables 5.1–5.3. Exogenous labeled Neo undergoes reductive transformations in insect eyes to form Car. The decrease in SpA of intermediate products of metabolism, Zea and Lut, testifies to this. Other pigments with carotenoid structures were not found by us. The obtained data indicate that pigment with an allenic structure of the ionone ring is capable of undergoing, in insect eyes, transformation into a carotenoid with a diene structure, without destruction of the basic carbon skeleton (Bartlett et al. 1969; Saakov 1989a,

Table 5.1 Kinetics of radioactivity inclusion from ^{14}C-neoxanthin into carotenoids of insect eyes

	Specific radioactivity of fractions (imp/min/μg carbon), $n = 3$, variation ±12 %			
	Eyes of bees		Eyes of dragonflies	
Pigment fraction	*Experiment 1*	*Experiment 2*	*Experiment 1*	*Experiment 2*
Lutein	22,000	23,000	31,000	20,000
Zeaxanthin	16,000	19,000	23,000	27,000
Carotene	9,300	8,700	7,600	8,200
Geraniol	1,600	1,800	1,900	2,100

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Table 5.2 Kinetics of radioactivity inclusion from ^{14}C-neoxanthin into isolated chloroplasts of pea

Pigment fraction	Specific radioactivity of pigment fractions (imp/min/μg carbon), $n = 4$, variation ±9 %			
	Experiment 1	*Experiment 2*	*Experiment 3*	*Experiment 4*
			+ Nigericin[a]	+ Salicylaldoxime[b]
Violaxanthin	64,000	76,000	46,000	1,450
Neoxanthin	97,000	101,000	114,000	109,000
Lutein	53,000	62,500	2,700	43,000
Zeaxanthin	28,000	31,000	1,600	24,000
Carotene	8,000	9,500	Traces	3,400

In experiments 1, 3 and 4, 350,000 imp/min was introduced; in experiment 2, 300,000 imp/min was introduced

[a]In the sample, 250 nM of nigericin was added in the presence of 50 nM KCl

[b]Concentration of salicylaldoxime in experiment 4 was 10^{-2} M per sample

1990a, 2003b). However, the detection of radioactivity in geraniol (geraniol pyrophosphate), a possible predecessor of carotenoids, suggests destruction of the initial carbon skeleton at further steps of metabolism.

A further task was specification of the mechanism of geraniol formation, namely, as a result of direct Neo disintegration, or, as consequence, at subsequent steps of disintegration of the carbon skeleton of Car at the 15–15′ carbon atoms.

The medium for chloroplast isolation and the quantity of materials used in the experiment were according to published method (Saakov 1989a, 1990a). From the data in Table 5.2, it follows that in aqueous medium for chloroplast incubation, there is a possible reorganization of the unstable allenic structure at the seventh to eighth atom of carbon (=•=) in the first ionone Neo ring that accompanies both oxidization of Neo into Viol and its reduction, with formation of more reduced carotenoid forms with α- and β-structures of the ionone rings. The character of SpA change indicates the existence of Car in the final link of Neo transformation. Nigericin, an inhibitor of proton transport, substantially decreased the efficiency of a reductive vector of Neo transformation, whereas salicylaldoxime, retarding the return oxidizing reaction of xanthophyll, decreased the formation of Viol from Neo. The direct reaction of Neo reduction into Lut, Zea, and Car was not influenced significantly enough for interpretation.

To check data obtained for suspensions of isolated chloroplasts, *^{14}C*-Neo was infiltrated into string bean leaves for tracking of label movement in fractions of pigments of the live leaf. These results are presented in Table 5.3. From the data in Table 5.2, it follows that transformations of *^{14}C*-Neo in the intact leaf also have oxidizing and reductive vectors and respond to the influence of corresponding inhibitors, as found in chloroplasts. These experiments additionally supported our suggestion (Saakov et al. 1971b) that Neo is a key pigment specifying the orientation of the reduction reaction that forms derivatives with a β- or α-ionone ring. Another author (Gregory 1989) took the same position, considering Neo participation to be a necessary condition for activity of the xanthophyll cycle. Thus, the presented material

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Table 5.3 Kinetics of radioactivity inclusion from ^{14}C-neoxanthin, infiltrated into leaves, in carotenoid fractions of the leaf

	Specific radioactivity of pigment fractions (imp/min/μg carbon), $n = 4$, variation ±12 %				
	Experiment 1	*Experiment 2*	*Experiment 3*	*Experiment 3*	*Experiment 4*
Pigment fraction	Pea	Clover	String beans	+Nigericin[a], pea	+Salicylaldoxime[b], pea
Violaxanthin	33,000	41,000	27,000	19,000	500
Lutein	24,000	34,000	19,500	700	18,000
Zeaxanthin	14,000	19,000	13,000	500	16,000
Carotene	3,400	5,600	4,500	340	8,000

[a]In 20 mL of medium, 250,000 imp/min ^{14}C-Neo was introduced
[b]Cocentrations of nigericin an of salicylaldoxime were 10^{-2} M per sample

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proves the general biological law of Neo transformation into Zea in insects and plants. A question arises on the functional importance of these transformations in plants.

Demming-Adams (1990) indicated (but did not prove) the existence of the transformation Car → Zea through β-cryptoxanthin. They showed that the influence of intense light in normal air or of weak light under conditions of an oxygen lack (O_2 2 %, CO_2 0 %, i.e., weak protonation) leads to an increase in Zea content in photoinhibition in shade-loving *H. helix* and *N. oleander* at water deficiency. In these experiments, the nature of fluorescence quenching was explained by an increase in non-radiating dissipation of energy, caused by formation of alternative quenchers competing with RCs for excitation energy. The increase in Zea content in response to light was thought to be caused by an increase in dissipation of energy in antenna chlorophyll. Therefore, it was thought that Zea can directly operate as a fluorescence quencher. However, until now there has been no successful explanation of how Zea acts in this case.

There are data showing that treatment of leaves with dithiothreitol (DTT) causes inactivation of Viol de-epoxidation and, thus, the formation of Zea (Saakov 1998b). In this work, the absence of a significant influence of DTT on the ratio of fluorescence parameters F_v/F_m or on F_m increase was found for both objects of research, accenting the increase in F_v. During exposure to actinic light, F_0 varies considerably, which assumes incomplete work of the re-oxidation cycle of electron acceptors in RCs of PS-2. Besides, considerable distinction between the kinetics of F_0 curves and Zea content was shown. The kinetics of curves $(F_0^t/F_0)^{+DTT}$—$(F_0^t/F_0)^{-DTT}$ and of Zea quantity Zea^{-DTT}—Zea^{+DTT} highlights the discrepancy between the maxima of the considered curves and, possibly, the shift of energy dissipation to RCs. In consideration of reactions supporting thylakoid protonation with Zea formation, Heber, Bukhov, and coworkers (Bukhov et al. 2001; Heber et al. 2001) did not find that additional protonation induced Zea formation, and they preferred Mehler's reaction although it is inefficient at low temperatures. The authors also showed the formation of Zea funds in response to far-red light, when linear electron transport is slowed down and the Calvin cycle is inactive. Zea formation stops when an acceptor of photosystem 1 (PS-1) is opened for a linear electron flow. All this allowed Heber et al. (2001) to indicate the problematic character of the need for fast reversible formation of effective traps of energy in antenna of RCs of PS-2, which compete with RCs for energy capture. It is supposed that changes in energy dispersion occur faster than the reversible reactions Zea ↔ Viol. The selectivity of Zea participation is accented at dissipation of the energy excess according to the object taxonomy.

Thus, with direct methods we proved the participation of Neo funds in Zea formation and found a *generality* of reductive Neo transformations with Car formation in insects and plants. The material offered in this book allows us to state that, along with the xanthophyll cycle, Car and Lut take part in formation of Zea funds (Saakov 1968a, 1989a, 1990a). It became obvious that, simultaneously with metabolic oxidative–reductive transformations of carotenoids, the processes of their biosynthesis actively participate in creation of Zea funds (Saakov 2003b, 2004), because discovery of the new DOXP/MEP pathway of carotenoid biosynthesis (the international designation) led to explanations concerning the raised speeds of carotenoid biosynthesis due to interaction of 3-phosphoglyceric aldehyde and pyruvate.

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5.3 Metabolic Transformations of Labeled ^{14}C- or ^{3}H-Carotene in Animal Tissues

Earlier we ascertained the law of Car stepwise oxidization in chloroplasts of higher plants and cells of some algae (Saakov 1963c, 1965a, b, 1989a, b). Then began extensive research by Japanese scientists on the transformation of alimentary (food) carotenoids in animals (Davies et al. 1970; Katayama et al. 1972, 1974; Rodriguez et al. 1974; Tanaka et al. 1976; Gilchrist and Lee 1976). The use of material of various evolutionary and systematic relations, namely, *Carassius auratus*, *Chrysophrys major*, *Cyprinus carpio*, *Panulirus japonicus*, *Portunus trituberculatus*, *Penaeus japonicus* Bate, *Evynnis japonica* Tanaka, some representatives of *Crustaceae* (including *Artemia salina*), *Daphnia magna Straus*, *Daphnia pulex*, representatives of *Isopodes* (*Idotea montereyensis*, *I. resecata*, *I. granulosa*), the flamingo *Phoenicopteridae*, and others indicated the presence of a certain law regarding Car transformation through steps of intermediate oxidization. Similar results were obtained in modeling experiments for *Anacystis nidulans* (*Cyanophyceae*) (Halfen and Francis 1972). These experiments created the basis for supposition of the existence of pathways for oxidizing Car transformations into compounds having four atoms of oxygen in their molecules, in the form of 3,3′,4,4′-tetraketo-β-carotene (astacin, Asc) or 3,3′-dihydroxy-4,4′-diketo-β-carotene (astaxanthin, Ast). Additional proof in favor of this supposition was obtained by other experimental technique, as described in articles by Czygan (1966, 1968). Cultivating representatives of green algae in a medium lacking nitrogen, the author found active synthesis of the secondary carotenoids echinenone, Ast, and Asc. This pathway for Car transformation could appear only in the case of Car oxidization reactions, which are alien to representatives of *Chlorophyceae* under their habitual conditions of existence (Czygan 1968).

Existing experimental data concerning pathways of Car oxidization are necessary but not sufficient for making strict conclusions about the sequence of oxidization reactions for this pigment in representatives of fauna and green algae under extreme conditions. More reliable proof is the determination of SpAs of products of the intermediate steps of Car oxidization during introduction of alimentary preparations of labeled Car into an animal organism. The rest of this section is devoted to study of this problem.

Once again, we emphasize that obtaining reliable data depends on high radiochemical purity of the initial Car preparation used for assessment of the metabolic variety of oxidization pathways of the pigment in tissues. Car purification is always complicated by the accompanying colorless impurities. Purification was carried out using established techniques of thin layer, column, and paper chromatography (Saakov and Shiryaeva 1967, Saakov 1970b, 1989a, 1990a). Crawfish (*Astacus leptodactylus* Esch) served as the object of research. Labeled ^{14}C- or ^{3}H-Car (0.1 mL) in vegetable oil was introduced into an oral opening with a micropipette or syringe. Time of exposure to labeled preparation was taken from recommendations in the literature (Davies et al. 1970; Katayama et al. 1972; Rodriguez et al. 1974;

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Table 5.4 Dynamics of radioactivity inclusion from exogenous alimentary ^{14}C- and ^{3}H-carotene in fractions of pigments of crawfish

Fraction №	Fractions of isolated carotenoids	Specific activity(imp/min/μg carbon)		
		Experiment 1	*Experiment 2*	*Experiment 3*
From ^{14}C-Car				
1	Carotene	397 ± 24	430 ± 26	372 ± 26
2	Echinenone	198 ± 16	210 ± 21	194 ± 19
3	Canthaxanthin	117 ± 12	119 ± 18	92 ± 9
4	Astaxanthin with astacin	76 ± 11	79 ± 14	61 ± 11
From ^{3}H-Car				
1	Carotene	897 ± 34	832 ± 46	937 ± 37
2	Echinenone	383 ± 21	350 ± 34	405 ± 31
3	Canthaxanthin	250 ± 17	135 ± 14	218 ± 17
4	Astaxanthin with astacin	72 ± 9	67 ± 7	92 ± 11

For ^{14}C-Car, average coefficient of decrease in specific activity for three experiments from fraction 1 to 2 was 1.98; from fraction 2 to 3 was 1.85; from fraction 3 to 4 was 1.52; for ^{3}H-Car, from fraction 1 to 2 was 2.34; from fraction 2 to 3 was 1.99; and from fraction 3 to 4 was 2.64

Katayama and Miyahara 1974; Tanaka et al. 1976; Gilchrist and Lee 1976). After 10 days, shells and claws were fixed in an ethanol and acetone mixture (1:1) and then crushed and pigments extracted. Pigments were purified by consecutive chromatography in a column, on paper, and in a thin layer (Kleinig and Czygan 1969; Saakov 1970b) with preliminary saponification in 15 % KOH. Each experiment was repeated three or four times. Into each object, an identical quantity of labeled Car was introduced. ^{14}C-Car in a sample of 0.1 mL contained 30,000 imp/min with SpA of 1,430 imp/min/μg carbon; for ^{3}H-Car, 55,000 imp/min was introduced with SpA of 3,700 imp/min/μg hydrogen. Data on possible ways of transformation of alimentary labeled Car are summarized in Table 5.4.

After introduction of labeled ^{14}C- or ^{3}H-Car into crawfish, the radioactive label was found in all fractions of isolated carotenoids. Measurement of SpA and calculation of coefficients of SpA decrease for pigment preparations indicated the stepwise oxidizing transformation of exogenous labeled Car. A number of measurements of the SpA indicated that the Ast and Asc fraction is the end product of the pathway of oxidizing Car transformations when its carbon skeleton still remains. Thus, the results in Table 5.4 give direct necessary and sufficient proof of stepwise oxidizing transformations of Car in animal organisms into products with four atoms of oxygen in the pigment molecule.

This book does not discuss the multilateral and confused problem of the functional importance of carotenoids, however coupled with their oxidizing metabolism. However, in the process of analysis of our experimental data and that published by other authors, we face a question of the metabolic variety of Car oxidization pathways in tissues of vertebrate and invertebrate animals, cells of algae and bacteria, and the chloroplasts and chromoplasts of higher plants. Existing data (Saakov 1965a, 1989a, b; Czygan 1966, 1968; Davies et al. 1970; Katayama et al. 1972; Katayama and Miyahara 1974; Rodriguez et al. 1974; Tanaka et al. 1976;

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Gilchrist and Lee 1976) assume that similar functional loadings can be carried out by carotenoids of various types. Earlier we and a number of authors (Czygan 1966; Hager 1980; Saakov 1989a, b) discussed the problem of the variety of reactions of carotenoid transformation in various taxonomic groups of animals and plants. Originally these data were summarized in works (Saakov 1963c, 1965a, 1968b) in which the existence of cyclic reactions of carotenoids in chloroplasts was proved. Development of our conceptions about the pathways of oxidation–reduction reactions of Car and some of its derivative forms in animals and plants is described (Saakov and Baranov 1987; Saakov 1989a, b). Improvements in the experimental technique and analysis of pigments from representatives of a number of taxonomic groups indicated the existence of cyclic transformations of acetylene carotenoids in *Euglenophyceae*, *Bacillariophyceae*, *Xanthophyceae*, etc. Transformations of carotenoids with allene groups of fucoxanthin and pyridinine type were found in *Dinophyceae* and *Chrysophyceae*. Neoxanthin of higher plants is related to these pigments. The presence of fucoxanthin in brown diatomeae and in some other algae suggests that this pigment is a potential component of cyclic reactions of xanthophylls. Organisms standing at various steps of the evolutionary ladder possess a variety of cyclic transformations of carotenoids. For example, chromatophores of photobacteria (*Rhodopseudomonas* sp. and *Rh. gelatinosa*) are capable of reversible reaction of oxygen binding after a change in illumination conditions. In response to light, the quantity of spheroidenone increases and the content of spheroidene decreases. In darkness this process is reversible. In *Cyanophyceae* and *Rhodophyceae*, the presence of echinenone and myxoxanthophyll suggests the possibility of a sequence of carotenoid oxidization by means of Ast formation or through the mechanism of transformation of spheroidenone into spheroidene (Saakov and Baranov 1987). The cycle of acetylene xanthophylls is characterized by transformation of diadinoxanthin in diatoxanthin, and this process is connected with transformation of the epoxy group, probably through the mechanism of the Viol cycle in higher plants and *Chlorophyceae*.

So, in evolutionary advanced organisms (*Eucaryota*), Car oxidization occurs in compounds containing four oxygen atoms in hydroxyl, keto, or epoxy groups (Fig. 5.24), whereas in *Photobacteria*, *Cyanophyceae*, *Cryptophyceae*, and *Rhodophyceae*, transformations of carotenoids having only two oxygen atoms in their molecules are observed.

Thus, the results presented in this section serve as direct proof of stepwise metabolic reconstruction, in an animal organism, of alimentary labeled Car into compounds with three or four oxygen atoms, which corresponds to data obtained for vegetative objects. The methodological part of the experiments was substantially facilitated by the use of derived spectra of Car. From the point of view of evolutionary biochemistry, the considered oxidation–reduction transformations in the carotenoid system in a cell indicate the phylogenetic generality of pathways of pigment metabolism in animals and plants.

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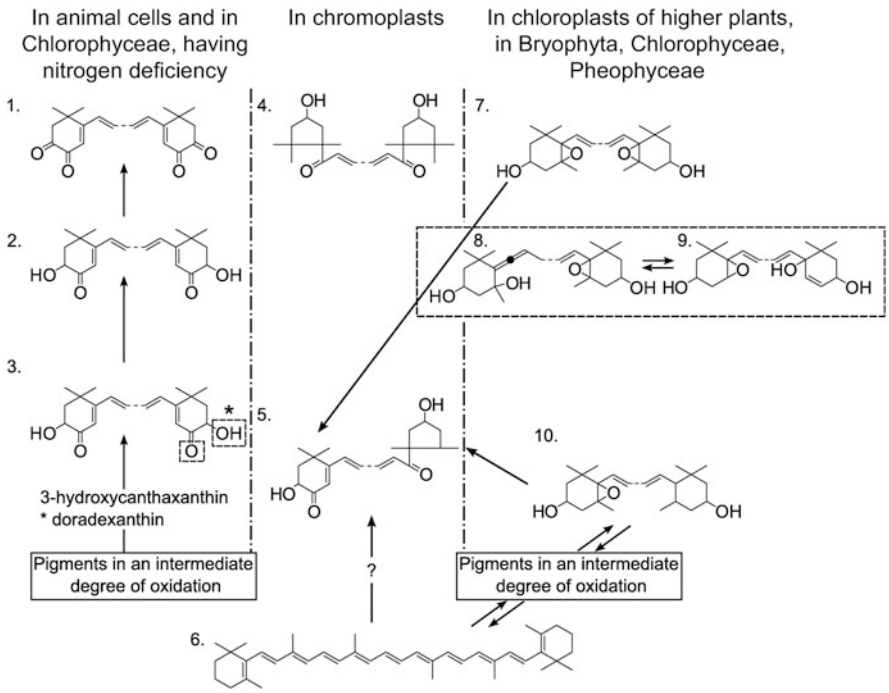

Fig. 5.24 Ways of carotene oxidization in cells of animals, chloroplasts of higher plants and algae, and chromoplasts: (*1*) astacin; (*2*) astaxanthin; *3* 3-oxycanthaxanthin or doradexanthin; (*4*) capsorubin; (*5*) capsanthin; (*6*) β-carotene; (*7*) violaxanthin; (*8*) neoxanthin; (*9*) trollixanthin; (*10*) antheraxanthin or epoxylutein

5.3.1 Unexpected Synthesis of Carotenoids from ^{14}C-Mevalonic Acid Pyrophosphate in an Animal Organism

Investigation of the possible pathways of carotenoid biosynthesis in animal organisms is an example of how slowly the mysteries of nature are unraveled.

For a long time it was thought that cells of higher and lower animal organisms are not able to synthesize carotenoids de novo. Over the years, this opinion was petrified into categorical presentation in authoritative material of the professor from Liverpool T.W. Goodwin (1953, 1954, 1955a, b; 1959, 1960, 1965, 1969, 1971a, b). In Chap. 1 we pointed out the methodological errors of Goodwin and coworkers, but these errors had their roots in earlier works (Goodwin 1958, 1959) on the study of carbon incorporation from $C^{14}O_2$, 2-C^{14}-mevalonate, and 2-C^{14}-acetate in a number of carotinogenic systems.

At present, it is considered that carotenoids are precursors of the chromophore of visual pigment (rhodopsin). In insects, a certain correlation is found between the chemical structure of carotenoids and that of the chromophore (retinoid); namely,

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insects with a prevalence of oxycarotenoids in the eye xanthophylls (Zea, Lut, β,β-cryptoxanthin) have 11-*cis*-3-oxyretinal as the chromophore of visual pigment, but the presence in eyes of a high concentration of β-Car predetermines the chromophore 11-*cis*-retinal in the pigment structure. For a long time the conception existed that insects (as for many other investigated animals and the apex of evolution, human beings) are not able to synthesize carotenoids de novo and therefore depend on the presence of carotenoids in food. Numerous experiments with various representatives of *Diptera* and *Lepidoptera* showed that cultivation of insects on a diet with carotenoid (retinoid) deficiency leads to dramatic changes in their visual system. The absolute sensitivity of the eye decreased by several orders; the content of rhodopsin and its apoprotein opsin also decreased; and there was a decrease in the content of other proteins involved in the visual cycle (phospholipase C, retinoid-binding protein). Most induced failures can be prevented or completely treated by carotenoid (retinoid) "therapy." The use of different *Drosophila* mutants with defects of retinoid (carotenoid) metabolism opened new approaches for the investigation of pathways of transformation of the chromophore of insect visual pigments, as discussed earlier (Shukolyukov and Saakov 2001).

We carried out a study of the visual system of the most ancient representative of insects, *Periplaneta americana*, on the assumption of finding in these evolutionary ancient organisms the existence of specific biochemical visual mechanisms, which could be lost, retained, or functionally modified in the course of evolution. Therefore, the study of interrelationships between carotenoids and the visual system of the examined insect is of special interest. However, before investigating the role of carotenoids as precursors of the chromophore of visual pigments of *Periplaneta*, it was necessary to obtain experimental answers to the following biological questions:

1. Does *P. americana* depend on exogenous carotenoids like some other representatives of *Diptera* and *Lepidoptera* and possess the ability to accumulate these pigments?
2. In which organs or tissues does carotenoid concentration occur?
3. What are the qualitative and quantitative contents of carotenoids in locations of their accumulation or at the excess of these carotenoids in food?
4. How long can the animal culture in the experiment exist on a carotenoid-deficient diet?
5. Has the American cockroach (Periplaneta am.) the ability to synthesize *endogenous* carotenoids from the exogenous labeled precursor of its synthesis, ^{14}C-mevalonic acid pyrophosphate?
6. What is the chemical nature of the substance containing the radioactive label after exposure of insects to labeled precursor?

Colonies of American cockroach were represented by animals of the wild type and white-eye mutants. The latter are characterized by the absence of shielding ommochrome pigment in the ommatidium and were autosomal recessive. Insects were kept at a constant temperature of 25 °C and the natural photoperiod. We describe the methodology of the work and underline its features, although the principles are described in Chap. 3.

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Two qualitatively different diets were used, which (as we suggested) should lead to two different levels of carotenoid content. The carotenoid-deficient diet was white bread, hercules, sugar, and water; the carotenoid-excessive diet was white bread, cereals, sugar containing an excess of carrot juice, and water.

As the radioactive precursor of carotenoids, 5-pyrophosphate-*R*[5-^{14}C]-mevalonic acid was used (Amersham, UK; SpA, 58 mCi/mmol). Labeled preparation of volume 19 μL (0.2 μCi, 3.4 nmol) was introduced laterally between the third and fourth ventral segments using a microsyringe. As control, physiological solution of the same volume was introduced into animals of the same size. For extraction of carotenoids, batches of animals were used on the morning of days 1, 2, 3, 4, and 7 after injection. To prevent photodestruction of pigments, the work was performed under far-red light during extraction and further chromatography.

Material for the analysis (10–120 eyes, 5–60 intestinal tracts) was weighed and stored at −15 °C until the moment of use. On the day of analysis, tissues were thawed out and porphyrized with anhydrous Na_2SO_4 in a small mortar and then extracted six times with 1–2 mL acetone. To the total acetone extract was added 2–4 mL hexane or petroleum ether (fraction 40–70 °C) and 2–3 mL 5 % NaCl. Samples were mixed and centrifuged for 5 min at 5,000 *g*. The epi-phase (less dense phase) was collected, and extraction from the water phase using hexane or petroleum ether was repeated three times. The total epi-phase was washed three times with NaCl solution for removal of acetone traces, concentrated in vacuum to 400–3,000 μL, and predried with sodium sulfate. Thus, a non-purified extract of carotenoids was obtained. Then, the extract was saponified and purified for the first time in a column containing aluminum oxide and cellulose.

Because of the low content, isotopic dilutions were carried out in two experimental variants and resulted in enrichment of the radioactive material with the non-radioactive one. Such an approach provided a high concentration of carotenoids in samples, sufficient for three or four chromatography steps (see Sect. 3.5.4.1). After repeated saponification with 10 % KOH in methanol, the washing and drying of epi-phase extracts were carried in a column containing aluminum oxide (1 × 18 cm, volume about 10 mL) or cellulose (sigmacell, Sigma, USA) that had been previously equilibrated with petroleum ether or hexane. Adsorbed material was eluted with 30 mL petroleum ether (or hexane) and then with 45 mL 3 % acetone in petroleum ether. Pigments were identified using absorption spectra, and some fractions were centrifuged three or four times after concentration to provide the maximal degree of purity.

After the fourth act of chromatography, when the elution profile showed a symmetrical carotenoid peak and, coinciding with it, a symmetrical peak of radioactivity count, fractions were used as the purified carotenoid sample (Sect. 3.6.4.1). Aliquots of 400–1,000 μL were evaporated to the dry state and then mixed with 5 mL of scintillation cocktail (LKB, Sweden) and counted for 10 min using the counter 1209 Rackbeta (LKB). Results were expressed in impulses per minute per microgram (imp/min/μg) of carotenoid.

The concentration of carotenoids was calculated according to the formula $C = A \times V \times f \times 10/2{,}500$, where C is the carotenoid content in milligrams, A the

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absorption of the probe at 450 nm, V is the total volume in milliliters, f is the dilution factor, and 2,500 is the averaged specific coefficient of absorption for carotenoids ($A^{1\%}_{1cm,\ 453}$). The carotenoid content in tissue was expressed in micrograms of carotenoids per gram of wet tissue.

Preliminary screening of organs and tissues showed that in *P. americana* the maximal quantity of carotenoids is found in eyes and intestinal tract Analysis of non-purified extracts of eyes and intestinal tracts of animals maintained on the carotenoid-excessive diet showed that the main pigment is the relatively nonpolar carotenoid with a slightly expressed structure (λ_{max} in hexane at 425, 450, and 476 nm), which was identified as bicyclic β-Car. At repeated chromatographic purifications of intestinal extracts of carotene, it was possible to separate two minor carotenoids from the main spot (or band) of β-Car, one of which (less polar) we classified as α-Car (λ_{max} in hexane at 420, 442, and 472 nm) and the second (more polar and migrating more slowly) as aliphatic ξ-Car (λ_{max} in hexane at 395, 415, 440, and 470 nm) (see Fig. 5.18). It is important to note that spectral characterizations were of slightly changing character from experiment to experiment.

We emphasize that it was not possible to detect other carotenoids with high reliability, except for β-Car in the eyes of animals maintained on the carotenoid-excessive diet and also in the eyes and intestinal tract of animals on the carotenoid-deficient diet.

Qualitative comparison of the carotenoid levels in the eyes and intestines of animals kept for more than 6 months on the two types of diets revealed an almost 13-fold increase in the carotenoid content in intestines of animals on a carotenoid-excessive diet in comparison with animals on a carotenoid-deficient diet. At the same time, there were no differences in carotenoid content in the eyes of animals of both groups.

These results suggest that, on the one hand, *P. americana* has the ability to accumulate (deposit) a huge amount of carotenoids. On the other hand, it is possible that, with the help of an unknown mechanism, the animal can keep a stable and, probably, evolutionary reasonable physiological level of these substances in eyes and that this level does not depend on the quantity of carotenoids arriving from outside. Comparison of data obtained earlier (Shukolyukov and Denisova 1992) for the representative of *Diptera (Calliphora erythrocephala)* with the results of the present work reveals a number of differences: First, the main carotenoid of *P. americana* is β-Car not xanthophyll–Zea as in *Calliphora erythrocephala*. Second, it was not possible to change the amount of carotenoids in eyes of *P. americana* by varying the content of these pigments in the food ration, as was possible for *Calliphora erythrocephala*.

The evolutionary mysteries of nature start with the maintenance of a constant level of carotenoids in eyes of *P. americana* that do not receive these pigments from food. Experiments showed that a carotenoid-deficient diet for 3 years (i.e., three to four generations of animals, the lifetime of one animal being 9–13 months) does not result in a decrease in the carotenoid level in eyes and intestines. This fact led us to the supposition (hypothesis) that the cockroach is able to synthesize carotenoids de novo. An endogenous supply of carotenoids due to the "maternal effect," when a definite quantity of carotenoids is transferred to next generations with the maternal egg, should finish earlier than 3 years.

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If the processes of carotenoid biosynthesis do occur in *P. americana*, then according to our hypothesis, the introduced labeled precursor of these pigments should be involved in the corresponding synthetic reactions, and the radioactive label should end up in molecules of newly formed carotenoids. Let us call these molecules "young molecules" analogously to newly synthesized chlorophyll molecules. It is also interesting from the point of view of their functional activity (Shlyk 1971; Gaponenko 1976, p. 240 Influence of external factors on the chlorophyll metabolism).

Experiments showed that introduction of labeled precursor of carotenoids (3.4 nmol) into experimental animals led to changes in the Car content in eyes at 2 and 4 days after injection. Still, there is difficulty for such cyclicism. However, there is no doubt that introduction of the mentioned (nonphysiological) concentration of exogenous pyrophosphate of mevalonic acid (from which isopentyl diphosphate, the main structural element of the carbon skeleton of terpenoids, is formed) influences in a complex way the further reactions of dimerization, dehydration, and oxidization, which result in the final formation of individual carotenoids. In any case, changes in carotenoid biosynthesis at 2 and 4 days after injection suggest activation of the synthesis of these substances in *P. americana*.

In the process of extraction, saponification, and subsequent chromatography, the SpA of preparations continuously decreases, but later reaches a stable level according to methodologies described in Sect 3.6.4.1. The elution profile or spots of β-Car isolated from eyes or intestine coincide with the scan of radioactivity distribution on a chromatogram (see Sect. 3.6.4.1). This indicates that the substance containing the carbon label can be classified as a carotenoid. If we assume the correctness of Goodwin's analyses (Goodwin and Williams 1965a, b; Williams et al. 1967; Goodwin 1958, 1961, 1965, 1969) about separate pathways for the synthesis of α-Car and β-Car, then the quantitatively more significant product β-Car is the product of carotenoid synthesis de novo from labeled precursor.

Thus, a positive answer to the main question of this research was obtained. *For the first time, the possibility of synthesis of "young molecules" of carotenoid, more precisely of β-Car, by animal objects was established*. This supports the possibility of maintenance of *P. americana* vision, under conditions when the food ration does not contain exogenous carotenoids, through de novo synthesis. Experimental data obtained in the process of work allowed us to make the following conclusions:

1. In contrast to other insects, *P. americana* does not need a supply of exogenous carotenoids. This allows them to live on a carotenoid-deficient diet and, correspondingly, to maintain a stable level of carotenoids in the eye for an indefinitely long time.
2. With an excess of carotenoids in food, most of the pigments are concentrated in the intestinal tract.
3. The dominating carotenoid of the eyes and the intestinal tract is β-Car, in "young" (i.e., synthesized) molecules of which the radioactive label from ^{14}C-mevalonic acid pyrophosphate, the precursor of its synthesis, becomes concentrated.

The performed experiments allow us to express the statement that, in contrast to representatives of *Diptera* and *Lepidoptera*, the cockroach *P. americana* possesses two specific physiological mechanisms for optimization of visual function. On the

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one hand, with an excess of carotenoids in the ration, animals are able to accumulate carotenoids in the intestinal tract and to become independent of its presence in food. On the other hand, with a many-year deficiency of carotenoids in the food ration, *P. americana* are able to obtain carotenoids due to synthesis of pigments de novo and to become independent of its content in food. The result of these two mechanisms, which developed in the process of evolution, is a stable level of eye carotenoids that does not change with an excess of carotenoids in food nor with a deficiency.

It is still unclear whether *P. americana* has a set of enzymes necessary for synthesis of carotenoids or whether these compounds are produced by bacterial symbiotes that live in the digestive tract of *P. americana* and help the insect to biosynthesize pigment. The suggestion that microorganisms play a role in carotenoid synthesis in insects (*Musca domestica*) was expressed by a doctoral student of Prof. N. Krinsky (and now a professor), T. Goldsmith.

The journal *Science and Life*, *Russian Science News* (http://informatica.ru/eng/2001/2001-09-21-0327_e/htm), and the newspaper *Communications* (*Izvestiya*) from 3 to 8 November 2001 all positively reacted to the originality of our published material. As the newspaper *Communications* wrote, "the discovery of St. Petersburg biologists caused a wide response in the scientific word," because as well as its contribution to fundamental biology, the work of S. Shukolyukov and V. Saakov is also a contribution to evolutionary theory. Analogous responses appear on the following Internet sites:

1. http://www.reactivereports.com/21/
2. http://unisci.com/stories/20013/0927016.htm
3. http://www.acdlabs.com/webzine/21
4. http://www.protein.bio.mso/biokhimiya/contents/v66/abs/66050663.htm
5. http://www.google.com.ru/search?q=cache:lyPEJstgRl8C:vnexpress.net/Vienam/Khoahoc/2001/10/3B9B5029/+Saakov+VS+&hl=ru&ie=UTF-8&inlang=ru
6. http://www.google.com/search?q=cache:kppHKdZwgC4C:lat.subscribe.ru/archive/rest.mys*0*.ery.news/200110/05064702.html+Saakov+VS+&hl=ru&8&inlang=ru&e=619
7. http://www.google.com.ru/search?q=cache:Hrao89kf6LIJ:www.reactivereports.com./21/
8. http://www.google.com.ru/search?q=cache:Hrao89kf6LIJ: www.reactivereports.com./21/++SAAKOV=ru&Ir=&strip=1
9. http://www.reactivereports.com./21/21_4.html
10. http://www.altavista.com/web/results?q=V.+S.+Saakov+%22american+cockroach%22&. . .
11. http://reo.niii.ac.jp/journal/HtmlIndicate/Contents/SUP0000001000/JOU0001000133/ISS. . .
12. http://www.google.com.ru/search?q=cache:6U43JgKvEi4C:www.informnauka.ru/eng/2001/2001-09-21-0327_e.htm+Saakov+VS+&hl=ru&ie+UTF-8&inlang=ru
13. http://www.informnauka.ru/eng/2001/2001-09-21-0327_e.htm
14. Akkmulytor Novostei 02.10.2001 Istochnik: Izvestiy. RU

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Such an active reaction on the Internet was unexpected and at the same time pleasant, because the publicity (including a report on St. Petersburg television) made it possible to carry out further experiments (Sect. 5.2) on incorporation of Neo-^{14}C in Car and geraniol pyrophosphate isolated from the eyes of *Apis mellifera* and *Odonata* sp.

Our experiments once again emphasize the difficulty of investigation into the mysteries of nature and into the development of mechanisms of evolutionary biochemistry and sometimes put in doubt established points of view and postulates.

5.4 Importance of Derivative Spectrophotometry for Study of Alternative Ways of Carotenoid Biosynthesis in *Procaryota* and *Eucaryota*

In the last 25 years, enough experimental data has appeared to question the generalization of the acetate/mevalonate (Ac/MVA) pathway of isoprenoid biosynthesis in representatives of flora and fauna. However, experiments performed at the end of the 1950s by a known expert in the field of carotenoid biosynthesis (Goodwin 1958) brought elements of doubt regarding the unambiguity of the Ac/MVA pathway of isopentyl pyrophosphate (IPP) synthesis, defended by him as the unique and correct pathway for synthesis of vegetative carotenoids (tetraterpenes). This persistent assurance of T.W. Goodwin in the unconditionality of domination of the Ac/MVA pathway of isoprenoid biosynthesis delayed for at least 30 years world research (including USSR) into the mechanisms of their biosynthesis in different evolutionary groups of vegetative and bacterial organisms. As has already happened many times in science, the theater of wild absurdity proceeded for some time. But researchers from France and Germany (Roberts and Perkins 1966; Rohmer et al. 1993; Lichtenthaler et al. 1997a, b; Rohmer 1999, Lichtenthaler 2000), well familiar to Goodwin, destroyed the long-held theory with their experiments.

The experiments of Costes (1963a, b, 1968) forced researchers to look in a new way at the synthetic sequence of Viol, epoxylutein, and Zea and to put forward a hypothesis about the formation of Car by reduction of xanthophylls. We studied the regeneration (renovation) of xanthophylls, and our data indicated the existence of alternative predecessors of carotenoids, in which the carbon of photosynthesis was included in the first minutes of exposure to light (Costes 1968; Lutsenko and Saakov 1971, 1972, 1973).

Despite the complexities of publication, we stated our idea about the existence of various pools of precursors for epoxyxanthophylls and Car and about the presence of a special pathway of epoxyxanthophyll synthesis involving primary products of carbon metabolism such as phosphoglyceric acid (PGA), phosphoenolpyruvate (PEP), etc. We also considered the possibility of oxidizing reamination of leucine and deaminization of glycocol, alanine, serine, and valine with their subsequent decarboxylation and formation of acetate funds. Decarboxylation of phosphotrioses and oxidizing decarboxylation of oxaloacetic acid (OAA), pyruvic (acetylformic) acid (Pyr), and PGA lead to a similar result. Moreover, at that time not only our studies

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but also aspirations of researchers from different countries were focused on the substantiation of formation of acetate as a basic precursor of acetyl coenzyme A (AcCoA) in the synthesis of plastid carotenoids. There were experiments showing that exogenous ^{14}C-Pyr was quickly included both into extraplastid isoprenoids and into tetraterpenes of chloroplasts (Roberts and Perkins 1962; Lutsenko and Saakov 1973).

At the same time, because of the 40-year domination of another point of view and excessive care of expert reviewers of journals, the interpretation of results in the majority of works was reduced to a substantiation of formation pathways of funds of acetate, AcCoA, and MVA as precursors of both cytosol and plastid IPP. Nevertheless, 38 years ago we considered that the decarboxylation of Pyr, PGA, 3-phosphoglyceric aldehyde (3-PGal), and OAA and also the pathway $CO_2 \rightarrow$ glycolate $\rightarrow$ glyoxylate $\rightarrow$ glycine $\rightarrow$ serine $\rightarrow$ Pyr $\rightarrow$ AcCoA $\rightarrow$ terpenoids could be plastid pathways of isoprenoid formation. The same concerned the interpretation of suitability of pathways for movement of the label from ribulose biphosphate $\rightarrow$ 3PGA $\rightarrow$ 2PGA $\rightarrow$ PEP and the use of PEP and acetate funds for IPP biosynthesis, and further for carotenoid formation (Costes 1968; Lutsenko and Saakov 1972, 1973). In spite of that, fast inclusion of carbon from $^{14}CO_2$ into carotenoids during the process of photosynthesis was ascertained, and very low inclusion of MVA into β-Car and other plastid carotenoids was found. By contrast, the issue of active MVA inclusion into cytoplasmic sterols (Goodwin 1958) was dominated for many years by the view that the Ac/MVA pathway was predominant in the synthesis of plastid carotenoids. Some questions arose in articles (Costes 1968; Lutsenko and Saakov 1971, 1972, 1973) that were in advance of their time, but only recognized later.

The history of development of the doctrine about carotenoid biosynthesis pathways confirms our correctness. For some nonscientific reasons, our concept was initially stated in a cautious and vague form (Lutsenko and Saakov 1972, 1973) and was later developed in a more fundamental paper (Lutsenko and Saakov 1971). We returned again to consideration of alternative ways of carotenoid biosynthesis in a later publication (Saakov 2003b).

Ideas formulated by us (Lutsenko and Saakov 1971, 1972, 1973) found their acknowledgment after many years. After publication of some results (Costes 1963a, b, 1965; Lutsenko and Saakov 1971, 1972, 1973), articles started to appear that provided enough evidence to put in doubt the conception and theoretical statements of Goodwin about the existence of one main way of synthesis of carotenoids and other cellular components of an isoprenoid nature, realized only through the Ac/MVA pathway.

For an explanation and details about the position of the authors of this book, we return to the 1950s and 1960s, when many scientific collectives were discussing the primary product of photosynthesis, that is, the primary compound formed after the incorporation of carbon from carbon dioxide in the process of intake of the latter by a green plant. Scientists were arguing and proposing various schemes for a long time, until the future Nobel laureate from the University of California Radiation Laboratory (Melvin Calvin) carried out experiments in which he gradually shortened the exposure of experimental plants to CO_2 and simultaneously increased the specific

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radioactivity of the used carbon dioxide. We think that Prof. Goodwin knew about the methods of Prof. Calvin's work, but for some reasons in his investigations, he didn't use the methodological approach of Prof. Calvin and continued to perform long exposures of biological objects to the radioactive substrate, which wasn't correct for studies of biosynthetic reactions. We have already discussed this in Chap. 1.

It is regretful that, even in our time, the experimentally unfounded schemes, propositions, fabrications, and free interpretations can be met in research on the complexity of isoprenoid biosynthesis. But these interpretations and schemes are not methodologically reliably confirmed by experiment (Woitsch and Romer 2005). Note that S. Romer (Konstanz, Germany) should not be confused with Prof. M. Rohmer (Strasbourg, France). The absence of strict and methodologically exact experiments means that the opinions of authors remain at a superficial and precocious level. New facts are beyond the matrix of this scrupulous scientific approach, and the substantial part of verity is fancifully distorted.

In this connection, we have already emphasized that in this book we prefer to cite experimental works that are grounded on a serious methodological base (Lichtenthaler et al. 1995, 1997a,b; Schwender et al. 1996, 1997, 1999, 2001; Rohmer 1998; Rohmer et al. 1988, 1989, 1996, 1997, 1998, 2000). The combination of modern physical and chemical research methods, with competent inhibitor analysis, is original in the listed and other works of these scientific groups. Thus, from the beginning of the 1990s, occasional works (Roberts and Perkins 1966) were supported by a series of publications by two groups of researchers under the leadership of Prof. H. K. Lichtenthaler (The Technical University, Karlsruhe, Germany) and Prof. M. Rohmer (Louis Pasteur University, Strasbourg, France). In one of the initial works of this series of investigations (Schwender et al. 1997), performed with the alga *Scenedesmus obliquus* by introduction of ^{13}C-glucose into its cells under heterotrophic conditions and the application of ^{13}C NMR spectroscopy for the analysis, the authors established an important fact. The prenyl part of molecules of plastid pigments (chlorophyll, plastoquinone-9, β-Car, Lut, and also, we underline, in cytoplasmic sterols) is not formed through the classical (accepted by Goodwin) Ac/MVA pathway (AcCoA → mevalonate → isopentyl diphosphate). Instead, the biosynthesis occurs through glyceraldehyde 3-phosphate/Pyr, that is, through the reaction sequence earlier found in *Eubacteria* (Flesch and Rohmer 1988; Rohmer et al. 1989, 1993, 1996).

The subsequent conversion of IPP is coupled to condensation of the bicarbonate unit appearing at decarboxylation of pyrophosphate and forming a five-carbon skeleton (Fig. 5.25a, b). It was found that the carbon chain (Fig. 5.25a, b) is labeled in the same way as the ^{13}C isotope in phytol, plastoquinone-9, β-Car, and other xanthophylls. The final formulation of conceptions described in the works of these groups of authors was made in reviews (Lichtenthaler 1999, 2000a, b, 2007).

We direct the reader's attention to the fact that before presentation of illustrations from the works of Lichtenthaler and colleagues in this part of the monograph, we obtained his written permission to use them, with citations to the original publications (Figs. 5.26 and 5.27).

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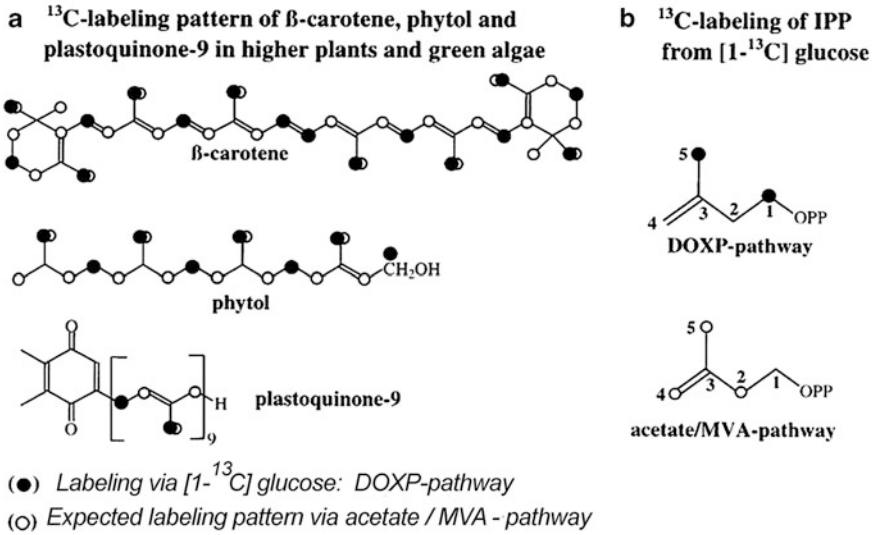

Fig. 5.25 Hypothetical scheme for the biosynthesis of IPP from pyruvate and glyceraldehyde 3-phosphate (*GA-3-P*) in *Scenedesmus*. *Points* indicate label from [1-^{13}*C*]-glucose; *TTP* thiamine pyrophosphate (Schwender. . ..Lichtenthaler 1996, 1999, 2001; Lichtenthaler 2000a, b)

The presence of inconsistent results that contrasted with the generalization of the Ac/MVA pathway promoted researchers from Germany and France to simultaneously formulate a new way of isoprenoid biosynthesis as consequence of 3-PGal and Pyr interaction with formation of l-deoxy-D-xylulose-5-phosphate (DOXP) (Roberts and Perkins 1966; Rohmer et al. 1993; Lichtenthaler et al. 1997a, b; Rohmer 1999; Lichtenthaler 2000a, b). 1-Deoxy-D-xylulose (DOX) was considered to be the precursor of a new non-mevalonate pathway of isoprenoid biosynthesis. The following step of synthesis was supposed to be the transformation of DOXP into 2-C-methyl-D-erythritol-4-phosphate (MEP). From here, the specified sequence of isoprenoid formation received the international name of the DOXP/MEP pathway. Further, MEP through 4-(cytidine-5′-diphospho)-2-C-methyl-D-erythritol (1) turns into 4-(cytidine-5′-diphospho)-2-C-methyl-D-erythritol-2-phosphate (2) and 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (3). Last steps of biosynthesis between (3) and IPP are not exactly established, but are possibly connected with two dehydrogenases and two NADPH reductases (Rohmer 1999). The data obtained reliably proved the DOXP/MEP pathway for eubacteria (*Mycobacterium*, *Vibrio cholerae*), cyanobacteria, green and red algae, diatomeae, and higher plants. Synthesis of plastid Lut and β-Car, the phytol part of chlorophyll (Moskvin and Saakov 1970), plastoquinone-9, and isoprene occur through this pathway.

There were almost 40 years of sharp discussion between researchers from different countries, first, because of ignorance and, second, because of reciprocal misunderstanding that plants possess both plastid (DOXP/MEP) and cytosol (Ac/MVA) pathways of isoprenoid biosynthesis. Now, the question on the origin

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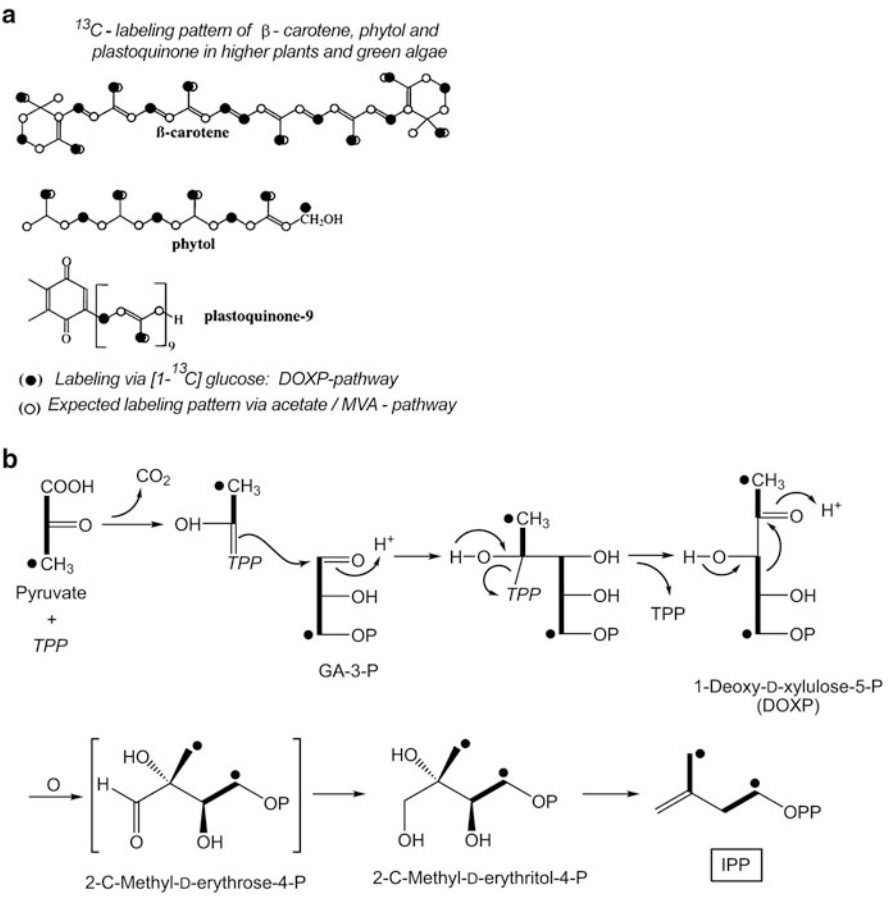

Fig. 5.26 (**a**) Biosynthesis of isoprenoid precursor from IPP (isopentenyl pyrophosphate) and glyceraldehyde 3-phosphate (*GA-3-P*) via non-mevalonate DOXP pathway. The C atoms *1*, *2*, and *4* of IPP derive from GA-3-P and the C atoms *3* and *5* from pyruvate. (**b**) ^{13}C-labeling pattern in isoprenoids of *Scenedesmus obliquus* after growth on [1-^{13}C]-glucose. *I* phytol; *II* plastoquinone-9; *III* β-carotene; *IV* lutein (Zeidler. . ..Lichtenthaler 1997, 1998, 1999; Schwender. . .Lichtenthaler 1996, 1999)

of cytosol IPP funds on the basis of the Ac/MVA pathway (used for synthesis of sesquiterpene and sterol) and of plastid IPP funds involved in synthesis of prenyl lipids has been answered (Lichtenthaler et al. 1997a, b; Rohmer 1999). Besides, the complexity of this problem consists in that, for example, in *Euglena* the Ac/MVA pathway operates of synthesizing sterols and plastid isoprenoids. In fungi carotenoids are synthesized only through Ac/MVA. Thus, despite some taxonomical features of some objects and their different evolutionary stages, experiments on the fast inclusion of the label from $^{14}CO_2$ (Saakov 1970b) gave the explanation. Data on Lut and Car formation are described in works on substantiation of the new pathway of biosynthesis, and data on synthesis of carotenoids possessing epoxy

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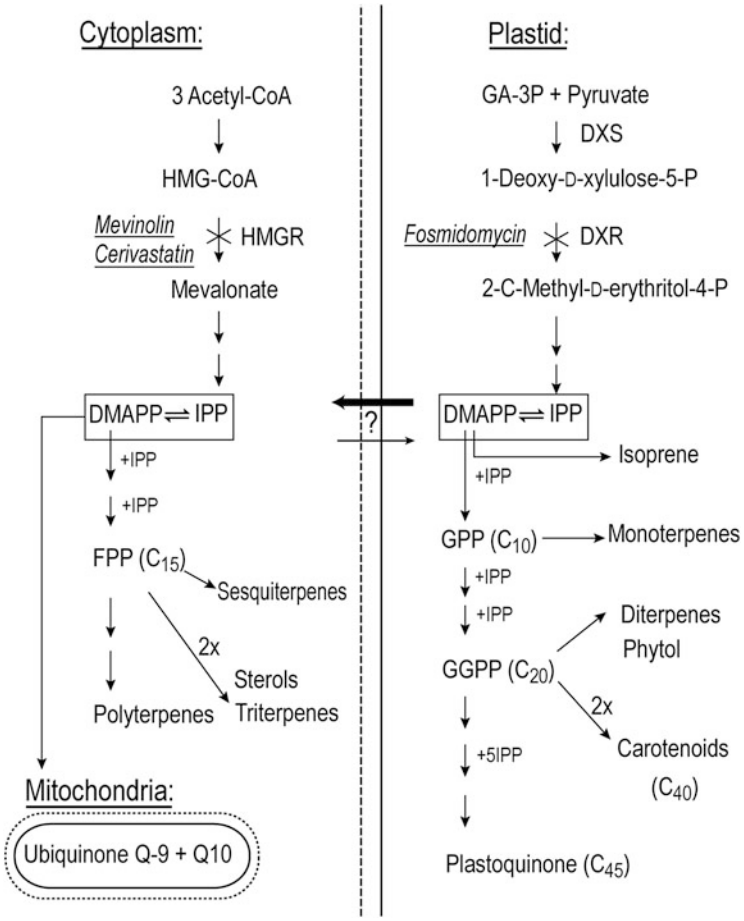

Fig. 5.27 Compartmentation of isoprenoid biosynthesis in the plant cell. *Right*: Plastid DOX/MEP pathway for the biosynthesis of the active C5 unit (IPP) for chlorophylls (phytol side chain), carotenoids, etc. *Left*: The cytosol Ac/MVA pathway of IPP biosynthesis for the formation of sterols and prenyl side chain of the mitochondrial ubiquinones (Q-9; Q-110) (Schwender. . .Lichtenthaler 1999, 2001; Lichtenthaler 2000a, b, 2007)

groups (Viol) are contained in a small number of publications (Costes 1963a, b, 1968; Saakov 1970b; Lutsenko and Saakov 1971, 1972) and demanded their development by a new methodological step.

The great interest in this field of research conditioned the scrupulous attention to the methodological basis of works (Roberts and Perkins 1966; Rohmer et al. 1993; Lichtenthaler et al. 1997a, b; Rohmer 1999) (as well as those not included in the reference list) connected with the need for high radiochemical purification of isolated preparations. For this purpose, we carried out comparative research into the biosynthesis processes of Viol, Lut, and Car in other biological objects

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using the experience accumulated by us and other researchers on radiochemical purification of plastid carotenoids and on their exact spectral identification with the help of techniques developed earlier (Saakov 1970b; Aleinikov 1974; 2000a, b). Experimental experience indicated a large number of colorless impurities accompanying dyed spots of carotenoids (Saakov and Shiryaeva 1967). The authors of the DOXP/MEP pathway did not focus their attention on this part of experiment at all, and it is exactly this part of the methodological work that is a weak point. We emphasize that we developed a measurement technique that is substantially free from this disadvantage, and it was shown that the phenomena of xanthophyll conversion are not conditioned by dyed or colorless impurities (Saakov and Shiryaeva 1967; Saakov 1970a; Saakov and Nasarova 1970).

We performed verifying work using leaves of *Phaseolus vulgaris*, *Nicotiana tabacum*, and mixotrophically grown *Scenedesmus obliquus* cells. The chamber for photosynthesis contained a CO_2 concentration of 0.3 % with total activity 200 MBq. Cells of *Scenedesmus* were resuspended in phosphate buffer containing labeled glucose (120 MBq per vessel). Upon termination of exposure, the glucose solution was sucked out with a vacuum pump through Millipore filters (0.3 μm). Objects were fixed with liquid nitrogen. Extracts of pigments were saponified with 15 % KOH in methanol, and carotenoids were dissolved in a mixture of petroleum and diethyl ethers. Extracts were put on thin layer plates or paper and were chromatographed using published techniques (Saakov 1970b; Aleinikov 1974). After five or six consecutive chromatography steps, the SpA of preparations was determined. For uniformity of calculations, we used normalization of SpA of measured fractions per microgram of carbon in 100 s.

In preliminary experiments, we investigated DSHO for Lut and Car in polar and nonpolar solvents, as presented in Sect. 5.1. Derived spectra for Viol were studied earlier (Saakov 2002a, b). For suppression of pathways of mevalonate biosynthesis, the antibiotic mevinolin was used, which inhibits sterol biosynthesis but does not influence carotenoid formation in plastids of higher plants and green algae. For suppression of plastid carotenoid biosynthesis, fosmidomycin was applied, which effectively blocks a link of DOXP/MEP recombination (Roberts and Perkins 1966; Rohmer et al. 1993; Lichtenthaler et al. 1997a, b; Rohmer 1999; Lichtenthaler 2000a, b). The radioactivity measurement was carried out using the scintillation counter Intertechnique (France).

Results of performed experiments are summed in Table 5.5 and in Fig. 5.28.

It is possible to make the following conclusions from the presented data:

1. There is fast inclusion of the radioactive label in all tested fractions of pigments, which indicates high speed of updating of preparations.
2. Speed of inclusion of radioactivity from glucose and carbonic acid is higher in Viol preparations.
3. SpA distinctions in Viol preparations and, accordingly, in Lut and Car preparations decrease with time.
4. There is a gradual accumulation of updated, labeled molecules of pigments, that, most likely, is accompanied by disintegration of old non-labeled funds. Over

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Table 5.5 Dynamics of specific activity change of carotenoid preparations in dependence on duration of object incubation in the radioactive medium (imp/min/μg carbon in a pigment molecule, variation ±10 % for $n = 3$)

Exp. №	Object, source of ^{14}C, inhibitor	Pigment fraction	Duration of exposure on labeled substrate (min)									
			5	10	20	30	60	120	240	300	600	1,200
1	*Scenedesmus obliquus*, glucose	Viol	1,450	1,837	2,350	2,900	3,800	5,100	6,210	8,400	9,000	9,200
		Lut	270	330	450	805	1,300	2,100	2,800	3,500	4,710	5,100
		Car	290	360	440	790	1,270	2,450	3,100	3,670	4,520	4,400
2	*Scenedesmus obliquus*, glucose + mevinolin	Viol	1,270	1,640	2,200	2,850	3,650	4,800	5,870	8,050	8,670	8,900
		Lut	230	320	470	840	1,470	2,320	2,650	3,640	4,860	5,460
		Car	240	380	430	790	1,390	2,640	2,930	3,720	4,670	5,300
3	*Phaseolus vulgaris* + $^{14}CO_2$	Viol	2,870	3,790	4,680	5,990	6,780	7,900	9,340	10,560	11,450	12,600
		Lut	440	560	830	1,450	2,200	3,460	4,800	5,200	6,340	7,560
		Car	510	580	870	1,330	2,150	2,960	4,780	5,300	6,890	7,870
4	*Phaseolus vulgaris* + mevinolin + $^{14}CO_2$	Viol	2,560	3,580	4,200	5,350	6,270	6,900	8,400	8,900	9,470	10,450
		Lut	378	470	680	980	1,300	1,900	3,700	4,150	5,200	6,890
		Car	470	530	820	1,170	1,900	2,680	4,470	4,920	6,230	7,280
5	*Nicotiana tabacum* + $^{14}CO_2$	Viol	2,300	2,700	3,540	4,690	5,470	6,340	7,600	8,470	9,420	10,560
		Lut	380	510	770	1,290	1,980	2,920	4,170	4,470	5,610	6,430
		Car	435	510	750	970	1,860	2,560	3,260	3,670	4,860	5,430
6	*Nicotiana tabacum* + mevinolin + $^{14}CO_2$	Viol	2,260	2,530	3,270	4,130	4,980	5,870	7,290	7,980	8,880	9,350
		Lut	370	490	710	1,130	1,760	2,650	3,820	4,100	5,230	5,980
		Car	370	390	680	840	1,530	2,150	2,830	3,340	4,360	4,970
7	*Nicotiana tabacum* + fosmidomycin + $^{14}CO_2$	Viol	450	490	690	820	970	1,110	1,190	1,420	1,550	1,940
		Lut	66	89	155	190	242	360	429	434	795	840
		Car	74	93	137	164	193	310	364	396	654	687

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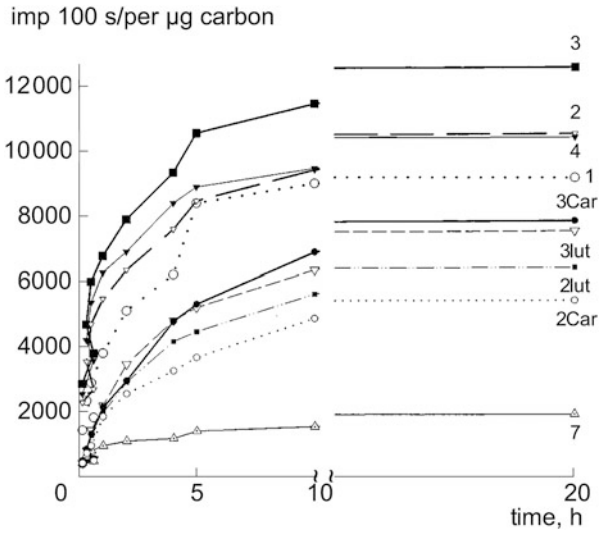

Fig. 5.28 Dynamics of SpA change for radiochemically purified carotenoids in the process of their renovation, using different substrates: (*1*) Viol, *Scenedesmus*, ^{14}C-glucose; (*2*) Viol, *Ph. vulgaris*, $^{14}CO_2$; (*3*) Viol, *Ph. vulgaris*, $^{14}CO_2$; (*4*) Viol, *Ph. vulgaris*, mevinolin, $^{14}CO_2$; (*7*) Viol, *Ph. vulgaris*, fosmidomycin, $^{14}CO_2$; (2Car) carotene, *N. tabacum*, $^{14}CO_2$; (3Car) carotene, *Ph. vulgaris*, $^{14}CO_2$; (2Lut) lutein, *N. tabacum*, $^{14}CO_2$; (3Lut) lutein, *N. tabacum*, $^{14}CO_2$. *Abscissa* time of incubation in labeled medium

10–12 h there is a tendency for the SpAs of Lut and Car to reach a plateau, because the increment of SpA fractions is within the limits of measurement error.

5. After 20 h of incubation in radioactive medium, the SpA of Viol practically reaches a plateau, which assumes achievement of a dynamic balance in updating of pigment funds and suggests that this time interval is necessary for full updating of Viol funds.
6. With certain caution, conclusion 5 is also valid for funds of Lut and Car.
7. Carbon from CO_2 more actively goes into plastids of objects than carbon from glucose, which indicates the participation in xanthophyll synthesis of primary products of photosynthesis such as Pyr, 3-PGal, PGA, etc. Chloroplasts possess their own compartments for IPP synthesis in parallel with IPP biosynthesis in cytoplasm.
8. Mevinolin action did not interfere with synthesis of Viol, Lut, and Car; therefore, it did not suppress the vigorous activity of the DOXP/MEP pathway, as confirmed in Table 5.5. SpA of Viol remains higher than those of Lut and Car for 20. This suggests a high updating speed for Viol funds.

The data in Table 5.5 and Fig. 5.28 give the chance to plan and to consider a number of probable ways of ^{14}C label migration before its inclusion in the DOXP/MEP pathway (Scheme 5.1):

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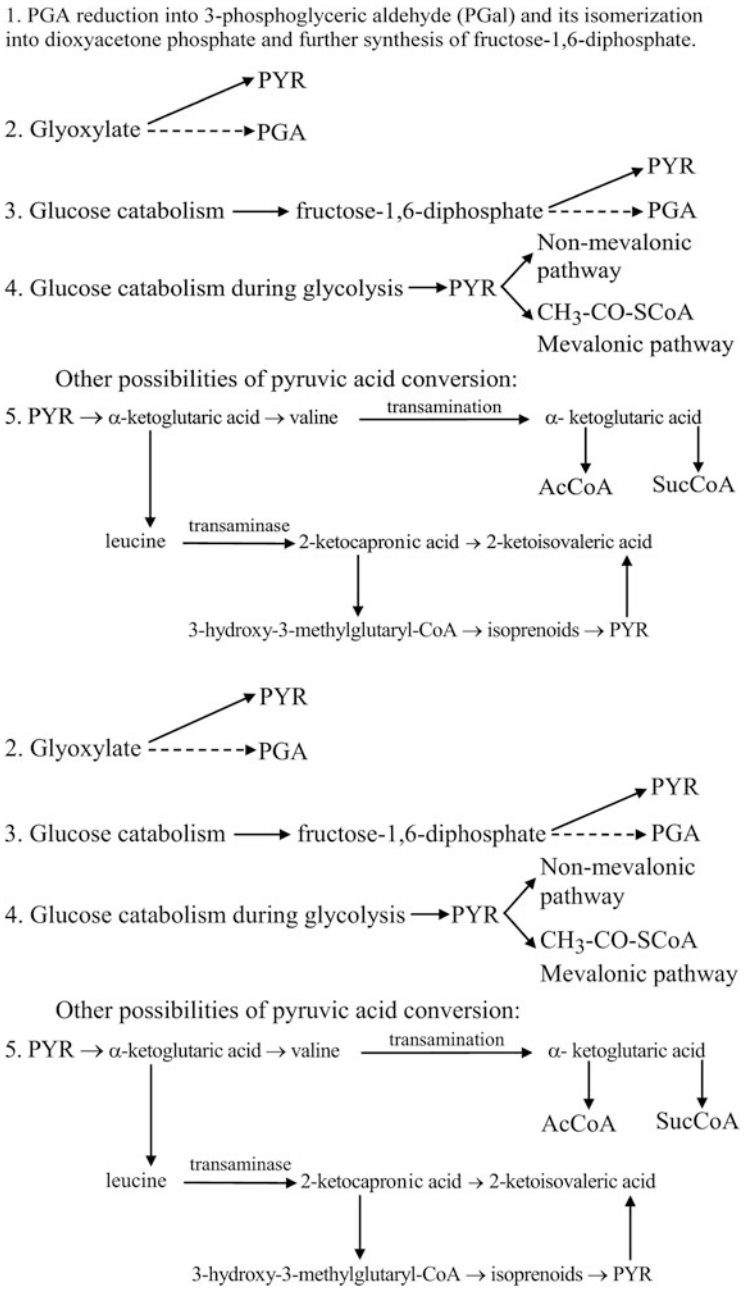

Scheme 5.1 PGA reduction into 3-PGal and its isomerization into dioxyacetone phosphate and further synthesis of fructose-1,6-diphosphate

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Thus, the reality of inclusion of the label from $^{14}CO_2$ or from glucose does not raise doubts, but there are several probable ways of migration of the label. At the same time, the high radioactivity of Viol in the first minutes of exposure in labeled medium suggests that not all ways of carotenoid biosynthesis have been thoroughly revealed and it is possible to expect unexpected surprises. It is enough to recall for how many years and how many researchers puzzled over the primary product of inclusion of the carbon label in photosynthesis.

Results of mevinolin action are in accordance with its influence on the DOXP/MEP pathway described earlier (Lichtenthaler et al. 1997a, b; Rohmer 1999), and they do not interfere with the synthesis of Viol, Lut, and Car. The absence of 3-hydroxy-3-methylglutaryl-CoA (HMG) is assumed because inactivation of the corresponding reductase (HMGR) with mevinolin inhibits a step of HMG reduction with specific inactivation of HMGR. So mevinolin did not suppress the vigorous activity of DOXP/MEP pathway. Refinement of mevinolin action in a cytosol pathway of isoprenoid biosynthesis is a subject for further work.

There is a separate question about the influence of fosmidomycin. The inhibitor does not catastrophically affect the speed of label inclusion into Viol and does not completely suppress the system of updating of its funds, which suggests that our assumption of the presence of other probable pathways of Viol biosynthesis is not deprived of its basis. Thus, it is not necessary to ignore the presence of a second carboxylating system in plants (this pathway accounts for up to 20 % of absorbed $^{14}CO_2$) and the fast formation of OAA from PEP funds and further of aspartic and malic acids. Furthermore, the label does not skip PGA with an increase in exposure time. Features of photosynthesis in C3 and C4 plants indicate that synthesis of malic acid is secondary with respect to PGA. Therefore, advanced comparative research into the pathways of IPP biosynthesis in C3 and C4 plants is necessary.

The dichotomy of two independent ways of biosynthesis of IPP funds into cytoplasmic and plastid funds explains the complexities appearing in interpretation of data: the fast inclusion of the label into Car, Lut, Zea, Viol, and Neo and the dependence of obtained results both on the conditions of autotrophic, mixotrophic, or heterotrophic feed and on the origin of the radioactive label (CO_2, alanine, acetate, glucose, glycine, etc.). In the case of CO_2, the label is quickly found in plastid isoprenoids β-Car, Viol, Lut, Zea, Neo, epoxylutein, phytol of chlorophyll, plastoquinone-9, and isoprene. These data are fair for C3 and C4 plants. Naturally, acetate can be used in the DOXP/MEP pathway of biosynthesis, but for this purpose, it should be involved in the glyoxylate cycle of tricarbonic acids for synthesis of PEP and further of Pyr and 3-PGal.

Proponents of the DOXP/MEP pathway do not focus the attention of the reader on the source of thiamine pyrophosphate (TPP) for two steps of the reaction, that is, interaction of two-carbon Pyr with 3-PGal and synthesis of 1-deoxy-D-xylulose-5-phosphate (DOXP), nor for two places of reaction in the case of valine synthesis through acetolactate (Lichtenthaler et al. 1997a, b; Rohmer 1999). However, 40 years ago, at the multinational symposium “Biochemistry and biophysics of photosynthesis” (Irkutsk, near Baikal see; arranged by Prof. Dr. Saakov VS, 1970),

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we reported the influence of light on TPP and thiamine triphosphate (TTP) phosphorylation in chloroplasts and highlighted the role of TTP in decarboxylation reactions. We also described the requirement for TTP and TPP participation in transketolase reactions of transport of two-carbon residues in the Calvin cycle and in glycolysis, and also the TTP connection with carotenoid metabolism (Sysoev et al. 1971).

The new DOXP/MEP pathway of IPP formation from Pyr and 3-PGal in plastids possesses, apparently, features that have remained in the evolutionary process of metabolism of their prokaryotic and endosymbiotic ancestors. Kinds of *Archaebacteria* are different because enzymes catalyzing the DOXP/MEP pathway were not found and the presence of only 3-hydroxy-3-methylglutaryl-CoA (HMG) has been established. Mevinolin inhibits the stage of HMG reduction by inactivating HMGR. At the same time, the presence of the Ac/MVA pathway is established in *Staphylococcus carnosus*, *Myxococcus fulvus*, *Flavobacterium* sp., *Chloroflexus aurantiacus*, in thermophiles *Caldariella acidopholia*, and in halophytes *Halobacterium halobium*, *Haloarcula japonica*, etc. (Roberts and Perkins 1966; Rohmer et al. 1993; Lichtenthaler et al. 1997a, b; Rohmer 1999; Lichtenthaler 2000a, b). The list of prokaryotes possessing Ac/MVA and DOXP/MEP pathways of biosynthesis can be found in a review on the site of the Institute of Genomic Research (http://www.tigr.org). Thus, in *Procaryota* there is no uniformity of pathways of isoprenoid biosynthesis. For fungi and yeast, the presence of only the Ac/MVA pathway was shown. At the same time, *Chlorophyta* are characterized by identical ways of isoprenoid biosynthesis in cytosol and in plastids through the DOXP/MEP scheme (*Prasinophyceae*, *Chlorophyceae*, *Ulvophyceae*), and also in *Bacillariophyta* and *Rhodophyta*. In *Zygnematales*, *Klebsormidiales*, *Charales*, and others, the cytosol synthesis of isoprenoids is carried out through the Ac/MVA pathway and plastid synthesis through the DOXP/MEP pathway. In *Mammalia* and *Nematoda*, isoprenoid synthesis is only through the Ac/MVA pathway. Recently, we presented data on the possibility of Car synthesis through the mevalonate pathway in the cockroach *Periplaneta americana*, but our data do not exclude the possibility of other biosynthesis branches conditioned by the initial carbon substratum (Shukolyukov and Saakov 2001). In higher plants, there is no less difficult a picture of isoprenoid biosynthetic pathways. For example, *Lemna gibba*, *Daucus carota*, *Hordeum vulgare*, and *Catharanthus roseus* possess both the cytosol Ac/MVA pathway and the plastid DOXP/MEP pathway of isoprenoid synthesis, and a large variety of other higher plants (*Populus nigra*, *Ginkgo biloba*, *Lycopersicon esculentum*, *Hedera helix*, etc.) are characterized by the DOXP/MEP pathway.

The above-mentioned facts help explain the reason for misunderstandings between researchers concerning the biosynthesis pathways for plastid and cytosol isoprenoid. Theoretical questions about the biosynthesis of isoprene compounds have enabled practical methods to be offered in the struggle against the malarial parasite *Plasmodium* and pathogenic eubacteria (i.e., the introduction of drugs containing an inhibitor of isoprenoid synthesis).

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On the basis of the stated, it is possible to conclude the following:

1. In the process of active updating of funds of plastid carotenoids, there is differentiation regarding speeds of inclusion of the radioactive label from exogenous substrates.
2. At the initial stages of object exposure to labeled substrate, the label penetration speed into Viol funds is significantly higher than into funds of Lut and Car.
3. Time of reaching of the dynamic balance in the processes of synthesis and disintegration of carotenoid funds is about 10–15 h.
4. The speed of carbon inclusion in carotenoid skeletons is higher from $^{14}CO_2$ than from glucose.

The analysis of non-realized alternatives often allows a more precise and deeper understanding of what has actually occurred. In reality, because of the self-conceit of the reviewer of the above-mentioned work and because of worship of Goodwin's infallible authority, one more discovery was pushed to the back in our science.

So it is possible to conclude the high informative value of *comparison* of biochemical processes of carotenoids from the point of view of the phylogenetic generality of evolutionary pathways of metabolism and pigment biosynthesis in animals and plants. DSHO served as a reliable tool for gaining knowledge.

It is not so difficult, when resulting conclusions are known, to come back to the beginning of a dispute and to make assessments post factum. It was much more difficult to understand and to place emphasis correctly in the totality of arisen questions, in expediency of the antagonism of scientific opinions, and in interpretation of experimental facts while keeping ourselves at the given historical moment and within the framework of scientific courtesy.

5.5 Possibility of Participation of α-Ketoglutaric Acid Funds in Carotenoid Biosynthesis in Chloroplasts

After detection of the light de-epoxidation reaction (DER) of violaxanthin–xanthin during study of the conditions of realization, inhibition, and reconstruction of the DER, many hypotheses were suggested, some correct and some not (Moster and Quackenbush 1952a, b; Cholnoky et al. 1956, 1957; Yamamoto et al. 1962; Sapozhnikov 1973; Saakov 1990d, 1971b, 1973b; Hager 1967a, 1966). However, there were no questions about what occurs with the *other set of carotenoids in chloroplasts*, where the Zea formed in de-epoxidation either disappears or, as postulated, goes to Lut (up to 55 % of the carotenoid fraction). Because of Lut, there were many sharp disputes (Yamamoto et al. 1962; Sapozhnikov 1973; Saakov 1990d; Hager 1967a, 1966; Donohue et al. 1967). Another question was absolutely omitted: How should the classical ratio Car/xanthophyll(s), discovered by Willstätter and Stoll (1913) and changing depending on external conditions, be considered? The lability of this ratio already assumes the existence of interconversions between carotenoids.

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We mentioned initial works on the given problem because a habit had already appeared to accept a priori (often thoughtlessly) that the reactions of the xanthophyll cycle are *something separate* from the reactions in chloroplasts, forgetting the existence of data on the interrelation of oxidation–reduction reactions of carotenoid metabolism and biosynthesis in vegetative and animal cells. These data accented the connection between Car transformations and the formation of Lut and Zea and showed a generality of Car oxidizing transformations in green algae and animals without damage to the initial carbon skeleton of Car. Further, these works indicated the presence of transformation reactions uniting carotenoids in the integral system (Saakov 1990b) and answered the question about what occurs and where the dihydroxy derivatives of Viol (Zea and Lut) formed in the DER disappear. Neither Lut nor Zea can remain infinitely in chloroplasts, considering that the speed of the DER is ten times higher than the speed of the return dark reaction of epoxidation of Zea or Lut (Yamamoto et al. 1962; Sapozhnikov 1973). Does epoxidation of Zea or Lut occur in light or is there a constant feed of spent Viol funds through its de novo synthesis from precursor pools, in which phototrophic or heterotrophic carbon is quickly included without participation of the carbon skeletons of Lut and Zea? Probably, slow Zea epoxidation and biosynthesis of Zea and Viol go simultaneously and independently of de-epoxidation of Viol. There is not yet an answer to the question about the kinetics of these reactions. All these specified questions were missing from the attention field of the majority of researchers dealing with the xanthophyll cycle (Lichtenthaler 1999).

The existence of a DER for Viol in light and the corresponding accumulation of Zea automatically exclude questions of interrelation and of the state of other carotenoids in chloroplasts, which were not considered. There was no answer to the question about the presence of a DER for Viol in the presence of dithiothreitol; only the oppression of Zea formation was highlighted. Nobody specified whether the decrease in Zea content occurs due to inhibition of the mechanism of Zea biosynthesis or due to the transformation Viol → Zea. Similarly, there was no answer to the question of the functional expediency of a return low-speed epoxidation reaction Zea → Viol, because the process of formation of new "young" Viol molecules is quick (Saakov 1990d, 2003b). The habit of accepting the established view became unstable when the carotenoid system started to be considered as a versatile set of components instead of only three separate xanthophylls (Saakov 1990d; Depka et al. 1998). What occurs with Lut, Car, Neo, and lutein epoxide is a theme that is not discussed in research on the interconversions Viol → Ant → Zea and back. It is especially interesting, taking into account the high speeds of their biosynthesis (Lichtenthaler 1999; Schwender et al. 2001).

To answer questions about pigment transformation and in the light of data about new ways of carotenoid biosynthesis (DOXP/MEP) (Lichtenthaler 1999; Schwender et al. 2001), it was important to assess the dynamics of inclusion of different key products of cell metabolism in carotenoid molecules and to assess the speed of updating of their funds during creation of new young molecules of Car and xanthophylls. The importance of this task was greater because of the extremely poor number of methodologically correct works on the study of carotenoid updating (Saakov 2003b). Therefore, an assessment was made of the possibility of using

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compounds such as α-ketoglutaric acid (α-KGA) for synthesis of carotenoid funds. α-KGA along with pyruvic acid is a key metabolite of reactions of tricarbonic and dicarbonic acids, re-aminization of amino acids, heterotrophic assimilation of CO_2, decarboxylation of oxalo-amber acid, transformations of pyruvic acids, and glucose catabolism (Saakov 2003b), that is, α-KGA funds could be the source of a carbon skeleton for carotenoids of various structures and degrees of oxidization.

Preparations of 2α-keto-[5-^{14}C]-glutaric acid from the firm Amersham (England) were used. As objects of research, leaves of *Phaseolus vulgaris*, chloroplasts of *Picea excelsa*, and suspensions of *Scenedesmus obliquus* served. Labeled preparation was introduced into a nutrient incubation medium or infiltrated into disk cuttings from leaves in a syringe (Aleinikov 1974). Radiochemical purification of xanthophylls and in particular of Car was performed using the published technique (Saakov and Shiryaeva 1967; Saakov 1990d; 2003b). For the additional control of purification criteria of carotenoid preparations, derived spectra of fourth to twelfth orders (D^{IV}–D^{XII}) were used (Saakov 2002a, b, 2003b). The expediency of registration of D^{XII} spectrum arises during isolation of especially pure preparations, as shown by comparison of curves 3 and 4 in Fig. 5.3 (Saakov 2002a, b, 2003b). Digitalization of spectra was carried out with programs *Grafula* 3 and *Graph Digitizer* 2.16. Construction and differentiation of spectra were performed with programs *Origin* 6.1 and *Spectra Calc* and the plotting with *SigmaPlot* 2000.

During measurement of speeds of inclusion of the radioactive label (RL) from α-KGA funds, the controls were experiments with addition of fosmidomycin, which inhibits a recombination link of DOXP and MEP. The second inhibitor was the antibiotic mevinolin, which blocks the mevalonate pathway of prenyl lipid biosynthesis in cytoplasm and does not influence carotenoid biosynthesis in chloroplasts (Lichtenthaler 1999; Schwender et al. 2001; Saakov 2003b). As an additional control for measurement of the speed of Viol and Neo biosynthesis, the data on intensity of label inclusion in Lut and Car served. Calculations of the SpA of preparations corresponded to those described (Saakov 2003b).

The results of experiments are presented in Table 5.6 and in Fig. 5.29, from which it is possible to make the following conclusions: High speed of RL inclusion is found at incubation with α-KGA in all studied carotenoid fractions within the first minutes of objects being exposed to the radioactive medium. The same fact, but using another method, was confirmed in other works (Saakov 1990d, 2003b). Average speeds of α-KGA penetration in suspensions of chloroplasts and *Scenedesmus* in pigment fractions are higher than after its infiltration in leaf cuttings of string beans. Reduction of the slope angle of curves of RL inclusion speed into Viol, Neo, Lut, and Car from α-KGA corresponds to a number of the listed pigment fractions and assumes that the speed of α-KGA inclusion into Viol and Neo is significantly higher than into Lut and Car. In the process of RL inclusion, there is no convergence of Viol SpA with those of Lut and Car, and this contrasts with changes in the SpA of pigments during CO_2 inclusion (Saakov 2003b). In the case of objects incubated with fosmidomycin, the trend of considered curves is similar.

The obtained results were in accordance with published material (Saakov 2003b) showing that during the influence of fosmidomycin on a leaf of a string bean, the label from $^{14}CO_2$ nevertheless arrives in carotenoid funds, although in considerably

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Table 5.6 Dynamics of specific activity change for violaxanthin, neoxanthin, lutein, and β-carotene in dependence on duration of exposure in the medium with α-ketoglutaric acid

Exp. №	Conditions and object of study	Pigment fraction	Time of exposure with α-KGA (min); data in imp/100s/μg carbon in a pigment molecule, variation ±8 % for $n = 4$							
			3	9	18	36	72	180	360	600
1	*Ph. vulgaris*, in 10 mL of solution for infiltration of 250 μCi acid	Viol	1,850	2,700	4,450	5,700	7,900	8,400	8,600	8,750
		Neo	1,270	2,220	3,200	4,600	5,100	5,900	6,200	6,370
		Lut	730	1,140	4,600	3,250	3,940	4,610	4,760	4,820
		Car	730	1,090	1,560	3,100	3,700	4,360	4,520	4,640
2	Chloroplasts of *P. excelsa* in 10 mL of 250 μCi acid	Viol	2,100	3,200	4,880	6,200	9,000	9,500	9,800	9,950
		Neo	1,600	2,400	3,500	5,250	5,950	6,500	6,800	6,900
		Lut	820	1,400	1,750	3,750	4,500	5,120	5,300	5,400
		Car	830	1,300	1,630	3,450	4,200	4,850	4,950	5,100
3	*Sc. obliquus*, in 10 mL of suspension of 250 μCi acid	Viol	1,960	2,970	4,640	5,780	8,100	8,520	8,750	8,890
		Neo	1,520	2,370	3,350	490	520	6,340	6,940	7,200
		Lut	670	1,320	1,630	3,320	4,100	4,900	4,950	5,200
		Car	650	1,240	1,570	3,120	3,850	4,450	4,560	4,920
4	Fosmidomycin, *Ph.vulgaris*, infiltration as in exp. № 1	Viol	370	485	580	910	990	1,210	1,300	1,410
		Neo	310	345	410	860	895	980	1,150	1,210
		Lut	99	115	165	220	265	330	426	560
		Car	84	103	160	190	245	290	390	520
5	Fosmidomycin, chloroplasts of *P. excelsa*, conditions as in exp. № 2	Viol	270	350	450	610	715	830	860	870
		Neo	220	270	360	475	590	670	720	710
		Lut	75	95	113	210	280	330	360	370
		Car	66	85	105	170	215	275	315	320
6	Mevinolin, other conditions and the object as in exp. № 2	Viol	2,240	3,210	4,600	5,900	7,900	8,150	8,270	8,350
		Neo	1,580	2,240	3,270	4,990	5,500	6,900	6,120	6,240
		Lut	790	1,200	1,620	3,100	4,250	4,750	5,160	5,270
		Car	800	1,200	1,400	2,800	3,940	4,570	4,830	4,990

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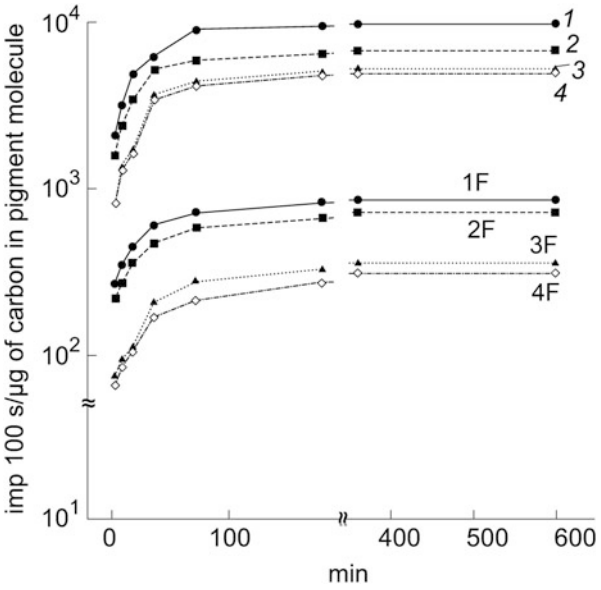

Fig. 5.29 Dynamics of inclusion of exogenous α-ketoglutaric-^{14}C acid in the fraction of radiochemically purified carotenoids of pea chloroplasts (*P. excelsa*): (*1*) Viol; (*2*) Neo; (*3*) Lut; (*4*) Car. The variant with addition of fosmidomycin (*F*): *1F* Viol; *2F* Neo; *3F* Lut; *4F* Car. *Abscissa* time of incubation with labeled preparation

lower quantity. However, this result contrasts with the level of suppression of pigment biosynthesis described in other works (Lichtenthaler 1999; Schwender et al. 2001). It could be that, in the case of fosmidomycin influence, unknown alternative pathways for formation of IPP precursor continue to function. Lower SpAs of Lut and Car compared with Viol and Neo were also observed by us earlier (Saakov 2003b). We state the conception that either synthesis of Viol and Neo is primary in relation to Lut and Car or IPP precursors with smaller SpAs participate in biosynthesis of Lut and Car (i.e., an alternative biosynthesis pathway is supposed).

Raised SpA of Viol and Neo were also observed in studies of RL inclusion from $^{14}CO_2$ (Saakov 1990d, 2003b). The stable domination of SpA of Viol over Neo indicates that it is first in the biosynthesis queue. The later SpA maximization in Viol biosynthesis compared with Lut and Car attracts attention; it is similar, with some stipulation, for Neo (Fig. 5.29, curve 2). After 80–100 min of incubation with α-KGA, the SpA of carotenoids approaches a plateau; the SpA increment in all pigments fractions is slight, which indicates the process of label saturation of carotenoid funds and assumes the appearance of a dynamic balance in biosynthesis speeds and, possibly, disintegration of pigments. Accumulation of updated labeled funds of the α-derivative of Lut occurs faster than during inclusion of RL from CO_2 (Yamamoto et al. 1972).

α-KGA is at a crossroad in the metabolic pathways of di- and tricarbonic acids and can turn into pyruvic acid. The latter gives rise to the DOXP/MEP pathway of carotenoid synthesis (Saakov 2003b; Lichtenthaler 1999). On the other hand, connection of α-KGA with the metabolism of glutamine, glutamic acid, and oxalo-amber acids accents the interdependence of reactions of protein and lipid metabolism and the possibility of their participation in carotenoid synthesis. The results presented in Table 5.6 and in Fig. 5.29 prove with a high degree of reliability

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that carbon funds of α-KGA can be used for carotenoid biosynthesis. Whether the biosynthesis mechanism occurs only through the DOXP/MEP scheme or there are alternative pathways (as indicated by indirect data of the present work) for label inclusion into carbon skeletons of xanthophylls and Car is a subject for further research.

The data presented in this section testify convincingly enough in favor of the biosynthesis of Viol, Neo, Lut, and Car through mechanisms for chloroplast carotenoids other than the mevalonate pathway, although the latter was strenuously embedded for many years (Saakov 1990d, 2003b; Lichtenthaler 1999; Schwender et al. 2001). At the same time, perfection of methodological approaches does not exclude detection of new pathways for the synthesis of components of the difficult system of chloroplast carotenoids. Further, assessment of SpA levels of considered pigments assumes a similarity in biosynthesis speeds for Zea and α-Car. Possibly, this is true for pairs Zea and Lut (β- and α-dihydroxy carotene derivatives) and α- and β-Car. It seems more difficult in the case of mono-epoxy Neo, Ant, and lutein epoxide. If the last two differ only in the β- and α-structures of second ionone rings and have similar SpA levels, then for Neo the presence of an allenic structure in the first ionone ring (=•=) is characteristic. Features of the Neo structure suggest the specificity of its biosynthesis and different SpAs of the carbon skeleton compared with Lut and Zea.

Existing data assume that, in the evolutionary process, photosynthesizing eukaryotes have preserved the DOXP/MEP pathway, which had been initially introduced by precursors of chloroplasts (i.e., endosymbiotically similar cyanobacteria) into chloroplasts of green algae and higher plants. Perhaps, decoding the features of carotenoid biosynthesis could end the long-term disputes concerning their function.

Demonstrative results have been obtained about the participation of α-KGA funds in carotenoid biosynthesis and about the primacy of synthesis of Viol and Neo compared with Car and Lut, and also about the time of updating of their funds in chloroplasts. It was shown that the time needed to reach a dynamic balance in the process of synthesis and disintegration of carotenoids during use of α-KGA (especially in isolated chloroplasts and in unicell algae) is shorter than in the case of absorption of gaseous $^{14}CO_2$.

5.6 Malic Acid as the Source for Carotenoids Synthesis in Plants with C4-Way of Carbon in Photosynthesis

After publication of material about the existence of a new pathway of carotenoid biosynthesis in chloroplasts, called the DOXP/MEP pathway because of the names of key products, the inconsistency of Goodwin's point of view about the general nature of the Ac/MVA pathway for biosynthesis of carotenoids, steroids, and other isoprene compounds in cytosols and chloroplasts became clear (Rohmer et al. 1993; Rohmer 1999; Lichtenthaler 1999; Saakov 2003b). The absence of critical consideration of the totality of available experimental material on the synthesis of isoprenoids in cytosols and chloroplasts of representatives of various taxonomical groups, and the frequently blind belief of Russian and European researchers in the

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infallibility of Goodwin's school, detained for almost 40 years the development of research on carotenoid biosynthesis and updating in chloroplasts. Discovery of the interaction of 3-PGal with Pyr, with formation of DOXP and transformation of the latter under the influence of DOXP reductoisomerase into MEP, ended the long hegemony of Goodwin's ideas and divided the pathways of IPP synthesis into cytosol and chloroplast pathways. It promoted further research, both in the area of updating of funds of chloroplast carotenoids and in the search for a new substrate for their biosynthesis (Saakov 2004). But any nonstandard opinion, decision, or discovery causes the activation of opponents. The 50–60-year development of inconsistent, and sometimes tendentiously fitted, research on carotenoid biosynthesis, and also of the diurnal and seasonal dynamics of their content in a cell, could be an extremely curious theme for the Institute of History of Natural Sciences.

With the appearance of new data, the specification of ways of biosynthesis, updating, and transformation of Viol, Neo, Ant, Zea, Lut, Car, and epoxylutein transferred the research to a qualitatively new level. Therefore, in assessing the updating of carotenoid molecules, there was first study of the inclusion dynamics for various key products of chloroplast metabolism, which are connected with formation of Pyr and 3-PGal funds. Initial works in this field are considered in Sect. 5.5.

In the photosynthetic process in C4 plants, the fast speed of carboxylation of PEP through OAA results in formation of malate (Mal) and aspartate (Asp), which can be transported to the Calvin cycle, where their decarboxylation occurs with regeneration of Pyr (Rubin and Gavrilenko 1977; Hoffmann 1987). Thus, it was possible to assume that the label from Mal goes to Pyr and the segregating carbon atom goes through PGA into 3-PGal. Besides, Mal can be involved in the citric acid cylce, resulting in formation of α-KGA. The possibility of α-KGA participation in the biosynthesis of chloroplast carotenoids was shown earlier (Saakov 2004) (see Sect. 5.5).

The possibility of inclusion of the radioactive label from ^{14}C-malic acid (Amersham, CFB-42) into molecules of the main carotenoids in chloroplasts of plants possessing the C4 pathway of carbon photosynthesis was investigated. As objects of study, we used leaves of 3- to 4-week-old sprouts of *Zea mays*, *Sorghum sudanense*, *Amaranthus retroflexus*, leaves of *Bryophyllum*, and also leaves of *Miscanthus sinensis* and *Atriplex hortensis*.

Labeled ^{14}C-malic acid (Saakov 2003b) was infiltrated in a syringe into cuttings from leaves and the leaves left in Petri dishes in the labeled solution for the period of exposure to light of 1,500 μE/(m^2s). After fixation of the material with liquid nitrogen, the radiochemical purification of xanthophylls and Car was performed using an established technique (Saakov and Shiryaeva 1967; Saakov and Nasarova 1970; Saakov 1970b, 1989a). Preparations of pigments were stored in liquid nitrogen; their radioactivity was counted with the scintillation counter Intertechnique (France). For suppression of biosynthesis of plastid carotenoids, we applied fosmidomycin, blocking the link of recombination DOXP/MEP at the formation of IPP (Rohmer et al. 1993; Lichtenthaler et al. 1997a, b; Lichtenthaler 2000a, b).

Table 5.7 presents the results of research on the dynamics of ^{14}C-Mal inclusion in fractions of various carotenoids as a function of exposure time in labeled substrate. Analyzing the data in Table 5.7, it is possible to make following conclusions: First, the radioactive label from Mal reliably goes to fractions of the main

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Table 5.7 Dynamics of specific activity change for violaxanthin, neoxanthin, lutein, and β-carotene in dependence on duration of exposure in the medium with malic acid

Exp. №	Conditions and object of study with malic acid	Pigment fraction	Time of exposure with malic acid (min); data in imp/100s/μg carbon in a pigment molecule, variation ±12 % for $n = 5$					
			3	10	15	25	60	120
1	Leaf cuttings of *Zea mays* in 10 mL of solution for infiltration of 1 mCi acid	Viol	675	823	1,342	1,846	2,200	2,400
		Neo	530	726	987	1,432	1,657	1,956
		Lut	275	374	630	976	1,278	1,780
		Car	245	346	645	924	1,345	1,830
2	Leaf cuttings of *Sorghum sudanense* in 10 mL of solution for infiltration of 1 mCi acid	Viol	579	920	1,458	1,926	2,435	2,657
		Neo	497	823	1,134	1,489	1,897	2,365
		Lut	324	435	775	1,112	1,576	1,987
		Car	289	443	780	976	1,489	1,968
3	Leaf cuttings of *Amaranthus retroflexus* in 10 mL of solution for infiltration of 1 mCi acid	Viol	714	856	1,468	2,234	2,469	2,564
		Neo	587	846	1,223	1,765	1,998	2,345
		Lut	364	431	694	1,198	1,778	2,134
		Car	296	415	812	1,154	1,653	1,876
4	Leaves of *Atriplex hortensis* in 10 mL of solution for infiltration of 1 mCi acid	Viol	645	879	1,376	1,987	2,365	2,678
		Neo	611	934	1,342	1,933	2,314	2,524
		Lut	411	498	832	1,297	1,897	2,233
		Car	312	465	932	1,314	1,968	2,295
5	Leaves of *Bryophyllum* in 10 mL of solution for infiltration of 1 mCi acid	Viol	324	579	1,124	1,658	1,799	1,935
		Neo	287	473	882	1,436	1,767	1,879
		Lut	224	362	637	873	1,236	1,465
		Car	239	376	689	936	1,344	1,567
6	Leaves of *Miscanthus sinensis* in 10 mL of solution for infiltration of 1 mCi acid	Viol	578	890	1,567	1,874	2,279	2,476
		Neo	467	1,012	1,263	1,679	1,926	2,265
		Lut	443	521	711	1,387	1,911	2,022
		Car	357	497	968	1,421	1,879	2,189
7	*Zea mays*, acid infiltration, as in Exp. № 1 + fosmidomycin	Viol	92	130	324	375	427	456
		Neo	88	170	193	346	391	412
		Lut	65	129	145	211	245	268
		Car	64	131	156	221	254	246
8	*Sorghum sudanense*, acid infiltration, as in exp. № 2 + fosmidomycin	Viol	112	145	156	390	435	467
		Neo	79	95	187	326	411	467
		Lut	49	88	156	263	289	297
		Car	55	123	144	223	267	298
9	*Atriplex hortensis*, acid infiltration, as in exp. № 4 + fosmidomycin	Viol	145	187	297	356	423	435
		Neo	98	178	221	365	422	455
		Lut	66	97	167	243	287	321
		Car	72	101	165	236	273	318

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carotenoids of chloroplasts. Second, the time for the hit of initial radioactivity to enter into pigments is short enough and corresponds to the time inherent to Mal metabolism in chloroplasts of C4 plants (Hoffmann 1987). Third, the fast increase in SpA of pigments in the first 3–25 min decreases in the period between the first and the second hours of exposure. SpA increment in the second hour (as the average of the first six experiments presented in Table 5.7) is equal to 8.3 % for Viol, 13.5 % for Neo, 17.5 % for Lut, and 18.1 % for Car. These results indicate the faster saturation of Viol funds with radioactive label and, obviously, faster fund updating of Viol, which is the key xanthophyll in the violaxanthin cycle of light reactions (Saakov 1990d). Neo has the second fastest updating speed; this pigment has a role as an intermediate in the violaxanthin cycle (Saakov 1966, 1973b, 1990b, d). Updating of Lut and Car, final compounds of violaxanthin reduction, occurs more slowly and with similar speed. The obtained data correspond to results on inclusion of labeled CO_2 and glucose into carotenoids (Saakov 1990d, 2003b) and also of α-KGA (Saakov 2004). From the data in Table 5.7, it also follows that, as described earlier (Saakov 2003b, 2004), fosmidomycin does not completely suppress synthesis of Car and xanthophylls in the chosen objects of research. Possibly, in this case, as discussed earlier (Saakov 2003b), the specificity of C4 plants manifests because they possess a second carboxylating system in combination with additional Mal funds.

Zea mays and *S. sudanense* are from the first group of C4 plants (Hoffmann 1987; Rubin and Gavrilenko 1977), the mesophyll chloroplasts of which have a granular structure that is manifested less clearly in bundle chloroplasts. In the first group of C4 plants, the role of Mal as the CO_2 source for reactions of the Calvin cycle is most significantly manifested during its transition from mesophyll to bundle chloroplasts, with Pyr formation from the Mal skeleton. The latter, except in PEP formation, can interact with 3-PGal in the mesophyll and form the substrate for IPP synthesis. Liberating $^{14}CO_2$ is included into the 3-PGal of bundle cells through ribulose biphosphate. So there is a second branch of IPP formation, through which exogenous radioactive label goes into IPP.

In another group of C4 plants, into which it is possible to include *A. retroflexus* and *Atriplex hortensis*, the mitochondria of bundle cells have an additional role during decarboxylation of aspartic acid (Asp) and Mal with formation of Pyr and liberation of CO_2. The latter, also interacting with ribulose biphosphate, creates a second branch of IPP formation (Hoffmann 1987). There is easy exchange of Mal between cytoplasm and chloroplasts and its fast transformation into OAA, as described by U. Heber (Würzburg, Germany), G. Krause (Düsseldorf, Germany), and K. Santarius (Germany) (Hoffmann 1987). In this process, the typical compartmentation of reactions in bundle cells occurs. In addition, part of the Pyr funds from the cytoplasm of bundle cells goes via alanine formation into the mesophyll cytoplasm with PEP regeneration. Thus, it is not excluded that the interaction of two branches of IPP biosynthesis promotes fast inclusion of the label from Mal into xanthophyll funds. The speeds of metabolism of malic acid and Viol de-epoxidation are quite comparable (Rubin and Gavrilenko 1977; Hoffmann 1987). Mal is also capable of leaving the mitochondria and being oxidized in the cytoplasm to OAA, with subsequent formation of Pyr and PEP. Obviously, the mechanism of shuttle of

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Mal, connected with OAA formation, promotes the transformation of Mal into Asp and, via alanine formation, participation in the formation of Pyr in mesophyll cells.

Thus, in chloroplasts of C4 plants, there are a number of possibilities of fast ^{14}C incorporation into the carbon skeletons of carotenoids with usage of different substrates (Saakov 2003b, 2004), one of which is Mal. The processes of carotenoid biosynthesis are closely combined with their oxidation–reduction transformations in cells of animals and plants (Saakov 1990b, d). These are especially concerned with replenishments of funds for the light reaction of Viol de-epoxidation (Saakov 1966, 1973a, 1990b) and of Zea funds, to which a protective role during energy dissipation has been strenuously attributed (Lichtenthaler 1999).

From the data in Table 5.7, the primary inclusion of label in molecules of Viol and Neo in the first minutes of exposure to radioactive substrate is visible, which coincides with the material presented in Sect. 5.5 on the inclusion of labels from α-KGA into xanthophylls (see Fig. 5.29).

It became obvious that, simultaneously with metabolic oxidation–reduction transformations of carotenoids, the processes of Zea biosynthesis (Saakov 2003b, 2004) actively participate in creation of Zea funds, because after ascertaining the new DOXP/MEP pathway of carotenoid biosynthesis, there were explanations about the raised speeds of their biosynthesis due to interaction of 3-PGal with Pyr.

Summing up the consideration of data in Sects. 5.2–5.6, it is necessary to notice that during research into the metabolism and biosynthesis of carotenoids in the twentieth century, interesting hypotheses repeatedly appeared. Thus, ideas that at first sight seemed very paradoxical turned out to be the most true. However, to prove such new hypotheses is always more difficult than to state them. For example, for about 100 years researchers could not clarify the function of the small thymus gland, which starts to disappear after about 12 years of life. Then, it was found that the major mechanisms of immunity are hidden in this gland. It is still not clear how much exists in common in the carotenoid metabolism of phylogenetically different representatives of *Procaryota* and *Eucaryota* (Saakov and Baranov 1987).

In describing some debatable moments of the history of our science, we have tried not to oversimplify the difficult covering up of feelings, which involuntarily accumulates over many years of scientific activity and which obviously accompanies the scientists standing on the edge of development of an area of science. Eventually, history arbitrates opponents and has its say.

The Viol transformation into Zea (Saakov 1989a, 1990a) is revealed in green algae (*Chlorophyta*) in orders *Volvocales* (family *Polyblepharidaceae*, *Chlamydomonadaceae*, *Volvocaceae*), *Ulotrichales* (family *Chaetophoraceae*, *Oedogoniaceae*), *Coccales* (family *Protosiphonacea*, *Oocystaceae*, *Scenedesmaceae*, *Hydrodictyaceae*), *Zygnematales* (*Zygnema*), and *Charales* (*Characeae*). The presence of Neo and β-Car in representatives of listed families of seaweed testifies in favor of the possibility of Viol reduction into Car through intermediate steps of de-epoxidation (Saakov et al. 1971b).

In dinoflagellates *Heterocontae*, the cyclic transformation of diadinoxanthin (3,3′-dihydroxy-7,8-dehydro-β-carotene-5′,6′-monoepoxide; Diad) into diatoxanthin (3,3′-dihydroxy-7,8-dehydro-β-carotene; Diato) (Saakov 1971b) is revealed. Such

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light-induced transformations of the monoepoxide Diad were found by Hager in orders *Heterococcales* (*Mischococcales*, genera *Botrydium*, *Bumilleropsis*), *Heterothrichales* (genera *Tribonema*, *Bumilleria*, *Heterococcus*, *Heterothrix*), and *Heterosiphonales* (*Botrydiales*, *Botrydium*). A special position in *Botrydiales* is occupied by *Vaucheria*. According to the classification of Kursanov and coworkers (Saakov 1990d), *Vaucheria* is from *Siphonales* (*Bryopsidales*, *Chlorophyta*), but according to the set of pigments, it can be considered as *Heterocontae*. According to Hager, the de-epoxidation reaction in *Vaucheria* is connected with transformation of the Diad cycle (Saakov 1971b) (Figs. 5.30, 5.31).

However, H. Kleinig (Kleinig and Czygan 1969) accents the existence of a Ant ↔ Zea cycle in this alga. These data are supported by the experiments of N.I. Krinsky on conversion of Ant into Zea in lyophilized *Euglena* cells after addition of NADPH and FMN (Goodwin 1971). The transformation of Ant into Zea has been shown (Sagromsky and Saakov 1970; Saakov 1989b).

At the same time, the transformation cycle of Viol that is characteristic for *Chlorophyceae* is found in genus *Pleurochloris* (*Heterococcales*). A distinctive feature of representatives of the listed algae is the presence of β-Car and Neo. The presence of these two pigments creates preconditions for functioning of the whole carotenoid cycle and for Diato reduction into β-Car. Suppression of the de-epoxidation of Diads by inhibitors of PS-2 is the same as in *Chlorophyta*. Unlike *Chlorophyta*, the de-epoxidation of Diad pigment is inhibited by salicylaldoxime. It is remarkable that vaucheriaxanthin was found in representatives of *Heterocontae*. The formula assumed for it contains four hydroxyls and one epoxy group. It is possible that the participation of vaucheriaxanthin in xanthophyll transformations in *Heterocontae* will be discovered.

In performed experiments, there were no revealed transformations in *Cyanophyta* and *Rhodophyta* characteristic for epoxyxanthophylls (Saakov and Baranov 1987; Saakov 1990b). This is explained by the absence of epoxyxanthophylls in the given algae. In *Oscillatoria*, the existence of light-induced changes in phycoerythrin content was accented. In *Cyanophyta* and *Rhodophyta*, the presence of β-Car, echinenone, and myxoxanthophylls allows us to hope that *further cyclic reactions of xanthophylls* will be detected. Experiments on *Rhodopseudomonas sphaeroides* support this thesis: Anaerobically grown cells produce *spheroidene*, and during culture aeration, this pigment turns into *spheroidenone*. The same pigment turns into spheroidene under the influence of light (Shneour 1962; Goodwin 1971).

In representatives of *Phaeophyta* (*Laminariaceae*), the cyclic de-epoxidation of Viol was found. Similar transformations of xanthophylls were ascertained in flagellates *Chrisomonadales* (*Ochromonadaceae*), but another cyclic reaction was noted in flagellates *Isochrysidaceae*, namely, de-epoxidation of Diad. In diatomeae *Bacillariophyceae* (*Naviculaceae*, *Discaceae*), transformation of xanthophylls occurs according to Diad type. The possibility of Diato participation in carotenoid metabolism in diatomeae (Saakov 1989b) was confirmed experimentally (Saakov 2005a). Hager discovered the cyclic de-epoxidation of Diad in *Euglenophyta* (*Euglenaceae*: *Euglena*, *Trachelomonas*). Because of insufficient experimental

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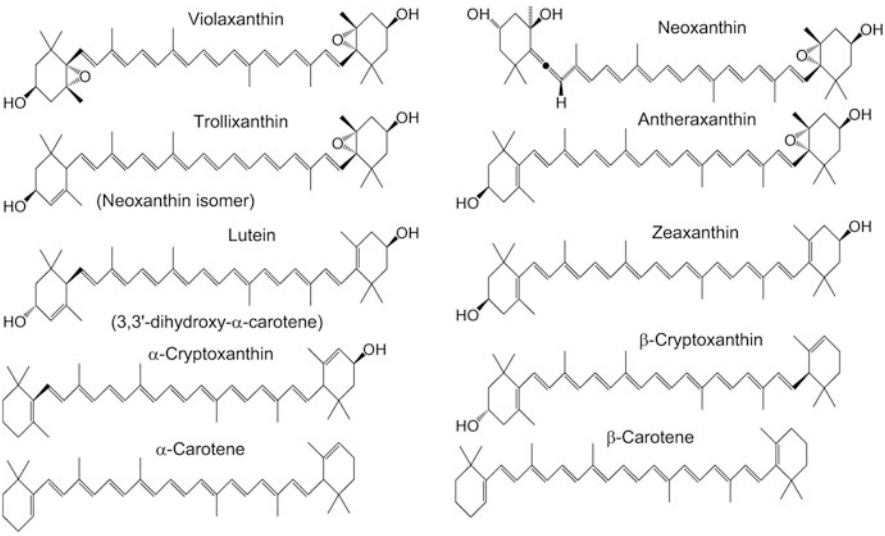

Fig. 5.30 Formulae of carotenoids probably taking part in light reactions of chloroplasts

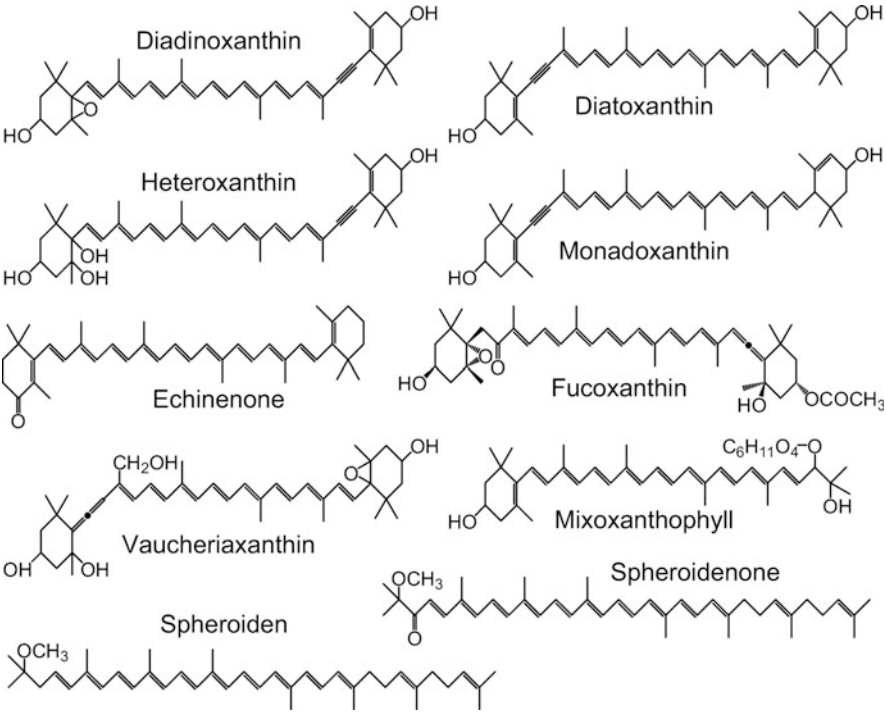

Fig. 5.31 Formulae of carotenoids taking part in cycles of reactions in algae and bacteria

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data and their discrepancies, the existence of the Diad cycle cannot be considered as strictly proven in *Euglena gracilis*.

The presence of monoepoxide fucoxanthin (Yamashita et al. 1969a, b) in brown algae (*Laminariaceae*) and also in *Bacillariophyceae*, *Ochromonadaceae*, and *Isochrysidaceae* allows us to consider this pigment as a potential component of cyclic reactions of xanthophylls. The presence of β-Car in studied seaweeds (except *Cryptomonas*) does not exclude the possibility of existence of deeper reductive transformations of xanthophylls.

Thus, the presented material is the certificate of competency of the assumption stated by us 40 years ago, namely, the existence of a cycle of carotenoid reactions in inferior plants (Saakov 1967). Both the reductive cycle of Viol and the light reaction of Diad are connected with de-epoxidation of the initial pigment.

The data presented in Sect. 5.7 indicate the connection of transformations of xanthophylls with the electron transport chain (ETC) of photosynthesis. If we assume that epoxyxanthophylls have a function in the mechanism of photophosphorylation (Bershtein et al. 1969, 1971; Petrenko et al. 1970), then the presence of the DER in evolutionary advanced photosynthetics allows us to connect its necessity with the realization of photophosphorylation. Similarly, we can connect de-epoxidation with the reactions of light defense as an obligatory sign of evolutionary developed autotrophic function. This is confirmed by data on *Antirrhinum*, and also on a variety of mutants and revertants of *Chlamydomonas*, and sets of mutants of *Scenedesmus* (Baranov et al. 1975; Kvitko et al. 1976, 1977; Saakov and Baranov 1987). Thus, possibly, blocking one of the steps of the cycle of carotenoid reactions (or their absence because of genetic blocks) can promote photosensitivity (Shneour 1962; Baranov et al. 1975) caused by damage to the activity of the carotenoid cycle and of components of chlorophyll–protein complex 2 in salad and green mutants and, correspondingly, of chlorophyll–protein complex 1 in yellow-green and orange mutants. This material highlights the common biological importance and specificity of de-epoxidation for the majority of autotrophic organisms.

5.7 Indication of the De-epoxidation Reaction with the Help of Derived Spectra

5.7.1 Coupling of the De-epoxidation Reaction of Xanthophylls with Changes in D^{II} Spectra at $\lambda = 460–470$ nm

Hager (1966, 1969) presented data on the influence of a number of inhibitors of photosystems and of uncouplers of photophosphorylation on the light reaction of xanthophyll transformation. Collectives of the Ukraine Institute of Organic Chemistry and Institute of Plant Physiology, both from the Academy of Science (Kiev) in

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Ukraine, found a dependence between the change in the ratio of chloroplast carotenoids in the photophosphorylation process with flavin mononucleotide (FMN) and a change in inorganic phosphate levels (Bershtein et al. 1969, 1971; Petrenko et al. 1970). During the first moments (1–3 min) of illumination of a chloroplast suspension, there is a sharp decrease in the violaxanthin content . The content of Lut (its fraction, apparently, also contains Zea) also gradually decreases in the first minutes (1–6 min) of illumination and then increases somewhat. The Car quantity sharply increases from the first minutes of light reaction (i.e., from the beginning of the photophosphorylation process). The authors suggested a dependence between the quantitative changes in carotenoids with time and the change in content of inorganic phosphate in the photophosphorylation process (Bershtein et al. 1969, 1971; Petrenko et al. 1970). It would be a great error to suppose that, as a whole, the balance of Lut did not change even though the content of Car increased considerably. This analysis by chemists possessing outstanding qualifications confirmed our conceptions about Viol reduction into Car in the process of de-epoxidation, as specified earlier in Sects. 5.2 and 5.3.

Thanks to the methodological possibilities of derivative spectrophotometry, it was revealed that, for the leaf of mutants lacking chlorophyll *b*, D^{II} changes in optical density (OD) in the region $\lambda = 460$–470 nm in response to the influence of light can be caused by the light reaction of xanthophylls (Meister and Maslova 1968; Sagromsky and Saakov 1970). These spectral changes we named the "$E_{460–470}$ effect." This section is devoted to description of this phenomenon and of spectral changes in the native leaf combined with it.

The specificity of the applied technique gives the possibility to measure the directivity of the xanthophyll reaction in the *intact leaf* (in vivo) over short intervals of time and without the errors and loss of pigments that are characteristic of chromatographic analysis. It is necessary to emphasize the possibility of consecutive measurements of direct (light) and return (dark) reactions in the same leaf, without removing it from the spectrophotometer cell. Application of derivative spectrophotometry allowed us to carry out research on the influence of inhibitors on the system of light reactions of xanthophylls, to study the behavior of this system during the influence of the complex of inhibitors and reductants, and the influence of uncouplers of photophosphorylation at a new methodological level and much more widely (Vernon and Zaug 1960; Losada et al. 1961; Wessels 1964).

As the object of research, we used leaves of barley mutants 2,800, 2,807, and 3,613 from the cultivar *Donaria*, received from the Central Institute of Genetics and Crop Plant Research of the Academy of Sciences, GDR (Zentralinstitut für Genetik und Kulturpflanzenforschung AdW, Gatersleben, GDR, renamed Institut für Pflanzengenetik und Kulturpflanzenforschung, IPK, after German reunification). The specific feature of these mutants is the absence of chlorophyll *b* in their plastid apparatus. The absence of chlorophyll *b* allows us to be assured that the bands of its absorption in the blue region do not mask the absorption spectra of xanthophylls. Figure 5.32 presents the D^{II} of absorption spectrum for the initial normal leaf of barley containing chlorophylls *a* and *b* and the D^{II} for the mutant 2,800. Numerous registrations of the spectra of intact leaves of the specified mutants showed their

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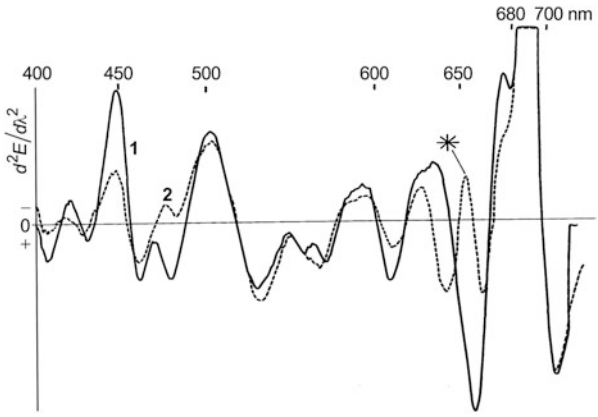

Fig. 5.32 D^{II} spectra of absorption of the initial barley cultivar *Donaria* (*1*) and of its mutant № 2807 (*2*) lacking chlorophyll *b*. *Asterisk* indicates the absorption maximum characteristic for chlorophyll *b* in the red region

identity. The work was started on the basis of studies at the Department of Physical Physiology of the Central Institute of Genetics and Crop Plant Research of the Academy of Sciences, now IPK (Gatersleben, GDR) (Saakov 1973a, b).

Inhibitors, ATP, and reductants were introduced into blades of barley leaves with the method of vacuum infiltration in a syringe. As a result of infiltration of metabolite solutions, the light scattering by the leaf decreased and the light transmission coefficient increased. Optimum duration of light or dark exposure of leaves after infiltration of inhibitor or metabolite was 10 min. It was experimentally found that longer exposures with inhibitors (30–60 min) caused secondary reactions of chloroplasts, manifested in the case of pigment complexes as a decrease in chlorophyll quantity and as opposite sign changes in the contents of epoxy and hydroxyl xanthophylls (Saakov 1971a, b; Saakov and Hoffmann 1974).

Directly before illumination, for a leaf infiltrated with metabolite and previously kept for 12–16 h in darkness to shift the epoxidation reaction to the dark reaction, the recording of the second derivative of its OD was performed in the range 400–500 nm. Then, the leaf was exposed for 10 min to light of 35,000 lx. Upon termination of light exposure, repeated recording of the D^{II} of absorption spectrum of the leaf was performed in the blue-green region. The presence or absence of a xanthophyll reaction was determined from the change in peak height in the region $\lambda = 460$–470 nm before and after illumination (ΔE_{460}). In experiments on the effects of inhibitors and other metabolites, we used as the control similar mutant leaves infiltrated with water. The average statistically reliable value for the light reaction of a leaf infiltrated with water (for the selected interval of amplification) was equal to five points on the scale of the recorder (each point corresponds to 0.1 relative OD units). In experimental work, it was revealed that at the given amplification scale, it is possible to reliably judge the realization of a light reaction of xanthophylls if the change in peak height at $\lambda = 460$ nm is three divisions of the recorder tape (0.3 relative units).

Figure 5.33 shows the efficiency of the DER of xanthophylls for control leaves of the barley mutant and for similar leaves infiltrated with diuron solution (DCMU,

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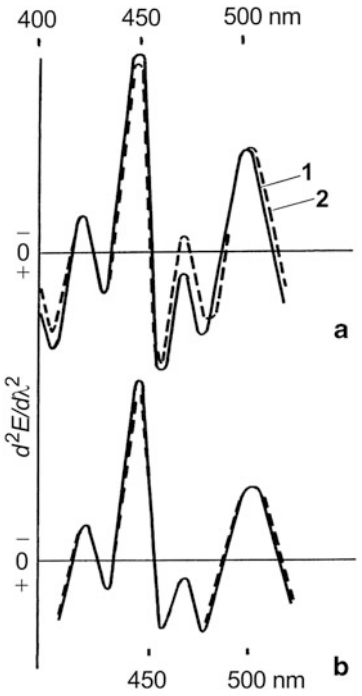

Fig. 5.33 Influence of light on the amplitude change of the peak at $\lambda = 460 - 470$ nm: (**a**) without the inhibitor; (**b**) in the presence of 5×10^{-5} M diuron; (*1*) before illumination; (*2*) after 10 min of light impact

10^{-5} M, 10 min). It can be seen that this concentration of inhibitor inactivates the light reaction of xanthophylls. A series of experiments showed that concentrations of inhibitor lower than 5×10^{-6} M used for infiltration in the leaf do not influence the yield of light reaction even after long exposure (30–60 min). DCMU concentrations of about 5×10^{-5} M and 10^{-4} M (for 5–7 min of exposure) inhibit this reaction. Similarly, damage of the light reaction of xanthophylls occurs in response to infiltration of the leaf with *o*-phenanthroline (10^{-3} M and 5×10^{-4} M) and with hydroxylamine (10^{-2} M and 10^{-3} M). Oppression of the light reaction of xanthophylls was not revealed during research on the inhibiting influence of salicylaldoxime on the ETC of photosynthesis in the plastocyanin step (Saakov 1971a, b; Saakov and Hoffmann 1974).

The data, obtained by us with the help of D^{II} measurement, on the short-term influence of a photosynthesis inhibitor on the light reaction of xanthophylls in an intact leaf, substantially correspond to the effect of inhibitor on the light reaction of xanthophylls in a chloroplast suspension (Saakov 1971a, b). On the basis of our and literature data, it was possible to assume the interaction between the xanthophyll system and ETC of photosynthesis, somewhere between plastoquinone and plastocyanin.

Inactivation of the light reaction of xanthophylls by inhibitors of PS-2 was demonstrated in various laboratories and using various research techniques; therefore, it is possible to consider this as reliably established (Saakov 1990d).

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In the process of research into the ETC of photosynthesis, it became known that the inhibiting influence of DCMU on photosynthetic reduction of $NADP^+$ in isolated chloroplasts can be neutralized by addition of dichlorophenolindophenol with ascorbate (DCPIP + AS) to the incubation medium (Vernon and Zaug 1960; Losada et al. 1961; Wessels 1964). This causes NADP reduction using electrons of the system DCPIP + AS (Vernon and Zaug 1960). It was also shown that *p*-phenylenediamines (PPD) with substituted nitrogen can be used in photosynthetic reduction of NADP as reductants in combination with ascorbate, removing the inhibiting influence of DCMU (Trebst 1963, 1966; Trebst and Pistorius 1965), for example, 2,3,5,6-tetramethyl-*p*-phenylenediamine (diaminodurol, DAD) and *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD). DAD has more negative potential than TMPD and DCPIP and is a much better donor of electrons (Witt et al. 1961; Wessels 1964; Trebst 1964, 1966; Arntzen et al. 1971). A number of research studies describe the connection between the removal of the DCMU inhibiting influence, when using DCPIP + AS, and the photosynthetic formation of ATP (Vernon and Zaug 1960; Losada et al. 1961; Witt et al. 1961; Trebst and Pistorius 1965, Trebst 1966). The DCMU inhibition of NADP reduction in chloroplasts is also removed by DAD, and this process is accompanied by photophosphorylation with ATP formation. At the same time, reduction of $NADP^+$ using derivatives of PPD is not connected with photosynthetic formation of ATP (Trebst and Pistorius 1965; Trebst 1966, 1963).

On the basis of the presented data, the conclusion was made that derivatives of PPD are electron donors in ETC after the photophosphorylation link. Hager presented data about the possibility of removal of the DCMU inhibiting influence on the light reaction of xanthophylls by the presence of hexylresorcinol or vitamin K_5 (4-amino-2-methyl-1-naphthol hydrochloride). Both these compounds provide a cyclic flow of electrons in the process of cyclic photophosphorylation (Hager 1966, 1969; Trebst 1966). Thus, the listed experiments allowed us to suppose that, by applying various combinations of inhibitors of photosystems and of reductants, it is possible to disjoint the ETC of photosynthesis and to create real preconditions for investigating where in the ETC the investigated system of xanthophylls is connected.

Earlier, as the result of a series of preliminary experiments, an interesting fact was discovered (carried out in 1969–1970 at IPK, Gatersleben). Leaf infiltration with DAD solution (10^{-3} M) causes stimulation of the change in the peak in the region of 460 nm in response to light (Fig. 5.34). Observable changes are obviously more intensive (average value from 20 experiments is 0.43 (Saakov 1971a)) than those of the water control (0.234) (see Table 5.8). Data from the experiments were statistically processed, and the effect of stimulation of the light reaction of xanthophylls does not raise doubts. We analogously checked the influence of ascorbate (2×10^{-2} M and 10^{-3} M) and the combination of ascorbate (10^{-2} M) and PPD (10^{-3} M) on the D^{II} change in the region of 460 nm in response to the action of light on the leaf. When ascorbate and the mixture PPD + AS were infiltrated into the leaf, the efficiency of the DER did not significantly differ from the reaction yield in the water control.

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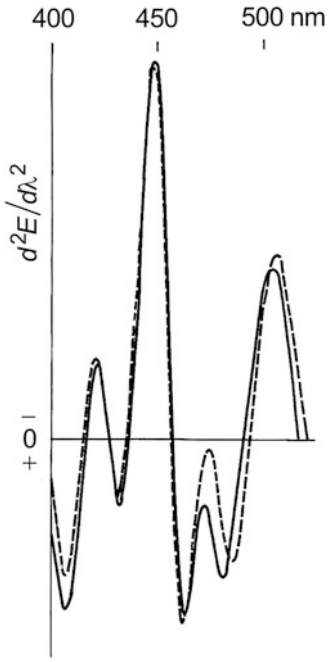

Fig. 5.34 Influence of infiltration of diaminodurol (10^{-3} M) on the amplitude of peak change in the region 460 – 470 nm; the inhibition effect is absent

Table 5.8 Effect of diaminodurol (DAD) on the efficiency of D^{II} change for the leaf under the influence of light

Experiment №	Duration of DAD infiltration before illumination of the leaf (min)	Changes of ΔE_{460} after light influence	Values of ΔE_{500} before and after illumination	Effect of the de-epoxidation reaction $\Delta E_{460}/\Delta E_{500}$
1	10	0.6	2.7	0.22
2	10	1.0	1.4	0.71
3	10	0.7	2.8	0.25
4	13	0.9	2.8	0.32
7	10	1.0	2.1	0.48
11	5	0.8	2.5	0.32
13	5	0.9	1.4	0.64
16	5	0.7	2.1	0.33
18	5	0.8	1.5	0.53
20	5	1.0	1.6	0.62
Average value of 20 experiments	≅ 0.8		≅ 0.43	

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Table 5.9 Influence of diuron (DCMU) and of artificial systems of electron donors on the effect of absorption change at $\lambda = 460$ nm (Saakov 1971b; Saakov and Hoffmann 1974)

Infiltrated inhibitors and electron donors	Number of experiments	E_{460}/E_{500}	Difference from control	Significance level ρ (%)
Water control	16	0.234	–	–
DCMU (10^{-4} M)	8	0.030	−0.204	0.1
DCMU (5×10^{-4} M) + diaminodurol (5×10^{-4} M) + AS[a]	27	0.280	+0.046	> 20.0
DCMU (5×10^{-5} M) + 2,6-dichlorophenolindophenol (DCPIP 10^{-4} M) + AS	6	0.250	+0.016	> 20.0
DCMU (5×10^{-5} M) + hexylresorcin (7.25×10^{-4} M) + AS	8	0.400	+0.166	1.9
DCMU (5×10^{-5} M) + phenazine methosulfate (5×10^{-5} M) + AS	10	0.262	+0.028	> 20.0
DCMU (5×10^{-4} M) + DCPIP (10^{-4} M) + AS	7	0.053	−0.181	3
DCMU (5×10^{-5} M) + *p*-phenylenediamine (5×10^{-4} M) + AS	15	0.038	−0.196	0.1

[a]Ascorbate (AS) concentration was 10^{-2} M in all experiments

We could be assured that DAD, ascorbate, and PPD do not break the light reaction of xanthophylls. This created real preconditions and conditions for studying the behavior of the light reaction of xanthophylls in systems containing inhibitors and donors of electrons.

As can be seen from Table 5.9, simultaneous introduction in the leaf of DCMU, DAD (5×10^{-4} M), and ascorbate (10^{-2} M) demonstrated that, despite high concentrations of the inhibitor, in light there are D^{II} changes in the region of 460 nm. Efficiency of the reaction of violaxanthin de-epoxidation after simultaneous introduction of the inhibitor and donors of electrons is close to the efficiency of the reaction after leaf infiltration with water. Results of experiments were statistically processed, and their reliability is convincing enough. Results of our experiments testify that electron flow to the place of realization of the xanthophyll reaction started to appear after the ETC link of photosynthesis, inhibited with DCMU. The obtained data correspond to the ideas and results of Trebst on reactivation of photophosphorylation (Trebst and Pistorius 1965). Thus, the data presented in Table 5.9 could be interpreted as indicating the simultaneous realization of the reaction of xanthophylls with photosynthetic ATP formation.

When working with the yellow-green mutant *Botrydiopsis alpina*, lacking chlorophyll *b*, Fork (1969, 1987) observed absorptional (Fig. 5.35) changes caused by reactions of carotenoids. The author determined that the combination of donors of electrons and ascorbate promotes positive changes in the difference spectra, initially suppressed by the inhibitor DCMU.

In experiments with DCMU and PPD (5×10^{-4} M) and ascorbate (10^{-2} M), the DER accompanied by D^{II} change was not found.

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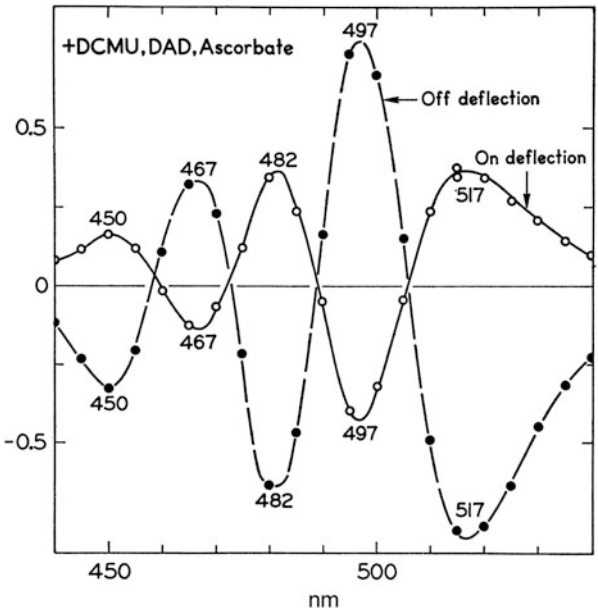

Fig. 5.35 Difference spectra for absorbance changes of *Botrydiopsis alpina* in DCMU, DAD, and ascorbate produced by a band of far-red actinic light (6.3×10^4 erg cm^{-2} s^{-1} between 690 and 750 nm). *Open circles* difference spectrum for the fast initial change produced upon illumination; *solid circles* difference spectrum for the change produced upon darkening. DCMU, 5×10^{-5} M; DAD, 6.6×10^{-5} M; sodium ascorbate, about 2×10^{-3} M (Fork 1969, 1987)

Thus, there is a basis to consider that simultaneous application of DCMU and of the reductant submitting electrons into the ETC of photosynthesis does not reactivate the light reaction of xanthophylls if applied after the place where photophosphorylation occurs. The obtained results testify to the possibility of the close connection of the light reaction of xanthophylls (namely, Viol de-epoxidation) with ETC activity. Behavior of the link of the xanthophyll system is rather similar to the behavior of the photophosphorylation link. The place of interface of the xanthophyll reaction is, apparently, between the point of electron receipt from the system DAD + AS and the point of receipt of electron flow from the system PPD + AS (Saakov 1971a, b; Saakov and Hoffmann 1974). Figure 5.36 presents the possible scheme of interaction of two photosystems and of electron transport (Saakov and Hoffmann 1974; Westerhoff 1974).

There was special interest in research into the xanthophyll reaction in the plastomutant albomaculata-1 obtained from *Antirrhinum majus* L. (Saakov et al. 1970a, b; Saakov et al. 1971a, b; Hagemann 1964). Mutant samples were kindly given by F. Herrmann (Halle, Germany). Plastids of this mutant are capable of becoming green in deep shade, but at normal illumination, they discolor. Despite this, the mutant has rather a high content of chlorophyll (38 % compared with the initial cultivar) and the normal ratio of chlorophylls *a* and *b*. At the same time, the mutant is not capable of photosynthetic assimilation of CO_2. The ability of plastids of this mutant to carry out the Hill reaction with DCPIP and with ferricyanide indicates that damage to the mechanism of photosynthesis of the mutant does not depend on the activity of PS-2. In the mutant, normal functioning of enzymatic systems of the Calvin cycle was found. Research on chlorophylls in vivo with the

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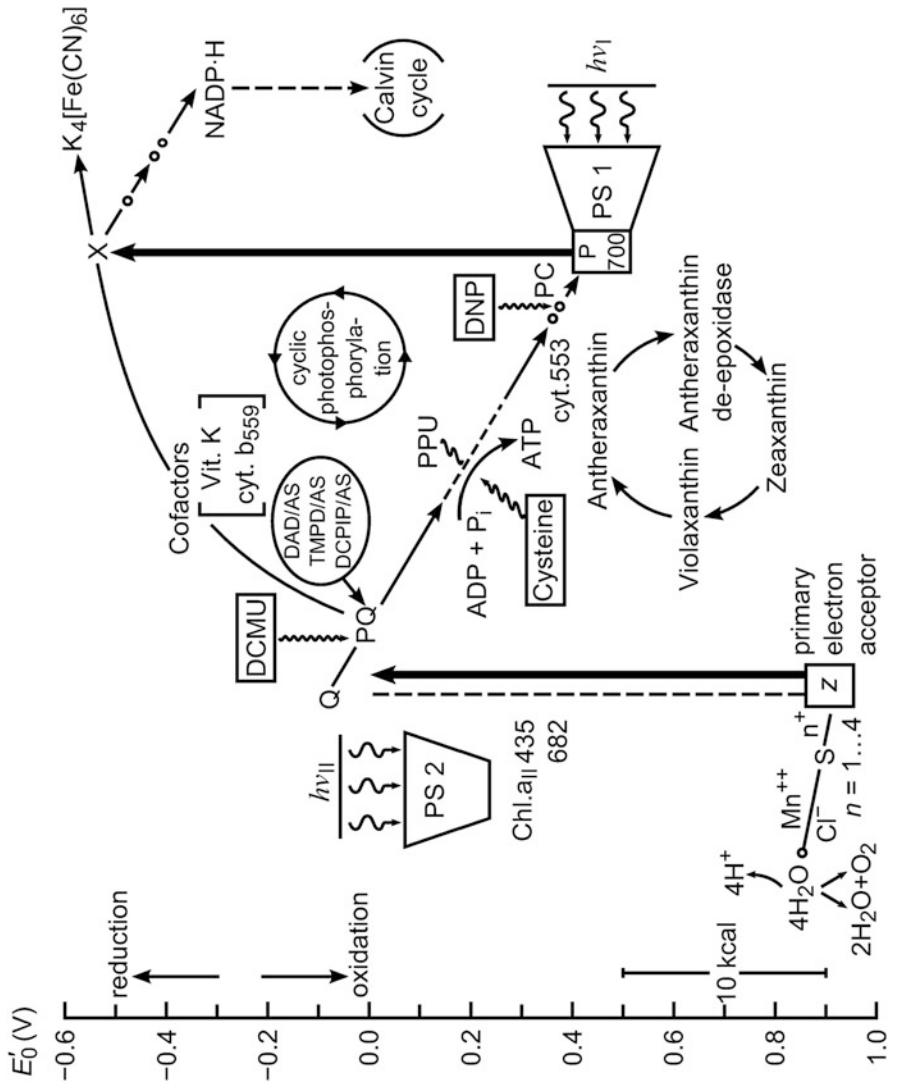

Fig. 5.36 Scheme of photosystems, inhibitors and electron donors

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help of derivative spectrophotometry showed that the maximum of the long-wave form of chlorophyll *a* (Chl_{680}) is shifted towards the short-wave part of the spectrum. These data, and also the absence of γ-component of lamellar proteins in plastids of the plastomutant, allowed us to assume the presence of a defect in PS-1 of this mutant (Sagromsky and Saakov 1970; Saakov 1971b, c). It is possible that damage can be located in the mechanism of photosynthesis of this mutant somewhere between PS-2 and the system of dark reactions. To check this, the D^{II} change of the mutant leaf at $\lambda = 460$ nm was investigated. Changes in the light reaction of xanthophylls were not revealed, as assessed by changes in D^{II} of the absorption spectrum under the influence of light on a non-photosynthesizing leaf of the mutant. Leaf infiltration with DAD solution (10^{-4} M), which usually increases the effect of the xanthophyll reaction, also did not cause changes in D^{II} of the leaf extinction. This fact is additional proof that, for occurrence of reactions of xanthophyll transformation, the normal cooperative realization of ETC functions between both photosystems is necessary; otherwise intervention by reductants would take place.

For specification of the place of interaction of the xanthophyll cycle with the ETC of photosynthesis, the influence of uncouplers of photophosphorylation on the process of light reaction of xanthophylls was investigated by assessment of the D^{II} spectrum. Results of experiments are presented in Table 5.10.

Table 5.10 Influence of ATP and of uncouplers of photophosphorylation on change of D^{II} signal in the region 460 nm in the intact leaf under light impact (Saakov 1971a; Saakov and Hoffmann 1974)

Infiltrated compounds	Time of infiltration before illumination (min)	Number of exp.	Average value ΔE_{460} under light impact	Difference from control	Significance levels (%)
Water control	10	8	0.510	–	–
ATP, 10^{-2} M	10	12	0.725	+0.215	0.7
Gramicidin D, 0.01 %	10–30	12	0.183	–0.327	0.1
Methylamine, 10^{-2} M	5–20	15	0.060	–0.450	0.1
NH_4Cl, 10^{-3} M	10–20	8	0.100	–0.410	0.1
CCCP, 10^{-4} M	5–20	21	–0.250	–0.760	0.01
CCCP, 10^{-4} M + cysteine, 10^{-3} M	10–20	12	0.450	–0.060	3
NaF, 2×10^{-3} M	10–40	13	0.190	–0.320	0.1
2,4-Dinitro-phenol 5×10^{-4} M	10–25	12	0.100	–0.410	0.1
Digitonin, 5×10^{-4} M	10–20	8	0.072	–0.438	0.1

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Experiments performed with infiltration into a leaf of gramicidin, methylamine, Chlorcarbonylcyanide phenylhydrazone (CCCP), ammonium chloride, and fluoric sodium (compounds that break the photophosphorylation process) showed suppression of the light reaction of transformation of xanthophylls. In this respect, the influence of CCCP and methylamine was especially interesting, because they are specific uncouplers of ATP formation in photophosphorylation (Heytler and Prichard 1962). The obtained results suggest that light de-epoxidation is connected with activity of the photophosphorylation process. It is known that cysteine reactivates the uncoupling action of CCCP on ATP formation (Porter and Anderson 1967). Experiments with simultaneous infiltration of cysteine and CCCP in the leaf demonstrated reactivation of the xanthophyll reaction, as assessed using spectra of the second derivative (E_{460}).

In connection with the listed experiments, it was interesting to check the influence of exogenous ATP on the realization of the light reaction of xanthophylls. The specificity of our analysis technique allowed us to assess ATP influence on this reaction in shorter periods than is usual in biochemical research (Porter and Anderson 1967). Results of experiments are presented in Table 5.10, from which it can be seen that the light reaction of D^{II} change (ΔE_{460}) considerably increases in the presence of ATP in comparison with the efficiency of reaction in the water control. It is possible to suggest that exogenous ATP accelerates the light reaction of de-epoxidation of xanthophylls. It is premature, however, to speak about the definiteness of this conclusion because, from the point of view of the group of researchers from Kiev (Bershtein et al. 1969, 1971; Petrenko et al. 1970), exogenous ATP can be the source of phosphate ion for its transfer by epoxyxanthophylls in the photophosphorylation process.

Thus, considering the efficiency of realization of the light reaction of xanthophylls in the intact leaf under the influence of various inhibitors of photosynthetic systems and of their combination with reductants, it is possible to make the following conclusions. Light de-epoxidation of xanthophylls depends on the inhibiting influence of compounds that switch off the activity of PS-2. The inhibitor of PS-1, salicylaldoxime, does not break the DER. These data suggest that the system of reactions of xanthophylls is connected to ETC of photosynthesis between plastoquinone and plastocyanin. Assessment of the influence of specific uncouplers of photophosphorylation on the investigated reaction indicates the connection of xanthophylls with the ETC of photosynthesis through the photophosphorylation link. If epoxyxanthophylls have a function in the mechanism of photophosphorylation (Bershtein et al. 1969, 1971; Petrenko et al. 1970), then the presence of the DER in evolutionary advanced photosynthetics allows us to connect its necessity with the realization of the photophosphorylating function, which is proved by data on *Antirrhinum*. The general biological importance and specificity of the DER for autotrophic organisms are emphasized. In consideration of this reaction of xanthophylls, the possibility of using derived spectra for the characterization of the course of chemical and biochemical reactions is demonstrated.

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5.7.2 *Derivative Spectrophotometry for Assessment of the Influence of Poisons and Herbicides as Extreme Factors of Environment*

Research into the influence of uncouplers during short-term exposure pushed us to more detailed research in this direction, with application of derivative spectrophotometry. In the great quantity of works performed with inhibitors, a number of which were known herbicides, only a few paid attention to the appeared reactions of the pigment complex. This, in turn, caused scantiness of data, contradiction of results, and a variety of interpretations. The influence of uncouplers is interesting not only from the theoretical point of view, but it also has a direct application for the assessment of chemical pesticides and herbicides and could be applied in extreme situations of anthropogenous influence (Saakov et al. 1971a, 1978a, b, Saakov and Nazarova 1972; Saakov 1973a, b, 1976; Saakov and Hoffmann 1974), either for monitoring of vegetative cenosis or of water surfaces of seas and oceans from flying objects (airplanes, balloons and artificial Earth satellites).

Herbicides show selectivity of action, suppressing one plant and not significantly influencing others. Data about an increase in the toxic action of inhibitors under the influence of light have been obtained (Yamashita et al. 1969; Paromenskaya 1970). The spectrum of influence often has two maxima, at 428 and 658 nm (Ashton 1965; Ashton et al. 1966). The features of usual spectrophotometry give substantially limited analytical possibilities for the inspection of intact leaves. Registration of derived spectra considerably expands the analytical possibilities of experimenters. An example of use of this method for the long-term study of the influence of inhibitors and herbicides on leaves is described in this section and is partially demonstrated in Figs. 5.37 and 5.38.

First of all, the figures show the clear specificity of inhibitor action on the change in the spectrum compared with water control. The change in the region of 460–470 nm attracts attention (Fig. 5.37). Under the influence of the majority of investigated inhibitors and chemical pesticides, changes in the spectrum started from this region. In the process of disappearance of the peak at 470 nm, oppositely directed changes in peaks at 420 and at 450 nm occur, and also a peak at 650 nm appears under KCN influence. These shifts of the spectral bands are rather specific for KCN (an inhibitor of cytochrome oxidases, blocking the functions of plastocyanin) (Fig. 5.36b) and only partially inherent to the influence of cyanic mercury (Fig. 5.36c). Chromatographic analysis showed that the appearance of a peak at 650 nm under KCN influence on the leaf is not accompanied by formation of forms of chlorophyll *b* and indicates pheophytinization of initial funds of chlorophyll *a*. The considered effect was not seen under the influence of cyanic mercury or sodium azide (Saakov 1973a).

Figure 5.38 presents the dynamics of the change in the peak in the region of 460–470 nm under a long influence of diuron, which blocks electron transport and between electron acceptors Q and PQ (i.e., oxidization of plastoquinone). The character of the change in the peak at 460 nm corresponds to data in previous

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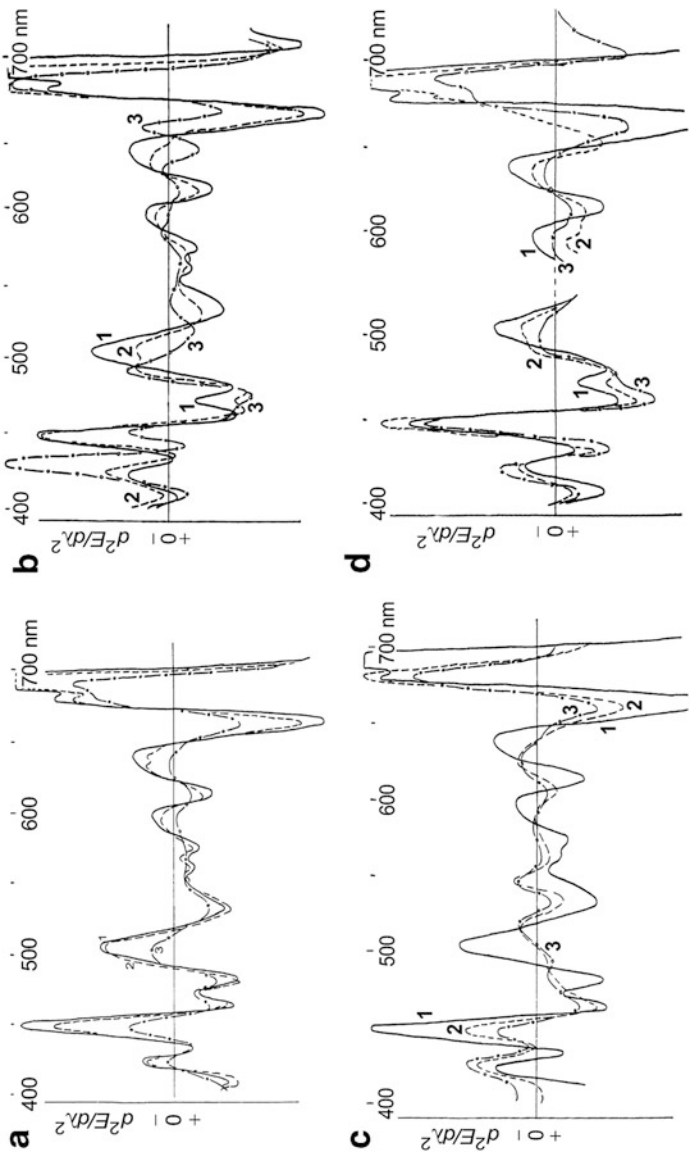

Fig. 5.37 Second derivatives of absorption spectra of native leaves of barley mutants lacking chlorophyll *b*. (**a**) Influence of water on the shift of spectral bands in the D^{II} spectrum for the intact leaf of mutant № 3613 (*1* immediately after infiltration of water; *2* after 72 h; *3* after 116 h). (**b**) Influence of KCN (10^{-3} M) on the shift of spectral bands in the D^{II} spectrum for the intact leaf of mutant № 2807 (*1* immediately after infiltration of KCN; *2* after 18 h; *3* after 87 h). (**c**) Influence of the solution of cyanic mercury (10^{-2} M) on the shift of spectral bands in the D^{II} spectrum for the intact leaf of mutant № 2800 (*1* immediately after infiltration of $Hg(CN)_2$ solution; *2* after 68 h; *3* after 99 h). (**d**) Influence of the CCCP solution (10^{-3} M) on the shift of spectral bands in the D^{II} spectrum for the intact leaf of mutant № 3613 (*1* immediately after infiltration; *2* after 5 h; *3* after 20 h)

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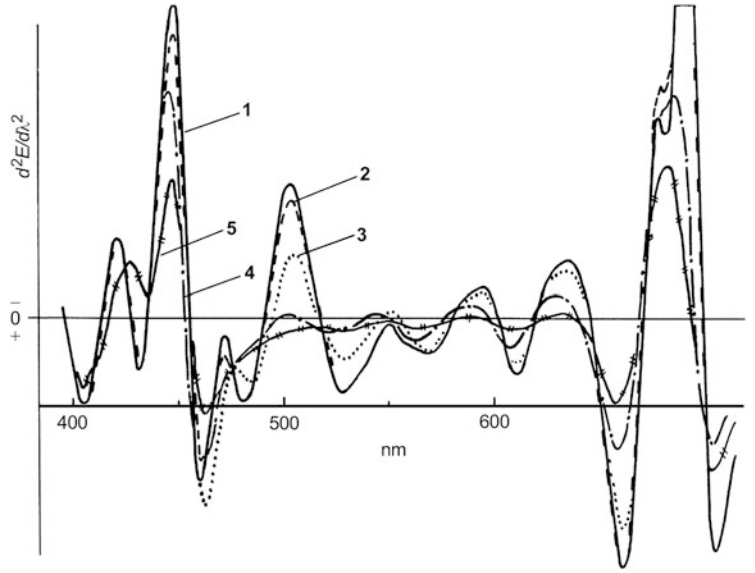

Fig. 5.38 Influence of salicylaldoxime solution (10^{-2} M) on the change of picture of the D^{II} spectrum for the intact leaf of mutant № 3613: (*1*) immediately after infiltration of salicylaldoxime solution; (*2*) after 69 h; (*3*) after 120 h; (*4*) curve after 143 h SA action; (*5*) curve after 155 h SA action

figures. A similar picture of change in the peak at 460 nm was observed in the case of a long influence of chloromercuribenzoate, sodium azide, methylamine, and gramicidin. Our results coincide with the material of other works (Arnon 1961; Trebst 1964; Wessels 1964; De Kiewiet et al. 1965; Yamashita and Butler 1968; Yamashita et al. 1969a, b; Yamashita and Konishi 1969; Arntzen et al. 1971). Similar data on the influence of diuron and CCCP on photo bleaching of carotenoids were obtained by Japanese researchers (Fujita and Suzuki 1973; Westerhoff 1974). The main check of our data was done by Prof. H. Sagromsky (1974), who supervised the Department of Physical Physiology of the Central Institute of Genetics and Crop Plant Research of Academy of Sciences, IPK (GDR), where this work was started. Prof. Sagromsky checked all our results with German carefulness, showing that under KCN influence, a product appears with $\lambda = 650$ nm, but its formation is not connected with chlorophyll *b*. It is the product of transformations of chlorophyll *a*, and its formation is stimulated with light and does not occur in solution (Saakov 1990d).

Let us consider the extraction of pigments with a mixture of solvents, which Prof. Sagromsky offered to perform for us (Saakov and Shiryaeva 1967; Sagromsky and Saakov 1970). Complexities, tested by experts on diatomeae algae (*Bacillariophyceae*), connected with decoloration of an algal skeleton are easily removed by the use of the described mixture of solvents directly at the sample table of the microscope. In this case, on one side of the coating glass, the experimenter holds a

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piece of filter paper and on the other side holds a pipette with the mixture of solvents. Gradually taking up the solvent with the filter paper, new solvent is simultaneously supplied from the pipette. Within several minutes, the culture of diatomeae becomes colorless. This method was widely used for the first time in expeditionary work in Posyet (in 1965). This city-port in the far east of Russia is named in honor of the adjutant general of Emperor Alexander III and later the full admiral of the Russian navy, Konstantin Nikolaevich Possuet. He came from the ancient French house Possuet de Rossiet, whose representatives served Emperor Peter I, and the admiral K.N. Possuet was the chairman of the Committee of the modern Russian flag design approval. We apologize for a small digression to history, but even the Russian reader does not always knows where Posyet city is located.

German colleagues checked our data together with known algologist, Professor Dr. Rieth (Gatersleben, DDR).

Under the influence of CCCP, disappearance of the spectrum band in the region 460–470 nm occurs much faster than in the case of the other considered inhibitors (Fig. 5.39). Bands in blue area of the chlorophyll spectrum remain. A similar picture of spectra change of the intact leaf of mutants was observed under the influence of salicylaldoxime (10^{-2} M), chloromercuribenzoate (10^{-3} M), and *o*-phenanthroline (5×10^{-2} and 10^{-3} M). The chelate former *o*-phenanthroline is a weaker inhibitor of the oxygen link in PS-2 than diuron.

The known herbicide diuron also results in radical changes in the spectrum structure in the blue region and only affects the red region of spectrum very slightly. Perhaps this example can explain that fact that, after the use of defoliants in Vietnam, plants still remained green for some time. From comparison of the effects of considered inhibitors, it can be seen that the uncoupler of photophosphorylation, CCCP, is the most effective; it starts to manifest its influence after only 10 min of impact on the pigment complex (Fig. 5.39).

The dynamics of peak change in the region 460–470 nm under a long influence of diuron is presented in Fig. 5.39. The character of the change in the peak at 460 nm corresponds to the data in previous figures. This very indicative picture of the change in the peak at 460 nm was observed in the case of a long influence of chloromercuribenzoate [an inhibitor of sulfo-groups and photophosphorylation (Arnon 1961)], sodium azide, methylamine, and gramicidin (Saakov et al. 1971a). A few years later, similar data on the action of diuron and CCCP on photobleaching of carotenoids were obtained by Japanese researchers (Fujita and Suzuki 1973a, b).

Unfortunately, the character of change in the D^{II} spectra shows only the integrated picture of damage to the pigment–protein complex in the leaf in response to the influence of inhibitor. Thus, infiltration of inhibitors into the leaf induces changes in the structure of the D^{II} spectrum, manifested in disappearance of some bands of the spectral structure in blue and red regions. Occurrence of specific changes in the spectral structure, inherent to a specific inhibitor, is possible.

The specificity of change in separate fractions of pigments can be revealed with the help of chromatographic analysis and is described in Sect. 3.7.3.

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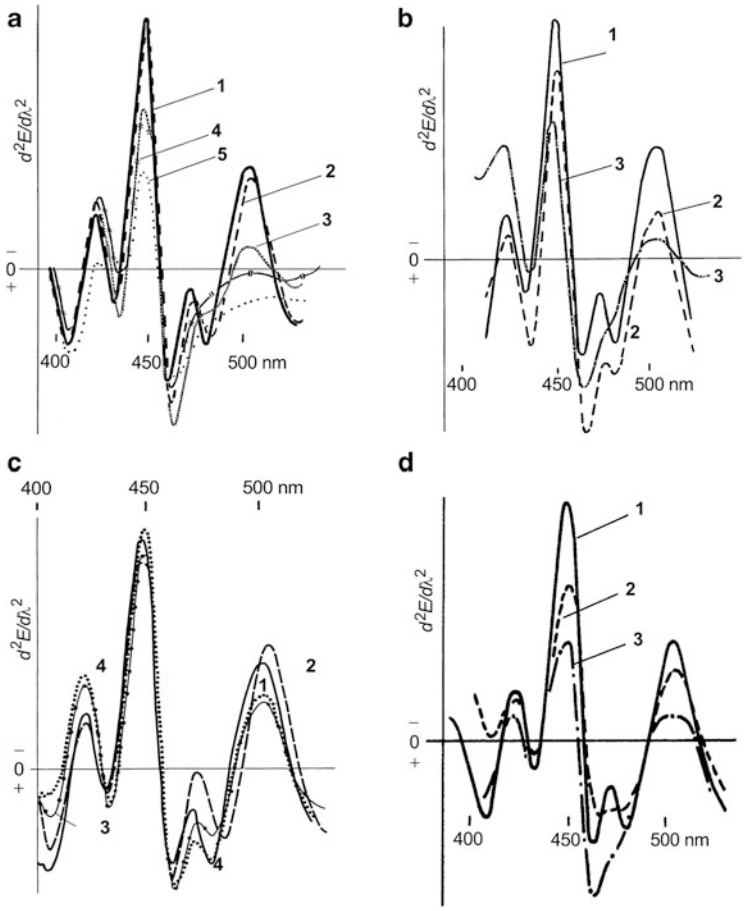

Fig. 5.39 The character of peak change in the region 460−470 nm in native leaves of barley mutant lacking chlorophyll *b*, under the influence of inhibitors of photosystems. (**a**) Influence of salicylaldoxime (10^{-2} M) on mutant №2800 (*1* the initial spectrum; *2* after 40 h; *3* after 64 h; *4* after 89 h; *5* after 120 h); (**b**) Influence of chloro carbonyl cyanide phenylhydrazone (0.0025 % solution), mutant №2800 (*1* the initial spectrum; (*2*) after 15 h; *3* after 48 h); (**c**) Influence of sodium azide (10^{-3} M), mutant №2807 (*1* the initial spectrum; *2* after 10 h; *3* after 15 h; *4* after 20 h). (**d**) Influence of diuron (10^{-4} M), mutant №3613 (*1* the initial spectrum; *2* after 5 h; (*3*) after 16 h)

5.7.3 Features of the Influence of Photosystem Inhibitors and of Photophosphorylation Uncouplers on the Dynamics of Pigment Content

Taking into consideration data on changes in the region $\lambda = 460$–470 nm with Viol de-epoxidation and also considering Hager's results obtained for chloroplasts

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(Hager and Bertenrath 1962; Hager 1966, 1967b, 1969; Hager and Mayer-Bertenrath 1967), we checked the change in the quantitative content of various fractions of carotenoids under a long influence of inhibitors on leaves or seaweed suspensions (Saakov et al. 1971a; Saakov and Nazarova 1972; Saakov 1973a, b; Nazarova 1974; Baranov et al. 1975, 1976).

Research into the character of change of separate fractions of pigments in a vegetative organism under the influence of photosystem inhibitors and photophosphorylation uncouplers are closely connected with the problem of photo- and heterotrophicity of vegetative objects and have been reviewed in various publications (Saakov et al. 1971a, 1978b; Saakov and Nazarova 1972; Nazarova 1974; Baranov et al. 1975, 1976). Suppression of the photosystems of the leaf and of *Euglena* by inhibitors causes damage to the phototrophic process. However, in *Euglena* - facultative autotroph can also exist using heterotrophic assimilation of elements from the nutrient medium. We have described this way of fast transfer of *Euglena* cells from autotrophic feed type to heterotrophic type (Saakov and Nazarova 1972). In addition, as objects of research, we used corn sprouts as representatives of C4 plants phyletically far from *Euglena*. Experiments with these *obligate* and *facultative* photosynthesizing organisms allowed us to obtain a more complete picture of the variability of the pigment system (Saakov 1990d; Nazarova 1974).

From Figs. 5.40 and 5.41, the reader can see the similarities of the influence of various inhibitors on pigment systems of phyletically remote objects. In *Euglena*, Neo funds disappeared with the greatest speed, followed by Ant (probably it is Diads from the diadinoxanthin ↔ diatoxanthin cycle). The spectrum of Neo in acetone has peaks at $\lambda = 428$, 448, and 478 nm and the furanoid form at $\lambda = 432$ and 458 nm. The content of pigment named by us as *Z*-xanthophyll simultaneously increased (the spectrum in acetone has peaks at $\lambda = 410$, 431, and 457 nm). Oppositely directed courses of dynamics of Ant, Neo, and, correspondingly, *Z*-xanthophyll allowed us to assume formation of the latter from funds of mono-epoxyxanthophylls.

Considering the dynamics of changes in pigment content in the representative of C4 plants (corn sprouts), a picture substantially similar to carotenoid dynamics in *Euglena* arises.

First of all, the Viol content decreases, and disintegration of Neo occurs at lower speed. The Lut content tends to decrease, but the speed of its disintegration is intense only in the case of salicylaldoxime influence. Car is characterized by a raised resistance to the influence of inhibitors. Addition of reductants to the system decreases the decay rates of Viol and Neo; contents of Lut and Car practically remain invariable. Apparently, in corn leaves, as well as in *Euglena* suspensions, addition of reductants promotes preservation of the initial structure of the pigment complex, interfaced with autotrophicity of the object.

From analysis of the quantitative change in pigments, it is possible to reliably conclude that there is a connection between changes to the spectral curve contour D^{II} in the presented graphs (Fig. 5.40) and disintegration of the system of xanthophylls containing epoxy groups.

Thus, use of derived spectra gives the possibility of fast observation of changes in photosystems in obligate photosynthesis as well as changes during the fast

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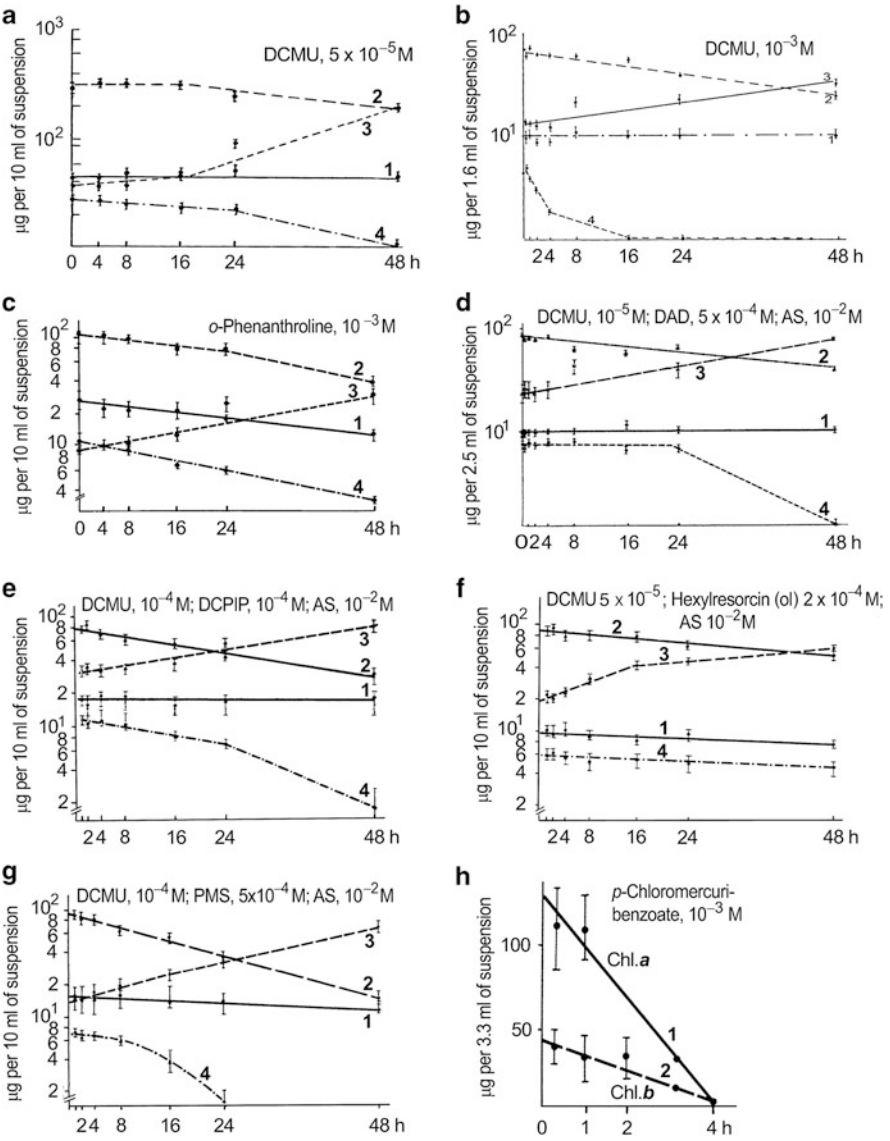

Fig. 5.40 (**a–h**) Influence of the indicated inhibitors and donors of electrons on the dynamics of change of the pigment content in *Euglenae*: (*1*) carotene; (*2*) antheraxanthin; (*3*) Z-xanthophyll; (*4*) neoxanthin

transfer of facultative photosynthesis, which is found in *Euglena*, to a heterotrophic type of existence. We attract special attention to this fact. Practically, this approach can be used for the land or air monitoring of vegetative cenosis or for scanning water surfaces in cases of various negative anthropogenous incidents.

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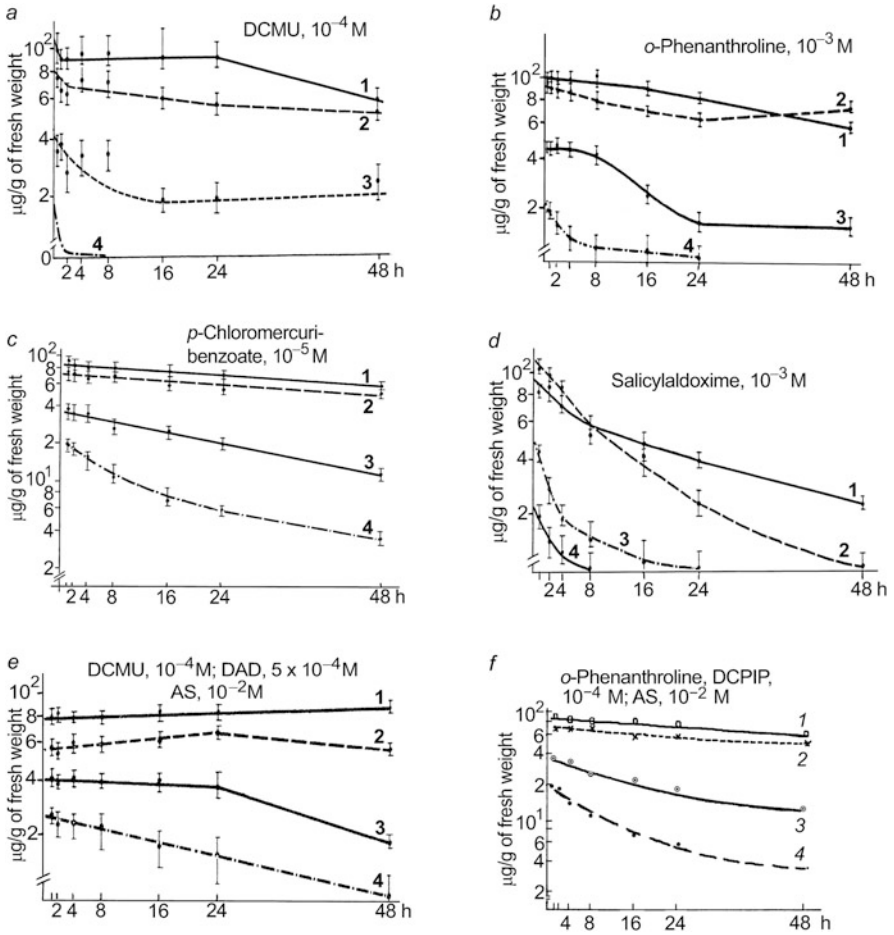

Fig. 5.41 Influence of inhibitors and donors of electrons on the dynamics of change of the pigment content in corn: (*1*) carotene; (*2*) lutein; (*3*) violaxanthin; (*4*) neoxanthin

Along with the influence of inhibitors, the influence of light and temperature, moisture deficiency, salt stress, and some anthropogenous influences on changes in the D^{II} spectrum in blue and red regions were investigated (Gribanovski-Sassu 1972; Baranov et al. 1974, 1976; Saakov 1976; Saakov and Udovenko 1976; Kvitko et al. 1976, 1977; Saakov and Baranov 1987; Saakov and Leontjev 1988). Characteristic changes in the blue spectral region near 470–480 nm were revealed, and they were very similar to changes in the D^{II} spectrum of mutants of barley Donaria induced by inhibitor action.

In research into photosensitive mutants of *Chlamydomonas* and their revertants, the band at 480 nm was found in the D^{II} spectrum of revertant r6-149. Cultivation of revertant r7-149 in medium containing thiamine promotes its light resistance and appearance of the band in the blue region at $\lambda = 482$ nm (Saakov and Baranov 1987).

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The same picture of appearance of a peak $\lambda = 480$ nm is characteristic for revertant r12-135 after its cultivation in medium containing thiamine. Appearance of a peak at 480 nm is possibly caused by the influence of thiamine (during biosynthesis reactions; see Sect. 5.4) on the light-dependent reduction of oxidized carotenoids (Baranov et al. 1975). The idea was suggested that thiamine-dependent light resistance of mutants and also the formation or the best manifestation of bands at $\lambda = 480.0$, 570.0, 650.0, and 673.0 nm, in response to the presence of thiamine, are caused by increased processes of biosynthesis of pigments and by the appearance of autotrophicity.

As a working hypothesis, the following are possible: First, as a result of mutations, the number of centers of pigment biosynthesis decreases or the activity of each of them decreases. Second, addition of thiamine to the medium creates the preconditions for involvement of a qualitatively different energetic system of the cell (Sysoev et al. 1971; Hoffmann 1987; Rohmer 1999; Lichtenthaler 2000a, b) that take part in the activation of processes of biosynthesis of protein and carotenoids, activating Pyr transformations and the appearance of autotrophicity.

In revertants, absorption in the carotenoid region increases, and their chlorophyll ratios become normalized. Sometimes (revertant r2-2) the increase in absorption in the region of 480 nm is observed without substantial changes in the region of chlorophyll absorption. Additional argument in favor of the stated is the fast reaction of the absorption band relating to carotenoids (Baranov et al. 1975; Kvitko et al. 1976, 1977; Saakov and Baranov 1987).

Incubation of *Euglena* cells and of corn (seedlings) sprouts with diuron in combination with electron donors halts the destruction of pigments induced by the inhibitor, and also change in the peak $E_{460–470}$ is observable.

Study of the damages to the *Euglena* pigment complex caused by the influence of an inhibitor allows a parallel to be drawn between the induced destruction of pigments and changes in the pigment composition in the case of formation of the etiolated form of algae caused by the influence of high temperature and antibiotics. It is known that reversible greening (the return to autotrophicity) of algae is accompanied by a reduction in the quantitative and qualitative composition of pigments (Smilie and Rigopoulus 1962; Stern et al. 1964; Dolphin 1970; Gribanovski-Sassu 1972; Westerhoff 1974). Data which have been obtained indicating that the connection between the activity of autotrophic assimilation of carbonic acid and the secretion of oxygen depends on the presence of Neo in the becoming-green form of algae (Krinsky 1968). Other studies also testify the same (Stolbova 1971; Temper and Kvitko 1971).

In the process of studying the influence of inhibitors on *Euglena* suspension, Lozina-Lozinskii and Zaar (1961) found that inhibitor concentrations that halted the work of photosystems do not cause the destruction of cells but lead to their reversible immobilization. After removal of the inhibitor, seaweeds transferred to habitual conditions of existence, developed normally, and regained the mobility and color of before the experiment. Changes in the pigment complex in *Euglena* and corn sprouts, found by us, substantially coincide with a picture of degradation of the *Euglena* photosynthetic apparatus during the change to a heterotrophic condition of existence, as described by V. I. Polyanskii and by other scientists (Polyanskii 1948;

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Stern et al. 1964). In our work, the suppression of photosystems with inhibitors causes damage to the mechanism of phototrophicity, and *Euglena*, remaining green for a long time, changes to a heterotrophic feed type. We emphasize that in our experiments the transfer to a heterotrophic condition of existence occurs in a very short time (minutes), whereas the usual ways of algae transfer to another feed type (connected with the influence of darkness, temperature, and other substances) demand a preliminary longer time of influence on algae suspensions before the occurrence of visible changes in the structure of the photosynthetic apparatus.

As can be seen from the above, changes induced in the D^{II} spectrum, corresponding to changes in the pigment composition in algae and corn sprouts, are rather similar to the spectral shifts found by us in studying the transition from the heterotrophic feed type to mixotrophic type in a number of mutants of *Scenedesmus* and *Chlamydomonas* (Nazarova 1974; Baranov et al. 1975; Kvitko et al. 1976, 1977; Saakov and Baranov 1987). The revelation of new forms of pigments (Zea, X-397, Z-xanthophyll, pigment 650) is, apparently, specific for a deviation from normal phototrophic activity of the plastid apparatus (Sauer and Calvin 1962). In favor of this conclusion, the experiments of Mandelli (1969) indicate a decrease in Diad content in a dark variant of *Amphidium* grown in light and in darkness. This Diad decrease was accompanied by the appearance of xanthophylls of dihydrostructure, which were absent in the light control. The oppositely directed course of dynamics of the content of derivative forms of pigments supposes the representation of the directivity of Diad transformation.

Thus, the material of this section has allowed us to show the high sensitivity of the band of the derived spectrum in the region of 470 nm to the influence of inhibitors and substances causing extreme stress. Further, the lability of the response to inhibitors of the system of epoxide-containing xanthophylls is revealed. This shows the coupling of quantitative changes of the content of epoxide-containing xanthophylls with the signal $E_{460\text{-}470}$ in the D^{II} spectrum. From changes in the derived spectrum in the region of 460–470 nm, it is possible to speak about the negative reaction of plants to external influences.

5.7.4 Coupling of Xanthophyll Transformations with Chloroplast Energetics

5.7.4.1 Direct Experiments on Suppression and Reactivation of Transformations of Labeled Carotenoids

The experiments described above show the correctness of conceptions about the reduction of Viol directly to Car (Saakov 1963b, c; 1965a; 1968c; 1970; Bershtein et al. 1969, 1971; Petrenko et al. 1970; Saakov 1989a, b) and serve as direct proof of Godnev's ideas about transformation of xanthophylls into Car (Saakov 1968a). These data correspond to the representations of Willstätter and Stoll (1913, 1918). Thus, the reaction of Viol (conversion) transformation into Car (i.e., DER) was proved reliably, but there was much less available data about the process of Car oxidization and the appearance of xanthophylls.

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Indirect data about the interface of the reactions of carotenoid transformations with the photophosphorylation link in chloroplasts were obtained as a result of studying various spectral effects (see Sect. 5.7.1). Thereupon, reliable *direct* proof of the connection between carotenoid transformations and the photophosphorylation process are of interest not only from the point of view of the theory, but also to ascertain test reactions showing the state of energetic structures in a chloroplast. It is also interesting from the point of view of evolutionary comparative biochemistry because the totality of literature data indicates the similarity of degree of Car oxidization in animal tissues, chromoplasts, and chloroplasts of higher plants and brown and green algae (correspondingly, *Phaeophyceae* and *Chlorophyceae*) (Saakov 1990a, b).

Accepting the working hypothesis that there is a connection between changes in the region $\lambda = 460$–470 nm and the DER of Viol, and also taking into account Hager's data (Hager 1966, 1967b, 1969; Hager and Mayer-Bertenrath 1967), we performed additional studies of the transformation of labeled pigments. Changes in the SpAs of xanthophyll fractions were investigated after introduction of exogenous labeled Viol and Car into chloroplasts. We also looked at the influence on this process of uncouplers and inhibitors of PS-1 in the assumption that it would give *direct* proof of the dependence of oxidation–reduction transformations of carotenoids, introduced from the outside, on the activity of ETC of photosynthesis.

Chloroplasts were isolated from the leaves of pea *Pisum sativum* using the accepted technique (Saakov 1989b). Each experiment was repeated three or four times. Each sample contained an identical quantity of chloroplasts and labeled pigments (^{14}C-Viol 20,000 imp/min, ^{14}C-Car 30,000 imp/min). In some experiments, we used preparations labeled with tritium (Saakov 1989b). Radiochemical purification of carotenoids was carried out with the help of an established technique (Saakov and Nasarova 1970; Saakov 1989b, 1990a) and using derived spectra as one criterion of the radiochemical purity of preparations. Data on the inhibition of pathways of carotenoid metabolism under the influence of uncouplers and inhibitors of PS-1 are summarized in Tables 5.11 and 5.12.

Table 5.11 Distribution of radioactivity inclusion from exogenous labeled violaxanthin in fractions of carotenoids of pea chloroplasts in the presence of nigericin ($n = 3$–4)[a]

Fraction of isolated carotenoids	Specific activity (imp/min/μg carbon)			
	Experiment 1	*Experiment 2*	*Experiment 3*	*Experiment 4*
Violaxanthin	92 ± 11	88 ± 9	95 ± 7	111 ± 9
Neoxanthin	7 ± 3	8 ± 3	6 ± 2	8 ± 3
Lutein epoxide	Traces	Traces	Traces	Traces
Zeaxanthin				
Lutein				
Carotene	0	0	0	0

[a]In all experiments, there was 20–23 μg chlorophyll per 1 mL of suspension. Duration of light exposure was 30 min, illumination 20,000 lx. In experiments 1 and 2 simultaneously 200 nM nigericin and 50 mM potassium chloride were introduced. In experiments 3 and 4, besides the corresponding quantity of nigericin and KCl, diaminodurol (5×10^{-4} M) and ascorbate (10^{-2} M) were introduced

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Table 5.12 Distribution of radioactivity from exogenous labeled violaxanthin in fractions of carotenoids in the presence of chloro carbonyl cyanide phenylhydrazone (CCCP) ($n = 4$)[a]

Fraction of isolated carotenoids	Specific activity (imp/min/μg carbon)				
	Control	*Experiment 1*	*Experiment 2*	*Experiment 3*	*Experiment 4*
Violaxanthin	94 ± 8	92 ± 11	95 ± 7	89 ± 9	97 ± 11
Neoxanthin	57 ± 10	7 ± 3	6 ± 2	42 ± 6	47 ± 7
Lutein epoxide	46 ± 6	Traces	Traces	33 ± 5	39 ± 6
Zeaxanthin	34 ± 7			17 ± 6	28 ± 4
Lutein	31 ± 6			18 ± 4	25 ± 6
Carotene	16 ± 4	0	0	8 ± 2	14 ± 3

[a]Experimental conditions: exposure 30 min, illumination 20,000 lx, CCCP concentration 0.01 % in all four experiments; experiment 3, combination of CCCP with cysteine (5×10^{-5} M); experiment 4, combination of CCCP with cysteine (5×10^{-5} M) and DAD (5×10^{-4} M) and ascorbate (10^{-2} M)

As shown in Sect. 5.7.1, in research on the suppression of D^{II} signals of absorption for intact barley leaves, it was found that some photosystem inhibitors and uncouplers are capable of suppressing the reactions of xanthophylls (Saakov 1971c). There were necessary, but not sufficient, data to unequivocally show the presence of interrelation between the carotenoid system and the energetics of chloroplasts. Table 5.11 shows direct proof that protonophore nigericin (an inhibitor of proton transport) promotes the suppression of Viol transformation into Car. These data coincide with results on thermal inactivation of chloroplasts, which inhibits xanthophyll transformation (Saakov 1990a). Earlier it was shown that reconstruction of the ETC promotes recovery of de-epoxidation of xanthophylls, assessed with D^{II} or with changes in the difference spectrum (Saakov 1971c). As can be seen from Table 5.11, in the case of application of nigericin and DAD + AS, recovery of the DER of Viol does not occur.

Experiments on the suppression of D^{II} signal in the region of 470 nm in the presence of CCCP, and its reconstruction in the presence of CCCP and cysteine (Saakov 1971b), gave hope of the possibility of reconstruction of direct transformation of labeled exogenous pigments in an incubation medium containing cysteine and CCCP. The data supporting this possibility are presented in Table 5.12. From the data in Table 5.12, it follows that after addition of cysteine, which is easily oxidized, there is reconstruction of the photophosphorylation link. Simultaneously, activity of the DER of Viol equals the control level (experiment 4) in response to a combination of cysteine, DAD, and ascorbate, which corresponds to the classical scheme of reconstruction of the photophosphorylation link (Saakov 1989a, 1990a).

According to previous work (Saakov 1971a), it was possible to expect that salicylaldoxime does not inhibit the direct DER, but suppresses the return reaction of epoxidation. However, data of the mentioned work only indirectly proved this idea. The direct experiments presented in Table 5.13 reliably confirm the stated conception. This means that the oxidizing reactions of Car possibly reach the stage of formation of dihydroxy derivatives of xanthophylls, but are interrupted by inhibition at the stage of formation of epoxy forms of xanthophylls. Thus, it is possible to

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Table 5.13 Distribution of radioactivity from exogenous labeled carotene in xanthophylls of chloroplasts in the presence of salicylaldoxime ($n = 3$)[a]

Fraction of isolated carotenoids	Specific activity (imp/min/μg carbon)		
	Experiment 1	*Experiment 2*	*Experiment 3*
Violaxanthin	0	0	0
Neoxanthin			
Lutein epoxide	Traces	Traces	Traces
Zeaxanthin	16 ± 2	9 ± 3	8 ± 2
Lutein	17 ± 4	11 ± 3	17 ± 5
Carotene	197 ± 11	173 ± 16	211 ± 13

[a]Exposure 30 min, illumination 7,000 lx. Chloroplast suspension contained 23 μg of chlorophyll per 1 mL. Salicylaldoxime concentration in experiment 1 was 10^{-2} M and in experiments 2 and 3 10^{-3} M

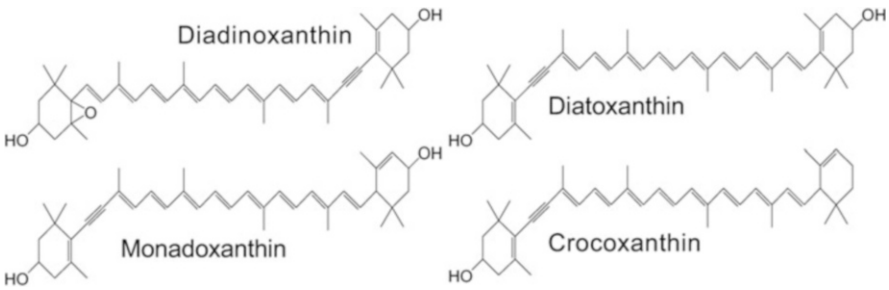

Fig. 5.42 Formulae of carotenoids participating in cycles of de-epoxidation through a type of de-epoxidation of diadinoxanthin in a number of algae

suppose that cutting the photophosphorylation link by salicylaldoxime also disrupts the process of epoxidation of xanthophylls (Saakov and Hoffmann 1974).

The presented results allow us to make following conclusions: Direct experimental proof of connection of the DER with the photophosphorylating function of chloroplasts is obtained. Data on research of the D^{II} signal (Saakov 1971c) and of direct Car transformations in chloroplasts (Saakov 1989a, 1990a) indicate that the process of epoxidation of xanthophylls depends on activity of the ETC, which is also proved by the direct experiments of the presented research. Our data allow us to consider the reactions of carotenoid transformations and the $E_{460–470}$ effect in derived spectra as a test reaction showing the activity of the ETC in chloroplasts. Results of previous works (Saakov 1989a, 1990a, b) promoted the creation of schemes of oxidation–reduction transformations of Car and Viol in various evolutionary distanced taxonomic groups (see Figs. 5.34 and 5.42). The combination of previous and present experiments allows us to suppose with great conviction that in the chloroplasts and chromoplasts of plants, oxidizing transformations of Car are performed up to the formation of compounds having four oxygen atoms in a pigment molecule.

In higher plants and brown and green algae, such pigments are Neo, trollixanthin, and Viol; in chromoplasts they are capsorubin and capsanthin; in

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cells of green algae grown under nitrogen deficiency and also in fish and invertebrates, they are Asc, Ast, and oxycanthaxanthin (as shown in Sect. 5.3). However, only in chloroplasts of higher plants and some algae are there pigments containing one or two epoxy groups. Higher plants and green and brown algae possess a functioning cycle of Viol de-epoxidation through Neo into Car. In a number of algae (euglenae (*Euglenophyceae*), dinophyta (*Dinophyceae*), golden (*Chrysophyceae*), diatomeae (*Bacillariophyceae*), yellow-green (*Xanthophyceae*), etc.), another cycle of reactions is found, functioning through a type of de-epoxidation of Diad (Saakov and Baranov 1987).

Because the listed algae are autotrophic organisms, but occupy another evolutionary niche, then by analogy with the Viol cycle of de-epoxidation, it is possible to assume that the de-epoxidation level of Diad also indicates the activity of the ETC. Because heteroxanthins have four hydroxyl groups (its key role is conditioned by two hydroxyl groups in positions 5′ and 6′), Diad, possessing also an acetylene bond between the seventh and eighth atoms of carbon, turns into Diato and monadoxanthin (with two end hydroxyls). Monadoxanthin, losing one hydroxyl group, turns into crocoxanthin (one hydroxyl and an acetylene bond). The product of reduction of crocoxanthin has not been found and is a problem for further research. Thus, there is a real possibility of a new Diad cycle (Fig. 5.42) functioning through a mechanism similar to the cycle of reductive de-epoxidation of Viol.

So, in this section, we have presented direct proof of the connection of the DER of Viol with the energetics of chloroplasts. We also suggested that in the listed representatives of some families of autotrophic organisms, there exists a Diad–crocoxanthin cycle that functions through a mechanism similar to the cycle of de-epoxidation of Viol.

5.7.4.2 Coupling of Transformations of Xanthophylls with Chloroplast Energetics

The number of experimental proofs of the fact that a decrease in Viol quantity is accompanied by an increase in the Car content has gradually increased (Saakov 1965a; Costes 1965; Kutyurin et al. 1969). Numerous more recent data testify in favor of this point of view, obtained in studying of the ratio of Car to xanthophylls (Hoffmann 1987). They are supported by the studies of Trebst (Depka et al. 1998) and Yasnikov (Bershtein et al. 1969, 1971; Petrenko et al. 1970).

Not limiting ourselves to statement of the fact of label penetration from Viol into Car or from Car into Viol, we tracked changes in the SpAs of carotenoids involved in the reactions of transformation of the carotenoid cycle, with the help of introduction of labeled preparations of Viol and Car into chloroplasts from the outside.

Chloroplasts were isolated from the leaves of *Pisum sativum* according to a published technique (Siefermann and Yamamoto 1974) at a temperature of 0 to –3 °C. Under these conditions, chloroplasts keep their activity over 4 h. Three or four repetitions of each experiment were made. Each sample contained an identical quantity of chloroplasts and labeled pigments (^{14}C-Viol 20,000 imp/min; ^{14}C-Car

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Table 5.14 Dynamics of radioactivity inclusion from exogenous labeled violaxanthin in fractions of chloroplast carotenoids[a]

Fraction of isolated carotenoids	Specific activity (imp/min/μg carbon or hydrogen)			
	Experiment 1	*Experiment 2*	*Experiment 3*	*Experiment 4*
Violaxanthin	66 ± 11	72 ± 9	425 ± 26	487 ± 37
Neoxanthin	32 ± 6	33 ± 4	317 ± 25	386 ± 31
Lutein epoxide	28 ± 5	29 ± 7	267 ± 21	278 ± 32
Zeaxanthin	19 ± 4	18 ± 3	245 ± 19	249 ± 24
Lutein	18 ± 4	22 ± 6	156 ± 17	167 ± 19
Carotene	12 ± 7	17 ± 6	134 ± 18	145 ± 21

[a]Exposure duration, illumination, labeled pigment: experiment 1–30 min, 35,000 lx, ^{14}C-Viol; experiment 2–30 min, 30,000 lx, ^{14}C-Viol; experiment 3–25 min, 20,000 lx, ^{3}H-Viol; experiment 4–25 min, 18,000 lx, ^{3}H-Viol

30,000 imp/min). Specific activity of Viol at the beginning of the experiment was equal to 850 imp/min/μg carbon, and the SpA of Car was 960 imp/min/μg carbon. In some experiments, we used Viol and Car labeled with tritium (SpA of Viol 9,700 imp/min/μg hydrogen; SpA of Car 9,300 imp/min/μg hydrogen).

Earlier it was shown that ^{14}C-Viol introduced from the outside is involved in the reactions of carotenoid transformations after only 15 min (Saakov 1970b). Data on the possible ways of transformation of Viol and Car are presented in Table 5.3. After introduction of labeled Viol into a suspension of illuminated chloroplasts, the radioactivity was found in all fractions of investigated carotenoids. Measurement of the SpA of preparations of pigments assumed the existence of stepwise transformation of ^{3}H- or ^{14}C-Viol. A series of changes in the SpAs of carotenoids allows us to conclude that Car is the end product in the pathway of reductive transformation of Viol in chloroplasts, when the basic carbon skeleton of the pigment still remains (Table 5.14).

Research of the D^{II} of the absorption spectrum of intact leaves of barley mutant lacking chlorophyll *b* showed that specific inhibitors of PS-2 cause significant changes in the blue part of the spectrum. They, in particular, suppress the light-induced effect at 470 nm that is connected with reactions of transformation of carotenoids (see Sect. 5.7.1). The effect at 470 nm is reconstructed under conditions promoting recovery of the activity of the ETC and photophosphorylation (Saakov and Hoffmann 1974).

Data of experiments are summarized in Table 5.15. Results show that diuron substantially damages the reaction of de-epoxidation of exogenous Viol and that reconstruction of the ETC promotes the reduction of Viol. These data completely confirm results obtained on the basis of measurement of derived spectra (Saakov and Hoffmann 1974) and give direct proof of the retardation of the de-epoxidation of Viol by inhibitors of PS-2.

Before the experiments of Hager (1966), it was considered that the return reaction of epoxidation of hydroxylated derivatives of xanthophylls in a suspension of isolated chloroplasts does not take place. Hager highlighted the necessity of NADPH together with oxygen for epoxidation of xanthophylls.

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Table 5.15 Dynamics of inclusion of labeled violaxanthin in chloroplast carotenoids in the presence of diuron and electron donors[a]

Fraction of isolated carotenoids	Specific activity (imp/min/μg carbon)		
	Experiment 1	*Experiment 2*	*Experiment 3*
Violaxanthin	78 ± 7	76 ± 8	63 ± 13
Neoxanthin	3 ± 2	47 ± 11	48 ± 13
Lutein epoxide	4 ± 2	28 ± 4	22 ± 4
Zeaxanthin	4 ± 1	24 ± 6	12 ± 6
Lutein	0	24 ± 8	22 ± 8
Carotene		12 ± 3	16 ± 4

[a]Experimental conditions: exposure 30 min, ^{14}C-Viol. Experiment 1–35,000 lx, diuron 10^{-4} M; experiments 2 and 3–30,000 lx, diuron 10^{-4} M, diaminodurol 5×10^{-4} M, ascorbate 10^{-2} M

Table 5.16 Dynamics of radioactivity inclusion from labeled carotene into xanthophylls of chloroplast suspension in the presence of NADPH[a]

Fraction of isolated carotenoids	Specific activity (imp/min/μg carbon or hydrogen)			
	Experiment 1	*Experiment 2*	*Experiment 3*	*Experiment 4*
Violaxanthin	12 ± 5	10 ± 2	195 ± 16	172 ± 14
Neoxanthin	12 ± 4	13 ± 2	211 ± 17	198 ± 18
Lutein epoxide	21 ± 5	13 ± 6	370 ± 28	327 ± 26
Zeaxanthin	39 ± 6	26 ± 6	387 ± 28	334 ± 27
Lutein	78 ± 12	71 ± 11	411 ± 27	378 ± 28
Carotene	192 ± 9	173 ± 17	521 ± 29	487 ± 31

[a]Exposure duration, illumination, labeled pigment: experiment 1–30 min, 6,000 lx, 15 μM NADPH, ^{14}C-Car; experiment 2–35 min, 7,000 lx, 20 μM NADPH, ^{14}C-Car; experiment 3–30 min, 6,000 lx, 20 μM NADPH, ^{3}H-Car; experiment 4–40 min, 6,000 lx, 20 μM NADPH, ^{3}H-Car

Data showing the possibility of direct oxidizing transformations of labeled molecules of Car in a suspension of chloroplasts are presented in Table 5.16. Unlike ours, the experiments of Hager were limited by only quantitative measurement of the content of pigments. From Table 5.16, it can be seen that in both the light and shaded variants of the experiment, the radioactive label was found in carotenoids of different degrees of oxidization. The assessment of SpA of fractions of carotenoids of pea chloroplasts allows us to conclude that the presence of NADPH promotes transformation of labeled Car into Viol. The low SpA of preparations of Viol and Neo indicates that both pigments are at the final steps of the reaction of Car oxidization.

From the results in Table 5.1 and 5.16, one more feature of xanthophyll transformations follows, namely, there is direct proof of transformation of xanthophylls with 5,6,7,8-diene structure into xanthophylls with 6,7,8-allenic structure and vice versa.

In chromoplasts of *Capsicum annuum* the transformation of ^{3}H- or ^{14}C-Viol into capsorubin, and ^{3}H-Ant into capsanthin, has been demonstrated (Camara and Moneger 1981). From this article, the possibility of similar transformation in

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chromoplasts follows, that is, Ant into Viol and Zea into Ant. Thus, as earlier with chloroplasts (Saakov 1963a, b, c, 1964, 1965a) and later with chromoplasts, the earlier experiments on Viol reduction were confirmed. This statement is sufficiently proven for *Chlorophyceae* and *Phaeophyceae*.

Additional proof of the correctness of our conceptions comes from experiments on the metabolism of labeled preparations of carotenoids in animal organisms (Hata and Hata 1975; Tanaka et al. 1976). Animals are capable of transforming alimentary pigments and reserving the resultant products of metabolism (Tanaka et al. 1976). In a large series from many works by Chichester and coauthors (Katayama et al. 1972, 1974; Tanaka et al. 1976), performed with evolutionary various materials, the transformation of β-Car into Ast was revealed. In animals *Carassius auratus*, *Chrysophrys major*, *Cyprinus carpio*, *Panulirus japonicus*, *Portunus trituberculatus*, *Penaeus japonicus* Bate, and *Evynnis japonica* Tanaka, the transformation of ^{3}H-Car through a number of intermediate products of different oxidative degrees into Ast and Asc was ascertained. Other data (Tanaka et al. 1976; Hata and Hata 1975) present proof of the transformation of Lut and Zea into Ast and doradexanthin, respectively, that is, active oxidization of Car occurs up to compounds containing four atoms of oxygen, as in Viol. These results have something in common and correspond to conceptions described in Sect. 5.3.

The analysis of our own and literature data allows us to suggest a scheme (Fig. 5.43) of interrelation of pathways for transformation of the main carotenoids in flora and fauna.

Thus, the solution to the problem of carotenoid transformations in green and animal cells is closely connected with the use of carbon skeletons of plastid

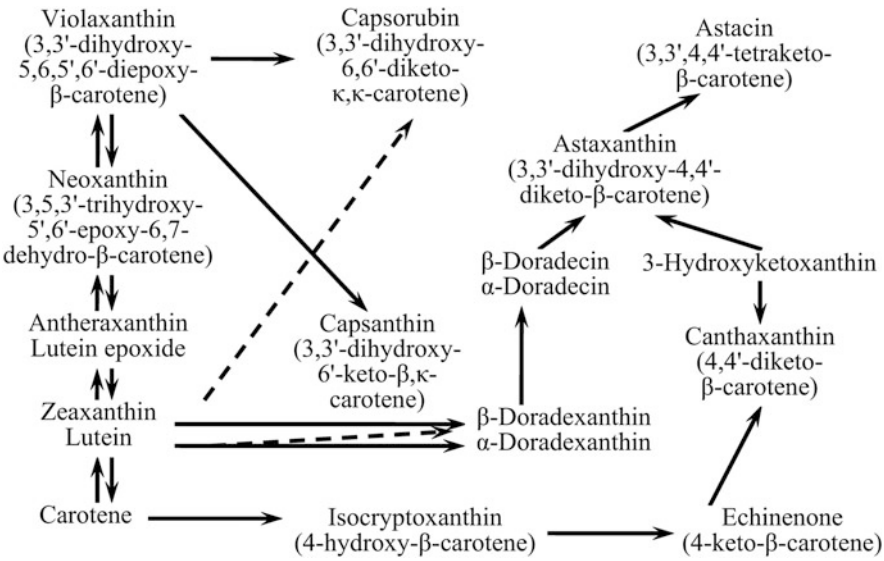

Fig. 5.43 Scheme of xanthophyll transformations in vegetative and animal organisms

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carotenoids and of alimentary carotenoids in animals, and it is of general biological interest because all oxidized forms of carotenoids in animals and plants contain four atoms of oxygen.

5.7.5 *Speculations Based on Experiments on the Exchange of Water Oxygen with Oxygen-Containing Xanthophyll Groups*

Reactions of xanthophyll interconversion considered in previous sections involve the separation of hydroxyl and epoxy groups containing oxygen. The easy oxidizability of carotenoids became the reason for supposing that the xanthophyll system is probably coupled to the transfer of photosynthetic oxygen.

It appears reasonable to assume that the reversible conversions of xanthophylls under the influence of light are probably the system at the place of one of question marks in the Calvin scheme (Anderson et al. 1960). For such a suggestion, there were premises originated in many works (Sapozhnikov et al. 1961, 1963, 1965, 1967a, b, 1969, 1973). The main idea was borrowed by Sapozhnikov from the work of Dorough and Calvin (1951). Such a system was attractive because, since the time of discovery of the source of photosynthetic oxygen (Vinogradov and Teis 1941, 1947; Vinigradov 1962; Kutyurin 1965; Kutyurin et al. 1969; Budzikiewicz and Inhoffen 1969), the intermediate systems of the oxygen transfer and oxidization in chloroplasts had not been reliably established (Bassham and Calvin 1957; Egneus 1971; Gerster et al. 1971; Kutyurin 1971).

The mechanism of oxygen oxidization and of its liberation is still the most complicated subdivision in the study of photosynthesis. For this there should be a methodologically supported level of experiment. In most works of this direction, indirect data are present, interpretation of which leads to ambiguous conclusions promoting the appearance of unscientific speculations. In this book, we have not discussed the theoretical premises and details of the variety of experiments investigating systems oxidizing water. This is not the scope of our monograph (Sisakyan 2010).

We discuss the *probable* or, more properly, *speculative* participation of oxidation–reduction reactions of xanthophylls as the, presumably, main link between oxidization of water and liberation of molecular oxygen. More precisely, at a methodologically high level of experimentation, we try to assess the probability of inclusion of water oxygen in oxygen-containing xanthophyll groups. The reliable establishment of this fact is *primary*, and the probable interpretation of the functional meaning of this fact is *secondary* and, more often, strained. In the above-cited publications, a number of schemes and hypotheses are proposed, some of which were belied by deeper experiments or left aside in favor of conceptions more accepted by society. However, there was none able to pretend to be the final solution of the problem.

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5.7.5.1 Influence of Light on the Activity of Water Oxygen Inclusion into Molecules of Violaxanthin and Lutein

The mere fact of detection of the oxygen of O^{18}-labeled water in the total fraction of xanthophylls (Dorough and Calvin 1951) gave an impulse to an experimental and methodological race, to which the group of D.I. Sapozhnikov came late. By some quirk of fate, one of the authors of this book was with the group, and the theme of *investigation of water oxygen inclusion in xanthophylls* was placed in his postgraduate plan. This means that the further narrative concerns not only the literature analysis, but reflects the state, efforts, and emotional stresses of the stakeholder and the main experimenter responsible for the performance of experiments. Because the main analytical work on the detection of the O^{18} isotope was carried out at the cyclotron in the Ioffe Physical-Technical Institute of the USSR Academy of Sciences (PhTI AS), the assigned responsible executor from the group was one of the authors of the present monograph. Apart from this, all the difficulties in the work performance, learning the methodology of analysis, and evolution of human relationships during the period of work the postgraduate student saw from inside the system. The postgraduate project work approved by the academic board turned into impersonal activity of the staff group, whose level of knowledge was quite remote from the problems investigated according to the research theme. Over 50 years, the poignancy of some peculiarities of the work has worn off, but it is not possible to conceal the principal moments.

In the context of this section, we partly return to the methodological events described in Chaps. 1 and 3 (see Sects. 3.6 and 3.6.4). We discuss specific experiments in which the postgraduate student performed most of the analyses and calculations.

The tactics of experimental setup and the rational realization of tasks in the frame of existent methodological possibilities, or introduction of new experimental approaches, could be discussed only with the group of physicists of PhTI AS, since members of Sapozhnikov's group were not physicists and could not participate in technical discussion. Because the methodological approach defines the success of a researcher, then without its detailed discussion (see Sect. 5.6), a set of necessary but not sufficient conditions for success can arise and lead to irrational ideas.

In initial experiments, two kinds of objects were used: vegetative shoot apexes of *Elodea* of approximately 1 cm length and suspensions of *Chlorella*, grown in the laboratory of Prof. O. V. Zalenskii. *Elodea* (140 apexes) was slightly predried in air to remove external water, and then it was placed in the opening of an organic-glass disk, which was then situated in a Petri dish containing heavy-oxygen water (23 % enrichment with O^{18}). The intensity of illumination in the experiment was approximately 20,000 lx. The temperature during exposure (2 h) (compared with selected second exposures in Chap. 1, Figs. 1.2 and 1.3) ranged from 20 to 25 °C. Periods of illumination (15 min) were alternated with 5-min periods of darkness.

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In experiments with *Chlorella*, centrifuged alga cells were resuspended in 5 mL H_2O^{18} (17 % enrichment with O^{18}) in vessels with plane-parallel walls (Saakov 1963c). Illumination of a vessel was approximately 20,000 lx. Light exposure was for 75 min. Interchange of light and dark periods was as described above. Extraction and chromatographic purification of pigments were performed according to accepted techniques (see Chap. 3).

The following preparations were isolated and purified: (1) Viol isolated from objects exposed to light in H_2O^{18}, designated here as ${V^{18}}_L$; (2) Viol isolated from objects kept in the dark in H_2O^{18} (${V^{18}}_D$); and (3) Viol isolated from objects kept in normal water non-enriched with O^{18}, in light (${V^{16}}_L$). Thus, variants ${V^{18}}_D$ and ${V^{16}}_L$ served as additional controls for the variant ${V^{18}}_L$ and allowed assessment of the stimulating role of light on the incorporation of O^{18} in a Viol molecule (see Fig. 3.89)

First experiments carried out with the leaves of *Elodea* allowed us to reliably determine incorporation of the stable isotope O^{18} in a Viol molecule (Fig. 5.44) (Sapozhnikov et al. 1961). Results of measurements both of β-spectra and γ-spectra allowed us to conclude with a high degree of possibility that the induced radioactivity in preparations was caused by radiation of the F^{18} isotope. From extrapolation to the point of origin, it followed that the initial radioactivity of the light variant exceeded the background value by 6.5 times. Thus, data about the *principal inclusion of water oxygen in Viol* were obtained, but the groups where oxygen was

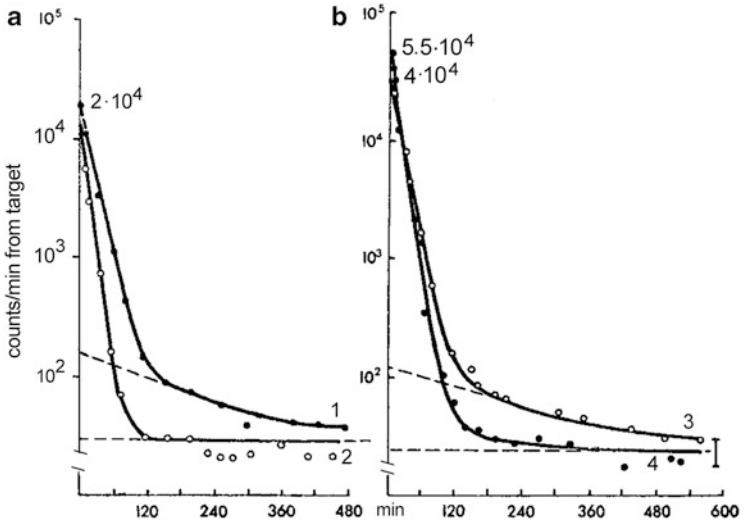

Fig. 5.44 Comparative dynamics (changes) of the radioactivity of targets containing violaxanthin that had been obtained from *Chlorella* sustained on heavy water (O^{18}). (**a**) (*1*) Viol-18 from the light variant reflects (rejects) the decay of fluorine atoms; (*2*) Viol-18 from the dark variant. (**b**) (*3*) Viol-18 from the light variant; (*4*) light variant but only the violaxanthin-16* (Viol-16). *Ordinate* number of pulses from the target in about a minute; *abscissa* time in minutes after irradiation by protons

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included were not determined. However, the suggestion about the positive influence of light was supported.

Weightier information about the predominant incorporation of water oxygen, in light, into preparations of Viol was obtained by us in further work (Sapozhnikov et al. 1964). In these experiments, the used water was enriched with the O^{18} isotope to 68 %.

In contrast to previous experiments, the following fractions of pigments were irradiated by protons: Car ($C^{18}{}_L$ and $C^{18}{}_D$), Lut ($L^{18}{}_L$ and $L^{18}{}_D$), and Viol ($V^{18}{}_L$ and $V^{18}{}_D$). We calculated the SpA of various fractions for 100 μg of substance and per 46 μC of charge transferred by protons irradiating the target, which were normalized to 240 min after the end of irradiation. In all cases, the background was subtracted from results of measurements. The resulting data confirmed previous results (Sapozhnikov et al. 1961). The effectiveness of O^{18} inclusion into Viol was three times higher than in the previous work, which is very possibly connected with the higher percentage of water enrichment with O^{18}.

A reliable difference between light and dark preparations of Lut was not found. The insignificantly induced radioactivity in fractions $L^{18}{}_L$ and $L^{18}{}_D$ was probably caused by oxygen incorporation into hydroxyl groups of the pigment in the process of biosynthesis. Comparison of preparations $V^{18}{}_L$ and $V^{18}{}_D$ with preparations $L^{18}{}_L$ and $L^{18}{}_D$ allowed us, at the present stage of research, to conclude that the oxygen of hydroxyl groups in ionone rings of Lut is not actively changed to water oxygen in darkness or in light, which corresponds to the ideas of Yamamoto et al. (1962) developed at those times.

The comparison of levels of the induced radioactivity of Viol and Lut fractions allowed us to find the conditions necessary for a conclusion about the incorporation of water oxygen in epoxy groups of Viol. In total, the SpAs of Viol fractions significantly exceeded levels of the induced radioactivity of Lut fractions, and this process was activated by light. The presented results were necessary for vindication of the hypothesis that xanthophylls play a role in the transfer and oxidization of photosynthetic oxygen, but *they were not in any way sufficient*.

In various works (Saakov 1963c; Saakov et al. 1970a; Saakov et al. 1970c), we underlined the ambiguousness of the above-stated ideas about the expressed direction of incorporation of water oxygen into the epoxy groups of Viol, although in some publications (Sapozhnikov et al. 1961, 1964, 1965a, b) exactly this very attractive aspect of the problem was discussed (Sapozhnikov 1961, 1965, 1967a, b, 1969).

For not always understandable reasons, in the series of experiments performed in those years, only the inclusion of water oxygen into some xanthophyll fractions was registered, but there was not always high validity of the difference between activities of fractions, so we could not unequivocally determine the groups incorporating water oxygen. However, at that time, when scientists of two countries competed in their achievements, some deviations from 100 % probability were permitted. For example, some conclusions in the works of Yamamoto et al. (1962a, b) were made on the basis of logical assumptions, because the authors indicated that they had failed to obtain the quantity of one or another pigment

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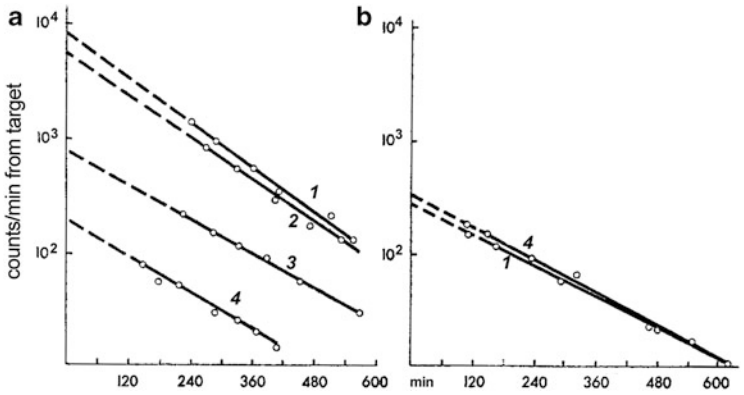

Fig. 5.45 Inclusion of oxygen O^{18} from labeled water into violaxanthin and lutein in response to action of light on a plant. *Straight lines* reflect the dynamics (changes) of the half-life period of the isotope F^{18} in molecules of xanthophylls, induced by proton action: (**a**) positive experience; (**b**) negative experience. (*1*, *2*) Violaxanthin, light variant; (*3*) violaxanthin, dark variant; (*4*) lutein, light variant

necessary for analysis. It was not surprising to us because we are familiar with the backbreaking work involved in the isolation and qualitative purification of xanthophyll preparations.

Figure 5.45 presents typical data from positive and negative experiments on the selective insertion of water oxygen into xanthophylls. The left part (Fig. 5.45a) shows the results of an experiment in which the expected effect of prevailing oxygen incorporation from water into Viol (curves 1 and 2) under the influence of light is visible (calculated per 100 μg of pigment and 250 μC of charge transferred by protons). Note the active dark incorporation of water oxygen into Viol compared with Lut preparations. It is not improbable that this is the consequence of oxygen biosynthetic incorporation or the aftereffect of isotopic exchange of oxygen of epoxy groups with water oxygen in intact cells, as suggested by Sapozhnikov and Kutyurin (1967).

Figure 5.45b shows a typical experiment in which the radioactivities of Viol and Lut light preparations practically coincide with each other. An analogous level of radioactivity is shown by the dark preparations of the mentioned xanthophylls.

Earlier, the supervisor of the work avoided discussing these results because of administrative reasons. Later research (Saakov et al. 1969, 1970a; Saakov et al. 1970a) allowed us to again return to discussion of this question.

Results of the experiment are reflected in Table 5.17. In contrast to previous works (Saakov 1964; Sapozhnikov et al. 1961, 1964), the described experiments were carried out with the use of a statistical sample uncommon for experiments of such kind and pigment preparations purified five or six times . For oxygen detection, the nuclear reaction $O^{18}(\alpha,n\gamma)Ne^{21}$ (Sect. 3.6.2) was applied, which was substantially gentler for targets placed on substrates. The obtained data allowed us to also consider the incorporation of water oxygen into the hydroxyl groups of

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Table 5.17 Value of induced radioactivity in fractions of xanthophylls of higher plants, calculated per 100 μg of pigment and 1 μC of charge (relative units)

	Pigment fractions			
Object	Violaxanthin	Lutein	Neoxanthin	Lutein epoxide
Pea	243	208	345	–
	245	219	–	–
Corn	350	260	300	230

xanthophylls as real. In favor of this *assumption* were the insignificant differences in the SpAs of various xanthophylls. This assumption became an additional argument against the absolutization of the stained and publicized position (Sapozhnikov 1961, 1965, 1967a, b). But there is a hidden contradiction, which we discuss below.

To summarize, science is developed in time and space. This means that a discovery made in one country can be confirmed or refuted in another. This state opens the way to definite fabrications and juggling of facts, which in some degree reflect the original assertions.

Now then, what was exactly found in the course of the first experimental series? First, water oxygen is incorporated into the oxygen groups of ionone rings of xanthophylls. Second, light activates processes of oxygen inclusion. At this point the *accuracy ends and assumptions arise*. Experimenters presumed that the possibility of water oxygen incorporation into xanthophylls was a result of slow metabolic processes or biosynthetic reactions during long exposures. To improve the above-stated position, it was necessary to assess the rates of xanthophyll biosynthesis (and at that time it meant to conflict with the accepted theories and statements of Professor Goodwin and coworkers). It was necessary to calculate *the probability of oxygen transport in the course of the direct (light) and back (dark) reactions of the xanthophyll cycle and its adequacy for elimination of photosynthetic oxygen in the required time interval*. All this was not solved and the theoretical statements of Sapozhnikov (1961, 1965, 1967a, b, 1969, 1973) remained unsettled for many years.

5.7.5.2 Influence of Light on Incorporation of Water Oxygen into Neoxanthin

On the grounds that in experiments with labeled preparations of xanthophylls the conversion of Viol in Neo was observed (Saakov 1963b, c, 1965a, b, 1966, Saakov 1968a, 1970a) and that Neo contains two hydroxyl groups and one epoxy group, there arose an interest in tracing the different possibilities of water oxygen inclusion into the Neo molecule.

For the purpose of examination, two portions of *Chlorella* alga in water enriched with the O^{18} isotope to 20 % (and occasionally to 40 %) were exposed to light and dark (Saakov 1963a, b, c, d, 1964, 1965a, b). The additional control was an algae sample exposed to light in water containing a natural content of oxygen isotopes.

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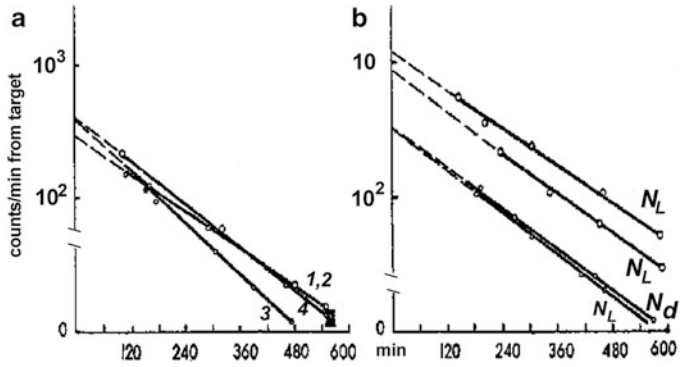

Fig. 5.46 Inclusion of O^{18} from labeled water into xanthophylls in response to action of light on a plant. *Left*: Inclusion of oxygen in violaxanthin and lutein illustrates the absence of an authentic difference in the degree of inclusion of O^{18} into epoxy and hydroxy groups of pigments. This is an example of an unsuccessful experience in studying the inclusion of oxygen of water into violaxanthin. There is no authentic difference between light and dark preparations, and also there is no difference compared with the radioactivity of the lutein preparation. *Right*: Unstable inclusion of O^{18} into neoxanthin; three parallel tests of light variant of neoxanthin (N_L) are shown and one test of the dark variant of neoxanthin (N_D)

In the experiment, three parallel probes of the Neo light preparation were used (Fig. 5.46).

Below are the values of the induced activity (in impulses per minute) of experimental and control preparations at 240 min after the end of proton irradiation, calculated per 100 μg of substance and per 258 μC of charge transferred by protons irradiating Neo (where L indicates in the light and D in the dark):

$N^{18}{}_L$	$N^{18}{}_L$	$N^{18}{}_L$	$N^{18}{}_D$	$N^{16}{}_L$
360	240	92	90	90 imp/min

Figure 5.46 shows the dynamics of radioactive decay of irradiated Neo preparations of the half-period $T_{½} = 110 \pm 5$ min.

Comparison of the activities of experimental and control targets (normalized to 240 min after the end of irradiation) shows that the activity of two parallel targets of the variant $N^{18}{}_L$ exceeds that of control $N^{18}{}_L$ and $N^{16}{}_L$ by 2.5–4 times. Extrapolation to the origin point corresponding to the end of irradiation at the cyclotron points to the presence of induced oxygen activity in two parallel targets of the variant $N^{18}{}_L$, which is 25–30 times the background value.

Thus, the presented data allowed us to suppose the existence of *light activation* of the incorporation of water oxygen into Neo molecules (Saakov 1964). This preliminary conclusion did not contradict the positions of other researchers (Yamamoto et al. 1962 other publications of Prof. Yamamoto et al). However, our experiments did not unequivocally show in which oxygen-containing group of the

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pigment the water oxygen is included. The influence of light only stimulated the processes of water oxygen incorporation.

Presented in this section are data and material from Chap. 3 (on methodology), and also an outline of existent literature, which point to the contradictions in results on investigation of water oxygen and molecular oxygen in xanthophyll molecules. The revealed differences are probably caused by methodological difficulties in determination of the oxygen isotopic content in xanthophyll molecules. Due to difficulties in isolation of radiochemically pure quantities of xanthophylls sufficient for analysis, scientists have to work with minute quantities of substances that handicap the obtaining of material for parallel determinations (Shneour 1961, 1962a; Yamamoto et al. 1962). For this reason, the verification of physical and biochemical sides of the experiment is complicated and prompted the application of very sensitive methods for detection of trace quantities of O^{18} isotope. The high cost of these studies meant that researchers tended to introduce the largest possible number of experimental variants, at the cost of their certainty.

In any case, in a significant number of works of this direction, the rational sufficiency of the experimental evidential base was absent, which led to desirable rather than real results (Sapozhnikov 1969; Shneour 1962).

In Chap. 3 we underlined that in the majority of works, *the rules of radiochemical purification* of preparations were not observed, and this can significantly distort the final result. For example, in one work (Sapozhnikov et al. 1964) the induced radioactivity of Car oxygen was detected, although the Car molecule does not contain oxygen atoms. Sapozhnikov did not reject these data as unrealizable fact, but included them in the publication. The absence of parallel experiments does not allow unequivocal conclusions to be made, and readers have to rely only on the decency of the researcher. Control determinations of pigment fractions not enriched with O^{18} isotope were rarely introduced, and during the work with gaseous O^{18}, a control with H_2O^{16} and H_2O^{18} was not introduced (Yamamoto and Chichester 1965; Takeguchi and Yamamoto 1968). More frequently in European and American works, the measurement of one sample without additional parallel determinations of probes was performed. At the same time, as our many years of experience show, the results of analysis of several parallel samples can *inexplicably* differ between each other (Saakov 1963c, 1964), which is presumably the consequence of instability of the physical part of the experiment (Dorough and Calvin 1951; Shneour and Calvin 1962). Moreover, there was quite significant variability of the enrichment of initial labeled preparations of molecular oxygen and water with O^{18} (3–70 %) and variations in the duration of exposure on the labeled substrate (from seconds to 2 h). Also, the vegetative objects applied in experiments differed significantly in their characteristics. As the consequence of a certain mess in works of this direction, the publication of Yamamoto and Chichester (1965) appeared.

Without doubt, for further successful work and to obtain comparable research data from various countries, it is necessary to develop a matrix of common research approaches to the experimental setup and to the selection of optimal analytical methods, in the absence of which, this theme remains unresolvable.

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5.7.5.3 Influence of Light on Incorporation of Water Oxygen into Antheraxanthin (Diadinoxanthin) and Zeaxanthin (Diatoxanthin) Isolated from *Euglena gracilis*

In connection with the above, a series of experiments was performed using the new methodological basis for detection of oxygen isotopes and purification of pigment preparations (Saakov et al. 1970, 1971; Saakov and Nasarova 1970, 1972).

The centrifuged precipitate of *Euglena* cells was resuspended in heavy-oxygen water (60 % enrichment with O^{18}). One part of the suspension was exposed to light (30 min, 40,000 lx), and the second part was kept in darkness (20 °C). Each fraction of pigments taken in the experiment was radiochemically purified (Saakov and Nasarova 1970). The radiochemical purity of preparations (e.g., of Car) was testified by the fact that the level of induced radioactivity did not exceed the background value in preparations of the pigment isolated from the variant exposed to H_2O^{18} (see Table 5.18). For the detection of the O^{18} isotope, α-particles were used (see Chap. 3).

From the data in Table 5.18, it follows that the inclusion of O^{18} isotope is sufficiently reliably detected in light in parallel preparations of Ant. This is the foundation for concluding that there is more intensive exchange and incorporation of water oxygen in the Ant molecule in the light variant than in the dark variant. An analogous statement can be made concerning Zea preparations. The ratio of the values of induced radioactivity for Ant is $Ant^{18}{}_{\text{light}}/Ant^{18}{}_{\text{dark}} = 1.37$ and, correspondingly, $Zea^{18}{}_{\text{light}}/Zea^{18}{}_{\text{dark}} = 1.52$.

If we compare the SpAs of $Ant^{18}{}_{\text{light}}$ and $Zea^{18}{}_{\text{light}}$, then the ratio 1.03 is obtained, and for the comparison of dark variants of these pigments, 1.14. The values of SpA ratios are very close to each other, and therefore it would be hasty to make any conclusions about the more intensive exchange of oxygen-containing groups of Ant or about the larger number of oxygen atoms incorporated in the Ant molecule.

Similarly, the withdrawal made by us (Sect. 5.7.5.1 and 5.7.5.2) about the light stimulation of incorporation of water oxygen into Viol and Neo molecules can be

Table 5.18 Values of induced radioactivity in fractions of *Euglena gracilis* xanthophylls, calculated per 100 μg of pigment and 1 μC of charge (relative units)

Variant of experiment and fraction of pigment	Parallel probes						Average probe
	I	II	III	IV	V	VI	
$Ant^{18}{}_{\text{light}}$	180	205	170	200	149	169	179 ± 19
$Ant^{18}{}_{\text{dark}}$	128	139	145	128	113		130 ± 9
$Zea^{18}{}_{\text{light}}$	188	154	180				174 ± 13
$Zea^{18}{}_{\text{dark}}$	118	111	112				114 ± 3
$Ant^{16}{}_{\text{light}}$	6	8					7 ± 1
$Zea^{16}{}_{\text{light}}$	4	6	8				6 ± 2
$Car^{18}{}_{\text{light}}$	6	12	2				7 ± 5

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extended to Ant and Zea. Summarizing, it is possible to express the idea about the incorporation of water oxygen into xanthophylls in light.

On the basis of earlier known data, we could expect that the SpA of Ant preparations would be higher than those of Zea preparations because the Ant molecule also contains an epoxy group, the oxygen of which could be exchanged with water oxygen (Yamamoto et al. 1962; Sapozhnikov 1969, 1973). At the same time, after the comparison of SpA of light variants of the two considered pigments and of their dark probes, we have the following ratios: SpA $Ant^{18}{}_{\text{light}}$/SpA $Zea^{18}{}_{\text{light}} = 1.03$ and SpA $Ant^{18}{}_{\text{dark}}$/SpA $Zea^{18}{}_{\text{dark}} = 1.14$.

These figures do not allow us to be optimistic about oxygen inclusion in the epoxy group of Ant under the influence of light. Taking into account the chemical structure of the considered xanthophylls and assuming the same velocity of the oxygen exchange for epoxy and hydroxyl groups, it could be expected that the ratio of SpA of these xanthophylls would be 3:2.

The radioactivity of samples exposed to the labeled substrate was 20–25 times greater than the radioactivity of the background, non-enriched with O^{18}, preparations. At the same time, comparing the data obtained by us (Table 5.17) with the results of another work (Kutyurin et al. 1967; Sapozhnikov et al. 1967), we showed little real participation of the oxygen of epoxy groups in the exchange with water oxygen. Although, without more evidential experiments, it is premature to *reject the participation of epoxy groups of xanthophylls in the water oxygen exchange*. Maybe, some subsequent mystery of nature is hidden here. Probably, investigation of the participation of a rather small part of the epoxide xanthophyll pool (young molecules, just formed in the process of biosynthesis) (Saakov and Nasarova 1970) can clarify the mysterious participation or non-participation of epoxide xanthophylls in the oxidization of water oxygen, as pictured by Calvin and coworkers (Anderson et al. 1960). Our *negative* stance on the role of xanthophylls containing epoxy groups composing the *main* way of oxidization of water oxygen was expressed earlier (Saakov 1965a, b). The author assumed the presence of parallel and, regretfully, still unknown ways and mechanisms of oxygen oxidization, the contribution of which to the total amount of liberated photosynthetic oxygen is low (Saakov 1965a, b).

5.7.6 *Derivative Spectrophotometry for the Analysis of Pigments of Blood and Its State*

The related chemical structure of the base of the chlorophyll molecule and of blood heme allowed us to assume the expediency of application of derivative spectrophotometry for analysis of blood and for the detection of blood minimum quantities in a mixture of substances. The first experiments in this direction showed the prospects of our ideas (Saakov et al. 1973) and the expediency of application of the method for assessment of various influences on the blood (Saakov et al. 1978a).

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Figure 5.47 shows the absorption spectrum of blood solution in water, and Fig. 5.48 shows the D^{II} recording. Comparison of curves of spectra D and D^{II} assumes the existence of spectral discreteness of the behavior of chemically identical hemoglobin molecules, possibly caused by a difference in the character of interaction of the pigment molecule with protein or lipoid complexes. The presence of heterogeneous funds and chlorophyll forms in plastids no longer raises doubts (Krasnovskii and Kosobutskaya 1953; French 1962). The fine structure of D^{I} and D^{II} spectra of absorption bands of chlorophyll allows us to isolate in vivo spectral forms differing in their reactions to the influence of external physical and chemical agents (Shlyk and Sukhover 1968; Litvin 1965). The influence of chemical and physicochemical agents leads to changes in the absorption spectrum of

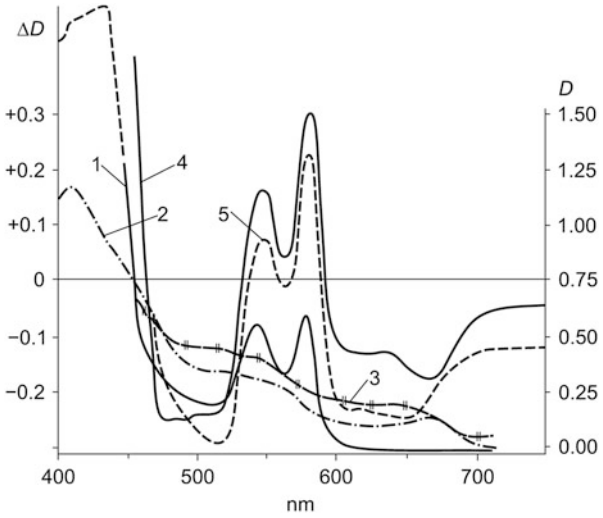

Fig. 5.47 Absorption spectra of solutions: (*1*) blood; (*2*) hematin; (*3*) blood treated with citric acid; (*4*) "blood minus hematin"; (*5*) "blood minus blood treated with citric acid"

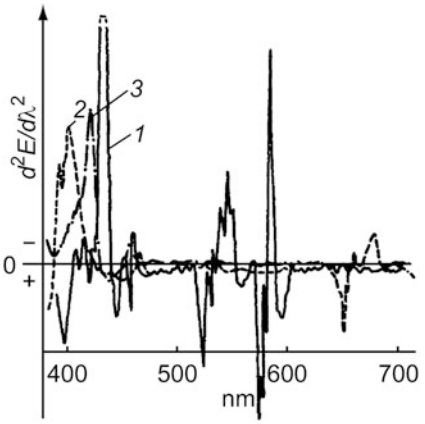

Fig. 5.48 The second derivative of the absorption spectrum: (*1*) blood; (*2*) hematin; (*3*) blood treated with citric acid

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blood that are especially noticeable using classical lines of absorption of hematin, kathemoglobin, carboxyhemoglobin, and methemoglobin.

The spectral changes after interaction of blood with 5 % hydrochloric (hematin) and 1 M citric acids are shown in Fig. 5.47. In the absorption spectrum, there are no reliable distinctions in the character of curves corresponding to the blood state after its treatment with mineral and organic acids. However, on curves of the D^{II} spectrum, there are distinctions in the character of influence of specified acids, and the curves are more convex (Fig. 5.48). It does not raise doubts that specific lines of the D^{II} spectra of parahematins, kathemoglobin, oxy- and carboxy-hemoglobin, and methemoglobin would reveal features of their spectral forms if we were to register D^{IV} and D^{VIII} spectra.

By analogy to the considered differential spectra of chlorophyll and its analogues, we performed measurements of the difference spectra "blood minus blood treated with hydrochloric and citric acids" (Fig. 5.49). Simultaneously, we recorded the second derivative of the difference spectra (ΔD^{II}). From the analysis of difference absorption curves, it can be seen that the difference in spectra "blood minus blood treated with hydrochloric or citric acid" manifests in a change in the ratio of maxima heights (we name them α and β) and equals 0.54 for hydrochloric acid and 0.34 for citric acid.

On the ΔD^{II} curve "blood minus blood treated with citric acid," a number of structures invisible on the difference absorption spectrum are noticeable in the region 530–550 nm. On the ΔD^{II} curve "blood minus hematin," peaks appear in

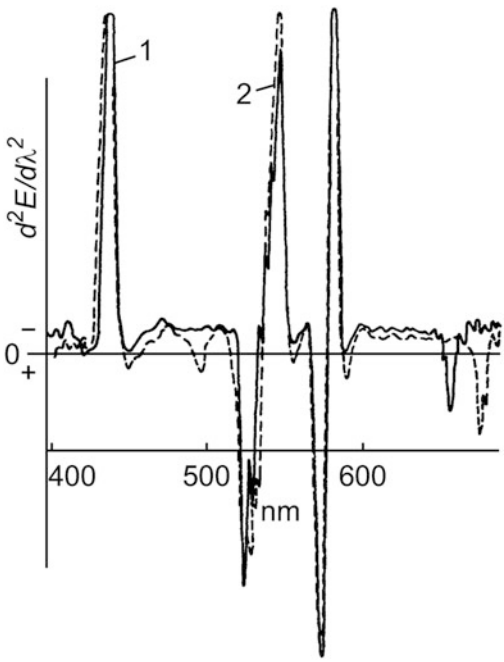

Fig. 5.49 The second derivative of the difference absorption spectrum: (*1*) "blood minus blood treated with citric acid"; (*2*) "blood minus hematin"

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regions 500 and 670–680 nm, and the fine spectral structure in the form of reproducible spikes in the region 530–540 nm comes to light.

Our further work in this direction was concerned with various topics, such as the role of Ca^{2+}- and Co^{2+}-dependent conformations of proteins of rat blood serum in the regulation of blood osmolality (Natochin et al. 1985); comparative analysis of blood plasma after transition of animals from diapause to the awakening state (Monin et al. 1985); assessment of age features of osmotic and ionic homeostasis in spontaneous hypertensive rats (Sokolova et al. 1991); and also with changes in osmolality, the concentration of monovalent cations, and structures of proteins of blood plasma under extreme (hyperbaric) influences (Sokolova et al. 1992).

In a slightly different direction, our other research topics were application of derivative spectrophotometry for analysis of the content of oxyhemoglobin, methemoglobin, and bilirubin in cerebrospinal liquid and in blood (Stroes and van Rijn 1987; Taulier et al. 1987); assessment of changes in hemoglobin quantity in blood plasma and in urine (Taulier et al. 1986, 1987); selective detection of carboxyhemoglobin in the presence of methemoglobin, sulfhemoglobin, and alkaline and acidic hematin in cadaveric and fresh blood (Siek and Rieders 1975); determination of methemoglobin and total hemoglobin in toxicological research (Cruz-Landeira et al. 2002); and detection of bilirubin in the presence of hemoglobin (Soloni et al. 1986). The separate direction of analytical works included the determination and content analysis in blood and blood serum of amino acids tyrosine, tryptophan, and phenylalanine using their D^{II} spectra (Nozaki 1990; Ichikawa and Terada 1981; Szczepaniak and Dudka 1993; Fell 1979, 1980) and quantitative assessment of the glucose content of whole blood (Shen et al. 2003). With success, we applied difference derivative spectrophotometry for simultaneous determination of hemoglobin and co-porphyrin in blood (Ficheux et al. 1989a, b).

5.7.7 *Application of the Method of Differentiation of Spectral Curves for Decoding of Electrocardiograms for the Analysis of Heart Activity*

As noted in Chap. 2, differentiation of any hardware signal expressed by a curve with differing extrema of positive or negative orientation is possible. Thereupon, we paid attention to electrocardiograph signals. Registration of biopotentials arising in muscles, including biocurrents in cardiac muscle, is named cardiography. The registered curve, the electrocardiogram (ECG), consists of five basic peaks (waves): *P*, *Q*, *R*, *S*, and *T*. The atrial wave is *P* and *QRS* is the gastric complex. There is an opinion that the nature of peaks *Q*, *R*, *S*, and *T* is not completely known. Scientists suppose that peak *Q* is interfaced with excitation of internal surface of ventricles, right papillary muscle, and heart top. At the same time, the peak *R* is caused by excitation of the surface and base of both ventricles. By the moment of maximum amplitude of peak *S*, both ventricles are excited. In other words, in this case there is

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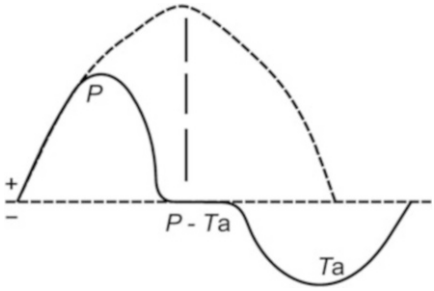

Fig. 5.50 Curves of depolarization of both atria

no difference in potentials between various parts of heart ventricles, and it corresponds to the rectilinear part of *ST*. The size of peak *T* is interfaced, possibly, with re-polarization of biochemical processes occurring after excitation of ventricles.

On an electrocardiogram, the electric activity of atriums is shown by the atrial complex, in which they distinguish wave *P*, segment *P–Ta*, and wave *Ta*. On a normal electrocardiogram, as a rule, only the first element of the atrial complex is presented, the wave *P*, which corresponds to non-simultaneous beginning of excitation of both atriums and to their simultaneous depolarization. The positive direction of wave *P* (Fig. 5.50) is caused by earlier appearance of a monophase curve of the right atrium and corresponds to depolarization of the right atrium. The top of *P* corresponds to the beginning of depolarization of the left atrium. The descending bend of wave *P* is formed because of depolarization of the left atrium.

The segment *P–Ta*, located on an isoline (the habitual zero line in a derived spectrum, the line of zero potential; see Chap. 1, Figs. 1.10 and 1.11), presents parts of the second phase of re-polarization synchronous for both atriums. Normal peaks (teeth) *P*, *R*, and *T* are positive, and teeth *Q* and *S* are negative. In some heart diseases, the described peaks (teeth) can change sign. For example, for cardiac infarction, the wave *T* becomes negative, and for other diseases (heart disease, stenosis of mitral valve), the teeth (e.g., peak *P*) are bifurcated. The wave *Ta* is the final phase of re-polarization of atriums, mainly of the left atrium. Its downward direction is caused by a delayed monophase of a curve of the left atrium.

Thus, the external electrocardiogram is the first derivative of the transmembrane potential of action and is a curve of speed change of difference of potentials registered with transmembrane recording in places of attachment of electrodes. Because registration of spike potential through a capacitor system results in differentiation, the cellular membrane possessing a certain capacity serves as such system.

The theoretical, methodological, and hardware approaches and solutions used in derivative spectroscopy seemed to us to be very appropriate for their application in the development of a hardware–methodological complex, with the corresponding software, intended for an increase in objectivity, reliability, and diagnostic reliability of the information contained in an electrocardiographic curve for the analysis of heart activity.

In summarizing this chapter, it is necessary to notice that, in the process of writing, the authors faced the need to describe various, debatable points of view on

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the processes of biosynthesis and metabolism of pigments in evolutionary different groups of animals and plants. The contrasting points of view of researchers were a result of methodological bases corresponding to the time. Clarification of questions was promoted by the development of the method of derivative spectrophotometry and by its combination with radiochemical approaches using isotopic techniques.

Together with consideration of some moments of biochemical history, we should not forget features of USSR history, when each scientific disagreement with the established opinion was treated as a deviation from the general line of the communistic party. It was more than inconvenient to write frankly about the lacks and to assess the harm for our science, although such a state of affairs did not exclude harm for science as a whole. Such a situation existed in spite of the fact that I.V. Stalin warned graduates of military academies on 5 May 1941: "The States perish, if they shut eyes to defects, delight in their successes, repose on their laurels" (Documents of Foreign Policy/Historical-Documentary Department of the Ministry of Foreign Affairs of Russian Federation, 1995, vol 23, book 2, p. 650).

The experience of solving the problems formulated in this chapter, with the help of hardware for derivative spectrophotometry when researching biological systems, allows us to look with optimism at the expansion of fields of application for derived spectra in the food and pharmaceutical industry, in analytical chemistry, and in the biochemistry of cellular structures.

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Chapter 6
Conclusion

The book is being published at a time when all peoples of the Earth celebrate the 70th anniversary of victory over fascism achieved by Russia and the allied armies of the USA, England, and France. This book is the result of many years of peaceful work and scientific contact between the peoples of Europe and America.

We pay tribute to those lost and injured at the fronts at that war. It was the heroism and steadfastness of the armed forces of the allies, and especially Russia, that enabled our generation of now elderly researchers to pursue science and write our books in peace.

Three of our authors were singed by war hardships. The head author stayed in Leningrad throughout the siege. He suffered from dystrophy, scurvy, and temporary loss of vision as a result of contusion caused by the burst of a fascist shell (Melua 1999).

Professor Eugen Rozengart faced the years of war as a child in sanitary trains collecting wounded soldiers from the front. He repeatedly came under air bombardment of these Red Cross trains.

Fortunately, Prof. Alex Krivchenko lived at that time in Siberia, far from the front, but the years of war famine left a mark on him as well.

The authors of our group are very grateful to our country and leaders of the government for the possibility of peaceful practice in their selected specialities.

Thus, we come to the end of this book. At this point, we can take the liberty of digressing from strict scientific treatment of phenomena and give a broad picture of the history and reasons for writing this book.

Quite intensive development and expansion of biophysical research methods in biochemistry and physiological sciences promoted the creation of the newest diversified diagnostic equipment. In this regard, some broad thinking and the private initiative of European and overseas firms ensured the mass production of very precise spectrophotometric equipment and devices for fluorescence analysis, which successfully conquered the world market. Studies of fluorescence can provide information about the functional state of living systems without damaging them. Fluorescence methods also allow researchers to carry out many tasks of

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ecological control within a short time interval. This helps to obtain new scientific information, and new information provides money, which is well understood in Western countries. Russia, however, makes no headway. All progress depends on the activity of individual scientists.

Except for the appearance of scientific information of high confidence in the flow of interesting Russian scientific developments, some works or even "scientific" directions appear that dominate for some time in the scientific environment but are then refuted as absolutely pseudoscientific theories and experiments that distract the collectives of scientific coworkers from proper scientific work.

In this connection, in the first chapter, we critically considered a number of works, performed at the most modern methodological level of analysis by a group of "researchers," which seem to be far from the development of Russian science. The desire of this group was not to clarify scientific verity and discover the mysteries of nature, but only the aspiration to be art and part in the application of the modern (and very expensive) new methods of scientific research. The result was rather sad. However, such a state of affairs arose not only at nonprofessional usage of the radioactivation method of oxygen detection. The problem was deeper and wider.

Almost half a century researchers of Europe and USA debated on xanthophyll transformations. From works of Yamamoto et al. (USA, 1962a, b, c, 1967); Hager (1957, 1966, 1967a, b, 1969, 1975, 1980 FRG), Hager and Perz (1970), Hager and Stransky (1970a, b, c), Krinsky (1962, 1964, 1966, 1968, 1971), Krinsky and Goldsmith (1960 USA), Latowski et al. (2000, 2002 Pln), Moster and Quackenbush (1952a, b Hol), Niyogi et al. (1997a, b, 1998, 2001), Havaux and Niyogi (1999 USA) it was well established that there is no direct transformation Viol into Lut as it was thought earlier.

To explain the errors that can arise in works using radioactive isotopes, and also with incorrect application of radioactivation analysis, we dedicated the third chapter of this book to consideration of the methodological approach for obtaining radiochemically pure preparations of pigments and the rules of use of nuclear reactions of activation. Without application of the described and suggested rules, the performance of analytical works is just scientific madness, leading to consciously obtained wrong results and data.

We will not discuss the theoretical explanations of the functional role of violaxanthin de-epoxidation or the interpretation of invalid results now. On behalf of the USSR Communistic Party, it's member working in Sapozhnikov's group blessed the scientific activity of the chief. At that time, her word in the party committee of the institute or the city district steered the investigations of the research group in the "*right*" direction.

The position of indifference and sometimes of some euphoria in reports and presentations to senior executives impeded its dispelling. All of this influenced on the attitude and orientation of work of young researchers in central scientific organizations, and in various scientific subdivisions geographically far from the center the positive perception of the scientific nonsense took place. As a consequence, for example, in Yakutia even now the reaction Viol → Lut is considered as the frost tolerance test for coniferous plants.

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The area of investigation of carotenoid reactions was, for regret, developed by researches who didn't have the necessary base in this field. It was reflected in critical publications of American and European scientists cited in Chapters of this monograph. Russia is a large country and sometimes modern knowledge doesn't pass quickly from one region to others.

But in total, the problem of biochemical transformations of carotenoids was interesting for many researchers. However, on various reasons some aspects weren't discussed for decades.

We give due to leaders of opposite opinions, but it is not possible not to take into account scientific achievments of investigators from USA, Germany, France, United Kingdom, Holland and other countries. In a modern science researchers should not stiffen with the representations as in a piece of ice but should develop these representations according to new methodical opportunities and approaches. Those who does not listen to the new data of a science lose an acuteness (a witticism) of perception (recognition) and curdle (solidity in the point of view), losing all.

The quick expansion in Europe and the USA of the pulse-amplitude modulated fluorescence method developed by the Würzburg scientific school attracted the attention of a wide range of biochemists and biophysicists. In our country, regretfully, similar fluorometers are not still produced. This is despite the fact that, in contrast to the usual fluorescence methods, PAMF can be used in light and actively applied during various technogeneous and natural catastrophes for assessing the degree of damage to plant cenosis, as can the specified method of derivative spectrophotometry.

Not having the possibility to work in Russia with the application of this method, we used the opportunity of overseas trips and performed experimental work in German laboratories. The experience of this work is concentrated in the second chapter of this book. In our earlier publications, we have expressed our acknowledgment of these laboratories.

Using various factors of extreme influence on the photosynthetic apparatus, we assured ourselves of the identity of the primary responses of autotrophic structures to the influence of the investigated natural and physical-chemical agents. We underline the fact that the exactly identical responses of the photosynthetic structures of *Eucaryota* and *Procaryota* in a set of various objects, and the related large amount of experimental material, allowed us to formulate a theoretical basis for the theory of energetic resistance of autotrophic tissues to extreme influences. Our conceptions coincided with a variety of overseas material dispersed in numerous publications and obtained for plant material of different systematic and evolutionary origin. Regretfully, Russian plant breeders and specialists in plant resistance are not practically familiar with the works of biophysicists of this research direction.

Material in the fourth and fifth chapters demonstrates the wide range of positive applications of derivative spectrophotometry in comparative biochemical studies of various directions.

The future prospects of the method of derivative spectrophotometry in biology and biochemistry are already described in a large number of review articles and in two monographs (Talsky 1994, see Chap. 3; Saakov et al. 2013, see Chap. 5). But

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Russian industry still does not produce suitable devices, although modern methods allow cheap and reliable equipment to be made, coupled to a built-in computer. We buy devices in Europe and pay a lot of money, which becomes less available in institutes from year to year. To develop experimental science only "with the help of fingers" is not possible.

From here, taking into account the positive response to the earlier published book (Saakov et al. 2013, see Chap. 5), we have included material on the application of derivative spectrophotometry in comparative biochemical, pharmaceutical, and physiological research.

By deepening and improving our scientific studies; and by publishing the corresponding scientific results in leading Russian and European journals, we have aimed to prove the significance of Russian studies at the international level.

We will carefully consider any reader's wishes, productive comments, and criticism.

With respect to readers,

Authoring team

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