APPLICATIONS OF SPECTROSCOPY IN BIOLOGY AND BIOCHEMISTRY*

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THE subject matter of this paper will not at all involve any detailed review of the spectra of various biologically important objects. Besides the fact that the number of such objects studied up to now is so great that it is impossible to review all of this material within the framework of a paper, it seems to me that such a review by itself would hardly be of interest to most of the participants in this conference.

Hence, I shall limit the theme, on the one hand, to an attempt to characterize briefly those peculiarities of biological objects and of the purposes of studying them, which make it necessary to develop specific methods of determining and analyzing the spectra of these objects. On the other hand, I shall attempt to show by some examples the great possibilities for spectroscopy in revealing the physical mechanisms of a series of fundamental biological processes.

1. Above all, I must emphasize the fact that biological objects not only are exceedingly complex systems (immeasurably more complex than those which we find in technology or in the inorganic world), but it is their complexity—in the coordinated occurrence of a multitude of interconnected reactions, in the continual exchange of matter and energy with the environment, in the continual process of the creation and decomposition of very complex specialized structures—which comprises the specificity of life processes and the principal difference between living and nonliving matter. Hence, however important the study may be of the composition and structure of the separate substances which we may isolate from the organism in a more or less unchanged form, such studies are only a preliminary stage from the broad biological point of view. The ultimate goal is always the physico-chemical study of living matter in the intact form, under normal physiological conditions of functioning.

On the other hand, we must keep in mind the fact that, except for some special problems, e.g., those associated with the presence of trace elements, the purpose of the physico-chemical study

of biological materials is not the determination of the overall atomic composition of the molecules of these substances, but the study of the details of their chemical structure: the structural chemical formulas, the stereochemical relations, the presence of various functional groups, the distribution of bonds, the presence or absence of double bonds, or especially of hydrogen bonds, etc.

For these reasons, the ordinary destructive methods of spectroscopy (spark, flame, and arc emission spectroscopy) find only very limited application in biological and biochemical studies. The basic role is played here by absorption spectroscopy. Among the methods of emission spectroscopy, the study of photoluminescence, and in particular, of the chemiluminescence of biological materials and living objects, has the greatest significance. In such cases, the influence of the exciting agent or radiation is sufficiently mild that it may be considered as not altering essentially the properties and structure of the object.

2. The spectral analysis of biological materials is based in principle, of course, on the study of the absorption spectra of these materials in some region of the spectrum, ultraviolet, visible, or infrared. Examples may be given for each of these regions of the highly successful application of spectroscopic methods for the identification of certain substances. In the ultraviolet region, the classic example of achievement in spectroscopy is the work which was carried out in the 'twenties by Pohl, which permitted the study of the very important process of the formation of vitamin D from ergosterol by the action of ultraviolet light. In the visible region, the study of absorption spectra has provided an especially large amount of valuable data in the study of the very important pigments, hemoglobin and chlorophyll. Numerous applications of spectrophotometry in the visible region and descriptions of the corresponding methods may be found in a series of books and review articles specially devoted to this question; this relieves me of the necessity of going further into detail about this subject.

At the same time, however, I must point out that in recent years the youngest branch of spectroscopy, infrared spectroscopy, has acquired the

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greatest significance in biological and biochemical research. The intensive development of this branch has occurred basically during the years following World War II. It is related both to spectroscopic analysis (the identification of various substances by the "fingerprints" which are contained in their infrared absorption spectra), and in an even greater degree to the analysis of spectra, that is, to the methods of establishing an empirical or theoretical relation between certain features of the spectra and the structures of the corresponding molecules.

The reasons why infrared spectroscopy in particular has increasingly become the general line of development of spectroscopic methods in biology and biochemistry are based on certain specific characteristics of biological materials, to which I would like to call your attention at this point.

These substances may be classified into a series of fundamental classes according to their functional significance and their biological activity. Such groups are, for example, the proteins, which include many hormones and all of the enzymes which catalyze and direct the course of biochemical reactions; the nucleic acids, which play a decisive role in the processes of auto-reproduction and biosynthesis, and which carry within themselves the basic store of genetic information; the steroid hormones, which control the course of very important life processes; the fatlike substances—the lipids, etc. All of these groups are quite sharply distinguished by their chemical compositions and structures; this is reflected in the structure of their electronic levels and in their absorption spectra in the ultraviolet and sometimes in the visible region. Thus, for example, proteins show a characteristic absorption maximum at about 280 mμ, while the absorption maximum for nucleic acids is at about 260 mμ. This fact is often used for the detection of proteins or nucleic acids in various objects.

However, each group contains a multitude of individual substances which are sharply distinguished by their biological activities and significance, whereas they are relatively slightly distinguished by their compositions and structures. Thus, there have been counted among the proteins at present up to 100,000 individual substances, which possess the most varied functions in the organism. For example, collagen, which enters into the composition of ligaments, bones, and skin, serves as a basis for the skeleton by means of its mechanical properties. The function of hemoglobin, which is contained within red blood cells, is that it combines with oxygen in the lungs and liberates it in the tissues. The muscle protein myosin plays a fundamental role in the process of transformation of the

potential energy of the chemical bonds of certain compounds into the mechanical work performed by the muscle. Such enzymes as pepsin, trypsin, and many others catalyze and direct the process of digestion, while cytochrome c and a series of other oxidation-reduction enzymes regulate the oxidation of nutrients in the cells, etc. This list could be continued almost to infinity. How may these proteins, which differ so much in their biological functions, be distinguished from each other chemically?[5]

In a very schematic first approximation, a protein can be represented as a long polypeptide chain containing various amino-acid residues as side chains bearing various chemical functions. The basic unit of this chain, repeated in all its links, is the amide group C-CO-NH-C, indicated in Fig. 1
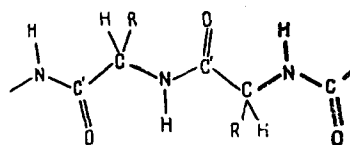

FIG. 1. Diagram of the structure of a polypeptide chain. The amide group C-CO-NH-C is indicated at the right of the figure by heavy lines. The letter R denotes atoms or groups of atoms in the side chains.

by heavy lines. This group is coplanar to an accuracy of several hundredths of an Angstrom unit, that is, all of its atoms lie in the same plane. The carbon atoms C' belong to each separate group, while the atoms C belong to both adjacent groups, and form links between them. This angular "hinge" carbon atom is connected with the corresponding atoms of the adjacent amide group by single bonds, so that, generally speaking, these groups may be rotated with respect to each other about these bonds by various angles. However, the distribution of these groups which is energetically most favorable and hence realizable in nature is that which makes it possible to form a hydrogen bond between the nitrogen atom in one group and the oxygen atom in another group, the distance between these atoms being 2.79 A. A consequence of this is the fact that in most cases protein molecules have the helical configuration shown in Fig. 2. The angular carbon atoms which form the links between the amide groups are connected to the side-chains of the amino-acid residues, which extend into the third dimension, that is, project out of the plane of the amide groups.

As distinct from many other organic polymers, for example, cellulose or rubber, whose molecules consist of a repetition of one and the same atomic grouping, the different amino-acid residues in proteins may occur in highly varied sequences. One of
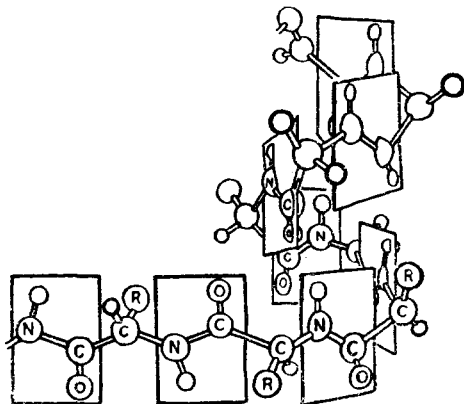
FIG. 2. Structural configuration of the polypeptide chain, the so-called α-helix. The rectangles indicate the individual planar amide groups. R indicates the side chains, which extend out of the plane of the amide groups into the third dimension. For clarity, in the first two amide groups in the lower left portion of the drawing, the rotation angle of one of these groups with respect to the other in the third dimension is not taken into account.

the most striking facts established by biochemists is that, of the great number of existing amino acids, one and the same set of 20 amino acids enters into the composition of the proteins of all organisms known to us. Several additional amino acids have been found only in the proteins of certain simple organisms. Apparently, during the course of evolution, an extraordinary process took place of standardization and selection of the minimum set of amino acids necessary and sufficient for construction of all of the functionally necessary proteins.

It is not possible to establish a simple connection between the amino-acid composition of some given protein and its biological function. On the one hand, cases are known in which proteins having identical biological activities, for example the hormone insulin, obtained from different animals (cow, sheep, or pig) are distinctly different in their amino-acid composition. On the other hand, it would seem that often very small changes in the amino-acid composition lead to radical, and sometimes catastrophic, changes in the functions of proteins. Apparently, the chemical functions of each residue contained within a protein molecule are different from its functions in the pure state, and are largely determined by its environment, i.e., by the properties of the molecule as a whole. Decisive significance is not possessed so much by the amino-acid composition by itself, as by the sequence distribution of the individual residues along the whole chain, or within its separate biologically important regions.

A very impressive example of such a profound

influence of small chemical changes is the solution of the riddle of the so-called "sickle-cell" disease, which has a great number of victims every year among the population of Central and South Africa. The name of the disease is associated with the fact that when it is present, peculiar changes are observed in the shapes of the erythrocytes (the red blood cells), which acquire the form of a sickle instead of the normal ellipsoidal form.

Like a number of other diseases (such as, for example, hemophilia or Mediterranean anemia) sickle-cell disease is hereditary; that is, it is genetically caused by definite changes in the chromosomes. One-eighth of the children born are doomed to death of sickle-cell disease at an early age.

Recently, Ingram[1] carried out a study of the amino-acid composition of the proteins of the erythrocytes of healthy persons and persons having sickle-cell disease. It was found that, out of about 300 amino acids, only one was different in the sufferers from the disease from that found in normal persons. This small change is sufficient to cause the catastrophic consequences indicated.

Another very brilliant example of the unusual biological sensitivity to small chemical changes is given by the so-called steroid hormones, which are secreted by various endocrine glands, and are one of the very important factors regulating the course of fundamental physiological and psychic processes.

The general chemical structure of these important compounds is characterized by the presence of four condensed rings (Fig. 3). To this basic struc-
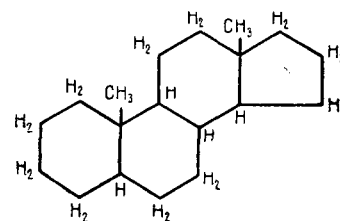

FIG. 3. General structural formula for the steroid hormones.

ture are connected other functional groups, and relatively small changes in these groups appear to be sufficient to change the function of the hormone radically. In Fig. 4 are shown the structures of four steroid hormones. The hormone estradiol (Fig. 4a) is produced by the ovaries of women, and is basically responsible for the physical and psychic aspects of the female sex, while the hormone testosterone (Fig. 4b) plays the same role with respect to the male sex. From the figure it is obvious that these great functional differences between the two cited hormones are associated with very small regroupings of the chemical bonds within them.
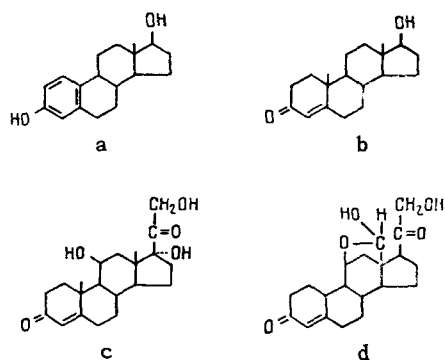
FIG. 4. Structural formulas of certain steroid hormones: a — estradiol, b — testosterone, c — hydrocortisone, d — aldosterone.

It is quite understandable that these small changes are insufficient in most cases to bring about changes in the electronic levels of the molecules which could be detected in their absorption spectra. On the contrary, in the infrared spectrum, which is a reflection of the frequencies of vibration and rotation of the individual groups of atoms in the molecule, these changes are often made manifest with complete distinctness. As an illustration, in Figs. 5 and 6 are given the infrared absorption spectra of two steroid hormones, androsterone and etiocholanolone, a hormone which is closely connected with the development of cancer, and besides,
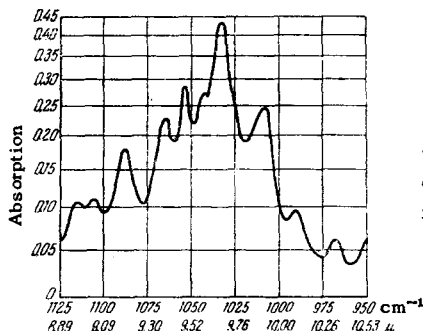

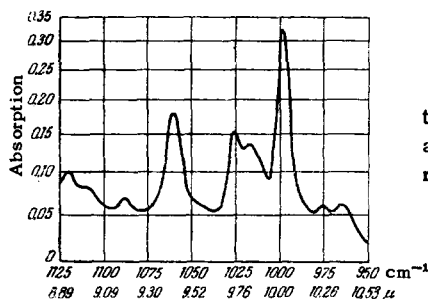FIG. 5. Absorption spectrum of pure etiocholanolone in the range 8.89 – 10.53 μ.

FIG. 6. Absorption spectrum of pure androsterone in the range 8.89 – 10.53 μ.

was discovered and identified by its infrared absorption spectrum. Although these spectra were taken over a relatively narrow spectral range, from 8.89 to 10.53μ, nevertheless, one can easily see from the figures how rich they are in detail,

and how characteristic they are of the substances being studied, in spite of chemical similarity.

Patterns which are as rich in details are also given by the infrared absorption spectra of many other biological substances. As an example, in Fig. 7 is given the spectrum of adenosine in the region from 800 to 1400 cm^{-1}, as obtained by Morales and Cecchini.[2] An analogous pattern is
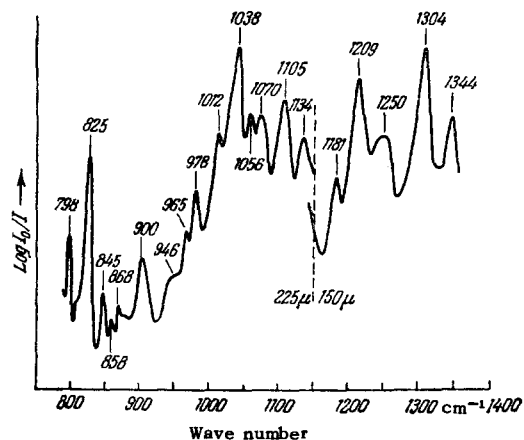

FIG. 7. Absorption spectrum of adenosine in the range 800 – 1400 cm^{-1} = 12.5 – 7 μ.

given by actomyosin, the fundamental protein component of muscle, which plays a basic role in the process of contraction. According to the data of Lecomte and his associates,[3] it has been possible to detect in the spectrum of frog muscle definite changes which depend on whether the muscle has previously been subjected to isometric contraction.

Not only the spectra of the separate substances extracted from the biological substrate, but sometimes also the spectra of the substrate itself show a highly characteristic structure, and may be deciphered. As an example, in Fig. 8 is shown the spectrum of a section of human adrenal glands (curve 1), and of various extracts from it, in the region from 2000 to 650 cm^{-1}. The various groups whose presence in this tissue may be established by comparison of these spectra are indicated below. Here we undoubtedly have an example of a highly successful spectral analysis of such a complex object for many components. In a number of cases, it has been possible to identify by infrared absorption spectra not only the genera and species of bacteria, but even the separate varieties within a species.

If we now go to the ultraviolet absorption spectra of biological substances, we find a picture which is barren by comparison. The strongest and most distinct absorption bands in the ultraviolet are those of the aromatic amino acids, tryptophan, tyrosine, and phenylalanine; and also those of the
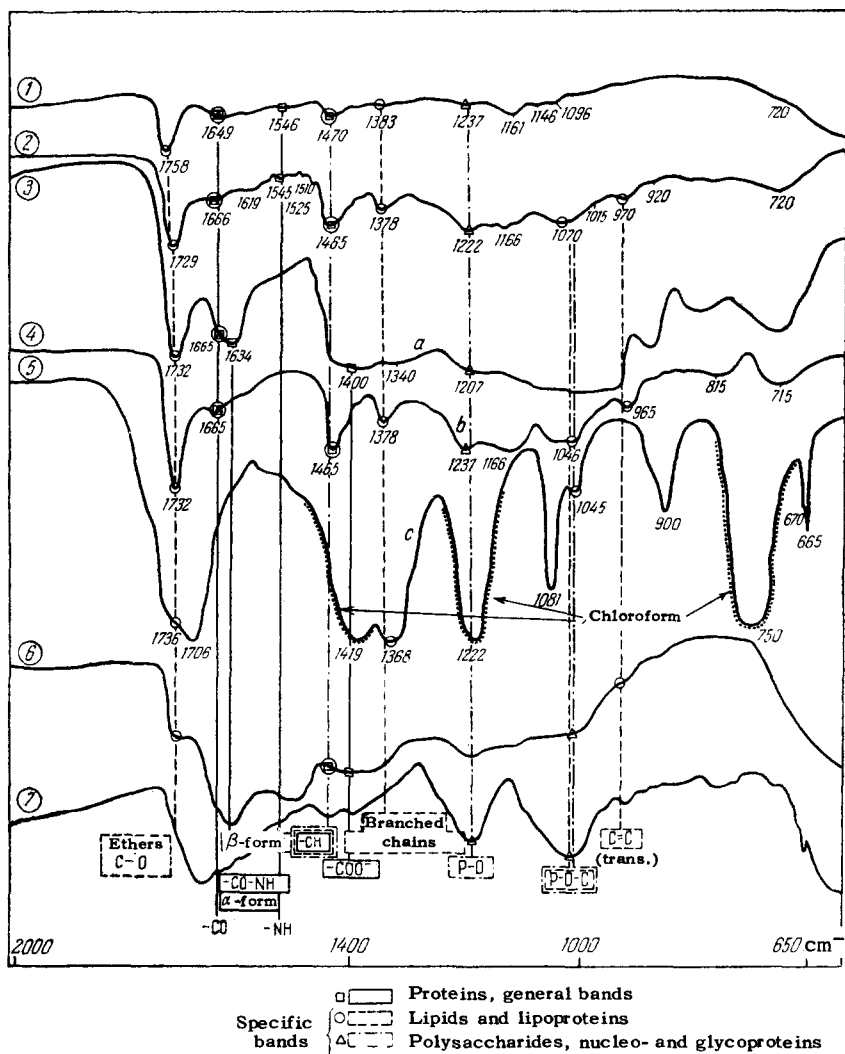
FIG. 8. Infrared absorption spectra of adrenal gland tissue and various extracts from it: 1 — lyophilized tissue, 2 — total lipid extract, 3 — cytoplasmic fraction obtained by ultracentrifugation in sucrose, 4 — the total lipid extract of the same, 5 — the cholesterin glyceride fraction of the same, 6 — total brei, 7 — nucleic acid. Taken from Wegmann.[4]

□▭	Proteins, general bands
○▭	Lipids and lipoproteins
△▭	Polysaccharides, nucleo- and glycoproteins

Specific bands

nucleic acids. The spectra of these substances, as given by Thorell[5] are shown in Fig. 9. One may easily see how devoid these spectra are of structural details which would permit the deciphering of their structure or the identification of these compounds in the composition of a material in which they occur. It is understandable that, in a biological substrate where numerous substances exist together, the signals given by the separate components, or by separate atomic groups in complex molecules, are lost in the general "noise", and the patterns obtained seldom make it possible to draw any conclusions, except the most general.

For example, the ultraviolet absorption spectra of some biological substances, taken from the paper of Sandritter,[6] are given in Fig. 10. What may be stated from these curves ? The maximum near 280 mμ on curves 1 and 2 indicates that proteins are the chief component in the corresponding objects, whereas the maximum at about 260 mμ on curve 3 indicates the presence of a large amount of nucleic acids; the shoulder on this curve at about

FIG. 9. Absorption spectra of certain biologically important substances in the ultraviolet: 1 — nucleic acid, 2 — tryptophan, 3 — tyrosine, 4 — phenylalanine (ratio = 0.4 : 1 : 1 : 1), 5 — other amino acids, 6 — Rayleigh scattering, 7 — reflection and refraction losses.
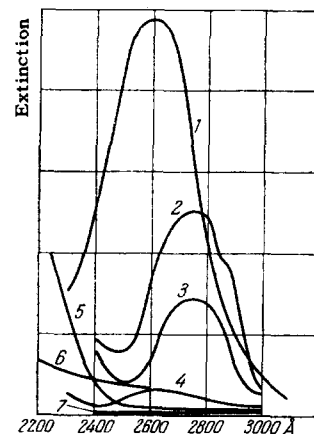
280 mμ shows the presence of tyrosine and tryptophan, whereas the secondary maximum on curve 1 at about 330 mμ is evidence of the presence of thyroglobulin, a protein containing tyrosine. One may say of the nucleolus (curve 4) that it contains both a protein component and nucleic acids. The information, as we see, is very general and clearly insufficient.
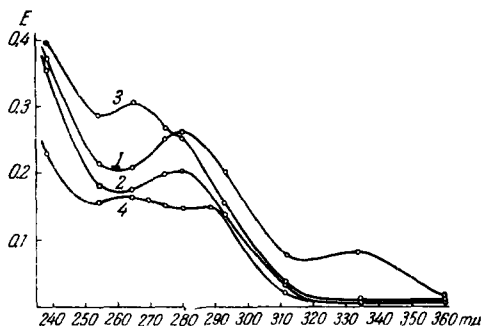
FIG. 10. Absorption spectra in the ultraviolet: 1 — colloid from the thyroid gland, 2 — cytoplasm of the parafollicular cells, 3 — cytoplasm of the follicular cells, 4 — nucleolus.

FIG. 11. Three structures for penicillin which were considered possible from the data of chemical studies.

1 — Oxazolone structure

2 — Tricyclic structure

3 — β-lactam structure

Speaking in the language of radiotechnology which is prevalent nowadays, we may say that for biological objects the "signal-to-noise ratio" is significantly more favorable in the infrared region than in the ultraviolet. This is the basic reason for the dominant significance of infrared spectroscopy in biological and biochemical research.

3. The wealth of information in the infrared spectrum and the presence in it of highly characteristic "fingerprints" of a large number of biologically important compounds have a great significance above all for the spectroscopic analysis of these compounds, i. e., for their detection and identification. However, these factors are no less important in the analysis of the spectra i.e., in the deciphering of the complex structures of these compounds by means of their spectra. The possibilities of application of infrared spectroscopy in the solution of these difficult problems were first indicated in the determination of the structure of.penicillin, the isolation of which opened a new era in medicine—the era of antibiotics. This problem was so important that, during the war years, a very broad joint effort of large groups of research workers was organized in England and the United States. The project was set up approximately in the same way as in the development of the atomic projects. The classical methods of chemistry led to the conclusion that penicillin may have one of the three structures shown in Fig. 11, but did not make it possible to make a final selection among these structures. It was considered that structure (1) was the most probable, while structure (2) was the least probable. Infrared spectra unequivocally indicated structure (3), the anomalous position of the absorption band of one of the carbonyl groups at about 5.62μ being of decisive significance. The same position of this band was then found in specially synthesized model compounds having the β-lactam structure. Finally, x-ray structure analysis confirmed the predictions of infrared spectroscopy.
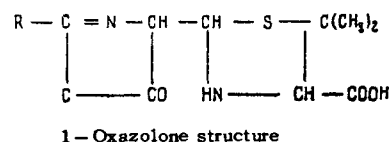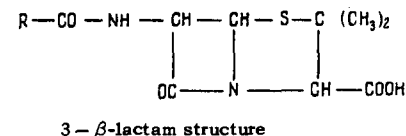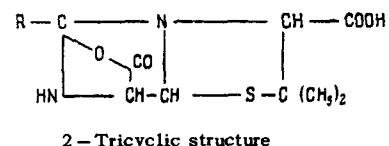
This outstanding achievement, along with the advances made in many branches of chemical technology, in particular in the analysis and deciphering of the structure of petroleum and petroleum products, attracted the attention of a large number of research workers, and served as the point of breakthrough in the development of infrared spectroscopy. Of course, the decisive role in this development was played by the intensive development in recent years of the design of optical apparatus. In particular, this has involved the designing of a series of highly automatic spectrophotometers for all regions of the spectrum. Thus the problem of obtaining and studying spectra was made accessible to a very large circle of workers in industrial, chemical, and medical institutions, who did not possess any special qualifications in physics. At the beginning of this century, there was no commercial production of apparatus for study of infrared spectra, and research in this region of the spectrum was possible only for a very limited number of physics laboratories and research workers who were in a position to build the necessary apparatus themselves. At present it is even somewhat strange to read that in 1909, when Twyman tried to get the Hilger firm to start commercial production of infrared spectroscopic apparatus, a number of specialists talked him out of it, indicating a lack of demand for such apparatus. During the period between the two world wars, a number of companies began to produce some kinds of infrared spectroscopic apparatus, but this apparatus, as before, required a high degree of specialization in the research workers. It resembled the modern automatic apparatus roughly in the same way as the linen covered "Blériot" of the period of the first world war resembled a modern airplane.

After the second world war, all of the leading optical instrument firms began with unusual vigor to develop and produce new and even more highly per-
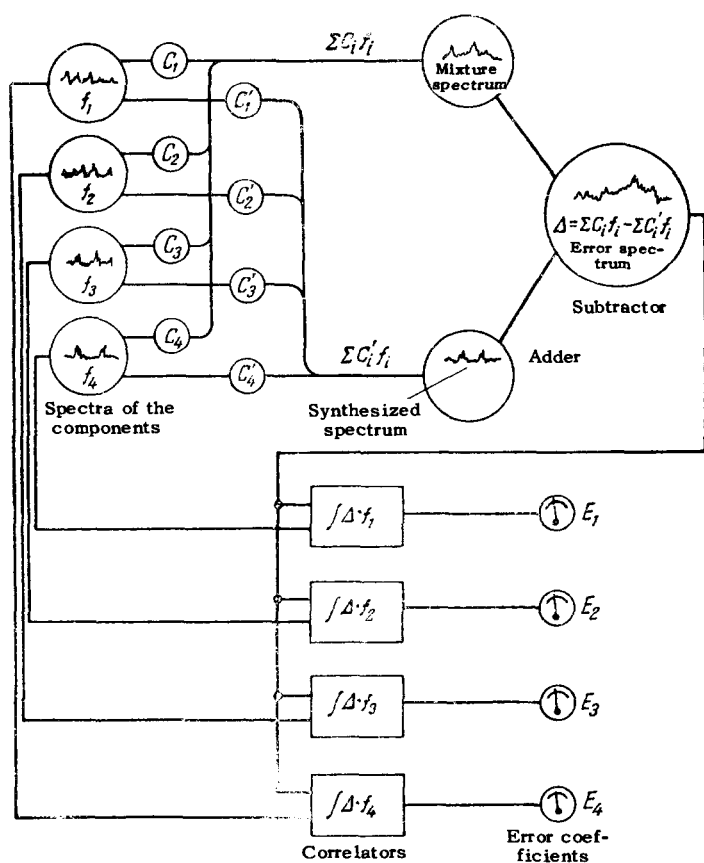
FIG. 12. Basic diagram of the computer of Rogoff for quantitative spectroscopic analysis.

fected models of spectroscopic apparatus, in particular for the infrared. This perfection in the technique of taking spectra spread the field of application of spectroscopy to an unusual degree. In turn, the increase in demand continued to stimulate research in the field of apparatus making. At present, this stage may be considered as being essentially completed. In all of the technically advanced countries of the world, including the Soviet Union, a large number of highly automatic spectroscopic instruments have been produced and have made work in the infrared region of the spectrum possible by any industrial or research laboratory. Substantial advances have also been made in the development of methods of sample preparation for the study of infrared absorption spectra. Of especial interest here is the method of incorporation of the substance being studied into a medium of powdered KBr or other crystals which are transparent in the infrared. This method was proposed in essence as early as 1928 by Maslakovets,[7] who obtained mixed crystals of the alkali halides (mainly KCl), in which the substance being studied was incorporated in low concentration. In recent years, this method has been developed and perfected abroad by a series of research workers,[8-13] who have widely implanted in infrared spectroscopic practice the method of pres-

sing the substance being studied in tablets of powdered KBr or other substances transparent in the infrared.

Of course, this does not mean that attention to the development of new models of spectroscopic instruments and to the perfection of methods of sample preparation may be lessened. On the contrary, the successful development of science, and above all, of organic chemistry, biology, and biochemistry, urgently requires the maximum intensificiation of this work, and the production of new, more perfected models of the instruments in sufficient quantity. Essentially, however, the principal difficulties have been overcome already, at least in the spectral region in use at present (i. e., approximately to 40μ). The technique of obtaining absorption spectra is no longer the bottleneck limiting the development of the science.

New difficulties now come to the forefront. On the one hand, they are associated with the fact that the unusually broad development of research in spectroscopy in general, and in the infrared in particular, has led to an accumulation of an almost immeasurable amount of data on the spectra of the most varied substances. For illustration it is sufficient to state that the latest great review of infrared spectroscopy, written by Lecomte and pub-

lished in Volume 26 of Handbuch der Physik, contains almost 700 pages in large format and in very fine type. One bibliography alone of papers on the application of infrared spectroscopy in biology and biochemistry, compiled by Clark and Chianta,[14] occupies approximately 50 pages. A serious problem arises of systematization and organization of the storage of this information so as to insure the possibility of effectively using it.

On the other hand, the complexity of infrared spectra and the wealth of information which they contain create quite significant difficulties. Although they open up great possibilities for the identification of biological compounds and for qualitative spectroscopic analysis, difficulties arise in the quantitative determination of the individual components of the mixture which have been detected in these spectra. With a manual technique of analyzing the spectra, such a quantitative analysis in very many cases appears practically unachievable. At the same time, it is the changes in the quantities of the separate components (often components occurring in very small amounts) which are of great biological interest. In particular, this is the case in the diagnosis of a number of diseases, for example cancer, or in the study of such irreversible processes as aging. Hence, the problem of building a special electronic computer for handling spectra and carrying out quantitative spectroscopic analysis has acquired the highest significance.

Insofar as I know, the first successful attempt to develop such a special electronic computer and to apply it for a concrete diagnostic purpose was made by Rogoff in the Federal Telecommunication Laboratories in the United States.[15] I shall take up this research in somewhat greater detail, since the problem of automation of quantitative spectroscopic analysis is of great significance not only for biological and biochemical research, but in general for all spectroscopic analysis.

Fig. 12 explains the fundamental idea proposed by Rogoff as the basis of the computer which he built. We shall assume that a qualitative analysis has created a basis for assuming the presence in the given material of a certain number of components, whose spectra f_1, f_2, f_3, ... are known. These spectra are introduced into the machine as given data. In the example in Fig. 12, there are four such components. If we assume that the spectra may be linearly superposed, i.e., that there is no interaction between the components, then we must select the values of the corresponding coefficients c_i so that the "synthetic spectrum" $\Sigma c_i f_i$ is practically indistinguishable from the spectrum of the material

being studied. These values of the coefficients are then the relative amounts of the components in the mixture.

Rogoff achieves the automation of the solution of this problem as follows. By assigning to the coefficients c_i arbitrary values c_i', we may compute on the machine the "synthetic spectrum" $\Sigma c_i' f_i$, and subtract it from the spectrum of the given material. The obtained difference spectrum is $\Delta = \Sigma c_i f_i - \Sigma c_i' f_i$. Rogoff calls this the "error spectrum." It is obvious that the best mathematical solution of the problem will be that which gives a minimum mean square deviation between the "synthetic" spectrum and the spectrum of the material being studied. In other words, we must vary the values of the coefficients c_i' until all of the correlation coefficients between each of the spectra of the components and the "error spectrum" vanish, i.e., until all of the integrals $E_i = \int \Delta f_i \, d\lambda$ vanish.

The values of these integrals are calculated by the computer itself; here, if a certain correlation coefficient is positive, this means that the assumed value of the corresponding coefficient c_i' is too high, and must be lowered. On the other hand, if a certain $E_i < 0$, then the corresponding coefficient c_i' must be increased. This permits the values of E_i and c_i' in the computer to be linked by a servomechanism which varies the values of the coefficients c_i' until all of the integrals E_i vanish.

Thus the computer gives the best mathematical approximation to the spectrum of the material being studied which may be obtained from the assumed starting spectra of the individual components. But is the "best" approximation obtained in this way correct? This depends on whether the selection of the assumed components is made correctly, i.e., on the correctness of the qualitative analysis. We note that the introduction of extra components which are actually absent in the given material has no effect, since the computer itself will give zero concentrations for these components. This permits one to be generous enough by introducing into the analysis all of the assumed or "suspected" components. But that is not all. The computer itself provides a control on the correctness of the qualitative analysis, and in some cases shows the way to correct it. In fact, if after we have obtained the best values of the coefficients c_i', the "error spectrum" vanishes for all frequencies, this is evidence of the correctness of the solution we have obtained, i.e., evidence that we have not omitted any of the actual components. If the residual error spectrum is significantly different from zero, this indicates an error in the choice of the starting spectra. Sometimes the pres-

ence in the residual error spectrum of certain characteristic absorption bands even suggests the way to improve this choice.

The first models of the Rogoff machine were analog computers, as is shown in Fig. 12. However, further work has shown that digital computers are more suitable. The spectra are introduced into the computer not in the form of the graphs with which we are familiar, but in the form of values of the absorption given in the binary system for various frequencies. All calculations are carried out in this system, the complete analysis being conducted in several minutes.

Besides the enormous saving in time and expenditure which permits one to apply the methods of quantitative spectroscopic analysis in many cases in which the application of these methods was practically impossible heretofore, the automation of these methods has two important advantages in principle. First, the analysis is carried out on the entire absorption spectrogram, rather than at separate selected wavelengths. The smoothing influence of the integral analysis significantly decreases errors which may occur in the analysis at separate points, and makes the results more accurate and reliable. In particular, it reduces the distortions associated with residual "noise" in the measuring instruments. Second, if there is a basis for the assumption that the spectroscopic analysis is being complicated in some region of the spectrum by interaction of the components and deviations from linearity in the superposition of the spectra, these regions may simply be omitted in the analysis. Infrared spectra are so rich in detail that the remaining information in most cases is quite sufficient for an accurate analysis.

The possibilities of the new method were convincingly demonstrated in a paper of Rogoff and Gallagher.[16] The problem was undertaken in this study of the accurate quantitative determination of certain steroid hormones in urine. This problem has an exceedingly important practical meaning, since the course of many diseases, in particular cancer, is associated with large disturbances in hormone activity in the organism, and is made manifest in large changes in the amounts of hormones produced by the organism. In cancer, even in the early stages before operation, an increase in the hormone content in the urine of severalfold is observed, as compared with the normal. After removal of the localized tumor, it falls to the normal value, but on resumption of the growth and spread of the neoplasm (the so-called "metastatic phase") the hormone content may exceed the normal by some hundredfold. At the same time, the quantity of these

substances is so small that their isolation in the pure state for quantitative analysis requires very complex and painstaking work, such that this analysis is almost inapplicable under clinical conditions.

In the work of Rogoff and Gallagher, the relative content of two steroid hormones, androsterone and etiocholanolone, in a certain fraction of the urine was studied. The spectra of these hormones are given in Figs. 5 and 6. All of the remaining components of the urine were considered as one general component, characterized in all of the analyses by one and the same general background spectrum (Fig. 13). This spectrum was obtained

FIG. 13 Difference spectrum of the background.
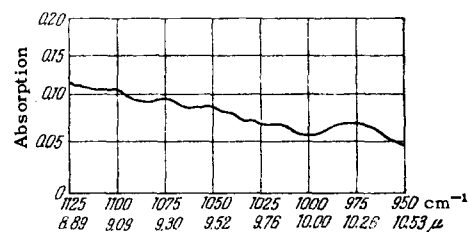
from one sample as the difference between the spectrum of the sample and the spectrum of a mixture of the two cited pure hormones. The relative concentrations of these hormones were taken such that there remained no noticeable traces of the steroid materials in the difference spectrogram. This method of combining a large number of components which are not of interest to us as a general background, and the presentation of those substances which are of interest against that background, is very original. It has, it seems to me, very great value as a method. It is possible that, in the given case, the constancy of this background for all of the samples studied may be explained by the general chemical method of selection of the fraction of the urine which was subjected to the spectroscopic analysis. Further, it is obvious that the development of this method is associated with the accumulation of a "library" of spectra of various compounds, and with the building of rapid computers which will be able to carry out the analysis with various "backgrounds."

The result of one of the analyses of Rogoff and Gallagher is shown in Fig. 14. The value indicated in the figure for the rms deviation of the "synthetic spectrum" from the original spectrum, 5.4% in the given case, was calculated by the computer itself. It seems to me, however, that a single glance at these curves is sufficient to convince any spectroscopist or analyst of the great possibilities of the computer technique of spectroscopic analysis.

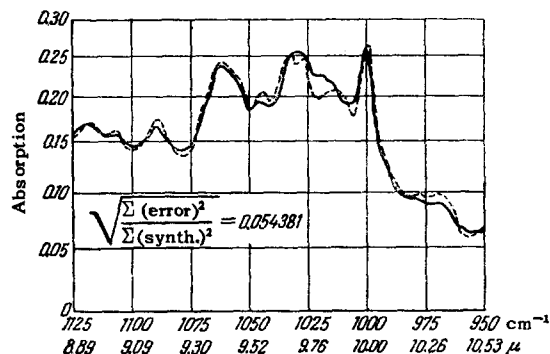In Figs. 15 and 16 are shown an external view of

FIG. 14. Solid curve: absorption spectrum of the urine sample being studied; dotted curve: synthetic spectrum calculated by the computer for values of the coefficients: $C_1 = 0.599719$ (androsterone); $C_2 = 0.412143$ (etiocholanolone); $C_3 = 0.555639$ (background).
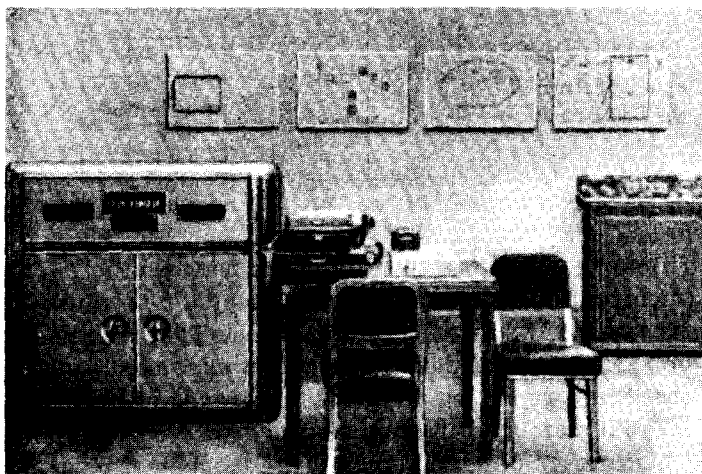

FIG. 15. External view of the analytical computer of Rogoff.

FIG. 16. General view of the infrared spectroscopy laboratory in the Sloan-Kettering Institute in New York. Rogoff machine at right.

Rogoff's computer and a general view of the infrared spectroscopy laboratory in the Sloan-Kettering Institute in New York where this machine was set up, and the studies described above were carried out. It cannot be doubted that the successful development of methods of spectroscopic analysis in the Soviet

Union, and their widespread implantation in the practice of research, medical, and industrial laboratories, urgently demand the most rapid development and production of similar machines by us. In connection with this, obviously, the necessity arises of developing and producing special accessories for infrared spectrophotometers which will give the results not in the form of the usual graphs, but rather in the form of a numerical record in the binary system. As was indicated by Rogoff, such a complete record of the spectrum from 650 to 5000 cm^{-1} may be kept on a piece of ordinary film 2.5-cm long, and may be read by the machine in a tenth of a second. With such a system of recording and keeping the information, many thousands of spectra may be kept in the computer for comparison and calculation.

4. In biological research it often becomes necessary to study the absorption in samples of very small dimensions, for instance in individual cells or in portions of them, in small regions in sections of inhomogeneous tissues, etc. The same type of measurement also often becomes necessary in biochemical research. Many biologically important substances are exceedingly active in infinitesimally small concentrations, and often the amount which the researcher has at his disposal is of the order of some micrograms. At the same time, if an ordinary spectrophotometer is used without special additional attachments, the dimensions of the sample must not be smaller than those of the entrance or exit slit of the spectroscopic instrument; that is, they must not be smaller than 1×10 mm. Even with a thickness of 25μ, the volume of such a sample may not be smaller than about 2.5×10^{-4} cm^3, which corresponds to a mass of $1/4$ mg for a substance of unit density. In practice, the minimum amount of material required in most cases for study with ordinary spectrophotometers is equal to 1 to 2 mg. Although the use of microcuvettes permits the lowering of this limit somewhat, this is still quite insufficient for many biological and biochemical studies. Hence, it became necessary here to create the so-called specialized microspectrophotometric methods of study, based on the combination of microscopes with spectrophotometers.

In principle, all these methods are based on the use of special micro-illuminators, which form a reduced image of the source on the specimen, and of objectives which restore the original directions of the rays, and form a magnified image of the specimen on the slit of the instrument or on a radiation detector. Such a system can be placed either ahead of the entrance slit of the spectroscopic instrument, or between the exit slit and the detector. In practice, the second position is more advantageous, since the

concentration of radiation from the light source, not yet resolved into a spectrum, on the specimen may result in undesirable heating. Besides, Cole and Jones[17] have shown that, if the specimen is pressed between two NaCl plates, the temperature of the specimen will not exceed 30 deg.

A very simple system of this sort, designed by Anderson and Woodall,[18] and produced as a standard attachment for the Baird spectrophotometer, consists of two silver chloride lenses. An image of the source is formed by the first lens on the specimen, with a linear reduction factor of 3; the second lens forms an image of the specimen on the slit of the spectroscopic instrument, with the same factor of magnification. The area of the specimen being studied may be reduced here approximately tenfold, that is, to the dimensions 0.33×3.3 mm. An essential defect of this system is the presence of large aberrations, especially chromatic. This is especially significant in infrared spectroscopy, where studies are commonly conducted over the very broad spectral range from 2.5 to 15μ, whereas the choice of materials for construction of an achromatic system is very limited. The large aperture required in these systems results in the appearance of aberrations other than the chromatic aberration.

Hence, the changeover to reflecting microscopic optics was of decisive significance to the development of microspectrophotometry. In the foreign literature, progress in this field is commonly associated with the paper of Burch,[19] published in 1947. However, we must note that even several years previous to this, reflecting microscope objectives were designed and built in this country in connection with the widely known studies of E. M. Brumberg in ultraviolet microscopy. Burch attained an improvement in the quality of reflecting objectives by using aspheric mirrors. However, apparently, this is not essential in practice for purposes of microspectroscopy, and completely satisfactory systems may be built also with spherical mirrors. The first microspectroscopic apparatus with reflecting optics of the Cassegrain type was built in 1949 by Barer, Cole, and Thompson.[20] In present day designs, systems of the Schwarzschild type are used more often. In Fig. 17, the path of the rays in this type of system is shown at the left, and at the right is shown an external view of the attachment for microspectrophotometry, produced as an accessory for the Perkin-Elmer infrared spectrophotometer. This apparatus is set up at the exit slit of the spectrophotometer. A reduced image of the exit slit S of the latter is formed by the condenser C on the object P. The magnified image formed by the objec-
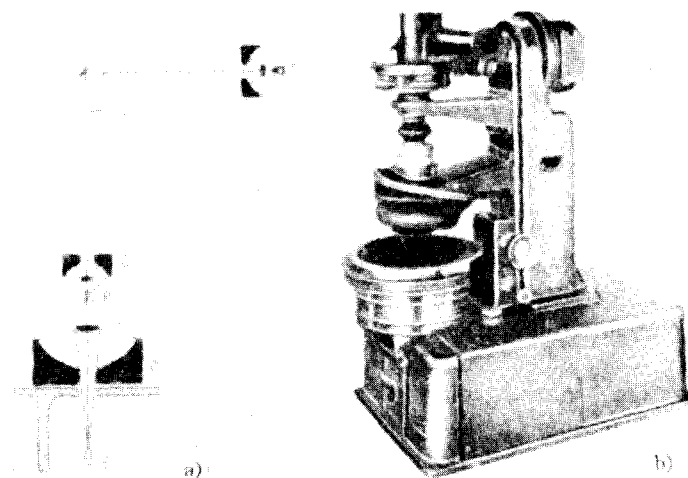

FIG. 17. Attachment for infrared microspectrophotometry by Perkin-Elmer: a — diagram, b — external view.[21]

tive O is located in the plane of the diaphragm B, which serves to exclude stray light. When necessary, templates having the shape of the specimen may be placed in this plane. After passing through the diaphragm, the rays are deflected by a plane mirror, and are focused by a reflecting condenser on the detector T (a thermocouple). The apparatus contains auxiliary optics and an auxiliary illuminator (at the exit slit of the monochromator) for adjustment of the position of the specimen. When the plane mirror is turned, the rays pass into a control eyepiece H, where the image of the specimen may be examined with a magnification of 175 ×.

The minimum dimensions of the specimen which can be studied by means of these systems are limited by two factors. The first is the finite resolving power of the microscope: obviously the specimen may not have dimensions smaller than the limit of resolution of the optics of the microscope. In the visible and ultraviolet regions of the spectrum, where microspectrophotometric methods have already been long in use, this "diffraction limit" is rather small and is approximately equal to 0.5μ; that is, it permits the study of individual cells and even of portions of them. The very strong absorption of certain biochemical substances in the ultraviolet has permitted the detection of these in cells in which they were present in amounts of 10^{-19} g.

In the infrared region the conditions are not so favorable. Here, because of the long wavelength, the "diffraction limit" restricts the minimum dimensions of the specimen to several microns. However, even this limit cannot be attained because of a restriction of an energetic nature. In order that the light bundle fill the entire collimator mirror, the ratio of the numerical aperture of the micro-illuminator to the numerical aperture of the monochromator must be equal to the magnification of the image

of the specimen at the slit of the apparatus. Since the numerical aperture of the monochromator is approximately equal to 0.1, while that of the micro-illuminator is near unity, the magnification must be approximately equal to 10. That is, the energetic requirement limits the dimensions of the specimen to values of the order of 0.1×1 mm.

The large gap mentioned between the "energetic" and "diffraction" limits on the dimensions of the specimen may be reduced somewhat by masking part of the area of the specimen, at the cost of reduction in the resolving power of the spectroscopic apparatus, and also by using more intense sources of infrared rays and more sensitive detectors. The details of calculations made in this regard may be found in the review by Wood[22] and in the article by Coates, Offner, and Siegler.[23]

In fact, in the most favorable case, in which the object was a long, thin fiber, it was possible to obtain a rather well-resolved absorption spectrum over the entire range from 2.5 to 15μ from a specimen of $17\text{-}\mu$ diameter and $650\text{-}\mu$ length. This makes it possible to record the spectrum of a material which is present in an amount of 10^{-7} g. For specimens of round cross section, there are data of a successful study of infrared spectra with a specimen diameter of 30μ.

In view of the indicated limitations and the weakness of absorption of most cell components in the infrared region, the applications of infrared microspectrophotometry are not so numerous as the applications in the ultraviolet. Nevertheless, we already have a number of interesting and important results. Thus, for example, on studying the infrared spectra of individual muscle cells, Wood[24] showed that they do not possess dichroism. This shows, on the one hand, that the contractile mechanism of muscle is built on a level larger than that of elementary polypeptide chains. On the other hand, it shows that the process of muscle contraction is more complex than a simple "folding" of polypeptide chains.

The application of methods of infrared microspectrophotometry has even greater possibilities in biochemical research, since these methods permit the study of the spectra of substances which are present in minimal quantities. Thus, for example, spectra have been obtained and identification performed of propionylcholine, a substance which plays an important role in the process of nerve conduction. This was done under conditions in which the entire amount of this substance which the experimenter had was $300 \mu g$[25,26]

Undoubtedly, the development of these methods, the production of the proper apparatus and the development of research in this field is an important problem for coordinated efforts by Soviet physicists, instrument makers, and biologists.

5. Passing over from the question of the composition and structure of the basic biological substrates to the question of the application of spectroscopic methods for study of the mechanism of fundamental biological processes, we must note above all that the topic here will be that of processes which are photobiological in the broad sense of the word. That is, the topic will be that of processes in which light is the agent eliciting, stimulating, or directing the course of biochemical reactions. These include such important processes as photosynthesis and vision, as well as the phenomena of phototaxis, phototropism, and photoperiodism, the various actions of light on enzymes and bacteria, photodynamic action, (i.e., the oxidation of biological systems by molecular oxygen, sensitized by fluorescent substances), and a number of other important processes.

The fact that these varied "photochemical" (in the broad sense of the word) biological actions of visible and ultraviolet light are possible is, above all, the result of the circumstance that the energy of the photons of these types of radiation corresponds to the energy of the typical bonds in the organic compounds which form the material substrate of life. The values of the energies of the most important valence bonds in organic compounds, such as the $C-N$, $C = O$, $C-H$ bonds, etc., lie between 2 and 6 ev. The dissociation of most covalent bonds requires energies of about 2.5 ev, while weaker bonds, such as hydrogen bonds, are characterized by still smaller energy values. If we recall that radiation with a wavelength 200 $m\mu$ corresponds to a photon energy of 6.2 ev, then it is clear that the absorption of ultraviolet or visible light may, without ionizing the atoms and molecules, bring about the breaking and rearrangement of the bonds within them. That is, absorption of these radiations may, in the broad sense of the word, initiate various photochemical reactions, leading to varied and far-reaching biological consequences.

Along with these photochemical transformations, the great significance of excited electronic states of molecules in the most varied biological processes has become increasingly clear in recent years. This question has been elucidated in detail in the books of Szent-Györgyi[27] and Reid,[28] which are to appear soon in Russian translation. I shall not go into them in more detail, although I must speak of some features of the conceptions of Szent-Györgyi in connection with the question of the role played in biological processes by the ability of molecules to luminesce.

Of the large number of varied and forward-looking applications of spectroscopic methods in re-

search devoted to the problems which I have indicated, I shall spend time only on those which have some specific character or which require the development of special methods and apparatus.

First of all, I must refer to the spectroscopic methods of study of the short-lived intermediate products which appear in photochemical reactions, and then either enter into a further chain of dark reactions or undergo the reverse transformation into the original reactants. The lifetime of these intermediate products may vary from several minutes to small fractions of a second. If they have the chemical nature of free radicals, then in many cases they may be successfully studied by the method of paramagnetic electronic resonance, to which a number of papers in this conference have been devoted. However, this is not necessary. In photosynthesis, for example, the intermediate products may be either the reduced form of chlorophyll, or its ionized forms, or triplet states, as well as other intermediate products which may be the precursors of the molecular oxygen which is produced by the plant. In all of these cases, the basic method of study of the intermediate products is the study of the changes on the absorption spectrum which appear when the material being studied is illuminated.

The "difference spectrum", which is obtained by comparison of the spectra of the illuminated with the nonilluminated specimen, may be considered as the spectroscopic characteristic of the intermediate product, and may be used for its identification. The study of the kinetics of the changes in absorption which appear on turning the light on or off enables us to follow the kinetics of the direct and reverse reactions of formation and decomposition of these products.

The method described here of obtaining a "difference spectrum" from the absorption of the illuminated and the nonilluminated material has been most widely and effectively applied in the studies of the photochemical reactions of chlorophyll, both in vitro and in its natural state in the photosynthetic apparatus of plants.

Apparently, Krasnovskii[29] was the first to apply the method of difference spectra in the study of the photochemical reactions of chlorophyll and its analogs in 1948. He showed that when solutions of chlorophyll or its analogs or derivatives in pyridine are subjected to brief illumination with red light in the presence of ascorbic acid or other hydrogen donors, characteristic changes appear in the absorption spectrum. These may be explained by the formation of photoproducts which have absorption maxima at shorter wavelengths. When the light is turned off, the reaction quickly proceeds in the re-
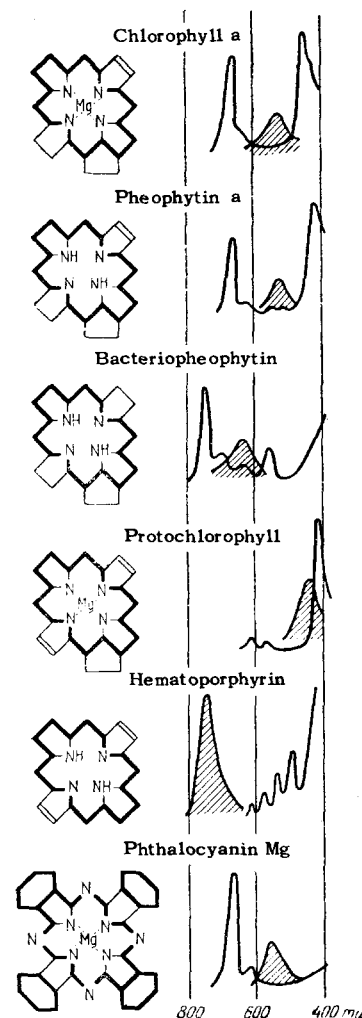

FIG. 18. Schematic diagram of the structure of the absorption spectra of chlorophyll and its derivatives and analogs, and the absorption spectra of the photoreduced forms of these pigments.

verse direction, and the original absorption spectrum reappears. These products are the photoreduced form of chlorophyll, which, according to the conceptions of Krasnovskii, plays a decisive role in the process of photosynthesis in vivo. According to his conceptions, it is the initial link in the complex chain of oxidation-reduction reactions whose final results are the assimilation of CO_2 and the liberation of oxygen. The difference spectra, or spectra of the reduced forms of chlorophyll and its analogs and derivatives, which were obtained by Krasnovskii and his associates are given in Fig. 18.

In 1952 Duysens[31] discovered analogous absorption changes which appeared upon illumination of a living object, the purple photosynthetic bacteria Rhodospirillum rubrum and Chromatium. Somewhat later, he and a number of other research workers studied the same sort of "difference spectra" in the one-celled green alga Chlorella (Fig. 20). The changes in the absorption spectra of the green leaves of higher plants upon illumination have been studied in the Soviet Union by Bell.[33]

The interpretation of the spectra of the inter-

mediate products which have been obtained is very difficult, and in many respects is still controversial, since the observed absorption changes may be due not only to the reversible formation of the reduced form of chlorophyll, as was assumed by Krasnovskii, but also to the transition of the excited chlorophyll molecules into the long-lived triplet state. This state, in the opinion of many investigators, in particular J. Franck,[34] plays a decisive role in the transformation of the energy of the light absorbed by the plant into the form of the potential chemical energy of various compounds. The absorption changes may also be due to the oxidation of certain substances, either to form radicals or saturated compounds. Thus, for example, Duysens[35] ascribes a series of bands in the difference spectrum of Chlorella (Fig. 19) to the oxidation of cytochromes, while others are ascribed to the transition of a
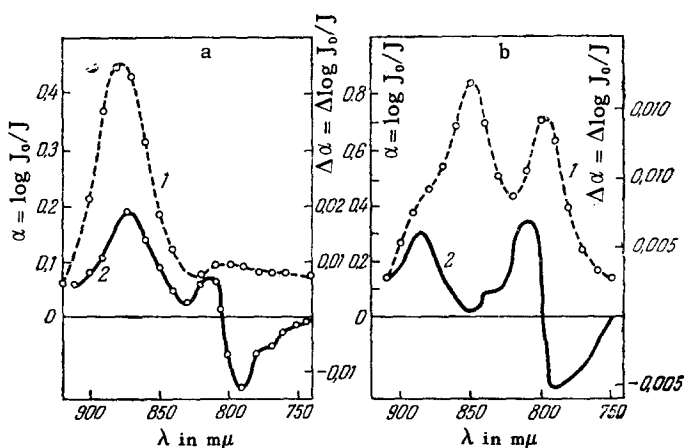

FIG. 19. Absorption spectra of purple bacteria and their changes: a — Rhodospirillum rubrum, b — Chromatium. Absorption spectra in the dark — dotted curve 1, with scale on axis of ordinates at left. Changes in the absorption spectrum upon illumination — solid curves 2, with magnified scale shown on axis of ordinates at right. Taken from Duysens.[31]

certain pigment (not yet identified) from the "dark" modification with an absorption maximum at about 478 mμ to the "light" modification with an absorption maximum at 515 mμ.

This entire complex of problems is exceedingly important in the explanation of the mechanism of the initial photochemical reaction in the process of photosynthesis. It is not surprising that in recent years it has been intensively studied by numerous groups of research workers (Livingston, Duysens, Witt, Strehler, Lynch, Coleman, Holt, Rabinowitch, Linschitz, etc.). A series of articles by these authors, giving a complete view of the present state of the problem, may be found in the book.[36]

The changes in the optical density of living ob-

jects or solutions which are associated with the formation of intermediate products are generally very small; they are measured in thousandths, as may be seen from Figs. 19 and 20. Hence, double-beam spectrophotometers and special instruments are generally used in the study of these spectra; these instruments make it possible to eliminate the influences of fluorescence and of scattering of the light being used to bring about the photochemical reaction.
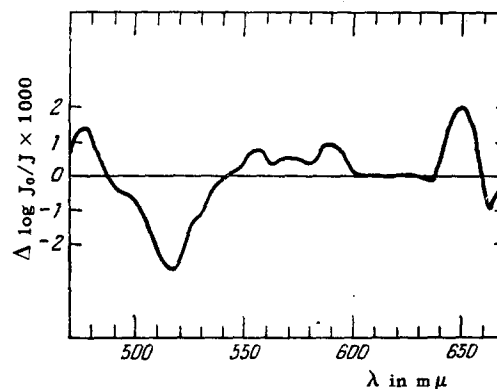

FIG. 20. Difference spectrum for absorption by illuminated and nonilluminated alga Chlorella. Taken from Strehler and Lang.[32]

The various experimental apparatus proposed by different authors for study of the spectra of intermediate products may be divided basically into two classes: 1) circulating systems, in which a suspension of the material being studied or a solution is illuminated for a certain period of time (from 5 to 60 sec) outside the spectrophotometer, and is then quickly transferred to a cuvette placed in the beam for measurement of absorption; and 2) systems in which the illumination is carried out continuously, while the measuring beam passes through the cuvette in a direction perpendicular to the beam used to bring about the reaction. A diagram of a typical apparatus of this type, as designed by Duysens, is shown in Fig. 21. Since the exciting light is significantly stronger than the test beam, in order to eliminate the scattered light and fluorescence, one must have recourse to crossed filters (f_2 and f_3 in Fig. 21), and modulate the measuring light beam, together with using resonance amplification of the photocurrent and detection with phase detectors; sometimes the spectrophotometer is located at a great distance from the cuvette.

Flash lamps have been widely applied in these studies in recent years; these give an exceedingly powerful brief illumination of the object, after which a time scan of the photomultiplier current is made on an oscillograph. The diagram of such an
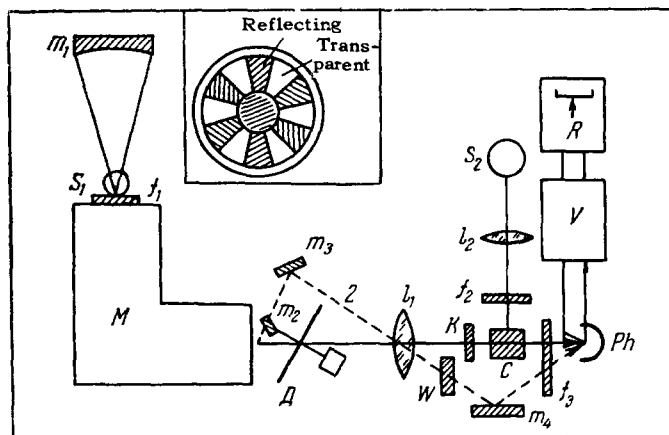
FIG. 21. Diagram of the apparatus of Duysens[37] for study of difference spectra with continuous illumination. S_1 and S_2 — light sources, m_1 — curved mirror concentrating the light from lamp S_1 on the entrance slit of the monochromator M. The disk D with alternating transparent and reflecting sectors (front view shown in the upper diagram) is attached to the shaft of a synchronous motor, and directs the measuring beam from the monochromator alternately along the direct path through the cuvette C containing the illuminated suspension of the alga, or around the cuvette by path 2 by means of mirrors m_2, m_3, and m_4; Ph — photomultiplier; V — photocurrent amplifier and phase-sensitive detector, R — measuring instrument, W — wedge for equalization of the intensities of the scanning beams, K — calibrated wire screen l_1, l_2 — lenses, f_1, f_2, f_3 — light filters.
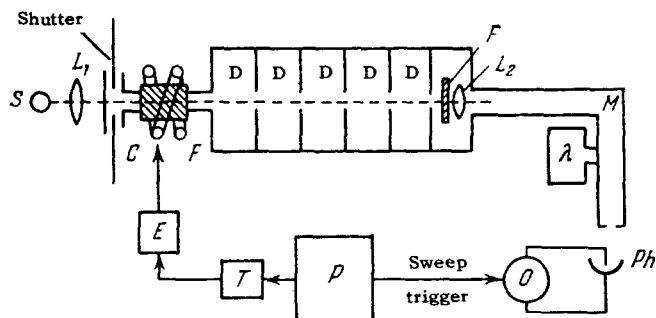

FIG. 22. Diagram of the apparatus of Linschitz and Abrahamson[38] for study of the changes in absorption upon momentary illumination by a flash lamp. S — light source for the measuring beam, L_1 — collimating lens, L_2 — lens focusing the light on the slit of the monochromator M, C — cuvette with specimen, F — flash lamp having the form of two turns of a helix surrounding the specimen, D — diaphragms for elimination of scattered light (distance between the specimen and the monochromator is 120 cm), F — light filter, Ph — photoelectronic multiplier, O — oscillograph, P — pulse generator, T — trigger circuit for the flash and delay line, E — energy source. The lamp operates at a voltage of 4 kv and capacitance of 24 mf, and gives a flash of duration 100 μ sec.

apparatus is shown in Fig. 22. Apparatus of this type makes it possible to follow the kinetics of the formation of intermediate products, for time periods of from 10^{-5} sec to several seconds.

On the whole, it cannot be doubted that the spectroscopic method which I have described for study of intermediate products of chemical reactions has very great possibilities and may give highly valuable results in studies not only of photosynthesis and other photobiochemical processes, but also in many "dark" chemical and biochemical processes. Here also, a broad field opens for fruitful teamwork of physicists, chemists, and biologists.

6. Another very important direction of development of spectroscopic methods of research on biological processes is the study of the so-called action spectra and the comparison of these with absorption spectra. By the action spectrum of radiation we mean the graph or function giving the relation of the effectiveness of the radiation for the process being studied to the frequency or wavelength of the radiation. In this definition we must emphasize first of all the words "for the process being studied." The actions of light are so varied and so specific that, obviously, there is no sense in comparing the effectiveness of the action of radiation for such processes as, say, photosynthesis and vision, or bactericidal action and phototropism, etc.

However, even with this reservation, the concept of "biological effectiveness" of a certain radiation requires a more precise definition. Phenomenologically, we always study the rate of change of some quantity M which quantitatively characterizes the given biological effect. For example, M may denote the amount of CO_2 assimilated in the process of photosynthesis or the amount of oxygen liberated, or the amount of pigment produced in suntan, the number of bacteria in a population subjected to radiation, or a value characterizing the inactivation of some enzyme, etc. Generally speaking, the rate of change of this quantity dM/dt varies in time and depends not only on the intensity and frequency of the radiation, but also on a whole series of other factors: physiological conditions in which the object occurs, composition and pH of the surrounding medium, temperature, length of illumination and of rest periods, etc. If, other factors being equal, we establish the fact that the same value of dM/dt is attained at an intensity I_1 of radiation of wavelength λ_1 and at an intensity I_2 of radiation of wavelength λ_2, we may then define the relative biological effectiveness of these two radiations. This is defined as the inverse ratio of the intensities:

$$\frac{\text{Relative effectiveness of } \lambda_1}{\text{Relative effectiveness of } \lambda_2} = \frac{I_2}{I_1}.$$

It is important to note that this definition has a real meaning only in the case that this ratio has one and the same value for any moment of time, either during illumination of the object or after cessation of the light action. This must be tested in each individual case, and is not always true.

The basis of every biological action of radiation is some "photochemical" reaction, in the broad sense of the word, i.e., the appearance of certain changes in the molecule which has absorbed a quantum of radiation. However, the connection between this initial reaction and the observed biological effect is ordinarily highly indirect. Between the act of absorption of light and the appearance of the final effect, there generally takes place a great number of very complex and interconnected chemical and biological reactions. Further, cases are possible in which one and the same biological effect may be associated with the absorption of light by different molecules, or with different changes which occur in one and the same molecule under the action of light.

We shall consider only the simplest case, in which this does not take place, and the effect of interest to us is determined only by a certain "damage" which takes place in certain definite molecules upon action of light. Then obviously, the macroscopic effect which we observe is some complex function, specific for the given process, of the rate of change in the number of "damaged" molecules (N^*):

$$\frac{dM}{dt} = g\left(\frac{dN^*}{dt}\right).$$

The probability of occurrence of the given "damage" is the product of the probability of absorption of a quantum of light by the given molecule and the probability that the result of absorption will be the required reaction. The first probability is ordinarily characterized in biological work not by the molar extinction coefficient, as is the custom in chemistry, but rather by the value of the "capture cross section" for the quantum s. The latter is related to the extinction coefficient by the simple relation:

$$s_{(cm^2/molecule)} = 3.83 \times 10^{-21} \, \varepsilon_{(moles/liter)}.$$

The probability of the given reaction in a molecule which has absorbed a quantum, i.e., the quantity

$$\Phi = \frac{(\text{number of reacting molecules})}{(\text{number of quanta absorbed})}$$

is called the quantum yield of the given reaction.

If we express the intensity of illumination not in energy units (power/cm^2), but rather by number of quanta per cm^2 per sec, then obviously

$$\frac{dN^*}{dt} = \Phi \cdot s \cdot I \cdot N,$$

where N is the total number of molecules of the given type in the object being irradiated, and

$$\frac{dM}{dt} = g\,(\Phi \cdot s \cdot I \cdot N).$$

From the definition given above for the relative biological effectiveness of radiation with wavelength λ, it follows that under these conditions the ratio I_2/I_1 is equal to the inverse ratio of the products $\Phi \cdot s$, that is:

$$\frac{\text{Relative effectiveness of } \lambda_1}{\text{Relative effectiveness of } \lambda_2} = \frac{\Phi_1 \cdot s_1}{\Phi_2 \cdot s_2}.$$

Hence, one can easily see that in the given case, in which the quantum yield of the given reaction does not depend on the wavelength of the absorbed light, the action spectrum must coincide in shape with the absorption spectrum of the substance in which the initial photochemical reaction takes place. Conversely, the coincidence of the action spectrum with the absorption spectrum of some particular substance occurring in the system shows two things. First, it shows that this is the substance which plays the role of initial link in the whole chain of biological reactions leading to the given effect. Second, it shows that the quantum yield of the initial photochemical reaction does not depend on the wavelength of the absorbed light.

Coincidences of this type between the action spectra of biological effects and the absorption spectra of certain substances have been known for a long time. For example, it is known that the sensitivity curve of the scotopic (twilight) visual apparatus of man coincides beautifully with the absorption spectrum of visual purple, the pigment contained in the optic nerve endings, the rods. This coincidence is the basis of the photochemical theory of vision. On the other hand, it has not been possible to find in the eye pigments whose absorption spectra coincide in form with the spectral sensitivity curves of the three fundamental color receptors required by the three-color theory of color vision of Young and Helmholtz. This is one of the most serious objections against the purely photochemical theory of color vision, and compels us to seek other explanations of this phenomenon.

Likewise, in the study of the action spectrum of photosynthesis in green plants which contain only chlorophyll as the active pigment, it has been possible to show that the action spectrum quite satisfactorily coincides with the absorption spectrum of chlorophyll (Fig. 23). This is the most convincing proof that, in these cases, the initial link in the reaction chain of photosynthesis is the absorption of
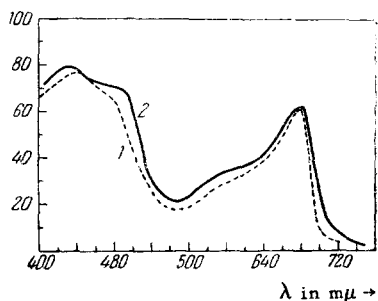
FIG. 23. Action spectrum for photosynthesis (curve 1) and absorption spectrum (curve 2) for the green alga Ulva taeniata. Taken from Haxo and Blinks.[39]

light by molecules of chlorophyll.

However, no matter how interesting those cases are in which the action spectrum coincides with the absorption spectrum of a photochemically active substance, the cases are even more interesting in which this coincidence does not take place. For example, in the study of photosynthesis in blue-green algae, which contain another pigment, phycocyanin, besides chlorophyll, it was shown that light absorbed by this pigment was just as effective both for photosynthesis and for excitation of the luminescence of chlorophyll as light absorbed by chlorophyll itself. In Fig. 24 are shown the ab-
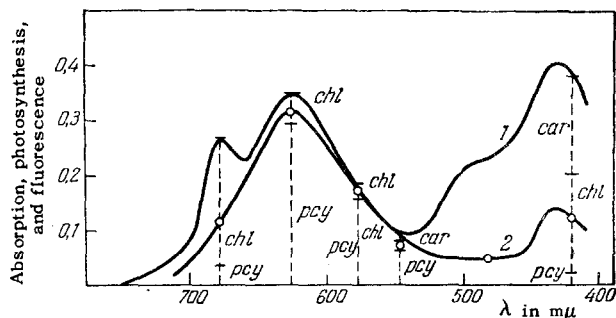

FIG. 24. Absorption spectra (curve 1) and action spectrum for photosynthesis (curve 2) for the blue-green alga Oscillatoria according to Duysens.[31]

sorption spectra and the action spectrum for the blue-green alga Oscillatoria. The circles on the latter curve indicate for a series of wavelengths the values of the relative effectiveness of the radiation in excitation of the fluorescence of chlorophyll in the plant. The marks on the ordinates at these wavelengths indicate the relative fractions of the energy which are absorbed by phycocyanin, chlorophyll, and carotenoids. For example, one may see that at a wavelength of about 680 mμ, an overwhelming fraction of the radiation (above 90%) is absorbed by chlorophyll, while at wavelengths of about 650 mμ or 580 mμ, almost all of the radiation is absorbed by phycocyanin. Nevertheless, the effectiveness of the absorbed light is the same,

both for photosynthesis and for excitation of the fluorescence of chlorophyll. This indicates that migration of energy from phycocyanin to chlorophyll takes place in the chloroplasts of the plant, i. e., transfer without dissipation of excitation energy of the phycocyanin molecules to the chlorophyll molecules at distances considerably greater than interatomic distances. Kinetic collisions between the energy donor and acceptor are excluded here. In the same figure is indicated the fraction of the light absorbed by carotenoids. This absorption is especially significant in the blue region of the spectrum around 400 mμ. As can be seen from the figure, the transfer of the energy absorbed by carotenoids to chlorophyll does not take place in the given case; this light participates neither in photosynthesis nor in the excitation of the fluorescence of chlorophyll.

The example given here is far from being the only case of the phenomenon of the migration of energy, i. e., the radiationless transfer of excitation energy from the site where it is absorbed to the site where it is utilized. These phenomena are apparently very widespread in biological processes, and the elucidation of their mechanism is of exceedingly great importance for the understanding of these processes. For this reason, much attention is being paid to them in modern physico-chemical biology. Not being in a position to go into more detail on these interesting phenomena, I shall cite only the book of Ril',[40] the series of reviews by Terenin,[41-43] and the review by Vladimirov and Konev,[44] which is especially devoted to the problem of energy migration in the protein molecule. I wish merely to emphasize that, in the study of phenomena of energy migration, spectroscopic methods, and in particular, the study of action spectra, are one of the most important tools of research, and often the only tool.

7. All that has been stated above refers only to the absorption methods of spectroscopy. We must briefly consider the emission methods. As has been noted already, of these, the methods which have the greatest importance in biological and biochemical research are the study of the photoluminescence and chemiluminescence of individual biological substances and of living objects.

I shall not at all deal here with the numerous and important applications of luminescence analysis and microscopy, to which a number of specialized books and reviews are devoted (see, for example references 45, 46). I shall call your attention only to certain specific applications of luminescence methods in the study of important biological processes.

I wish to note here one general circumstance.

Many biologically important luminescent substances are not only complex in their chemical compositions, but also "complex" in the special sense of luminescence, as applied to this word by B. S. Neporent. This means that they possess a large number of internal vibrational-rotational degrees of freedom, these degrees of freedom being so closely connected with the electronic excited states that an equilibrium distribution of the excited molecules over the vibration-rotation energy levels may be established in a time significantly smaller than the lifetime of the molecule in the excited state. As a result, the luminescence spectra of these "complex" molecules have the form of broad structureless bands, whose shapes are not characteristic of the given substances. Stepanov[47] has shown that the shape of the spectra of these molecules is universal; they differ only in the positions of the maxima, which are determined by the difference in energy between the corresponding energy levels.

It is true that under certain definite conditions, as has been shown by É. V. Shpol'skiĭ (see his article on p. 378, it is possible to resolve the spectra of certain compounds into a series of bands which are so narrow (almost spectral lines) that it becomes possible to carry out a vibrational analysis of the structure of these substances. Undoubtedly, this method may give valuable results in the study of biologically important substances as well. However, in most cases in the study of the luminescence of living objects and biological materials under ordinary conditions, we are dealing with broad, structureless, poorly characterized luminescence spectra. Of course, the purely spectroscopic study of these spectra may also give valuable indications for the understanding of processes which take place in the organism, or for the elucidation of the state in which the luminescing substance occurs. Thus, many important results have been obtained by the comparison of the luminescence spectra of chlorophyll in solutions and in plant cells. However, one may suppose that the study of the luminescence of biological objects and substances will give the most valuable results if it is not limited to the determination of the spectral intensity distribution of this radiation alone. Rather, the other characteristics should be brought into consideration: the yield, polarization, and duration of the luminescence, as well as its law of decay.

I shall limit myself merely to several examples which illustrate the effectiveness of such expanded, not purely spectroscopic, methods of studying luminescence. Arnold and Meek[48] have shown that the luminescence of chlorophyll in plant cells (the alga Chlorella) is practically depolarized (the de-

gree of polarization is not greater than 3%), while with the same method of excitation, the polarization of the luminescence of solutions of chlorophyll in castor oil is greater than 25%. We have had the opportunity to convince ourselves of the fact that the polarization of the fluorescence of isolated chloroplasts obtained from the leaves of beets and tobacco is also almost completely nil ($p \approx 2\%$). The viscosity of the chlorophyll-protein complexes in the grana is practically infinite, whereas the lifetime of the excited state is appreciably less than in solution. Hence, the possibility of depolarization of the fluorescence due to Brownian rotation of the molecules during the lifetime of the excited state is excluded. Thus, the only possible explanation of the phenomenon consists in the conception that concentration depolarization takes place in the plant, i. e., the transfer of energy from the chlorophyll molecule excited by the light to another molecule. Thus, these results are a very powerful argument in support of the conception of the so-called "photosynthetic unit," a complex of some thousands of chlorophyll molecules which are associated with one "reaction center, " and which take part in photosynthesis as a unit.

The fluorescence of riboflavin has been studied very intensively for purposes of analytical determination of this substance, but these studies were generally limited to measurements of the intensity and spectrum of the luminescence alone. G. Weber[49] has studied the polarization of this luminescence and has obtained a series of very important new results. He showed, for example, that the quenching of the fluorescence of riboflavin by various purines (adenosine, caffeine, etc.) is not accompanied by a shortening of the lifetime of the excited state, and consequently may be explained only by the formation of nonfluorescent complexes of riboflavin with these substances. Weber was also able to determine the heats of dissociation of these complexes from temperature studies. Analogous measurements made it possible for Weber to establish the fact that the lifetime of the excited state of the riboflavin molecule is not changed when the molecule is incorporated into the complex: flavin-adeninedinucleotide-protein.

The method developed by Weber[50] of study of the shapes and dimensions of macromolecules (proteins) by means of the polarization of the fluorescence of dye molecules absorbed on them is also of great interest. By using this method, Massey, Harrington, and Hartley[51] have detected physical differences between chymotrypsin and chymotrypsinogen.

Now for a final example. It is known that the

fluorescence yield of chlorophyll in solution attains values of 25 to 30%, whereas in the plant it is considerably less (2 to 3%).[52] Recently three authors[53-55] have independently carried out direct measurements of the lifetime of the excited state of chlorophyll in vitro and in vivo, and have shown that the decrease in the fluorescence yield in plants is accompanied by a parallel shortening of the lifetime. This shows that a competition takes place between paths of deactivation of the excited state of the chlorophyll molecule. On the one hand are the ordinary pathways of radiation and internal conversion, and on the other hand, there is the specific pathway of deactivation leading to the storing of the absorbed light energy in the form of the energy of the chemical products of photosynthesis. One may suppose that the beginning of this pathway is the transition of the chlorophyll molecule from the singlet state to the long-lived, metastable triplet state. This entire complex of problems is now being studied very intensively, and one may assume that these studies will lead us to a better conception of the initial photochemical stages of this process.

In conclusion, I wish to say a few words about the general conception of bioenergetic processes developed by Szent-Györgyi, and in this connection, about the role in biological processes of molecules which are capable of luminescence.

Basic to the conception of Szent-Györgyi is the hypothesis that the transition of the energy which is stored in potential form in the bonds of some chemical compound into the labile, kinetic form of the energy of vital activity takes place by means of the excited electronic states of certain molecules.

If we designate the energy stored in chemical bonds by (E), and the energy of excited electronic states by E*, then the process of photosynthesis may be written schematically in the form:

$$E^* \longrightarrow (E_1) \longrightarrow (E_2) \longrightarrow \ldots \longrightarrow (E_n),$$
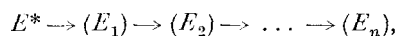
All other bioenergetic processes, in which this chemical energy is transformed into the energy of vital activities may, according to Szent-Györgyi, be written in the form:

$$(E_n) \longrightarrow (E_{n-1}) \longrightarrow \ldots \longrightarrow (E_1) \longrightarrow E^*.$$
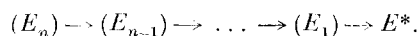
"All life," writes Szent-Györgyi, "is photosynthesis and its reversal." Here, reactions of the type $(E_k) \rightarrow (E_{k+1})$ are the ordinary chemical reactions of intermediary metabolism, in which energy is transferred from bond to bond, and from substance to substance. These may be understood within the framework of classical chemistry, and written in the form of combinations of letters symbolizing the atoms and lines symbolizing the bonds. As for the

processes of the types $E^* \rightarrow (E_1)$ or $(E_1) \rightarrow E$,* these are specific "quantum-biological" processes, whose mechanisms are not yet clear and are subject to further study. Transitions of the first type are the kernel of all those processes in which energy is accumulated and produces living matter. Those of the second type are the kernel of all those processes in which this energy leads to the activity of the living machine.

A specific peculiarity of luminescent molecules is the fact that excitation energy may be retained in them for times of the order of 10^{-8} to 10^{-9} sec, whereas in nonluminescent molecules, the transfer of the excitation energy among the vibrational-rotational degrees of freedom, and the transformation of the energy into heat, takes place in a considerably shorter time, of the order of 10^{-14} sec. The possibility of transforming the electronic excitation energy into chemical energy in processes of the type $E^* \rightarrow (E)$, as well as the possibility of transforming the energy E^* into the energy of vital activity (e. g., mechanical work in muscle contraction), is obviously considerably greater for molecules which are capable of luminescence. It is these molecules which must, in Szent-Györgyi's opinion, play the decisive role in bioenergetic processes.

Of course, this does not mean that these molecules actually luminesce under the conditions under which they occur in the organism. On the contrary, in most cases the processes of biological utilization of the excitation energy compete so successfully with the process of spontaneous emission that luminescence is practically absent, or shows a very small yield. For example, we have seen that the fluorescence yield of chlorophyll in the cell is one-tenth as great as in solution.

In most luminescent molecules, along with the ordinary singlet excited state characterized by values of the lifetime indicated above, there exists a metastable triplet state, for which the probability of spontaneous emission is several orders of magnitude smaller; the lifetimes of these states are correspondingly larger in the same proportion. From the chemical point of view, these molecules with two parallel electron spins are biradicals and are characterized by high reactivity. Both of these circumstances, high reactivity and long lifetime, are exceedingly favorable for the transformation of excitation energy into chemical energy. Terenin was the first to note these circumstances and to emphasize the great significance which triplet excited states have in all photochemical processes, and in particular, in photosynthesis and other photobiological processes. Szent-Györgyi has extended this

point of view to all the other bioenergetic processes; he considers that the first step in the pathway of transformation of electronic excitation energy into mechanical work or other forms of energy of vital activity is the transition from the singlet excited state into the metastable triplet state. This transition becomes possible under the conditions in the organism, according to Szent-Györgyi, because of adsorption of water in the cell on the branched macromolecules and structures. Thus, a considerable portion of the water becomes ordered and pseudocrystalline (in this regard, see the review by Privalov[56]).

The conception of Szent-Györgyi is still largely hypothetical, and requires testing and proof. However, independently of the degree in which it may be confirmed in all its universality, there is no doubt that electronic excited states, including the triplet state, play a leading role in very many processes, particularly photosynthesis. In the study of this very interesting complex of questions, leading us to the elucidation of the bioenergetic bases of life, the role of a many-sided study of luminescence processes will undoubtedly be very great; this should include both the purely spectroscopic study of the luminescence and the study of its other characteristics: yield, polarization, and kinetics.

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