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SPECTROSCOPY OF BIOLOGICAL SAMPLES AT ROOM AND LOW TEMPERATURES

During the last twenty years, visible progress has taken place in experimental biology, especially photobiology. An important role in this progress is due to the wide adoption of spectroscopy. Spectral methods are now indespensable in biological experiments. This is determined first of all by the high sensitivity of these methods, as well as by their specificity and the possibility to investigate biological processes quantitatively without damaging the natural integrity of the samples. Spectral methods are distinguished moreover by rapidity, allowing to study very fast processes.

Essentially, the use of spectral methods has led to progress in our knoledge of the mechanisms of the primary reactions of photosynthesis, the mechanisms of electron transport in respiration, and the mechanisms of vision. It is necessary to note especially the success actieved in the elucidation of the mechanisms of light reactions in photosynthesis [9, 36]. Due to the development of absorbance difference spectroscopy, it was possible to find the carriers of the photosynthetic electron transport chain. Flash difference spectroscopy has permitted the study of the kinetics of electron transsport. And, now, picosecond technique gives the possibility to study the charge separation in the reaction center.

The lectures at this school mainly concerned the physical aspects of spectroscopy and their possible application in biology. In a number of lectures (dr J. A m e s z, dr G. P a p a g e o r g i o u and dr P. W i l l i a m s), some results in photosynthesis obtained by means of spectral methods were considered. The title of our lecture suggested by the Organizing Committee is very extensive. The school is named "Spectroscopy in Biological Research", and so it may be advisable to discuss the peculiarities of the spectroscopy of biological material. I shall deal with some problems in photosynthesis solved by means of spectroscopy; photosynthetic material will be used to exemplity the biological sample.

I whould like to concentrate on the following questions:

1. Peculiarities of the measurement of absorption and fluorescence spectra of biological samples.

2. The principles of absorption difference spectroscopy and the sources of possible error in the course of measurement of light induced absorption changes of photosynthetic organisms.

3. Difference fluorometry and its application in biology.

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1. As is well known, the measurement of absorption spectra allows to elucidate the nature of absorbing substances as well as their content. In other words, absorption spectroscopy is an efficient method of qualitative and quantitative analysis [22, 29, 33].

The identification of the nature of absorbing substances is based on their specific spectral

properties. The position of the absorption maxima, their intensity and relation can provide information on the nature of the substance. This identification, which is very simple in the case of a one-component system, is essentially difficult when the sample is a mixture of two or more components. The spectral identification of different substances is more difficult in biological samples, which are multicomponent systems. This problem is complicated when the absorption spectra of the various substances overlap. For instance, in algae and green leaves, chlorophylls, carotenes, cytochromes, flavins and quinones absorb in the blue-green region of the spectrum. This makes difficult the simple identification of the absorption bands. At the same time, although absorption in the red region of plants is due to chlorophylls, the identification of that band is also complicated because the chlorophylls are present in several forms.

For the determination of the content of a substance in tissues, one carries out a selective extraction and then measures the absorption spectrum of the extracted pigment in a well defined solvent in order to assess its concentration.

Now concerning the peculiarities of the spectroscopy of biological substances. These are, essentially, the property to scatter light very efficiently as well as the irregular localization of the absorbing substances in the cells [1, 7, 28]. These properties have always to be taken into account when measuring correct absorption spectra of biological materials.

The light beam is partly absorbed but is partly lost because of reflection from the surface of the sample and because of light scattering in different directions. Only part of the measuring beam traverses the sample and reaches the detector. This could result in a mistaken conclusion of a greater absorbancy of the studied sample. This effect causes a general upward shift of the spectral curve that is especially visible in the region of minimal absorption. To measure the true absorption, it is necessary to determine the interference of light scattering in the value measured, or to reduce the scattering.

Therefore it is important to record all the light transmitted and scattered by the sample. To this aim, the sample is placed very close to the detector (it is desirable to have a photomultiplier with large photocathode). Besides, it is possible to use an integrating sphere in which the sample is placed, or to use light scattering plates (opal glass method).

The light scattering of biological samples is caused mainly by the difference in refractive index of the scattering parts of the cells and the medium. Artificial modification of the refraction index of the medium by an addition of glycerol, sucrose or other compounds diminishes the difference between the refractive indices and thus reduces the light scattering, because the sample is less turbid. Of course, the compounds added have to be inactive and cannot affect the spectral properties of the sample. Light scattering of green leaves could be essentially decreased due to vacuum filtration. The homogenates of tissues or leaves, isolated structures (mitochondria, chloroplasts, chromatophores) as well as the parts of such structures (e.g. protein-pigment complexes) have lower light scattering and are more suitable for spectral study.

To eliminate the error related to scattering, the measured values are corrected for the value of absorption in the spectral regions where real absorption is absent. Usually it is the value of absorption in the longwave part of the spectrum. Such correction is especially necessary if the measurement was performed without special devices to measure the contribution of light scattering.

Two main reasons cause distortion of the absorption spectra of biological samples

1. Light scattering and multiple relection increase the real optical path of the measuring beam and result in an apparent increasse in absorption. This effect, strongly pronounced in absorption minima, leads to a decrease of the difference between the maxima nad minima.

2. Coloured substances in biological systems are distinguished irregularly: they are concentrated in definite structures. Therefore one part of the light beam is strongly weakened while the other part could pass through the sample without absorption. This sieve effect, resulting in a decrease of the optical density, is more strongly apparent in absorption maxima [1, 7]. Both effects cause a decrease in absorption selectivity because of the broadening of the maxima and the flattening of the spectra [33].

Thus, spectroscopy at room temperature provides information about the presence in the material of definite pigments, about their content and state, as well as about changes of state.

The overlapping of absorption bands at room temperature results in a complication of the spectral curve: "shoulders" appear, and the absorption band broadens. The interpretation of such absorption bands is difficult. To solve this problem, use is made of low temperature spectroscopy. The cooling of the sample causes a narrowing of the absorption bands and the appearance of a fine structure that allows to reveal the positions of the maxima clearly, e.g. to study the presence of different types of cytochromes in biological material [26, 27, 37]. However, the measurement of absorption spectra at low temperature is complicated because cooling of the material results in crystallization of the solvent decreasing the light scattering. For the elimination of this effect it is necessary to diminish the thickness of the layer and, at the same time, to increase the sample concentration, or to use media freezing as glass. If there is no necessity to control the thickness of the sample layer at low temperature spectroscopy, the sample is placed on some transparent material.

Since deep cooling of the sample inhibits practically all biochemical processes and dark stages of photochemical reactions, it could be used for the investigation of light reactions of photobiological processes. At low temperatures, only photophysical processes take place; these could be separated from rapid chemical reactions and studied in detail. Such an approach is promising for the investigation of the primary photoact in reaction centers of photosystems. It has now been shown that charge separation in the reaction center occurs also at low temperatures and is reversible as a result of recombination of the primary donor and acceptor in the center [34].

When commercial spectrophotometers are not equipped with a low temperatures accessory a Dewar flask, completely transparent or with transparent zones for the measuring beam, can be used. Since biological samples are turbid at low temperatures, it is best to place the Dewar flask in an Ulbricht sphere [11]. To decrease the noise from bubbling of liquid nitrogen, the preliminarily cooled sample is placed in the Dewar flask under the surface of nitrogen. In this case the temperature of the sample is slightly higher than $-196^{\circ}C$, but the absorption spectrum is not distorted. The same approach is used in absorption difference spectroscopy at low temperatures.

Luminescent spectroscopy is an important method for the investigation of biological processes. By comparison with absorption methods, the fluorescent method is much more sensitive: the negligible changes in pigment state, poorly visible in the absorption spectrum, are revealed distinctly in the fluorescence spectrum. Therefore, on the basis of these spectra, it is possible to assess the state of pigments in biological systems and the changes in the state of the pigments [23, 25]. Measurements of changes in fluorescence yield and changes in fluorescence lifetime of chlorophyll allows to investigate the processes of excitation energy dissipation and the lifetime of the excitinic state of the emitted molecules. Sensibilized fluorescence provides information on energy migration between the different forms of the pigments. Thus the measurement of various parameters of fluorescence discloses the mechanisms of photobiological processes [18].

The fluorescent method is especially promising for the investigation of biological systems because the active sample as a whole is not demaged during the measurement. Using the fluorescent method it is possible to determine the concentrations of emitted molecules. At low concentrations, the absorption of the solution is proportional to the optical density and thus to the concentration of the fluorescing compound. However, at high concentrations the increase in content of the substance results in no increase in fluorescence intensity. Therefore, in concentrated solutions, the determination of the content of substances is no longer possible by the fluorescent method. At high concentrations the fluorescence is emitted from the superficial layer of the sample only and this causes a change in the amount of light reaching to photomultiplier. High concentrations can affect not only the fluorescence yield but also the fluorescence spectrum. Therefore it is advisable to use in experiments diluted solutions or samples with a small thickness of the layer.

One of the sources of possible error in fluorescence measurements resides in reabsorption when the emitted light is partly absorbed by the sample. Reabsorption results in a decrease in fluorescence intensity and a distortion of the fluorescence spectrum. This effect depends on the absorption of the sample and therefore becomes more strongly apparent in the fluorescence region corresponding to the absorption maximum. Weakening of fluorescence intensity in the shortwave region of the spectrum causes an apparent longwave shift of the fluorescence maximum. The influence of reabsorption is greater in cases of strong superposition of the absorption and fluorescence spectra. To eliminate reabsorption, it is necessary to measure the fluorescence in thin layers, using as far as possible a dilute sample; to excite and measure the fluorescence from the same side of the sample; and to excite the fluorescence with a wavelength corresponding to the absorption maximum, because in this case the exciting beam penetrates the sample layer less deeply. Another effect interfering in fluorescent measurements is the screening effect, which becomes apparent when luminescence is measured in the presence of an other colored substance absorbing in the region of the excitation wavelength. In this case the other substance, partly absorbing the excitinic light, causes a decrease in fluorescence yield. Therefore, to eliminate this effect, it is necessary to excite the fluorescence in a spectral region in which the other substance has no absorption band.

Because the fluorescence yield of biological material is quite small, highly sensitive apparatus is required. Besides, it is necessary to chose correctly the geometry of the optical system and to check for a possible contribution to the measured signal from fluorescence of the solvent or cuvetts (especially at low temperatures). The excitinic beam cannot be excessively intense so as to cause no changes in the properties of the sample.

The measurement of low temperature fluorescence spectra allows to obtain information concerning the properties of pigments and their state in biological systems. At room temperature, the fluorescence yield is small, but rises sharply at low temperatures. At the same time, the fine structure of the fluorescence spectrum becomes apparent. The use of low temperatures allows to inhibit the biochemical reactions as well as to protect the sample from the photochemical action of the excitinic beam. Due to low temperature spectrofluorometry, it was possible to establish the sequence of stages in the course of greening of etiolated leaves. Illumination of etiolated leaves during various periods of time and rapid cooling allowed to fix the different states of the pigments and to investigate them without interference from dark stages [24]. The detailed study of low temperature spectra of various types of algae and decomposition of the spectra allowed to establish the main forms of chlorophyll, common to all types of organisms studied [25]. Los temperature measurements permitted the observation of the fluorescence of bacteriochlorophyll – 850 in purple bacteria [12].

The intepretation of low temperature fluorescence spectra is diffucult, because a redistribution of emission takes place in the fluorescence maxima and new bands appear. Naturally, the question arises as to how far the low temperature spectrum reflects the normal state of the photosynthetic apparatus Only a comparison of the fluorescence spectra and photochemical activity of the samples allows to solve this problem.

Thus, accurate measurements allow to reveal the fine structure of the absorption and fluorescence spectra with more clarity at low temperatures. Sometimes, however, it is very difficult to reveal this structure, and the limit of resolution is determined by the properties of the sample and not by the apparatus. To solve this problem, the method of derivative spectroscopy has been developed, consisting in the differentiation of usual spectra in wavelength [10, 25]. Of especial advantage is the measurement of second derivative spectra because the shape and position of the maxima are similar to the initial spectrum and the half with of the second derivative is essentially narrower than that for the initial curve [25]. The measurement of derivative spectra, especially fluorescence spectra, at low temperatures provides new possibilities for revealing the fine structure of the spectrum. A combination of these two approaches can be used for measurements of derivative excitation spectra of fluorescence. However, the interpretation of derivative spectra is based with some difficulties.

2. The results obtained by means of absorption difference spectroscopy on photosynthetic organisms have been considered by dr A m e s z. I should like to remind briefly the main principles of this method and to enumerate some sources of possible error.

The methods of absorption difference spectroscopy have been developed the most strongly in biology because of the specificity of biological material, imposing the necessity of a sharp increase in sensitivity. The application of difference spectroscopy in biology allowed to measure more reliably and correctly very small changes in absorption due to the action of various agents on biological material [5, 8]. The methods of difference spectroscopy can be divided into two groups. Firstly, methods used for the recording of changes in the absorption of samples in the steady state (e.g. spectra "oxidized minus reduced"). Other methods are used to study the time evolution of processes, including very rapid processes [31].

Difference spectrophotometers can be double-beam and one-beam instruments. The first type is widely adopted for the automatic recording of difference spectra in the steady state; the second type-mainly for measurements of the kinetics of light induced absorption changes and difference spectra (point to point). The double-beam instruments have a high sensitivity and stability. There are two types of such instruments: split-beam and double-wavelength.

Split-beam spectrophotometers are used to record difference spectra of two, initially identical samples. After treatment of the samples (oxidation-reduction or otherwise), the absorption difference spectrum is recorded automatically. Double-wavelength spectrophotometers serve, as a rule, for kinetics investigations when the effect applied causes changes not only in absorption but also in light scattering. In this type of instrument, two beams with different wavelengths (from different monochromators) pass alternatively through a single sample. One of the beams has a wavelength corresponding to the maximal changes in absorption, whereas the other beam has a wavelength corresponding to the isobestic point or to the spectral field with minimal changes. With double-wavelength instruments, it is possible to measure the difference spectra free from contributions from variations in light scattering.

One-beam instruments are used mainly for kinetics studies of light induced absorption changes as well for the recording of difference spectra. These spectra are measured point to point because the time evolution of absorption changes is complex and depends on the spectral field measured. This type of instrument is widely adopted to the study of primary processes of photosynthesis. The weak monochromatic measuring beam, on traversing the cell with the sample, reaches the detector, the electric signal from which is compensated by a current from an outside source. Illumination of the sample with actinic light results in absorption changes and the appearing signal is amplified and recorded. To protect the detector from scattered actinic light, crossed filters are used [17]. For instance, when measuring the difference spectrum of photosynthetic material in the 600–400 nm region, the sample is illuminated with red light (more than 600 nm). Between the photomultiplier and the sample is placed a blue-green filter to cut-off the red actinic light. But when it is necessary to measure the difference spectrum in the red region (600–700 nm) where the chlorophylls absorb, a simple replacement of filters proves insufficient: blue actinic light excites intense red fluorescence of the photosynthetic material that passes through the red protective filter and is recorded by the photomultiplier.

For the elimination of interference of this fluorescence, it is possible to modulate the measuring and actinic beams at different frequencies. The amplifier has the same frequency as the measuring beam. But fluorescence from the actinic beam reaching the photomultiplier causes a sharp increase of the noise level, and that makes impossible the correct recording of small absorption changes. For the complete elimination of the recording of intense fluorescence excited by the actinic beam, use was made of phosphoroscopes: a sectoral disk [21], or a system of cylinders with slits [11, 13]. However, in this case, only photoproducts having a lifetime longer than the time between illumination and measurement are recorded.

The investigation of phototransformations of chlorophylls in plants, especially in the reaction center of photosystem 2, is a difficult problem. The changes in absorption accompanying these phototransformations are very small and could be measured only by using highly sensitive apparatus. Besides, they are very rapidly reversible. As it has been shown [11, 13], there are other methodical difficulties.

The weak monochromatic measuring beam excites fluorescence of the photosynthetic material and this fluorescence is recorded by the photomultiplier. The yield of fluorescence mekes up 10–15% of the total photosignal in the case of green algae. Under illumination by actinic light, a change takes place in the fluorescence yield, excited by the measuring beam, and this change in fluorescence can be mistaken for a change in absorption [6]. The same effect may be caused by delayed luminescence, which also is recorded by the photomultiplier in the course of difference spectra measurements [11]. The delayed luminescence has the same spectrum as the fluorescence. But in contrast to light-induced fluorescence changes, the value the signal from delayed luminescence does not depend on the wavelength of the measuring beam, and it is possible to correct the measured signal for the value. At the same time this could not be done for the signal from light-induced changes in fluorescence, excited by the measuring beam. It is possible to decrease strongly the value of this signal by placing an appropriate interference filter between the photomultiplier and the sample. When changing the wavelength of the measuring beam, it is necessary to exchange the interference filter. To reduce this effect, one can increase the distance between the sample and photomultiplier and use low voltage on the photomultiplier. However, both approaches cannot completyly eliminate the possibility of mistake in the measurement of light-induced absorption spectra, especially in the 680–690 region (photosystem 2).

There are other sources of possible mistakes when measuring light-induced absorption changes of photosynthetic organisms [17]. During measurements of light-induced absorption changes in some types of bacteria or algae, the actinic light can set these biological objects in motion and can cause changes in the chloroplast position in the cells. Both effects can affect the transmission of the sample in a way unrelated with the process studied. Addition of gelatine (6%) stabilizes the cells and prevents their motion. Actinic light can heat the sample resulting in a change in refractive index which can be mistaken for a change in absorption [32]. It is necessary to eliminate heating by actinic light. Besides, the settiling of large cells of isolated structures in the course of the measurement causes a clearing of the sample. To investigate such material, it is preferable to use a difference spectrophotometer with vertical beams. Finally, changes in light scattering of chloroplasts can be mistaken for changes in absorption.

Difference spectroscopy of biological samples at low temperature is a special field of research [22]. The measurement of low temperature light-iduced absorption spectra is an efficient method of investigation of the mechanisms of electron transport in reaction centers. The difficulty of such measurements resides in the stabilization of the sample. For low temperature measurements, the cuvettes with preliminarily cooled samples are placed in a special holder under the surface of liquid nitrogen to prevent bubbling and to decrease the noise level. To stabilize and to lower the temperature of the sample, the metal holder can be partly immersed in nitrogen.

Difference spectra "oxidized minus reduced" can be relatively simply measured by means of these cryostats. But the measurement of light-induced absorption changes presents additional difficulties because of the emission of glass at low temperatures.

Low temperature absorption spectroscopy allows to perform qualitative analyses of different types of cytochromes in cells or isolated structures. The sharp narrowing of the absorption bands and the increase in resolution under cooling give the possibility to analyze maxima overlapping at room temperature.

The interpretation of light-induced changes in absorption of photosynthetic organisms at low temperature is very difficult and sometimes results in erroneous conclusions. For example, bleaching at 555 nm in chloroplasts at $-196^{\circ}C$ has been attributed to photooxidation of cytochrome *f*, related to photosystem 1 [35]. Later, cytochrome *b*−559, localized in photosystem 2 [3], was shown to be responsible for this change in absorption.

Deep cooling of samples allows to block electron transport from the reaction center to secondary and other acceptors and to isolate the functioning of the primary acceptor [30, 34]. However, even at very low temperatures, in photosystem 1, besides the primary acceptor, reducing reversibly, other acceptors are reduced irreversibly. Repeated illumination of photosystem 1 causes a reduction of the promary acceptor only.

3. In some cases, the changes in concentration of different carriers of the electron transport chain are difficult or impossible to determine by absorption difference spectroscopy. If these substances are capable of fluorescence, their redox transformations will be accompanied by changes in fluorescence yield. Such a method of difference fluoremetry has been developed to record NADP redox changes [2]. The fluorescence of the sample (chloroplasts, mitochondria) is excited by light of wavelength 340 nm, corresponding to the absorption maximum of NADPH, and the integral fluorescence in the 400–480 nm region is measured. Reduction of NADP results in a higher fluorescence yield.

Difference fluorometry is widely used to measure the changes in fluorescence yield of chlorophylls of photosynthetic organisms. These changes reflect the functioning of the reaction centers and electron transport chain.

As is well known, the main part of chlorophylls in plants serves for light harvesting and transfer of absorbed energy to the reaction centers, where the transformation of light energy to chemical energy takes plase. Under illumination of green leaves after a long dark period the complex kinetics of variations in fluorescence yield becomes accessible to observation. This effect, found by K a u t s k y, was termed fluorescence induction [19]. Now it is known that the fluorescence yield of green plants is determined by the chlorophylls of photosystem 2 mainly, and depends on the redox state of the reaction center components [9]. The complex kinetics of fluorescence induction reflects the functioning of the photosynthetic electron transport chain and coupling processes [9, 18].

When the reaction center is open ($P.A$), energy harvested by antenna chlorophyll efficiently migrates to the center and is utilized there: the fluorescence yield is low. In closed states (P^+A or PA^-), the reaction center is incapable of the primary photoact, and the energy absorbed is emitted as fluorescence.

Instruments for the measurement of fluorescence induction can be divided into two main groups. One-beam instruments do not allow to distinguish constant and variable levels of fluorescence correctly [18]. Double-beam instruments are more convenient for the investigation of fluorescence induction [14, 15]. A weak monochromatic beam 480 nm, ($10\ erg\ cm^{-2}\ sec^{-1}$) excites the fluorescence of algae suspension or chloroplasts, which is integrally (more than 650 nm) recorded by a photomultiplier from the front part of sample. Illumination of the sample with the actinic beam results in redox transformations of the reaction center components and a transition to the closed state.

To eliminate the recording of fluorescence excited by the actinic beam, use is made of a phosphoroscope – a rotating cylinder with two slits. The photosignal due to delayed luminescence is eliminated by using double modulation: the weak monochromatic beam is modulated at a higher frequency (4600 cycles per second) than the actinic beam (100). The amplifier has a frequency corresponding to the fluorescence exciting beam.

Such apparatus allows to measure, with high sensitivity, small fluorescence changes light-on and light-off. Due to this information, we were able to discover the variable fluorescence of photosystem 1 caused by P 700 photooxidation or primary acceptor photoreduction [4, 16, 20]. Previously, fluorescence induction had been observed only for photosystem 2 and the photosystems of photosynthetic bacteria [9, 18]. The variable fluorescence of photosystem 1 is observed only on particles enriched by photosystem 1 [16, 20] or on chloroplasts without photosystem 2 [4].

Measurements of difference fluorescence spectra provide information about the contribution of various forms of chlorophyll in emission and about the changes in energy migration [9]. As a rule, difference fluorescence spectra of photosynthetic organisms are measured point to point because of the presence of constant and variable levels of fluorescence.

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In concluding it is necessary to note that, at present, practically maximal sensitivity and time resolution of spectral methods have been achieved. Future developments of spectroscopy in biological research can, maybe, lead to the simultaneous recording of several spectral parameters: absorption changes at different wavelengths, fluorescence changes, control of the redox potential of samples, and photochemical activity. The comparison of spectral and functional properties allows to elucidate the relation between different spectral changes and difinite processes. Another trend in the development of spectroscopy for biology can bear on

microspectrophotometry (e.g. the investigation of the spectral properties of individual chloroplasts). Such an approach could provide essentially new information concerning the kinetics of photoreactions in cells.

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SPETROSKOPIA PRÓBEK BIOLOGICZNYCH W TEMPERATURZE POKOJOWEJ I W NISKICH TEMPERATURACH

Streszczenie

Praca zawiera przegląd metod spektroskopowych stosowanych w biologii ze szczególnym uwzględnieniem spektrofluorymetrii. Omówiono technikę absorpcyjnej spektroskopii różnicowej oraz źródła błędów powstających przy zastosowaniu tej techniki do badania organizmów fotosyntetyzujących. Ponadto przedyskutowano technikę badań spektroskopowych materiałów biologicznych w niskich temperaturach.

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