PECULIARITIES OF ABSORPTION SPECTROPHOTOMETRY OF BIOLOGICAL OBJECTS

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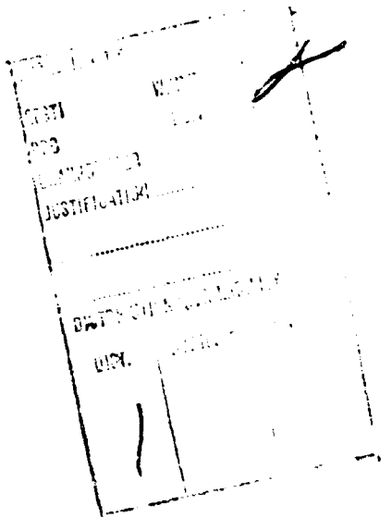

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I. Introduction

[Following is the translation of an article by L. N. Bell, published in the Russian-language periodical Biofizika (Biophysics), Vol X, No 2, 1965. Pages 374--385. Translation performed by Sp/7 Charles T. Ostertag, Jr.]

The mission of absorption spectrophotometry is the determination of the coefficient of absorption (absorbance) of a body in various bands of the spectrum, that is, a determination of its "absorption spectrum." The absorption coefficient of a body at a given wavelength is the ratio of the energy of radiation absorbed by the given body for a given time, to the energy of radiation of the same wavelength which fell on the body for the same time:

$$A = I_{abs.} / I_{incid.} = I_a / I_0$$

The value I_0 represents the power of the radiation reaching the body and bears the designation of the intensity of radiation reaching the body. Similarly, I_a is called the intensity of radiation absorbed by the body.[1]

Footnote 1. A known lack of coordination is observed in the terminology used in spectroscopy. In this article we are mainly adhering to the terminology established by GOST 7601-55 ("Physical Optics. Designation of Main Values").

If the body being investigated is homogeneous and certain other conditions are fulfilled, about which we will talk later, then the appearance of the absorption spectrum is determined mainly by the nature and amount of light absorbing substance, and this circumstance lies at the basis of the spectrophotometric method of quantitative and qualitative analysis, which has been used already for a long time in chemistry and biology.

At the present time, when all the more importance has been acquired by the investigation of the physical-chemical bases of biological phenomena, optical, and in particular spectroscopic, methods are being more extensively used in connection with the fact that they are particularly convenient for

intravitam observations. In contrast to the complex biochemical methods, the optical methods, as a rule, do not disrupt the normal course of biological processes and, consequently, reduce the probability of the appearance of artifacts during the investigation.

Up until recently spectrophotometry was used in biology mainly for the investigation of solutions of substances (pigments in particular) which were extracted from the organisms being studied. Since these solutions were usually homogeneous and do not scatter light, then the usual spectrophotometric methods were sufficient for conducting measurements. However, when carrying out observations directly on a biological object (blood, a leaf, tissues) a number of new moments emerged. The investigator is not always suspicous of these but disregarding them could involve serious after effects.

The main difference between biological objects and solutions in an optical sense is their heterogeneity. This circumstance influences the spectrophotometric mission in two respects. First of all, here the absorption coefficient and its spectrum are determined not only by the nature of the substance of which the object under investigation is made up, but by the nature of its distribution and also the distribution of the substance which is not absorbing, but scattering. Secondly, as a result of the scattering of light in the object, the usual (that is, suitable for non-scattering objects) methods of measuring the absorption coefficient often prove to be unsuitable.

Thus, we should distinguish two types of peculiarities in spectrophotometry of biological, that is, heterogeneous and scattering, objects: Peculiarities in the technique of measuring and peculiarities connected with a change in the absorption spectrum in comparison with the spectrum of the corresponding homogeneous and non-scattering object.

The aim of the present article is to examine the noted peculiarities. Here only those peculiarites will be analyzed which are encountered most often in biology and the nature of which is understandable to a degree which is sufficient for them to be taken into consideration. Besides this, we will examine only the spectrophotometry of the visible range of the spectrum, since it is primarily this range which plays a decisive role in nature, and mainly in the life of plants.

2. Some General Problems of Absorption Spectrophotometry

The ability of a body to retain incident light, that is, its ability to absorb radiation, depends not only on the nature of the absorbing substance (this dependency is the basis for the spectrophotometric method of analyzing substances), but also on the form and dimensions of the body under investigation, on the nature of the distribution of the absorbing and scattering substance in it, the scattering of light within the body and the direction of the incident rays.

The nature of the spectrophotometric mission may be diverse, depending on the goals of the investigation. Thus, in certain problems it may be necessary to determine the absorption coefficient of an object on the whole (as an example, an entire leaf). If the body is sufficiently homogeneous, then in a number of cases it may be sufficient to have a knowledge of the absorption coefficient of a specific sector of the object or a unit of volume of the medium from which it is made up. We encounter such a case, as an example, when investigating homogeneous suspensions or solutions. If the medium is made up of separate absorbing particles and a non-absorbing ("binding") substance, then the greatest interest may concern the stream of radiation absorbed by each particle separately. An example of a similar mission is found in the investigation of the photoenergetics of individual chloroplasts. Finally it is sometimes desirable to determine separately the absorption coefficient of the various substances which are components of the particle. This mission emerges in certain analytical investigations, when based on the measured absorption coefficient it is necessary to determine the nature of the absorbing substances or to follow the kinetics of their change. This mission is encountered in certain energetic problems, for example, when determining the photosynthetic effectiveness of various plant pigments.

And so it may be said concerning the determination of the absorption coefficient of a body, a medium, a particle or a substance. Not only the theoretical interpretation, but also the methods of experimental investigation of these missions are diverse [1]. In the present article we will mainly be concerned with the determination of the absorption coefficient of a body (object) on the whole, and with the factors which influence the form of its absorption spectrum. We note that the general problem of the spectroscopy of light scattering media both experimentally as well as in the peculiarities in the theoretical aspect is very complex and only in recent years it has begun to be developed as a new branch of physical optics.

We will now examine a brief problem concerning the peculiarities of measuring the absorption coefficient in the general situation of the incidence of light from an arbitrary direction on heterogeneous scattering objects of an arbitrary form.

As was already said, the absorption coefficient is defined as the relationship of two values -- the intensity of absorbed radiation and the intensity of radiation which falls on the body ($A = I_a / I_0$), and therefore its determination is reduced to the measurement of these two values or the measurement of two values equivalent to them.

Generally speaking, by using optical methods it is not possible to directly determine the amount of energy absorbed by a body. It is possible, however, to determine the intensity of rays which are not absorbed by the object I_t. We will designate these rays as outgoing (in certain cases they are called transient).

In general, outgoing rays (non-absorbed) consist of rays [illegible] not penetrate into the object and were reflected from its surface (1, figure 1), of rays which have penetrated into the object, been scattered and then emerged from it (2 and 3, figure 1), and of rays which had passed through the object without changing their direction (4, figure 1). In some cases these rays can be examined separately. For example, the mechanism of surface reflection is essentially different from the mechanism of "reflection" of rays which had entered the object and undergone scattering in it. In the present article we unite all the rays coming out of the object together regardless of the nature of their interaction with the substance of the body being investigated. Apparently, the amount of absorbed energy is equal to the difference between the intensity of incident radiation on the object and the intensity of radiation coming out of it:

$$I_a = I_0 - I_t$$

Consequently,

$$A = \frac{I_a}{I_0} = \frac{I_0 - I_t}{I_0} = 1 - \frac{I_t}{I_0}$$

The relationship of the intensity of outgoing rays to the intensity of incident rays on the object we designate as the transmission coefficient *: $T = I_t / I_0$ and, consequently, $A = 1 - T$.

Footnote * Since the transmission coefficient depends on the intensity of outgoing rays, part of which in general may not enter the object and therefore may not be passed by it, then the term "transmission" is somewhat unfortunate. Somewhat better would be the term "coefficient of scattered transmission," but since here also the word "transmission" is preserved, then we, not risking the introduction of a new term, will use the term mentioned in the text as the one which has received the widest distribution.

So in essence the definition of absorption coefficient is reduced to the definition of transmission coefficient.

As a rule the determination of the intensity of incident rays, I_0, is a simpler mission. The level of complexity of this measurement is determined by the nature of the spectrophotometric mission.

In certain cases the direction of incident light does not depend on the investigator. As an example we point out the determination of the absorption coefficient of leaves under field conditions, when the conditions of illumination and the form of the object are not assigned by the investigator. Thus, in the overall case the measuring of incident flux is a technically quite difficult mission. In principle this may be done with the help of a light sensitive device, having a sensitive surface which is identical in form to the surface of the object, but in practice this is very difficult to realize. An exception are spherical bodies which are exposed on all sides to light of the same intensity. For such bodies, it is possible with the help of special devices to measure the so-called integrical radiation exposure.

Another possible method is the successive measuring of light intensity based on all the elements of the surface of the object, which also is not a simple operation.

Thus, we see that measuring even such a value as the stream of incident light, which at first glance looks simple, may be accompanied with great difficulties.

Often however, with the peculiarities of laboratory work, the nature of the incident light on the object can be assigned by the investigator, and then the mission of determining I_0 is significantly simplified. It is most convenient to select the incident light in the form of a parallel pencil with an uniform intensity throughout the cross section, since in this case it is possible to use a plane light detector for measuring the intensity of the stream of incident light.

For determining the second value which is necessary for the determination of the absorption coefficient -- the stream of outgoing light -- in the general case one measurement with the help of the plane detector is insufficient, even with the incidence of parallel rays. Actually, due to scattering some of the rays do not reach the plane detector and therefore the latter does not register all the outgoing (non-absorbed) rays (figure 1). The transmission coefficient, measured by such (incorrect) a method, will be less than the true value, and consequently the absorption coefficient will be overestimated. Thus, the main condition which should be observed when measuring the stream of outgoing rays is that the light measuring device register all the rays which are coming out of the investigated object at various angles.[2]

Footnote 2. On the other hand there exists another difficulty in measuring the stream of scattered rays. This is conditioned by the fact that it is difficult to realize a plane detector, the sensitivity of which does not depend on the angle of incidence of the rays (there would be a "cosine dependency").

If the body does not scatter rays and the latter fall on the object in a parallel pencil, then the plane detector may be used for measuring I_0 and I_t, as this is done in the ordinary Bekman type spectrophotometers.

So from what has been stated it is clear the selection of the method for measuring I_t depends significantly on whether or not the object scatters light.

Spectrophotometric measuring of non-scattering media (solutions for example) has received quite extensive dissemination and therefore for many investigators the concepts and methods of primarily this branch of spectrophotometry are usual. However, sometimes these concepts and methods are used uncritically when investigating heterogeneous or scattering media, and

this often leads to inaccurate and sometimes even paradoxical results. It happens that the investigators do not always take into consideration (or know) the conditions of applicability even for those concepts which are used for describing homogeneous non-scattering media.

For this reason we will first review the method of spectroscopy of homogeneous non-scattering media.

3. Spectrophotometry of Non-scattering Objects

Methodically the problem of determining the intensity of rays coming out of the non-scattering object, I_t, is the same for a homogeneous and heterogeneous object, that is, for an object with an uniform or a non-uniform distribution of substance in the object. However, the results and their interpretation may be different in these two cases and therefore it is expedient to examine them separately.

A. Homogeneous objects. It was pointed out above that if the object does not scatter light, then the measurement of I_0 and I_t is a methodically quite simple problem under the conditions that parallel rays are falling. If the reverse is not stipulated, it will be further assumed that a parallel pencil of rays is falling on the object. Then it is possible to use a plane detector. In this, one calculation is made in the absence of the object, which gives I_0, and another in its presence, which gives I_t. Here the spectral curve of sensitivity of the detector has no importance if monochromatic light is falling on the object. The cross section of the object should not be simultaneously less than the section of the pencil of rays and the sensitive surface of the detector, since otherwise in the presence of the object some of the rays will reach the detector which had not passed through the object, and a lowered value for the absorption coefficient will be obtained. This inadmissible case is depicted in figure 2, a. Cases b, c, and d are permissible, since in the presence of the object only those rays reach the detector which have passed through the object. Cases c and d take place in ordinary spectrophotometers.

For a homogeneous non-scattering medium the law of Lambert--Buger holds true. This law says that the relative lessening of intensity ($-\Delta I/I$) of a narrow pencil (ray) of monochromatic light, passing through a layer with a small thickness Δx, is proportional to this thickness and does not depend either on the intensity of the light or the depth of the layer in the medium.

Mathematically the law of Lambert--Buger may be expressed in the following differential form.

$$-\Delta I/I = k\,\Delta x \qquad (1)$$

Here I is the intensity of light, falling[3] on the layer with the thickness Δx ; $-\Delta I$ is the lessening of the intensity of the light as a result of absorption. The coefficient k designates the index of absorption of the given substance[4]. It is the same physical parameter as, for an example, the index of refraction of the given substance. The numerical value of k and its dimensionality depend on the units in which the thickness of the layer is measured and not on the units of measurement for light intensity. Thus, if x is measured in centimeters, then the dimensionality of k will be cm^{-1}.

Physically the significance of the law of Lambert and Buger consists of the fact that the probability of the absorption of a photon of monochromatic radiation by a molecule does not depend on the density of the photons, that is, on the light intensity. Apparently this cannot take place if the number of excited molecules makes up a noticeable portion of the total number of molecules. Actually, the optical properties of excited molecules (more accurately, the probability of absorption of a photon) may be different from those of non-excited molecules. With very great light intensities the number of modified (excited) molecules may become appreciable. Such a possibility is especially probable if the time of life of the excited molecule is great, as in the case of fluorescent or phosphorescent molecules (when the period of the excited condition may reach up to seconds or minutes). For non-fluorescent substances the index of absorption, as the classic tests by Vavilov [2] showed, remains practically constant during a change of the intensity of the rays by 10^{20} times, from $2.5 \cdot 10^{-12}$ up to $2 \cdot 10^{8}$ erg/cm^{2}sec. For phosphorescent substances a lessening of the index of absorption was observed already at comparatively small intensities.

Footnote 3. More accurately, entering the layer.

Footnote 4. Often just this value is called the absorption coefficient; sometimes it is also called extinction or extinction coefficient. The accepted name is recommended by GOST.

The formula (1) expresses the law of Lambert-Buger in a differential form. For practical applications it is necessary to use the integral form of the law, obtained by means of integration of the equation (1):

$$I_2 = I_1 e^{-k(x_2 - x_1)} \qquad (2)$$

This formula shows that if onto the layer of subetance at a depth of x_1 there falls a narrow pencil of light with the intensity I_1 , then

the intensity of light at the depth of x_2 will be I_2, determined by the formula (2). If it is considered conditionally that a ray falls on a layer with $x_1 = 0$ (for example, on the surface of the object), then $x_2 - x_1 = x_2 = x$ will be equal to the path covered by the ray in the object and the formula takes on the usually encountered form (figure 3, a):

$$I = I_0 e^{-kx} \tag{3}$$

or $T = e^{-kx}$, where $T = I/I_0$ is the transmission coefficient. **

Footnote **. In those cases when the light is not scattered, instead of the term transmission coefficient, the term "transparency" is recommended. Thus, in the given case, T is the transparency.

The product $D = kx = -\ln T$ is called the optical density. In the case of solutions of stained substances (pigments) the absorption index k often turns out to be proportional to the concentration of pigment (Ber's law): $k = \varkappa c$, where $\varkappa$ is called the specific index of absorption and c is the concentration. Then the optical density

$$D = \varkappa cx = \varkappa\delta$$

is proportional to $\delta = cx$, that is, to the surface area of the stained substance. In practice the law of Lambert-Buger is usually represented in the form

$$T = 10^{-k'x} = 10^{-D'}$$

and in this case the optical density is defined as the negative decimal logarithm of the transmission coefficient: $D' = \lg \frac{1}{T}$. Here $D = 2{,}3 \cdot D'$.***

Footnote***. Strictly speaking, just D' is called the optical density. According to GOST, D, k, etc. should be designated as natural optical density, natural absorption index, etc.

Already from a simple examination of the formula (3) it is possible to make a number of conclusions relative to the conditions which should be satisfied for its practical application. Some of these requirements are relative to the nature of the incident rays, some -- to the properties of the object being investigated. These conditions are as follows.

1. Equality of the paths, covered by all the rays of the light stream in the object. In practice we are always dealing with light pencils with a finite cross section. In the formula (3) the length of the path x apparently should be the same for all the rays of the pencil. Namely

therefore, usually speaking we apply the law of Lambert-Buger to parallel rays, falling on a plane-parallel object. Actually, other configurations, generally speaking, are also permissible and in particular one such as that which is depicted in figure 3, b. The wide application of parallel rays and plane-parallel objects (or cuvettes for solutions) is explained exclusively by reasons of convenience.

Since in principle it is impossible to obtain absolutely parallel pencils of rays from a source with finite dimensions [3], then there will always be a certain error, caused by the difference between the path length of the light rays in the plane-parallel object and the thickness of the latter. Usually the greater the angular deviation of the rays, then the greater the dimensions of the source of light and the less the focal distance of the collimator lens. In the ordinary SP-4 spectrophotometer the direction of the rays is easily detected by placing a sheet of white paper on the path of the rays and moving it along the pencil of light. The width of the representation of the exit aperture of the monochromator is changed with this, and this designates the direction. Such a simple experiment shows that the angular deviation of the rays in the SF-4 spectrophotometer equals about 1.5°, which leads to a less than 0.1% scattering of rays in a cuvette. Consequently, the relative error in determining the optical density will also be less than 0.1%. The relative error in determining the transmission coefficient will depend on the absolute value of the latter. Thus, when $T = 0.3$ the relative error $\Delta T/T$ turns out to be equal to a 0.1%; when $T = 0.5$, $\Delta T/T = 0.06\%$ and when $T = 0.7$, $\Delta T/T = 0.03\%$. Thus, as the accuracy of ordinary spectrophotometers usually does not exceed 0.1%, then the error from a non-parallel pencil is immaterial, at any rate when there are no large optical densities.

2. The optical homogeneity of the object. The formula (3) may be obtained from the formula (1) only if the absorption index k does not depend on x, that is, on the point in the object. In order that the condition be fulfilled, it is sufficient that the body under investigation be homogeneous physically and chemically. The condition of such homogeneity, strictly speaking, is not necessary; in principle various substances may have the same k. However, in order that the law of Lambert-Buger be applied to such chemically heterogeneous substances, still another additional condition must be fulfilled: The refraction indices of the substances making up the body should also be the same or in any case should not be sharply shifted from point to point, which is necessary so that the body does not scatter light.

3. Monochromatism of light. This condition is necessary for fulfilling the differential law and all the more for the integral. Actually since the absorption index in a general case depends on the wavelength of light, then apparently for non-monochromatic light it is impossible to select such a coefficient k in the formula (1) which would not depend on the position of the layer in the object. In actuality, as the light penetrates the object the spectral composition of the light is changed and therefore for non-monochromatic light the effective value of k is also

changed. Therefore, the absorption index in the general case does not depend on the depth of the layer in the object; only in the event of strictly monochromatic light. An exception are the so-called gray bodies in which k does not depend on the wavelength, and therefore for them the formulas (1) and (3) are applicable even with the incidence of non-monochromatic light.

The light used for spectrophotometric measurements, strictly speaking is non-monochromatic and consists of rays lying in a certain interval of wavelengths. The distribution of light intensity based on wavelengths in prism spectrophotometers has approximately the form of an isosceles triangle (figure 4, b). The degree of monochromatism (spectral frequency) of the light depends on, in addition to other factors, the width of the input aperture of the monochromator. The narrower the aperture, then the more monochromatic the light which is given off, but the less its intensity. Therefore, the work is practically always done with not too narrow apertures, and consequently with pencils of light of a not very high spectral frequency. This circumstance may be of much importance when measuring an absorption coefficient in the vicinity of the acute maximum (or minimum) of absorption. The essence of the affair is easy to understand if you turn to figure 4. This depicts the absorption spectrum with an acute maximum (curve a) and the spectral distribution of the light given off in the spectrophotometer (curve b). Suppose it is required to measure the absorption coefficient at the point of the maximum. We will assume that the maximum of the spectral sector used coincides with the wavelength of the maximum of absorption. It is clear that each truly monochromatic ray will be absorbed in accordance with its absorption coefficient, which is depicted in figure 4. Actually the measured absorption coefficient will be a certain average value of the absorption coefficients of various wavelengths, given off by the monochromator; from figure 4 it can be seen that this resulting absorption coefficient will be less than the true maximum value. The deviation of the measured value from the true one will naturally depend on the form of the maximum of the absorption curve and on the form of the spectral range used in the measurements. It is necessary to keep this circumstance in mind during accurate measurements in the area of the acute maximums. Calculations show, for an example, that for a solution of chlorophyll a in acetone, having an optical density $D' = 0.47$ ($T = 0.34$) in the maximum of absorption (660 mμ), the measured value of the optical density with an aperture width of 0.02 mm practically corresponds with the true value; at the same time, with an aperture width of 0.1 mm $D' = 0.40$ is obtained, that is, a difference of 15%.

The influence of non-monochromatic light may be still more significant in a more general case when the absorption spectrum is determined of a substance which is found in a mixture with other stained substances. If at first the absorption spectrum of the whole mixture is measured and then the spectrum of all the components, with the exception of the one being investigated ("control" or "background"), then the desired spectrum is found by means of subtracting the second from the first, that is, it is a

difference spectrum. When using non-monochromatic light this difference spectrum may differ strongly from the true one in the vicinity of the maximums and with small optical densities for the substance being investigated [4].

It must also be noted that with non-monochromatic light the spectral selectivity of the detector may also influence the result of the measurements.

4. Absence of long-lived excited states for the molecules of the object. It was already stated that one of the most important conditions for the applicability of formula (1), and consequently the resulting formula (3), is the absence of long-lived excited states for the molecules of the object. Since many biological pigments possess photoluminescence and, consequently, may be found for a prolonged time in the excited state, then under certain conditions a deviation may be observed from the law of Lambert-Buger.

It can be shown that when calculating the time of the excited state of the absorbing molecules, τ, the usual Lambert-Buger formula (3) is replaced by the following:

$$I = \frac{I_0 e^{-kx}}{1 - \frac{I_0 k \tau (1-e^{-kx})}{h\nu\rho}} = \frac{I_0 e^{-kx}}{1 - N_0 \sigma \tau (1-e^{-kx})} \qquad (4)$$

Here I, I_0 and k, just as previously, denote correspondingly the intensity of transmitted and incident light and the absorption index; N_0 is the intensity of incident photons (the number of photons falling on 1 cm^2 in a second), σ is the molecular index of absorption of the absorbing substance: $\sigma = k/\rho$, where ρ is the number of molecules of the absorbing substance in 1 cm^3.

From formula (4) it is apparent that the deviation from the Lambert-Buger formula is determined by the factor

$$[1 - N_0 \sigma \tau (1-e^{-kx})]^{-1}$$

For definiteness we will consider that a essential deviation takes place if formula (4) deviates from formula (3) by more than 1%. Then the condition of the deviation from the Lambert-Buger formula is written thusly:

$$N_0 \sigma \tau (1-e^{-kx}) > 0.01$$

or for sufficiently great depths where the deviation is most noticeable

$$N_0 \sigma \tau > 0.01 \qquad (4')$$

In this manner an infraction of the Lambert-Buger law will be observed if on a strongly absorbing substance (σ sufficiently great) with continuously excited molecules (τ sufficiently great) there falls a sufficiently intense stream of light (N_0 is great).

The table presents the values of the light intensities (in erg/cm^2 · sec) which are necessary for carrying out the conditions (4') with different values of σ and τ . The least values of σ correspond to the minimum absorption of chlorophyll a (472 mμ), and the greatest -- to the maximum of absorption of chlorophyll a (660 mμ).

σ, cm^2	τ, sec: 10^{-8}	10^{-3}	1
$1.5 \cdot 10^{-16}$	$2.0 \cdot 10^{10}$	$2.0 \cdot 10^{5}$	$2.0 \cdot 10^{2}$
$1.7 \cdot 10^{-18}$	$2.5 \cdot 10^{12}$	$2.5 \cdot 10^{7}$	$2.5 \cdot 10^{4}$

The data of the table shows that even for strongly absorbing substances (red maximum of chlorophyll) a deviation from the Lambert-Buger law will be observed in ordinary fluorescing substances ($\tau = 10^{-8}$ sec) only with light intensities on an order of $2 \cdot 10^{10}$ erg/cm^{2}sec (in the tests by Vavilov [2] the greatest intensity was $2 \cdot 10^{8}$ erg/cm^{2}sec). For $\tau = 10^{-3}$ sec a deviation may take place already at intensities on the order of $2 \cdot 10^{-5}$ erg/cm^{2}sec which is half as much as the intensity of sun light (in the visible range) on a bright day.

B. Objects with an irregular distribution of the absorbing substance. In the previous section we examined homogeneous objects, that is, it was assumed that the absorbing substance was distributed uniformly throughout the entire volume of the object. Most often in biological objects this condition is not fulfilled, and the pigments are usually concentrated in small structural units (chloroplasts, erythrocytes, etc.) which are divided by the non-absorbing or weakly absorbing medium.

In connection with this the problem arises concerning the dependency of the value of the absorption coefficient A, the absorption spectrum A (λ), and the spectrum of optical density D (λ) of the object on the nature of the distribution of the pigment in it.

We will assume that the heterogeneity of distribution does not lead to the scattering of light. Generally speaking, heterogeneity of distribution of the absorbing substance is combined with the scattering of light, but since we want to clear up what effects may be caused by a certain non-uniformity in the concentration of pigment, then we will still consider that scattering does not take place. However, in certain cases it is possible to experimentally remove the influence of scattering. Thus, when working with suspensions of cells, it is possible to significantly decrease scattering by suspending the cells in solutions of the appropriate proteins. If the index of refraction of the solution is selected as close as possible to the index of refraction of the cells which are being investigated, then scattering is sharply decreased and almost a transparent suspension is obtained [5]. In similar "elucidated" suspensions the difference in the absorption spectrum and the spectrum of the corresponding extract of pigments is mainly conditioned by the non-uniformity in the distribution of the pigments, and also the possible change in their condition in living cells (for example, due to their bond with specific protein within the cell).

We note that the scattering of light also decreases sharply during the seepage of water in leaves [6].

1. The first peculiarity of the heterogeneous media under examination consists of the following: The absorption coefficient at any wavelength is always greater with an uniform distribution of the pigment than with a non-uniform. In particular, a homogeneous solution always absorbs greater than any other object of the same form containing the same amount of pigment which is distributed non-uniformly. Proof of this provision is given in the work /7/. The circumstance that more light passes through a non-uniform object than through an object with an uniform distribution of pigment confirms the inaccuracy of the work /8/, in which it is affirmed that the absorption coefficient of a system of absorbing tubes (calculation made for a system of blood vessels) does not depend on their number or diameter, but only on their volume, that is, the total amount of pigment.

The physical significance of the provision being discussed is easily cleared up by an examination of the following simple example. Assume that one cuvette is filled up with a homogeneous solution, and in another similar cuvette the pigment takes up only half of the volume (for example, the cuvette is divided by a membrane through which the molecules of the pigment do not pass). Suppose that on each half of the cuvette the stream I_0 falls and $I_i = e^{-\varkappa\delta_i}$ $(i = 1,2,3)$ passes (figure 5, a).

It is asserted that more light passes through an object with a non-uniform distribution of pigment, that is $I_2 + I_3 > 2I_1$, which is equivalent to $I_2 - I_1 > I_1 - I_3$.

If we turn to the lower of the curves on figure 5, b, which depict the dependency of transmitted light on the surface concentration of the pigment δ, then it is easy to see that this latter inequality actually takes place.

Physically this amounts to the fact that with a disruption of the uniformity of distribution of the pigment, more light begins to pass through the "enlightened" place (in our case this change equals $I_2 - I_1$) then is absorbed in the "darkened" places (a lessening of intensity, in our example of $I_1 - I_3$). The cause of this is concealed in the fact that the curve of transmission drops more rapidly at small values of surface density than at large ones, that is, it is transformed by a curvature downwards. If the law of light transmission was such that curve (δ) was transformed with the curvature upwards, then the result would be the reverse, that is heterogeneous objects would absorb most of all.

2. The second peculiarity, caused by the non-uniform distribution of the pigment, consists of the fact that the very form of the absorption curve is changed, that is, the absorption coefficient at various wavelengths is not increased by the same number of times with the disruption of homogeneity. Qualitatively it is possible to comprehend the reason for this if we again turn to figure 5, b, which depicts the curves of transmission for the stated object at two wavelengths, for which $\varkappa_1 > \varkappa_2$. Accepting the reasoning similar to that presented above, it is possible to be satisfied that with a disruption of the homogeneous distribution, for example in such a way that half of the object turns out to be without pigment ($\delta = 0$) and all of the pigment is concentrated in the other half of the object ($\delta'' = 2\delta_0$), an increase of light transmission will be greater for the more strongly absorbed wavelengths, that is

$$(I_2 + I_3 - 2I_1)_{\lambda_1} > (I_2' + I_3' - 2I_1')_{\lambda_2},$$

For a more quantitative demonstration of the "equalization" of the absorption spectrum with a disruption of the homogeneity of the object, we will examine a case when with the stated disruption a certain part of the light p passes through the absorbing medium, and the remaining part $1-p$ passes freely without absorption. Then if through D we designate the optical density of a homogeneous absorbing medium, it is possible to write:

$$T_{\text{homogeneous}} = e^{-D} \tag{5}$$

$$T_{\text{heterogeneous}} = (1-p) + pe^{-D/p} \tag{5'}$$

since, apparently, the concentration of absorbing substance in a heterogeneous medium is concentrated in less volume, and therefore the optical density increases by $1/p$ times. Figure 6 shows the dependency of the relationship of the absorption coefficient of a heterogeneous medium,

that is, $1-T_{hetero}$, to the absorption coefficient of a homogeneous medium, $1-T_{homog}$, as a function of optical density of the initial homogeneous medium with various values of ρ. It is apparent from figure 6 that the greater the degree of heterogeneity, that is the less ρ, then the greater is the difference in the absorption coefficients of the homogeneous and heterogeneous media at a given optical density. The absorption coefficient of a suspension is reduced very noticeably in the area of strong absorption, that is, there where the optical density is great. As a measure of the increase in optical density, the ratio $\frac{1-T_{hetero}}{1-T_{homog}}$ approaches ρ. In the area of weak absorption (small D) the absorption coefficients of homogeneous and heterogeneous media differ little. Consequently, a disruption of homogeneity leads to a strong reduction in the absorption band and to a relative decrease of the absorption coefficient in the area of weak absorption. The corresponding change in the absorption spectrum is depicted schematically in figure 7. There occurs a equalization, or leveling of the spectra of transmission and absorption due to the great decrease of the absorption coefficient in the area of strong absorption.

An example of the effect under discussion is the fact that the absorption coefficient of a leaf in the blue and the red ranges of the spectrum, that is, where the leaf absorbs most strongly, is less than the absorption coefficient of a solution of the isolated pigments with their same average surface density.

A special case of heterogeneous media are suspensions of stained particles. This case is specially interesting since it is often encountered in biology.

A suspension may be viewed as an exceptional case of a heterogeneous medium when it has a large number of strongly absorbing agglomerates of pigment. This case may be reduced to what was said above (see figure 6), if the total area of the cross sections of all the particles is less than the area of the base of the column of the suspension under investigation, which may be selected as equal to 1 cm^2. In other words, we consider that the "degree of covering" of the suspension $\rho = NS \ll 1$, where N is the number of particles in the column (surface density of the particles) and S is the area of a cross section of one particle. Physically this indicates that the absence of a mutual darkening of the particles is assumed.

Consequently, a leveling of the absorption spectrum, about which we spoke earlier, also takes place for suspensions. In particular, if the optical density of the pigment of the solution is so great that light practically does not pass through the solution, then following aggregating of the pigment part of the light may pass through the suspension without hindrance, in general not being absorbed. This "effect of passage" is one of the reasons why it is a mistake to consider that the absorption coefficient

of a structurized object (for example, a leaf) can be appraised based on the absorption coefficient of a solution of pigments isolated from it and having the same average optical density as the leaf. Even only for this reason the concept of "effective leaf", which is sometimes encountered in literature on the physiology of plants, is completely defective.

3. It is important to stress that the stated equalization of the absorption spectrum takes place without fail only with a strong disruption of homogeneity. With small disruptions in the uniformity of the distribution of the pigment, when the surface density of the pigment is changed insignificantly, the absorption curve may either level out or, just the opposite, "deepen", depending on the absolute value of the surface density of the pigment (δ) and on the ratio between specific indices of absorption at various wavelengths.

The essence of the matter is as follows. At small changes of surface density the increase of the coefficient of transmission depends on the curvature of the curve of transmission, that is, on how rapidly the slope of thi curve changes (figure 8).

Actually, if in the vicinity of a certain point the slope of the curve of transmission would be constant, then it is apparent that the increase of the surface density by a small value in one area of the object and the corresponding decrease in the other area would lead to equal and reverse changes in the coefficient of transmission. But namely because the curvature is greater with small values for the surface density, that is in the area of great transmission and the result is (just as with any disruption of homogeneity) that the total coefficient of transmission is increased: $\Delta T_1 > \Delta T_2$. The curvature for the curve of transmission is determined by the second derivative, $\partial^2 T/\partial \delta^2 = \varkappa^2 e - \varkappa\delta$ and therefore depends on the specific index of absorption $\varkappa$ and on the surface density δ .

The dependency of $\partial^2 T/\partial \delta^2$ on the specific index of absorption with the prescribed values of surface density has the form of a curve with a maximum (figure 9). The maximum value is achieved with the optical density $\varkappa\delta = 2$.

From an examination of figure 9 it is seen that for any two wavelengths λ_1 and λ_2 , of such that $\varkappa_2 > \varkappa_1$ (for example $\varkappa_1 = 1.33$ and $\varkappa_2 = 2.0$), the increase in the coefficient of transmission with a small disruption of homogeneity will be greater for the more strongly absorbable wave in the event of a relatively small surface density $\delta = 1$; a leveling of the absorption curve occurs. With a small density of the pigment ($\delta_2 = 1.5$) a small disruption of homogeneity leads to the fact that transmission is increased more strongly for the less absorbable light, that is, for light with $\varkappa_1 < \varkappa_2$, and consequently there takes place a "deepening" of the absorption curve.

Since with a set thickness for the object and a concentration of pigment, the values for the specific indices of absorption may be very diverse, then the relative values of the curvature will also be diverse and therefore the coefficients of absorption with the same wavelengths will draw together and with others will diverge. Similar effects may have importance when investigating small changes in the coefficient of transmission, as, for an example, when studying differential spectra [7].

4. Above we examined a change of the absorption coefficient, caused by the redistribution of the pigment of any one type. Often biological objects contain several various pigments. It is evident that if the redistribution of the pigments takes place in such a way that only the arrangement (order) of the pigments along the path of the light ray is changed and the surface density of each of these along the ray is not changed, then the total absorption coefficient of the object is not changed since the coefficient of transmission equals the product of the coefficients of transmission of the separate pigments (figure 10, a and b).

But if the redistribution of the absorbing substances leads to a change of surface density, then the coefficient of transmission is changed (figure 10, c).

In the cited example, transmission in case c is greater than in case a or b, since $\frac{T_1^2 + T_2^2}{2} > T_1 T_2$ which is equivalent to $(T_1 - T_2)^2 > 0^2$.
In general and here the theorem is applicable which states that the minimum coefficient of transmission takes place when the optical density is the same throughout the entire sample (the proof refers to a plane-parallel sample on which a parallel pencil of light falls perpendicularly).

5. In the preceding discussions it was assumed that the redistribution of pigments did not lead to a change of their specific absorption index. Actually with a strong drawing together (aggregating) of the molecules their interaction increases and the electron configuration of the atoms may be changed. As a result the optical properties of the molecules may also be changed. As a rule, aggregating leads to a broadening or narrowing of the absorption bands, shifting them to the long wave range and less often to separation into separate bands. Besides this, it is also possible that an interference of waves which are scattered by neighboring particles and other "cooperative phenomena" take place. [7]. These problems will not be examined in the present article. Nevertheless it is always necessary to keep in mind that a change in the degree of aggregating conditions a change in the absorption spectrum, caused not only by physical but also chemical reasons. A good example is the spectrum of pigments derived from leaves or cells of algae. Upon the extraction of the pigment a shift of the absorption band to the short wave range takes place as a result of a change in the physical-chemical condition of the pigment molecule; besides this, there is an increase of the absorption coefficient in the maxima (red and blue) as a result of the loss of passage

effect and a decrease of absorption in the area of weak absorption (green) as a result of the loss of scattering[5] (see example in figure 69, c /6/).

Footnote 5. The role of scattering will be examined below.

6. It was already noted that even with the incidence of a parallel pencil of light on a plane-parallel homogeneous object, the form of the absorption spectrum (in any case the relative values of the ordinates) depends not only on the nature of the absorbing substance, but also on its concentration and the thickness of the object. On the other hand, the form of a spectrum with the optical density $D = \lg T$ does not depend on the stated factors and therefore this spectrum is suitable in the best way possible for the identification of the absorbing substance. In truth, $D = k\varkappa$, and therefore a change in the thickness of the sample changes all the ordinates of the spectrum of optical density by the same number of times, and consequently does not distort the form of the spectrum. Besides, if the law of Beer takes place, that is $D = \varkappa c\varkappa$, where c is the concentration of pigment, then a change of the latter does not change the form of the spectrum of optical density. For this reason in analytical problems it is acceptable to work with the spectrum of optical density and not the spectrum of absorption.

In connection with this the problem arises concerning the expediency of using the spectrum of optical density in those cases when the object is not homogeneous. To begin with, it is evident that for a heterogeneous body the optical density, formally determined by the equation $D = -\lg T = -\ln(1-A)$, cannot be expressed through the index of absorption k and the thickness of the object $\varkappa$ by the formula $D = k\varkappa$, as this takes place for homogeneous objects, if only because k and $\varkappa$ are different for various parts of the object. Nevertheless, it can be proposed that in spite of the change in the form of the absorption spectrum during the disruption of the homogeneity of distribution of the absorbing substance, the form of the spectrum of optical density may be preserved and therefore it is expedient to work with this spectrum even in the case of a heterogeneous object. Actually the spectra of the optical density of a homogeneous and the corresponding heterogeneous objects are not the same. It is easy to be convinced of this by comparing $D_{\text{heterog}} = -\ln[(1-p)+pe^{-D/p}]$ and D (see formulae (5) and (5')). The nonproportionality of these two values denotes that their spectra are different.

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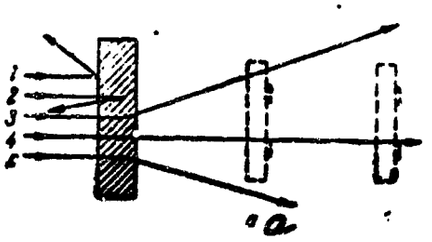

Figure 1. Scattering of rays in a heterogeneous object. The indications of the detector depend on its arrangement relative to the object.

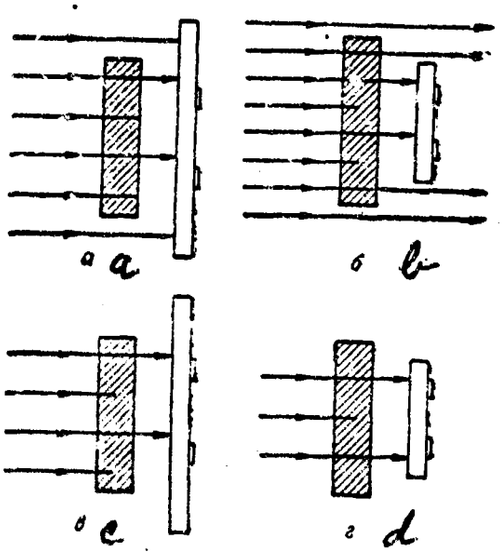

Figure 2. Various possible cases of using the plane detector for determining the absorption coefficient of a homogeneous object, onto which a parallel pencil of rays is falling.
Case a is inadmissible since it yields a lowered value for the absorption coefficient.

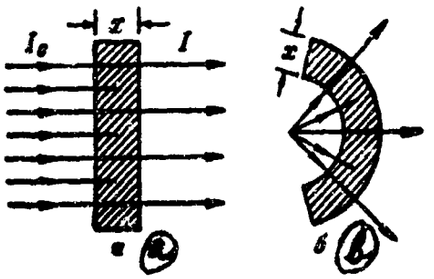

Figure 3.

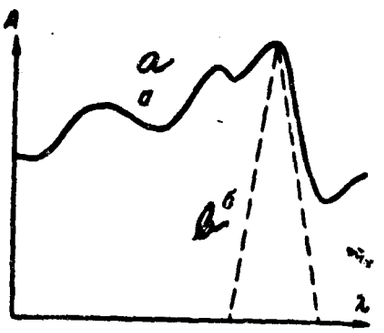

Figure 4. With a wide aperture on the spectrophotometer a wide range of the spectrum is given off (curve b) and the measured value of the absorption coefficient (A) in the vicinity of the acute maximum in the absorption spectrum a turns out to be understated.

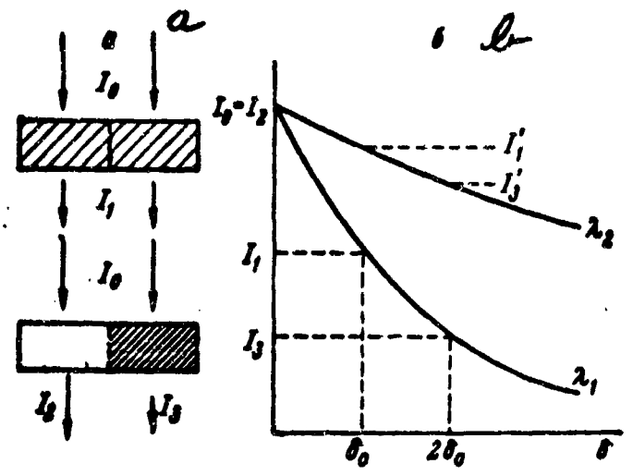

Figure 5. Curve of transmission for two wavelengths. Axis of ordinates -- intensity of passing light, axis of abscissae -- surface density.

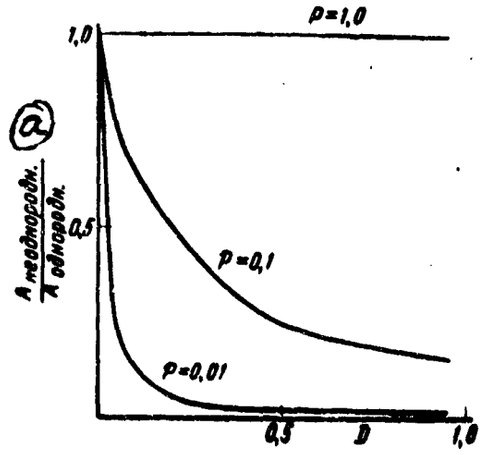

Figure 6. Relation of the coefficients of absorption of a heterogeneous object to the coefficient of absorption of an homogeneous object as the function of the optical density of the latter at various levels of disruption of homogeneity. $a = A_{heterogeneous} / A_{homog.}$

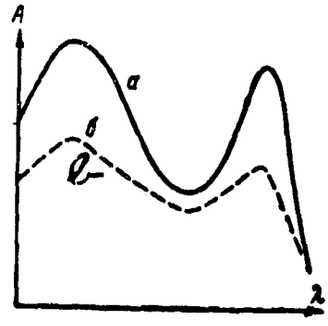

Figure 7. Spectrum of absorption of a homogeneous object - a; spectrum of absorption of a heterogeneous object - b.

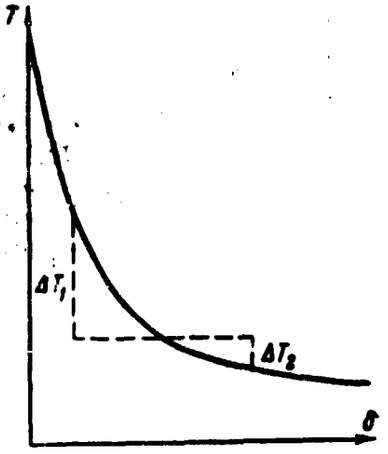

Figure 8. Change in the coefficient of transmission with small disruptions of the homogeneity of the distribution of pigment depending on the curvature of the curve of transmission $T(\delta)$

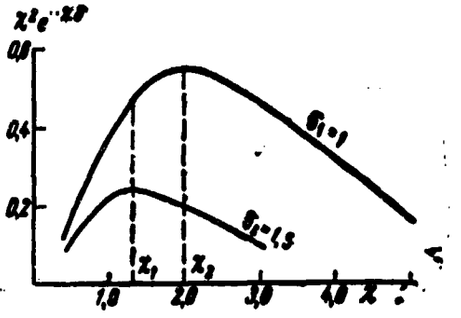

Figure 9. Dependency of the curvature of the curve of transmission on the specific index of absorption with various values of surface density of the pigment δ.

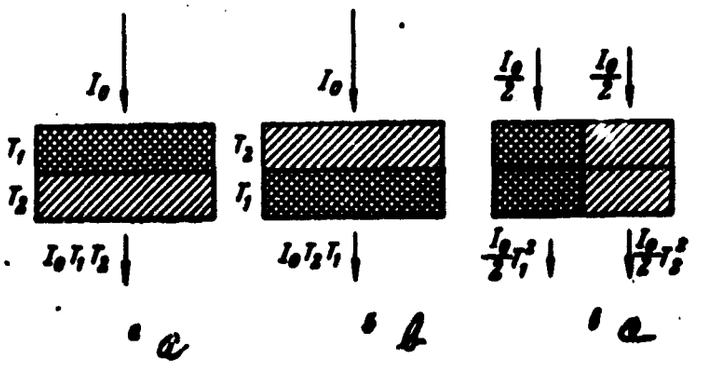

Figure 10.