SPECTROPHOTOMETRY OF INTACT BIOLOGICAL MATERIALS
ABSOLUTE AND RELATIVE MEASUREMENTS OF THEIR TRANSMISSION, REFLECTION AND ABSORPTION SPECTRA

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There has been more or less uncertainty as to the observed amount of light absorbed by translucent biological materials. The difficulties of the absolute measurement come from the optical characteristics of translucent materials, which contrast with the properties of transparent materials in the following respects.

Firstly, translucent materials reflect light, while transparent materials do not. The principle of absorption spectrophotometry of transparent materials is based on the fact that unabsorbed light comes out from them only in the form of transmitted light, so that the measurements of incident and transmitted light give us the amount of absorbed light. This principle does not apply to translucent materials, in which we have to measure the amount of reflected light, in addition to the above quantities, for calculating the amount of the absorbed light.

Secondly, incident parallel light is scattered by translucent materials. The diffuse nature of the transmitted and reflected light has made not only the absolute measurements but also the relative measurements more difficult than the measurement of the parallel light transmitted through transparent materials.

The purpose of this report is to present new techniques for measuring absolute and relative values of the transmittance and reflectance of biological samples, with a general theory on the spectrophotometry of translucent and non-transparent materials. Recently, Shibata, Benson and Calvin (1) developed a new method for observing the transmittance of translucent materials, using pieces of opal glass, and the method has been successfully applied for the measurement of biological samples (1-6). In the reports cited, however, the absoluteness of the value of the transmittance obtained by the method was not completely checked and the applications of the

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technique and its use as a relative measurement were mainly described. In the present study, the technique was further improved for obtaining absolute values and a complete theory on the technique was developed. The absoluteness of the transmittance of a sample observed by the improved method was checked in relation to the reflectance of the same sample. In the following description, the method will be called the opal glass transmission method. For observing absolute values of the diffuse reflectance of translucent samples of biological materials, another new method of using opal glass was recently presented by Shibata (7). This method will be referred to as the opal glass reflection method.

These newly developed methods have the following advantages. i) They are independent methods, which give us directly the absolute values of transmittance and reflectance. From the transmission and reflection spectra of a biological sample observed by these methods, the spectrum on the basis of true absorption can be accurately calculated. ii) They are simple and practicable, because the small pieces of opal glass can be used with most of the commonly available spectrophotometers.

For observing the relative value of the reflectance of biological samples simply and conveniently, the opal glass reflection method was modified. As will be seen later, the reflection spectra of various translucent or non-transparent materials observed by this modified method showed clear absorption bands. As one of the application of this technique, the state of carotenoids in vivo in roots or fruits was studied.

Instead of using the units of transmittance and reflectance, the logarithms of their reciprocals were mainly used. They are designated as attenuance and expressed by E throughout this study. The new word, attenuance, was adopted for translucent materials for expressing the quantity due to both absorption and scattering. It corresponds to absorbance (optical density) for transparent materials, where the quantity depends on only the absorption.

I. Principle of the Opal Glass Transmission Method

When the spectrum of a translucent biological material is measured with a commonly used spectrophotometer by the transmission method, usually the spectrum obtained has a rather high level in attenuance units, upon which are superimposed more or less indistinct diffuse absorption bands, whereas the spectrum of a solution of the pigments extracted from the sample has corresponding sharp absorption bands. A typical example is shown in Fig. 1 (7), where curve A is the spectrum of a suspension of the unicellular alga, Chlorella, and Curve C is the spectrum of the ethanol extracts from the suspension, both observed with a Cary Recording Spectrophotometer, a commonly used type.

By the opal glass transmission method, the spectrum of the suspension is greatly improved (Curve B in Fig. 1); namely i) the definition of the spectrum is remarkably increased, ii) the level (base line) where there is no absorption band is greatly reduced to a low horizontal line, although the
level is higher than the zero line of the spectrum, and iii) the attenuance measured is independent of the optical arrangement used.

![Absorption spectra of Chlorella suspension and extract](image)

**FIG. 1.** Absorption spectra of chlorella suspension and extract; reproduced from the figure in ref. 1.

Curve A, cell suspension, without opal plate; Curve B, the same suspension with opal plate; Curve C, alcohol extract from the suspension.

![Path of light in ordinary technique](image)

**FIG. 2.** Path of light in ordinary technique.

The principle of the opal glass transmission method can be explained easily, when it is known why poor results are obtained by ordinary techniques. Fig. 2 shows the path of light when the suspension is illuminated by parallel light. Roughly divided, two kinds of light are transmitted through the
suspension. One is parallel light, which has passed through only the suspending medium without hitting the microorganisms. This light will be called parallel transmitted light and its amount will be expressed by $I_p$. The other kind is diffused light, which has hit the cells of microorganisms. This light will be called diffuse transmitted light and its amount will be denoted by $I_d$. Therefore, the amount of total transmitted light ($I_t$) is

$$I_t = I_p + I_d$$

(1)

in addition to these light beams, we have reflected light coming out of the sample, which also has hit the cells of microorganisms. Obviously, the following relation exists between these beams;

$$I_0 = I_a + I_r + I_t$$

(2)

where $I_0$, $I_a$ and $I_r$ stand for the amounts of incident, absorbed and reflected light, respectively.

In ordinary spectrophotometers there is always a certain distance between the sample and the light detector (See Fig. 2). The intensity of the diffuse transmitted light decreases until it reaches the detector, while the parallel transmitted light does not decrease in intensity. Therefore, in the measurement by ordinary spectrophotometers, the light emerging in a direction not intercepted by the photocell is totally left out of account. What we are measuring in E units is the following quantity, which may be designated as quasi-attenuance ($pE_{pfd}$);

$$pE_{pfd} = \log \frac{I_0}{I_p + fI_d}; \quad 0 < f < 1$$

(3)

where $f$ is a constant between 0 and 1. The subscript $p$ to the left of $E$ indicates the nature of the reference incident light (parallel light), and subscripts $p$, $f$ and $d$ on the right side express the nature of the light transmitted by the sample and actually captured by the detector ($f$ and subscripts $p$ and $d$ in the denominator in the above equation).

When $f$ is equal to 0 or 1, equation (3) becomes equations (4) or (5), respectively;

$$pE_p = \log \frac{I_0}{I_p}$$

(4)

$$pE_{pd} = \log \frac{I_0}{I_p + I_d}$$

(5)

we shall call $pE_p$ and $pE_{pd}$, rectilinear attenuance and semi-integral attenuance with parallel illumination, respectively. $pE_p$ is the attenuance on the basis of parallel transmitted light and $pE_{pd}$ is the attenuance on the basis of total transmitted light.

The value of $f$ in equation (3) depends upon the geometry of the optical system, especially upon the distance between the sample and the light detector, and also depends on the diffuseness of the diffuse transmitted light. Under the condition employed in most commonly used spectrophotometers, the value of $f$ is quite small, a situation that makes the general level (base line) of measured spectra considerably higher than it would be
in the spectra in semi-integral attenuance units.

The lack of definition in the spectra by ordinary techniques is also due to the smallness of the factor, \( f \), in equation (3). As the diffuse transmitted light is the light which has hit the cells but was not absorbed, \( I_0/I_d \) varies with the wavelength depending upon the light absorption by the colored substances in suspended particles. On the other hand, \( I_0/I_p \) is independent of wavelength, since parallel transmitted light does not have any characteristics imparted to it by the pigments in suspended particles. Therefore, quasi-attenuance with a small value of \( f \) may not vary so markedly with wavelength as does semi-integral attenuance. As is clear from equation (3), the best value of \( f \) for obtaining a low base line and sharp absorption bands is 1.

The opal glass transmission method cited above (1) is a simple technique for measuring approximate values of semi-integral attenuance of translucent materials with parallel-light illumination. The principle is illustrated in Fig. 3. A plate of opal glass is attached to the detector side of the sample and reference cells, so that both the parallel and the diffuse light beams transmitted through a sample hit the opal plate and are completely diffused by the plate. Therefore, the same portion, \( \alpha \) of the parallel and diffuse transmitted lights hitting the plate goes into the detector after being diffused by the opal plate. As will be explained later, some of the multiply reflected light between the sample and the opal plate also goes into the detector in addition to the purely transmitted light. If we neglect the effect of these multiply reflected beams, the amount of light measured on the sample side is \( \alpha (I_p + I_d) \). By the same principle, the measured quantity on the reference side is \( \alpha I_0 \), provided that optical characteristics of the opal plate and the optical system on the reference side are the same as those on the sample side. Therefore, the observed attenuance by the opal glass transmission method is semi-integral attenuance, when the effect of multiple reflection is small.

To the same degree of approximation, semi-integral attenuance with diffuse-light illumination can be measured also by using opal glasses (Fig. 4).
An opal plate is attached to the monochromator side of both the sample and reference cells, so that the sample is illuminated by the light diffused by the opal plate. Since the light emerging from the sample and the reference cells has been completely diffused, the value of $E$ thus measured is semi-integral attenuance with diffuse-light illumination, $dE_{pd}$, neglecting the effect of multiple reflection*.

![Diagram of light path in opal glass transmission method](image)

**FIG. 4.** Path of light in opal glass transmission method for the observation of $dE_{pd}$.

So far the principle of the opal glass transmission method has been illustrated by the example of a dilute suspension. When the concentration of a suspension is high, there is no parallel transmitted light, so that the effect of using opal glass appears only as a reduction of the base-line height, with no increased definition of the spectrum. One example is the observation of cytochrome bands of yeast between 500-600 m\(\mu\). For this we need a highly concentrated suspension to detect the weak bands of the cytochromes, which are present in small quantities in yeast cells. However, in the observations of suspensions of most microorganisms, the optimum concentration is rather low, so that we have both parallel and diffuse transmitted light.

The transmitted light from biological materials other than suspensions of cells is more complicated. Such examples are seen in leaves, petals and slices of tissues. The light transmitted through the the rather transparent part of such samples is similar to parallel light transmitted by suspensions, and the transmitted light which has hit rather dense particles, like nuclei or chloroplasts, is highly diffused. Actually, the transmitted light is composed of many kinds of beams with different degrees of diffuseness. The use of opal glass is quite effective for such samples (1, 2, 6). The effectiveness can be explained in a similar way as for a dilute suspension, by increasing the number of terms in the denominator of equation (3).

* The transmitted light with diffuse-light illumination is completely diffused, having no parallel rays. However, it is composed of two kinds of light, that which has and has not hit the cells of suspended particles, as is the case with parallel-light illumination. From this point of view, subscript $p$ was retained in the expression of $dE_{pd}$. 
II. Correction for Multiple Reflection

For correcting the effect of multiple reflection, let us first estimate the amount of the effect in the measurement of $dE_{pd}$ with diffuse-light illumination. The amount of each multiply reflected beam is shown schematically in Fig. 5. In the actual system, the direction of the incident parallel beam is normal to the plane of the opal glass and only the incident light is parallel, although each light path is indicated by a single slanted arrow in the figure. Symbols $R$ and $T$ mean reflectance and transmittance for diffuse-light illumination, $R'$ and $T'$ for parallel-light illumination, and subscripts $1$ and $s$ refer to one sheet of opal glass and the sample, respectively. In Fig. 5, it is assumed that the light, once it goes through a sheet of opal glass, is completely diffused.

Summing up the amounts of all beams on the left hand side of Fig. 5, the following equation is obtained for the transmittance ($T_{1+s}$) of the system as a whole.

$$T_{1+s} = \frac{T_1 T_s}{1 - R_1 R_s}$$  \hspace{1cm} (6)

The transmittance of an opal plate on the reference side is obviously $T'_s$. Therefore, the value of attenuance measured by the opal glass transmission method with no correction for multiple reflection is

$$dE'_{pd} = \log \frac{1 - R_1 R_s}{T_s} = dE_{pd} + \log (1 - R_1 R_s)$$  \hspace{1cm} (7)

where a prime on $E$ indicates that the effect of multiple reflection is not corrected. The term $\log (1 - R_1 R_s)$ in the above equation is due to the effect mentioned above. As will be shown later, the value of $R_1$ is approximately 0.5. Therefore, the difference between $dE'_{pd}$ and $dE_{pd}$ is 0.3, when $R_s$ is 1.0. If $R_s$ is 0.1, the difference is only 0.02, so that the correction is significant only when the sample is highly light-reflecting.

The correction for multiple reflection was carried out experimentally. A gray film in addition to an opal plate was placed on both sample and reference sides. On the sample side, the gray film was sandwiched between
the sample and the opal plate. If we can assume that the gray film absorbs but does not reflect light, the observed attenuance \( dE'_{pd} \) with this system will be

\[
dE'_{pd} = \log \frac{1-R_1R_sT_f^2}{T} = dE_{pd} + \log (1-R_1R_sT_f^2)
\]

(8)

where \( T_f \) is the transmittance of the gray film with diffuse-light illumination. If \( T_f \) is small enough, the second term in the above equation will be practically zero. Therefore, \( dE'_{pd} \) is equal to \( dE_{pd} \) in this condition. In the experiment, a gray film with \( T_f = 0.14 \) was used. This means that \( R_1R_sT_f^2 \) is equal to 1 per cent of \( R_s \), since \( R_1 \) is approximately 0.5. Therefore, we can observe the absolute value of \( dE_{pd} \) within an error of 0.01 in \( E \) units, even when the reflectance of the sample is 100 per cent.

A rather great difference between \( dE'_{pd} \) and \( dE_{pd} \) is observed in the spectra of a variegated white ivy leaf. Curve A in Fig. 6 is the \( dE'_{pd} \) spectrum uncorrected for multiple reflection and Curve B is the \( dE_{pd} \) spectrum corrected for it. Although their attenuance values are different, the heights of the bands from their base lines and also the definition and positions of the bands in the two spectra are almost the same. Other examples of corrected \( dE_{pd} \) spectra are shown in Figs. 7 and 8. Curve B in Fig. 7 is the spectrum of a leaf of spinach (\textit{Spinacia oleracia}). The spectrum shows the clear absorption bands of chlorophyll \( a \) and a shoulder due to carotenoids. The position (676 m\( \mu \)) of the red peak of chlorophyll \( a \) agrees

![Fig. 6. The relation between the reflectance and transmission spectra of a white ivy leaf. Curve A; \( dE_{pd} \) spectrum uncorrected for multiple reflection. Curve B; \( dE_{pd} \) spectrum corrected for multiple reflection. Curve C; \( R_s \) spectrum. Curve D; \( \log (1-R_s)^{-1} \) spectrum. Curve F; \( dE_{pd} \) spectrum. The scale on the right is for Curve C.](chart)

well with that found in the uncorrected $dE_{pd}$ spectrum of a bean leaf previously observed (1). Curve B in Fig. 8 is the spectrum of a dark red leaf of *Coleus blumei* var. *Verschaffeltii* (variegated form), which has an absorption band due to anthocyanin at 541 m$\mu$.

![Fig. 7. The spectra of a spinach leaf.](image1)

Curves A, B, C and D are $R_s$, $dE_{pd}$, $\log(1-R_s)^{-1}$ and $d_rE_{pd}$ spectra, respectively. The scale on the right is for Curve A.

![Fig. 8. The spectra of a coleus leaf.](image2)

Curves A, B, C and D and $R_s$, $dE_{pd}$, $\log(1-R_s)^{-1}$ and $d_rE_{pd}$ respectively. The scale on the right is for Curve A.

By the same principle as before, we can determine the absolute value ($pE_{pd}$) of semi-integral attenuation with parallel-light illumination, using gray film and opal glasses. In this case again, only the base line of the
uncorrected $pE_{pd}'$ spectrum is raised slightly and no sharpening and shifting of the bands occur by the correction for multiple reflection. Therefore, for practical purposes of observing clear absorption bands, the observation of the uncorrected $pE_{pd}'$ spectrum with no gray film is the best among the observations of four semi-integral attenuances for parallel and diffuse incident lights, with and without the correction. Namely, the base line of the uncorrected $pE_{pd}'$ spectrum is the lowest and we can use the the narrowest slit width with no gray film, thus giving a wider usable wavelength range. However, in order to obtain the absolute value of semi-integral attenuation particularly of highly light-reflecting samples, a correction has to be included for the effect of multiple reflection.

III. Use of Rectilinear Attenuance for the Determination of Cell Concentration

Rectilinear attenuance defined by equation (4), can be used for the measurement of the concentration of suspended particles or cells. If $n$ is the number of cells per ml. of a suspension of a microorganism and the effective and average cross-sectional area of cells is $S$, the rectilinear attenuance of the suspension can be expressed as a function of these quantities:

$$pE_p = dnS$$

where $d$ is the thickness of the container. Therefore, dividing the observed value of $pE_p$ by $d$, we can obtain $nS$. The value of $nS$ is a measure of the concentration of the suspension, although the dimensions are different from that of $n$ or packed cell volume, which has been used as the measure of concentration of cell suspensions. The method of measuring $pE_p$ is superior to the nephelometric method of measuring scattered light, because the $pE_p$ measurement directly gives us the absolute value of $nS$, while nephelometric observation is based upon an empirical formula, which depends on the apparatus and the microorganisms to be measured. The experimental technique for the observation of $pE_p$ and the data on various microorganisms will be reported in a separate paper.

IV. Principles of the Opal Glass Reflection Method

The difference between reflected and diffuse transmitted light is a matter of angle of deflection, so that we can expect clear bands in the reflection spectra of biological samples. Rabideau, French and Holt (8) have succeeded in observing sharp bands in the reflectance spectra of plant materials with an integrating sphere, and Jacquez, Kuppenheim, Dimitroff, McKeehan and Huss (9-14) have developed the theory and observed also clear reflectance spectra of the skins of animals. Several techniques with an integrating sphere, which have been proposed so far, are reviewed by French and Young. (15). The principle of the opal glass reflection method (7) is different from that of the integrating sphere.
Before explaining the principle of the opal glass reflection method, let us illustrate first the apparatus we used for the measurement. A Beckman DK-2 Recording Spectrophotometer was used with an attachment which was set in place of the cell holder usually used for the observation of absorbance of solutions. The side view of the half of the attachment for the sample beam is shown in Fig. 9, where the path of light is indicated by arrows. The other half for the reference beam is similar, but no sample (s) is placed behind the opal glass (g). The light beam is raised by two prisms (p) and goes through a small window (w₁), which cuts down the beam size to a suitable cross section. After passing through the window, the light is reflected back by a front surface mirror (m) to a sheet of opal glass (g). The opal glass is the same kind as for the opal glass transmission method. It has an opalescent coating only on one side of the surface. The angle of the incident beam to the normal to the plane of the opal glass was 32°. Behind the opal plate, the sample or another opal plate was set with a block behind it covered with black velour paper. The diffusely reflected light from the opal glass was detected by the photomultiplier (d) in spectrophotometer through a window (w₂). With a sheet of opal glass in each beam, and no sample, we obtain an almost constant reading against wavelength.

Fig. 5 shows the amounts of the lights reflected from the system on the sample side with a sheet of opal glass and a light-reflecting sample superimposed. As is clear from this figure, the amount of total reflected light \( I_{1+s} \) is as follows;

\[
I_{1+s} = I_0R'_1 + \frac{T'_1T_1R_s}{1-R_1R_s}
\]  

(10)

if there is another sheet of identical opal plate in place of the sample, the amount of total reflected light \( I_2 \) from these two opal plates is expressed in the same way.

\[
I_2 = I_0R'_1 + \frac{T'_1T_rR_s}{1-R_1^2}
\]

(11)

Since the amount of the total reflected light \( I_1 \) from a sheet of opal plate is \( I_0R'_1 \), the difference between \( I_1 \) and \( I_{1+s} \) or \( I_2 \) is

\[
D_{1+s} = I_{1+s} - I_1 = \frac{I_0T'_1T_1R_s}{1-R_1R_s}
\]

(12)

\[
D_2 = I_2 - I_1 = \frac{I_0T'_1T_1R_s}{1-R_1^2}
\]

(13)
where $D_{1+s}$ and $D_2$ are those differences, respectively. Therefore,
\[
\frac{1}{R_s} = R_1 + \frac{D_2}{D_{1+s}} \left[ \frac{1}{R_1} + R_1 \right]
\]  
(14)

From this equation, we can calculate $R_s$, the absolute value of reflectance of the sample, if we know the values of $D_2/D_{1+s}$ and the reflectance of an opal plate, $R_1$ for diffuse light.

The observation of $R_1$ of an opal plate was carried out, with the same principle as for the correction for the multiple reflection. The ratio of the transmittance ($T_2$) of two opal plates to that ($T_{2+f}$) of two opal plates and a sheet of gray film inserted between them becomes as follows;
\[
\frac{T_2}{T_{2+f}} = \frac{1 - R_1^2 T_f^2}{(1 - R_1^2) T_f}
\]  
(15)
since the above ratio is directly observable and $T_f$ can be measured by the opal glass transmission method, we can calculate the value of $R_1$ by equation (15). The values of $R_1$ of an opal plate used for this study in the range between 380 and 750 m$\mu$ are listed in Table I.

**Table I**

<table>
<thead>
<tr>
<th>Wavelength (m$\mu$)</th>
<th>$R_1$</th>
<th>log ($1/R_1$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>750</td>
<td>0.451</td>
<td>0.346</td>
</tr>
<tr>
<td>700</td>
<td>0.459</td>
<td>0.338</td>
</tr>
<tr>
<td>650</td>
<td>0.468</td>
<td>0.329</td>
</tr>
<tr>
<td>600</td>
<td>0.476</td>
<td>0.322</td>
</tr>
<tr>
<td>550</td>
<td>0.485</td>
<td>0.314</td>
</tr>
<tr>
<td>500</td>
<td>0.493</td>
<td>0.307</td>
</tr>
<tr>
<td>450</td>
<td>0.500</td>
<td>0.301</td>
</tr>
<tr>
<td>400</td>
<td>0.504</td>
<td>0.297</td>
</tr>
<tr>
<td>380</td>
<td>0.499</td>
<td>0.302</td>
</tr>
</tbody>
</table>

Once $R_1$ of an opal plate is measured against wavelength, the only observation necessary for each sample is the measurement of $D_2/D_{1+s}$. For the observation of this ratio, the electronic recording system of the spectrophotometer was set so as to record the ratio of the intensity of the sample beam to that of the reference beam. Therefore, the relative values of $I_1$, $I_2$ and $I_{1+s}$ were recorded with a sheet of opal glass, two sheets superimposed, and a sheet of opal glass with the sample, respectively. A sheet of opal plate was always placed on the reference side for these measurements. Since we need only the ratio of $D_2$ to $D_{1+s}$, we can calculate the value from the relative values of $I_1$, $I_2$ and $I_{1+s}$. This feature of the observation makes it possible to expand the scale or to shift the zero level electronically, depending upon the sample. The value of diffuse reflectance of MgO observed by this method agreed fairly well with the values observed by
other methods (7).

V. Reflection Spectra of Leaves in Relation to Their Transmission Spectra

Leaves were chosen to illustrate the relation between reflection and transmission spectra for the following reasons. i) Leaves have rather high reflectance in the spectral range where there is no absorption. The condition is favorable for obtaining the relationship. ii) Leaves are thin enough to eliminate the possibility that some light may be lost from the side of the sample without being measured by either transmission or reflection measurement. iii) They require no container.

The reflectance spectrum of the same white ivy leaf as for the measurement of the \( \Delta E_{pd} \) spectrum was observed by the opal glass reflection method. The spectrum in \( R_s \) units is shown in Curve C in Fig. 6, where the scale of \( R_s \) is indicated on the right side of the figure. Now, we have the absolute values of the reflectance and the semi-integral attenuance of the same leaf. To correlate these values, a new quantity, \( R \)-corrected (Reflection corrected) attenuance was defined; that is the logarithm of the ratio of \( I_0 - I \) to \( I \). The difference between \( R \)-corrected and semi-integral attenuances is in the reference light. In \( R \)-corrected attenuance, the amount of the light, which actually goes into the sample without being reflected, was taken as the reference quantity. Therefore, \( R \)-corrected attenuance depends only upon the transmitting property of a sample, without being affected by the reflecting property. In other words, it is a sort of idealized quantity of semi-integral attenuance, which would be obtained from an idealized sample that does not reflect the incident light at all but nevertheless transmits the light in the same way as an actual sample does. \( R \)-corrected attenuance for parallel and diffuse incident light will be expressed by \( \Delta r E_{pd} \) and \( \Delta d E_{pd} \), respectively.

For diffuse incident light, we obtain the following equation;

\[
\Delta r E_{pd} = \log \frac{I_0 - I_r}{I_t} = \log \frac{I_0}{I_t} + \log \frac{I_0 - I_r}{I_0} = \log \left( \frac{I_0}{I_t} \right) + \log (1 - R_s) = \Delta E_{pd} - \log (1 - R_s)^{-1}
\]  

Equation (16) indicates that the value of \( \Delta r E_{pd} \) can be obtained by subtracting \( \log (1 - R_s)^{-1} \) from \( \Delta E_{pd} \). The spectrum of \( \log (1 - R_s)^{-1} \) of the white ivy leaf was calculated from Curve C in Fig 6 and is shown as Curve D in the figure. The comparison of Curves B and D shows clearly that these curves match where there is no absorption band beyond 700 m\( \mu \). As a consequence, the base line of the \( \Delta d E_{pd} \) spectrum (Curve F) coincides with the zero line. This fact proves that the existence of a certain height of base line in the \( \Delta E_{pd} \) spectrum is due to the reflection of the sample. It also proves that the values of \( R_s \) and \( \Delta E_{pd} \) observed by opal glass techniques were exact.

Other examples of the \( \Delta r E_{pd} \) spectra of spinach and Coleus leaves are shown in Figs. 7 and 8, where Curves A, B, C and D are their \( R_s, \Delta E_{pd}, \log (1 - R_s)^{-1} \) and \( \Delta r E_{pd} \) spectra, respectively. The \( \Delta E_{pd} \) and \( \log (1 - R_s)^{-1} \) spectra
in these figures match again where there is no absorption. The positions of the minima of the $R_s$ spectra and the maxima of the $dE_{pd}$ spectra were found to be the same as the maxima of the corresponding bands in the $dE_{pd}$ spectra. Therefore, the observation of a reflectance spectrum serves as the observation of a semi-integral attenuance spectrum, so far as the positions of the bands are concerned.

Using the data of the Coleus leaf, the relation between the transmittance and the reflectance was obtained. In Fig. 10, the reflectance was plotted against the transmittance calculated from the semi-integral attenuance. While points in this figure were obtained for wavelengths on both sides of the anthocyanin band, they fall on a smooth and slightly bent curve. Other plots of data from different leaves also show curves of a similar type but differently located. Since the bending is small, we can approximately substitute for this curve a straight line through the origin, which is indicated as a ditted line in Fig. 10. Namely, the ratio of $R_s$ to $T_s$ can be assumed to be approximately constant for each sample;

$$\frac{R_s}{T_s} = \beta$$  \hspace{1cm} (17)

where $\beta$ is a proportionality constant.

Recently, Duysens (16) reported a theory on the flattening effect in the spectra of cell suspensions. In order to estimate the amount of the effect, the height of a band in vivo in terms of R-corrected attenuance has to be known. If we can approximately use equation (17), the R-corrected attenuance spectrum of a sample can be obtained from the spectrum of semi-integral attenuance only, without measuring the reflectance. Namely, the height of the base line in the $dE_{pd}$ spectrum is equal to both $\log (1-R_s)^{-1}$ and $\log (1/T_s)$, where there is no absorption band. From those values, the
proportionality constant $\beta$ in equation (17) for the sample can be calculated. Therefore, we can calculate the value of $\log (1-R_s)^{-1}$ from the value of $\log (1/T_s)$ at any wavelength, so that we obtain the spectrum of R-corrected attenuance. The sizes of pigment particles in vivo evaluated from the flattening effect thus calculated will be reported separately.

**VI. Integral Attenuance Spectra of Leaves**

Integral attenuance ($dE_{\rho pd}$) with diffuse-light illumination, which is defined in equation (18), corresponds to the absorbance of transparent materials, in that we can calculate, if we know the amount of $I_0$, the absolute amount of absorbed light from it.

$$
\frac{dE_{\rho pd}}{I_0} = dE_{\rho pd} = \log (1+R_s/T_s)
$$

The integral attenuance ($\rho E_{\rho pd}$) for parallel incident light can be expressed in the same way. By equation (18), we can calculate the exact value of integral attenuance from the amount of $dE_{\rho pd}$, $T_s$ and $R_s$ obtained from semi-integral attenuance and reflectance measurements.

Here again, we can simplify the procedure of obtaining an integral attenuance spectrum of the sample, for which equation (17) holds. Namely, the second term, $\log (1+R_s/T_s)$ is equal to the height of the base line of the semi-integral attenuance spectrum. Since the value of $R_s/T_s$ can be considered to be independent of wavelengths for the sample, the integral attenuance spectrum can be obtained simply by subtracting the base line from the semi-integral attenuance spectrum. Probably this procedure has been used with no theoretical consideration in many studies for estimating the heights of the bands in the spectra of translucent materials. It is now clear, however, that it is the way to obtain integral attenuance spectra and is applicable only to the samples in which the value of $R_s/T_s$, is independent of wavelength.

**VII. Relative Reflectance Spectra of Biological Materials**

For obtaining the absolute value of reflectance by the opal glass reflection method, we need some calculations based on directly observed values. Furthermore, if the reflectance of a sample is low in the range of light absorption, it is hard to locate the absorption maximum clearly because of the small change of the reflectance with wavelength. In order to obtain sharp bands directly, even of low-reflecting samples, a way of recording the logarithm ($dE_r'$) of the reciprocal of the relative value of reflectance was devised. The logarithm ($dE_r$) of the reciprocal of the absolute value was designated as reflex attenuance;

$$
dE_r = \log (I_0/I_r) = \log (1/R_s)
$$

therefore, $dE_r'$ is

$$
dE_r' = \log (1/R'_s) = dE_r + C
$$

where $R'_s$ is the relative value of $R_s$ and $C$ is a constant which is independent of wavelength.
The same attachment unit as for absolute reflectance measurement was used but in a different way. The spectrophotometer (Beckman DK-2) was set so as to record the logarithm of the ratio of the intensity of the reference light to that of the sample light. A reference curve was first recorded with an opal plate for both sample and reference sides of the attachment. The result gives us a slightly sloping but almost straight line. When we observe a sample, it is placed alone, not together with an opal plate, on the sample side. In this case, the incident parallel light from the surface mirror (m) directly strikes the sample at a 32° angle to the normal to the plane of the sample. The specularly reflected light from the sample will hit the bottom of the attachment without being measured. Hence, the light which is reflected back to the detector is diffusely reflected light. If the intensity at the detector of the light from a sample is proportional to the absolute values of $R_s$, the increment of the reading with a sample above the reference curve is

$$\log \left( \frac{1}{R_s} \right) - \log \left( \frac{1}{R_r} \right) = dE_r + C - \log \left( \frac{1}{R_r} \right)$$  \hspace{1cm} (21)

It should be remarked, however, that such a proportionality independent of wavelength can not be generally expected from this optical arrangement.

The values of $C$ obtained from the observed increments may or may not be independent of wavelength.

The leaf of Coleus used for the absolute reflectance measurement was observed by this modified method. The observed values of $R'_s$ and $R_s$ are listed in Table II. The values of $R'_s$ in this table were calculated from the

<table>
<thead>
<tr>
<th>Wavelength ($\mu$)</th>
<th>$R_s$</th>
<th>$R'_s$</th>
<th>$R'_s/R_s$</th>
</tr>
</thead>
<tbody>
<tr>
<td>700</td>
<td>0.552</td>
<td>0.294</td>
<td>0.533</td>
</tr>
<tr>
<td>690</td>
<td>0.546</td>
<td>0.292</td>
<td>0.535</td>
</tr>
<tr>
<td>680</td>
<td>0.538</td>
<td>0.290</td>
<td>0.539</td>
</tr>
<tr>
<td>670</td>
<td>0.529</td>
<td>0.286</td>
<td>0.541</td>
</tr>
<tr>
<td>660</td>
<td>0.510</td>
<td>0.279</td>
<td>0.547</td>
</tr>
<tr>
<td>650</td>
<td>0.486</td>
<td>0.264</td>
<td>0.543</td>
</tr>
<tr>
<td>640</td>
<td>0.442</td>
<td>0.242</td>
<td>0.548</td>
</tr>
<tr>
<td>630</td>
<td>0.390</td>
<td>0.212</td>
<td>0.544</td>
</tr>
<tr>
<td>620</td>
<td>0.327</td>
<td>0.178</td>
<td>0.544</td>
</tr>
<tr>
<td>610</td>
<td>0.262</td>
<td>0.145</td>
<td>0.554</td>
</tr>
<tr>
<td>600</td>
<td>0.207</td>
<td>0.113</td>
<td>0.546</td>
</tr>
<tr>
<td>590</td>
<td>0.157</td>
<td>0.087</td>
<td>0.554</td>
</tr>
<tr>
<td>580</td>
<td>0.120</td>
<td>0.063</td>
<td>0.525</td>
</tr>
<tr>
<td>570</td>
<td>0.090</td>
<td>0.046</td>
<td>0.511</td>
</tr>
<tr>
<td>560</td>
<td>0.082</td>
<td>0.036</td>
<td>0.439</td>
</tr>
<tr>
<td>550</td>
<td>0.079</td>
<td>0.031</td>
<td>0.392</td>
</tr>
</tbody>
</table>
observed increments described above and the values of \( \log(1/R_t) \) in Table I. The constancy of the ratios, \( R_t'/R_s \) listed in the last column shows that the observed values of \( R_t' \) are truly relative values of \( R_s \). The change of the ratio near 550 m\( \mu \) seems to be due to the error in the observation of the small values of \( R_s \) and some non-uniformity of the sample, which might have been placed in slightly different positions in the two experiments. With more experiments with different samples, it was found that the observed value of \( R_t' \) is a relative value of \( R_s \), insofar as the sample is a highly light-diffusing material. Almost all biological non-transparent samples diffuse light sufficiently to observe the relative value of \( R_t \) by this method, but translucent materials with high transmittance do not diffuse light enough for applying this method. However, for the samples of the latter kind, the opal glass transmission method can be used effectively for obtaining clear absorption bands.

For practical purposes, we need not even correct for the change of \( \log(1/R_t) \) with wavelength, since it is small compared with the large change of \( \log(1/R_t') \) near the range of light absorption. The recorded spectrum itself with no correction for the change of \( \log(1/R_t) \) shows clear bands, from

<table>
<thead>
<tr>
<th>Figure and curve</th>
<th>Sample</th>
<th>Position of band (m( \mu ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>11A</td>
<td>Pink petal of sweet pea</td>
<td>531</td>
</tr>
<tr>
<td>11B</td>
<td>Violet petal of sweet pea</td>
<td>568</td>
</tr>
<tr>
<td>11C</td>
<td>Root of red radish</td>
<td>532</td>
</tr>
<tr>
<td>11D</td>
<td>Petal of hellebore</td>
<td>435, 533, 676</td>
</tr>
<tr>
<td>11F</td>
<td>Flowers of ceanothus</td>
<td>(490), 530, 574, 624, (675)</td>
</tr>
<tr>
<td>12A</td>
<td>Cake of yeast</td>
<td>412, (525), (558), (595), (640)</td>
</tr>
<tr>
<td>12B</td>
<td>Suspension of yeast with ascorbic acid</td>
<td>417, (445), (475), 520, 548, (556), 600</td>
</tr>
<tr>
<td>13A</td>
<td>Inner seed coat of squash</td>
<td>407, (488), (544), 589, 648</td>
</tr>
<tr>
<td>13B</td>
<td>Endosperm of pole bean</td>
<td>407</td>
</tr>
<tr>
<td>13C</td>
<td>&quot;</td>
<td>407, (422)</td>
</tr>
<tr>
<td>13D</td>
<td>&quot;</td>
<td>407, 422, (446), 478</td>
</tr>
<tr>
<td>14A</td>
<td>Crystal of ( \beta )-carotene</td>
<td>430, 460, 486, 528</td>
</tr>
<tr>
<td>14B</td>
<td>Benzene solution of ( \beta )-carotene</td>
<td>436, 463, 492</td>
</tr>
<tr>
<td>14C</td>
<td>Root of carrot</td>
<td>428, 452, 482, 515</td>
</tr>
<tr>
<td>15A</td>
<td>Crystal of lycopene</td>
<td>(460), 484, (515), (570)</td>
</tr>
<tr>
<td>15B</td>
<td>Flesh of watermelon</td>
<td>407, 435, (457), 483, 517, 562</td>
</tr>
<tr>
<td>15C</td>
<td>Benzene extract of watermelon</td>
<td>(430), 457, 483, 517</td>
</tr>
<tr>
<td>15D</td>
<td>Ethanol extract of watermelon</td>
<td>400, 427</td>
</tr>
</tbody>
</table>
which we can estimate the exact positions of the bands. The spectra, which will be shown in this section are not corrected for the change and also the zero line for each spectrum was chosen arbitrarily. The positions of the main peaks identified in these spectra are shown in Table III.

The most useful application of this technique is the observation of the spectra of samples which can hardly be observed by the transmission method. Those are non-transparent materials or translucent materials with low transmittance. The $dE_r'$ spectra of various petals are shown in Fig. 11.

![Image](image_url)

**Fig. 11.** The $dE_r'$ spectra of a pink petal of sweet pea (Curve A), a violet petal of sweet pea (Curve B), a root of red radish (Curve C), a petal of hellebore (Curve D) and flowers of ceanothus (Curve F). The scale on the right is for Curve C. The base line for Curve F was raised arbitrarily to avoid confusion.

Curves A and B represent the spectra of the petals of pink and violet flowers of *Lathyrus odoratus* (sweet pea). These petals show distinctly different absorption spectra with maxima at 531 and 568 m.$\mu$. The spectrum (Curve C) of the surface of the red root of *Raphanus sativus* (red radish) seems to indicate the presence of the same kind of pigment (532 m.$\mu$) as that of the pink petal of sweet pea, with a small amount of an additional pigment, which appears as a shoulder at the longer wavelength side of the main band. In the spectrum (Curve D) of the petal of *Helleborus orientalis* (hellebore), we can see the presence of chlorophyll $a$ in addition to an anthocyanin. The spectrum of the flowers of *Ceanothus* (California “Lilac”) has three main
bands (Curve F). An ethanol extract from the flowers showed a similar absorption spectrum. The curve is symmetrical in shape and resemble the absorption spectrum curve of a carotenoid. But, a carotenoid spectrum which is located at such a long wavelength has not yet been found. Chemical studies may lead to the finding of a new pigment in *Ceanothus*.

Curve A in Fig. 12 shows the $dE_r'$ spectrum of a cake of commercial Fleishman's yeast. The bands of oxidized cytochromes are seen in the spectrum. The yeast was suspened in water, 16.5 g. wet weight per 100 ml. with a small amount of ascorbic acid. The spectrum (Curve B) shows the bands of reduced cytochromes. The soret band in the reduced form was found to be at 417 m$m$ instead of at 412 m$m$ as in the oxidized form.

The $dE_r'$ spectra of seeds are shown in Fig 13. The seed of *Cucurbita pepo* (squash) has a thin and brownish-green inner seed coat. The spectrum (Curve A) of the intact inner seed coat was observed without peeling the coat. The red band at 648 m$m$ seems to consist of two bands with a shoulder at the shorter wavelength side of the band. Two similar components have been found in etiolated leaves (2), in which the corresponding bands were identified at 650 and 636 m$m$ in vivo and the band at 650 m$m$ was due to protochlorophyll $a$. According to Koski, French and Smith, (17), the action spectrum of the transformation of protochlorophyll $a$ to chlorophyll
a has bands at 650, 593, 550 and 445 m\(\mu\). These studies indicate that the band at 648 m\(\mu\) is due to protochlorophyll \(a\). The small shift toward shorter wavelength may arise from the overlapping effect of the additional band at 636 m\(\mu\). The other bands at 585 and 545 m\(\mu\) and a small hump at 445 m\(\mu\) on Curve A seem to be also due to protochlorophyll \(a\) (2). High bands at 407 and 485 m\(\mu\) are probably due to cytochromes and carotenoids, respectively.

![Graph](image)

**FIG. 13.** The \(dE_r\) spectra of seeds; inner seed coat of squash (Curve A), endosperm of pole bean in the original dry state of the seed (Curve B), endosperm of pole bean after 24 hours in water (curve C) and cotyledon of pole bean plant cultured for ten days in the dark (Curve D).

Curve B in Fig 13 shows the \(dE_r\) spectrum of the inside surface of the endosperm of *Phaseolus vulgaris* var. Ferry-Morse's Pole bean, No. 191. It was observed in the dry form of the endosperm, which has a sharp band at 407 m\(\mu\). The seeds of the pole bean were placed on a wet paper for 24 hours and the \(dE_r\) spectrum of the endosperm was observed again (Curve C). In this spectrum, we can see a band developed around 422 m\(\mu\). This band is, therefore, due to the initial pigment formed in the germination process. Similar seeds were cultured in the dark under soil. The etiolated leaves came out from the cotyledons after a week. After ten days, the cotyledon was picked from the plant and the \(dE_r\) spectrum of the inner surface was observed (Curve D). The spectrum shows the increase of the band at 422 m\(\mu\), and also new bands at 446 and 478 m\(\mu\), which are probably
due to some carotenoids formed in the culturing process. But, no protochlorophyll band was observed in the red region. This indicates that protochlorophyll a is formed only in the embryo of the leaf. Similar studies with different kinds of bean seeds also showed the similar process as that in the bean No. 191.

As a separate experiment, Shibata (3) observed the absorption spectra of suspensions of crystalline carotenes and compared them with their spectra in solution. It was found that there is an additional band in the spectrum of the suspension besides the bands which correspond to the bands in solution. This additional band exists at the longer wavelength side of the other bands. It was also found that the position of this band depends upon the size of the crystals in the suspension much more than do the positions of the other bands. The observed spectra of β-carotene are reproduced in Curves A and B in Fig. 14. From the shape of those curves, it is clear that the band at 528 mμ is the additional one.

Many investigations have been done on the state of carotenoids in vivo, since the earliest observation by Schimper (18) until the most recent studies by Straus (19). The results were reviewed by Straus (20). These studies showed that, in their natural state, some carotenoids may exist as crystals. However, these results were obtained mainly by microscopic observations, which seem to need support from other kinds of observations for the conclusive solution of the problem. With the hope that the additional band found in the suspension of the crystals of β-carotene may found in the spectrum of some intact biological materials, the $dE'$ spectrum of the
root of *Daucus carota* var. sativa (carrot) was observed. The spectrum (Curve C in Fig. 14) shows the additional band at 515 m\(\mu\). The deviation of the position from the value, 528 m\(\mu\), previously obtained in the suspension of the crystals is probably due to the difference in size of the crystals.

Similar experiments were carried out on the state of lycopene *in vivo*. In the experiment with \(\beta\)-carotene (3), the suspension was prepared by sonic treatment of the crystals in water with a drop of a soap solution. A different method was adopted for making fine crystals of lycopene. A petroleum ether solution of lycopene was evaporated drop by drop in a small area on a filter paper (Whatman No. 2), in order to obtain fine crystals on the paper. This sample was used for the semi-integral attenuance measurement using a plain filter paper as the reference (1). Curve A in Fig. 15 shows the spectrum.

![Fig. 15. Spectra of lycopene.](image)

Curve A; \(pE'_{pd}\) spectrum of crystalline lycopene on filter paper, Curve B, \(dE'\) spectrum of flesh of watermelon, Curves C and D, absorbance spectra of benzene and ethanol extracts from watermelon.

which exhibits an additional band at 570 m\(\mu\). Curve B in the figure indicates the \(dE'\) spectrum of the red flesh of *Cucurbita pepo*, watermelon. Curves C and D are the absorption spectra of the benzene and alcohol extracts from the watermelon, respectively. The comparison of these curves shows that the band at 562 m\(\mu\) on Curve B corresponds to the characteristic band of lycopene crystals at 570 m\(\mu\). A similar band was found at 560 m\(\mu\) also in the \(dE'\) spectra of the fruits of *Lycopersicon esculentum* (tomato) and pink
fleshed variety of Citrus maxima (grape fruits). These spectroscopic observations support the point of view that some carotenoids exist in a crystalline form in their natural states. The additional band of carotenoids was observed only in the spectrum of fruits or roots, but not in leaves, petals and microorganisms examined.

DISCUSSION

The names, symbols and definitions of attenuances required for the spectroscopic measurements of translucent or non-transparent materials are summarized in Table IV. It will be worth while to stress the importance of defining these attenuances, since in many spectroscopic studies of translucent biological materials, the word absorbance (optical density) has been carelessly used. Without any detailed description of the optical arrangement used or any comment on the definition, it could be any attenuance except R-corrected and reflex attenuance listed in Table IV. At least six attenuances listed in the table are required for avoiding such a confusion. Various spectroscopic observations on biological samples with this new point of view will solve many problems without leaving any ambiguity as to the observed values of attenuances.

In this report, the theories and techniques of opal glass transmission and reflection methods were described. From both experimental and theoretical points of view, the opal glass methods for absolute measurement
are certainly simple, compared with the classical use of an integrating sphere. Probably the only question left about opal glass methods would be whether an opal plate is a complete light diffuser or not. However, if the effect of this factor were serious, we would not have obtained such good agreement between the values of semi-integral attenuance and \( \log (1-R_t)^{-1} \) of leaves, in the range where there was no absorption. The observations (7) on the reflectance of MgO also support the conclusion that the effect is within the experimental error even if it exists. Of course, the angular distribution of the intensity of the light transmitted through the opal plate will tell how complete the diffusing property is.

For the practical purpose of obtaining clear absorption bands, the opal glass transmission method previously proposed (1) and the modified opal glass reflection method for observing \( \log (1/R_t') \) will be most useful. For translucent materials, the transmission method can be applied. If a sample is non-transparent or low light-transmitting, the reflection method can be used. Therefore, we can observe the bands of any biological sample clearly and simply, regardless of the optical property of the sample. These methods for relative measurement are even simpler and will be more useful in many biological researches than the opal glass methods for absolute measurement. However, the principles of the methods for relative measurement are based upon the theories of opal glass methods for absolute measurement and the experimental results presented in this report.

**SUMMARY**

1. The theories and techniques of measuring the absolute values of the transmittance and reflectance of translucent biological materials with opal glass plates are presented. These methods were called opal glass transmission and reflection methods. They are simple and easy to practice with spectrophotometers commonly used for the measurement of transparent materials. From the transmission and reflection spectra of leaves observed by these methods, it was proved that they give us the exact values of the transmittance and reflectance.

2. From the results of the measurements by opal glass transmission and reflection methods, it was found that we need to define at least six quantities, which describe the optical properties of translucent or non-transparent biological materials. The way and use of evaluating these properties are described, especially paying attention to the absolute measurement of the light absorbed by those samples.

3. By modifying the opal glass reflection method, we can observe the logarithm of the reciprocal of the relative value of reflectance, even more simply than by opal glass reflection method for absolute measurement. The measurements by the modified method showed clear absorption bands of biological non-transparent materials. As one of the application of the method, the state of carotenoids in vivo was studied. The results indicated that some carotenoids exists in their crystalline state in roots or fruits.
SPECTROPHOTOMETRY OF BIOLOGICAL MATERIALS

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