SPECTROPHOTOMETRIC STUDY OF SUSPENSIONS OF PIGMENTED PARTICLES*

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INTRODUCTION

Study of light absorption by pigments present in non-transparent media is a problem frequently encountered in biochemical investigations. The pigments may be solutes in a liquid containing light-scattering particles or, as is usually the case, they may be within cells or sub-cellular particles.

Four general methods have been developed for obtaining absorption spectra of suspensions in such a way as to increase the ratio of light absorbed by pigments to light scattered. By such methods the absorption maxima characteristic of the pigments stand out more clearly against a background of non-selective scattering.

1. The earliest method was one devised by us[1] for visual spectroscopic study of the γ-bands of cytochrome components in a colloidal heart muscle preparation. In order to reduce light scattering this preparation was suspended in concentrated solutions of sucrose, in glycerol, or was treated with bile salts such as taurocholate or desoxycholate. By similar treatments such tissue preparations have become amenable to spectrophotometric study[2,3]. By suspending red blood cells (RBC) in a concentrated solution of dextrin RUBINSTEIN AND RAVIKOVICH[4] found that the γ-band of oxyhaemoglobin at 414 mμ, which is feeble in the spectrum of RBC suspended in isotonic salt solutions, was intensified so that it became comparable with that of the lysed cells. BARER[5] suspended RBC in a protein solution of the same refractive index and obtained an almost clear fluid with an absorption spectrum quantitatively close to that of dissolved oxyhaemoglobin. The same method was also extended to highly pigmented micro-organisms[6,7].

2. TAMIYA, SHIBATA and their colleagues[8,9] placed opalescent screens against the emergent optical surfaces of spectrophotometer cuvettes containing RBC or strongly pigmented micro-organisms. With this set-up the recorded absorption bands of intracellular pigments (carotene, chlorophyll, haemoglobin) were found to be sharpened and comparable with those of the pigments extracted in suitable solvents.

3. The application of the integrating sphere to spectrophotometry has been developed widely in the study of intracellular pigments in living tissues, RBC and micro-organisms[10,11] and also in chloroplasts and intact leaves[12]. The results have been similar to those produced by the first two methods. A principle which can be

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considered as analogous to the integrating sphere is that of reflectance spectrometry. By this method the absorption bands of cytochrome have been detected in micro-organisms and in certain mammalian cells[13,14].

4. The fourth method is one based upon the sharpening and intensification of absorption bands at liquid air temperature. This principle has been applied with considerable advantage to visual spectroscopy of cytochrome pigments[1,15] and has recently been extended to spectrophotometric studies of these pigments[16,17].

So far the above developments have not reached the stage where they can provide absorption data on intracellular or particulate pigments which can be quantitatively related to the extinction coefficients of the pigments and thus to their concentrations. This ideal can be approached by method 1 but only by using non-physiological suspending media which modify the physical properties, and often largely inhibit the catalytic activities, of the suspended material. The essential problem in quantitative absorption studies of scattering media is to deduce E_p, the extinction, that would be recorded if the scattering material were removed and the pigments dispersed in true solution. The relationship between E_p and E_t, the measured total extinction of the material, can be expressed in its simplest form as

$$E_t = E_p + E_s$$

where E_s, the "scattering extinction", measures the decrease in light energy reaching the photocell as a result solely of light scattering by the interposed material. This argument is oversimplified inasmuch as it assumes that under conditions where light is not scattered by a particulate suspension, *e.g.* when particles and medium have the same refractive index, the experimental absorption curve will not differ from that of the pigment in solution. It can be shown mathematically that, independently of scattering, the particulate pigment should exhibit absorption bands which are flattened in comparison with the dissolved pigment. In certain cases this can be demonstrated[18] and DUYSENS[19] has been able to correct the absorption curve of a *Chlorella* suspension so as to obtain the "unflattened" curve characteristic of the intracellular chlorophyll. With such an organism the scattering contribution to the measured extinction is very small.

The validity of any method for evaluating E_p from E_t can only be tested if E_p can be independently measured. For this reason the customary experimental materials have been either RBC suspensions, which yield clear solutions of unmodified oxyhaemoglobin (HbO_2) on addition of lytic agents, or known mixtures of coloured solutions and white scattering particles.

A purely empirical approach was that of DRABKIN AND SINGER[20] who examined suspensions of cream particles in HbO_2 solution, and also RBC suspensions, within the range 500–630 mμ. The relationship $E_t = E_p + E_s$ was valid for the former type of suspension but with RBC the relationship was less simple and could be expressed as

$$E_t = f_1 + f_2 E_p$$

where $f_1 = a(Nl)^n/[1 + a(Nl)^n]$, $\log f_2 = b + m \log (Nl)$ and a, b, m, n are empirical constants (see also Table I).

FROMAGEOT and his associates[21,22] made use of calculations by WURMSER[23] who had deduced the relationship

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$$\frac{I_0}{I} = 1 + K_s l$$

for a purely scattering medium. When, as in FROMAGEOT's experiments, $K_s l \gg 1$ and when an absorbing pigment is also present

$$E_t = K_p l' + \log K_s l$$

where l' is the optical depth corrected for the vol. fraction occupied by pigment-free particles. Measurement at two values of l (and l') enabled the constants K_p and K_s to be determined. In the case of $BaSO_4$ suspended in $KMnO_4$ solution it was possible to deduce K_p and hence the absorption curve of the clear, $BaSO_4$-free solution. However, application of the same principles to RBC suspensions yielded "solution curves" which were higher, by a constant increment of K_p, than the experimental curve for the lysed cell suspensions.

When a clear solution is examined in a conventional spectrophotometer values of E_p are independent of the position of the cuvette along the optic axis. On the other hand it has been pointed out by several workers[9,21] that E_t of a suspension will vary with the distance (x) between cuvette and photocell since the fraction of the total scattered light reaching the photocell will be a function of x. In fact, the various constants derived by these workers[20,21] depend not only upon the position of the cuvette but also upon the design of the spectrophotometer.

The method of scattering correction described in the present paper is based upon the variation of E_t with x, and by an extrapolation procedure E_s is eliminated.

TABLE I

EXPLANATION OF SYMBOLS

*I_0: Energy flux of light reaching the photocell via the blank cuvette
*I: Energy flux of light reaching the photocell via the experimental cuvette
Z: $[I_0/I]^{0.5}$
E: Extinction (optical density) = $\log_{10}[I_0/I]$
ε: Molecular extinction coefficient = E/lc
l: Optical depth
c: Molar concentration of solute
N: Number of light-scattering particles per unit volume
x: Distance between incident surface of cuvette (in cuvette compartment of spectrophotometer) and photocell envelope (Fig. 1)

Subscripts:

p' clear pigment solution
s' scattering medium devoid of pigments
t' medium which both absorbs and scatters light

* With a light-scattering medium in the cuvette the usual term "light intensity" as a definition of I becomes ambiguous. According to the scattering properties of the medium a given light flux may illuminate different areas of the sensitive element of the photocell. The photocell response, being a measure of the total incident flux, will thus no longer measure light intensity at any point on the sensitive element.

PRINCIPLE OF THE METHOD

Suppose a beam of monochromatic light is passing through a cuvette containing a purely scattering medium. If the light is uniformly scattered I_s will vary with x according to the inverse square law.

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i.e. $$I_s \propto \frac{1}{x^2} \text{ or } \left[\frac{I_0}{I_s}\right]^{0.5} \propto x \quad (1)$$

When the cuvette contains a non-scattering pigment solution the expression corresponding to (1) is

$$\left[\frac{I_0}{I_p}\right]^{0.5} = \text{constant}$$

If the cuvette contents both scatter and absorb light the relationship of I_0 to the light reaching the photocell (I_t) will be

$$Z = \left[\frac{I_0}{I_t}\right]^{0.5} = px + q$$

where p and q are constants. It follows that if E_t is measured with the cuvette at different distances from the photocell, a plot of antilog $0.5E$ against x should be a straight line. Furthermore, the point on the line corresponding to $x = 0$ should give antilog $0.5E$ corresponding to E_p. In practice it is only possible to measure x in terms of the distance between cuvette and photocell envelope. Thus the extrapolation will require to be made to a point $-r$ on the x scale where r is the distance between the photocell envelope and the light-sensitive element; in other words r is the "effective radius" of the photocell. The magnitude of r can be determined if the scattering solution can be clarified (*e.g.* lysis of RBC) and the absorption characteristics of the pigment in clear solution obtained. The procedure is exemplified in Table II and Fig. 3.

Extrapolation to $-r$ would in effect bring the cuvette to the centre of the photocell, and thus to occupy the same position as the sensitive element. This hypothetical arrangement would ensure maximal registering of scattered light and is broadly analogous to the integrating sphere.

EXPERIMENTAL PROCEDURE AND MATERIALS

A Hilger Uvispek spectrophotometer fitted with a glass prism was used. In addition to the normal (8 watt) tungsten lamp a high intensity (36 watt) tungsten lamp was fitted in the position normally occupied by the hydrogen arc lamp. The high intensity lamp was used only in experiments with heart muscle preparations.

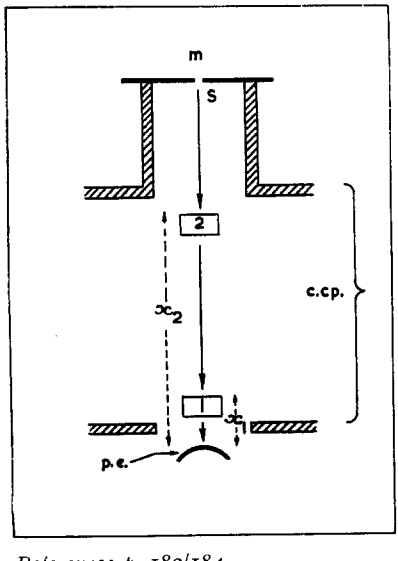

Fig. 1. The two extreme positions for a 5 mm cuvette within the cuvette compartment of the Uvispek spectrophotometer. c.cp., cuvette compartment; m, monochromator; p.e., photocell envelope; s, exit slit. $x_1 = 13$mm, $x_2 = 61$ mm.

Suspensions and solutions were examined in 5 mm cuvettes the positions of which could be varied within the cuvette compartment between the two extreme positions shown in Fig. 1: these correspond to $x_1 = 13$ mm and $x_2 = 61$ mm. The distance x was measured from the incident surface of the cuvette to the photocell envelope. Extinctions were measured either against a blank cuvette containing water or against a 1 mm cuvette containing Whatman 3MM filter paper soaked in liquid paraffin[9], a correction being applied for the measured E_s of the paper. Measurements reported in detail here were made within the region 620–480 mμ. A few experiments at lower wavelengths will be briefly described.

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The same U.V.-sensitive photocell was used for all wavelengths up to 620 mμ since scattering solutions, as would be expected, give rise to different E_t values with different photocells.

RBC suspensions. Defibrinated horse blood was centrifuged, the red cells washed three times with 0.9% NaCl and resuspended in saline to the required concentration. The concentration of HbO_2 in the suspension was determined, after lysis with a trace of saponin, from the value $\varepsilon_{577} = 14{,}200$ where molarity is referred to haem.

Scattering HbO_2 solutions. To clear lysed RBC suspensions was added sufficient milk to treble the value of E_{577} when the cuvette was in position 2 (Fig. 1). A trace of trishydroxymethyl-amino-methane buffer (pH 8) was added to prevent a slow precipitation of milk protein.

Heart muscle preparation (succinic oxidase system). This was prepared from horse heart[24] and the washed iso-electric precipitate suspended in an equal volume of a solution of 200 g sucrose in 100 ml 0.25 *M* phosphate buffer, pH 7.3. After 12 hours' storage at 5° the colloidal suspension was strained through glass wool.

RESULTS

A. Experiments at wavelengths above 480 mμ

1. *RBC suspensions*

The absorption curves within the range 500–600 mμ of a suspension containing 75 μ*M* HbO_2 were determined with the cuvette at five positions within the cuvette compartment. These curves are shown in Fig. 2. the values of x in mm being indicated against the curves. Each curve is based upon readings at the same set of 17 wavelengths. The curve *S* in Fig. 2, which is that of the clear solution obtained after addition of saponin, was independent of x. In Table II are given the data obtained at 600 mμ. A plot of Z against x for the cell suspension at this wavelength yields a

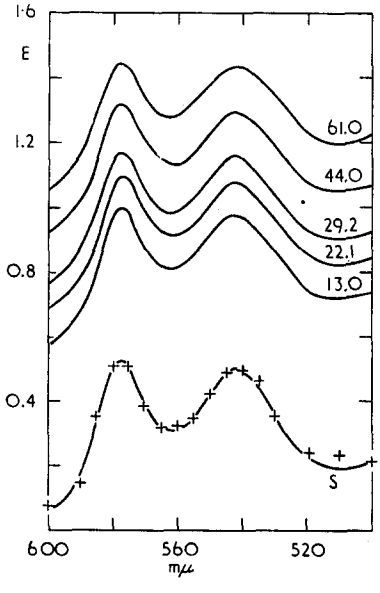

Fig. 2. Absorption curves of an RBC suspension measured with the cuvette at different distances from the photocell. The numbers on the curves are the corresponding values of x in mm (Fig. 1). The curve *S* was obtained after addition of saponin. The crosses represent the E values obtained by extrapolation to $x = -r$ where r = effective radius of photocell.

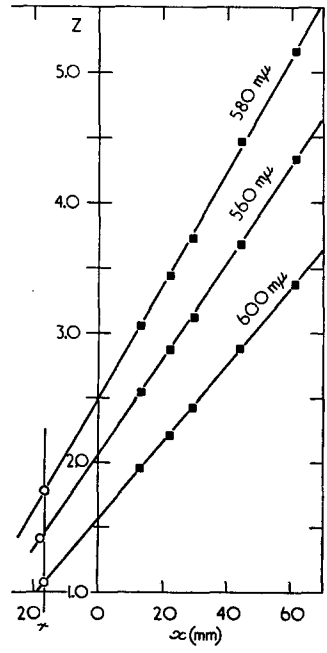

Fig. 3. Extrapolation at 3 wavelengths of Z values, obtained from upper 5 curves in Fig. 2, to $x = -r$.

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TABLE II

ABSORPTION DATA FOR A SUSPENSION OF RBC BEFORE AND AFTER LYSIS. EFFECT OF VARYING x, THE DISTANCE BETWEEN CUVETTE AND PHOTOCELL

	RBC suspension					*Lysed suspension*
x (mm)	13.0	22.1	29.2	44.0	61.0	13–61
E	0.580	0.689	0.768	0.921	1.060	0.061
$Z = \left[\frac{I_0}{I}\right]^{0.5}$	1.95	2.21	2.42	2.89	3.39	1.07

straight line (Fig. 3) to which is added an additional point (open circle) representing the value of Z for the lysed suspension. This point represents in effect the point of intersection of the Z–x plots for the suspension and for the clear solution obtained after lysis since in the latter case the plot would be a horizontal line passing through the open circle. The distance between this point of intersection and the vertical axis gives the value of r at 600 mμ. Similar plots at 560 and 580 mμ are also given in Fig. 3 and two further values of r are so obtained. The mean value of r, based upon the 17 wavelengths at which readings were made, was 16.2 mm. Since the standard deviation of r was only 0.8 mm, the values of E_p calculated from the intercepts of the 17 straight line plots with the vertical line at $x = -16.2$ mm lie close to the experimental curve S. These calculated values are denoted by crosses in Fig. 2. The value of r was almost independent of HbO_2 concentration from 40 to 150 μM, the variation not exceeding $\pm$ 1 mm. Below 40 μM, the extrapolation becomes uncertain.

2. *HbO_2 solution in a light-scattering medium*

When a solution of HbO_2 (69 μM) containing fat-free milk was examined in the same way the values of r at eleven wavelengths were in good agreement but the mean value was now 24.5 mm. In the spectrum of the HbO_2–milk mixture the β-band (542 mμ) was slightly higher than the α-band (577 mμ). Nevertheless the extrapolation to -24.5 gave points corresponding to the solution curve where the α-band is slightly higher than the β-band. This result indicates that the higher α-band in an HbO_2–milk mixture results not from any milk pigments but from a somewhat greater light scattering by milk at 542 mμ than at 577 mμ. This conclusion was supported by absorption measurements on diluted milk and from the finding that the flavine content of the diluted fat-free milk was unmeasurably small.

3. *Heart muscle preparations*

The foregoing experiments demonstrated that r varies with the type of scattering material and this fact raises the problem of the determination of r for heart muscle. The use of sucrose-phosphate as a suspending medium for HMP gives rise to a much less opaque material than does suspension in the customary phosphate buffer. A further marked clarification can be obtained by addition of sodium cholate[25] but this substance, like other bile salts, slowly denatures some of the endogenous cytochrome pigments. Nevertheless complete spectrophotometric curves in the visible region could be obtained before changes became apparent. Although scattering is diminished by addition of cholate a marked Tyndall effect persists.

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Since there is no known way of rendering HMP completely transparent without denaturation of all cytochrome components except cytochrome *c*, determinations of *r* were based upon parallel experiments with two types of HMP: (P) 9 vols. HMP + 1 vol. 0.25*M*-Na_2HPO_4 and (C) 9 vols. HMP + 1 vol. sodium cholate. The sodium cholate was a solution containing 40% w/v cholic acid plus suffient NaOH to bring the pH to 7.4. Both solutions were reduced in the cuvettes by addition of 25 m*M*-$Na_2S_2O_4$ and immediately covered with a layer of liquid paraffin. The spectrophotometric records, which were obtained without delay, are shown in Fig. 4.

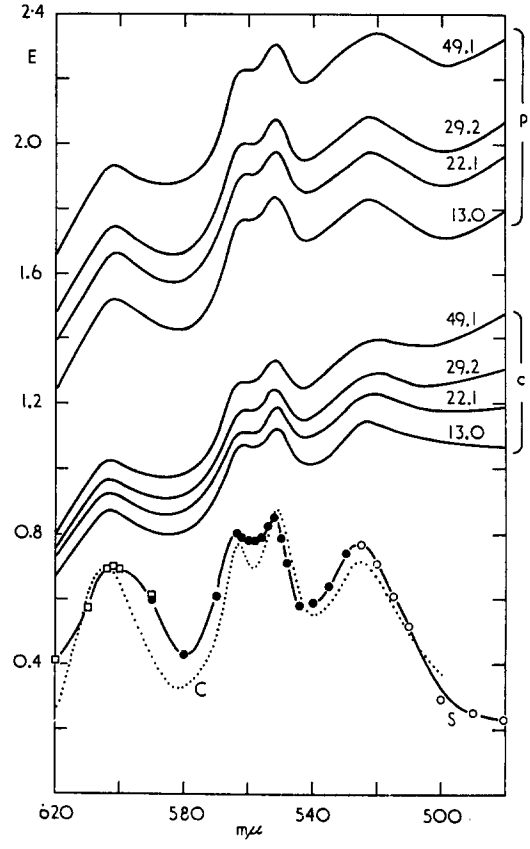

Fig. 4. Absorption curves of HMP suspended in sucrose-phosphate (P) and sucrose–phosphate–cholate (C), with the cuvettes situated at different distances from the photocell (see legend to Fig. 2). Extrapolation as described in the text gives the points □ ● ○ from which curve *S* is drawn. The dotted line *C* is the estimated absorption curve of the mixture of cytochromes in HMP if they could be obtained as a clear solution.

Within the range 530–580 mμ both types of HMP yielded straight line plots of *Z* against *x*. The procedure for the eight *E* values at 552 mμ is shown in Fig. 5 (full lines) and the intersection of the two lines was taken as indicating the magnitude of *Z* at 552 mμ when scatter is eliminated. The mean value of *r* for 15 wavelengths within the 580–530 mμ range was 21.8 mm. The values of E_p were calculated as before from the intercepts of the *P* lines, as in Fig. 5, with the vertical line at $x = -21.8$ mm. These are plotted (●) in Fig. 4 as part of the derived curve *S* of the intracellular pigments. Similar treatment of the results below 530 mμ yielded straight lines for *P* but not for *C*. The *P* lines were therefore extrapolated to the same value of *r* (21.8) to give points (○) for the S curve. At wavelengths above 590 mμ straight lines were obtained for neither preparation. By trial it was found that straight lines resulted from plotting $(I_0/I)^{0.8}$ against *x* and the extrapolation derived from readings obtained at 610 mμ is shown in Fig. 5 (broken lines). In these cases the mean value of *r* was 11 mm. The points obtained by this procedure are also recorded (□) in Fig. 4. At 590 mμ both extrapolation procedures yielded approximately straight lines.

B. Limitations of the method at lower wavelengths (400–460 mμ)

When RBC suspensions are examined in conventional spectrophotometers the γ-band (414 mμ) is found to be very weak or even absent[26] although the α and β bands are

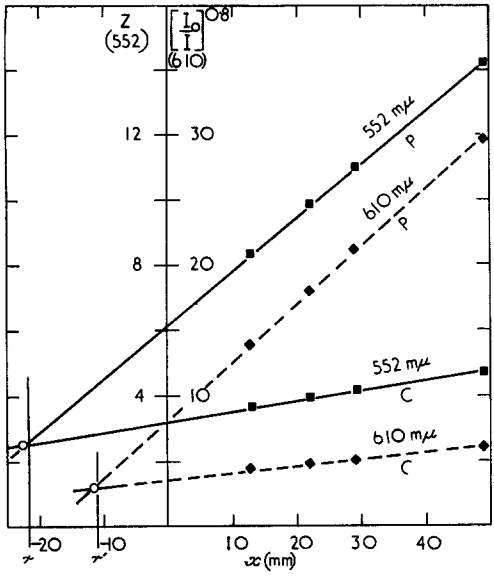

Fig. 5. Extrapolation, at 2 wavelengths, of E values obtained from the upper 8 curves in Fig. 4.

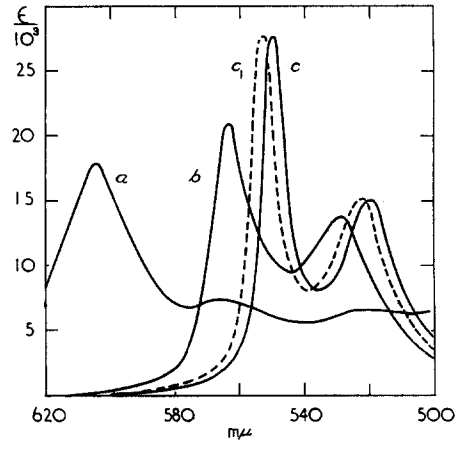

Fig. 6. Absolute absorption spectra of cytochromes a ($= a + a_3$), b, c, c_1.

very similar in shape to those of the equivalent HbO_2 solution (Fig. 2). This anomaly was originally ascribed to the existence within RBC of a stromatin–HbO_2 compound[26] although it has since been shown that the effect is purely physical[27,28]. Thus JOPE[28] using a spectrograph in which light passed vertically through the cuvette found that a uniform suspension of RBC showed no γ-band. However, if the cells were allowed to settle under gravity the thin layer now exhibited a very strong γ-band. A pronounced γ-band was also revealed in the spectrum of a single cell. In addition, the γ-band can be brought into prominence by suspending RBC in media of high refractive index (see p. 173). LOTHIAN AND LEWIS[18] have considered two extreme cases of the spectrophotometry of RBC suspensions: (i) where no scattered radiation is received and (ii) where all scattered radiation is received. In the former case, by considering the interference between wave-fronts transmitted by cells and isotonic medium, these authors have calculated that for wavelengths in the region of 360 mμ there will be destructive interference which will increase with the transparency of the cells. Under such conditions the measured E_t should exhibit a minimum at 414 mμ. Curves of this type could in fact be obtained. For case (ii) it was calculated that the γ-band, though present, would be depressed. On this basis it is understandable that in spectra of RBC suspensions obtained with a conventional spectrophotometer the γ-band may be weak or even absent[27,28].

Attempts to apply the present method to RBC suspensions at 400–460 mμ have so far been unsuccessful. Below 460 mμ the magnitude of r increases with decreasing wavelength and passes through a maximum at the wavelength of the γ-peak. It is remarkable that milk–HbO_2 mixtures exhibit similar variations in r although a normal γ-band can be detected spectrophotometrically. Such mixtures, however, differ fundamentally from RBC suspensions in that the pigment is dissolved in the medium in which the particles are suspended. Furthermore that scattering particles

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are much smaller than RBC. Examination of HMP over the same range of wavelengths was also impractical not only because r was again variable but also because of the great increase in light scattering at lower wavelengths (see Fig. 4) which made the extrapolation uncertain.

DISCUSSION

It is fortunate that the spectral region 500–600 mμ within which the present method of scattering correction is reliable, is also the region which is most important for the spectroscopic study of haematin pigments. It is within this range that reactions of haemoproteins are reflected by the most marked changes in spectral pattern. DUYSENS' method of correcting for "flattening" of absorption bands, which occurs when the pigment is intracellular, may be valid for a much wider range of wavelengths. Thus with a *Chlorella* suspension the correction was successfully applied over the range 420–720 mμ[19]. However, it must be borne in mind that DUYSENS' method corrects for flattening but not for scattering which, in the case of the *Chlorella* suspension, was quite small in comparison with light absorption. With the materials used in this paper light scattering, in terms of measured extinction, is large compared with the extinction changes due to flattening.

With RBC suspensions the extrapolation procedure eliminates not only the effects of light scattering but also the flattening of absorption bands. This flattening is not very marked for RBC within the visible spectrum. For example, in Fig. 2 the difference between E_{577} (α-maximum) and E_{562} (minimum between α and β bands) is 0.238 for the S curve, 0.184 for the "13" curve and 0.166 for the "61" curve. Thus flattening increases with x, *i.e.* as the proportion of scattered light received by the photocell decreases. These findings conform with the deductions of DUYSENS[19] that flattening is a property of the light transmitted, rather than scattered, by a suspension of pigmented particles.

The extrapolation to zero distance between cuvette and light detector surface of the photocell becomes an arbitrary procedure with a cuvette of finite optical depth. It is possible that differences in the values of r for RBC and for milk–HbO_2 mixtures could be reduced by the use of very thin cuvettes. Such cuvettes would, however, greatly restrict the applicability of the method to biological materials.

The heart muscle preparation containing the succinic oxidase system (HMP) consists of particles[24] which are considerably smaller than the erythrocyte. They are derived from heart muscle sarcosomes which have disintegrated in hypotonic media[29]. The theoretical upper limit of their size is thus that of the sarcosome (1–2 μ) while the lower limit appears, from ultracentrifugal data[30,31], to be about 0.05 μ. From the extensive work of MIE and others[32] on the angular distribution of light scattered by particles of a size comparable with that of the wavelength of the light, it can be assumed that the distribution may well be non-uniform in the case of HMP. Non-uniformity of scattering can be considered a possible reason for the non-linearity of Z–x plots for HMP at certain wavelengths.

The derived curve S for the components of cytochrome in HMP (Fig. 4) gives evidence for flattening of absorption bands in the experimental curves. As already stated, a precise experimental check upon the validity of the S curve cannot be made. On the other hand, from the available data (i) on the absolute absorption curves of

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the individual cytochrome pigments and (ii) on the probable concentrations of these pigments in HMP it is possible to derive a curve which should be an approximation to the S curve if the extrapolations give a true correction for scattering. Some discrepancies between such a curve and the S curve obtained by extrapolation will be inevitable since there is more haematin present in HMP than can be accounted for by the cytochromes present[33,34].

The five known cytochrome pigments in HMP are a, a_3, b, c, and c_1. Since a and a_3 appear to have virtually identical spectra in the visible region we shall in this paper use the term cytochrome a for the mixture of a and a_3 in HMP. The following data are available on the cytochromes of HMP.

Cytochromes c and c_1

The absolute absorption curve of cytochrome c has been published by KEILIN AND SLATER[35]. A method for estimating cytochrome c in HMP by spectroscopic comparison of HMP with standard solutions of cytochrome c[34] was applied to the sample used in the present work and a value of 30 μM was obtained. Since the absorption bands of cytochromes c and c_1 are very close this method in fact estimates $c + c_1$[3]. On the basis of a previous spectrophotometric analysis of HMP[3] the ratio $c:c_1$ appears to be ~ 2. This calculation is based upon the assumption that the absolute heights of the α-bands of cytochromes c (550 mμ) and c_1 (553 mμ) are identical: an assumption which is reasonable on the basis of the close similarity of the two pigments. The value of ε_{550} for cytochrome c is 27,700[35]. Variations in the $c:c_1$ ratio will have little effect upon the form of the calculated curve for the mixed pigments of HMP so long as the total concentration (30 μM) is fixed. The concentrations of cytochromes c and c_1 will therefore be taken as 20 and 10 μM respectively.

Cytochrome b

This pigment was obtained in a state of spectral purity by HÜBSCHER, KIESE AND NICOLAS[36] who give a value of 20,800 for ε_{max} (563 mμ). The estimation of cytochrome b in HMP is based upon spectroscopic comparison with the cytochrome c in the HMP. In developing this method SLATER[34] assumed that when the b and c bands in HMP appear equal, the concentrations of the two cytochromes are identical. However the values of ε_{max} are different for the two pigments and a corresponding correction should be applied in the estimation of cytochrome b. In the present instance the b and c bands were of equal intensity. Hence the concentration of cytochrome b = concentration of cytochromes $(c + c_1) \times 27{,}700/20{,}800 = 40$ μM

Cytochrome a

The separation of this pigment from heart muscle as a clarified solution was effected by DANNENBERG AND KIESE[37] and the value of ε_{max} (604 mμ) was given as 19,500. This value is certainly too high since the solution still showed a Tyndall effect and its absorption at 640 mμ was still marked. From comparison with the spectra of other cytochromes the absorption at the latter wavelength should be very small and by analogy with the absorption curve of the pyridine haemochromogen of cytochrome a[38] a reasonable value for $\varepsilon_{640}/\varepsilon_{max}$ for cytochrome a would be 0.05. This condition can be satisfied by applying a constant correction, throughout the visible spectrum, of -1800 to the values of E, provided by the experiments of DANNENBERG AND KIESE. This lowering of the absorption curve, which can be

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considered as a correction for light scattering, results in a value of 17,700 for ε_{max}. CHANCE[39] has attempted to estimate cytochrome *a* in tissue preparations by assuming that its spectrum in the red region is identical with that of verdoperoxidase. There is, however, no experimental basis for this assumption. Were haemin *a* more readily available[40], the concentration of cytochrome *a* in HMP could be readily determined by visual spectroscopic comparison of a standard solution of pyridine haemochromogen *a* with HMP in which the cytochrome components had been converted into the corresponding pyridine haemochromogens. Since no other cytochrome component of HMP absorbs light appreciably at 640 mμ, it was assumed that the height of the *S* curve (Fig. 4) at this wavelength could be taken as a measure of the cytochrome *a* concentration. In this way a value of 80 μM was obtained.

On the basis of the above absorption data the absolute absorption curves of cytochromes *a*, *b*, *c* and c_1 are plotted in Fig. 6. On the assumption that the μ-molar concentrations of these pigments are 80, 40, 20 and 10 respectively, the calculated absorption curve of the mixture of HMP cytochromes is plotted (as a dotted line) in Fig. 4. The agreement between this calculated curve, *C*, and the *S* curve is, under the circumstances, quite good. By making the heights equal at 604 mμ, a marked discrepancy is produced at 600–580 mμ. However, within this region the extrapolation procedure is abnormal. A difference curve S *minus* C would show a broad maximum at 585–590 mμ and a narrower one at 558 mμ. While it is not possible to associate the former with known haem compounds, except possibly with denatured cytochrome *a*, it is perhaps of interest that the latter maximum corresponds to the absorption peak of deoxygenated myoglobin: a very probable contaminant of HMP which has been treated with reducing agents. On the other hand the differences between the *S* and *C* curves may in part represent an incomplete correction for the flattening of the experimental curves of Fig. 4.

SUMMARY

A method is described by which the spectrophotometric curve of a suspension of pigmented particles can be corrected for light scattering. A series of absorption curves is determined with the cuvette at different distances from the photocell and an extrapolation to zero distance between the cuvette and the light-sensitive element is performed. In the case of red blood cell suspensions the corrected curves were in quantitative agreement with those of the oxyhaemoglobin solutions resulting from lysis of the cell suspensions. Similar experiments with the cytochrome system of a heart muscle preparation yielded a corrected curve which was in reasonable agreement with a curve based upon the concentrations and spectral characteristics of the cytochrome components in the preparation.

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METHODS FOR THE QUALITATIVE AND QUANTITATIVE DETERMINATIONS OF TETROSES BY TWO NEW SPECIFIC COLOR REACTIONS*

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Studies of the role of tetrose in the intermediary metabolism and interconversion of sugars in animal and plant cells suggested the development of simple and sensitive color reactions for their qualitative and quantitative determination. The present report deals with two such reactions.

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