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# DERIVATION OF PHOTOCHROME ABSORPTION SPECTRA FROM ABSORBANCE DIFFERENCE MEASUREMENTS

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**Abstract**—A method is presented with the aid of which the absorption spectrum of at least one of the two states of a photochrome can be calculated from experimental difference spectra. It is shown that the method can be applied to (biological) photochromes contained in inhomogeneous media together with absorbing, non-photochromic impurities. This medium may have the properties of an optical waveguide.

## INTRODUCTION

The spectral absorbance of biological pigments as a rule is determined from purified solutions. A drawback of purification, however, is that the extraction procedure may alter the pigment properties (Bowmaker, 1973; Goldsmith and Bruno, 1973; Kendrick and Spruit, 1973; Schwemer and Paulsen, 1973). Furthermore, the isolation procedure may have a low yield and the extract may include impurities that cannot be removed (Dartnall, 1957). With microspectrophotometry of *in situ* pigments the latter difficulties are avoided (Liebman, 1972), but then the estimation of accurate absolute spectra can be wearisome. On the other hand, difference spectra generally are accepted as an adequate technique to study those pigments that participate in photochemical processes. Absolute spectra then are derived from indirect evidence such as sensitivity measurements (Höglund *et al.*, 1973), (Dartnall, 1957) nomograms (Hamdorf *et al.*, 1973), or action spectra.

In the present paper we argue that difference spectra in certain instances are sufficient to enable calculation of photopigment absorbance spectra. We demonstrate this claim on pigments showing photochromism.

Originally we developed the calculation method in the course of our work on fly rhodopsin (Stavenga *et al.*, 1973). Obviously the present method will be applicable to other invertebrate photopigments also, since most probably the photochemistry of invertebrate visual pigments is quite uniform (Goldsmith, 1972). Moreover, as will be shown, the technique can be utilized in studies on a large class of (biological) photochromes.

## PHOTOCHROMISM

Photochromism is defined as a reversible change of a single chemical substance between two states having different absorption spectra, such a change being induced in at least one direction by the action of electromagnetic radiation (Brown, 1971):

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B \tag{1}$$

*A* and *B* represent the two thermostable states of the photochromic pigment *P*. If the concentrations of substances *A*, *B* and *P* are $c_A$, $c_B$, and $c_P$, respectively, then

$$c_A + c_B = c_P = \text{constant} \tag{2}$$

It will be advantageous to employ fractions defined by

$$f_A = c_A/c_P \quad \text{and} \quad f_B = c_B/c_P \tag{3}$$

It follows from Eq. 1 that the fractions at equilibrium are given by

$$f_{Be} = (1 + k_2/k_1)^{-1} = 1 - f_{Ae} \tag{4}$$

if $k_1$ and $k_2$ are the rate constants (at equilibrium) for the conversions *A* to *B* and *B* to *A*, respectively. The rate constants can be written explicitly, but first we will introduce some quantities common in spectrophotometric practice.

## DIFFERENCE SPECTRA OF SAMPLES CONTAINING PHOTOCHROMES

We consider a pigment *P* immersed in a medium, of which a sample of thickness *L* is studied spectrophotometrically.

The transmittance and absorbance of the sample are defined by

$$T(\lambda) = I_L(\lambda)/I_0(\lambda) \tag{5}$$

and

$$E(\lambda) = -\log_{10} T(\lambda) \qquad (6)$$

respectively, where $\lambda$ is the wavelength and $I_0$ and $I_L$ are the incident and the transmitted light fluxes, respectively. The absorption coefficient of the medium at location $x$, $\kappa(\lambda, x)$ is equal to

$$\kappa(\lambda, x) = \kappa_P(\lambda, x) + \kappa_S(\lambda, x) \qquad (7)$$

when, apart from the pigment $P$, other substances are contained in the medium, the sum of their absorption coefficients being $\kappa_S$.

We provisionally assume that scattering is negligible and that Beer's law holds, or

$$T(\lambda) = \exp\left[-\int_0^L \kappa(\lambda, x)\,dx\right] \qquad (8)$$

Subsequently we consider two situations in which $P$ has absorption coefficients $\kappa_{P0}$ and $\kappa_{P1}$, respectively.

The difference spectrum of the two situations is then defined as the change in spectral absorbance

$$D(\lambda) = E_1(\lambda) - E_0(\lambda) \qquad (9)$$

This results, with $g = \log_{10} e$, in

$$D(\lambda) = g \int_0^L [\kappa_{P1}(\lambda, x) - \kappa_{P0}(\lambda, x)]dx \qquad (10)$$

Relation 10 demonstrates one of the advantages of difference spectra, since by this technique interference from photostable pigments $S$ is eliminated.

To obtain difference spectra experimentally, only transmission measurements are required, as follows from definitions 5, 6 and 7:

$$D(\lambda) = \log_{10} I_{L0}(\lambda)/I_{L1}(\lambda) \qquad (11)$$

So, difference spectra are of use in those cases where either impurities cannot be avoided or the incident light flux $I_0$ is difficult to estimate accurately. Both situations sometimes occur simultaneously.

We will now derive a general expression for the difference spectra which can be measured from the sample if the pigment $P$ is the photochrome of the preceding section.

The absorption coefficient $\kappa_P$ can be written as

$$\kappa_P(\lambda, x) = \alpha_P(\lambda, x) . c_P(x) \qquad (12)$$

where $\alpha_P$ is the molecular absorption coefficient and $c_P$ the number of molecules of photochrome per unit volume.

$\alpha_P$ depends on the mixture of states $A$ and $B$ by

$$\alpha_P(\lambda, x) = f_A(x)\alpha_A(\lambda) + f_B(x)\alpha_B(\lambda) \qquad (13)$$

($\alpha_A$ and $\alpha_B$ obviously are the molecular absorption coefficients of states $A$ and $B$, respectively).

With relations 2, 3, 12 and 13 we derive from Eq. 10 that the difference spectra are represented by

$$D(\lambda) = g[\alpha_B(\lambda) - \alpha_A(\lambda)] \int_0^L [f_{B1}(x) - f_{B0}(x)]c_P(x)\,dx \qquad (14)$$

in which $f_{B1}$ and $f_{B0}$ represent the fractions of $B$ in the two situations. From this expression we conclude that difference spectra obtained from a sample containing a photochrome are always proportional to the difference in the molecular absorption coefficients $\alpha_A$ and $\alpha_B$, irrespective of inhomogeneities in the pigment concentration and distribution of the fractions $f_{B1}$ and $f_{B0}$ throughout the sample.

Explicit expressions for the fractions have to be derived for further development of Eq. 14. We shall turn to this in the next section where we treat a particular case of photochromism; it is assumed that no dark reactions are involved.

## PHOTOEQUILIBRIA BETWEEN STATES A AND B INTERCONVERTIBLE BY LIGHT ONLY

A sample containing a photochromic substance is illuminated with monochromatic light of (stimulus) wavelength $\lambda_s$. The rate constants governing the photochemical process are given by

$$\kappa_1(\lambda_s, x) = \alpha_A(\lambda_s)\gamma_A(\lambda_s)I(\lambda_s, x) \qquad (15a)$$

and

$$\kappa_2(\lambda_s, x) = \alpha_B(\lambda_s)\gamma_B(\lambda_s)I(\lambda_s, x) \qquad (15b)$$

$\gamma_A$ is the quantum efficiency of $A$, i.e. the probability that an absorbed quantum by a molecule in state $A$ induces a conversion to state $B$; $\gamma_B$ is the corresponding quantity for the opposite conversion; $I$ is the quantum flux.

With the relative quantum efficiency $\phi$, defined by

$$\phi(\lambda) = \gamma_B(\lambda)/\gamma_A(\lambda) \qquad (16)$$

the equilibrium fraction of state $B$ is conveniently presented, with Eqs. 4, 15, and 16, by

$$f_{Be}(\lambda_s) = \left[1 + \frac{\alpha_B(\lambda_s)}{\alpha_A(\lambda_s)}\phi(\lambda_s)\right]^{-1} \qquad (17)$$

From Eq. 17 the reason for selecting monochromatic

illumination clearly emerges. The equilibrium created by monochromatic light is the same at all locations in the sample. This will not generally hold for polychromatic irradiation.

To avoid confusion between stimulus wavelength $\lambda_s$ and test wavelength $\lambda$ (Eq. 14), the latter will henceforth be denoted $\lambda_t$. We assume that the equilibrium at $\lambda_s$ has been reached, starting from a situation where $f_{B0}(x) = 0$. Then with $f_{B1}(x) = f_{Be}(\lambda_s)$ we can write Eq. 14 as

$$D_e(\lambda_t, \lambda_s) = N f_{Be}(\lambda_s)[\alpha_B(\lambda_t) - \alpha_A(\lambda_t)] \qquad (18)$$

where

$$N = g \int_0^L c_P(x)\, dx \qquad (19)$$

We remark that $f_{Be} = 0$ means that $\alpha_A/\alpha_B = 0$; this will be approximated at long wavelengths if the absorption peak of $B$ is located at a longer wavelength than that of $A$ (see Fig. 1).

Expression 18 states the following; if we start from the situation that all molecules of a photochrome in a sample are in state $A$ and subsequently a photoequilibrium is established at wavelength $\lambda_s$, then the result is a change in absorbance proportional to the fraction of molecules transferred into state $B$ times the difference in the molecular absorption coefficients of the two states.

In experimental practice Eq. 18 represents a family of difference spectra as a function of $\lambda_t$ with independent parameter $\lambda_s$. The fraction $f_{Be}$ is the proportionality factor of the separate spectra. Both expressions 17 and 18



Figure 1.

contain the molecular absorption coefficients $\alpha_A$ and $\alpha_B$. This provides the possibility to separate these quantities.

## CALCULATION OF ABSORPTION COEFFICIENTS FROM DIFFERENCE SPECTRA

Essential to the calculation procedure for the absorption coefficients $\alpha_A$ and $\alpha_B$, which we present now, is the supposition that the states $A$ and $B$ possess an isosbestic point. This is the wavelength $\lambda_{iso}$, defined by

$$\alpha_A(\lambda_{iso}) = \alpha_B(\lambda_{iso}) \qquad (20)$$

The isosbestic point is highly recognizable since here the family of difference spectra cross each other, see Eq. 18. Moreover, the isosbestic point provides the clue to separate $\alpha_A$ and $\alpha_B$; at this wavelength the absorption coefficients cancel from Eq. 17:

$$f_{Be}(\lambda_{iso}) = [1 + \phi(\lambda_{iso})]^{-1} \qquad (21)$$

Therefore let us relate the equilibrium fraction $f_{Be}(\lambda_s)$ to this value by

$$Q(\lambda_s) = f_{Be}(\lambda_s)/f_{Be}(\lambda_{iso}) \qquad (22)$$

According to Eq. 18:

$$Q(\lambda_s) = D_e(\lambda_t, \lambda_s)/D_e(\lambda_t, \lambda_{iso}) \qquad (23)$$

It is important to note that this function is independent of $\lambda_t$ and therefore can be determined experimentally at any fixed test wavelength. On the other hand, having once determined for a certain $\lambda_s$ the value of $Q(\lambda_s)$, the difference spectrum corresponding to that wavelength $D_e(\lambda_t, \lambda_s)$ yields the function $D_{iso}(\lambda_t) = D_e(\lambda_t, \lambda_{iso})$ as follows from Eq. 23, where

$$D_{iso}(\lambda_t) = D_e(\lambda_t, \lambda_s)/Q(\lambda_s) \qquad (24)$$

With the aid of Eqs. 18 and 21, and replacing $\lambda_t$ by $\lambda$, this equation can be written explicitly as

$$D_{iso}(\lambda) = N[1 + \phi(\lambda_{iso})]^{-1}[\alpha_B(\lambda) - \alpha_A(\lambda)] \qquad (25)$$

Subsequently we obtain from Eqs. 17, 21 and 22, while dropping the suffix $s$,

$$Q(\lambda) = [1 + \phi(\lambda_{iso})]\left[1 + \frac{\alpha_B(\lambda)}{\alpha_A(\lambda)}\phi(\lambda)\right]^{-1} \qquad (26)$$

The desired absorption coefficients $\alpha_A$ and $\alpha_B$ can now be solved from Eqs. 25 and 26:

$$\alpha_A(\lambda) = N^{-1}D_{iso}(\lambda)\phi(\lambda)Q(\lambda)\left[1 - Q(\lambda)\frac{1 + \phi(\lambda)}{1 + \phi(\lambda_{iso})}\right]^{-1} \qquad (27)$$
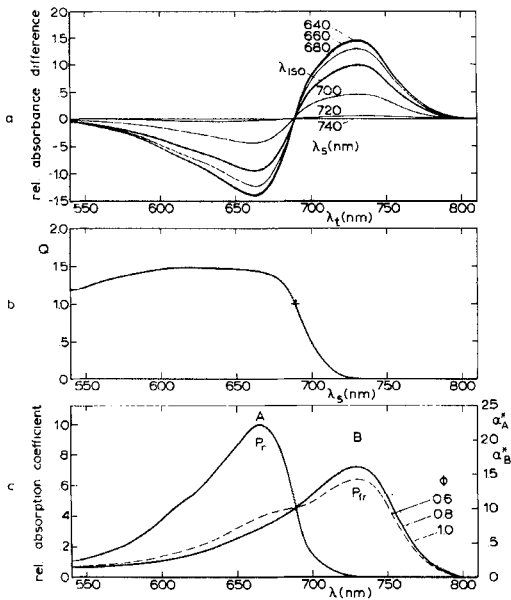
and

$$\alpha_B(\lambda) = \alpha_A(\lambda)[1 + \phi(\lambda_{iso}) - Q(\lambda)][\phi(\lambda)Q(\lambda)]^{-1} \quad (28)$$

An important simplification is achieved by assuming that, in a substantial wavelength range, the relative quantum efficiency is constant:

$$\phi(\lambda) = \phi(\lambda_{iso}) = \phi \quad (29)$$

Hence

$$\alpha_A(\lambda) = N^{-1}D_{iso}(\lambda)\phi Q(\lambda)[1 - Q(\lambda)]^{-1} \quad (30)$$

and

$$\alpha_B(\lambda) = N^{-1}D_{iso}(\lambda)[1 + \phi - Q(\lambda)][1 - Q(\lambda)]^{-1} \quad (31)$$

$D_{iso}(\lambda)$ and $Q(\lambda)$ in Eqs. 30 and 31 are the experimental data, resulting from transmission measurements.

The sample constant $N$ embodies the total amount of molecules of photochrome in the sample, (see Eq. 19). Although $N$ usually will be unknown it is of course removed from the normalized absorption spectra. It is useful to note that upon normalization the relative quantum efficiency $\phi$ vanishes also from the absorption spectrum of state $A$. A similar result for state $B$ cannot be drawn, however, owing to the more complex dependence of $\alpha_B$ on $\phi$. Therefore, in order to obtain the spectrum of $B$ the value of $\phi$ has to be determined separately. This is achieved in the most direct way if a wavelength $\lambda_d$ can be found where $\alpha_B(\lambda_d)/\alpha_A(\lambda_d) \simeq 0$. According to Eqs. 26 and 29 it follows then that

$$\phi = Q(\lambda_d) - 1 \quad (32)$$

Usually, however, a wavelength $\lambda_d$ cannot be found. Other methods to obtain the relative quantum efficiency will be treated elsewhere (in preparation).

The method is now demonstrated for the example of the pigment phytochrome. Starting from published spectra of $P_r$ and $P_{fr}$, corresponding to our $A$ and $B$ respectively, i.e. the solid lines in Fig. 1c, which are taken from Spruit and Spruit (1972), and a relative quantum efficiency of $\phi = 0.8$ (cf. Hartmann, 1966), we have constructed the expected photoequilibrium difference spectra $D_e(\lambda_t, \lambda_s)$ for a number of stimulus wavelengths (Fig. 1a). Figure 1b gives $Q(\lambda_s)$, which may be compared with Fig. 3 of Hartmann (1966). We calculate

$$\alpha_A^*(\lambda) = \alpha_A(\lambda)/\alpha_A(\lambda_{iso}), \quad (33)$$

the normalized absorption spectrum, from Eq. 30. The result is shown in Fig. 1c. As we remarked earlier, this is independent of the value chosen for $\phi$. On the other hand,

we obtain from Eq. 31:

$$\alpha_B^*(\lambda) = \alpha_B(\lambda)/\alpha_B(\lambda_{iso}) \quad (34)$$

which still depends on $\phi$. Figure 1c shows how the spectrum of $B$, calculated from Eq. 33 is affected by adjustment of $\phi$.

For the present we conclude that, in the case of an inhomogeneous medium containing both a photochromic substance and photostable impurities, spectral transmission measurements are sufficient to calculate unambiguously the absorption spectrum of at least one of the two photochrome states.

## DISCUSSION

We have considered the case of a photochromic substance localized in a sample together with other absorbing, photostable, impurities. It is supposed that (i) the two photochrome states $A$ and $B$ are converted into each other only after light absorption, (ii) the relative quantum efficiency $\phi$ defined in Eq. 16, is independent of wavelength, and (iii) a wavelength exists where the absorption by state $A$ is negligible with respect to that of state $B$. At photoequilibrium, established at this latter wavelength, all photochrome molecules are in state $A$. To this situation all other photoequilibria are related by the expression for the difference spectra, Eq. 18. Accordingly we have derived that the normalized spectrum of state $A$ can be calculated from experimental difference spectra. Also, if the relative quantum efficiency is known, the normalized spectrum of $B$ can be obtained.

Basic to the calculation method has been the assumption that Beer's law holds in the sample. Actually the example of Fig. 1, phytochrome, is embodied in vivo in turbid plant tissue (Spruit, 1972; Schmidt et al., 1973). Absorbance measurements from turbid media, in which Beer's law is no longer valid, generally involve major complications. We shall discuss the applicability of the calculation method for this case in a separate paper (in preparation). Yet, it will be clear that if the turbidity of a medium is small and the scattering coefficient is independent of the photochrome, this coefficient then is removed from the difference spectra. So in this simple situation the procedure can be executed identically as in the non-scattering case.

Another special case occurs if the photochrome is contained in an optical waveguide, as is the case with the visual pigments of invertebrates (Hamdorf et al., 1973; Stavenga et al., 1973). It is derived in the Appendix that this does not impose serious restrictions to our calculation technique.

We wish to point out here that the procedure outlined above is especially useful for the investigation of biological photochromes, since biological tissues usually

require techniques causing minimal damage. A large number of biological photochromes are already known, but new fields probably will emerge since new substances are discovered continually, e.g. bacterio-rhodopsin (Oesterhelt and Stoeckenius, 1971), a photo-reversible algae pigment resembling phytochrome (Scheibe, 1972), and photochromes in microorganisms (Rubin et al., 1972). Our investigations even may be of interest to another novelty in biological photochemistry, namely enzyme and polymer studies in which azodyes are applied (cf. Bieth et al., 1969; Van der Veen et al., 1974).

It may be pointed out that most biological photochromes do not fit the simple case of photochromism used in this paper since they usually have a so-called dark process. The latter can, however, be neglected at high irradiation intensities. Nevertheless a dark process sometimes can be exploited in the estimation of the quantum efficiency. This will be discussed in a future paper, having the kinetics of photochromes as a special topic.

In the foregoing we have made efforts to present a clear and unequivocal formalism for the photochemistry of photochromes. We hope that this approach will meet with approval since a unification of nomenclature will make the studies in different fields more accessible. As examples where related treatments in dissimilar terminology can be found, we mention the papers of Hamdorf et al.(1968 and 1973), Schwemer (1969), and Höglund et al. (1973), dealing with invertebrate visual pigments, and the investigations on phytochrome by Butler et al. (1964) and Hartmann (1966).

### APPENDIX: PHOTOCHROMES IN OPTICAL WAVEGUIDES

Suppose the (non-turbid) absorbing medium is the medium within a dielectric or optical waveguide and let the surrounding medium be transparent. The transmittance of a waveguide of length $L$ is then (Snyder and Richmond, 1972)

$$T(\lambda) = \exp\left[ -\int_0^L \eta(\lambda, x)\kappa(\lambda, x)\,dx \right] \tag{35}$$

$\eta$ is the fraction of light power propagated within the boundary of the waveguide. This quantity depends, besides wavelength, on cross-section and refractive indices of the media within and surrounding the waveguide. Knowing the parameters, its value can be calculated from waveguide optics. Useful data for the case of circular-cylindrical dielectric waveguides have been supplied by Biernson and Kinsley (1965). We remark that the general treatment of optical waveguides is not in principle invalidated by irregularities, as for instance in shape or absorption (Snyder, 1972; Snyder and Richmond, 1972).

It is readily seen that the formulae derived for the absorption coefficients are unaffected, if we replace $N$, defined in Eq. 19 by

$$N(\lambda) = g\int_0^L \eta(\lambda, x)c_P(x)\,dx \tag{36}$$

In the case of visual photoreceptors, $c_P(x) = c_p$ = constant. Then

$$N(\lambda) = gc_P\int_0^L \eta(\lambda, x)\,dx \tag{37}$$

The latter integral is discussed by Snyder and Pask (1973) in relation to tapering visual photoreceptors.

In conclusion, if difference spectra are obtained of photochromes in optical waveguides, calculation of the absorption spectra can be executed unrestricted if the sample constant is merely redefined.

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