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DIFFERENCE SPECTRUM DISTORTION IN NON-HOMOGENEOUS PIGMENT ASSOCIATIONS: ABNORMAL PHYTOCHROME SPECTRA *IN VIVO*

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SUMMARY

Difference spectra for phototransformation of phytochrome *in vivo* may vary considerably in shape, in peak position and in the ratio of the heights of red to far-red peaks. Assuming non-homogeneous distributions of pigments, spectra could be calculated for a number of models of plant cells containing both phytochrome and chlorophyll. Some models satisfactorily account for the observed spectra and provide some information about the intracellular pigment distribution. The theory may also find application in the interpretation of difference spectra of other naturally occurring pigment associations.

INTRODUCTION

In 1967 we published a short note on an abnormal difference spectrum for phototransformation of phytochrome, observed in leaves of dark-grown peas[1]. This spectrum was characterised by a peak in the red at 651 nm and a ratio of the magnitudes of red to far-red peaks of −0.42. In sections of internodes of the same plant these values were 665 nm and −0.88, respectively. On the basis of these findings we tentatively suggested that the phytochromes in pea leaves and in pea stems might be different pigments.

Our report was critisised by Hillman[2], who could not reproduce our results and found red peak positions as well as peak height ratios close to those normally observed in plants as well as in pigment extracts. Since we had regularly encountered such abnormal spectra in leaves of peas, grown in the laboratory under standard conditions, we examined the effects of variations in the treatment of plants and the preparation of the samples. It soon became clear that it is possible to observe difference spectra with considerably different positions and relative magnitudes of their red peaks. This was even more drastically apparent in leaves of dark grown bean seedlings and in cotyledons of mustard, which were next included in our study. Spectra of these materials showed very low red peaks compared to the absorption changes at 730 nm. It was evidently unattractive to ascribe such spectra to an equal number of different

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forms of phytochrome, the more so since the far-red peaks of all of them fell within the range 730–733 nm.

The spectra deviating most from the "normal" type were observed in materials that initially contained appreciable quantities of protochlorophyll. It appeared desirable, therefore, to consider the possibility of interference from either protochlorophyll or chlorophyll.

INTERFERENCE FROM OTHER PIGMENTS

One way to approach this problem is as follows. Our spectra were measured in a double-beam spectrophotometer as the difference between the absorbances of two samples of the same material. At the start of an experiment both samples were irradiated with sufficient red light to convert all protochlorophyll to chlorophyll. This was followed by a dark period of 30 min at room temperature, during which most of the spectral shifts associated with chlorophyll[3] had an opportunity to reach equilibrium. After this period, far-red light was given to one of the samples and the accompanying spectral changes were measured during a subsequent period of about 20 min. During the dark intervals, an appreciable fraction of the protochlorophyll initially present, is regenerated[4]. Since both samples had received an identical treatment up to the end of the first 30-min dark period, the quantity of both protochlorophyll and chlorophyll in them should have been equal and should not have affected the difference spectrum. However, from the moment that one of the samples had been irradiated with far-red light, the two were no longer identical. If far-red light treatment affects the rate of protochlorophyll regeneration, differential formation of this compound during the 20-min period required for recording a spectrum would then contribute to the observed difference spectrum. A closer examination of the data contradicts this explanation, however. Differential protochlorophyll regeneration between the two samples should either increase the ratio of the red to far-red peaks while shifting the red maximum in the direction of 650 nm, *i.e.* to shorter wavelengths, or it should decrease the peak height ratio while shifting the maximum upwards, depending on whether protochlorophyll regeneration is accelerated or slowed down by far red. Both possibilities are at variance with the observed spectra. Since we have also demonstrated[4] that the rate of dark regeneration of protochlorophyll in peas is exactly equal after red and after far-red irradiation, it is evidently unattractive to consider differential protochlorophyll regeneration as a factor when attempting to explain the abnormal spectra.

It therefore remains for us to examine the possibility of interference from chlorophyll. Our initial reluctance to consider light absorption in accessory pigments as responsible for distorted difference spectra stems from the following argument. Let $A_1, \ldots A_n$ represent the absorbances due to each of the absorbing Components 1, ... n in a mixture of pigments. The total absorbance is:

$$A_t = A_1 + \ldots A_n$$

Assuming that actinic irradiation bleaches Component m, and m only, the change in absorbance of the mixture is then:

$$\Delta A_t = - A_m$$

the absorbances of the inactive components cancelling out. A difference spectrum of a phototransformable component mixed with an arbitrary quantity of photochemically inactive substances, however large their absorbances, should equal the difference spectrum of the pure component both in shape and in magnitude (as long as the solution transmits sufficient light to make measurement possible, of course). For this reason we hesitated to accept chlorophyll interference as the explanation for the distortion of the phytochrome difference spectra until it occurred to us that the reasoning given above may not at all be valid if the various pigments are not present as a homogeneous solution. In the following we will give equations which allow us to calculate absorbances for a number of such inhomogeneous pigment distributions. It will be seen that, in certain pigment configurations, difference spectra are indeed influenced by the presence of other absorbing substances.

DIFFERENCE SPECTRA OF INHOMOGENEOUS ASSEMBLAGES OF PIGMENTS

Two pigments can be distributed non-homogeneously over a given volume in an infinite number of ways. We will assume that each is either inside or outside a single cubical subfraction ("particle") of a cubical plant cell. Within the two fractions of the cell volume, the pigments were assumed to be in homogeneous solution. In this way, eight different patterns of distribution result (Fig. 1). If the direction of the light beam is normal to a cube face, it is a matter of elementary mathematics to calculate the total transmission of the cube. A differential absorbance is next obtained by subtracting the total absorbance of the cell with phytochrome completely in the far-red absorbing form from the absorbance with the pigment in the red form.

To illustrate the procedure, consider the case of a single pigment, present inside a particle with fractional volume a^3. The total transmission of the cell is given by

$$T = 1 - a^2 + a^2 T_p$$

where T_p is the transmission of the particle. If the pigment in the latter were to be distributed homogeneously over the whole cell volume, a transmission T_0 would result and:

$$T_p = T_0^{1/a^2}$$

Hence:

$$A = -\log T = -\log [1 - a^2(1 - T_0^{1/a^2})]$$

Absorbances for the models with two pigments in the eight distributions shown in Fig. 1 can be calculated in a similar way. The absorbances of chlorophyll, P_r and P_{fr} at a given wavelength, when these pigments are dispersed homogeneously in the whole cell volume, will be indicated by C_0, R_0 and F_0, respectively, and the absorbances of these pigments at their absorption maxima by $C_0(\max)$, $R_0(\max)$ and $F_0(\max)$. It can be checked easily that the absorbance changes upon phytochrome phototransformation, ΔA, for the eight models are given by the following equations:

$$\Delta A = \log [a^2 10^{-F_0/a^2} + 1 - a^2] - \log [a^2 10^{-R_0/a^2} + 1 - a^2] \quad \text{(A)}$$

$$\Delta A = R_0 - F_0 \quad \text{(A')}$$

$$\Delta A = \log[(1-a^2)10^{-C_0 a/1-a^3} + a^2 10^{-F_0/a^2}] - \\ - \log[(1-a^2)10^{-C_0 a/1-a^3} + a^2 10^{-R_0/a^2}] \quad \text{(B)}$$

$$\Delta A = \log[a^2 10^{-C_0/a^2} + (1-a^2)10^{-F_0 a/1-a^3}] - \\ - \log[a^2 10^{-C_0/a^2} + (1-a^2)10^{-R_0 a/1-a^3}] + (R_0 - F_0)(1-a)/1-a^3 \quad \text{(B')}$$

$$\Delta A = \log[a^2 10^{-(C_0+F_0)/a^2} + 1 - a^2] - \log[a^2 10^{-(C_0+R_0)/a^2} + 1 - a^2] \quad \text{(C)}$$

$$\Delta A = \log[a^2 10^{(C_0+F_0)a/1-a^3} + 1 - a^2] - \\ - \log[a^2 10^{(C_0+R_0)a/1-a^3} + 1 - a^2] + (R_0 - F_0)/1 - a^3 \quad \text{(C')}$$

$$\Delta A = R_0 - F_0 \quad \text{(D)}$$

$$\Delta A = \log[a^2 10^{F_0 a/1-a^3} + 1 - a^2] - \\ - \log[a^2 10^{R_0 a/1-a^3} + 1 - a^2] + (R_0 - F_0)/1 - a^3 \quad \text{(D')}$$

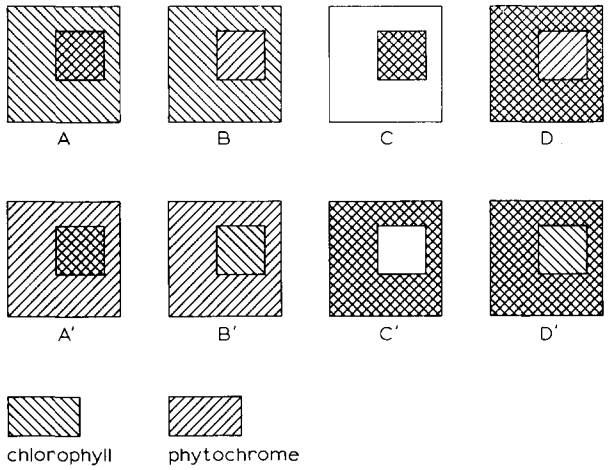

Fig. 1. Cross sections through the eight cell models.

It should be noted that some equations become identical for certain values of the parameters. Eqns A, A', D and D' do not contain C_0: difference spectra for these models are free from chlorophyll interference. Moreover, Eqns A' and D give phytochrome difference spectra, identical with those of homogeneous solutions of the pigment as to form and magnitude. Models A and D' give spectra, distorted by the "sieve effect"[5] at values of a approaching zero or unity, respectively. Fig. 3 can serve as an example. Note further that for $a = 1$, Models C' and D' give undistorted phytochrome spectra, one third the magnitude of that of a completely homogeneous dispersion. We will consider Models A, A', D and D' as trivial and concentrate the discussion mainly on the remaining four: B, B', C and C'.

The same equations can of course be applied to flat layers of one cell thickness. To obtain absorbances of a sample composed of N such layers, stacked at random, we follow the derivation given by Duysens[5] for the transmission of a suspension of

absorbing particles. Our model differs from the one of Duysens in that the number of particles in a column, parallel to the direction of the light cannot exceed N. We will limit the discussion to a model with only one pigment which is contained inside the particle and will assume that analogous results can be obtained for the two-pigment models. The probability that there are k particles ($k \leq N$) in a column with base a^2 is:

$$(a^2)^k(1 - a^2)^{N-k}$$

This can be reached in $\binom{N}{k}$ ways. The chance that the whole cross section contains a column containing k particles is, therefore,

$$\binom{N}{k}(a^2)^k(1 - a^2)^{N-k}$$

If T_p is the transmission of a single particle, the total transmission of the sample will be

$$T_N = \sum_{k=0}^{k=N} \binom{N}{k}(T_p a^2)^k(1 - a^2)^{N-k}$$
$$= (T_p a^2 + 1 - a^2)^N$$

and the absorbance of the sample:

$$A_N = -N \log [1 - a^2(1 - T_p)]$$

Introducing T_0, we obtain:

$$A_N = -N \log [1 - a^2(1 - T_0^{1/Na^2})]$$

For $N = 1$, this yields the same expression that was derived above in a straightforward way. It can be seen that the same expression would also be obtained if the light leaving each single cell layer were "homogenised" before entering the next layer. From this point of view, multiple scatter in the sample, which so far has been left out of consideration, would not be expected to affect the form of the ΔA equations directly.

If $1 - T_0 \ll 1$, following Duysens, we obtain:

$$A_N = 0.434\, Na^2(1 - T_0^{1/Na^2})$$

Introducing $a^2_n = Na^2$, this becomes:

$$A_N = 0.434\, a_n^2(1 - T_0^{1/a_n^2})$$

This equation is formally analogous to the absorbance of a single cell or layer of cells under the same restriction of high transmission:

$$A_1 = 0.434\, a^2(1 - T_0^{1/a^2})$$

This analogy justifies the use of the equations derived for the single cell models also for multilayer models. It necessitates the redefinition of the meaning of the parameter a, however. At higher absorbances where the approximations are no longer valid, deviations between single and multilayer models will occur, but these are of a quantitative rather than a qualitative nature. Since we have made many more assumptions in the derivations, such as absence of multiple scatter and selective scatter, unrealistic shapes of cells and absorbing particles *etc.*, we feel that little can be gained

at this stage from attempts to develop complete ΔA equations for multilayer models and we will base our calculations on the single cell equations exclusively. Before doing so, it is necessary to discuss briefly the expected influence of multiple scatter and variation in the thickness of the sample upon the spectra predicted from the models.

In the presence of multiple scatter, the effective number of cells, traversed by the light, is larger than the geometric number N, which should then be replaced by a statistical quantity $\bar{N}$ ($\bar{N} > N$). We define:

$$\bar{N} = f \cdot N, \qquad a_n = a \cdot f^{\frac{1}{2}} N^{\frac{1}{2}}$$

where f is a factor accounting for the increase in effective path length as a result of multiple scatter (see Butler[6]).

Further, it can be shown that for increasing N, the absorbance of the sample approaches that of a homogeneous dispersion of the total pigment in the sample volume:

$$A_N(N \to \infty) = -\log T_0$$

We may expect, therefore, that increase in sample thickness is accompanied first by an apparent increase in the value of the parameter a and finally by a gradual disappearance of the chlorophyll interference. At infinite thickness (when the absorbance becomes infinite!) the difference spectrum for phytochrome phototransformation should approach the "normal" shape. This effect of sample depth may be one of the factors explaining discrepancies between results obtained with different methods of measurement.

In order to calculate difference spectra from the equations, we will have to make assumptions about $R_0(\lambda)$, $F_0(\lambda)$ and $C_0(\lambda)$. We have adopted the absorption spectra shown in Fig. 2. Those for phytochrome were derived from the data of Kroes[7]

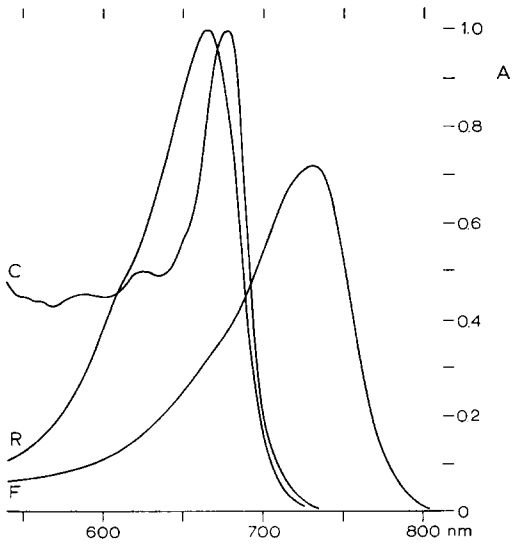

Fig. 2. Absorption spectra of "chlorophyll" (C), P_r (R) and P_{fr} (F) upon which the calculations are based.

on highly purified solutions of oat phytochrome. Since difference spectra calculated from absorption spectra of phytochrome solutions are displaced somewhat to shorter wavelengths as compared with difference spectra *in vivo*, we have corrected the spectra of Kroes by shifting the one for P_{fr} over 6 nm and the one for P_r over 5 nm upwards. The spectrum of "chlorophyll" was an absorption spectrum of a layer of "white" flower petals of *Viola*, measured in a Cary-14 spectrophotometer with scattered transmission accessory. Some results of computer calculations are shown in Figs 3–6 for

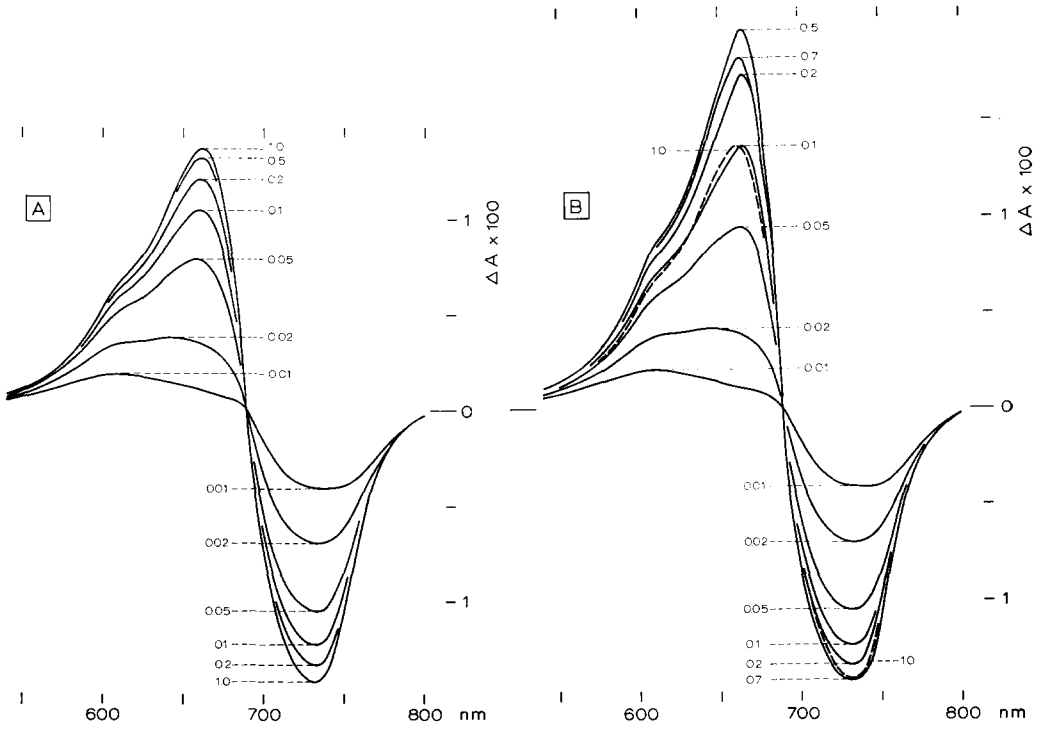

Fig. 3. Calculated phytochrome difference spectra for Model A. $R_0(\max) = 0.02$. Parameter: a^2.

Fig. 4. Calculated phytochrome difference spectra for Model B. $R_0(\max) = 0.02$; $C_0(\max) = 0.5$. Parameter: a^2.

some combinations of parameters, applied to Models A, B, B′ and C. From these Curves, peak height ratios, P, can be calculated. P is the quotient of the magnitudes of the difference spectrum maximum in the red divided by that in the far red. Results are shown in Figs 7 and 9. In Models B and B′ the values of $-P$ always turn out to be higher, and in Models C and C′ to be lower, at the same value of parameter a, than the corresponding values in the absence of chlorophyll ($C_0 = 0$; $P = -0.96$). Peak wavelengths are also dependent on a and C_0. The red peak is especially sensitive in Models C and C′. As an example, the red peak wavelengths calculated for Models B and C are shown in Fig. 8. Shifts in the far-red maximum are of less importance since the chlorophyll absorption is small in this region of the spectrum. They amount to some 5 nm at most. There is some influence on the bandwidths of the far-red peaks, however.

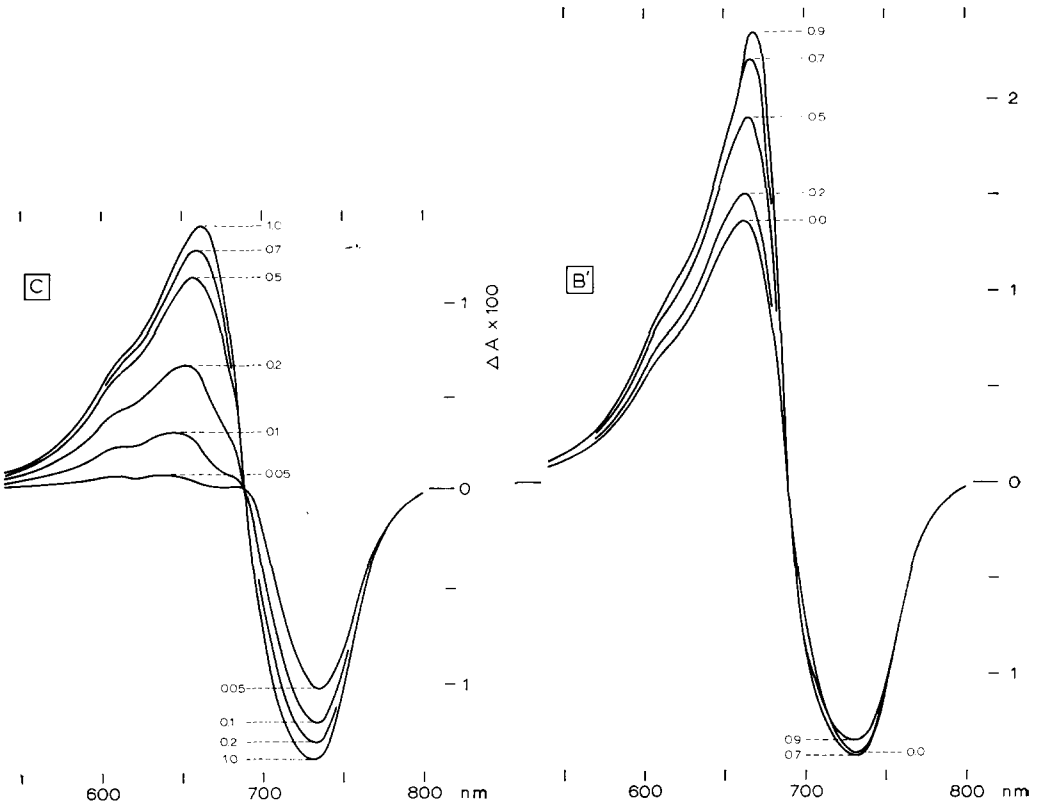

Fig. 5. Calculated phytochrome difference spectra for Model C. $R_0(\max) = 0.02$; $C_0(\max) = 0.1$. Parameter: a^2.

Fig. 6. Calculated phytochrome difference spectra for Model B'. $R_0(\max) = 0.02$; $C_0(\max) = 0.5$. Parameter: a^2.

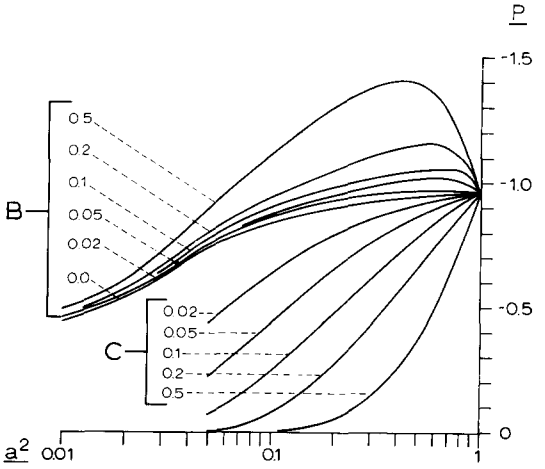

Fig. 7. Ratios (P) of heights of red to far-red difference spectrum peaks for Models B and C. $R_0(\max) = 0.02$. Parameter: $C_0(\max)$.

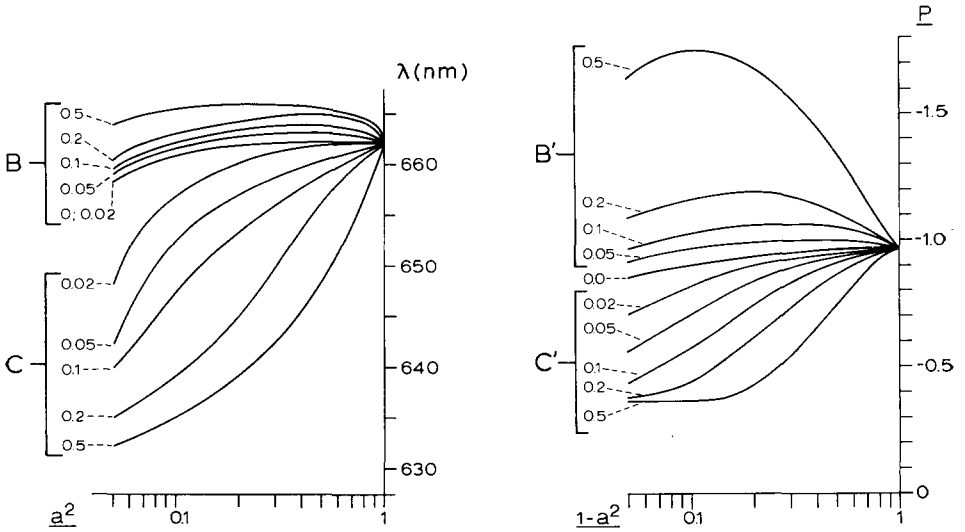

Fig. 8. Wavelengths of red peaks in the difference spectra for Models B and C. R_0(max) = 0.02. Parameter: C_0(max).

Fig. 9. Peak height ratios (P) for Models B′ and C′. R_0(max) = 0.02. Parameter: C_0(max). Since the influence of chlorophyll is most pronounced around $a^2 = 0.9$, the curves have been plotted against $1-a^2$.

The values of P and λ_{max} as a function of parameter a are the resultant of two sieve effects. Light absorption in chlorophyll is responsible for one of them, and in phytochrome for the other. Decreasing the volume fraction in which the phytochrome is contained leads to lower values of both $-P$ and λ_{max} as illustrated by Figs 7 and 8, curves marked "o". In Models B and B′, the opposite effect results from the concomitant changes in the volume fractions of the chlorophyll. The combined effects result in the occurrence of maxima in the values of P as a function of a. It will then be clear that a decrease in R_0 results in an increase in $-P$ at constant a and C_0. All other parameters equal, the distortion in the phytochrome difference spectra should increase in Models B and B′, and decrease in Models C and C′ with decreasing absolute amounts of phytochrome present in the cells.

It should be stressed that the shapes of the calculated spectra depend upon the assumed absorption spectra of the pigments. Comparison with difference spectra observed *in vivo* would require precise knowledge of the spectra of the pigments in the sample and for this reason usually can be of a qualitative nature only. Examples of *in vivo* spectra are given in Figs 10–12. Those of Figs 10 and 11 were measured in a Cary-14 spectrophotometer adapted for sample irradiation, and those of Fig. 12 in a dual-wavelength spectrophotometer. The instruments and the experimental methods have been described earlier[8]. Photometric linearity, *i.e.* additivity of absorbances at any setting within the range of the Cary-14, was checked by means of a calibrated attenuator of 0.025 ΔA that could be introduced into the sample beam during measurement. Results were completely satisfactory.

The assumption, made in the derivation of the equations for the absorbance differences, *viz.* that all cells inside the sample contain the pigments in the distri-

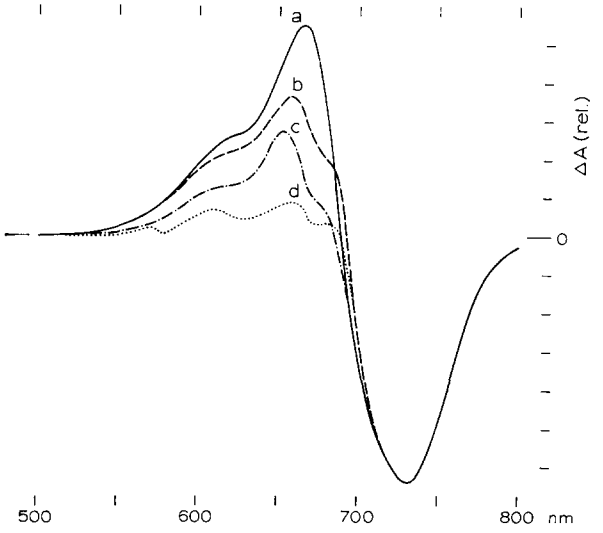

Fig. 10. Examples of phytochrome difference spectra observed *in vivo*. Isolated leaves of dark grown pea, subjected to various short pre-irradiations producing a range of chlorophyll levels.

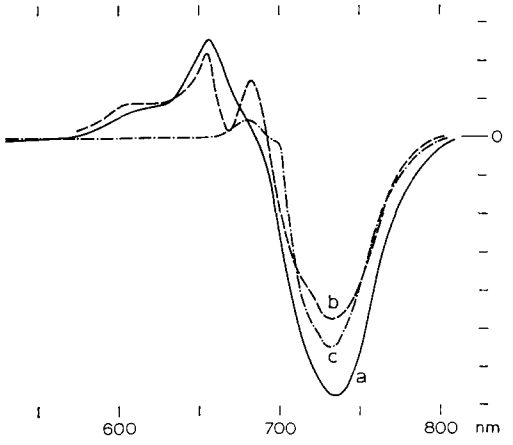

Fig. 11. Phytochrome difference spectra of cotyledons of dark grown *Sinapis* (a and b) and leaves of bean (c). Samples were pre-irradiated before measurement.

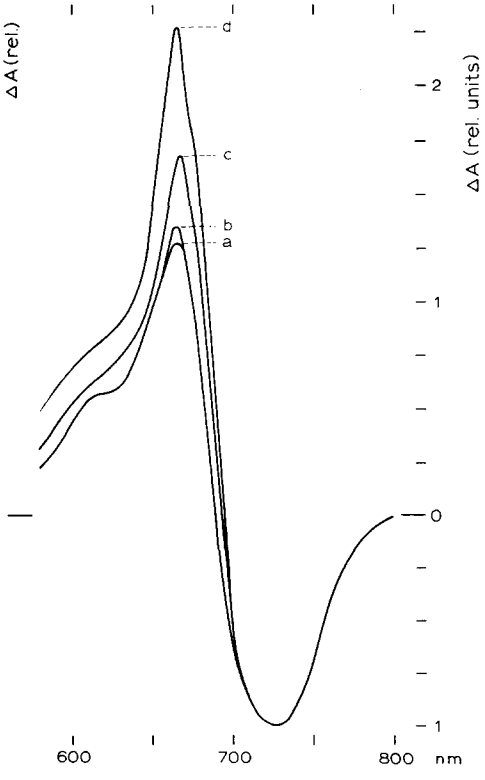

Fig. 12. Phytochrome difference spectra of isolated stem sections of seedlings, exposed to pre-irradiations with red light during the growth period. a, dark only; b, 7.5 h red light followed by 24 h dark; c, 15 h red light + 24 h dark; d, 30 h red light + 24 h dark. The spectra were normalised at 730 nm and were corrected for reference filter transmission at this wavelength (from ref. 9, with permission of Dr Grill).

butions shown in Fig. 1, may not be realistic. We may briefly consider one more set of models, obtained from those discussed above, by mixing the cells with pigments at random with a quantity of "empty" cells. This introduces one more parameter, the mixing ratio, and makes calculations rather cumbersome. We have noted, however, that such models can be treated as suspensions of absorbing particles, the absorbance of each separate particle being given by one of Eqns A–D'. This leads to the well known "sieve effect" again, resulting in a decrease of the magnitude of ΔA and a flattening of the difference spectrum peaks, analogous to the example given in Fig. 3. As long as the volume fraction occupied by the "empty" cells is not very large, the effect upon the difference spectra can be expected to be moderate.

DISCUSSION

All phytochrome difference spectra we have measured so far in samples of leaves and cotyledons that contain appreciable amounts of chlorophyll, show P values deviating from those expected for pure phytochrome solutions. Moreover, they may show more or less pronounced shoulders in the 685-nm region and their red peaks fall below 662 nm. Since it is most unlikely that there are as many spectroscopically different phytochromes as there are difference spectra observed, we believe that the theoretical considerations discussed above support the conclusion that the abnormal spectra result from inhomogeneous distributions of the pigments in the samples. Models C and C' account for spectra such as those of Figs 10 and 11 very well. We conclude, therefore, that they indicate a close spatial association of phytochrome and chlorophyll in a relatively small fraction of the sample volume. Local concentrations of pigments may then be considerable and it would be desirable to take into account selective scatter. This can hardly be done in a quantitative way and we will restrict ourselves to the remark that in passing through the wavelength regions where the absorbing particles have absorption bands, light scatter as well as penetration of light through the particles may vary rapidly with wavelength. This effect could be expected to sharpen bands in the difference spectra considerably and may be a factor contributing to the formation of spectra such as those of cotyledons of *Sinapis* (Fig. 11b).

On the other hand, in this Laboratory Dr Grill has recently studied the effect of accumulation of chlorophyll on the phytochrome difference spectra in some detail[9]. Whereas in leaves spectra characterised by low P values, shoulders around 685 nm and red peaks displaced towards shorter wavelengths, such as described above, were observed, the opposite was found in hypocotyl tissue of plants grown in light. These organs do not easily form chlorophyll and prolonged irradiation is required to accumulate an appreciable quantity. Material treated in this way showed P values considerably in excess of one and red peaks hardly different from the normal position (Fig. 12). This type of spectrum agrees with the predictions from Models B and B', suggesting that chlorophyll and phytochrome in such material occupy separate, "packages".

Although these findings seem to suggest that in leaf and cotyledon material, phytochrome is associated with the proplastids, we doubt whether the theory in its present form is sufficiently accurate to warrant such a conclusion. All we can say is that the pigment distribution in stem material differs markedly from that in leafy tissue.

If phytochrome difference spectra are measured repeatedly with the same sample of a material that rapidly regenerates protochlorophyll in the dark, the spectral shifts associated with the chlorophyll transformations (such as the Shibata[3] shift) as well as chlorophyll build-up following each actinic irradiation can be followed qualitatively in the spectra in a way that is in agreement with the model calculations. There is a rough inverse correlation between P values and total chlorophyll in the samples. This explains why P values in mustard cotyledons and leaves of beans which contain much more protochlorophyll than peas, are invariably lower than those observed in the latter plant. The correlation is not very good, however, suggesting that the situation is more complex than is accounted for by our models. One factor may be that during measurement, the volume of the proplastids may change. There are reasons that we cannot discuss here in detail, leading us to assume that phytochrome translocation processes follow the illumination of dark-grown plants. Both processes affect the value of a as well as the pigment distribution pattern at otherwise constant total quantities of phytochrome. For these reasons the apparent total quantity of phytochrome as derived from absorbance measurements as well as the shape of the difference spectra may change in the course of an experiment and according to the theory developed in this paper, this does not necessarily imply a change in total phytochrome in the sample.

Previously we have measured difference spectra of phytochrome solutions obtained from pea leaves[10]. These show peaks at about 650 nm under certain conditions, a result we interpreted as supporting our previous hypothesis that pea leaves contain a form of phytochrome different from the normal one. In the mean time we have become aware that, contrary to our earlier conclusions, the extracts contained some native protochlorophyll which is photochemically transformable by red light. This explains the difference spectrum peak at 650 nm upon the first actinic irradiation, invalidating our earlier conclusion. If anything, these difference spectra point to the presence in the extracts of "classical" phytochrome.

Summarising, we can say that observation of difference spectra *in vivo*, deviating from the shape expected for a particular pigment, in itself does not provide conclusive evidence for the occurrence of different pigment forms. In such cases, the possibility of interference by other, unreactive, pigments should be considered. The spectra can then furnish some information about the pigment distribution pattern. The application of our model calculations of course is not limited to the field of phytochrome research but should have general significance for all difference spectrophotometry of optically inhomogeneous materials containing more than one pigment. For instance in the study of photoreactions of photosynthetic pigments, where close spatial associations are known to occur, the shape of difference spectra of components may be distorted appreciably by nonphotoreactive fractions of the pigments associated with them. In the case of phytochrome, we believe that difference spectra in Angiosperms should be interpreted as being distorted to varying degrees by interference from chlorophyll. They do not provide evidence for different forms of phytochrome, as long as such forms have not been shown to exist by isolation and measurement *in vitro*.

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