Title

THE DEVELOPMENT OF PHOTOSYNTHETIC MEMBRANES IN GREENING LEAVES. II. CHANGES IN FLUORESCENCE SPECTRA AND POLARIZATION ACCOMPANYING THE PROTOCHLOROPHYLLIDE TO CHLOROPHYLLIDE CONVERSION IN HOLOCHROME PREPARATIONS

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SUMMARY

The photoreduction of protochlorophyllide to chlorophyllide in holochrome preparations from etiolated bean seedlings (Phaseolus vulgaris L.) produces a chlorophyllide holochrome that exhibits polarization of the chlorophyllide fluorescence at 7°C. If the holochrome is suspended in 2 M sucrose in order to prevent the usual dark spectral shifts that follow photoconversion, then the fluorescence polarization observed is about 50% of the value for chlorophyll a in a viscous solvent. In the absence of sucrose, on the other hand, the chlorophyllide fluorescence is 100% polarized by the time it is first measured (ca. 20 min). These observations, together with the circular dichroism spectra measured in Part I of the study[1], lead to the conclusion that protochlorophyllide and, when first formed, chlorophyllide are present in an aggregated, probably dimeric, form in the holochrome. Following the photoconversion, the chlorophyllide association rapidly breaks up and forms a protein particle that is monomeric in chlorophyllide. The relationship of this rearrangement to the process of membrane development in greening plastids is discussed.

Abbreviations: Chl, chlorophyll; Chlide, chlorophyllide; PChl, protochlorophyll; PChlide, protochlorophyllide; PCH, protochlorophyllide holochrome; CH, chlorophyllide holochrome; CD, circular dichroism.

The first illumination of dark grown (etiolated) seedlings of angiosperms induces a sequence of profound changes, including the formation of chlorophyll, a thorough reorganization of the internal structure of the etioplast and the steps leading to the formation of the photosynthetic energy-conversion apparatus. The first part of this study[1] described the evidence of absorption and circular dichroism studies bearing on this major pigment-protein reorganization.

Fluorescence intensity and, especially, fluorescence depolarization also provide sensitive indications of pigment interactions and the changes in these interactions accompanying proplastid development. Latimer and Smith observed strong fluorescence polarization, comparable to that of Chl a in a viscous solvent, from the Chlide produced via the photoconversion of protochlorophyllide in the holochrome[2]. Goedheer and Smith applied increasing durations of illumination to etiolated leaves and observed a progressive decrease in the polarization of fluorescence of the holochrome extracts prepared from the leaves[3]. Because their results extrapolated to a value indicating 50% of the maximum polarization at the first stage following photoconversion, they concluded that the observed depolarization resulted from rotation of the Chlide molecules within the holochrome framework. This was subsequently shown not to be the case by Losev and Gurinovitch, who observed strong (80-100%) fluorescence polarization in leaves at either 20° or -100°C immediately following photoconversion[4]. They attributed the polarization decrease upon longer illumination to excitation transfer among associated Chlide molecules, rather than to rotational diffusion.

During the greening process the yield of Chlide fluorescence decreases approximately 10-fold, starting at a level characteristic of Chl a in organic solvents[5]. The intensity decrease presumably results from quenching processes that become more probable as the concentration and extent of aggregation of Chl increases in the developing plastid.

Fluorescence excitation spectra offer evidence of the occurrence or absence of energy transfer amongst different pigment molecules. For example, carotenoids present in etioplasts are not effective in transferring excitation energy for the photoreduction of PChlide[6,7], nor are they effective in stimulating PChlide or (for several hours following photoreduction) Chlide fluorescence[4,5]. Carotenoids are absent in the purified PChlide holochrome preparation[8].

We present here a detailed study of the fluorescence excitation and emission spectra, relative fluorescence yields and fluorescence polarization of holochrome preparations before and following the PChlide to Chlide conversion. These observations supplement the circular dichroism spectra presented in Part I of this research[1], and they provide clear support for the molecular interpretation of the CD spectra.

EXPERIMENTAL

Holochrome preparations

The preparation of PCH and CH from etiolated bean seedlings was carried out as described previously[1]. In the present study PCH(suc) was prepared by diluting PCH with an equal volume of 4 M sucrose, 0.02 M tris-Cl. CH(suc) was formed by maximal transformation of PCH(suc) samples at 0°C using brief illumination from a 150 W floodlamp[1].

Fluorescence measurements

An Aminco-Bowman spectrophotofluorometer (American Instrument Co., Baltimore, Md.) was modified as follows: The emission monochromator grating was replaced with one blazed for optimal efficiency at 700 nm. Wavelength calibration was carried out using a low pressure mercury arc. The excitation beam was modulated using a vibrating slit operating at 200 Hz (American Time Products, Woodside, N. Y., Type 40 Light Chopper) and phase detected (Princeton Applied Research Corp., Princeton, N. J.; Model 210 selective amplifier, Model 220 lock-in amplifier and Model 221 high-voltage power supply). A red-sensitive photomultiplier (RCA 7102, Type S-1 photocathode) was cooled by solid CO_2. Excitation and emission spectra were recorded using an XY-recorder (Moseley/Hewlett Packard, Palo Alto, California, Model 2D-2A) and were not corrected for wavelength variation of the efficiency of the optical system. Monochromator bandwidths (at half maxima) noted in the figure captions were estimated from the widths of the peaks resulting from light scattering. Sample temperatures were controlled to about 7°C. Cuvettes with four clear sides had square cross-sections of 10.0 mm I.D.

Excitation spectra were recorded using a supplementary sharp-cut glass filter (Corning C.S. 3-73) in the emission beam just before the photomultiplier. Fluorescence polarization spectra were recorded using similar filters (Corning C.S. 3-68 or 3-66). Glan-type crystal polarizers (American Instrument Co.) were used in both the excitation and emission beams. Polarization values were measured and corrected as described by Houssier and Sauer[9].

RESULTS

Fluorescence emission spectra

The measurement of fluorescence spectra of PCH and PCH(suc) is complicated by the efficient phototransformation caused by the excitation light. The fluorescence spectra at 7°C were measured with no more than 12% accompanying transformation through the introduction of neutral density filters (transmission 0.008) into the excitation beam. Typical fluorescence emission spectra for PCH(suc) and CH(suc) are shown in Fig. 1. The corresponding absorption spectra are shown in Fig. 2. The extent of transformation of PCH(suc) during the fluorescence measurement is estimated from the absorbance at 678 nm due to Chlide. Comparison of Figs. 1 and 2 shows that the Chlide formed initially has a substantially greater fluorescence efficiency (> 3x at 685 nm) than does the Chlide in the fully transformed CH(suc).

The shorter wavelength emission peak of PCH(suc) occurs at 642 nm, whereas that of the residual (inactive PChlide in CH(suc) occurs at 637 nm. The difference results from the loss of the weakly fluorescent, active PChlide in the latter material and perhaps, in part, from some decrease in self-absorption within the sample. Based on the corresponding absorbance changes, the uncorrected relative fluorescence efficiencies of inactive and active PChlide in PCH(suc) are in the approximate ratio 3.3:1.

The emission spectrum of this same sample of CH(suc) measured using unattenuated excitation light is shown in Fig. 3. In addition to the maxima at 637 (inactive PChlide) and 684 (Chlide), there appears a distinct shoulder at 745 nm. The latter is undoubtedly a vibrational component (0→1) of the Chlide emission.

Fluorescence excitation spectra

The excitation spectra of CH(suc) fluorescence measured at 637, 685 and 745 nm are shown in Fig. 4. The emission at the latter two wavelengths result from identical excitation spectra, whereas the fluorescence at 637 nm has a distinctly different excitation spectrum. These observations confirm the association of the 745 nm shoulder with the Chlide emission. They also argue against the occurrence of substantial excitation transfer from inactive PChlide to Chlide in CH(suc).

In the absence of sucrose, the transformed holochrome, CH, undergoes a dark shift in the absorption maximum from 678 to 674 nm during about 20 min at room temperature. This absorption shift and the concomitant change in the fluorescence emission spectrum are depicted in Fig. 5. The decrease in magnitude by 8% in the emission spectrum is accompanied by a small decrease in the absorption and is probably not significant.

Fluorescence polarization

The fluorescence polarization of CH was studied using a fully converted sample with A (1 cm) = 0.15 at the red maximum. Although the sample was maintained at about 5°C, the dark shift of the absorption from 677 to 673 nm continued during the course of the measurements. The polarization values, $p = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$, for emission at 680 nm are plotted as a function of excitation wavelength in Fig. 6a. Because there was no measurable change in the polarization values during the course of the dark shift, the measurements at different times are not distinguished from one another on the plot. The p values and their dependence on excitation wavelength agree generally with those obtained for Chl a in viscous solvents; however, there are significant differences among the several published polarization spectra[10-13].

The fluorescence polarization spectrum of CH(suc) under the same conditions is shown in Fig. 6b*. In the wavelength regions where comparisons can be made, the p values of CH(suc) are approximately half those of CH. The wavelength dependences are qualitatively similar, however.

The polarization of the PCH(suc) fluorescence at 642 nm was measured using neutral density filters (transmission 0.008) in the excitation beam in order to retard the photoconversion. With the excitation wavelength at 440 nm, p values of $+0.06 \pm 0.14$ were observed. The large uncertainty resulted primarily from the low signal level under these experimental conditions. PChl a in mineral oil exhibits a polarization of +0.23 at the corresponding wavelengths[9].

DISCUSSION

Protochlorophyllide holochrome fluorescence

The protochlorophyll(ide) molecules in etiolated leaves or holochrome preparations occur in at least three distinguishable spectral forms[14,15]. These are best characterized at liquid nitrogen temperatures, where the resolution of the components and the fluorescence efficiencies are enhanced. The fluorescence emission spectrum at 7°C shown in Fig. 1 for PCH(suc) contains a small component (~10%) of Chlide a formed during the fluorescence measurement. Nevertheless,

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*The presence of the asymmetric sucrose molecule introduces some rotation of the plane of polarized light. This was calculated to be less than 2° under the conditions of the experiment.

there is an apparent shift of the PChlide fluorescence peak from 642 to 637 nm and a decrease in its magnitude in the fully converted CH(suc) preparation. The Chlide a fluorescence should not interfere significantly in this short wavelength region. The simplest explanation of the spectra is that the short wavelength component, P_{630} (inactive), is strongly fluorescent, whereas the longer wavelength component, P_{639} (active), is at most weakly fluorescent at 7°C. If the fluorescence emission at 642 nm originates in the component absorbing at 639 nm, then its 3 nm Stokes shift would be significantly smaller than those (6-8 nm) observed for P_{630}, C_{678} or C_{674}. It may be, therefore, that this emission at 7°C results from a hidden component corresponding to the central absorption band observed by Dujardin and Sironval at liquid nitrogen temperature in etiolated leaves[14]. At -196°C, where the PChlide is unable to undergo photoconversion, the long wavelength component becomes highly fluorescent[14-18], and the intermediate wavelength component transmits its excitation rather than fluorescing[14].

While the polarization of PChlide fluorescence in PCH(suc) could not be measured accurately under the conditions of low excitation intensity necessary to prevent photoconversion, the observed value p = 0.06 is low in comparison with the value p = 0.23 for isolated PChl a in mineral oil[9]. Presumably, the measurement for PCH(suc) applies primarily to the fluorescence from PChlide (inactive).

Butler and Briggs have attributed the two principal spectral forms of PChlide to monomeric (short wavelength) and aggregated (long wavelength) species[19]. The low fluorescence yield of P_{639} relative to that of P_{630} at room temperature may result from quenching by the aggregates in the

former case. The CD spectrum of PCH reported in Part I of this study is interpretable on this basis[1]. In the region from 600 to 700 nm, there appears to be a (+,-) CD centered near 630 nm that could be attributed to monomeric PChlide superimposed on a more complex CD pattern (- at the longest wavelengths) which results from the aggregated P_{639} form.

Chlorophyllide holochrome fluorescence

Light absorbed by active PClide leads to the formation of Chlide with a high quantum efficiency[20]. However, the process does not follow simple first-order kinetics, but appears to result from the sum of two first-order processes[21]. The underlying reason for this behavior is still obscure[22].

Comparison of Figs. 1 and 2 indicates that the Chlide formed during the initial 10% of photoconversion of PCH(suc) is over three times more fluorescent than is the Chlide of the fully converted CH(suc) at 7°C. This can be understood on the basis of a model in which active PChlide in PCH(suc) occurs in an aggregated (dimeric?) form. If the initial stages of the photoconversion result in the transformation of only one of the aggregated PChlide (active) molecules, then the resulting Chlide molecules initially occur singly and their fluorescence is not quenched. In the fully converted CH(suc), the Chlide molecules are now essentially all in an aggregated state[1] and their fluorescence is partly quenched at 7°C. The role of the sucrose is probably similar to that of glycerol in high concentrations in preventing the subsequent rearrangements that lead to a blue shift of the absorption and fluorescence maxima[16].

At 7°C we find (Fig. 6) a relatively low polarization of the Chlide fluorescence from CH(suc). This is consistent with the presence of Chlide

aggregates (dimers) in this preparation. By contrast, Latimer and Smith[2] and Losev and Gurinovitch[4] found essentially complete polarization of Chlide fluorescence at the earliest stages of PChlide to Chlide conversion. We conclude that the stage of the photoconversion corresponding to our CH(suc) preparation was passed through too quickly to be observed by these workers.

The distinctive excitation spectra of fluorescence emitted either at 637 or at 685 (745) nm are consistent with the conclusions that photons absorbed by PChlide (inactive) in leaves or in CH are ineffective in stimulating Chlide fluorescence[23,24]. This may be either because the two species are spatially separated from one another, or because the fluorescence of PChlide (inactive) itself occurs rapidly at 7°C in comparison with excitation transfer. The observation[15] that excitation transfer from PChlide to Chlide does occur at -196°C favors the second explanation.

Weber has formulated a method of enumerating the components having distinct absorption and fluorescence spectra in complex systems[25]. The number of components is determined from the rank of a matrix of sufficient size to account for the fluorescence intensities of the system measured at several excitation and emission wavelengths. By application of Weber's method to the spectra of CH(suc) shown in Figs. 3 and 4, we found that there are two, but not three, distinct fluorescent components in the completely photoconverted holochrome at 7°C. These components are PChlide (inactive) and Chlide.

The excitation transfer from PChlide to Chlide at liquid nitrogen temperature reported by Kahn et al.[15] and by Thorne[26] may result from the

longer range of interaction associated with Förster transfer[27]. Tweet et al.[28] and Trosper et al.[29] report distances of transfer of the order of 65-85 Å in Chl a monolayers, whereas the weak exciton interaction responsible for the multiple CD of PCH(suc) and CH(suc) would certainly be negligible at these distances[30]. It is likely, therefore, that the low temperature fluorescence properties of post-etiolated leaves results from the transfer of excitation via a Förster mechanism among nearby holochrome particles. Whether there exists in the leaves a particular cooperating unit of 20 PChlide molecules, as proposed by Thorn[26], must be determined by further experiments.

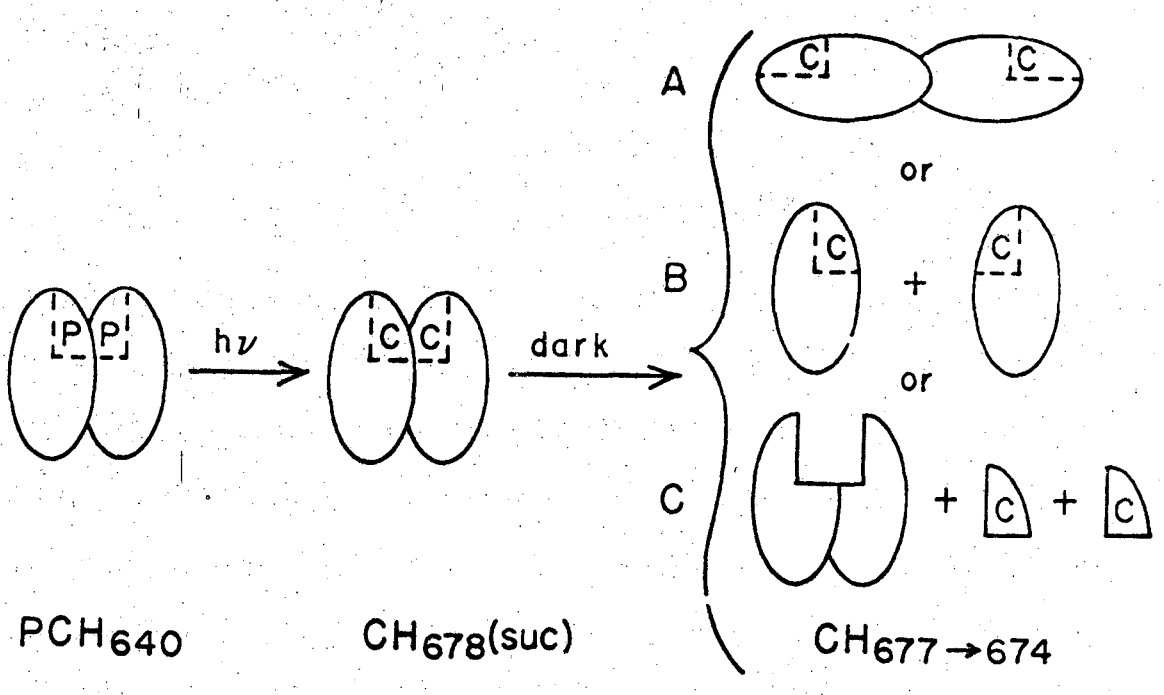
In the absence of sucrose, the fully converted CH exhibits a blue shift in the Chlide absorption maximum from 678 to 674 nm (Fig. 5), a parallel shift in the emission maxima from 684 to 682 nm, and an accompanying transformation from a double to a single CD[1]. The shape of the fluorescence emission spectrum is unchanged during this process, and its intensity is slightly decreased (Fig. 5). The fluorescence polarization (Fig. 6), on the other hand, is increased by about a factor of two in CH compared with that of CH(suc). The polarization of Chlide fluorescence in CH is indistinguishable from that of Chl a in viscous solvents[11-13]. The strongly polarized fluorescence is consistent with the conclusion reached on the basis of the CD spectrum, that CH contains essentially monomeric Chlide, at least by the time these measurements can be made at 7°C. The increase in fluorescence polarization and the changes in the CD spectra are much more direct evidence of this decrease in aggregation than were the blue shifts in absorption and fluorescence maxima, previously the only evidence available to support the proposal[18,31].

The monomeric form of Chlide in CH is presumably responsible for the high polarization values previously reported[2,4], although our values of polarization excited at 405 nm are somewhat higher than those reported by Latimer and Smith[2].

The one observation that appears to be inconsistent with the picture of Chlide disaggregation accompanying the blue shift of the absorption is our failure to observe a concomitant increase in fluorescence efficiency in CH (Fig. 5). The simplest explanation of this is that the increase in efficiency has already occurred by the time we are able to make the first fluorescence measurements on CH. Goedheer found the fluorescence efficiency of Chlide in greening bean leaves initially to be comparable to that of Chl a in methanol[5]. It will be of interest to determine whether a lower value can be detected if measurements are made very rapidly following a strong flash of actinic light.

CONCLUSION

From the measurements of absorption, fluorescence, excitation, emission and polarization spectra, together with the CD spectra, a consistent picture emerges of the initial stages of PChlide to Chlide conversion in etiolated plants. If we accept the stoichiometric data of Schopfer and Siegelman[8], the process can be described as follows:



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The PChlide holochrome, PCH_{640}, contains (at least) two PChlide molecules that interact sufficiently at close range to give a negative CD for the long wavelength band. These must be on the same holochrome particle. Immediately following photoconversion, the CH_{678}(suc) produced contains two strongly interacting Chlide molecules, on the basis both of CD and fluorescence polarization spectra. The siting of the pigment on the holochrome protein may be essentially unchanged from that in PCH_{640}. Very rapidly following photoconversion in the absence of high concentrations of sucrose or glycerol the Chlide holochrome undergoes a rearrangement or dissociation. This results in the disappearance of the double CD feature, a two-fold increase in the fluorescence depolarization and eventually a shift of the absorption and fluorescence emission maxima

to shorter wavelengths. The scheme above indicates three possible explanations: A) a rearrangement of the CH particle leading to decreased interaction between the Chlide molecules, B) dissociation of CH into two equivalent parts, and C) dissociation of small Chlide-protein fragments from the major protein component. The alternatives are not mutually exclusive, for structure A could precede B or C. This would account for the observation that the CD and fluorescence depolarization changes appear to occur more rapidly than does the blue shift in the absorption band. Alternative C would be consistent with the observations of Boardman[32] and of Bogorad et al.[33] that the final Chlide-containing species has a molecular weight much different from that of the holochrome protein. In the intact etioplast, the major protein component of the holochrome would then be available for the placement of two more PChlide molecules[34] and the Chlide-protein fragments could be transferred into the newly forming lamellar structures.

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FIGURE CAPTIONS

Fig. 1. Fluorescence emission spectra of PCH(suc) and of CH(suc) at 7°C. Excitation wavelength, 440 nm. Monochromator bandwidths, 20 nm. The PCH(suc) spectrum exhibits a peak at 685 nm owing to a small amount of Chlide formed by the exciting light during the process of obtaining the emission spectrum.

Fig. 2. Absorption spectra of PCH(suc) before fluorescence emission spectrum shown in Fig. 1 (- - - -), PCH(suc) after fluorescence emission spectrum (— - —) and CH(suc) after complete photoconversion (———). Optical pathlength, 1.0 cm.

Fig. 3. Fluorescence emission spectrum of CH(suc) at 7°C. Excitation wavelength, 440 nm; excitation intensity 125X greater than that used in Fig. 1. Monochromator bandwidths, 18 nm.

Fig. 4. Fluorescence excitation spectra of CH(suc) at 7°C at the emission wavelengths 637 nm (— - —), 685 nm (- - - -), and 745 nm (———). Monochromator bandwidths, 16 nm. Instrument gain has been adjusted to facilitate comparison of the spectra.

Fig. 5. Absorption and emission spectra of CH at the beginning (———) and at the end (- - - -) of the dark shift following photoconversion. Excitation wavelength, 440 nm.

Fig. 6. Fluorescence polarization spectra at 7°C. a) CH. Emission wavelength, 680 nm. b) CH(suc). Emission wavelength, 682 nm. Monochromator bandwidths, 25 nm.

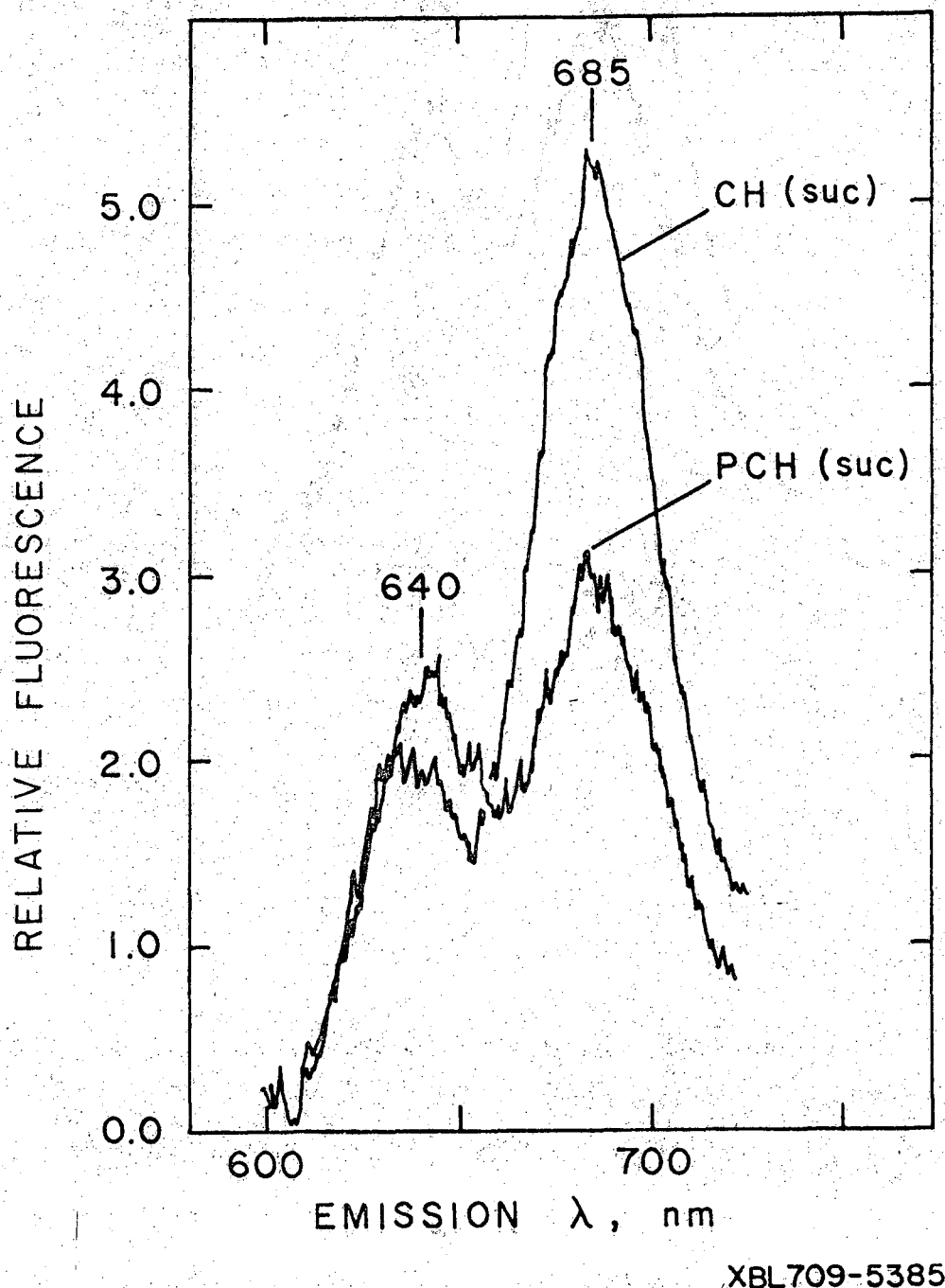

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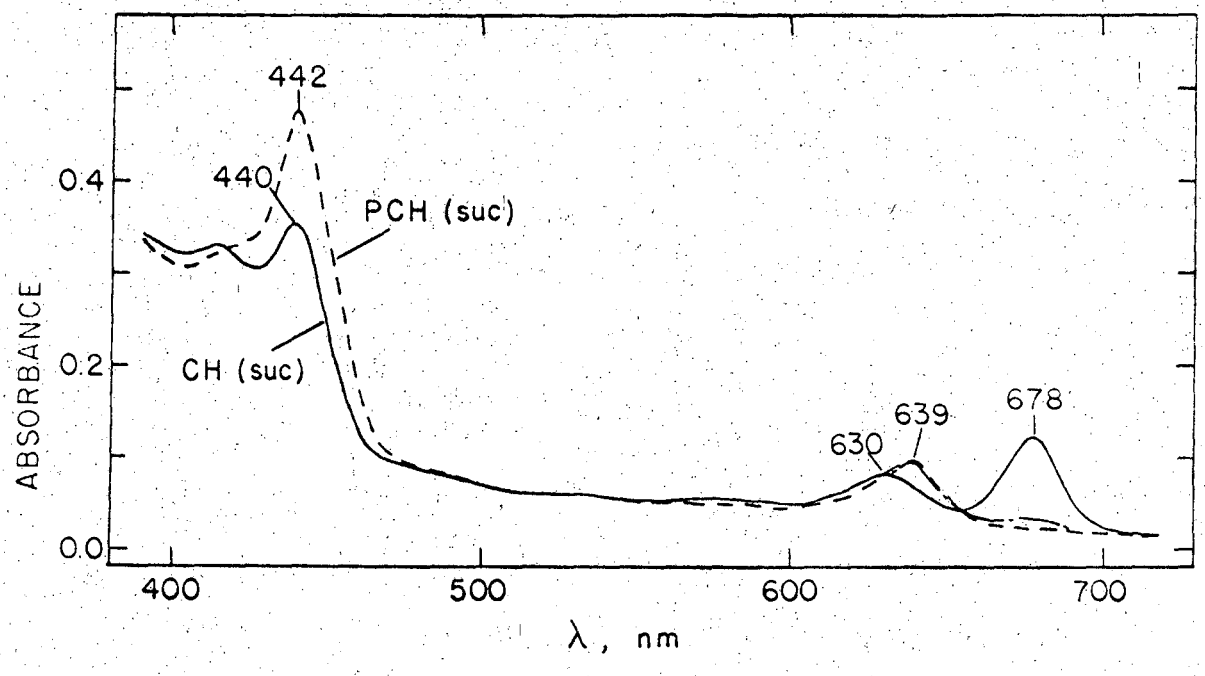
Fig. 1



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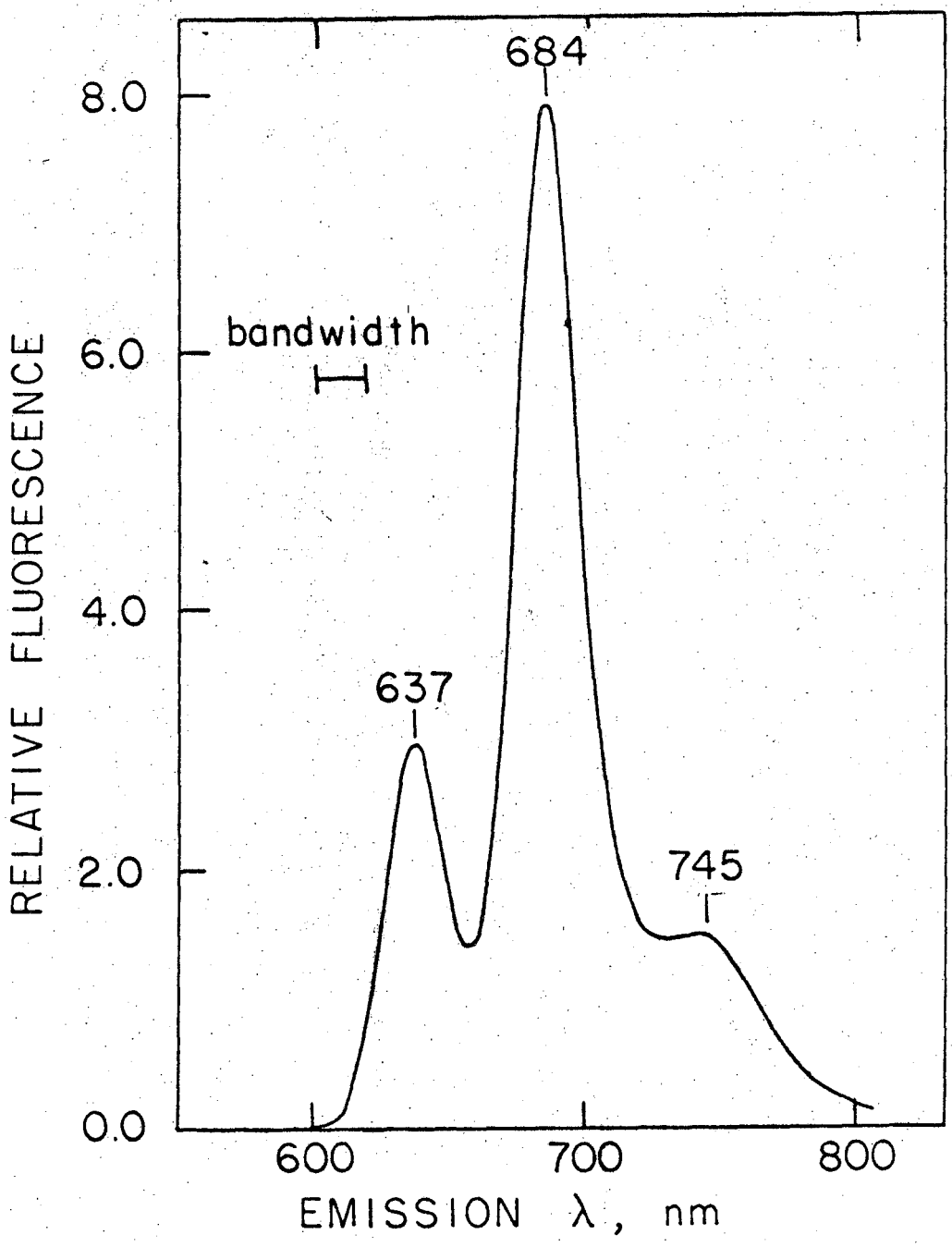

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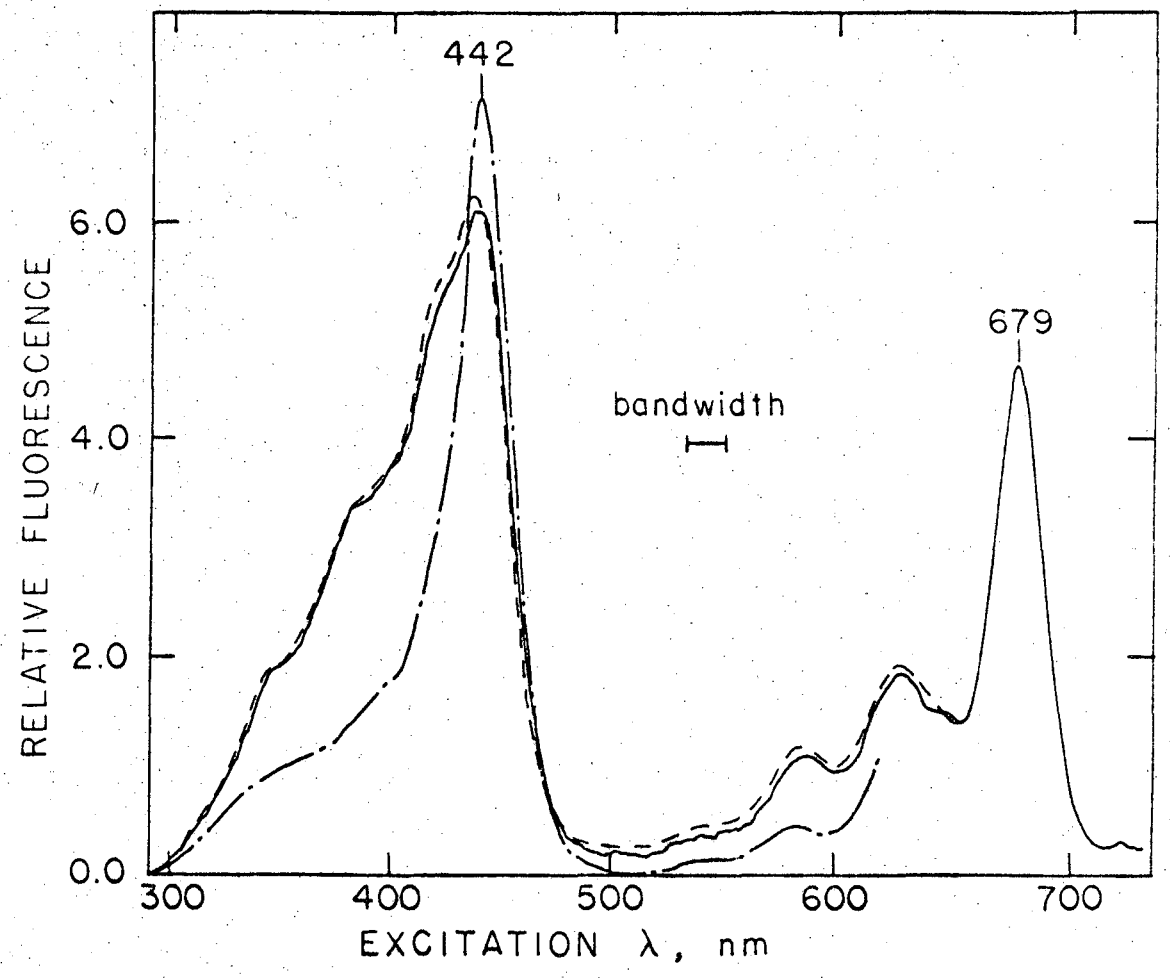

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Fig. 4

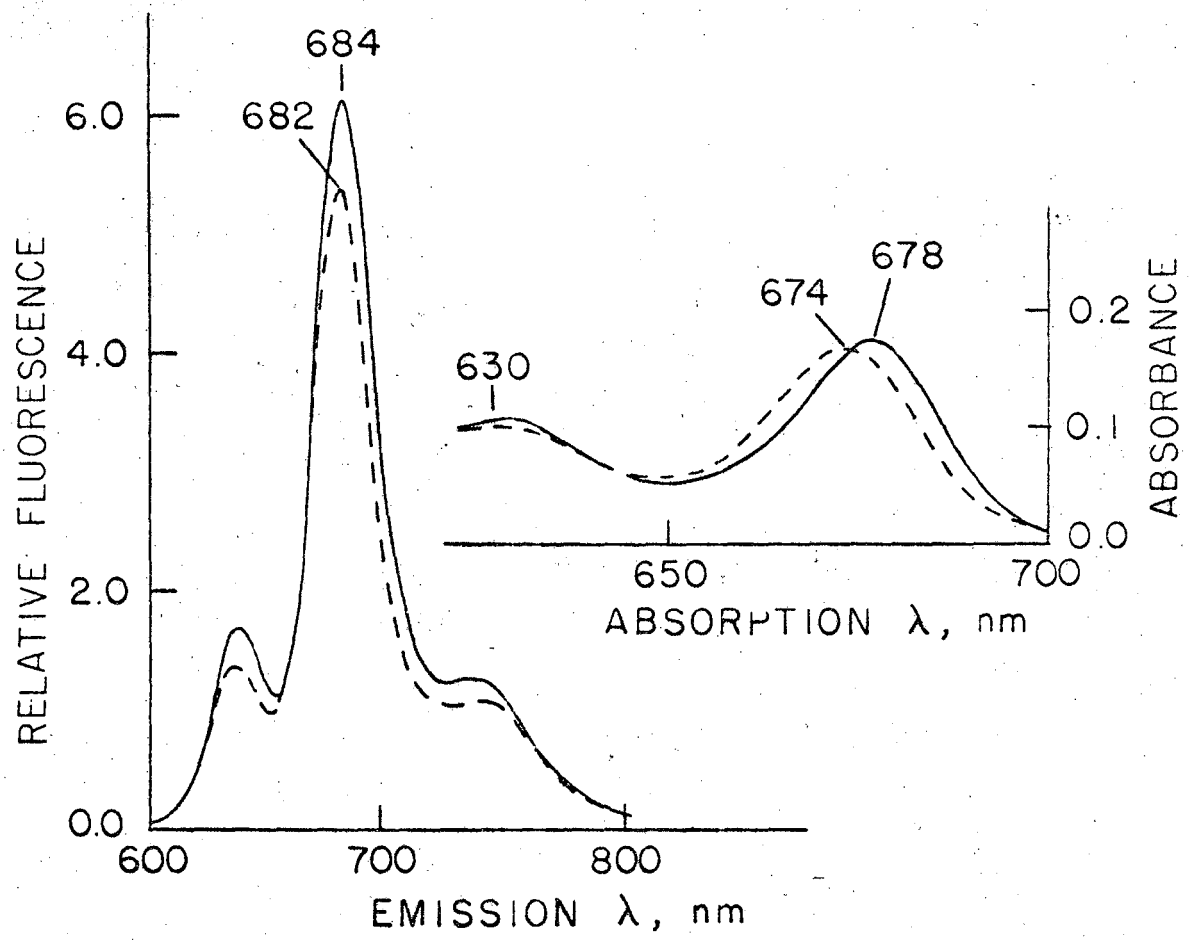

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Fig. 5

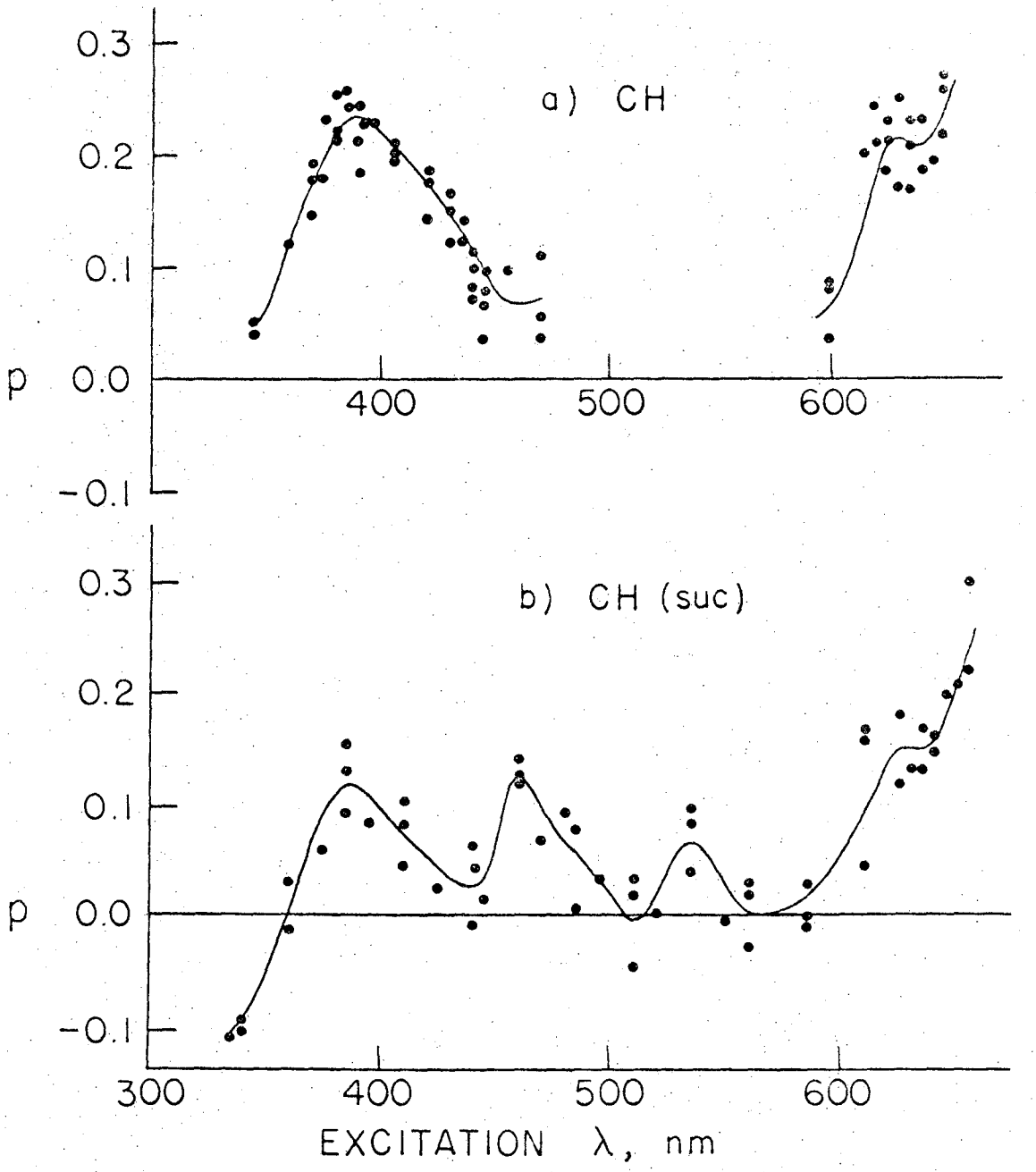

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Fig. 6

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