Title

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G. Douglas Vaughan
(Ph. D. thesis)

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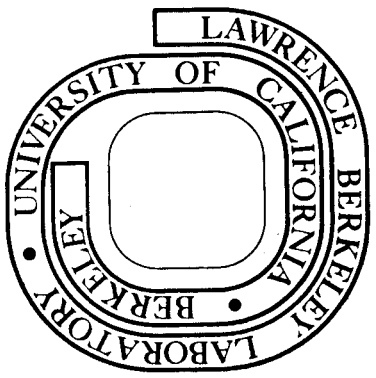

LBL-4621

DISCLAIMER

CONTENTS

A child said What is the grass? *fetching it to me*
with full hands;
How could I answer the child? I do not know what
it is any more than he.

. . . . .

I believe a leaf of grass is no less than the journey-
work of the stars.

Walt Whitman
Song of Myself

STUDIES ON THE PHOTOCONVERSION OF PROTOCHLOROPHYLLIDE TO CHLOROPHYLLIDE IN ETIOLATED PLANT MATERIAL

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November 1975

ABSTRACT

The synthesis of chlorophyll (Chl) in angiosperms is dependent upon the photochemical conversion of protochlorophyllide (PChlide) to chlorophyllide (Chlide). This study attempts to elucidate the kinetics of the photochemistry, as well as some mechanistic aspects of this reaction and the spectroscopically observable changes that immediately follow it. Experiments were performed on intact leaves, on organelles (etioplasts) extracted from leaves, and on aqueous suspensions of photoactive PChlide-protein complexes (holochrome).

Measurement of (1) Chlide fluorescence excited at wavelengths where both Chlide and PChlide absorb, (2) Chlide fluorescence yield, and (3) polarization of Chlide fluorescence, during the photoconversion reaction in holochrome preparations from bean and barley, suggest that the photochemistry is intrinsically

first-order, complicated by efficient energy transfer from PChlide to Chlide within aggregated pigment arrays. The fluorescence polarization results also demonstrate that energy transfer occurs among Chlide pigments following photoconversion. Energy transfer occurs effectively in bean holochrome, less effectively in barley extracts, and vanishes in saponin-treated barley extract. I observed no manifestations of excitonic interaction. (Contrasting data from other investigators is presented.)

Attempts were made to measure photoinduced linear dichroism in bean holochrome, both in dried gelatin films and in solution. The failure to measure such dichroism is most readily explained by the effective depolarization of exciting illumination through energy transfer among aggregated PChlide molecules before photoconversion.

Following the photochemistry in intact tissue at room temperature, a sequence of spectral shifts occurs. An attempt was made to characterize these shifts by observing them at temperatures between -30°C and +6°C in leaves exposed to various doses of exciting illumination. The results are consistent with a model in which PChlide undergoes a single photochemical reaction followed by various relaxations or reorientations that depend upon the temperature and the extent of photoconversion.

ACKNOWLEDGMENTS

I owe a debt of gratitude not likely to be repaid to my research adviser, Professor Kenneth Sauer. His insightful guidance was keen and imaginative. Even more personally indispensible were his patience and unflagging encouragement, and an infectious enthusiasm that spread even among the most effectively inoculated cynics.

My thanks also to Drs. Paul Mathis, Ruth Alscher, and Ora Canaani for their gracious help and collaboration during some of the experiments described herein.

Finally, a deep bow to the friends, coworkers, laboratory staff, and other fearless troopers (whose names I shan't attempt to list, for fear of heinous omissions) who made five toilsome years seem passing well spent.

I. INTRODUCTION

Whereas protochlorophyll (PChl), or its unphytylated derivative protochlorophyllide (PChlide), are found in a variety of botanical tissue (including gymnosperm seedlings, some algae, mutant strains of bacteria, dark grown Euglena, some seed coats, and a variety of root tissue), it is only in angiosperms that its photoreduction plays a critical role in the evolution of photosynthetic membranes (Boardman, 1966a). When most angiosperms are germinated and grown in darkness, the seedlings possess neither photosynthetic competence, nor any measurable chlorophyll (Chl). Instead, among the pigments is PChlide, which, only upon illumination, is reduced to chlorophyllide (Chlide) and subsequently to Chl.

Thus, as an element of a biophysical process of some importance, the photochemical conversion of PChlide to Chlide is a reaction of interest. Perhaps more interesting, however, is the possibility that the photochemical nature of this reaction might be used as a key to studying the structural aspects of the functioning photosynthetic apparatus. No such functional unit exists in angiosperm seedlings grown in the dark; instead, the plant contains organelles known as etioplasts whose substructure is clearly distinguishable from that of chloroplasts (Kirk, 1970; Kirk and Tilney-Bassett, 1967). It is only upon exposure to light and the consequent production of Chlide, hence Chl, that

the plastids evolve the functional and ultrastructural characteristics of mature chloroplasts. Hence, one can visualize the possibility of approaching an understanding of the photosynthetic apparatus by observing its evolution in etiolated material with whatever techniques the imagination suggests. That the evolution of chloroplasts from etioplasts may be somewhat artifactual, in the sense that neither etioplasts nor readily measurable quantities of PChlide have been observed in angiosperms germinated under normal conditions, does not diminish the importance of this approach to the investigation of the fully functional photosynthetic membrane. Whether such an approach to the study of photosynthetic membranes might ultimately be fruitful or not, its contemplation serves as ample justification for seeking answers to fundamental questions about the nature of the photoreduction of PChlide. (The above, of course, need serve only those -- among whom I do not number myself -- not content with human curiosity, love of order and beauty, and other aesthetic justifications for basic research as reasons for undertaking this study.)

Systematic study of the PChlide to Chlide conversion began in the 1950's with Krasnovskii and Kosobutskaya's (1952), and then Smith and Benitez's (1953,1954), isolation of a soluble, photoactive pigment-protein complex; Smith and coworkers' (Smith and Benitez, 1954) kinetic studies; and Shibata's (1957) spectral studies. Each of these studies initiated a series of investigations in various laboratories with subsequent observations and conclusions supplementing and often supplanting the earlier ones. To outline the current understanding of the PChlide/Chlide system,

we might follow chronologically three main lines of experimental developments during the past two decades.

Smith and Benitez (1954) were the first to attempt to determine the kinetics of the photoconversion. Using intact etiolated barley they found the reaction to be most suitably fitted with a second-order rate law, leading them to postulate the requirement for molecular collisions in vivo. Boardman (1962b) pursued Smith's kinetic measurements using a purified holochrome preparation from bean. He found that the data were best fitted with a sum of two exponentials. This result nicely eliminated the requirement for molecular collisions and suggested instead either two environmentally distinct PChlide species with different rate constants for conversion, or the requirement for consecutive photochemical reactions for the reduction of each PChlide molecule. Later, a perplexing and still unexplained result was reported by Sironval, et al.(1968). They observed first-order kinetics when the exciting wavelength was greater than 647 nm, but a requirement for two exponentials to fit the kinetic data at shorter wavelengths. They suggested two distinct types of PChlide-protein complexes, only one of which was excited at longer wavelengths. Finally, and most recently, the suggestion has been made (Nielsen and Kahn, 1973; Thorne and Boardman, 1972; Vaughan and Sauer, 1974) that the photochemistry is first-order but is complicated by the possibility that excited PChlide can transfer its energy to nearby, previously converted, Chlide molecules. The requirement in this formulation for two or more pigments on each pigment-protein complex leads to a discussion of the evolving picture of the structural nature of the

isolated holochrome.

Soluble, active pigment complexes, dubbed holochrome, were first isolated in glycerol from barley (Smith and Benitez, 1953; Smith and Ahrne, 1955; Butler and Briggs, 1966; Henningsen and Kahn, 1971) and in phosphate buffer from bean (Krasnovskii and Kosobutskaya, 1952). The molecular weight of the bean holochrome was first estimated from sedimentation measurements as 400,000; later revised to 700,000 (see Smith, 1960). Smith further concluded, on the basis of spectrally determined pigment to protein ratios, that one pigment inhabits each holochrome molecule. Boardman (1962a) obtained a molecular weight of 600,000 for holochrome from bean and concluded that each unit contains one or two pigment molecules. On the basis of his studies on the holochrome, he further characterized the reaction as photoenzymatic because of the rigorous requirement for the pigment-protein association in order to observe photoreduction, and because of the apparent absence of a dialyzable reductant. More extensive purification procedures on the bean holochrome yielded an entity of molecular weight of 550,000 (Schopfer and Siegelman, 1968), composed of two apparently equivalent subunits and containing at least two PChlide molecules. Treatment of crude holochrome with a detergent, saponin, produced smaller subunits (63,000 for barley, 100,000 for bean) which retained photoactivity and exhibited first-order kinetics (Henningsen and Kahn, 1971). Kahn, et al. (1970), using low-temperature fluorescence spectroscopy, estimated the extent of energy transfer from PChlide to Chlide after partial conversion of a holochrome preparation.

They concluded that the results require at least four chromophores on each holochrome unit.

The world of spectroscopy is perhaps the most chaotic one with regard to the PChlide/Chlide system. Increasingly sophisticated measurements have made it increasingly difficult to offer a concise picture of the spectral changes that can be observed during the photoconversion process in intact tissue. Shibata made the first systematic observations in 1957. He reported two forms of PChl(ide), with red absorption maxima at 636 nm and 650 nm. The latter (active) form was transformed by light to Chl(ide) absorbing at 684 nm. A slow spectral shift to 673 nm followed in the dark. Gassman, et al. (1968), and Bonner (1969) observed an intermediate form absorbing at 678 nm following the photoreduction, with a dark spectral shift to 684 nm occurring in about 30 seconds at room temperature. These three spectral shifts (one photochemical, two not so) are those readily observed in living tissue, and the two dark shifts have elicited a variety of explanations as to their origin. The rapid shift from 678 nm to 684 nm has been attributed to environmental changes (Gassman, et al., 1968) as well as to mere protein relaxation in the absence of any physical environmental change (Thorne, 1971). The second dark shift has been variously ascribed to metabolic chemical changes and orientation changes (Thorne, 1971), to phytylation (Sironval, et al., 1965) -- there is also contradictory data (Boardman, 1966b), to disaggregation of the Chl(ide) from the protein complex (Butler and Briggs, 1966; Bogorad, et al., 1968), and to disruption of Chl(ide) aggregates

(Butler and Briggs, 1966; Mathis and Sauer, 1973).

The picture, however, appears to be more complex still. Kahn, et al. (1970), using low temperature fluorescence spectroscopy, found two different photoactive forms of PChlide absorbing at 637 nm and 650 nm, both being converted upon illumination to Chlide absorbing at 680 nm. A peak for inactive PChl(ide) was also observed at 628 nm. Finally, there is evidence that there exists still another form of Chlide, occurring prior to the appearance of $Chlide_{678}$. The absorption maximum of this intermediate has been variously reported as 675-676 nm (Litvin and Belyaeva, 1968, 1971a, 1971b; Mathis and Sauer, 1973) and as 668 nm (Thorne, 1971). The conversion of this species to $Chlide_{678}$, as well as the production of the intermediate from PChlide have been reported to be photochemical reactions.

To conclude, I shall mention one further relevant area of research -- the investigation into the nature and extent of PChlide and Chlide aggregation. As outlined above, recent investigations have suggested perhaps four or five pigments per holochrome unit in isolated holochrome (Kahn, et al., 1970). Working with intact bean leaves, however, Thorne (1971) concluded from a rather cryptic analysis that 20 pigment molecules were associated closely enough to permit excitation transfer at 77°K. Finally, a convincing argument has been made that pigment associations are of the weak excitonic type, leading to complex circular dichroism (CD) spectra of isolated holochrome (Mathis and Sauer, 1972).

In view of the rather complex picture outlined above, one

modest goal of any investigator in the area of PChlide/Chlide conversion might be to lend some order to the situation -- not only by experimentation but also by some detached reasoning. I shall risk being judged, at least in part, in terms of this ideal.

II. KINETICS AND PIGMENT AGGREGATION

The nature and extent of PChlide and Chlide aggregation is apparently bound up with the complexity of the kinetics of the photoconversion reaction. Mathis and Sauer (1973) explained the kinetics in terms of the need for two consecutive photochemical reactions to convert excitonically paired PChlide to excitonically paired Chlide. [While they did not suggest mechanistic explanations, Thorne (1971) and Litvin and Belyaeva (1968, 1971a, 1971b) likewise concluded that two consecutive photochemical reactions are responsible for the complex kinetics.] However, Nielsen and Kahn (1973) and Thorne and Boardman (1972) suggested excitation transfer between PChlide and Chlide as the complicating factor in an intrinsically first-order photochemical process.

The experiments described here were aimed at determining whether the photoconversion reaction is essentially first-order, complicated by energy transfer from PChlide to Chlide, or more complex. The extent of energy transfer was measured by observing Chlide fluorescence excited at several wavelengths then assessing the contribution to the fluorescence of energy absorption by PChlide. Intrinsic Chlide fluorescence (fluorescence excited by direct energy absorption by Chlide) was also measured in an attempt to detect evidence of an intermediate species in the photoconversion reaction. Finally, Chlide fluorescence polarization was measured during photoconversion as a means of estimating

the extent of pigment aggregation.

A. Materials and Methods

Kidney beans, disinfected with Arasan (DuPont), were planted in vermiculite which had been briefly soaked in water. The seedlings were then grown in darkness at about 22°C for 11-13 days. Harvesting was done at room temperature under a green safelight; subsequent operations were carried out at 4°C in darkness or under the safelight. Each preparation was derived from leaves of the same age, and no variation among preparations that might be attributed to leaf age differences was detected.

The samples containing bean leaf holochrome were prepared by grinding about 20 gm of leaves in 10 ml glycerol and 30 ml sucrose-tricine buffer consisting of 0.1 M tricine, pH 8.0, 0.4 M sucrose, for a total of 5 min in a Waring Blendor. The 5 min homogenization period was divided into several shorter intervals to prevent overheating the sample. The brei was then filtered through four layers of cheesecloth and centrifuged for 30 min at 20,000 x g. The supernatant was dialyzed for 12 hr against buffer diluted 10-fold, recentrifuged for 90 min at 20,000 x g, and concentrated by ultrafiltration against polyethylene glycol 6000. The resultant preparation was not further purified. It was made to 1.2 M sucrose with a solution of 2.0 M sucrose in 0.1 M tricine, pH 8.0. The photoconvertible PChlide in the holochrome had a red absorption maximum at 640 nm; the absorption maximum moved to 678 nm following photoconversion (see Fig 1). No subsequent spectral shifts were observed at room

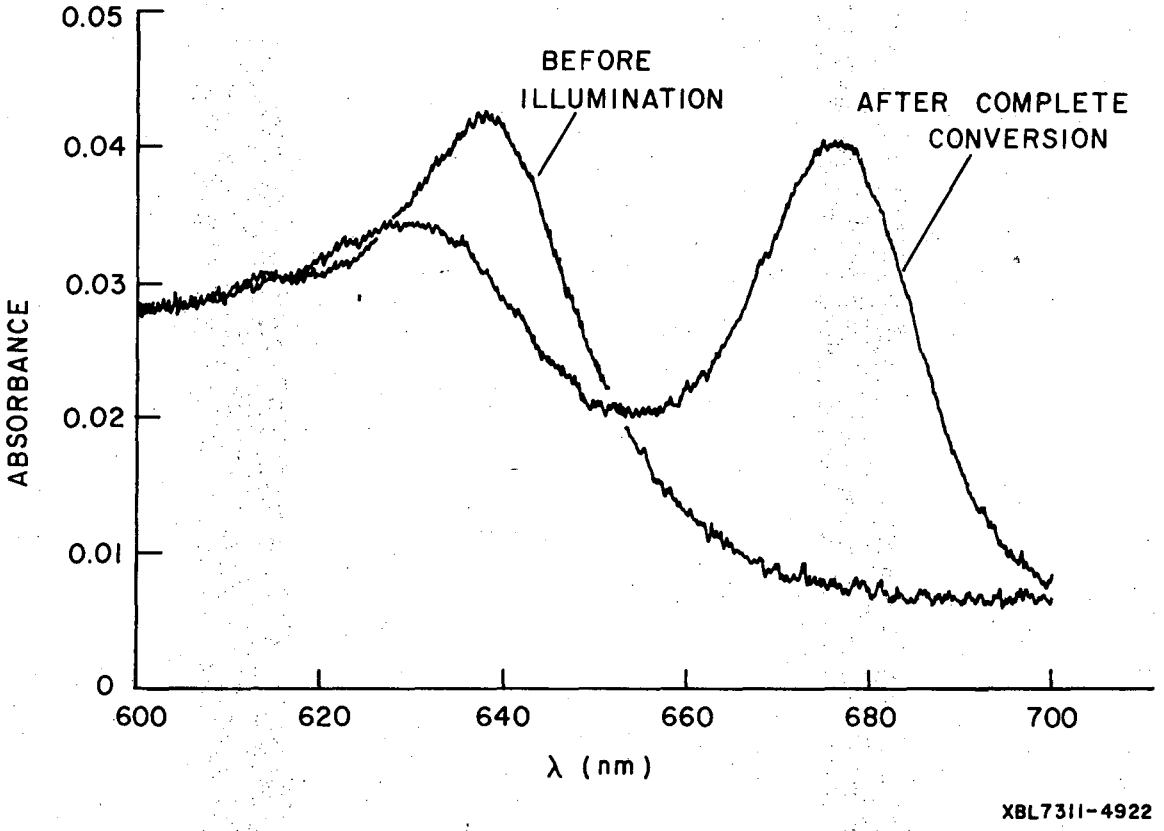


Fig 1. The absorption spectrum of an etiolated bean leaf homogenate in sucrose, before and after photoconversion.

temperature.

Etioplasts were prepared from etiolated bean leaves by grinding about 16 gm of leaves in 50 ml buffer solution consisting of 0.1 M tricine, pH 7.5, 0.4 sucrose, in a Waring Blendor for 45 sec. The homogenate was then filtered through eight layers of cheesecloth and centrifuged at 350 x g for 8 min. The pellet was washed twice with buffer, then resuspended in a minimum amount of 70% buffer/30% glycerol solution. The etioplast preparation showed an absorption maximum at 650 nm, shifting to 681 nm when photoconverted at -10°C (see Fig 2a).

Barley was grown in vermiculite and watered initially with Hoagland's solution. The terminal 10 cm of the shoots were harvested in the dark after 7 days' growth at 22°C. The plant material was then finely chopped with a scalpel, and frozen and finely ground at -76°C. The resultant powder was then ground with glycerol (5 ml/gm plant material) in a Waring Blendor for two 1 min bursts. The homogenate was then filtered through four layers of cheesecloth and spun for 60 min at 20,000 x g in an SS34 rotor. The final supernatant, referred to as glycerol extract, showed an absorption maximum at 650 nm (see Fig 2b), though some photoconvertible PChlide absorbing at about 640 nm was sometimes evident.

Saponin extract was prepared by diluting the glycerol extract 1:1 with buffer containing 0.1 M tricine, pH 8.5, 5 mM dithiothreitol, 1.5% saponin. The extraction procedure used with the barley was necessary because of its extreme sensitivity to all aqueous isolation techniques.

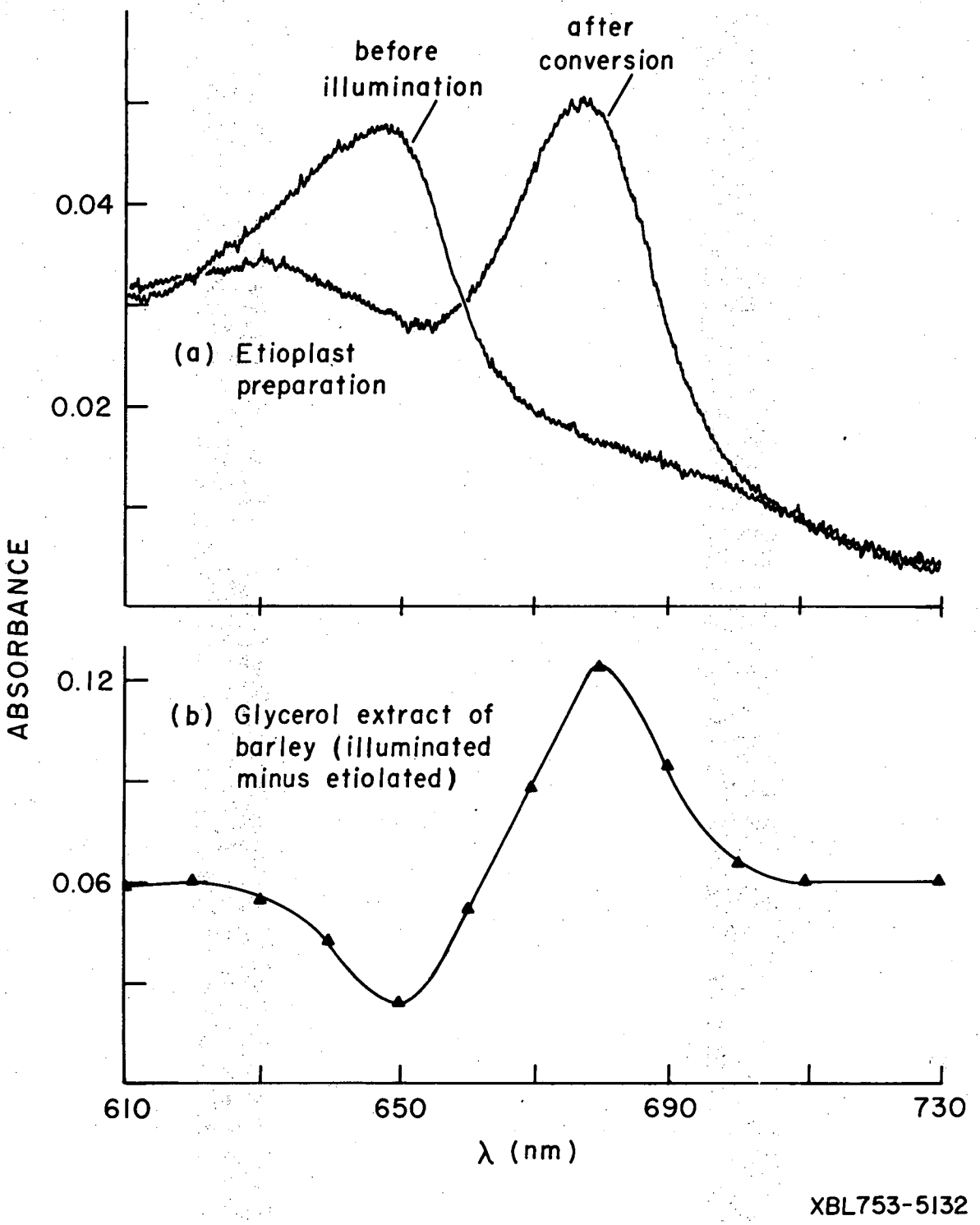


Fig 2. (a) The absorption spectrum of a bean leaf etioplast preparation, before and after complete photoconversion. (b) The difference spectrum (completely converted minus unilluminated) of a glycerol extract of barley. The ordinate indicates absorbance relative to an arbitrary baseline.

The fluorescence of Chlide was usually measured in a Perkin-Elmer MPF-2A fluorometer. The emission monochromator was set at 685 nm (20 nm bandwidth) for the experiments in Figs 3 and 4. For the rest of the experiments, the monochromator was set at 690 nm (40 nm bandwidth) and a 690 nm interference filter was placed in the emission beam. The wavelength and bandwidth of the excitation beam are given in the figure legends. The actinic light passed through several pieces of translucent tape so that the entire front surface of the cuvette containing the sample received the same illumination. The sample cuvette was covered with reflecting aluminum foil on the surface(s) opposite the excitation and/or emission beams, and was cooled when necessary by passing cold N_2 gas or cooled water through the sample block. In some of the experiments to be described, Chlide fluorescence was also measured in a Cary 14 spectrometer with the Model 1462 scattering accessory. In these instances, fluorescence was excited with 440 nm illumination which passed through translucent tape and was measured through a Kodak #70 Wratten filter that blocked the actinic beam as well as all emission below about 660 nm. For fluorescence measurements, the Cary 14 was operated in the reference mode with a linear (%T) slidewire. The jacketed sample cuvette was cooled with cold N_2 gas.

Absorption measurements were made on the Cary 14 with an expanded scale (0.0 - 0.2 absorbance unit) slidewire. The standard sample compartment was used except for the absorption spectra of etioplast suspensions, where the Model 1462 scattered

transmission accessory was used.

Fluorescence polarization measurements were made using the Perkin-Elmer instrument as described above. Fluorescence polarization measurements on solutions of fluorescein demonstrated that the presence of the aluminum foil had no effect on the results. A Polaroid polarizer was oriented in the excitation beam normal to the plane defined by the excitation and emission beams. A second polarizer was used to analyze the emission into parallel and perpendicular components. The excitation monochromator was set at 670 nm (3.5 or 4 nm bandwidth, depending upon the experiment). Corrections were applied to the polarization results as described by Houssier and Sauer (1969).

B. Results

(1) Kinetics

Etiolated bean leaf holochrome preparations in 1.2 M sucrose were photoconverted in the fluorometer at room temperature with illumination at various wavelengths. As the photoconversion proceeded, the fluorescence of Chlide excited by the actinic beam was measured continuously. At regular intervals the sample was removed to a spectrophotometer and the absorption spectrum recorded. At the end of the experiment, complete conversion was effected by exposing the sample to intense illumination at 640 nm for about 5 min. A final fluorescence value and a final absorption spectrum were then recorded. Thus, both absorption changes, and Chlide fluorescence at 685 nm were obtained as a function of time of illumination. The results of five such

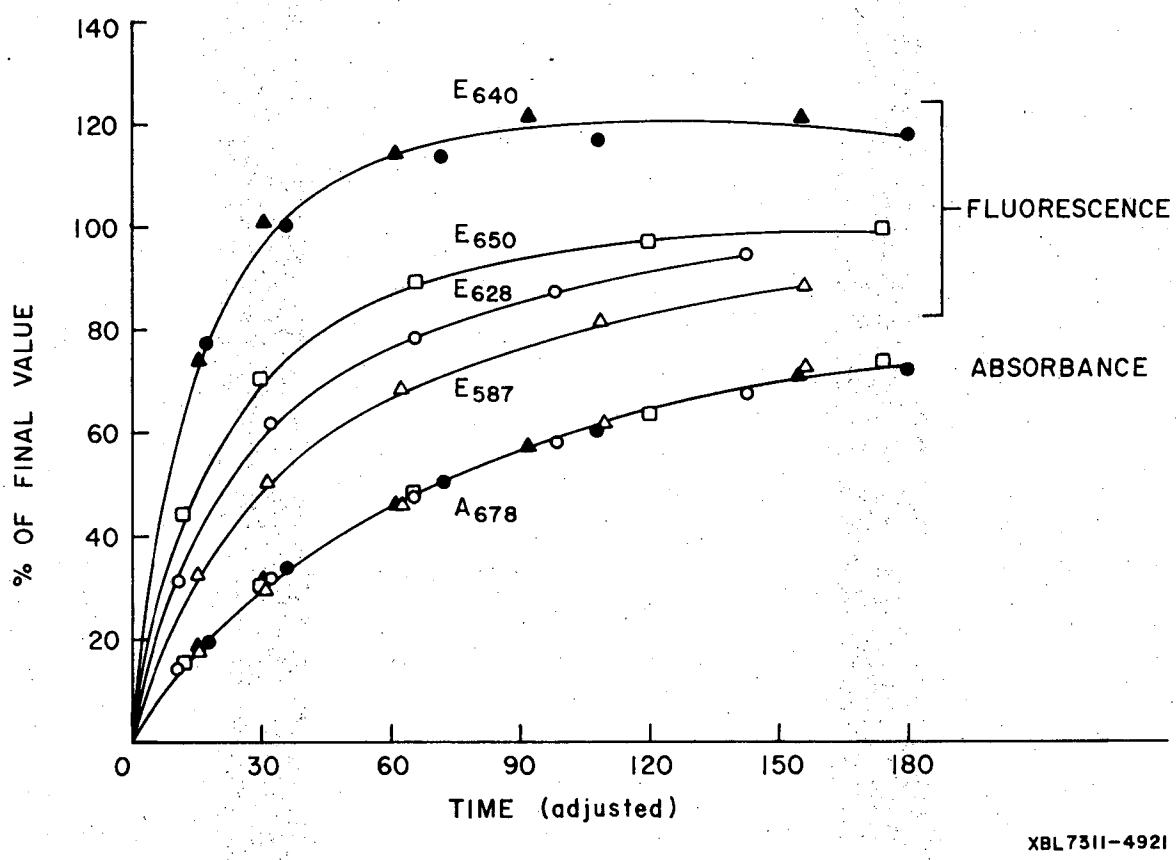


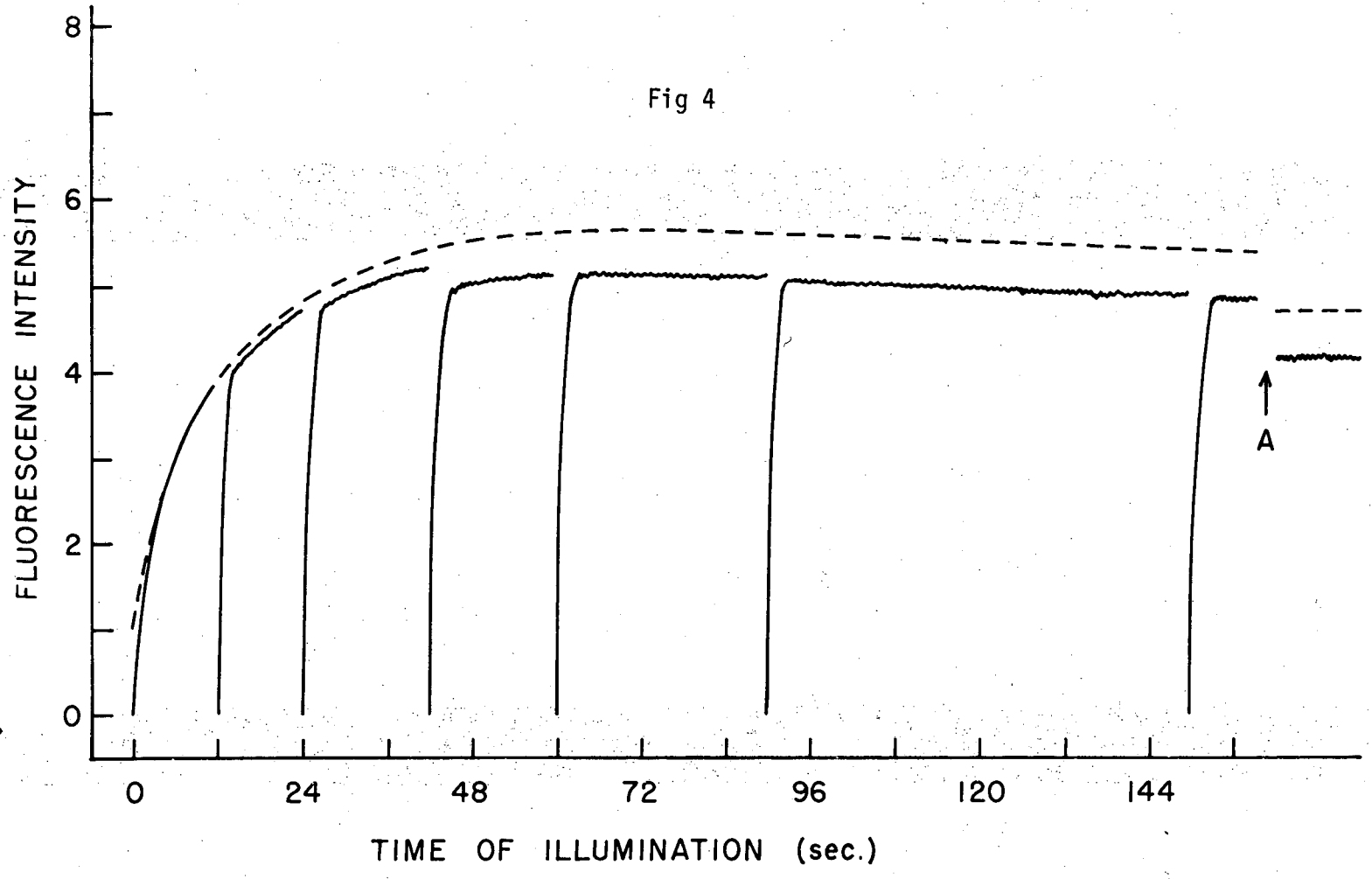
Fig 3. Absorbance and fluorescence of Chlide in bean leaf homogenates as a function of time of illumination at room temperature. Upper four curves from top to bottom: Chlide fluorescence at 685 nm excited at 640 nm (8 nm bandwidth), 650 nm (10 nm), 628 nm (8 nm), 587 nm (14 nm), respectively. Lower curve: absorbance change at 678 nm. Each symbol type represents a single experiment in which absorbance change and fluorescence were measured. The half-time for each experiment, as measured by A_{678}, has been adjusted to the same value by multiplying each time scale by the appropriate factor. The true half-times of the experiments were between 70 sec and 120 sec.

experiments are shown in Fig 3, each symbol type representing a single experiment in which absorption at 678 nm and Chlide fluorescence at 685 nm (excited at the indicated wavelength) were measured. The half-times of the experiments varied by a factor of two owing to variations in light intensity at the sample and differences in effectiveness among the actinic wavelengths in promoting photoconversion. The time scales, therefore, were linearly adjusted so that the curves representing absorption change coincided (see figure legend).

The presence of 1.2 M sucrose in my samples stabilized the 678 nm form of Chlide, as reported previously for a somewhat higher sucrose concentration (Mathis and Sauer, 1972). However, a small fluorescence decrease attributable to dark processes was observed, although it was much less pronounced than in the absence of sucrose. To illustrate how the corrections were made for this small fluorescence decrease, Fig 4 depicts a typical fluorescence trace vs time of illumination. The discontinuities occur at those times when the sample was removed from the fluorometer to allow recording of the absorption spectrum. These intervals were long (about 5 min) compared to the times of illumination. Consequently, I concluded that, during the course of the brief illumination periods, no significant decrease occurred in the fluorescence of previously formed Chlide. Thus, the corrected fluorescence at any time was taken as the measured fluorescence plus the sum of decreases observed during the intervals between illumination periods. The dashed line in Fig 4 illustrates the level of corrected fluorescence. (Although this

Fig 4. A typical trace of Chlide fluorescence at 685 nm excited at 640 nm vs time of illumination at room temperature. The discontinuities represent the points at which absorption spectra were taken. The decrease in fluorescence between intervals of illumination was assumed to result from a dark process that slightly reduced the fluorescence yield of Chlide. The broken line is the fluorescence intensity corrected for this dark decay (see text). At A, complete photoconversion was effected.

Fig 4



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method of correction cannot be defended rigorously, I believe that the conclusions based on these measurements would not be materially altered even in the absence of any correction.)

In general, experimental curves such as those represented in Fig 3 (with the exception of the curve representing Chlide fluorescence excited at 640 nm) could be computer fitted with high accuracy with a sum of two exponentials. However, there are several indications that such fits are fortuitous. First, for a reaction involving two simple first-order steps, whether they occur consecutively (e.g., Mathis and Sauer, 1972) or concurrently (e.g., Boardman, 1962b), all measured properties of Chlide such as absorbance or fluorescence intensity will be resolvable in terms of the same two first-order rate constants. However, this was never the case in experiments such as those illustrated in Fig 3. Second, curves were artificially generated by sums of three or four exponentials in which the rate constants differed by factors as large as five. The computer program used could usually fit such curves with only two exponentials and with remarkable precision. Third, the curve representing Chlide fluorescence excited at 640 nm could not be fitted by the sum or difference of any two exponentials.

Fig 3 also illustrates another striking phenomenon. The curves representing Chlide fluorescence excited at 587 nm, 628 nm, 640 nm, and 650 nm all rise more rapidly than does the curve representing Chlide absorbance, which may be taken as a measure of Chlide concentration (Mathis and Sauer, 1972). It is noteworthy that this disparity is greatest at 640 nm, the absorption maximum of PChlide.

Glycerol extract of barley was subjected to a similar experiment, but with different results. A sample of the extract was converted with 650 nm illumination at about 0°C while continuously monitoring fluorescence intensity. At intervals, the photoconversion was stopped and a measurement was made of the fluorescence intensity excited at 670 nm, where only Chlide absorbs. (It will later be demonstrated that fluorescence excited solely by Chlide absorption is a direct measure of the Chlide concentration.) As Fig 5 shows for three separate experiments, the curve representing Chlide fluorescence excited at the PChlide maximum lies very near the curve representing Chlide fluorescence excited at 670 nm. With saponin extract the correspondence is even closer, though some disparity remains.

A contrast with bean holochrome is also seen when the photoconversion kinetics for barley extracts is examined. Fig 6 compares the progress of photoconversion in bean holochrome, glycerol extract of barley, and saponin extract of barley, plotted as first-order reactions. The complexity of the reaction in bean is obvious; however, both barley extracts show little or no deviation from first-order behavior.

(2) Chlide fluorescence yield

To confirm that the observations described above for bean holochrome were not affected by a real change in the intrinsic quantum yield of Chlide fluorescence during photoconversion, an experiment was conducted in which Chlide fluorescence (at 690 nm) excited at 670 nm was measured at room temperature in addition to absorbance at 678 nm and fluorescence excited at 640 nm.

Fig 5. Fluorescence at 690 nm of glycerol extract of barley excited at two wavelengths as a function of time of illumination at about 0°C. Upper curve: fluorescence excited at 650 nm (10 nm bandwidth); lower curve: fluorescence excited at 670 nm (4 nm bandwidth). The different symbol types represent three independent experiments, in each of which fluorescence was excited at both wavelengths. The time scales of the experiments were adjusted linearly so that the E_{670} curves coincided. The true half-times of the experiments were between 35 sec and 150 sec.

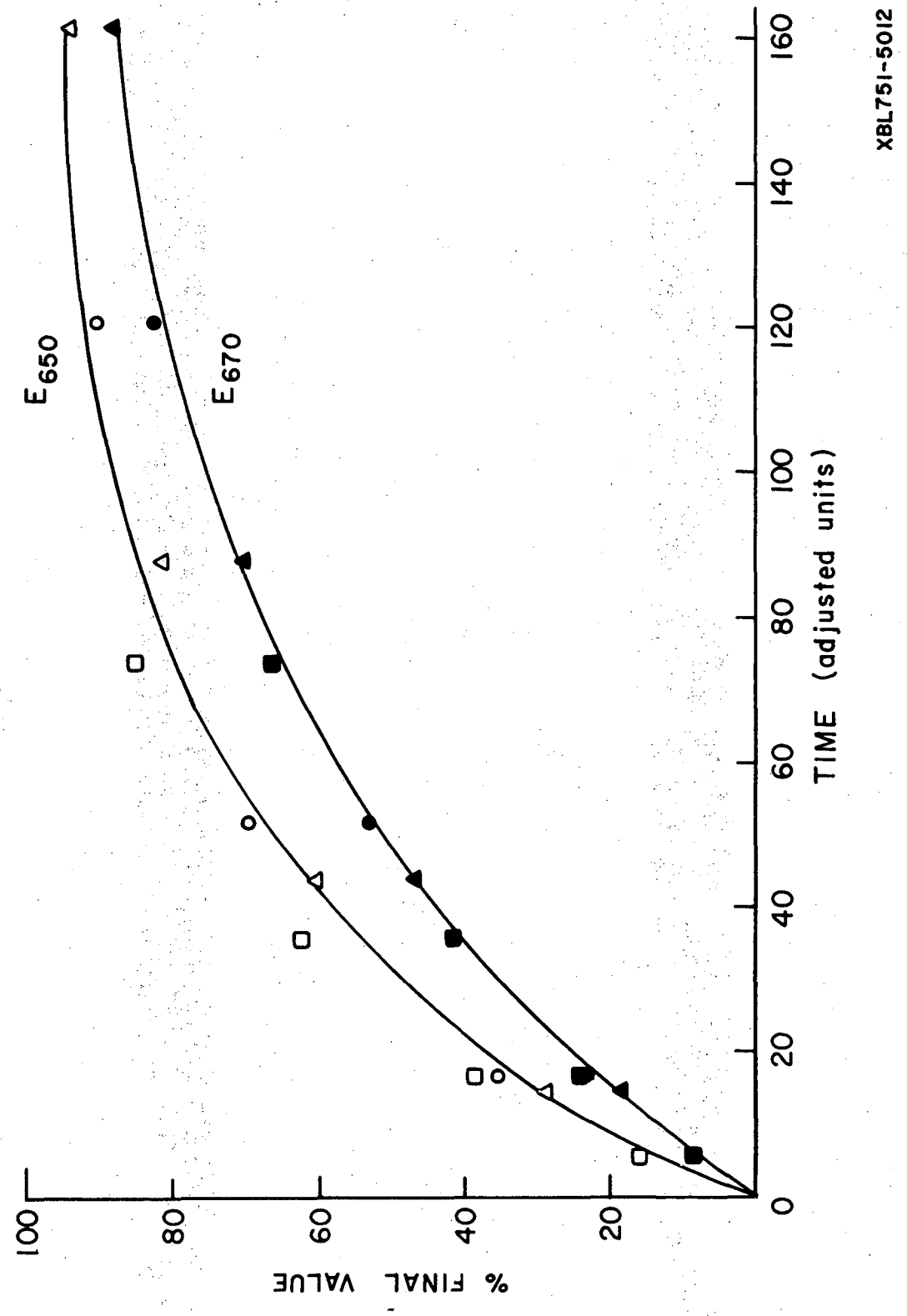
E650
E670
% FINAL VALUE
TIME (adjusted units)
0
20
40
60
80
100
120
140
160
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Fig 5

Fig 6. The photoconversion of bean holochrome, glycerol extract of barley, and saponin extract of barley, plotted as the logarithm of remaining active PChlide as a function of time of illumination. Simple first-order reactions will appear as straight lines. The curve representing the conversion of the glycerol extract of barley contains points from three experiments. (The conditions of the experiments illustrated were various; the figure is intended only to indicate any deviation from first-order behavior.)

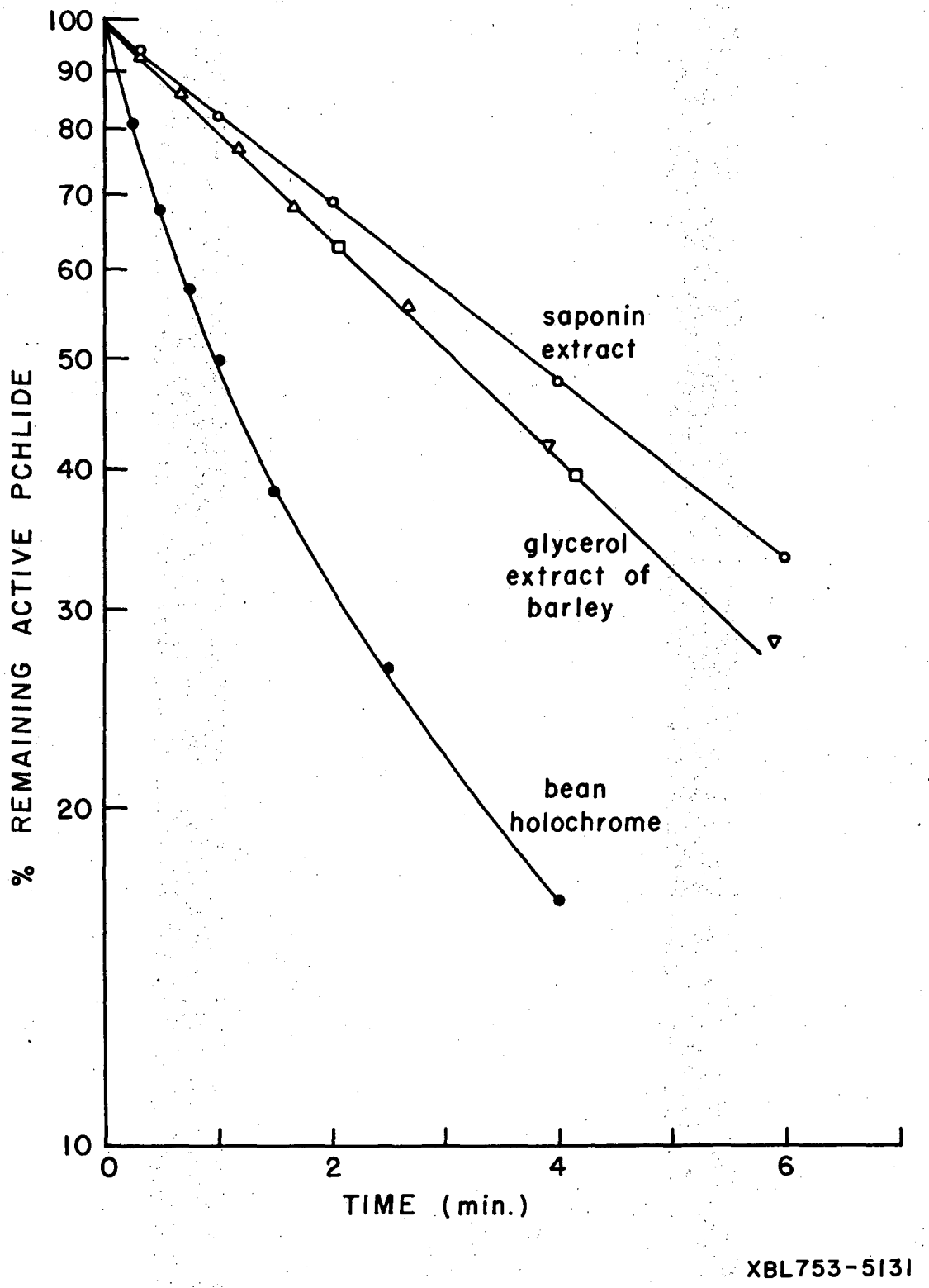

100
90
80
70
60
50
40
30
20
10
% REMAINING ACTIVE PCHLIDE
0
2
4
6
TIME (min.)
saponin extract
glycerol extract of barley
bean holochrome
XBL753-5131

Fig 6

Excitation of the holochrome preparation at 670 nm caused no photoconversion, and Chlide may be considered to be the only absorbing pigment. The results of two such experiments are plotted in Fig 7. From the coincidence of the curves representing Chlide absorption at 678 nm and the intrinsic Chlide fluorescence (E_{670}), I conclude that the intrinsic quantum yield of Chlide fluorescence is constant during the course of photoconversion in the sucrose holochrome preparation. To extend this conclusion to etioplast preparations, a somewhat more complex set of experiments was necessary. In order to prevent subsequent dark shifts in the Chlide absorption, it was necessary to cool the etioplasts to -10°C; thus it became inconvenient to move the preparation from one instrument to another during the course of a single experiment. Therefore, fluorescence at 690 nm excited at 440 nm and at 670 nm were measured during photoconversion in the fluorometer, and fluorescence excited at 440 nm and absorbance were measured on a second sample under similar conditions in the Cary spectrophotometer. The results of four such experiments on the fluorometer and two on the Cary 14 are summarized in Fig 8. The six curves representing Chlide fluorescence excited at 440 nm (one from each of the six experiments) have been made to coincide by multiplying the time scales of the six experiments by appropriate factors. This allows the direct comparison of the time courses of intrinsic Chlide fluorescence (excited at 670 nm) and Chlide absorbance at 681 nm. As with the holochrome (Fig 7), this comparison reveals that the quantum yield of Chlide fluorescence remains substantially constant during the photoconversion.

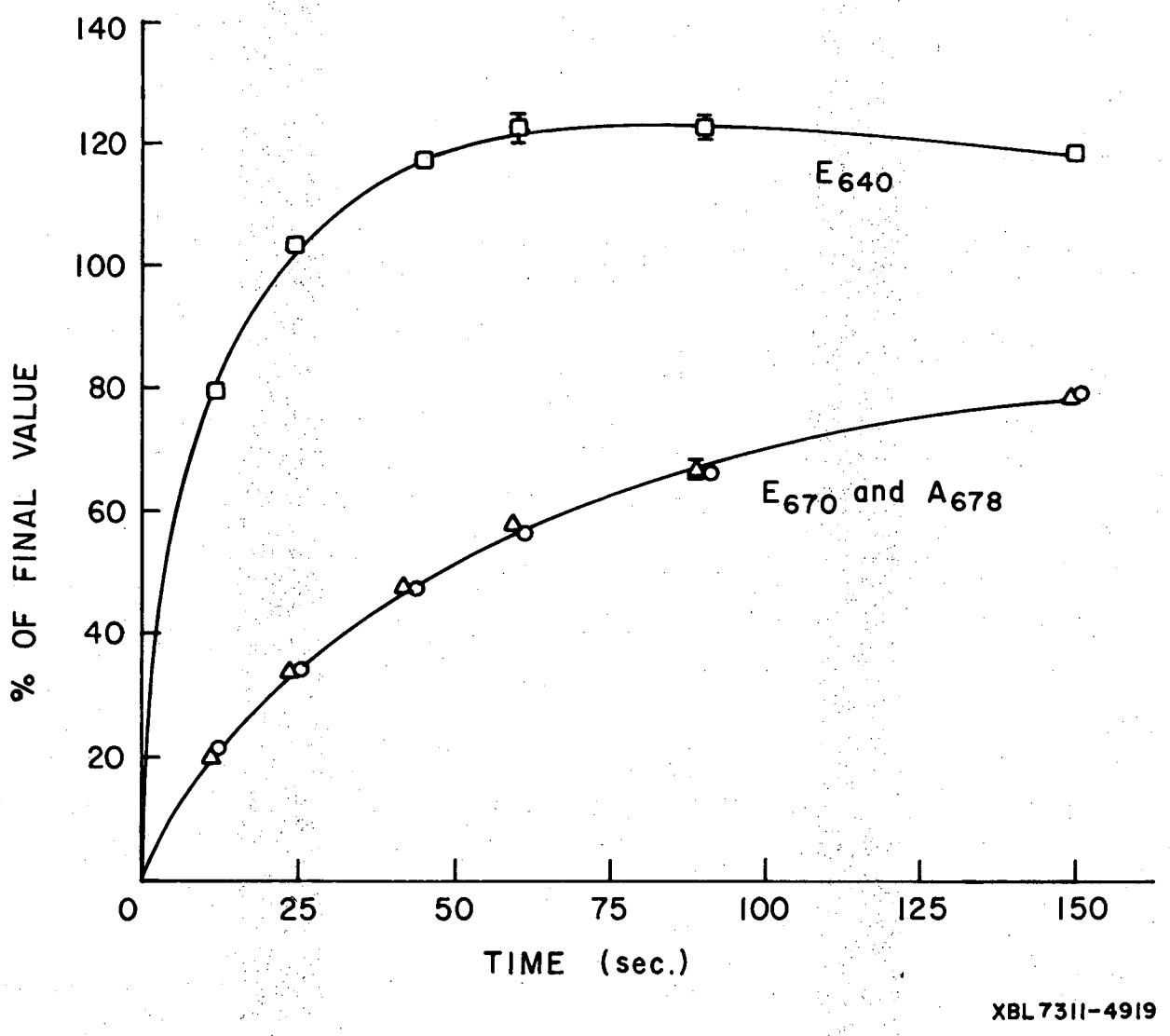


Fig 7. Absorbance and fluorescence of Chlide in bean leaf homogenate as a function of time of illumination at room temperature. The symbols represent the averages of two experiments. The range of experimental values is shown by the error bars. Δ, Chlide fluorescence at 690 nm excited at 670 nm (3.5 nm bandwidth); O, absorbance change at 678 nm; □, Chlide fluorescence at 690 nm excited at 640 nm (10 nm bandwidth).

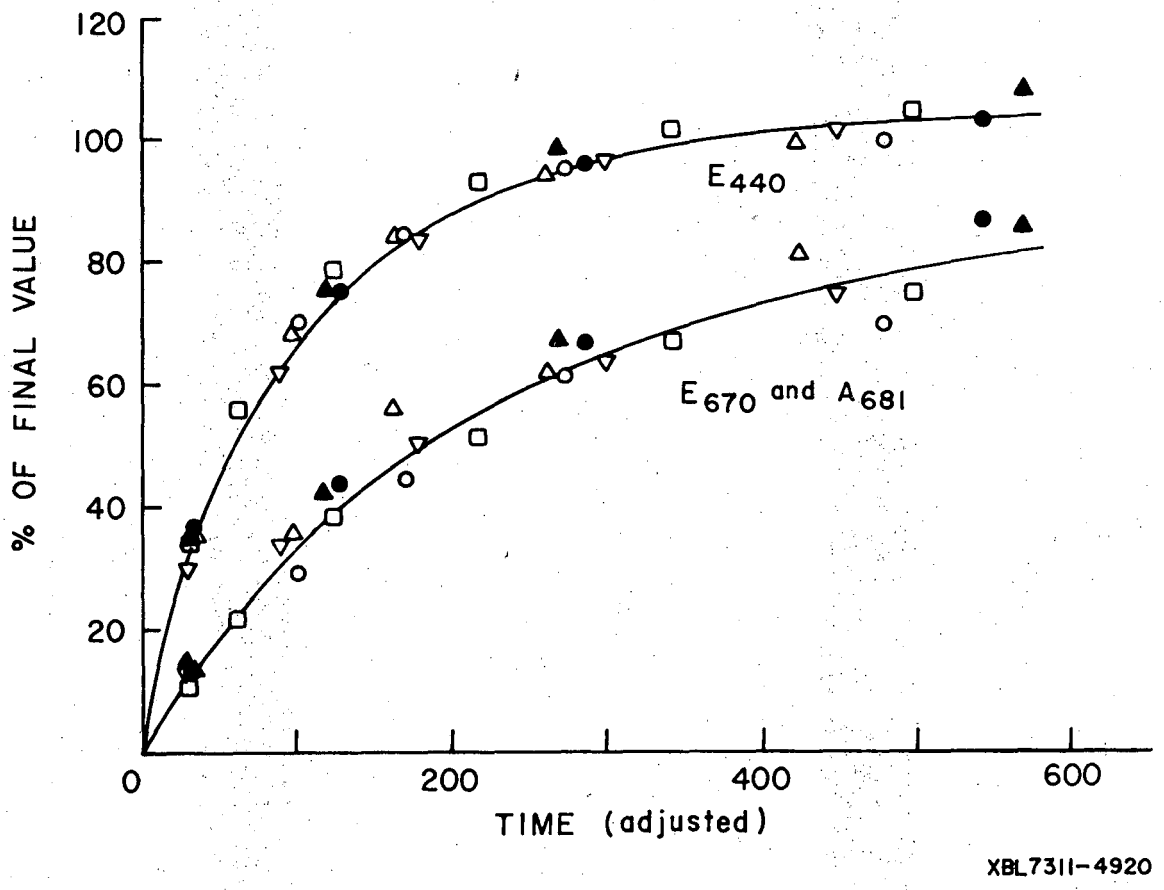


Fig 8. Fluorescence and absorbance of Chlide as a function of time of illumination of etioplast preparations at -10°C. Each symbol type represents a separate measurement: the solid symbols represent measurements on the Cary 14 of absorbance at 681 nm (lower curve) and Chlide fluorescence excited at 440 nm (10 nm bandwidth) (upper curve); the open symbols represent measurements on the fluorometer of Chlide fluorescence at 690 nm excited at 670 nm (3.5 nm bandwidth) (lower curve) and fluorescence at 690 nm excited at 440 nm (5 nm bandwidth) (upper curve). The time scale for each experiment has been multiplied by an appropriate factor so that the six curves representing fluorescence excited at 440 nm coincide.

(I am inclined to extend these conclusions to other plant material -- notably the barley extracts -- although no additional experiments were performed.)

(3) Polarization of fluorescence

The polarization of Chlide fluorescence excited at 670 nm was measured at room temperature during the course of photoconversion of a crude bean leaf holochrome preparation and at about 0°C for barley extracts. The fluorescence polarization may be expressed as

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} - I_{\perp}} \qquad (1)$$

where $I_{\parallel}$ and $I_{\perp}$ are the measured intensities of fluorescence polarized parallel and perpendicular to the polarized actinic beam. The results for two such experiments on bean holochrome are plotted in Fig 9. The error bars represent the approximate experimental uncertainty in each measurement. The results indicate that there may have been a systematic difference between the two sample preparations used; however, a significant trend in the fluorescence polarization is obvious. The polarization decreases from a value of 0.38±0.08 at 6% conversion to 0.17±0.05 at 50% conversion and 0.11±0.03 at 100% conversion. In the glycerol extract of barley, depolarization of Chlide is also observed, though it is less pronounced than in the bean holochrome. Fig 10 illustrates the results of three experiments on the glycerol extract; the initial polarization value of about 0.30 decreases smoothly to 0.15±0.01 at 100% conversion. For the saponin extract of barley, the situation is markedly different;

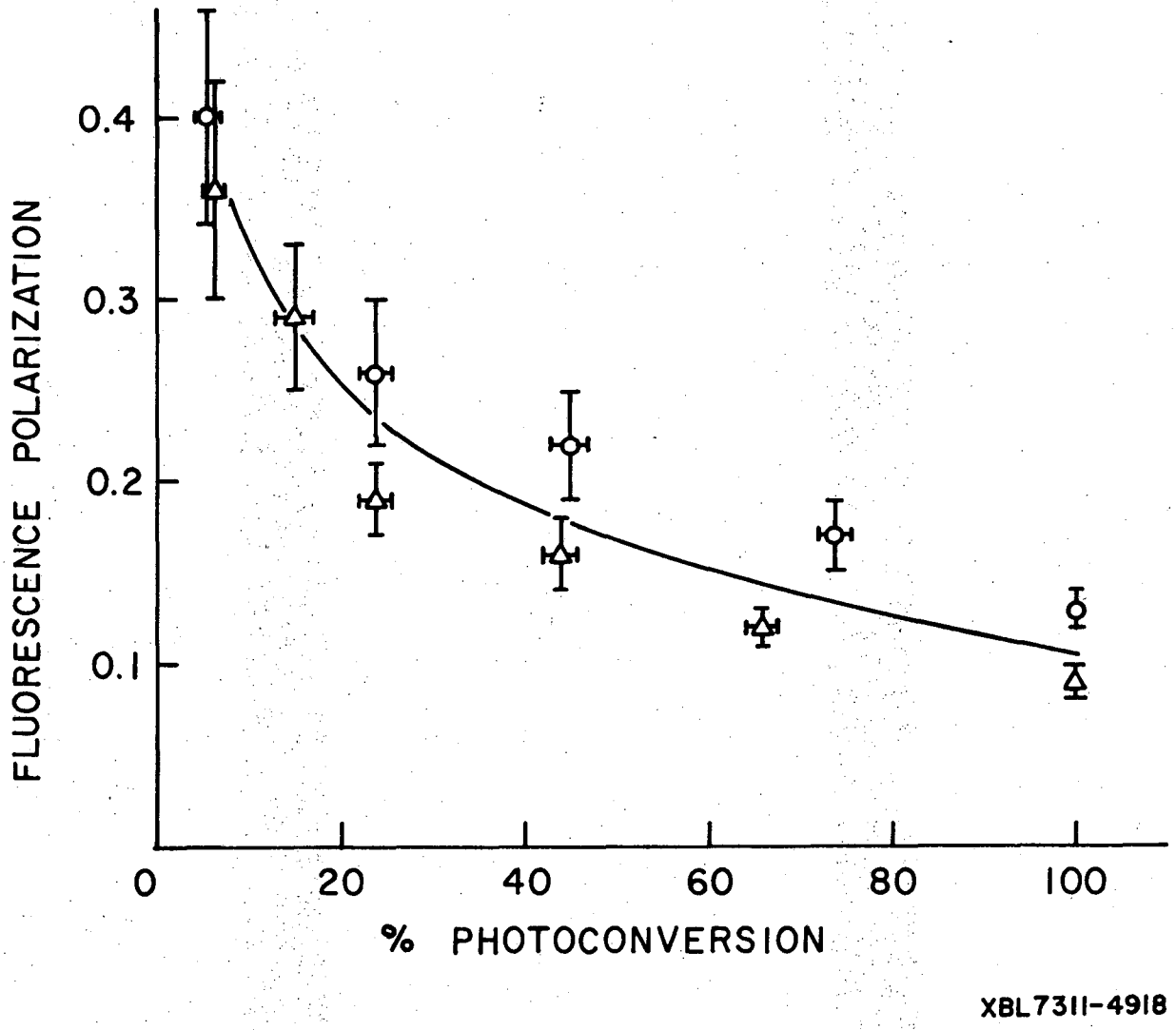


Fig 9. Polarization of Chlide fluorescence at 690 nm excited at 670 nm (4 nm bandwidth) as a function of extent of photoconversion of bean homogenate preparation. Each symbol type represents a separate experiment at room temperature. The error bars represent the estimated uncertainty in each measurement.

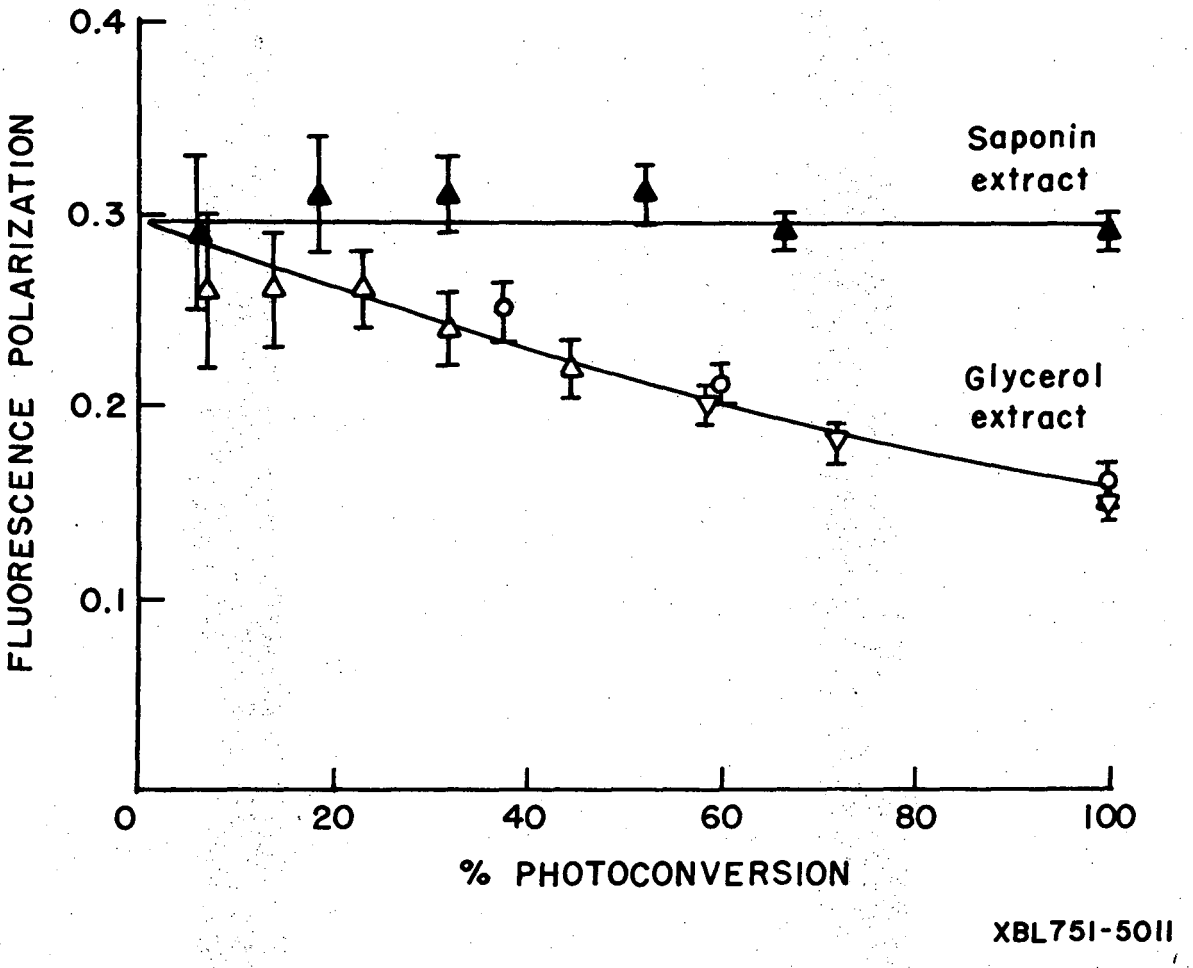


Fig 10. Polarization of Chlide fluorescence at 690 nm excited at 670 nm (4 nm bandwidth) as a function of extent of photoconversion of glycerol and saponin extracts of barley. The lower curve represents three separate experiments on glycerol extracts; the upper curve a single experiment on saponin extract. Experiments were done at about 0°C. Error bars represent estimated uncertainties.

the polarization value of 0.30 remains unchanged during the photoconversion reaction (Fig 10).

C. Discussion

The fluorescence and fluorescence polarization results observed in the above investigations admit of a rather simple and self-consistent explanation. The observations suggest that, at or near room temperature: (1) under conditions that inhibit post-conversion dark shifts, the PChlide and Chlide pigments are substantially associated during and following photoconversion in both barley and bean extracts; (2) the kinetics of the photoconversion PChlide to Chlide is intrinsically first-order; however, under conditions that permit energy transfer from PChlide to Chlide, this process competes with the conversion reaction, thus complicating the observed kinetics (see also Thorne and Boardman, 1972; Nielsen and Kahn, 1973); (3) the pigments are aggregated in bean holochrome, allowing extensive energy transfer from PChlide to Chlide and among Chlide molecules; (4) pigment aggregates in glycerol extracts of barley differ from those in bean holochrome in extent and/or mutual orientation and, thus, show little energy transfer from PChlide to Chlide but substantial excitation transfer among Chlide molecules; (5) saponin treatment of barley-extracts disaggregates the pigments so that no excitation transfer is possible (see Henningsen and Kahn, 1971). These conclusions will be borne out in the discussion that follows.

The kinetics for the photoconversion of bean holochrome

shows considerable deviation from first-order behavior. This observation and that illustrated in Fig 3 are most readily explained by the occurrence of excitation transfer from PChlide to Chlide at room temperature. The relatively high levels of Chlide fluorescence excited at 587 nm, 628 nm, 640 nm, and 650 nm during the early stages of photoconversion arise from the fact that, while some of the actinic illumination absorbed by PChlide at these wavelengths promotes photoconversion, some of the energy is transferred to the newly formed Chlide. Thus the Chlide fluorescence excited at wavelengths shorter than 660 nm is dependent not only upon the concentration of Chlide but also upon the concentration of photoactive PChlide. When Chlide emission is excited by direct absorption of actinic illumination, and at the low sample concentration used, Chlide fluorescence is proportional to the concentration of the pigment and shows the same time course as absorption (see Fig 7). Therefore, the disparity between the curve representing Chlide absorption and those representing fluorescence in Fig 3 arises from the contribution of energy absorbed by active PChlide and transferred to the Chlide. This is confirmed by the observation that this disparity is greatest at 640 nm, the absorption maximum of the PChlide in the holochrome preparation, and is smallest at 587 nm and 628 nm, where the excitation spectrum of Chlide fluorescence in the completely converted holochrome has maxima. The above conclusions can be used to calculate energy transfer efficiencies from the curve representing Chlide fluorescence excited at 650 nm and that representing Chlide absorbance (Fig 3).

Of the energy absorbed directly by the active PChlide, let E_t be that fraction transferred to Chlide. Then E_t can be calculated from the relation

$$E_t = \frac{E_{650} - E_{670}}{E_{670}} \cdot \frac{\varepsilon_{650}^{Chlide} \cdot [Chlide]}{\varepsilon_{650}^{PChlide} \cdot [PChlide]} \qquad (2)$$

where E_{650} and E_{670} are the intensities, expressed as a fraction of the values observed at 100% conversion, of Chlide fluorescence excited at 650 nm and 670 nm; $\varepsilon_{650}^{Chlide}$ and $\varepsilon_{650}^{PChlide}$ are the extinction coefficients for Chlide and active PChlide at 650 nm; and the brackets denote concentrations. The values for E_{650} are taken directly from Fig 3; the values for E_{670} are assumed to be equal to those for A_{678} in Fig 3 (see Fig 7). The derivation of Eqn 2 is included in Appendix 1. The straightforward calculation of E_t, however, is beset by two uncertainties. First, since the bandwidth of the actinic light was substantial (10 nm), the relative values of the extinction coefficients at 650 nm do not rigorously represent the relative absorption efficiencies of the two pigments. Second, the inactive PChlide, with an absorption maximum at about 630 nm, makes a small contribution to the absorption in the region of 650 nm, thus making the evaluation of the relative values of the extinction coefficients difficult. With cognizance of these uncertainties, the efficiency of energy transfer, E_t, has been estimated from the above equation using $\varepsilon_{650}^{Chlide}/\varepsilon_{650}^{PChlide} = 0.58 \pm 0.08$. This value was obtained from spectra such as that in Fig 1 by estimating the absorbances at 650 nm of active PChlide before photoconversion and Chlide

after photoconversion. The calculation of E_t at several values of percentage conversion is summarized in Table 1.

Thorne and Boardman (1972) and Nielsen and Kahn (1973) have suggested that the probability of energy transfer from PChlide to Chlide is linearly dependent upon the concentration of Chlide. With this assumption, the rate law for the deactivation of excited PChlide is

$$-\frac{d[PChlide^*]}{dt} = k_t\,[PChlide^*][Chlide] + k_f[PChlide^*] \qquad (3)$$

where k_t is the rate constant for energy transfer from PChlide to Chlide and k_f is the sum of rate constants for all first-order deexcitation processes, including photoconversion. The efficiency of excitation transfer, expressed as a fraction of the total excitation of PChlide, is then

$$E_t^{theory} = \frac{k_t[Chlide]}{k_t[Chlide] + k_f} \qquad (4)$$

Hence, E_t^{theory} can be calculated at various values of the percent conversion if one value of E_t is used for calibration. Using the value of E_t at 10% conversion, therefore, transfer efficiencies at further conversion were calculated. These theoretically derived values for transfer efficiency, E_t^{theory}, are given in Table 1. The agreement between the latter values and the experimentally observed values for E_t provides strong empirical support for the kinetic formulation of Nielsen and Kahn (1973).

In the absence of rotational diffusion, the polarization of emission from isolated, randomly oriented absorption dipoles

Table 1. THE EFFICIENCY, E_t, OF ENERGY TRANSFER FROM PCHLIDE TO CHLIDE AT ROOM TEMPERATURE IN BEAN HOLOCHROME, EXPRESSED AS PERCENTAGE OF THE ENERGY ABSORBED BY ACTIVE PCHLIDE.

E_{650} and E_{670} are the values (relative to those at 100% conversion) of Chlide emission at 685 nm excited at 650 nm and 670 nm, as abstracted from Fig 3 (E_{670} is assumed to be proportional to A_{678}, see text and Fig 7); [Chlide]/[PChlide] is the ratio of the concentrations of Chlide and active PChlide. E_t is then calculated for $\varepsilon_{650}^{Chlide}/\varepsilon_{650}^{PChlide} = 0.58\pm0.08$ according to Eqn 2. E_t^{theory} are the calculated values for the efficiency of energy transfer based on E_t at 10% conversion and the assumption that the probability of transfer increases linearly with the concentration of Chlide.

Percent Conversion	$\frac{E_{650} - E_{670}}{E_{670}}$	$\frac{[Chlide]}{[PChlide]}$	E_t	E_t^{theory}
10	2.00	0.11	0.13±0.02	(0.13)
25	1.42	0.33	0.27±0.05	0.27
50	0.79	1.00	0.46±0.07	0.43
75	0.32	3.00	0.56±0.08	0.53

is 0.50. As expected, the polarization of Chlide fluorescence in crude holochrome approaches this value at low conversion. With increasing conversion, the progressive decrease in polarization values suggests that absorbed energy is transferred among pigments before emission. Since the extent of fluorescence depolarization is dependent upon the relative orientation of pigment dipoles and upon the efficiency of energy transfer among the pigments, the number of molecules among which excitation transfer occurs cannot be deduced from the fluorescence polarization results. However, the results in Fig 9 require that aggregates permitting efficient intermolecular energy transfer contain at least four pigment molecules. Approximately 75% of the polarization decrease observed during the course of photoconversion occurs before 50% conversion is reached. This result cannot be explained by a model involving three or fewer pigment molecules, even if dipole orientations and excitation transfer efficiencies are chosen to maximize depolarization. Indeed, the results are most adequately fitted if average group sizes are assumed to be five or larger. The fluorescence polarization of Chlide-protein holochrome in 2 M sucrose has been measured by Schultz and Sauer (1972). They concluded that the observed depolarization (relative to monomeric Chlide in viscous solvents) was due to energy transfer between pigments on the holochrome protein. The present results at 100% conversion agree qualitatively with those of Schultz and Sauer (1972), though the depolarization observed in the present work is more pronounced. One possible reason for this disparity is that the crude sucrose

holochrome preparation used in the present work contains larger protein aggregates than the purified holochrome preparation of Schultz and Sauer. Thus, more extensive energy transfer may be possible. Measurements of Chlide fluorescence polarization in which no substantial depolarization was observed (Latimer and Smith, 1958; Losev and Gurinovitch, 1969) were probably performed on preparations in which pigment disaggregation had occurred following photoconversion (see Schultz and Sauer, 1972).

Two additional comments are warranted with regard to the fluorescence depolarization experiments on bean holochrome. First, since the data at less than 50% conversion are not highly reliable, the curve drawn in Fig 9 might well be questioned. Indeed, to relax the rigorous requirement for the aggregate group size to three, a curve can be drawn such that 65% of the total decrease of fluorescence occurs during the initial 50% of photoconversion. However, again in this case, one must make some rather unlikely assumptions about the aggregated pigments: they must be oriented so that their absorption dipoles are very nearly perpendicular to one another, energy transfer must be very nearly perfectly efficient, yet photoconversion quantum efficiency must not decrease markedly as conversion proceeds to completion. Second, while the above discussions have assumed aggregated pigment molecules which do not manifest excitonic interactions, some discussion is due the behavior expected of excitonically coupled dimers (Mathis and Sauer, 1972). According to this model, at 50% conversion, at least 1/2 of the Chlide remains in the form of an associated PChlide-Chlide pair (showing no depolarization).

Consequently, no more than 1/2 of the total fluorescence polarization decrease should be observed during the first 50% of the reaction. This is not consistent with observations.

Experiments done on barley extracts yielded considerably different results from those with bean, and, in most cases, helped confirm the explanations given above for the bean holochrome results by serving as control experiments. Saponin treatment has been previously reported to yield monomeric holochrome subunits exhibiting simple first-order kinetics (Henningsen and Kahn, 1971). My studies fully support these findings. Fig 6 demonstrates the first-order kinetics for the photoconversion of saponin treated barley extract. The absence of PChlide-Chlide energy transfer as a competing reaction was confirmed in experiments similar to those illustrated in Fig 5, where the curves representing Chlide fluorescence excited at 670 nm (where only Chlide absorbs) and that excited at 650 nm (where PChlide absorbs strongly) very nearly coincide. Further, the absence of Chlide-Chlide energy transfer is demonstrated in Fig 10, where the polarization of Chlide fluorescence is constant during the progress of photoconversion. The glycerol extract of barley differs from the saponin treated extract in only one regard. The former shows considerable depolarization of Chlide fluorescence as photoconversion proceeds, suggesting that the pigments are sufficiently aggregated to permit energy transfer among Chlide molecules prior to emission. However, from the very nearly first-order kinetics exhibited (Fig 6) and from the near-coincidence of the curves E_{670} and E_{650} in Fig 5, one must conclude that PChlide-Chlide excitation transfer is not

an effective competitor with photoconversion at 0°C.

The contrasts between the fluorescence depolarization curves for bean holochrome (Fig 9) and glycerol extract of barley (Fig 10) are also worth noting. Although the measurements at low conversion are subject to large errors owing to the small fluorescence signals, there appears to be a significant difference between the values for polarization extrapolated to 0% conversion in the two preparations. In bean holochrome, fluorescence polarization appears to approach a value greater than 0.40, whereas, for barley (with or without saponin), the value is about 0.30. Since the viscosity of the barley preparations was about 25 times that of the bean holochrome preparation -- about 180 centipoise (measured) vs about 7.5 centipoise (estimated) -- the difference is not likely to be a result of greater rotational diffusion in the barley, even were it a somewhat smaller species. Indeed, even if the Chlide pigment has considerable freedom of movement independent of the holochrome protein, such freedom should not be manifest in fluorescence polarization measurements in glycerol, unless the local viscosity is substantially less than the bulk value of 180 centipoise. (The notion of a less rigid barley holochrome unit is, however, not inconsistent with its lability to most aqueous isolation techniques.)

A second difference between the fluorescence polarization results for these two preparations is the extent of depolarization during photoconversion. The bean holochrome shows a precipitous decrease in fluorescence polarization at low conversion and the value at complete conversion is only about 25% of the initial

value. In barley, the depolarization is less pronounced with the final value about 50% of the initial. Indeed, while aggregates of four or five pigment molecules are necessary to explain the behavior of bean holochrome, only three are necessary for barley. Clearly, however, these data do not demonstrate that pigment group sizes differ in the two cases. In fact it might be more reasonable to assume that the pigment molecules are merely arranged differently in the barley, thus permitting only limited energy transfer among molecules. This suggestion is supported by the finding that energy is not effectively transferred from PChlide to Chlide at 0°C (cf Fig 5 in contrast with Fig 3).

Finally, some comment is necessary with regard to the constancy of the intrinsic quantum yield of fluorescence of Chlide during photoconversion. This finding does not confirm a model in which closely coupled dimers of Chlide exist (Mathis and Sauer, 1972). Such dimers would be expected to display a fluorescence efficiency different from that of the monomers (i.e., mixed Chlide-PChlide dimers) present at partial conversion. Changes in the shape or position of the absorption band during photoconversion are also absent (Mathis and Sauer, 1972). In the absence of closely coupled dimers, however, the CD spectra of Mathis and Sauer cannot be readily explained. This apparent discrepancy remains unresolved at present.

III. LINEAR DICHROISM

If a population of immobile, isolated absorption dipoles is partially photoconverted with linearly polarized light to a second species of identically oriented oscillators, the newly produced population will be linearly dichroic (see, for example, Junge, 1972; Cone, 1972; Brown, 1972). In the apparatus diagrammed schematically in Fig 11, the dichroic ratio is defined as

$$P = \frac{I_z - I_x}{I_z + I_x} \qquad (5)$$

where I_z and I_x represent relative extinctions of the new species measured with z- and x-polarized measuring beams, respectively. The dichroic ratio will approach 0.50 in the ideal case, if photoconversion is effected with z-polarized exciting light. [This and subsequent values for the dichroic ratio were obtained using the general equations of Albrecht (1961). Values for selected cases are tabulated in Table 2.] The analogy with fluorescence polarization is obvious. If the photoconversion is accomplished with unpolarized light, the dichroic ratio will be 0.33. Even with planar (rather than linear) oscillators and with unpolarized photoconversion illumination, a non-zero dichroic ratio approaching 0.077 will be observed. (The results are different in many cases when the photoconversion is effected along the axis of the measuring beam; however, for simple absorption dipoles and a linearly polarized conversion beam, the dichroic ratio still approaches 0.50.)

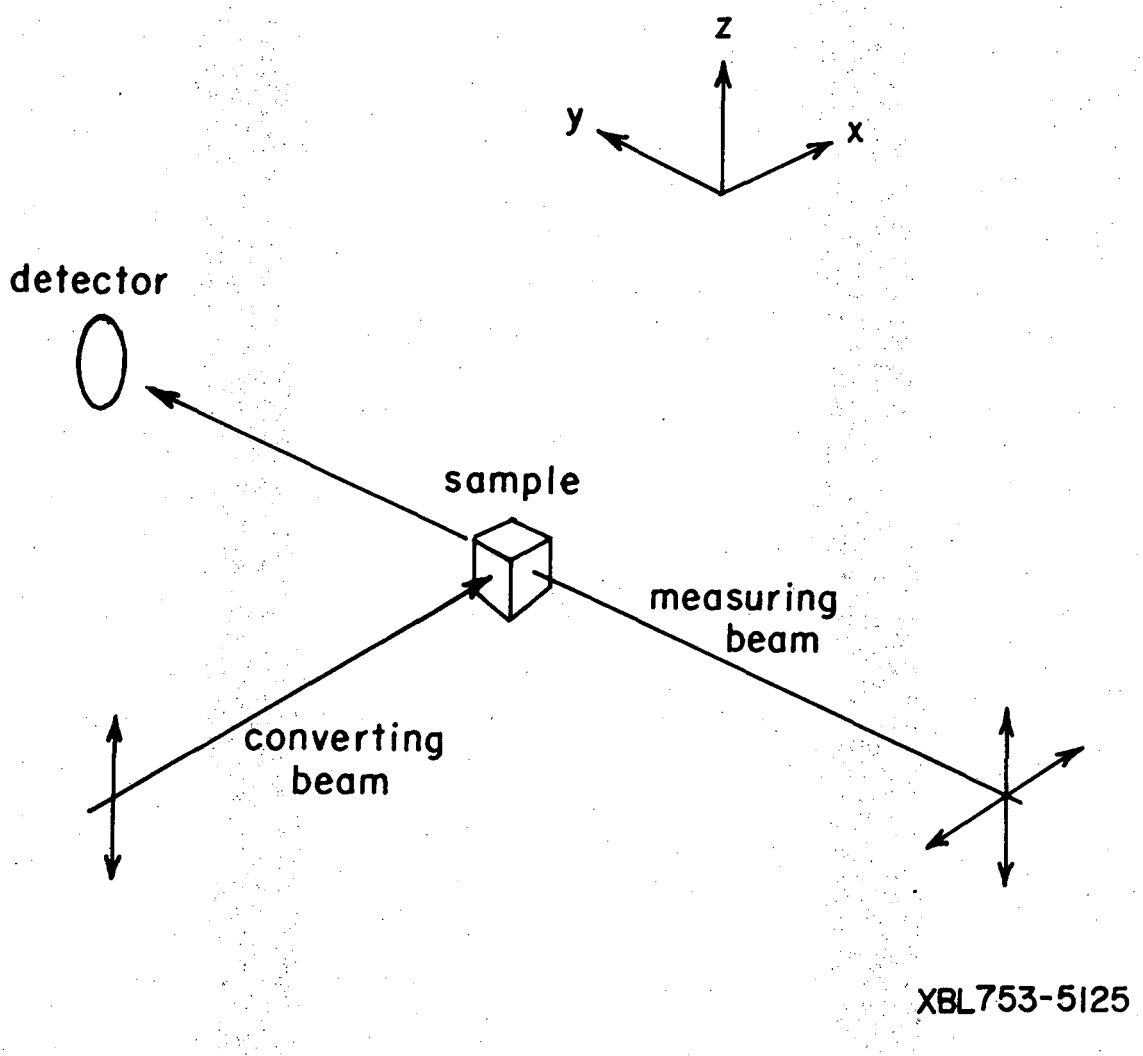


Fig 11. Schematic of an apparatus designed to measure linear dichroism induced by partial photoconversion of the sample by a linearly polarized converting beam. Conversion is effected by a z-polarized beam propagated in the +x direction. Measurement of absorbance changes is made by a beam propagated in the +y direction, either x- or z-polarized.

Table 2. CALULATED DICHROIC RATIOS FOR SELECTED SYSTEMS.

Single oscillator refers to an isotropic array of molecules in each of which the transition excited by the converting beam and that observed by the measuring beam are parallel. Perpendicular pair may be visualized as a similar array where in each molecule these two transitions are mutually perpendicular. Planar molecule refers to an array where, in each molecule, excitation of two mutually perpendicular transitions may occur, but observation of only one such transition is made. $f_{\parallel}$ and $f_{\perp}$ represent the extinctions of the excitation-absorbing oscillators parallel and perpendicular, respectively, to the oscillator observed by the measuring beam.

	z-polarized excitation	y-polarized excitation	unpolarized excitation
single oscillator	0.500	0.00	0.333
perpendicular pair	-0.333	0.00	-0.143
planar molecule ($f_{\parallel} = f_{\perp}$)	0.143	0.00	0.077
planar molecule ($f_{\parallel} = 3f_{\perp}$)	0.333	0.00	0.200

Therefore, if PChlide molecules behave as isolated species, their partial photoconversion with polarized or unpolarized light will produce a nonisotropic population of Chlide molecules. In a medium in which the pigments have been immobilized, the photoconversion of the PChlide and the spectroscopic observation of the Chlide can be conducted at one's leisure. However, in solution, the photoconversion and observation must be effected before rotational diffusion randomizes the Chlide orientations. Both of these approaches were attempted.

A. Materials and Methods

(1) Gelatin films

Bean holochrome was prepared as described in the previous section. For the preparation of gel films, a procedure similar to that of Wright, et al. (1972) was used. The holochrome was dialyzed against buffer containing 0.1 M tricine, pH 7.6, 0.1 M sucrose. This low-sucrose holochrome was then mixed with a 10% aqueous solution of purified calfskin gelatin (Eastman Chemical) in a ratio that was varied in different experiments from 3:2 to 8:3. Ten ml of the resulting mixture was layered into a Parafilm-coated petri dish and stored for two days in darkness at room temperature under house vacuum.

Absorption measurements, including those used for the calculation of dichroic ratios, were made in the Cary 14 spectrophotometer with the scattered transmission accessory. The gel films were cut into 2 cm x 1 cm pieces, then three or four thicknesses were affixed to a standard sample cuvette for spectrometry.

Absorption was usually measured from difference spectra -- illuminated sample vs unilluminated reference sample. Photoconversion was effected with the measuring beam (640 nm, 3.0 mm slit width) passing through a Polaroid polarizer. Polaroid filters were also used to polarize the measuring beam when desired during the actual absorption measurements. During the recording of the spectra, no measurable photoconversion occurred.

(2) Imidoester treatment

Attempts to cross-link the holochrome protein were made on bean holochrome preparations obtained as described earlier except that the buffer consisted of 0.2 M TAPS (tris [Hydroxymethyl] Methylamino-propane Sulfonic acid, Sigma Chemical), pH 8.4, 0.4 M sucrose. Preparations containing 0.1 M sucrose were obtained by dialysis against this buffer in 0.1 M sucrose. Various schemes were used to cross-link the holochrome, each involving the addition of dimethyl adipimidate dihydrochloride (DMA) or dimethyl suberimidate dihydrochloride (DMS) [Pierce Chemical Co.] to a stirred holochrome suspension at 0°C. From 4 mg to 9 mg of the imidate was added, either in aliquots or in one lot, for each 1 ml of holochrome solution. Stirring was usually continued for about 3 hr. Spectra were taken on the Cary 14.

A single sucrose density gradient was run with the cross-linked holochrome vs a control, untreated holochrome. The cross-linked holochrome was prepared by treating 10 ml of a 0.1 M sucrose dialysate with 60 mg DMS for 4 hr, then redialyzing against buffer in 0.1 M sucrose. The continuous gradient was prepared between 50 gm/l sucrose and 250 gm/l sucrose. The

cross-linked dialysate was run in one tube, the untreated dialysate in a second, for 8 hr at 24,000 rpm in an SW27 rotor.

(3) Transient dichroism

Glycerol extract of barley was obtained as outlined in the previous section. A glycerol extract of bean was prepared in an exactly analogous way. To decrease the water content, hence increase the viscosity of the preparation, 20 ml of the glycerol extract in a petri dish was placed in a desiccator under house vacuum for about 24 hr. The resulting material is referred to as desiccated glycerol extract. The viscosity (and thus the percentage of glycerol) of this desiccated extract was measured by comparing the flow time through a capillary with the flow times of known water-glycerol mixtures.

The apparatus used to measure transient linear dichroism is diagrammed in Fig 12. The Bausch and Lomb high intensity monochromator was set at 680 nm and its entrance and exit slits set at 2.5 mm and 1.9 mm, respectively. The tungsten lamp, nominally rated at 45 W (6.6 amps) was operated at 7.0 amps by a regulated DC power supply. A 680 nm interference filter (F2 in the figure) and the appropriate polarizing filter (F3) were placed in front of the EMI Model 9558 photomultiplier tube (PM). The PM voltage source was a Power Designs high voltage supply operated at a voltage (typically 400-450 volts) which yielded maximum signal while not saturating the PM response. The pre-amplified PM signal was observed and photographed on a Tektronix RM31 oscilloscope with a Type D plug-in unit. The partial photo-conversion was effected with an ILC xenon flash unit operated at

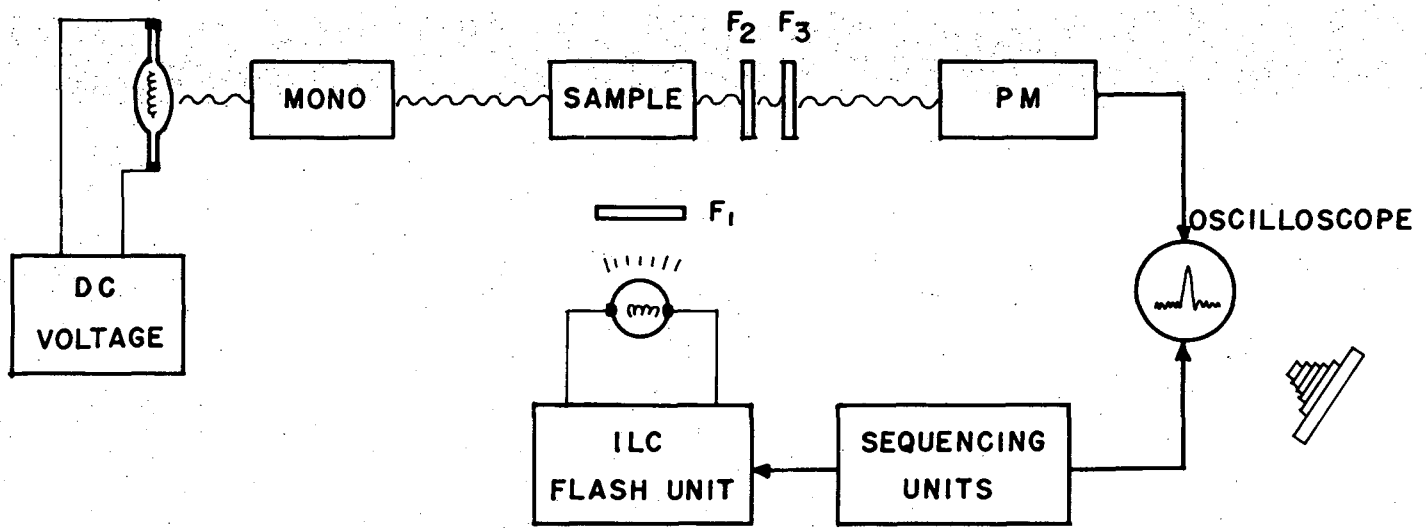


Fig 12. An apparatus for measuring transient linear dichroism. The components are discussed in the text.

2000 volts. The flash duration was about 50 μsec. A Corning 4-97 color filter (F1) was used in the flash lamp to minimize the flash intensity detected through F2 by the photomultiplier. Signal and pulse generators (Princeton Applied Research) were used to sequence the flash burst and the oscilloscope trace. Under typical experimental conditions, a single flash converted 10-20% of the sample from PChlide to Chlide. This resulted in a reduction in detected light intensity (i.e., increased absorbance) at 680 nm equal to about 1% of the total detected signal.

The sample was contained in a 1 cm x 1 cm cuvette (mirrored opposite the xenon flash) which in turn was held in a small cubical sample compartment with windows for the measuring beam and excitation flash. No means was established to cool samples in this apparatus. When measurements were desired at lower temperatures, the sample was precooled, then subjected to the experiment within 15 sec.

B. Results

(1) Linear dichroism in gelatin films

The activity of gelatin films, as measured by the total possible change in absorbance at the Chlide absorption maximum, corrected for dilution and path length differences, was typically about 75% that of the homogenates from which they were prepared. Further, the films retained this level of activity for at least a week if kept dry and at room temperature. By contrast, bean holochrome in 0.1 M sucrose rapidly loses its activity at room temperature. The films also showed unusual spectral characteristics.

When illuminated at room temperature, the films showed the usual reduction of absorbance at 640 nm, but the absorption increase associated with the appearance of Chlide appeared at 680 nm and exhibited no dark shifts.

When fractionally converted with linearly polarized light, the gelatin flims never exhibited measurable linear dichroism. (The accuracy of the measurements would have permitted the detection of dichroic ratios of the order of 0.01.)

(2) Cross-linking treatments

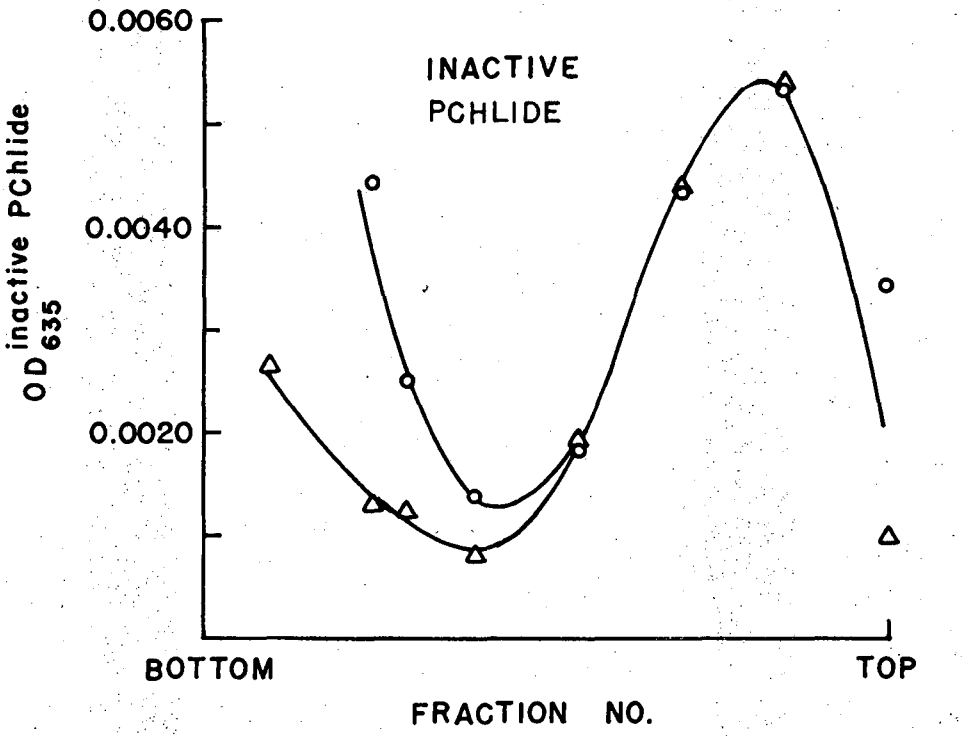
Gels were made from imidate cross-linked holochrome (0.2 M TAPS, 0.1 M sucrose) in the hope that the cross-linking might further immobilize the holochrome protein and enhance the possibility of observing linear dichroism. However, gels prepared with TAPS buffer lost all activity during the drying period.

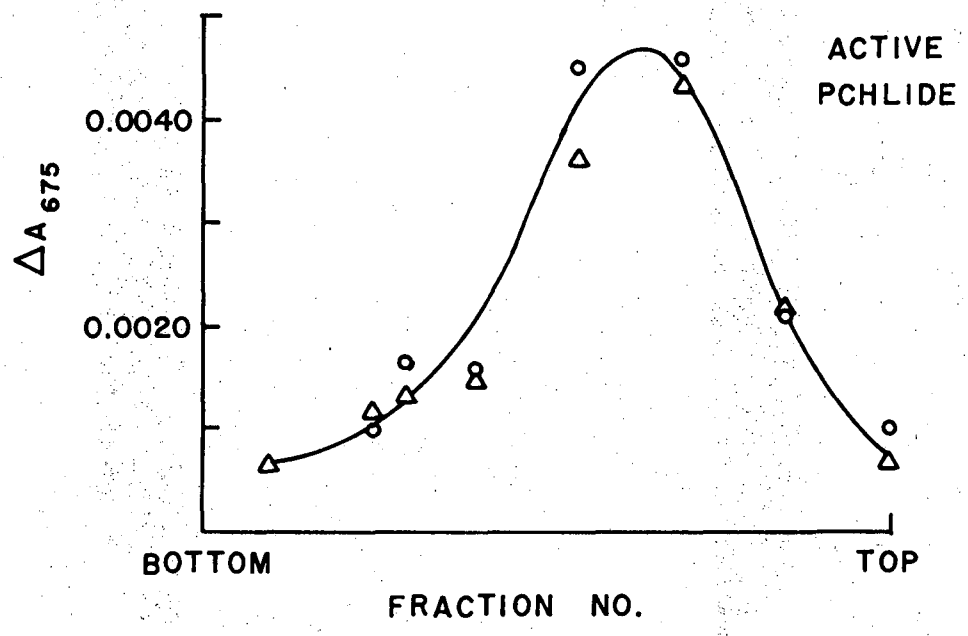
Holochrome preparations treated with up to 6 mg of diamidoester per ml of holochrome solution (about 20 mg imidate/mg holochrome protein) showed no detectable changes in activity or spectral characteristics. At room temperature, the Chlide holochrome displayed the usual dark shift from about 677 nm to 673 nm. The sucrose density gradient showed little significant difference between the cross-linked material and the control (Fig 13), though there does appear to be an increase in the amount of a heavy, inactive PChlide fraction upon treatment with imidate.

(3) Transient linear dichroism in solution

The desiccated glycerol extracts of barley and bean used in the transient dichroism experiments showed viscosities of 1.5-2.0 poise at room temperature (corresponding to 88-90% glycerol).

Fig 13. The results of a sucrose density gradient on redialyzed bean holochrome. The upper figure shows the distribution of inactive PChlide, measured by its absorbance at 635 nm; the lower figure illustrates the distribution of active PChlide, measured by the change in absorbance at 675 nm upon complete photoconversion. Δ, control dialysate; o, cross-linked dialysate.





XBL751-5014

Fig 13

Therefore, the viscosities of these preparations were estimated at 4-7 poise at 5°C. Earlier experiments were done on undesiccated extracts at about 5°C where viscosities were about 1.6 poise, and a single experiment was done at temperatures below 0°C (as the extract warmed from -76°C) where viscosities exceeded 10 poise.

Figs 14-16 depict typical experiments at about 5°C, performed on several separate samples. Each sample, in the presence of a continuous polarized measuring beam of the indicated orientation, was exposed to two or more actinic flashes which reduced the transmitted signal as the Chlide absorption at 680 nm increased. The absolute magnitude of the signal reduction varied irregularly among samples and does not reflect any phenomenon of interest. Dichroism, which would be detected as a transient of the sort indicated by the broken lines, was never consistently observed (see further discussion below).

C. Discussion

Unfortunately, the experiments in this section do not suggest clear and unambiguous conclusions. The absence of definitive controls which would render negative results meaningful compel an ambivalent discussion.

In the bean holochrome gels, the pigment molecules may be considered linear oscillators since only the Q_y transition of PChlide was excited by the 640 nm actinic beam (a conclusion drawn from the spacing of the Q_y and Q_x bands in Houssier and Sauer, 1969) and only the parallel Q_y transition of Chlide was analyzed for evidence of dichroism (see data for Chl in Houssier

Fig 14. Measurement of absorbance change at 680 nm in glycerol extracts of barley at about 5°C. Each pair of traces represents the first (top) and second (bottom) flashes on a single sample. The polarization of the measuring beam is shown in the figure for each of the four experiments. The dashed lines illustrate the behavior expected under these experimental conditions for a dichroic sample of molecular weight 600,000 (see text).

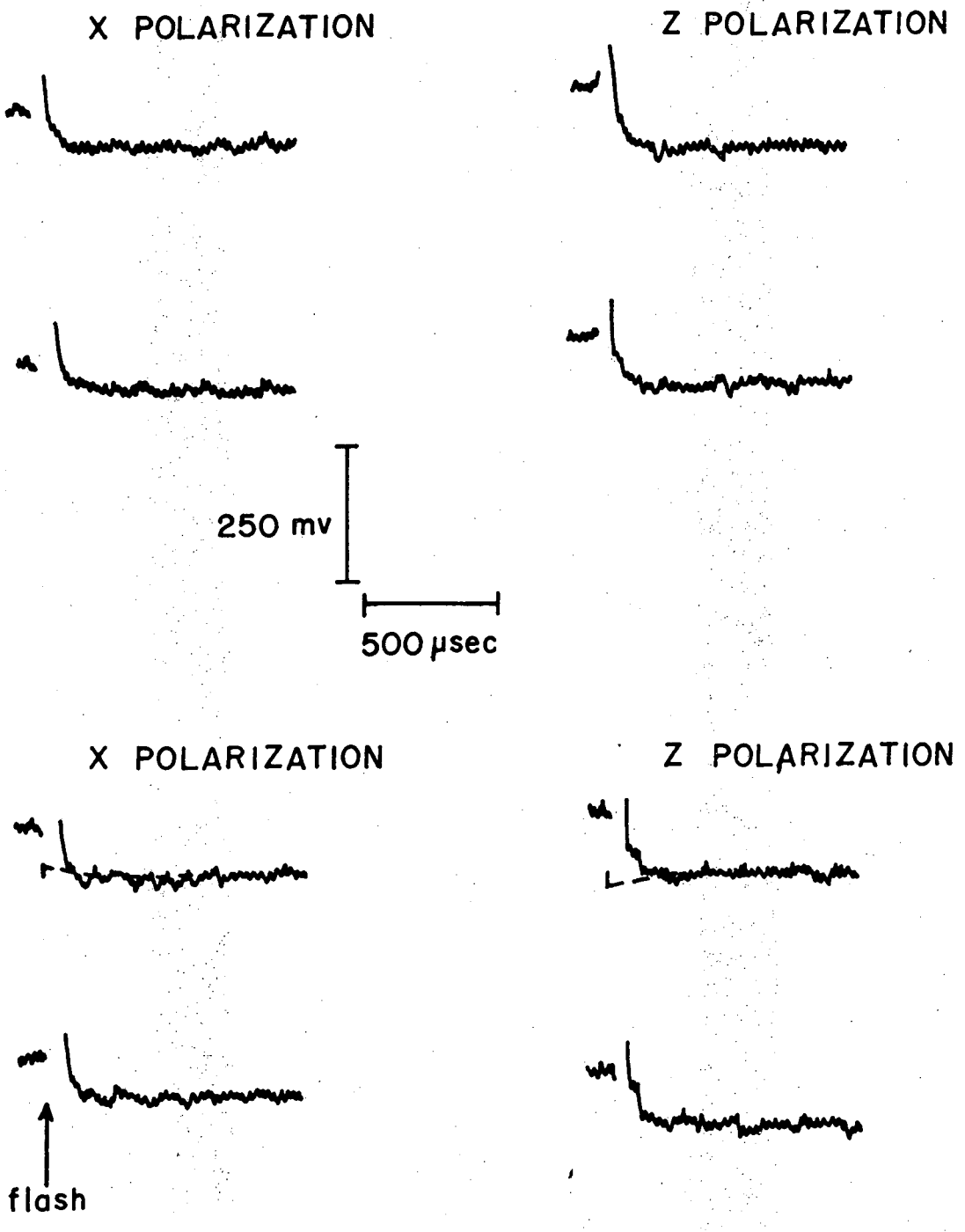

X POLARIZATION
Z POLARIZATION
250 mv
500 μsec
X POLARIZATION
Z POLARIZATION
flash
XBL753-5130

Fig 14

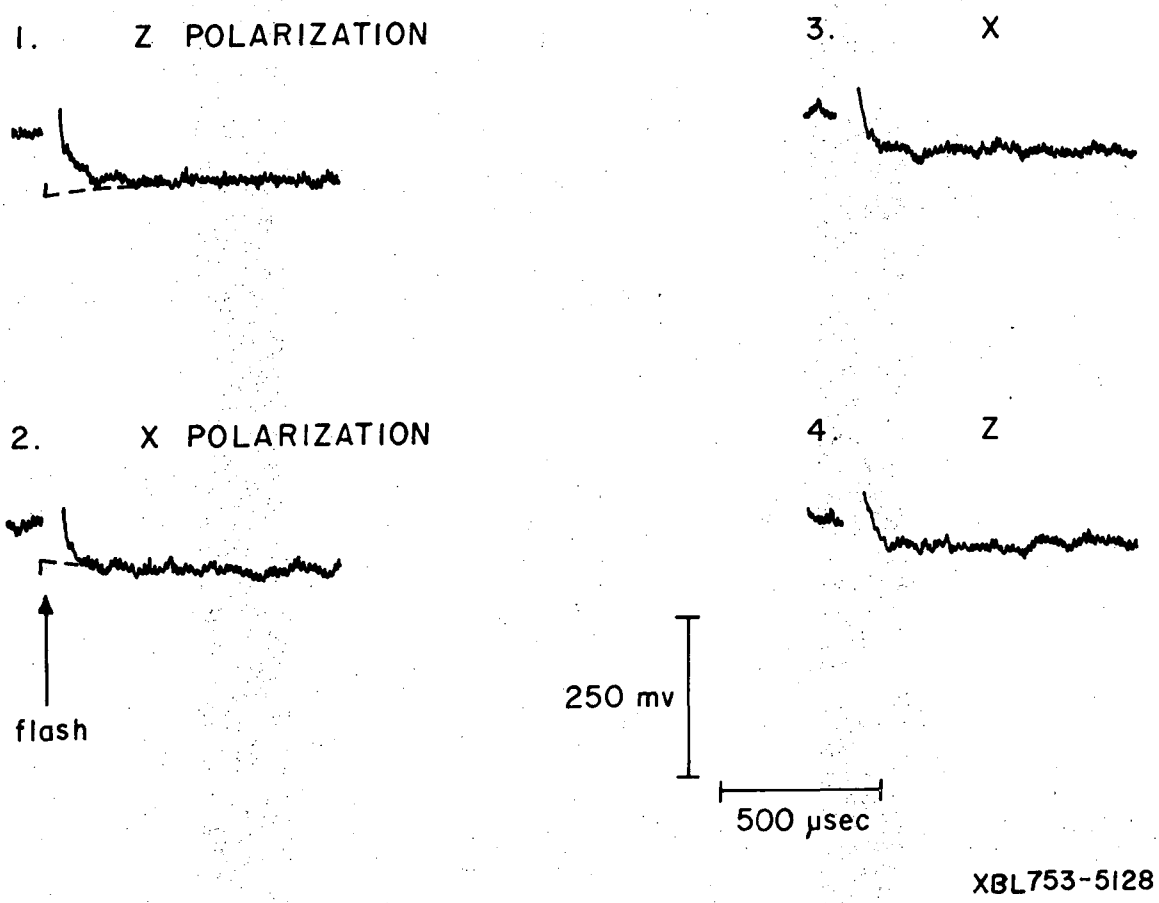
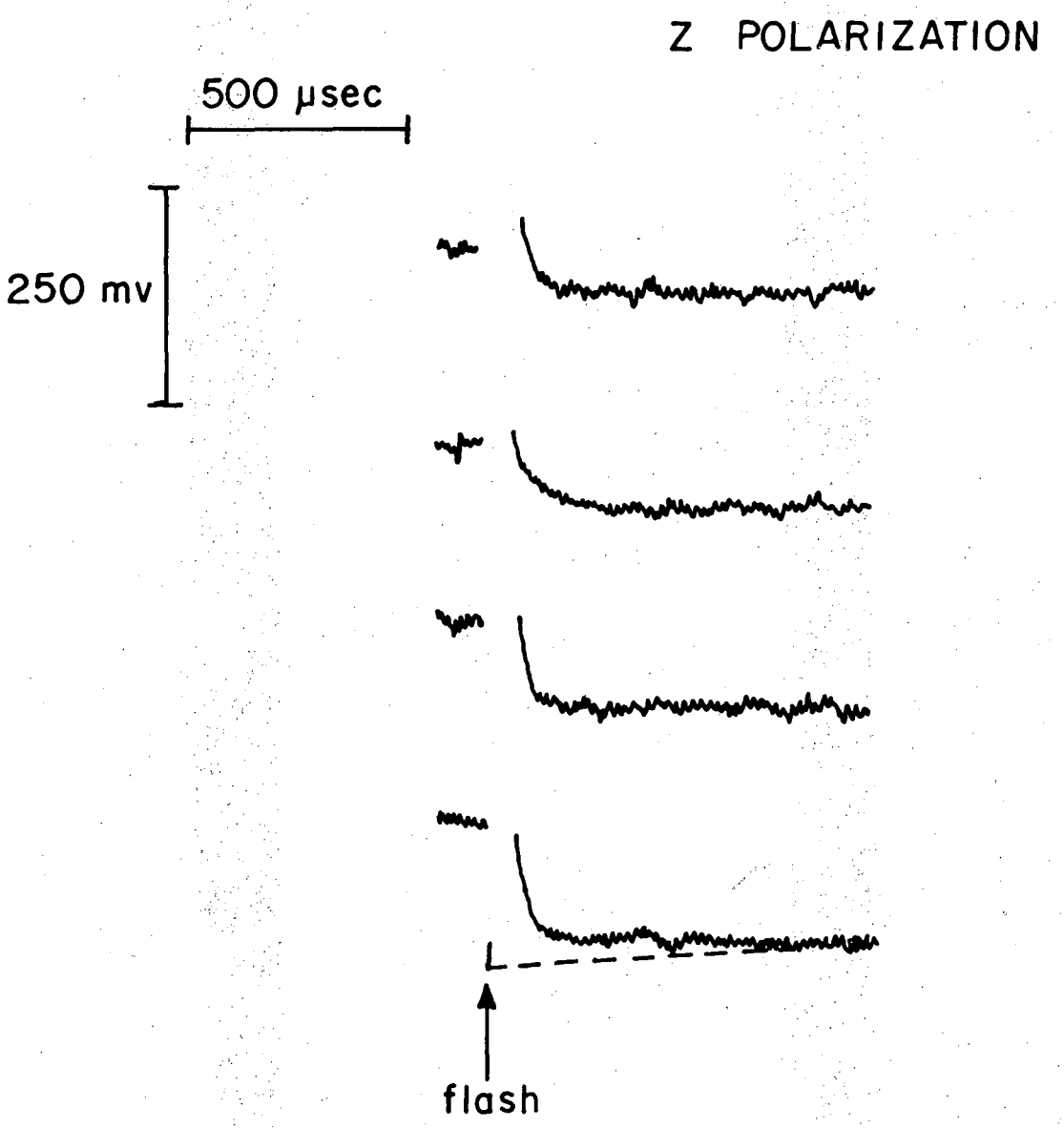


Fig 15. Measurement of absorbance change at 680 nm in desiccated glycerol extract of barley at about 5°C. The traces represent the first through fourth flashes on a single sample, with the measuring beam polarized as indicated. The dashed lines illustrate the behavior for a dichroic sample of molecular weight 300,000 (see text).



XBL753-5127

Fig 16. Measurement of absorbance change at 680 nm in desiccated glycerol extract of bean at about 5°C. The traces represent the first (bottom) through fourth (top) flashes on a single sample, with the measuring beam polarized parallel to the z-axis. The dashed line illustrates the behavior for a dichroic sample of molecular weight 600,000 (see text).

and Sauer, 1970). For this system, with the actinic and analyzing beams along the same axis, the dichroic ratio for ideal immobile oscillators will be 0.50 (Albrecht, 1961). The absence of measurable dichroism for bean holochrome films, therefore, implies either that excitation is depolarized (presumably by intermolecular transfer among PChlide molecules) before photoconversion occurs, hence producing a randomly oriented array of Chlide molecules, or that the pigments in the film were not immobile.

There is clear evidence that the holochrome in desiccated gel films does not exhibit some of the characteristics often associated with structural mobility of the protein, but it is problematical that this implies absolute immobility. The spectral shift toward the blue which is normally seen in holochrome preparations, and the shifts in intact leaves from 678 nm to 684 nm, then to 673 nm, are usually attributed to conformational changes (even disaggregation of subunits) (see, for example, Thorne, 1971; Butler and Briggs, 1966; Mathis and Sauer, 1973) or, at least, to subtle conformational relaxations (Thorne, 1971). No such spectral shifts were seen in the gel films; however, high concentrations of sucrose in holochrome preparations similarly stabilize the first spectral species of Chlide without immobilizing the molecule (see this work, and Mathis and Sauer, 1972). Inactivation of the holochrome is also likely due to denaturation or conformational disruption. Again, the film preparations showed high stability at room temperature, more dramatic even than the stabilizing effect of sucrose in solution.

More suggestive evidence that the holochrome protein was

immobilized in the gelatin film can be found in the study of Wright, et al. (1972). Using preparative procedures essentially identical to those described in the present work, Wright and coworkers were able to observe anisotropic orientations of rhodopsin and other proteins in dried gelatin films. The presence of sucrose in the holochrome preparation may, however, have reduced the effectiveness of the drying procedure in removing water from the sample. Whereas (by my calculations) Wright, et al., were able to reduce the volume of their samples about 20-fold during desiccation, I was able to effect only about a 10-fold reduction in volume with holochrome in 0.1 M sucrose.

Qualifications notwithstanding, the weight of suggestive evidence seems to indicate that the holochrome protein was at least partially immobilized by suspension in gelatin film, and, therefore, that the absence of linear dichroism is a result of effective energy transfer among PChlide molecules before photoconversion.

The transient dichroism experiments used unpolarized actinic flashes which excited both B_x and B_y transitions in PChlide molecules, while only the Q_y transition of the Chlide molecules was examined for dichroism. The dichroic ratio expected in the case of ideal, immobile oscillators, therefore, is less than 0.50, but can be estimated from the equations of Albrecht (1961). If we assume that the oscillator strength of B_y is three times that of B_x (see values in Houssier and Sauer, 1969), the dichroic ratio approaches 0.20 in the ideal case under these experimental conditions. (Even if the oscillator strengths are equal, the dichroic ratio will approach 0.077.)

It was important to insure that the transient phenomenon being sought occur during the time frame of experimental observation. The lamp artifact obscured things of interest during the initial 150 μsec of the experiment, whereas the AC-coupled oscilloscope sweep began to decay back to the baseline after about 10msec. To calculate the rotational correlation time for the holochrome protein, the equation of Einstein was used:

$$\tau = \frac{4\pi\eta r^3}{kT} \qquad (6)$$

where η is the viscosity of the medium, r the radius of the (spherical) molecule, k Boltzman's constant, and T the absolute temperature. Table 3 gives representative values for τ at different viscosities for three values of r (corresponding approximately to molecular weights of 100,000; 300,000; and 600,000 daltons). The true correlation times will reflect any non-rigidity of the holochrome molecule as well as a non-spherical shape, and hence may vary widely from the calculated values. However, since, with one exception, experiments described herein were done on samples with $\eta < 10$ poise, the conclusion must be drawn from Table 3 that linear dichroism would not be detected in a molecule of less than 300,000 daltons. The molecular weight of the bean holochrome in glycerol can be estimated at 600,000 based on its weight in aqueous solution, but the barley holochrome is not so well characterized. Therefore, negative results with barley holochrome must be interpreted with caution.

In addition, as with the bean holochrome gels, conclusions

Table 3. ROTATIONAL CORRELATION TIMES FOR SPHERICAL HOLOCHROME PROTEINS OF DIFFERENT SIZES AT SEVERAL SOLVENT VISCOSITIES.

The values of the rotational correlation time, τ, are calculated from Eqn 6 with T = 273°K. The viscosity, η, is expressed in poise; the radii, r, correspond to approximate molecular weights of 600,000; 300,000; and 100,000.

	VALUES FOR r		
η	68 Å	54 Å	38 Å
0.5	52 μsec	26 μsec	9 μsec
1.0	105	52	18
2.0	210	105	35
5.0	525	262	88
10.0	1050	525	175

from the transient dichroism experiments must be tempered by the absence of a dichroic control which might have demonstrated the meaningfulness of negative results. The primary difficulty in attempting a definitive explanation of the results in this section is the possibility, suggested above, that the correlation times might be much lower than those estimated. This situation might arise, even with large proteins, if the pigment or a small protein subunit containing the pigment is non-rigidly attached to the bulk of the protein. With this disclaimer, however, some conclusions are indicated.

The broken line in Fig 16 represents the transient one would expect to observe for a rigid holochrome of 600,000 molecular weight under the conditions of this experiment in the absence of any depolarizing mechanism except rotational diffusion. (The viscosity is taken as 6.5 poise.) The initial dichroic ratio of 0.20 decays with a calculated rotational correlation time of 650 μsec. The consistent failure of the desiccated glycerol extract of bean to show any transient phenomenon requires the presence of other depolarizing mechanisms. The most likely explanation appears to be that the excitation is effectively transferred among PChlide molecules before conversion to Chlide occurs, thus yielding a non-oriented population of Chlide molecules. Efficient transfer among as few as two or three PChlide molecules could eliminate any measurable transient orientation anisotropy of the generated Chlide population. (The stability of the bean holochrome during isolation and in solution, and the high initial fluorescence polarization value in Fig 9 certainly fail to support

the possibility that the absence of measurable dichroism results from a non-rigidity of the holochrome molecule.)

The experiments illustrated by Figs 14 (glycerol extract of barley) and 15 (desiccated glycerol extract of barley) do not, unfortunately, lend themselves so readily to analysis. There are problems on several fronts: (1) the molecular weight of the holochrome protein is unknown, hence the estimation of rotational correlation time is difficult; (2) the instability of the barley suggests the possibility of molecular non-rigidity; (3) the activity of the barley preparations was lower than with bean and the lower signal to noise made the detection of any real transient more difficult. Nevertheless, dashed lines are included in the figures to illustrate ideal transient behavior under the given conditions (η = 6.5 poise, r[assumed] = 54 Å, in Fig 15; η = 1.8 poise, r[assumed] = 68 Å, in Fig 14). No transients were consistently observed, though individual experiments (e.g., trace 2, Fig 15) gave ambiguous, or even apparently positive results.

IV. IN VIVO SPECTROSCOPY

The measurement of absorption and fluorescence spectra of intact tissue is perhaps the most direct way of analyzing the changes that accompany and follow illumination of etiolated tissue. Such measurements are fraught with difficulties, however, and the reports of various investigators have yielded a complex, nearly incomprehensible, picture of the PChlide to Chlide conversion.

As outlined in the Introduction, several shifts are readily observed in the absorption maximum of intact etiolated material following brief, saturating illumination (Shibata, 1957; Gassman, et al., 1968; Bonner, 1969). This sequence of spectral forms can be represented as follows:

$$\text{PChlide}_{650} \xrightarrow{h\nu} \text{Chlide}_{678} \xrightarrow[>-10°C]{\text{dark}} \text{Chlide}_{683} \xrightarrow[>0°C]{\text{dark}} \text{Chl(ide)}_{673}$$

[All investigators observe these species, although there remains some debate about the precise wavelengths of the spectral maxima and considerable variety in the nomenclature adopted to identify the several species. To simplify the discussion, the above notation will be used, even when discussing the observations of other investigators, unless there is some uncertainty about the identity of a species. Note will be taken when there is disagreement about the position of the absorption (or fluorescence

excitation) maxima.] In addition, several recent observations have suggested a more complicated picture:

(1) Kahn, et al. (1970), using absorption and fluorescence measurements made at 77°K, concluded that there are two forms of PChlide with absorption maxima at 637 nm and 650 nm. They further adduced evidence that energy transfer does not occur between these two distinct species at room temperature.

(2) Thorne (1971), making similar measurements in intact tissue, observed a stable species with a fluorescence excitation maximum at 668 nm following fractional conversion of PChlide. This new intermediate species was stable at room temperature in the dark, but presumably underwent further photoconversion to $Chlide_{678}$. Thorne also concluded that, in intact tissue, pigment groups within which effective energy transfer can occur at 77°K contain about 20 molecules.

(3) Litvin and Belyaeva (1968, 1971a, 1971b), in common with Thorne, suggested a scheme featuring two consecutive photochemical reactions. The Russian investigators also reported fluorescence excitation maxima in intact leaves at 77°K that differed substantially from the absorption maxima usually observed -- $Chlide_{678}$ showed a maximum at 680 nm, $Chlide_{683}$ at 685 nm, and $Chl(ide)_{673}$ at 670 nm.

(4) Mathis and Sauer (1973) concluded from absorption spectra of intact bean leaves taken between 0°C and room temperature that conversion occurs via two photochemical steps representing the sequential reduction of the two molecules in a PChlide dimer.

Clearly, the most striking aspect of these recent reports

is the suggestion that two consecutive photochemical reactions accompany the conversion of PChlide to Chlide. This argument is not in ready agreement with the notion that the reduction is a simple first-order reaction complicated by energy transfer among associated pigments (Nielsen and Kahn, 1973; Thorne and Boardman, 1972; this work). This section will attempt to reconcile these models -- at some obvious cost to the former.

A. Materials and Methods

Kidney beans, disinfected with Arasan (DuPont), were planted in vermiculite that had been briefly soaked in water. The seedlings were then grown in darkness at about 22°C; harvesting was done at room temperature under a green safelight. Absorption spectra were recorded and sample temperatures were measured as described previously (Mathis and Sauer, 1973). Photoconversion, when desired, was effected with the measuring beam of the spectrophotometer (650 nm, 3.0 mm slit).

B. Results

With an eye to systematizing the apparently disparate observations of other investigators and presenting a coherent picture of the spectral shifts associated with conversion of PChlide to Chlide, intact leaves were exposed to various doses of illumination at temperatures between -30°C and +6°C. The ages of the leaves varied, but age differences had much less effect on the observed spectral regimes than did the temperature or extent of photoconversion. Consequently, Table 3, which outlines the significant

Table 4. SHIFTS IN ABSORPTION MAXIMA OBSERVED IN ETIOLATED BEAN LEAVES FOLLOWING ILLUMINATION AT VARIOUS TEMPERATURES.

Leaves were converted to the extent indicated by exposure to the measuring beam of the spectrophotometer. See text for experimental details. Figures above the arrows denote the length of time (minutes) required for the indicated shift to occur.

TEMPERATURE (°C)	% CONVERSION	OBSERVATIONS (absorption maxima in nm)
-30	100	650 $\longrightarrow$ 680 (stable)
-21	25	650 $\longrightarrow$ 680 (stable)
	100	650 $\longrightarrow$ 680 (stable)
-14	10	650 $\longrightarrow$ 677-678 (stable)
-11	10	650 $\longrightarrow$ 678 $\xrightarrow{15'}$ 675 (stable)
	25	650 $\longrightarrow$ 678 $\xrightarrow{15'}$ 675 (stable)
	100	650 $\longrightarrow$ 678 $\xrightarrow{50'}$ 675
-7	20	650 $\longrightarrow$ 677 $\xrightarrow{10'}$ 675.5 $\xrightarrow{30'}$ 674.5
	100	650 $\longrightarrow$ 678 $\xrightarrow{50'}$ 676
-2.7	15	650 $\longrightarrow$ 681 $\xrightarrow{10'}$ 682.5 $\xrightarrow{40'}$ 682
	100	650 $\longrightarrow$ 682 $\xrightarrow{30'}$ 683
1.7	6	650 $\longrightarrow$ 677.5 $\xrightarrow{20'}$ 675.5 $\xrightarrow{30'}$ 674.5
	20	650 $\longrightarrow$ 682.5 (stable)
5.8	5	650 $\longrightarrow$ 674.5 $\xrightarrow{10'}$ 672.5 (stable)

results of these spectral shift experiments, reflects the effects of temperature and extent of conversion as observed in leaves 11±3 days old. Indeed, much older leaves showed the same behavior. The most noticeable feature of Table 4 is that a Chlide form absorbing at 675-676 nm -- observed by Litvin and Belyaeva (1971a, 1971b) at 77°C following photoconversion at room temperature and by Mathis and Sauer (1973) at physiological temperatures, and thought to represent an intermediate species phototransformable to $Chlide_{678}$ -- appears to be produced via a dark process following the appearance of $Chlide_{678}$.

To address the issue of an in vivo, photoactive $PChlide_{637}$ form (Kahn, et al., 1970), Fig 17 represents the difference (illuminated minus unilluminated) between spectra obtained before and after complete conversion of an intact leaf at room temperature.

C. Discussion

Wielding Occam's razor is a formidable responsibility. All the same, it is the province of scientists to do so, and this seems an appropriate time for someone to heft the ungainly instrument. The apparent complexity of recent spectral observations has been accompanied by several complex, often contradictory, attempts at explanation. However, it seems possible to postulate a single regime of spectral shifts (and, perhaps, even a simple, qualitative, mechanistic explanation) that will encompass most of the observations reported here and in the literature. Not surprisingly, in view of the authorship, this model agrees with a simple picture of a first-order photochemical mechanism. It

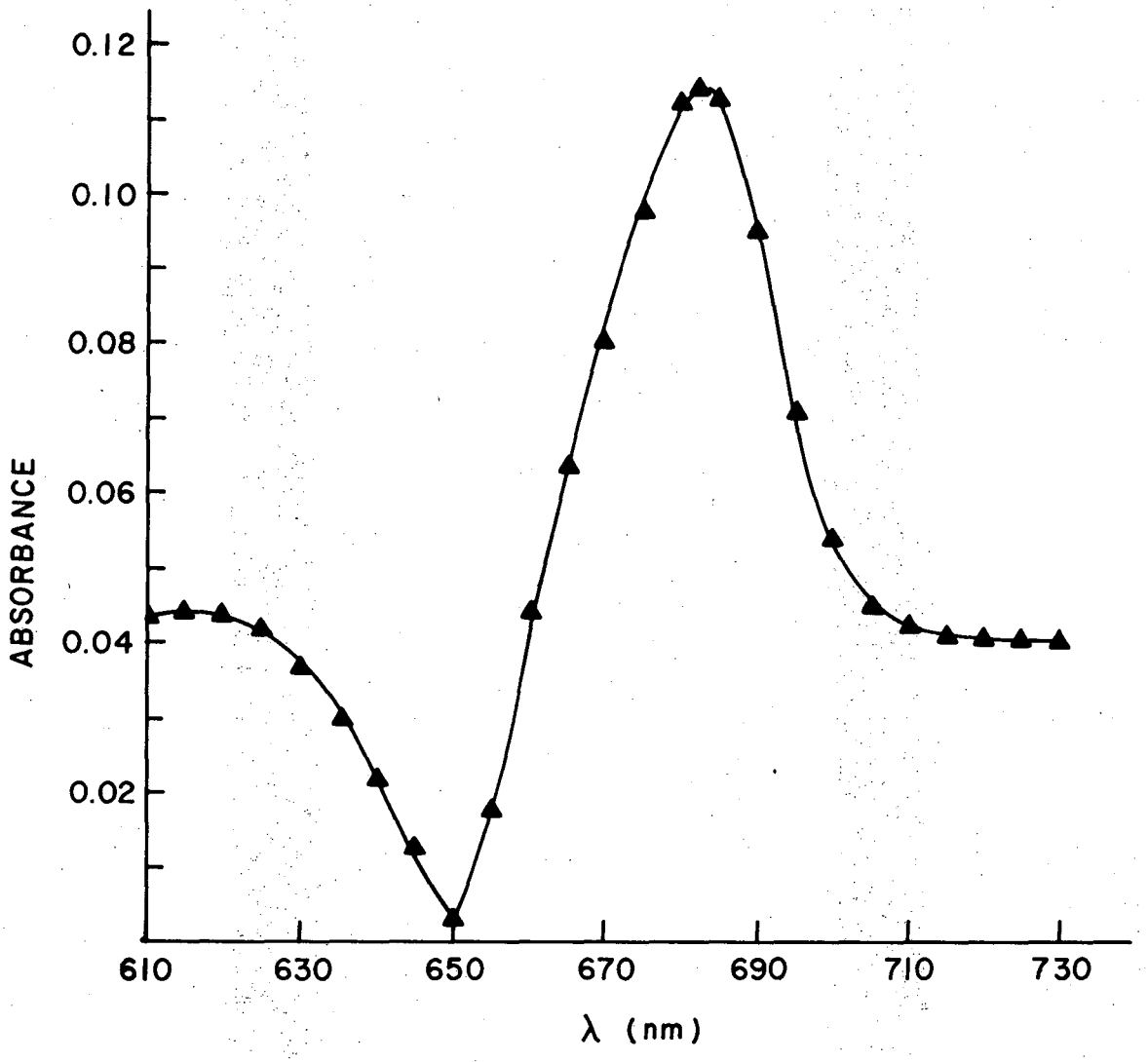


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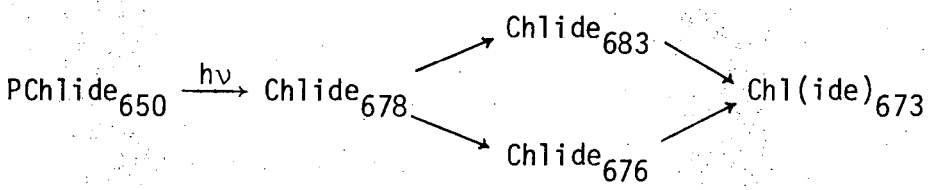
Fig 17. The difference spectrum (illuminated minus unilluminated) of an intact bean leaf at approximately -3°C. The photoconversion was about 90% complete; in the region of PChlide absorption, the shape of the difference spectrum did not depend upon the extent of photoconversion. The ordinate indicates absorbance relative to an arbitrary baseline.

also explains the observation of Litvin and Belyaeva (1971) and Mathis and Sauer (1973) while taking exception with their models; it fails to account explicitly for the intermediate species observed by Thorne (1971) -- though it attempts a justification -- and cannot justify the pattern of CD spectra observed in holochrome by Mathis and Sauer (1972). Despite the complexity of the regime of spectral shifts and the subtlety of the factors affecting the kinetics of the dark reactions, this mechanism abjures the requirements for multiple photochemical reactions (Mathis and Sauer, 1972, 1973; Litvin and Belyaeva, 1971a, 1971b; Thorne, 1971) or dimeric pigment associations (Mathis and Sauer, 1972, 1973). These complexities would not merely complicate the model; they are argued against persuasively by evidence presented earlier in this work and by the results of Thorne and Boardman (1972) and Nielsen and Kahn (1973). Nor is the need for complex photochemistry suggested by the spectral evidence.

The results illustrated in Table 4 are consistent with the following mechanism (the factors that affect the kinetics of the several dark reactions are discussed below):



When complete conversion is effected (requiring about 3 minutes illumination with the measuring beam of the spectrophotometer) below about -15°C, $Chlide_{678}$ is the only Chlide species observed, remaining stable in the dark for at least one hour. [The Chlide

species absorbing at 680 nm (Table 4) at temperatures below about -20°C is taken as not significantly different from $Chlide_{678}$. This argument cannot be rigorously defended since no attempt was made to prove the equivalence of "$Chlide_{680}$" and $Chlide_{678}$. Litvin and Belyaeva (1971a, 1971b), however, observed a 680 nm form in the absence of a 678 nm form at 77°K. In view of the structural changes that are likely to be induced at such low temperatures, it seems unwarranted to invoke a new Chlide species.] At temperatures between -15°C and about -6°C, the Chlide maximum shifts slowly from 678 nm to a stable position at 675-676 nm. At higher temperatures, $Chlide_{678}$ evolves predominately to $Chlide_{683}$ (and hence to $Chlide_{673}$ at temperatures above about 0°C). The shift from 678 nm to 683 nm was typically not observed above -6°C since several minutes elapsed between photoconversion and the recording of the spectra. Similarly, no pains were taken to document the shift from 683 nm to 673 nm above 0°C. These shifts are well described elsewhere (Shibata, 1957; Gassman, et al. 1968; Bonner, 1969; and later investigators). When only 15-25% conversion is effected, a similar picture emerges. However, the shift from 678 nm to 676 nm is generally more rapid. The shift from 678 nm to 683 nm continues to occur at temperatures above about -6°C. At or below 10% conversion, similar behavior is observed below about 0°C, with the dark shifts occurring more rapidly than at complete conversion; however, above 0°C, $Chlide_{683}$ never appears, while the shift from 678 nm to 676 nm (and ultimately to 673 nm) continues to be observed.

Although their fluorescence excitation maxima differ somewhat

from the absorption maxima here and in most of the literature, Litvin and Belyaeva (1971a, 1971b) appear to have observed the same species depicted in the scheme above. At low conversion (about 10%) they observe $Chlide_{676}$ -- their Chlide 684/676; whereas, at greater conversion (>25%), they see only $Chlide_{678}$ -- their Chlide 690/680. In their view, $Chlide_{676}$, owing to its early appearance at fractional conversion and its relative stability, falls between $PChlide_{650}$ and $Chlide_{678}$; and they further postulate that $Chlide_{676}$ is converted to $Chlide_{678}$ by a second photochemical reaction. The scheme illustrated above, however, will explain the observations of Litvin and Belyaeva (1971a, 1971b) if, at low fractional conversion, the dark shift from 678 nm to 676 nm is presumed to have occurred at or near room temperature before they recorded the low temperature spectra. On the contrary, the Litvin and Belyaeva scheme cannot explain the observed dark shift from 678 nm to 676 nm (Table 4). Litvin and Belyaeva also distinguished, on the basis of fluorescence emission maxima, between the terminal species obtained from $Chlide_{676}$ and that obtained from $Chlide_{683}$ (their Chlide 695/685). The experiments described herein cannot. (It is worth emphasizing again the disparity between the fluorescence excitation wavelengths observed by Litvin and Belyaeva at 77°K and the absorption maxima observed at or near room temperature. At liquid nitrogen temperature, the species that I identify as $Chlide_{678}$ appears to absorb at 680 nm, $Chl(ide)_{673}$ at 670 nm, and $Chlide_{683}$ at 685 nm.)

Mathis and Sauer (1973) also postulate a two-photochemical-reaction picture in which $Chlide_{676}$ precedes the appearance of

$Chlide_{678}$. Their evidence is the appearance of $Chlide_{676}$ as the first observed species at fractional conversion near 0°C. (For similar observations, see results for 5.8°C in Table 4.) However, as with Litvin and Belyaeva (1971a, 1971b), they do not directly observe the suggested photochemical reaction $Chlide_{676} \xrightarrow{h\nu} Chlide_{678}$. The present scheme argues instead that $Chlide_{676}$ is the result of a dark shift readily observed only below 0°C. This view can encompass the observations of Mathis and Sauer; their hypothesis cannot explain the shift $Chlide_{678} \xrightarrow{dark} Chlide_{676}$ observed below 0°C.

It is also important to observe that the results illustrated in Figs 7 and 8, demonstrating the constancy of Chlide fluorescence yield during the course of photoconversion in homogenate and etioplast preparations, argues against a distinct intermediate Chlide species in the photoconversion reaction. These results would require that such an intermediate exhibit the same fluorescence yield as the terminal species ($Chlide_{678}$). Alternatively, to preserve the two reaction hypothesis in intact tissue, it would be necessary to argue that the complexity of the mechanism disappears in holochrome and etioplast preparations. The principle of Occam's razor seems not well served by either unlikely explanation.

The intermediate form observed by Thorne (1971) is, however, a more difficult matter. Following conversion of about 5% of the PChlide in an intact leaf at room temperature, Thorne observed, at 77°K, a species with a fluorescence excitation maximum at 668 nm. This observation is unchanged if the converted leaf is left in

darkness at room temperature for several hours before the low temperature spectrum is measured. This contrasts with the observations of Litvin and Belyaeva (1971a, 1971b), Mathis and Sauer (1973), and this work, where such experiments produce a terminal species [Chl(ide)$_{673}$ in this work, Chl 675/670 in Litvin and Belyaeva]. I am therefore tempted to suggest that Thorne's intermediate is in fact identical with the terminal species Chl(ide)$_{673}$ -- a result of a delay between illumination at room temperature and effective cooling of the sample. This time delay could be sufficient to allow the spectral shift from 678 nm to 676 nm to 673 nm (see, for supportive evidence, Mathis and Sauer, 1973). The disparity between 668 nm and the longer wavelengths observed by other investigators can be accounted for only by the low temperatures employed by Thorne. [It is interesting -- if not illuminating -- to notice that, although they both measured spectra at 77°K, Litvin and Belyaeva (1971a, 1971b) and Thorne (1971) cannot agree on fluorescence excitation maxima. Thorne's maxima are generally shifted about 2 nm to the blue, except for his Chl E672 -- red-shifted from Litvin and Belyaeva's Chl 683/670.]

In summary, it appears that models involving two consecutive photoreactions (Litvin and Belyaeva, 1971a,1971b; Thorne, 1971; Mathis and Sauer, 1973) arose from the misidentification of Chlide$_{676}$ or Chl(ide)$_{673}$ as intermediate species between PChlide$_{650}$ and Chlide$_{678}$, rather than as species produced following the appearance of Chlide$_{678}$. This misidentification seems to have arisen largely from the speed of the dark shifts from 678 nm to shorter wavelengths in fractionally converted samples near room

temperature. Whether the $Chl(ide)_{673}$ species produced from $Chlide_{676}$ is identical to that produced from $Chlide_{683}$ is unclear. The work of Litvin and Belyaeva (1971a, 1971b) and Thorne (1971) suggest not, at least when subjected to low temperatures; this work leaves the question open.

It remains to comment on the observations of Kahn, et al. (1970). In intact leaves, difference spectra such as that reproduced in Fig 17 demonstrate that only a single form of PChlide, with an absorption maximum at 650 nm, is photoconverted to Chlide. Therefore it seems likely that the photoactive PChlide form absorbing at 637 nm seen by Kahn and coworkers was an artifact of the low temperatures employed during measurements. Disruption of the native tissue (such as in the preparation of a soluble holochrome preparation) frequently results in such a blue shift. Butler and Briggs (1966) made similar observations during freezing and thawing. The conclusion by Kahn, et al. (1970) that energy transfer does not occur at room temperature between $PChlide_{637}$ and $PChlide_{650}$ does not, fortunately, rule out the possibility of energy transfer among $PChlide_{650}$ molecules on a single native protein (or, for that matter, among $PChlide_{637}$ molecules on a single denatured protein).

In summary some generalizations may be attempted concerning the several spectral shifts observed in intact tissue. Most importantly, only a single, elemental shift ($PChlide_{650} \longrightarrow Chlide_{678}$) can be clearly attributed to photochemistry. Other observed species appear to be the products of dark reactions.

The kinetics of the shift from 678 nm to 675-676 nm shows,

at physiological temperatures, much less temperature sensitivity than subsequent dark shifts. Between about -15°C and +2°C, in samples where less than 10% of the PChlide was converted, this shift goes to completion in 15-20 minutes; above 2°C, it occurs with increasing rapidity. In completely converted samples, this shift occurs more slowly: at -11°C, it is complete only after about 50 minutes.

Thorne (1971) illustrated the temperature dependence of the dark shift from 678 nm to 683 nm (his shift III) by monitoring the associated change in fluorescence yield. His study agrees essentially with the observations presented herein. Below about -7°C, this shift does not occur; instead a blue shift is observed. Near 0°C, this shift competes effectively with the shift to 676 nm, resulting (at least when more than 15% of the pigment is photoconverted) in absorption maxima at intermediate wavelengths -- 681-683 nm. (See also the results of Mathis and Sauer, 1973.) Above 0°C, the shift to 683 nm occurs effectively within 3-4 minutes in completely converted tissue. At fractional conversion, however, the shift does not occur; the shift to 676 nm (and ultimately to 673 nm) continues to be observed.

Perhaps it would be permissible, on the basis of these observations, to toy with some qualitative pictures of the nature of the shifts to 683 nm and to 676 nm. One might visualize portions of the etioplast membranes as fairly densely populated with PChlide pigment-protein complexes. It is possible, therefore, to imagine that the extent of photoconversion might (owing, say, to steric interactions among the photoactive sites) have an

effect on the thermodynamics and kinetics of the available relaxation processes. This possibility will be exploited in the ensuing discussion.

The assumption that the shifts to 676 nm and 683 nm following photoconversion may be characterized as "relaxation" processes implies that illumination produces $Chlide_{678}$ existing in a somewhat strained relationship with a holochrome protein designed to accomodate $PChlide_{650}$. At temperatures above about 0°C, and if photoconversion is extensive, a thermally induced relaxation occurs in which the Chlide absorption maximum shifts to 683 nm. At lower temperatures, the "relaxed" 683 nm state of the Chlide pigment becomes kinetically unavailable, perhaps because of membrane or protein rigidity induced by ice formation. With each holochrome constrained to remain in a largely native state, some local conformational change at the enzymatic site of photoconversion would be left to produce the shift from 678 nm to 676 nm. (While this shift might reflect a relaxation of tertiary strain in the protein, it produces a blue shift in the pigment absorption maximum.) Presumably, this local conformational change is available above 0°C but is not kinetically competitive with the red shift to 683 nm.

At fractional conversion (about 10%), a somewhat different picture emerges: the relaxation to 683 nm is not observed. It is sufficient to explain this observation merely to propose that the shift to 676 nm becomes kinetically more viable as the extent of photoconversion is lessened. (Somewhat broadly, we may invoke steric considerations. Macroscopic allusions are attractive, but

perhaps not illuminating: one or two of a great many upstanding dominoes on a small table top might be easily tipped onto their faces, whereas to arrange all of the dominoes facedown on such a delimited surface might require more time and effort.) This increasing kinetic favorability is reflected not only in the disappearance of the 683 nm shift in fractionally converted samples above 0°C (now losing rather than winning the kinetic race), but also in the greater speed of the 676 nm shift in fractionally converted tissue compared to completely converted tissue at temperatures below 0°C (see Table 4).

Finally, one attractive, but unnecessary, additional explanation for the disappearance of the 683 nm relaxation in fractionally converted tissue above 0°C argues that the 683 nm state becomes *thermodynamically* unfavorable in such cases. With fewer than 10% of the pigment molecules converted to Chlide, one can imagine this relaxation being inhibited by the large population of remaining PChlide molecules -- quite content with the membrane or protein as it is. Each holochrome would then be thermodynamically constrained to remain in a largely native state. Only the local conformational changes leading to the 676 nm shift could occur. As mentioned above, however, this explanation is possible but not necessary: the kinetic arguments necessary to explain the relative velocities of the 676 nm shift in fractionally and completely converted samples below 0°C take care of the phenomena above 0°C as well.

V. CONCLUSION

The results of the experiments described in the preceding pages are consistent with a simple explanation of the photochemical conversion of PChlide to Chlide. In this concluding summary, I shall review this model. It is also important, however, to recall the experimental results of others that do not fully support the model proposed herein. To the extent possible, these conflicting results will be reconciled. I shall place special emphasis on the differences that remain in the hope that such consideration might improve the aim of future researches.

The present study has centered on holochrome preparations from bean. Each pigment-protein complex in such preparations appears to contain at least four aggregated pigment molecules. The photoconversion of PChlide to Chlide is intrinsically first order; there is no evidence of intermediates. The photochemistry is, however, complicated by the competing possibility of efficient energy transfer from PChlide to Chlide. Energy transfer also occurs efficiently among PChlide molecules and among Chlide molecules. There is no evidence to suggest that these characteristics should not be extrapolated to intact bean leaves, though, of course, aggregate sizes and the relative efficiencies of fluorescence, intermolecular energy transfer, and photochemistry might be expected to be different from that in holochrome. In holochrome preparations of barley (in glycerol), some differences were noted: energy transfer from PChlide to Chlide did not

compete effectively with the photochemistry, and the minimum pigment group size was three.

The following contrary, and seemingly contrary, results of other investigators can be divided into three categories: (1) studies of the stoichiometry of pigment-protein complexes and studies of pigment group sizes in vivo, (2) evidence of photochemical intermediates, and (3) evidence of non-first-order kinetics.

Schopfer and Siegelman (1968) identified two species in a purified PChlide holochrome preparation -- one with molecular weight 300,000 and a second with molecular weight 550,000. Based on the ratio of pigment to protein, they concluded that the larger species contained two pigment molecules. [This species is apparently that obtained most frequently using procedures comparable to those described in this work (see Smith, 1960; Boardman, 1962a).] This result is not consistent with the results depicted in Fig 9 which demand four aggregated pigments to explain the observed fluorescence depolarization. There seem to be two plausible explanations: (1) the preparations used in the experiments of the present study differed substantially from that of Schopfer and Siegelman -- this would not be surprising in view of the considerable pains the latter investigators took to purify their sample; (2) the preparation of Schopfer and Siegelman (despite their pains) contained a contaminating protein that artificially lowered the pigment:protein ratio -- suggestive support comes from Kahn, et al. (1970), who reported four pigments per holochrome protein, and Canaani (1975), who reported at least

two pigment molecules on each 300,000 dalton subunit (hence, presumably, four on each larger unit).

Based on the extent of excitation transfer at 77°K, Thorne (1971) concluded that at least 20 pigment molecules were associated in vivo. The analysis that led to this conclusion is rather unclear; nonetheless, it does not contradict the findings reported above for holochrome preparations.

Evidence for the existence of a photochemical intermediate in intact tissue has been presented by Litvin and Belyaeva (1968, 1971a, 1971b), Thorne (1971), and Mathis and Sauer (1973) As discussed earlier, each of these studies reported a new spectroscopic species following fractional photoconversion at physiological temperatures. No direct evidence was presented that the new species underwent further photochemistry. The experiments depicted in Table 4 provide suggestive evidence that the newly observed species are not photochemical intermediates, but rather are spectral forms produced by rapid dark processes following photoconversion. Fortunately, therefore, the results reflected in Table 4 and those of Litvin and Belyaeva (1968, 1971a, 1971b), Thorne (1971), and Mathis and Sauer (1973) are not irreconcilable; the proposals of a photochemical intermediate seem rather to have arisen from misidentification of the new species, an error due to the rapidity of the dark reactions above 0°C.

Mathis and Sauer (1972) concluded from CD spectra of bean holochrome preparations that PChlide pigments (prior to photoconversion) and Chlide pigments (following photoconversion) are associated as dimers. Further, by following the evolution of

the Chlide CD spectrum during photoconversion, they produced evidence that the transformation occurs in two distinct steps, thus:

$$\text{PChlide-PChlide} \xrightarrow{h\nu} \text{PChlide-Chlide} \xrightarrow{h\nu} \text{Chlide-Chlide}$$

where the heterodimer and the true Chlide dimer are distinguishable species. This notion is in serious conflict with the model proposed in the present study and cannot be readily reconciled with other observations. Contradictory evidence includes: (1) the presence of an isosbestic point between PChlide and the earliest observed final product [generally reported for all preparations since Shibata (1957)], (2) the constancy of Chlide fluorescence yield during photoconversion (Figs 7 and 8, this work), (3) the absence of variation in the shape or position of the Chlide absorption peak during photoconversion (Mathis and Sauer, 1972), and (4) the apparent adequacy of a first-order formulation to explain the kinetics of the photochemistry (Thorne and Boardman, 1972; Nielsen and Kahn, 1973; Vaughan and Sauer, 1974; this work). The notion of excitonic interaction, demanded by the observations of complex CD spectra, is inconsistent with these observations. This substantial conflict appears to be the only remaining difficulty between the model proposed in this work and the observations of other workers.

Smith and Benitez (1954) and Boardman (1962b) explained the superficially complex kinetics of the photoconversion as second-order and sum-of-two-first-orders, respectively. It has now been convincingly shown, however, that the perplexing kinetics arise

from intermolecular energy transfer competing with intrinsically first-order photochemistry (Thorne and Boardman, 1972; Nielsen and Kahn, 1973; Vaughan and Sauer, 1974; this work). Again, it is not the observations, but rather the conclusions, of the earlier workers that are in conflict with the current interpretations.

* * * * *

A number of questions remain to be answered before science can claim even a passable understanding of the phototransformation of PChlide to Chlide. To point to a few: What is the nature of the reductant? the mechanism of reduction? What is the origin of spectral differences among these pigments in solution, in native tissue, and in holochrome preparations? What are the origins of the post-illumination spectral shifts? Even more immediate, however, is the unresolved dilemma fostered by the complex CD spectra observed by Mathis and Sauer (1972). The reconciliation of these observations, suggesting excitonic interaction among pigments, with those implying otherwise lies perhaps as much in the province of theoretical model making as in that of experimental spectroscopy, and should broadly increase our understanding of pigment interactions in photosynthetic tissue.

But that is work for other hands.

APPENDIX 1

The Chlide emission represented by the curve labeled E_{650} in Fig 3 has two components. These arise from the fact that both PChlide and Chlide absorb at 650 nm. The first component is that portion of the emission produced by direct absorption by Chlide at 650 nm; this component presumably follows the same time course as A_{678} or E_{670} (see text and Fig 7). The second component is attributed to emission produced by 650 nm excitation of PChlide, the excitation energy being subsequently transferred to Chlide. (The small absorption by inactive PChlide at this wavelength, even if it results in energy transfer to Chlide or to active PChlide, does not affect this treatment.) Therefore, at any point during the photoconversion the intensity, per unit energy absorbed, of Chlide emission owing to absorption by PChlide is proportional to $(E_{650} - E_{670})/A_{650}^{PChlide}$, where $A_{650}^{PChlide}$ is the absorbance of PChlide, and, at low absorbances, is proportional to the energy absorbed by PChlide. Likewise, E_{670}/A_{650}^{Chlide} is proportional to the intensity, per unit energy absorbed, of Chlide emission owing to direct absorption by Chlide. Therefore,

$$E_t = \frac{(E_{650} - E_{670})/A_{650}^{PChlide}}{E_{670}/A_{650}^{Chlide}}$$

$$= \frac{(E_{650} - E_{670})}{E_{670}} \cdot \frac{\varepsilon_{650}^{Chlide}\,[Chlide]}{\varepsilon_{650}^{PChlide}\,[PChlide]}$$

is the ratio of the efficiency of excited PChlide in promoting Chlide emission to the efficiency of excited Chlide in producing Chlide emission. Hence, E_t is the extent of energy transfer from PChlide to Chlide, expressed as a fraction of the total excitation absorbed by PChlide. The values for E_t can range between zero (where no Chlide emission owing to PChlide absorption is observed) and unity (where PChlide absorption is as efficient as Chlide absorption in stimulating Chlide emission).

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