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ABSTRACT

The research described here has been directed toward the isolation and characterization of phytochrome from the chlorophyllous cells of light-grown higher plants and green algae. We have developed a simple procedure that separates chlorophyll from phytochrome in crude extracts from green tissue thus permitting spectral measurement of the phytochrome in such extracts for the first time. Spectral and immunochemical analysis of phytochrome from green oat tissue indicates the presence of two distinct species of the molecule: a minority species (~20%) that is recognized by antibodies directed against phytochrome from etiolated tissue and that has an apparent molecular mass of 124 kilodaltons (kD), the same as that of the native molecule from etiolated tissue; and a majority species (~80%) that is not recognized by anti-etiolated tissue phytochrome Ig and has a Pr absorbance maximum some 14 nm shorter than its etiolated tissue counterpart. Mixing experiments have established that these different molecular species preexist in the green cell and are not the result of posthomogenization modifications. The basis for these intracellular differences has not been determined but one possiblity consistent with other circumstantial evidence is that two genes are operating in green tissue: one producing residual amounts of the molecule that is present at high levels in etiolated tissue and the other producing a new phytochrome species yet to be fully characterized.

Attempts to purify the phytochrome from green tissue by the most obvious and direct method available to us, i.e. immunoaffinity chromatography, have been thwarted by the lack of immunological cross-reactivity referred to. Initial data from the use of more conventional chromatographic matrices indicate that it may be possible to separate the two green-tissue phytochrome species.

We have begun to identify monoclonal antibodies specific for antigenic sites distributed throughout the length of the etiolated-tissue phytochrome polypeptide. One use of these antibodies will be to search for as yet undetected antigenic sites common to both types of phytochrome in green tissue.

Axenic cultures of the alga Mesotaenium have been established and preliminary spectral analysis of phytochrome isolated from these cells has been carried out.

PROGRESS REPORT

I. INTRODUCTION

This research effort has been aimed at effecting the assay, purification and characterization of phytochrome from green tissue of higher plants and from green cells of algae. The strategy has been to develop suitable procedures with a higher plant (oats) while in parallel establishing conditions for growth of axenic algal (Mesotaenium) cultures and accumulating these cells for future analysis. As a result of these efforts we have developed a relatively simple protocol for the routine isolation and spectrophotometric assay of phytochrome from green tissue and have obtained an initial characterization of the photoreceptor. The data thus far gathered indicate the presence of at least two immunologically distinct types of phytochrome in green oat cells - a minor fraction (~20%) that is recognized by antibodies directed against etiolated oat phytochrome and a major fraction (~80%) that is not recognized by these antibodies. Spectral analysis indicates further that at least the major fraction has absorbance properties distinct from etiolated-tissue phytochrome.

II. SCOPE VS. ORIGINAL PROPOSAL

The scope of the research for this funding period has encompassed essentially all areas outlined in the original proposal. A new spectrophotometer has been built, installed and tested; the parameters involved in the use of polyethyleneimine (PEI) as a means of spectrally assaying phytochrome in green tissue extracts have been explored; attempts have been made to develop procedures to purify phytochrome from green tissue to homogeneity; a preliminary spectral and immunochemical characterization of the phytochrome has been made; and axenic algal cultures have been established.

III. INSTRUMENTATION

We have installed and have operational a microprocessor-based dual wavelength spectrophotometer for phytochrome assay that was designed and built by L. H. Pratt, University of Georgia, as part of a collaborative effort between our two laboratories. This instrument is superior to other such spectrophotometers designed for this purpose. The signal to noise ratio is greater, thereby providing greater sensitivity. Enhanced sensitivity is an important factor in attempts to measure phytochrome in green tissue extracts where it is present at only ~1% that in etiolated tissue.

IV. EXTRACTION PROCEDURES AND SPECTRAL ASSAY

Our observation that polyethyleneimine (PEI) precipitates chlorophyll from crude extracts leaving phytochrome in the supernatant was the key to developing a procedure for the spectrophotometric measurement of phytochrome in green tissue extracts (Fig. 1). This observation led to an initial report on the extraction, assay and preliminary characterization of phytochrome from green oat tissue (1). We subsequently discovered, however, in studies with etiolated tissue phytochrome, that the time-honored practice of maintaining phytochrome in the Pr form during extraction results in previously undetected limited proteolysis of the molecule that converts it from a 124 kilodalton (kD) monomer to a mixture of 118 and 114 kD polypeptides (2 , 15). The 6-10 kD polypeptide domain lost by this proteolysis appears to be cleaved from the N-terminus and

has been shown to be critical to the spectral and structural integrity of the molecule (11, 16-18). This proteolysis means that much of the data accumulated on the properties of purified phytochrome over the last decade was almost certainly obtained using degraded preparations. It thus became necessary to develop an isolation protocol that would preclude the proteolysis and having done so to go back and re-examine the properties of the chromoprotein in green tissue.

It has been found with etiolated tissue that the maintenance of phytochrome as Pfr and the inclusion of phenylmethylsulfonyl fluoride (PMSF) in the buffers eliminates proteolysis to the extent that a homogeneous monomer of 124 kD can be purified from oats, free of detectable degradation products (17). The 124 kD monomer is considered to be the native molecule by virtue of its comigration with the phytochrome in vitro translation product upon SDS gel electrophoresis (2).

We have now applied the same methodology to green tissue with the exception that the tissue is first freeze-dried and powdered to afford more effective and uniform cell breakage (Fig. 2) (14). This procedure provides yields of phytochrome 2-3 times higher than previously obtained (1) and permits the photoreceptor to be assayed spectrally without chlorophyll interference after the first two steps in the procedure - PEI and $(NH_4)_2SO_4$ precipitations (Fig. 3,4). PEI fractionation at the standard concentration of 0.5% (v/v) reduces chlorophyll in the PEI supernatant to a level that is ~0.07% of the crude extract and ~1.2% of the minus PEI control supernatant (Fig. 3). The level of phytochrome in light-grown oats is 1.5%-2% that in etiolated shoots as determined by this assay. This simple two-step protocol can now be implemented for the routine quantitation of phytochrome in green tissue in any laboratory with a standard Sorvall-type refrigerated centrifuge and a dual wavelength spectrophotometer.

V. SPECTRAL CHARACTERIZATION

The higher yields obtained with the modified protocol have permitted spectral analysis of the phytochrome in the $(NH_4)_2(SO)_4$ pellet in clear solution without the need to add a scattering agent such as $CaCO_3$ as was previously the case (1). The difference spectrum obtained exhibits a far-red absorbance maximum (λ^{FR}max) of 729 nm and a red absorbance maximum (λ^{R}max) of 652 nm (Fig. 5). The λ^{FR}max is comparable to that of 124 kD etiolated-tissue phytochrome where a λ^{FR}max of 730 nm is indicative of a lack of the limited proteolysis at the N-terminus referred to above (16). This result contrasts with our previous data obtained with green-tissue phytochrome extracted as Pr where the λ^{FR}max was 720 nm, suggestive of proteolysis (1). The green-tissue λ^{R}max, on the other hand, is similar to our previous observations and is some 14 nm shorter than for etiolated-tissue phytochrome. This spectral shift is not caused by the residual chlorophyll in the preparation (Fig. 5b) nor apparently by a posthomogenization modification of an initially etiolated- tissue-like phytochrome. The latter conclusion is based on the observation that when freeze-dried and powdered etiolated tissue is mixed with green-tissue powder in a ratio of 1:9 and the mixture is extracted and processed according to the standard protocol, the spectral properties of the etiolated-tissue phytochrome are unaltered i.e. λ^{R}max is still 666 nm (data not shown). This experiment is possible because etiolated tissue has about 100-times more phytochrome than green tissue per g dry weight. Thus 90% of the spectrally measurabe phytochrome in the mixed tissue extract is of

etiolated-tissue origin, exposed from the moment of extraction to an environment that is 90% of green tissue origin. It is noteworthy that the spectral properties measured here for phytochrome from green tissue bear a striking resemblance to those measured in vivo in light-grown, Sandoz-bleached tissue (9). Together these data strongly indicate the presence in light-grown tissue of a phytochrome species spectrally distinct from that predominanting in etiolated tissue.

A second spectral criterion signaling N-terminal proteolysis in etiolated-tissue phytochrome is rapid, dithionite-accelerated dark reversion (17). When applied to green-tissue phytochrome, this test indicates the presence of two populations: one (~20%) showing dark reversion kinetics parallel to those of 118/114 kD etiolated-tissue phytochrome; and the other (~80%) showing kinetics comparable to 124 kD etiolated-tissue phytochrome (Fig. 6). A mixing experiment similar to that described above tends to speak against the 20% rapidly reverting population representing an originally etiolated- tissue type molecule that was altered during extraction in the green-tissue homogenate (see "etiolated/green" curve, Fig. 6).

In the absence of dithionite, the green tissue phytochrome prepared with the modified protocol exhibits minimal and slow dark reversion. This result contrasts with our previous data on green tissue phytochrome extracted as Pr (1) where extremely rapid dark reversion occurred without dithionite.

VI. CRITICAL PARAMETERS AND PROBLEMS ASSOCIATED WITH THE SPECTRAL ASSAY

Although PEI fractionation dramatically reduces the chlorophyll content of phytochrome containing fractions, the residual chlorophyll is still at significant levels when compared to phytochrome in terms of absolute absorbance (Fig. 5). In order to define the limitations of the procedure for our own information and as a guideline for other investigators, we have examined in model experiments the question of how much chlorophyll can be tolerated in the green-tissue $(NH_4)_2SO_4$ precipitate and what effects, if any, that chlorophyll which is inevitably present has on the phytochrome spectra obtained.

A. Fluorescence or Screening?

These frequently cited potential problems are not apparent at the ratios of chlorophyll to phytochrome absorbance normally obtained in the $(NH_4)_2SO_4$ pellet (Fig 5). Virtually identical difference spectra are observed before and after addition of chlorophyll to purified etiolated-tissue phytochrome at a level simulating the ratio in the $(NH_4)_2SO_4$ pellet (Fig. 5b). This ratio expressed as $\frac{A667 - \Delta(\Delta A)653/730}{\Delta(\Delta A)653/730}$ has a value of 14 for the samples in Fig. 5. Thus, as indicated above, the observed differences in the spectra of etiolated- and green-tissue phytochrome are not artifacts caused by residual chlorophyll.

B. The Problem of Limited Irreversible Chlorophyll Bleaching

1. Increasing amounts of chlorophyll added to purified, etiolated-tissue phytochrome (118/114 kD) causes relatively minor interference with the difference spectrum up to a chlorophyll:phytochrome absorbance ratio about twice that normally observed in the $(NH_4)_2SO_4$ pellet from green-tissue

extracts (Fig. 7a,b,c). At ratios of about three times or greater the absolute magnitude of the irreversible bleaching of the chlorophyll induced by the red actinic irradiations becomes significant on the sensitivity scale needed to measure the phytochrome spectrum (Fig. 7d).

2. Irreversible bleaching is detectable in the green-tissue $(NH_4)_2SO_4$ pellet under two circumstances: (a) A significant bleaching occurs in response to the initial red actinic irradiation following a period of storage of the sample (Fig. 8a); and (b) A minor but discernable amount of irreversible bleaching is steadily "accumulated" with successive red actinic irradiations in a series of red/far red cycles (Fig. 8b,c).

Thus provided the ratios of chlorophyll to phytochrome absorbance indicated are not exceeded, and actinic red irradiations are used in a way that precludes or minimizes irreversible bleaching, then reliable spectral measurements can be made.

VII. IMMUNOCHEMICAL CHARACTERIZATION

We have found that polyclonal antibodies directed against 118/114 kD phytochrome from etiolated tissue exhibit only limited recognition of the phytochrome extracted from green tissue. Only 25% of the spectrally detectable phytochrome in green-tissue extracts can be immunoprecipitated in contrast to 100% for etiolated oat phytochrome; and five times more antiphytochrome Ig is required to give maximal immunoprecipitation for green-tissue than for etiolated-tissue phytochrome (Fig. 9). This result is not due to either (a) the presence in green-tissue extracts of components that interfere directly with the antibody-antigen interaction, or (b) the posthomogenization modification in the green-tissue extract of a phytochrome molecule that before extraction was the same as that predominating in etiolated tissue. This conclusion is based on the mixing experiments in Fig. 10. When resuspended and clarified $(NH_4)_2SO_4$ pellets prepared separately from green and from etiolated tissue are mixed just before immunoprecipitation, essentially all of the added etiolated tissue phytochrome is immunoadsorbed (Fig. 10c,d). Likewise, when etiolated-tissue powder is mixed with green-tissue powder such that an approximatly equal amount of phytochrome from each is present, and the extract of this mixture is processed according to the standard protocol, the total amount of spectral activity immunoprecipitated from the $(NH_4)_2SO_4$ pellet is equal to the sum of all of the etiolated-tissue phytochrome estimated to be present, plus the small fraction of the green-tissue phytochrome that is immunoprecipitable (Fig. 10e,f). These data indicate that green tissue contains a spectrally measurable phytochrome molecule that is immunochemically distinct from that in etiolated tissue and that this difference exists prior to extraction.

The subfraction of green-tissue phytochrome that does immunoprecipitate, on the other hand, is predominantly 124 kD with a less prominent 118 kD species also visible (Fig. 11, lane 3). This subfraction thus tends to resemble etiolated-tissue phytochrome as regards apparent molecular weight and at least partial immunochemical identity. No spectral analysis on the separate subfractions has yet been performed. Etiolated-tissue phytochrome recovered from the mixing experiments in Fig. 10 is also almost exclusively 124 kD (Fig. 11, lanes 2 and 4) consistent with the notion that any "etiolated-type" phytochrome present in green tissue at extraction would be expected to survive through the protocol without experiencing significant proteolysis. Taken

together the data suggest the presence of at least two populations of phytochrome in green, light-grown tissue: a minor fraction that appears related to the species familiar from etiolated tissue and a larger fraction apparently consisting of a new species immunologically unrelated to the etiolated-tissue molecule.

VIII. PHYTOCHROME BIOGENESIS

A full understanding of how phytochrome functions in the living cell must include an understanding of the regulation of the levels of the photoreceptor. It has long been known that these levels are under both developmental and light control. The much lower levels of phytochrome in light-grown than in dark-grown tissue has been explained in the past exclusively in terms of the much greater degradation rate of Pfr than of Pr, assuming a constant rate of Pr synthesis. Recent studies on the cell-free synthesis of phytochrome, however, have led us to reassess this view. We have observed that, whereas translatable phytochrome mRNA is readily detectable in etiolated tissue, none is detectable in green tissue in continuous light (10). Gottman and Schäfer (4) have reported similar observations. We have now found that phytochrome itself is responsible for this effect (3). The photoreceptor exerts negative feed-back control whereby conversion of Pr to Pfr causes a rapid decline in the level of translatable phytochrome mRNA. This decline represents a decrease in the abundance of phytochrome mRNA sequences as shown by northern blot analysis using a phytochrome cDNA clone that we have isolated (6). Thus Pfr formation causes not only a higher rate of phytochrome protein degradation but also a reduced rate of synthesis due to a reduction in the level of phytochrome mRNA. This effect appears to be reversible as translatable phytochrome mRNA levels have been reported to increase again upon depletion of Pfr when light-grown green plants are returned to the dark (5).

Although not an integral part of the present project these data have important implications for the interpretation of studies on green-tissue phytochrome. First, they are consistent with the notion of the existence of more than one type of phytochrome molecule in green tissue. The level of phytochrome measured spectrally in green tissue in continuous light (1-3% of the etiolated tissue level (8, 9)): (a) appears to be higher than expected of the combined effect of the more than 20-fold decrease in rate of synthesis (3) and 100-fold increase in rate of degradation (12) that should apparently result from P_{fr} formation; and (b) appears to be higher than expected given the lack of detectable levels of phytochrome apoprotein among the cell-free translation products of green-tissue poly(A) RNA (4, 10). As with our immunochemical characterization data (Fig. 9,10), one potential explanation of this apparent discrepancy is that the predominant phytochrome species detected in green tissue is a second gene product, immunologically distinct from that predominating in etiolated tissue. Most recently we have found, using our phytochrome cDNA as a probe in northern blot analysis of mRNA from green tissue, that there are in fact extremely low levels of a phytochrome mRNA homologous with the probe. This observation would be compatible with the presence of the minor fraction of etiolated-tissue-like 124 kD phytochrome detected in green-tissue extracts on western blots (Fig. 11). The absence of an observable *in vitro* translation product might result because the amount of mRNA is below the limits of detection of our assay system.

A second implication of this autoregulation of phytochrome mRNA levels is that in green plants grown under the natural light-dark diurnal cycle,

phytochrome levels will oscillate, not only because of the disparate rates of degradation of Pr and Pfr but also because the rate of synthesis will be expected to fluctuate as the level of mRNA fluctuates under Pfr control. Thus the manner in which plants are grown will be important for interpreting data on phytochrome from green tissue.

IX. PURIFICATION

It was our initial intention to utilize the immunoaffinity procedure of Hunt and Pratt (7) to purify phytochrome from green tissue. However, the discovery that the major phytochrome species in green tissue does not cross-react with anti-etiolated phytochrome Ig has precluded this approach. The alternative currently being explored is based on the procedure we have developed for etiolated tissue (17), namely, sequential PEI and $(NH_4)_2SO_4$ precipitations, as above, followed by hydroxylapatite and blue sepharose column chromatography. Initial data from the hydroxylapatite column step indicates that there are two chromatographically distinct species in the preparations. Whether these species correspond to the two populations detected immunologically and by dark reversion is currently under investigation.

X. MONOCLONAL ANTIBODIES

We have generated about 50 hybridoma cell lines that produce monoclonal antibodies against 124 kD phytochrome from etiolated tissue. Part of the present research effort has been devoted to this project because these reagents will be invaluable tools for dissecting the presently complex situation in green tissue. In mapping experiments we have thus far identified monoclonal lines specific for the N-terminus of the phytochrome polypeptide and others specific for other regions of the molecule. With a representative set of antibodies specific for antigenic sites distributed throughout the length of the polypeptide, we hope to be able to probe for potentially more subtle immunological relationships between green- and etiolated-tissue phytochrome that may have gone undetected with our present polyclonal antibodies.

XI. ALGAL CULTURE AND PHYTOCHROME EXTRACTION

Initially we invested considerable effort attempting to establish axenic cultures of Mesotaenium. Such cultures are needed as we found a carbon source was required in the medium to maximize algal growth. We have succeeded in isolating several axenic lines and have selected a fast-growing strain. The low rate of dry matter production of these algae (compared to higher plant systems) is a rate-limiting factor since large quantities of cellular material are required for phytochrome extraction. We have recently scaled up to the point where 3 x 14 liter cultures are being grown continuously and routinely. The growth curves of these cells show a doubling time of ~1.7 days. We are able to harvest 16g of freeze-dried cells every 6 days with this capacity. This is sufficient for some pilot trials. We have measured phytochrome in extracts from these cells and preliminary indications are that the data agree with a previous report (13). It is intended to scale up to very large batch cultures using culture vessels available in the Biochemistry Department on campus in order to ensure that the growth of the algal cells is not a limiting factor to the project.

XII. CONCLUSIONS

Green tissue extracts contain two types of phytochrome: (a) a minor fraction that resembles the molecule from etiolated tissue in that it cross-reacts with antibodies prepared against etiolated-tissue phytochrome and has an apparent molecular mass of 124 kD the same as native phytochrome from etiolated tissue; and (b) a major fraction that is not recognized by anti-etiolated tissue phytochrome Ig and has spectral properties distinct from those of native phytochrome from etiolated tissue. Mixing experiments establish that the distinctive properties of the major fraction do not arise by posthomogenization modification of "etiolated-tissue-type" molecules, thus indicating that the two phytochrome fractions pre-exist in green tissue cells.

Possible bases for intracellular differences between the phytochrome species that predominate in green and in etiolated tissues are: (a) the existence of distinct gene products, so different from one another that they exhibit distinctive spectral properties and little or no immunological cross-reactivity; and (b) identical gene products in both tissues with a certain percentage being subjected to posttranslational modifications in green tissue to the extent that immunological cross-reactivity is eliminated. Two categories of such modifications might be envisioned: (a) function-related such as glycosylation, phosphorylation, etc, leading to spectral alteration and "masking" of antigenic sites; and (b) degradation-related where it might be that a high proportion of the phytochrome pool is in various stages of degradation but still photoactive at any instant due to rapid turnover of the molecule. There is good precedent for immunorecognition of proteolytically derived but still photoactive degradation products of phytochrome from etiolated tissue. This might speak against the possibility that the predominant fraction of green tissue phytochrome is a pool of partial degradation products but is not a definitive argument. Similarly, if a large fraction of the phytochrome synthesized in green tissue were being rendered "invisible" to the antibodies by function-related, post-translational modification *in vivo*, it might be expected that the apoprotein synthesized *in vitro* would still be produced in more than adequate quantitites for detection by immunoprecipitation since it would not be subjected to these modifications. Thus the absence of detectable levels of immunorecognizable apoprotein among the *in vitro* translation products of green tissue poly(A) RNA (4, 10) seems more compatible with the notion that the low proportion of immunoprecipitable, etiolated-tissue-like, 124 kD phytochrome in green tissue extracts results from low levels of synthesis of this polypeptide, rather than from posttranslational modification of a major fraction of more abundant quantities of this polypeptide synthesized *in vivo*. The detection of very low levels of hybridizable phytochrome mRNA in green tissue with a cDNA probe is consistent with this notion. However, the data are insufficiently quantitative as yet to provide a definitive conclusion on this point.

Thus on the basis of available data, the possibility remains that two genes are operative in green tissue: one coding for the etiolated tissue-type 124 kD phytochrome polypeptide and the other for a second product of unknown characteristics.

XIII. CONTRIBUTIONS OF THIS RESEARCH EFFORT

This research program has thus far made two principle contributions: (a)

It has provided for the first time a simple procedure for the routine preparation and spectral assay of phytochrome in extracts from fully-green, light-grown tissue. (b) It has provided evidence that green tissue contains two phytochrome species - one similar to the well-characterized molecule from etiolated tissue, and the other an apparently new species with immunological and spectral properties distinct from those of the etiolated-tissue molecule. The stage is set for establishing the source of these observed differences and determining their functional significance to the green plant.

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XV. FIGURE LEGENDS

Fig. 1. Absorbance spectra of green oat shoot crude extract (1/100 dilution) and of the supernatant of the same extract following polyethyleneimine (PEI) precipitation.

Fig. 2. Standard protocol for the preparation of phytochrome-containing fractions from green tissue for spectral assay.

Fig. 3. Precipitation of chlorophyll and protein from extracts of green oat tissue with PEI. Aliquots of rehydrated oat tissue powder were adjusted to various concentrations of PEI, centrifuged at 27,000 xg for 30 min and the supernatants assayed for chlorophyll (●; A652 nm in 80% acetone) and for protein (■; Lowry et al.).

Fig. 4. $(NH_4)_2SO_4$ fractionation of phytochrome and total protein in the PEI supernatant of rehydrated green oat tissue powder extract. Various amounts of $(NH_4)_2SO_4$ were added to aliquots of the PEI supernatant, the precipitates were collected by centrifugation, resuspended in isolation buffer minus $(NH_4)_2SO_4$ and clarified by centrifugation. Phytochrome was measured in the clarified samples with a dual wavelength spectrophotometer using $CaCO_3$ as a scattering agent (●; 1 arbitrary unit = 1.6×10^{-4} $\Delta(\Delta A)$ 730/800 nm) and protein (■) was measured by the method of Lowry et al.

Fig. 5. Absorbance and difference spectra of extracts from green and from etiolated oat tissue following PEI and $(NH_4)_2SO_4$ precipitation. Spectra were recorded in clear samples in 1 cm cuvettes following resuspension and clarification of the $(NH_4)_2SO_4$ precipitates. (a) Direct comparison of comparable fractions from green and etiolated tissue at equivalent phytochrome absorbances. (b) Comparison of the spectra obtained for the same etiolated sample as in (a) before (="etiolated") and after (="etiol + chl") addition of chlorophyll to a level approximately equivalent to the amount residual in the green-oat fraction in (a).

Fig. 6. Dark reversion of phytochrome extracted from etiolated tissue, from green tissue and from a mixture of green and etiolated tissues that were combined before extraction. Data are plotted as phytochrome remaining as Pfr as a function of time in the dark after an initial red light pulse. All samples were adjusted to 5 mM dithionite to enhance the rate of dark reversion. Green (●): green-tissue phytochrome. Etiolated-124 (■): etiolated tissue fraction containing 124 kD phytochrome. Etiolated/green (*): fraction obtained by extracting a mixture of green and etiolated tissues in a ratio such that the spectral activity contributed by the etiolated-tissue phytochrome was 10-times that of the green tissue. Etiolated-114/118 (▲): immunopurified 114/118 kD phytochrome.

Fig. 7. Absorbance and difference spectra of purified etiolated oat shoot phytochrome (114/118 kD) with addition of increasing amounts of chlorophyll. For each panel the following sequence was employed in recording the spectra: the initial absorbance of the sample was recorded (scan lower right of each panel) with phytochrome as Pr; a difference spectrum was then measured on the same sample before any actinic irradiations were given i.e. a dark-minus-dark baseline control (D at right in each panel); a saturating red actinic irradiation was given and a difference spectrum obtained by subtracting the new

spectrum from the stored dark spectrum (R at right in each panel); the recorder pen was shifted to the left and the same difference spectrum once more recorded without further irradiation (R at left of each panel); a saturating far-red actinic irradiation was given and a difference spectrum once more recorded by subtracting the new spectrum from the original stored dark spectrum (R/FR at left of each panel). A. Phytochrome before addition of chlorophyll. B. Phytochrome plus 3 ul of chlorophyll containing crude extract; one cycle of R/FR irradiation with repeat baseline scans 5 and 20 min after FR. C. Phytochrome plus 6 ul of crude extract. D. Phytochrome plus 9 ul of crude extract; repeat baseline scans 5 min and 2 hr after final FR irradiation.

Fig. 8. Absorbance and difference spectra of green oat tissue $(NH_4)_2SO_4$ fraction. Extract prepared in early experiments as Pr (therefore probably 118/114 kD phytochrome) and measured with $CaCO_3$ as a scattering agent. General procedure for recording spectra as for Fig. 7. A. First R/FR actinic cycle applied to sample after a period of storage at -20°C. D. Third R/FR actinic cycle of same sample as in A. but with new "dark" baseline stored before red actinic irradiation. C. Fourth and fifth actinic cycles, same sample as in A. and B. but with new "dark" baseline stored before starting the cycle. The order of the scans is indicated by the circled members.

Fig. 9. Immunoprecipitation of phytochrome from green and from etiolated oat tissue with antiphytochrome Ig directed against etiolated-tissue phytochrome. Aliquots of resuspended $(NH_4)_2SO_4$ pellets from green (●,o) and from etiolated (■,□) tissue were incubated for 15 min on ice with either polyclonal anti-etiolated oat phytochrome Ig (MAB) or preimmune Ig (NIS), S. aureus cells were added as an immunoadsorbent and after a further 15 min were collected by centrifugation. Supernatants and pellets were assayed for phytochrome in a dual wavelength spectrophotometer with $CaCO_3$ as a scattering agent.

Fig. 10. Immunoprecipitation following mixing of phytochrome from green and etiolated tissue. Three types of samples were subjected to immunoprecipitation at the $(NH_4)_2SO_4$ pellet stage in the standard procedure with either polyclonal antiphytochrome Ig (M) or preimmune Ig (N). "Green": green tissue sample. "Mixed Extracts": Extracts prepared separately from green and from etiolated tissue were mixed immediately before immunoprecipitation in proportions such that each extract contributed equal amounts of spectrally measurable phytochrome. "Mixed tissues": Etiolated-tissue powder and green-tissue powder were mixed before extraction in a ratio such that each contributed approximately equal amounts of spectrally measurable phytochrome. The amount of each tissue type required to give equal amounts of phytochrome in the mixture was determined by separate extraction of aliquots of each tissue type. Immunoprecipitations were as in Fig. 3 using both antiphytochrome and preimmune Igs at 17 ug/ml. This level of antiphytochrome Ig precipitates 100% of the phytochrome in etiolated tissue samples (cf. Fig. 3). Phytochrome was measured as in Fig. 3 in supernatants (a-e) and pellets (b-f). 1 arbitrary unit = 3.3×10^{-4} $\Delta(\Delta A)$ 730-800 nm. Dotted line in d and f is the overall level of immunoprecipitable phytochrome expected in the mixed samples if the etiolated-tissue phytochrome present were to immunoprecipitate proportionally only to the same limited extent as the green-tissue phytochrome. The extent of the area above the dotted line indicates that essentially all of the phytochrome of etiolated-tissue origin has immunoprecipitated in each case.

Fig. 11. Western blot analysis of phytochrome from mixing experiment in Fig.

10. Samples were subjected to SDS polyacrylamide gel electrophoresis, transferred to nitrocellulose paper and the phytochrome visualized using polyclonal rabbit antiphytochrome IgG as primary antibody and alkaline phosphatase-conjugated goat anti-rabbit IgG as a second antibody. Lanes 1, 6 and 16: purified 124 kD phytochrome as marker. Lanes 2-8, 10: immunoprecipitates prepared using anti-etiolated oat phytochrome Ig. Lanes 11-15: immunoprecipitates prepared using preimmune Ig. Lanes 2 and 12: immunoprecipitates from `mixed extracts' (Fig. 10d). Lanes 3 and 13: immunoprecipitates from green oat tissue extracts (Fig. 10b). Lanes 4 and 14: immunoprecipitates from `mixed tissues' (Fig. 10f). Lanes 5 and 15: immunoprecipitates from etiolated oat tissue extracts. Lanes 7 and 8: immunoprecipitates from green oat tissue extract mixed just before application to the SDS gel with either purified 124 kD phytochrome (lane 7) or immunoaffinity purified 114/118 kD phytochrome (lane 8). Lane 9: immunoprecipitate from `mixed tissues' (Fig. 10f) combined just before gel electrophoresis, with purified 124 kD phytochrome. Lane 10: minus phytochrome control immunoprecipitate (from buffer). Equal amounts of phytochrome spectral activity were loaded onto lanes 1-9 and 16. Corresponding volumes of preimmune immunoprecipitates were then loaded in lanes 11-15 where appropriate. This procedure visualizes the rabbit Ig (broad bands at 40-50 kD in lanes 3, 7 and 8) used in the initial immunoprecipitation of green-tissue phytochrome (cf. control lane 10) as well as phytochrome itself at 124 kD.

Fig. 1

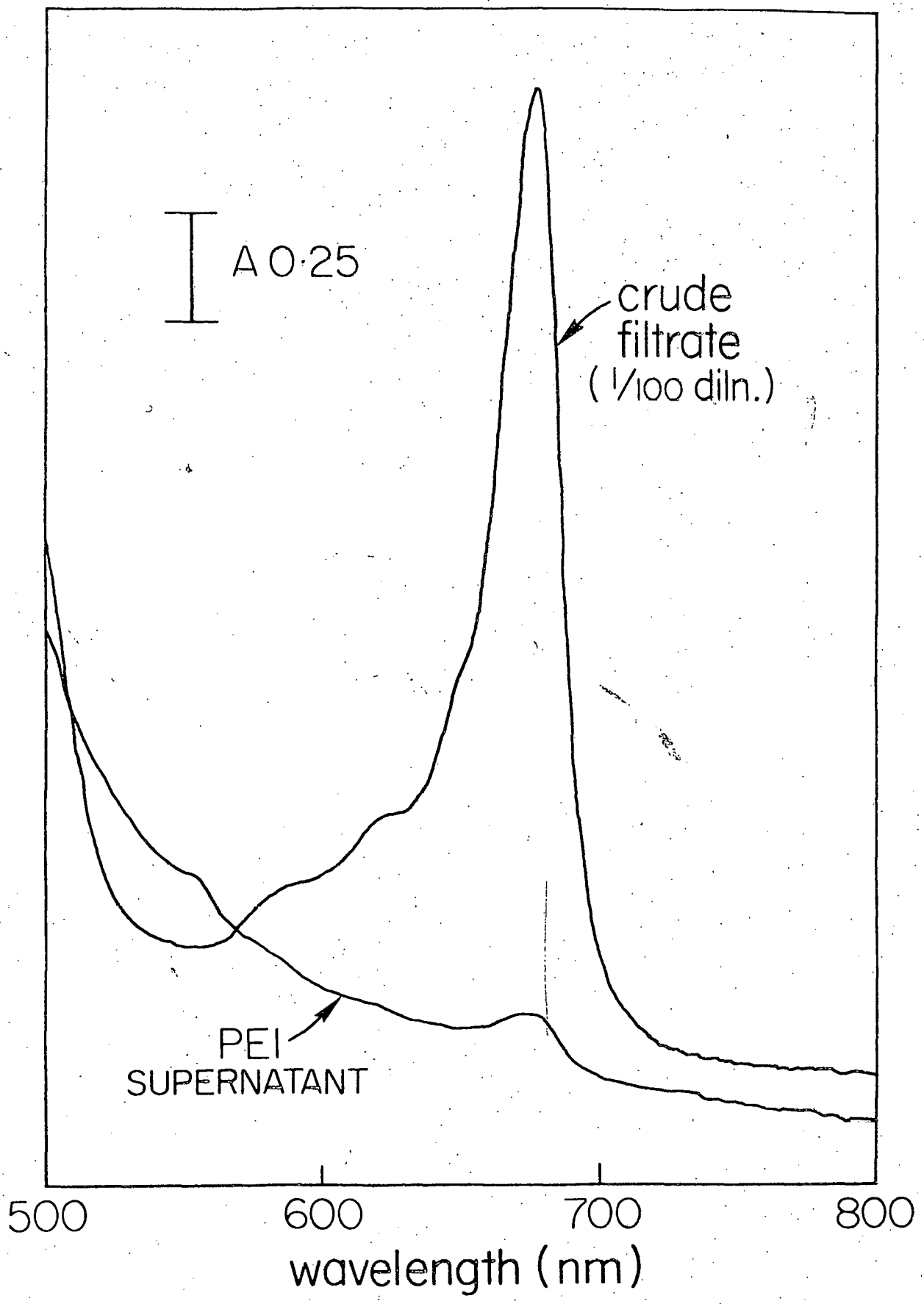

A 0·25
crude
filtrate
(1/100 diln.)
PEI
SUPERNATANT
500
600
700
800
wavelength (nm)

Fig. 2

STANDARD PROCEDURE

IRRADIATE 7D OLD GREEN TISSUE WITH RED LIGHT, LYOPHILIZE, GRIND TO A POWDER.

(9 G DRY WEIGHT = 100 G FRESH WEIGHT)

MIX POWDER WITH BUFFER, 9 GDW + 150 ML OF BUFFER A

ADD PEI (FINAL CONCENTATION 0.5% (v/v))

CENTRIFUGE 27,000 XG, 30 MIN, DISCARD PELLET

IRRADIATE PEI SUPERNATANT WITH RED LIGHT

ADD $(NH_4)_2\ SO_4$ (25G/100ML OF SUPERNATANT) ADJUST TO 4 MM PMSF

CENTRIFUGE 27,000 XG, 30 MIN, DISCARD SUPERNATANT

RESOLUBILIZE PELLET, 4 ML OF BUFFER A MINUS $(NH_4)_2SO_4$ PH 8.1

CENTRIFUGE 48,000 XG, 20 MIN, DISCARD PELLET

SUPERNATANT = "GREEN OAT EXTRACT".

("ETIOLATED OAT EXTRACT" PREPARED AS ABOVE AT 1/10 SCALE, RESOLUBILIZED IN 20 ML)

EXTRACT PH 7.8

BUFFER A

- 50 MM TRIS
- 75 MM $(NH_4)_2SO_4$
- 25% ETHYLENE GLYCOL
- 25 MM $NaHSO_3$
- 5 MM EDTA
- 2 MM PMSF
- ADJUST TO PH 8.4 (4°C)

Fig. 3

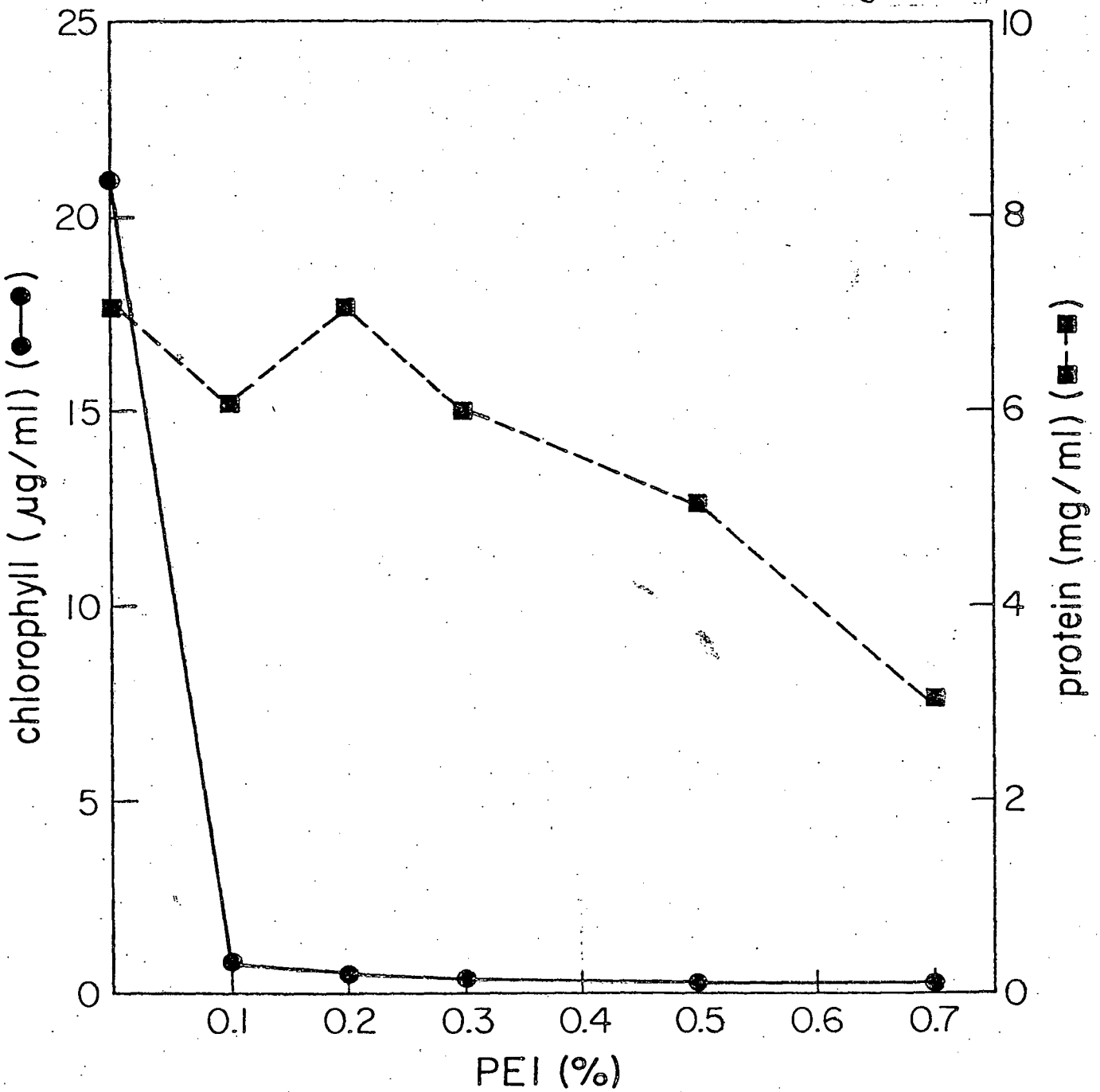

25
20
15
10
5
0
chlorophyll (μg/ml) (●—●)
10
8
6
4
2
0
protein (mg/ml) (■—■)
0.1
0.2
0.3
0.4
0.5
0.6
0.7
PEI (%)

Fig. 4

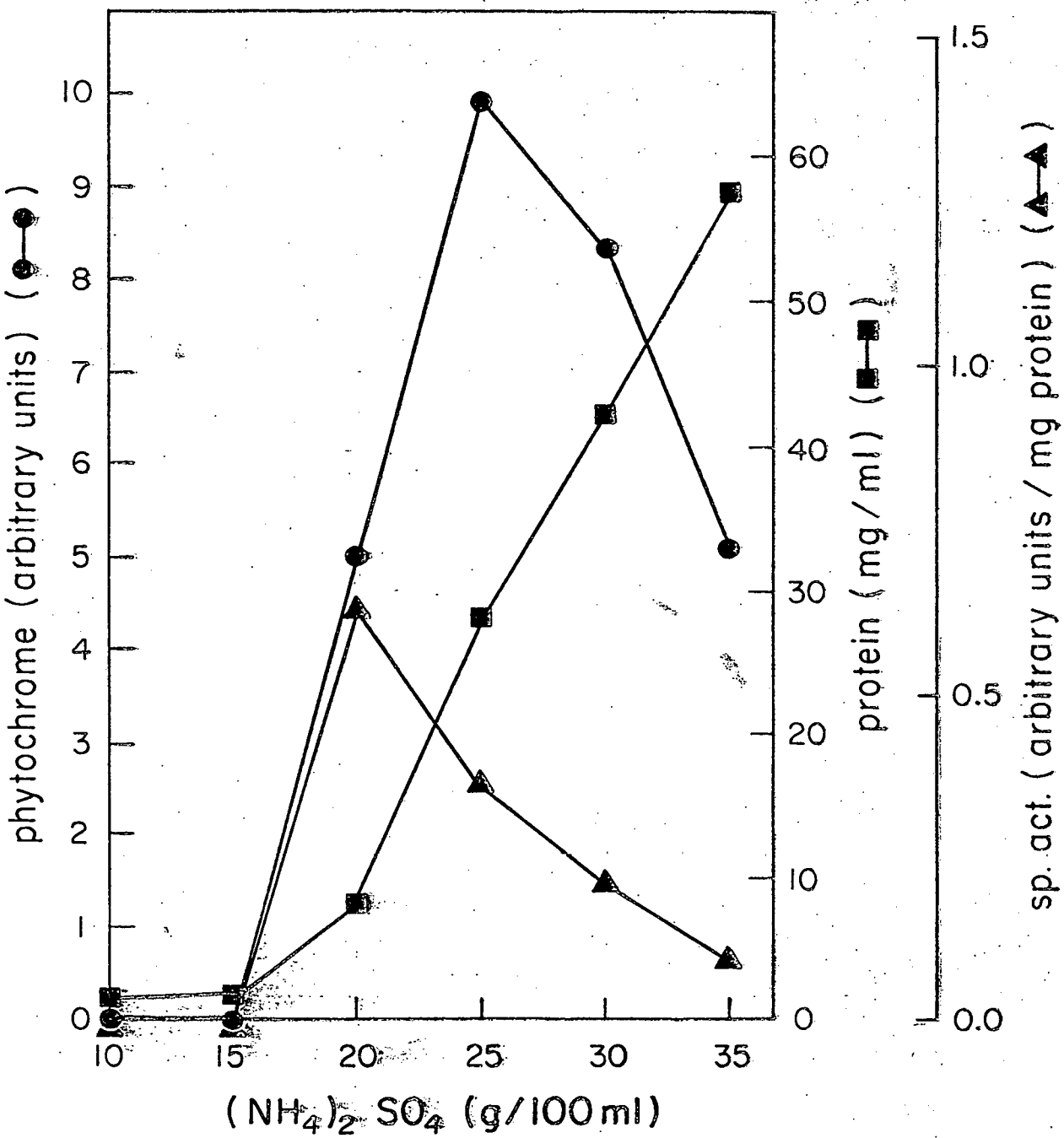

phytochrome (arbitrary units) (●—●)
protein (mg/ml) (■—■)
sp. act. (arbitrary units / mg protein) (▲—▲)
0
1
2
3
4
5
6
7
8
9
10
0
10
20
30
40
50
60
0.0
0.5
1.0
1.5
10
15
20
25
30
35
(NH4)2 SO4 (g/100 ml)

FIG. 5

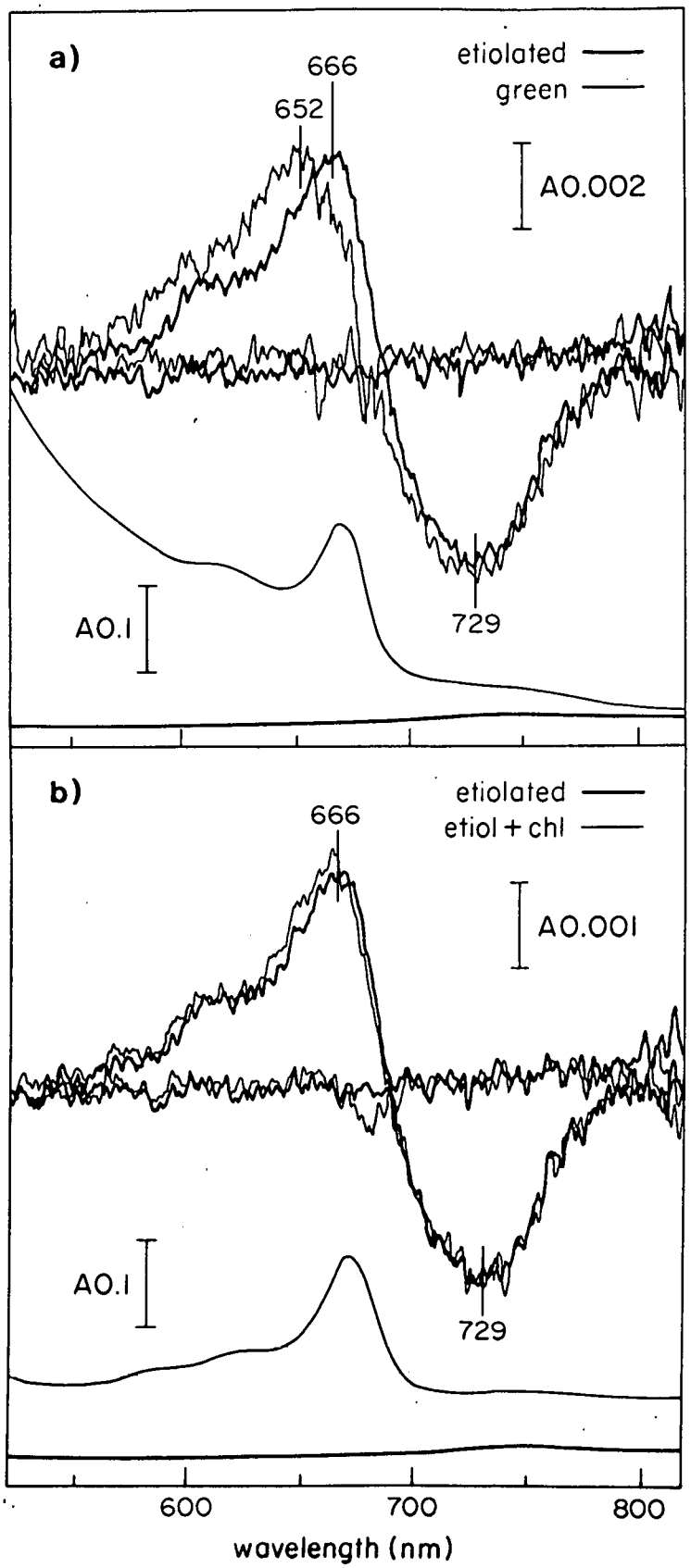
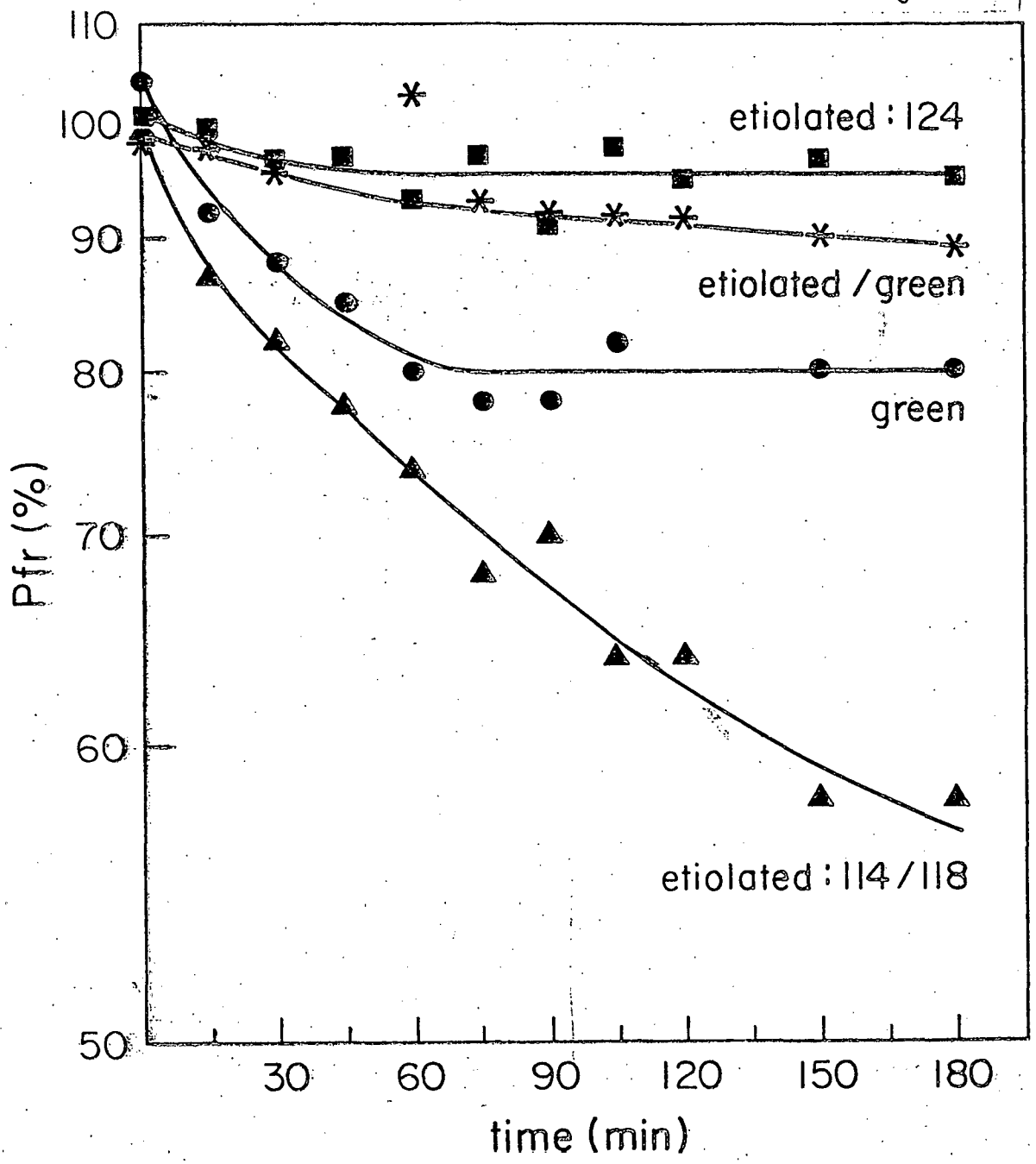

a)
666
652
etiolated
green
A0.002
A0.1
729
b)
etiolated
etiol + chl
666
A0.001
A0.1
729
600
700
800
wavelength (nm)

Fig. 6


110
100
90
80
70
60
50
Pfr (%)
30
60
90
120
150
180
time (min)
etiolated : 124
etiolated / green
green
etiolated : 114 / 118

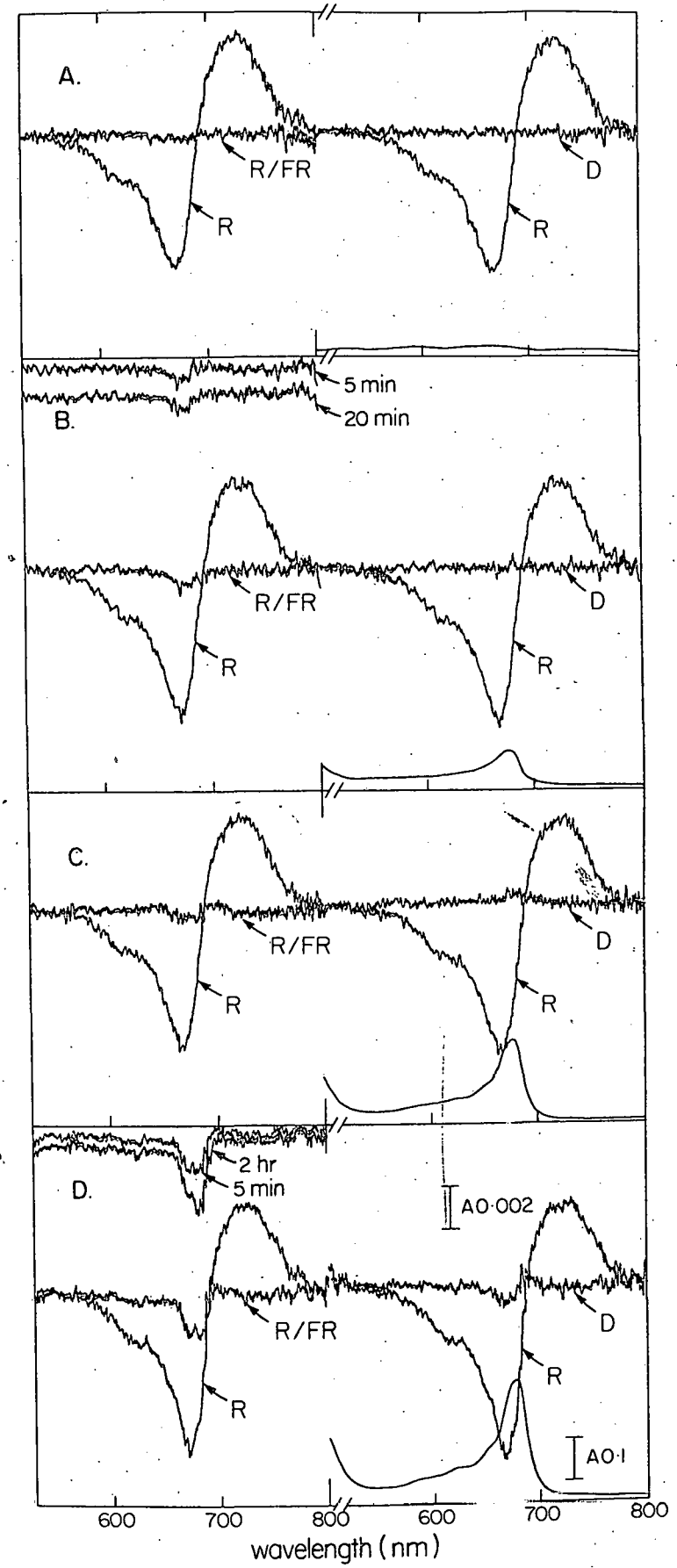

A.
R/FR
D
R
R
5 min
20 min
B.
R/FR
D
R
R
C.
R/FR
D
R
R
2 hr
5 min
D.
A0·002
R/FR
D
R
R
A0·1
600
700
800
600
700
800
wavelength (nm)

Fig. 7

Fig. 8

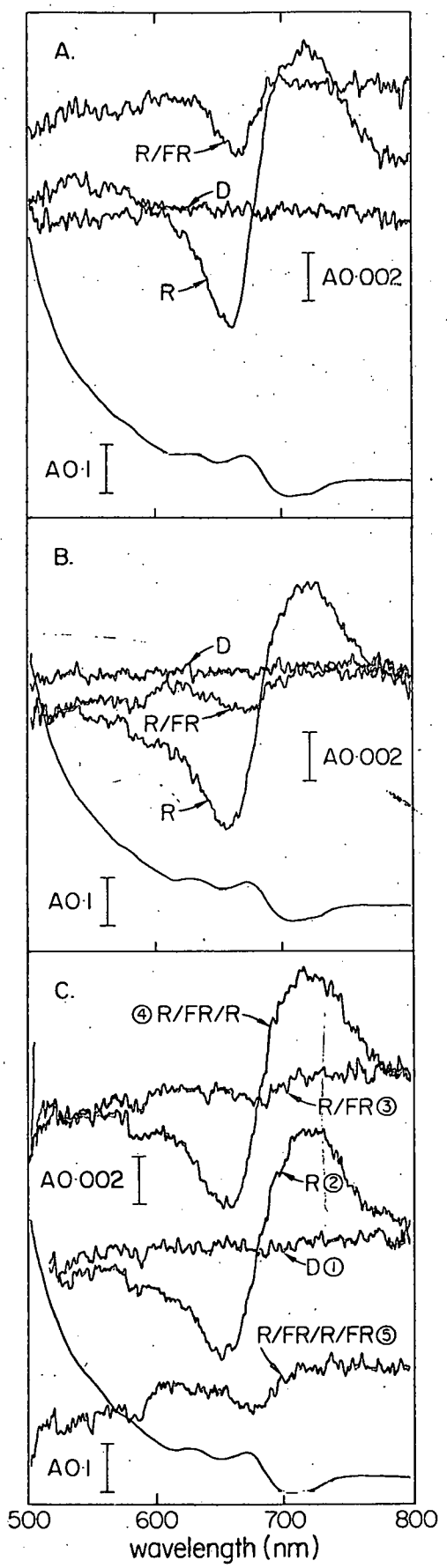

A.
R/FR
D
R
A0·002
A0·1
B.
D
R/FR
R
A0·002
A0·1
C.
④R/FR/R
R/FR③
A0·002
R②
D①
R/FR/R/FR⑤
A0·1
500
600
700
800
wavelength (nm)

Fig. 9

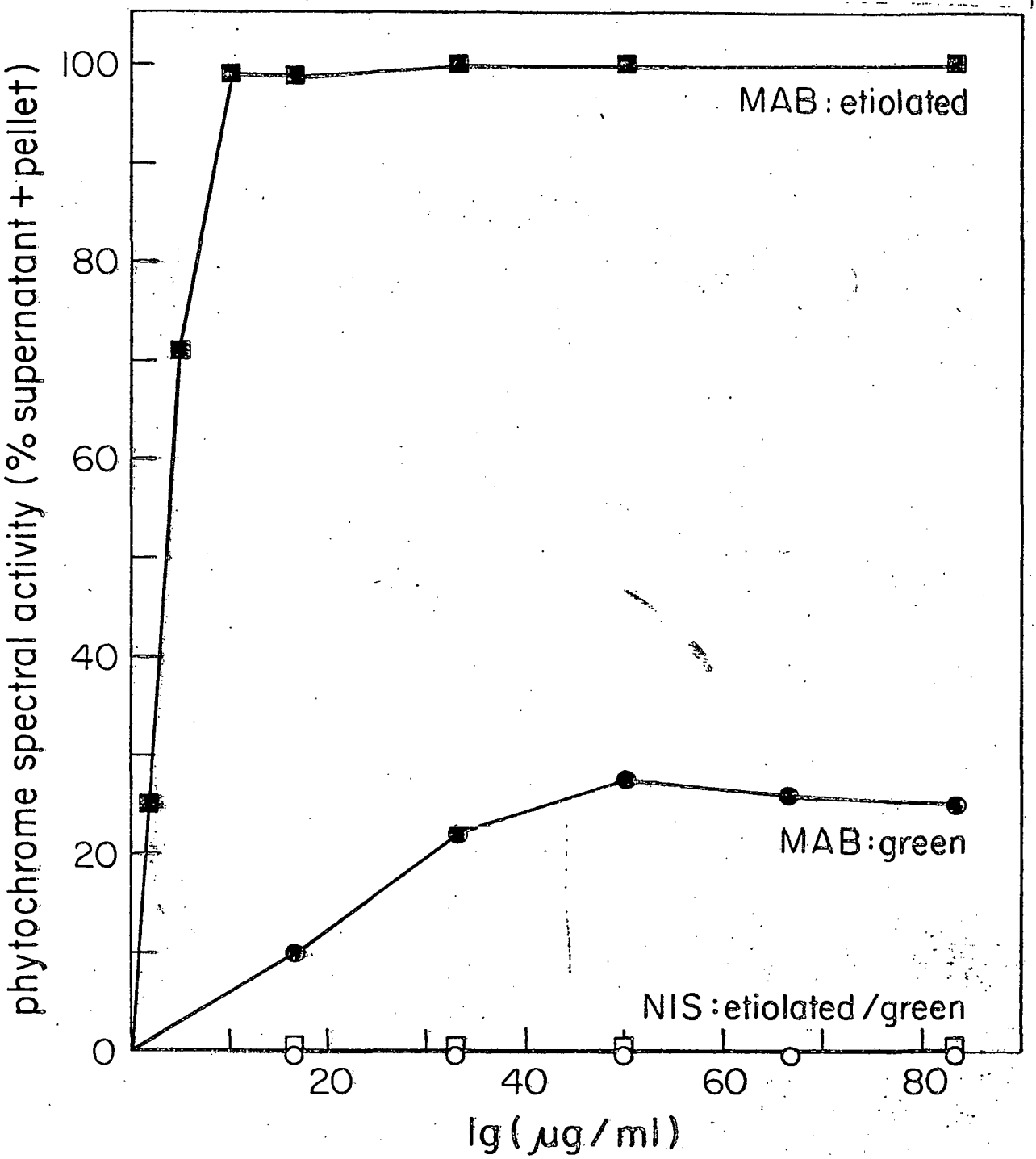

phytochrome spectral activity (% supernatant + pellet)
100
80
60
40
20
0
MAB : etiolated
MAB : green
NIS : etiolated / green
20
40
60
80
lg (µg / ml)

Fig. 10

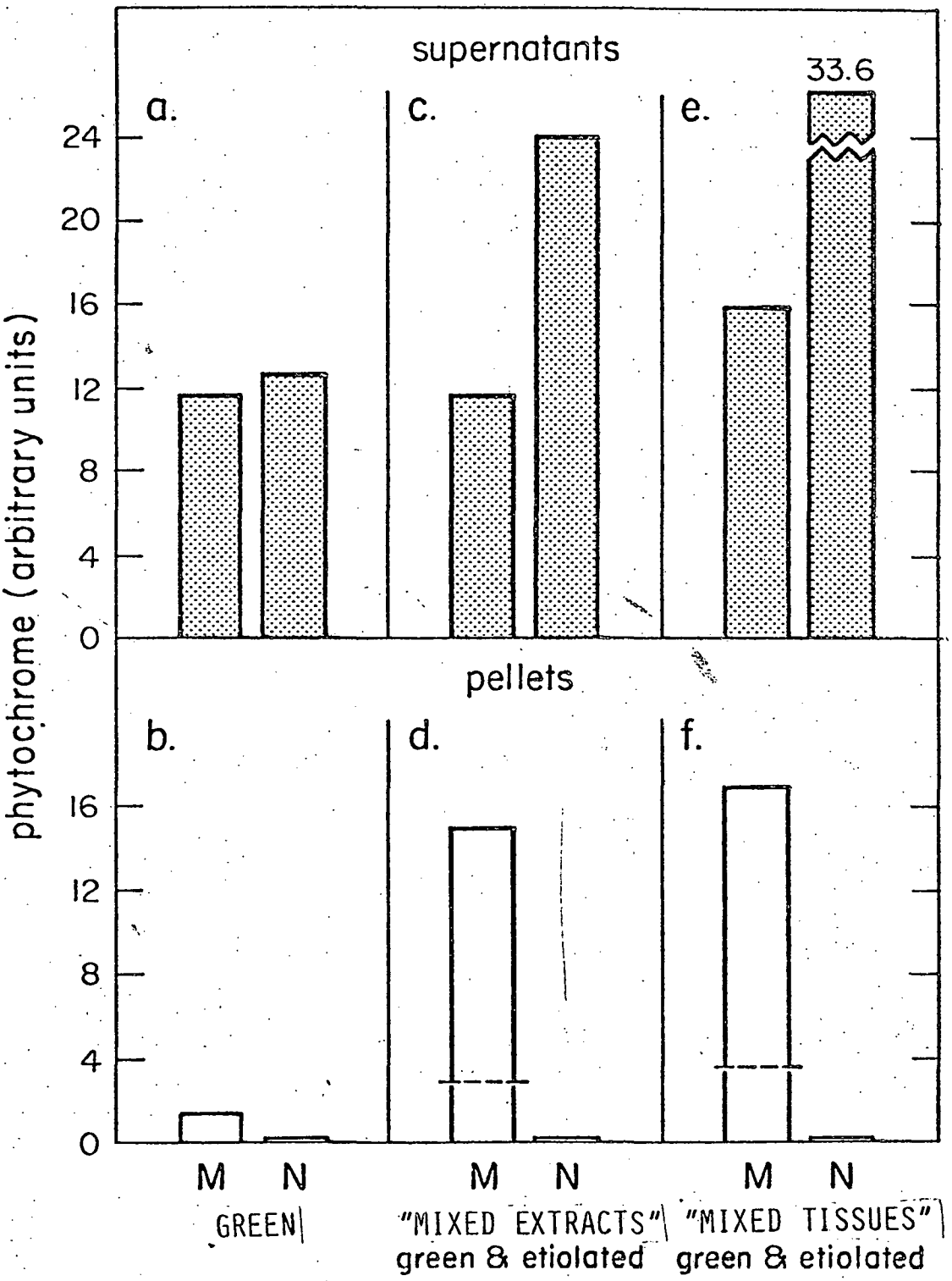
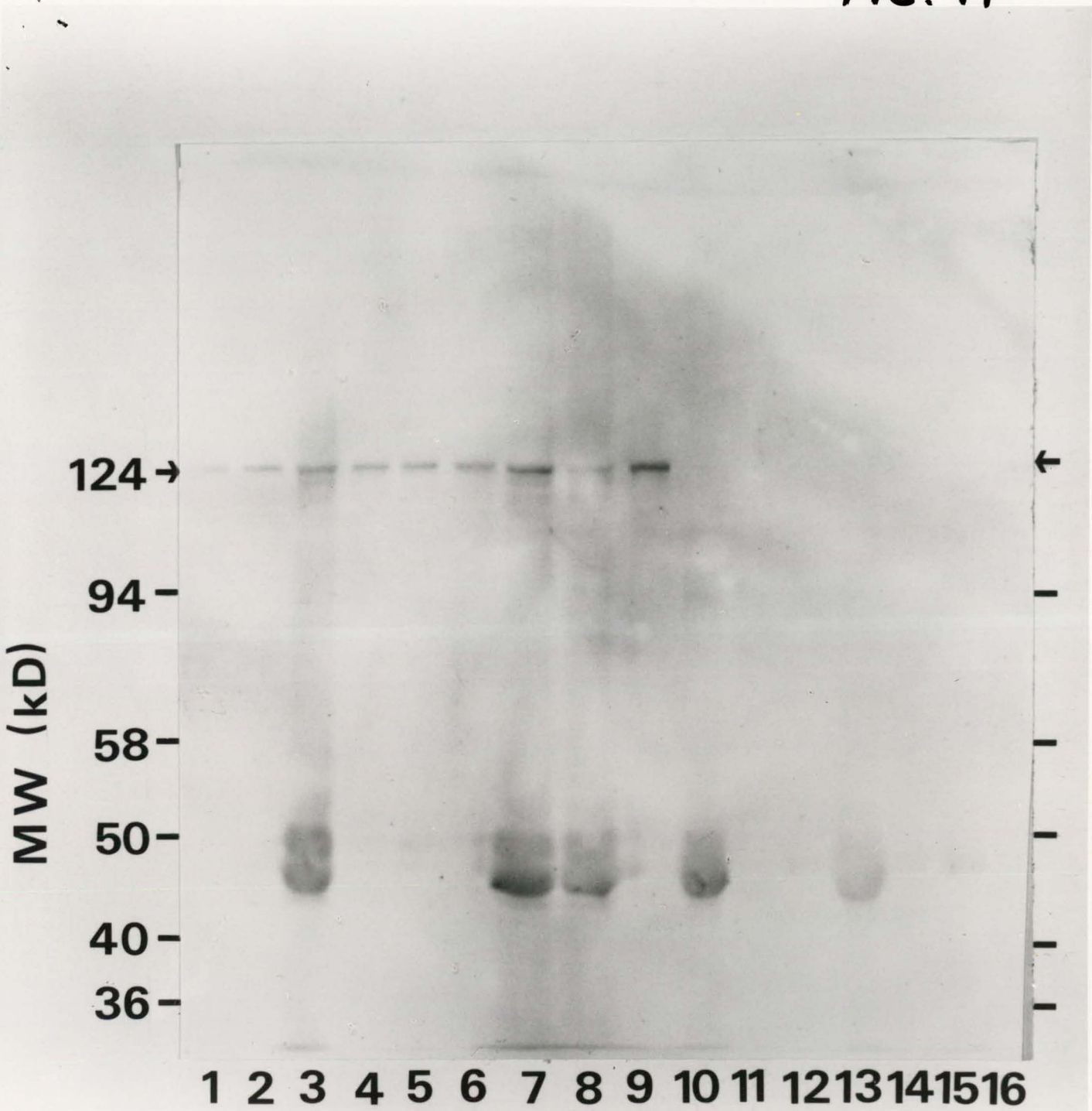

supernatants
33.6
a.
c.
e.
24
20
16
12
8
4
0
phytochrome (arbitrary units)
pellets
b.
d.
f.
16
12
8
4
0
M
N
M
N
M
N
GREEN
"MIXED EXTRACTS"
green & etiolated
"MIXED TISSUES"
green & etiolated

FIG. 11



XVI. PUBLICATIONS (ATTACHED)

1. Bolton, G.W. and Quail, P.H. 1981. A method for preparing green plant tissue extracts for spectrophotometric measurement of phytochrome. Plant Physiol. 67: S104.

2. Quail, P.H., Bolton, G.W., Hershey, H.P. and Vierstra, R.D. 1983a. Phytochrome: Molecular weight, in vitro translation and cDNA cloning. In: Current Topics in Plant Biochemistry and Physiology, Vol. 1, eds. D.D. Randall, D.G. Blevins, and R. Larson, University of Missouri, pp. 25-36.

3. Quail, P.H., Colbert, J.T., Hershey, H.P. and Vierstra, R.D. 1983b. Phytochrome: Molecular properties and biogenesis. Phil. Trans. Royal Soc. Lond., in press.

4. Tokuhisa, J.T. and Quail, P.H. 1983a. Spectral and immunochemical characterization of phytochrome isolated from light-grown Avena sativa L., Plant Physiol. 72: S85.

5. Tokuhisa, J.T. and Quail, P.H. 1983b. Spectral and immunochemical characterization of phytochrome isolated from light-grown Avena sativa L., Photochem. Photobiol. 37: S103.

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