Photochemistry and Photobiology, 1978, Vol. 27, pp. 81–105. Pergamon Press. Printed in Great Britain

REVIEW ARTICLE

MOLECULAR PROPERTIES OF PHYTOCHROME

LEE H. PRATT
Department of Biology, Vanderbilt University, Nashville, TN 37235, U.S.A.

(*Received* 1 *June* 1977; *accepted* 10 *August* 1977)

CONTENTS

I. Introduction
II. Assay methods
  (a) Spectral assay and units
  (b) Immunochemical assay
III. Purification
IV. Biochemical characterization
  (a) Protein moiety
  (b) Chromophore
V. Differences between Pr and Pfr
  (a) Protein moiety
  (b) Chromophore
VI. Photochemistry
  (a) Phototransformations
  (b) Photostationary equilibria
VII. Destruction
VIII. Reversion
IX. Pelletability
  (a) *In vitro*-induced association
  (b) *In vivo*-induced association
  (c) Interpretation
X. Mode of action?
XI. References

I. INTRODUCTION

The physiological experiments which led to the discovery of the photoreversible, morphogenically active plant chromoprotein phytochrome and to predictions concerning its molecular properties represent a classic example of a successful photobiological investigation (Hendricks, 1964). Biochemical and biophysical studies which followed verified many of these predictions. However, hypotheses concerning the molecular mode of action of phytochrome (Mohr, 1966; Borthwick *et al.*, 1969; Mancinelli and Rabino, 1975; Schäfer, 1975a) have not yet been definitively tested. A principal purpose of this review will be to summarize our present understanding of the molecular properties of phytochrome in order to provide a framework within which these latter hypotheses may be tested.

Numerous reviews (Briggs and Rice, 1972; Mohr, 1974; Quail, 1975a, 1976; Kendrick and Smith, 1976; Satter and Galston, 1976; Smith and Kendrick, 1976; Kendrick and Spruit, 1977; Marmé, 1977), monographs (Mohr, 1972; Smith, 1975) and symposium volumes (Mitrakos and Shropshire, 1972; Smith, 1976) concerning phytochrome have appeared in recent years. Because most of these reviews deal more with phytochrome-mediated events within intact tissues or whole organisms than with the molecular properties of the pigment itself, I shall emphasize those studies which involve direct *in vitro* biochemical or biophysical assay of phytochrome. In addition, since available reviews of the molecular properties of phytochrome generally cover literature up to about 1973, I shall further emphasize information which has appeared since that year.

II. ASSAY METHODS

(a) *Spectral assay and units*

The unique, photoreversible absorption properties of phytochrome (Fig. 1) form the basis for the original and still most widely used assay for phytochrome (Butler *et al.*, 1959). Upon absorption of light phytochrome, which is synthesized in a physiologically in-

* Abbreviations used: Chl, Chlorophyll; P_r, phytochrome in the red-absorbing form; P_{fr}, phytochrome in the far red-absorbing form; $P_{fr\infty}^{\lambda}$, proportion of phytochrome present as P_{fr} at photostationary equilibrium with light of wavelength λ.

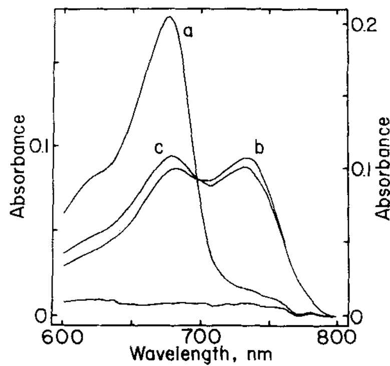

Figure 1. Absorption spectra for degraded (a, b) and undegraded (a, c) oat phytochrome following saturating far red (a) or red irradiation (b, c). Absorbance scale for degraded phytochrome is on left, for undegraded phytochrome on right. (After Pratt and Cundiff, 1975).

active red-absorbing form (P_r*; $\lambda_{max} \simeq 667$ nm), photoconverts to a physiologically active, far red-absorbing form (P_{fr}; $\lambda_{max} \simeq 730$ nm). P_{fr} upon absorption of light converts back to P_r. Since P_r and P_{fr} are indefinitely photointerconvertible, continuous irradiation leads to a photoequilibrium ratio of the two forms which is a function of actinic wavelength (Butler *et al.*, 1964a; Pratt, 1975b).

Phytochrome assay most commonly involves monitoring spectral changes with a dual-wavelength spectrophotometer designed for opaque light scattering samples (Butler, 1964) at *ca.* 667 nm and/or 730 nm as a function of alternating far red (which yields spectrum a in Fig. 1) and red (which yields spectrum b or c in Fig. 1) actinic irradiations. While general purpose dual-wavelength spectrophotometers are available for this assay, it is becoming increasingly common to use custom-built instruments as they may be made easily from inexpensive, off-the-shelf electronic components (Spruit, 1970; Kidd and Pratt, 1973; Pratt and Marmé, 1976; Schäfer, personal commun.; Rüdiger, personal commun.). Of particular interest is a design by Spruit (1971) which irradiates a sample repetitively in sequential fashion with two measuring wavelengths and an actinic wavelength permitting "quasi-continuous" measurement of light-induced absorbance changes. This "quasi-continuous" dual-wavelength spectrophotometer has proved especially useful for assay of intermediates of phytochrome phototransformation under high actinic fluence rates (Kendrick and Spruit, 1972, 1973a, c) using the approach first described by Briggs and Fork (1969). In the absence of interfering pigments and in non-light scattering samples, as for example during the purification of phytochrome (Pratt, 1973; Rice *et al.*, 1973), a simpler single-beam spectrophotometric assay at *ca.* 667 nm after far red actinic irradiation is generally adequate.

There is no standard definition of a phytochrome unit based on the spectral assay because each laboratory expresses its measurements differently. Some even use arbitrary units with no definition at all. For the purposes of this review I shall define one unit of phytochrome as that quantity which, in a volume of 1 ml and for a light path of 1 cm, has an $A_{667} = 1.0$ after actinic far red irradiation. Using absorption spectra for phytochrome after red and far red irradiation (Fig. 1), it is relatively simple to convert photoreversibility units to that just defined and, whenever possible, this conversion will be made below. For example, 1 unit of undegraded, purified phytochrome in a 1 ml sample and for a 1 cm light path will give a measured photoreversibility of 0.91 at 667 nm vs 730 nm [$(\Delta A_{667} + \Delta A_{730})/A_{667}$] and 0.42 at 730 nm vs 800 nm ($\Delta A_{730}/A_{667}$). Since arbitrary units neither permit comparison of data among different laboratories nor indicate how close the data are to typically-observed noise levels, their use should be discouraged. As a minimum, data should be in absolute absorbance units with a full description of the composition and geometry of the sample so that, in the absence of any empirical determination of the scattering factor of the sample (Butler, 1964; Kendrick and Smith, 1976), one can at least estimate phytochrome content and when necessary duplicate the sample assay conditions.

As is the case for any assay, the spectral assay suffers from a number of practical limitations. Most of these limitations, such as lack of sensitivity, inability to detect phytochrome apoprotein in the absence of a photoreversible chromophore, and screening by chlorophylls, have been widely acknowledged (Smith, 1975; Kendrick and Smith, 1976). However, two limitations have not received sufficient attention and will therefore be considered here: fluorescence artifacts generated by measuring light beams and the dependence of phytochrome extinction upon its environment.

A fluorescence-induced distortion is readily appreciated in a single wavelength assay for which the reference is nonfluorescent (e.g. air or buffer) (Clayton, 1970). However, when the reference fluoresces equally as well as the sample it is tempting to assume, in error, that the fluorescence problem is eliminated by subtraction in the resulting difference spectrum. A typical phytochrome-containing sample often contains chlorophylls which fluoresce in response to a measuring beam of appropriate wavelength (Govindjee and Govindjee, 1975). This is the case even for etiolated tissue since protochlorophyll (which is converted to Chl during spectral assay of phytochrome) content is generally much higher than phytochrome content. In addition, a spectrophotometer *intended* for phytochrome assay is very similar in design and operation to one constructed for fluorescence yield assays (Govindjee and Papageorgiou, 1971). Thus, it is generally not possible to separate transmitted from fluoresced light (Clayton, 1970).

Because certain features of many published phytochrome spectra indicate the presence of fluorescence artifacts, the problem will be discussed in detail using a specific example. Assume two light-scattering

samples, each containing the same amount of Chl but one with phytochrome present as P_r (sample) and the other with phytochrome present as P_{fr} (reference). The intensity of transmitted light (I_t) which is detected by the photomultiplier will be

$$I_t = \alpha(I_0 - I_a) = \alpha I_0 10^{-A}$$

where I_0 is the incident intensity, I_a is the proportion of I_0 absorbed by the sample or reference, A is the sum of Chl plus phytochrome absorbance for the sample or reference, and α is the proportion of nonabsorbed light which reaches the photomultiplier, the latter thereby correcting for other light losses such as backscatter. Since a fluorescing pigment, namely Chl, is present the photomultiplier will also detect fluoresced light (I_f). Ignoring (1) reabsorption of fluorescent light which will be negligible because it is at wavelengths beyond maximum pigment absorption, (2) any change in photomultiplier sensitivity as a function of wavelength, and (3) fluorescence by phytochrome which should be vanishingly small under these conditions, and assuming that a fluoresced photon has the same probability (α) of reaching the photomultiplier as a transmitted photon for a typical light scattering sample as is being considered here,

$$I_f = \alpha\phi I_a^{\mathrm{Chl}} = \alpha\phi I_0(1 - 10^{-A_{\mathrm{Chl}}})$$

where ϕ is the quantum yield for chlorophyll fluorescence, I_a^{Chl} is the proportion of I_0 absorbed by chlorophyll, and A_{Chl} is the absorbance arising from chlorophyll alone.

Given the above conditions, the apparent absorbance measured in a difference spectrum (A_m) will be,

$$A_m = A_m^{\mathrm{sam}} - A_m^{\mathrm{ref}} = \log\{[I_0/(I_t^{\mathrm{sam}} + I_f)]/[I_0/(I_t^{\mathrm{ref}} + I_f)]\}$$

where the superscripts sam and ref define the appropriate symbols as referring to the sample and reference, respectively. Substituting for I_t and I_f using the relationships given above and simplifying,

$$A_m = \log\{[10^{-A^{\mathrm{ref}}} + \phi(1-10^{-A_{\mathrm{Chl}}})]/[10^{-A^{\mathrm{sam}}} + \phi(1 - 10^{-A_{\mathrm{Chl}}})]\}.$$

With absorbance values from a phytochrome difference spectrum in the absence of interfering pigments (Fig. 2b) and absorbance values from a typical *in vivo* spectrum for Chl (Fig. 2a), predicted spectra based upon a conservative estimate of 0.03 for ϕ (Govindjee and Govindjee, 1975) may be calculated (Fig. 2c–e) for given Chl concentrations using the last equation given. If sufficient Chl is present to give a maximum absorbance value of 1.0, the P_r difference peak is only slightly reduced in magnitude and shifted by 2 or 3 nm to shorter wavelength (Fig. 2c). However, with increasing Chl content, the difference peak is shifted to increasingly shorter wavelengths, the magnitude of the peak in the red is further decreased, and a shoulder close to the baseline eventually appears in the region of 680 nm. As expected, no distortion is evident in the region of the P_{fr} peak where Chl absorbance is small. Because phytochrome-containing samples are commonly opaque and highly light-scattering the Chl absorbance values considered here are often expected. Also, since ϕ is highly variable and often much higher than the 0.03 assumed here (Papageorgiou, 1975), the fluorescence artifact should often be correspondingly more severe. While the above analysis has been with reference to simultaneous assay of sample and reference in a split-beam spectrophotometer, the artifact will also be present in a dual-wavelength spectrophotometric assay where the sample and reference measurements are separated in time as phytochrome is transformed back-and-forth between P_r and P_{fr}. With a dual-wavelength assay, interference by fluorescence will be seen as an apparent decrease in $\Delta(\Delta A)$ as Chl content increases while phytochrome content remains constant.

From the above analysis it is apparent that the different spectra obtained for phytochrome in pea leaf vs pea stem tissue (Spruit, 1967) need not indicate the existence of two forms of phytochrome as had originally been suggested. Instead, since more Chl is present in leaf as opposed to stem tissue (Pratt, unpublished spectra), the observed difference between the two spectra may readily be explained as a fluorescence artifact since the spectrum given for leaf tissue is similar to Fig. 2d. In addition, spectra which have led to the conclusion that phytochrome is present in an inhomogeneous distribution within the cell (Spruit, 1972; Spruit and Spruit, 1972) are perhaps more similar to those presented here (Fig. 2c–e) than to those calculated based upon the hypothesis that the pig-

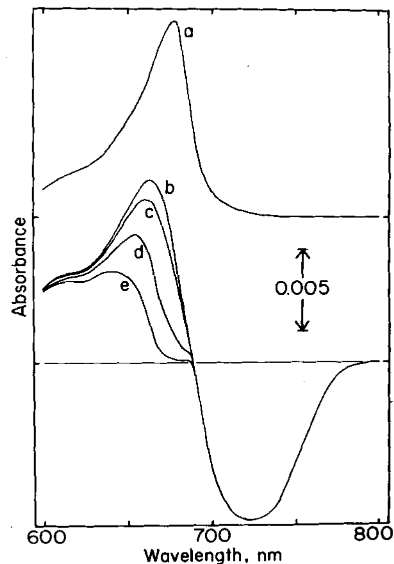

Figure 2. The predicted effect of chlorophyll fluorescence on phytochrome difference spectra. *In vivo* absorption spectrum for chlorophyll (a) taken from Butler and Hopkins, 1970. *In vitro* P_r vs P_{fr} difference spectrum (b) is previously unpublished. Predicted spectra are in the presence of chlorophyll sufficient to yield maximum absorbance of 1.0 (c), 2.0 (d) and 3.0 (e). Details in text.

ments (phytochrome and Chl) were non-homogeneously distributed. There is no apparent need to speculate about pigment distribution from such spectra. Similarly, distortions in phytochrome spectra which appear as Chl content increases during greening (Grill, 1972) may also be explained as fluorescence artifacts. One final example which introduces another parameter to be considered derives from spectra of phytochrome transformation intermediates at cryogenic temperatures (Spruit and Kendrick, 1973; Kendrick and Spruit, 1976). The difference between a spectrum recorded at 203 K and one recorded at 77 K (Fig. 4.1 in Kendrick and Spruit, 1976) may arise from an expected increase in Chl fluorescence yield as temperature is decreased (Govindjee and Govindjee, 1975) thus providing an explanation for the difference in the two spectra which does not require proposing the existence of different intermediates at the two temperatures.

Phytochrome also fluoresces, with increasing yield both as temperature is lowered and as the sample "ages" (Song *et al.*, 1975). Thus, it is possible to obtain fluorescence artifacts using highly purified phytochrome samples, especially at cryogenic temperatures. Such spectra would also show a shift toward shorter wavelength for the P_r difference peak and a distortion toward the baseline near the peak of P_r extinction. Anomalous spectra of this sort have been observed (Pratt, unpublished observations).

In the absence of the precise information needed for a mathematical prediction of whether fluorescence is to be expected as an artifact, one is left only with the possibility of empirically demonstrating that fluorescence is in practice not significant. This is most easily done by decreasing pigment content of the sample by a known factor (by dilution or by a decrease in sample thickness) and observing that the resultant difference spectrum is decreased in magnitude by the same proportional amount at every wavelength or that the magnitude of a photoreversibility measurement is correspondingly attenuated. In the absence of such a demonstration, it is often not possible to exclude fluorescence as a potential artifact and interpretation of data may become tenuous. As discussed above, fluorescence artifacts should be anticipated whenever spectral assay utilizes concentrated phytochrome preparations, is done in the presence of chlorophylls and/or is performed at cryogenic temperatures.

The second limitation in spectral assay of phytochrome derives from the long recognized sensitivity of phytochrome extinction to a wide variety of conditions (Butler *et al.*, 1964b; Lisansky and Galston, 1974, 1976; Pratt and Cundiff, 1975). Of special interest are the recent observations that phytochrome, particularly as P_{fr}, undergoes large and often reversible changes in extinction in the presence of concentrations of divalent cations such as Cu^{2+}, Co^{2+} and Zn^{2+} as low as 0.08 m*M* (Lisansky and Galston, 1974; Pratt and Cundiff, 1975) and as a function of its state of hydration (Tobin *et al.*, 1973; Balangé, 1974). Highly purified preparations of undegraded phytochrome have also been found to undergo reversible changes in P_{fr} extinction (Pratt and Cundiff, 1975) which do not seem to be related in any way to the stability of the chromoprotein. A kinetic analysis of phytochrome samples with unusually low P_{fr} extinction indicated that the samples contained either two populations of phytochrome, one with very low far red extinction, or one population in which each molecule possessed both one or more high extinction chromophores and one or more low extinction chromophores (Pratt, 1975a). Thus, there is even more reason to question the routine assumption that measured photoreversibility is proportional to phytochrome content as others have already pointed out (Spruit, 1972; Kendrick and Smith, 1976). In particular, since reversible changes in phytochrome extinction are most commonly observed in the far red region, and since many investigators rely solely upon spectral measurements in the far red, it would be prudent if unusual spectral data are obtained to confirm spectral changes observed in the far red region by independent assay in the red region.

(b) *Immunochemical assay*

Availability of highly purified phytochrome samples has permitted the production of highly specific antisera (Hopkins and Butler, 1970; Pratt, 1973; Cundiff and Pratt, 1973; Rice and Briggs, 1973a) which form the basis for a wide variety of immunochemical assays, examples of which will be discussed below. Since limitations inherent in the use of immunochemical assays including the inability (a) to determine whether detected antigens are biologically active and (b) to discriminate between P_r and P_{fr} (Pratt, 1973; Cundiff and Pratt, 1975a), are quite different from those associated with spectral assays, a combination of both assay methods in attacking a particular problem can provide much more information than either method alone (Pratt *et al.*, 1976).

Immunochemical assays offer at least three important advantages. First they are independent of the presence of a chromophore and of pigment photoreversibility. They can thus be used as an absolute measure of phytochrome apoprotein (Pratt *et al.*, 1974). Second, they offer much higher sensitivity which permits the detection of smaller absolute quantities of phytochrome. A radioimmunoassay for phytochrome has recently been developed which is sensitive to about 1 ng (Hunt and Pratt, 1977) and, using a higher specific activity label, the assay has a potential sensitivity of about 1 pg (Parker, 1976). Higher sensitivity also permits greater resolution with respect to its distribution, both within tissues (Pratt and Coleman, 1974) and within cells (Coleman and Pratt, 1974a). Third, antisera may be used to probe the structure of the protein moiety of phytochrome as a function of its form (Hopkins and Butler, 1970)

or as a function of its interactions with other cellular components.

III. PURIFICATION

Since the report of Siegelman and Firer (1964) first describing a substantial purification of phytochrome, several laboratories have purified the chromoprotein to a high degree of homogeneity (Mumford and Jenner, 1966; Correll *et al.*, 1968b; Hopkins and Butler, 1970; Pratt and Coleman, 1971; Rice *et al.*, 1973; Roux *et al.*, 1975). Initial reports indicated that phytochrome obtained from oats (Mumford and Jenner, 1966) was substantially different than that obtained from rye (Correll *et al.*, 1968c). In particular, oat phytochrome was apparently of lower molecular weight and different subunit composition. Investigation of these differences led to the realization that phytochrome is highly susceptible to endoproteases (Gardner *et al.*, 1971), that oat extracts are relatively rich in endoprotease activity (Pike and Briggs, 1972a), and thus that the apparent differences between oat and rye phytochrome were largely artifactual (see Briggs *et al.* (1972) and Briggs and Rice (1972) for discussions). Subsequent immunochemical investigations, using antisera against both native and degraded forms of phytochrome confirmed that low molecular weight forms of phytochrome arose as a consequence of proteolysis rather than disaggregation of the native molecule and did not reflect inherent differences in phytochromes obtained from different sources (Pratt, 1973; Cundiff and Pratt, 1973, 1975a, b). Because of this proteolysis problem and because most *in vitro* investigations utilized oat phytochrome, one must assume that much of what we know about the biochemical and biophysical properties of phytochrome in fact represent properties of a degraded form of the chromoprotein (see Briggs and Rice (1972) for discussion). In order to properly evaluate future work with phytochrome *in vitro*, it is thus imperative that the molecular form with which one is working (see Section IV) be defined not only at the time of preparation but at the completion of each critical experiment.

The most thoroughly documented protocol for the purification of undegraded phytochrome is that described by Rice *et al.* (1973) for use with rye tissue. This protocol, which is derived from earlier procedures (Siegelman and Firer, 1964; Hopkins and Butler, 1970) with modifications to minimize proteolysis and accommodate differences in properties between the degraded and undegraded forms of phytochrome, utilizes sequential column chromatography with brushite, diethylaminoethyl-cellulose, hydroxylapatite, Bio-Gel A1.5 M and Sephadex G-200. The product has an apparent molecular weight of about 400,000 daltons based upon calibrated gel exclusion chromatography and is homogeneous by gel electrophoresis (Rice and Briggs, 1973b). The purity of such a homogeneous preparation, expressed as the specific absorbance ratio (A_{667}/A_{280} after far red irradiation) is about 0.7 (Table 1). Modification of the Rice *et al.* protocol to further minimize proteolysis during purification has made it possible to purify undegraded phytochrome from a number of different tissues including endoprotease-rich oats (Pratt, 1973; Cundiff and Pratt, 1973, Table 1). While it has been difficult to obtain completely homogeneous preparations of undegraded oat phytochrome, sequential chromatography with brushite, diethylaminoethyl-cellulose and Sephadex G-200 is a simple, reproducible means of obtaining preparations of high purity (Table 1). It is not certain that pure oat phytochrome has the same specific absorbance ratio as pure rye phytochrome since Cundiff (1973) reported that an undegraded oat preparation with a specific absorbance ratio of 0.50 (Table 1) was homogeneous as judged by sodium dodecyl sulfate gel electrophoresis (cf. also Fig. 3). The use of Bio-Gel P-300 as a final purification step as recommended by Cundiff (1973) and Pratt (1973) is no longer satisfactory since the characteristics of this gel have been changed by the manufacturer (Pratt, unpublished observation). Sephadex G-200 is now used as a substitute.

Two other approaches to phytochrome purification, in addition to the straightforward method referred to above (Rice *et al.*, 1973), have also been attempted. Balangé and Rollin (1973) have described preparative isoelectric focusing of phytochrome purified previously through brushite and hydroxylapatite

Table 1. Purification of undegraded phytochrome from rye and oat tissue

Fraction	Rye* Phytochrome (units/kg)	Rye* S.A.R.‖	Oat† Phytochrome (units/kg)	Oat† S.A.R.	Oat‡ Phytochrome (units/kg)	Oat‡ S.A.R.
Crude extract	14.5	0.00021	ND§	ND	ND	ND
Brushite pool	8.9	0.016	16.9	0.022	15.7	ND
$(NH_4)_2SO_4$ fractionation (200 g/l)	6.8¶	0.065¶	8.0	0.050	9.2	0.044
Diethylaminoethyl-cellulose pool	4.1	0.192	3.0	0.115	2.6	0.138
Hydroxylapatite pool	2.48	0.290	1.63	0.270		
Bio-Gel P-300 pool			0.20	0.500		
Agarose (Bio-Gel A1.5 M) pool	1.45	0.565				
Sephadex G-200 pool	0.73	0.695			1.26	0.286

*Data from Rice *et al.* (1973). †Data from Cundiff (1973). ‡Unpublished data of R. Hunt (1976). ‖Specific absorbance ratio. §Not determined. ¶Calculated values.

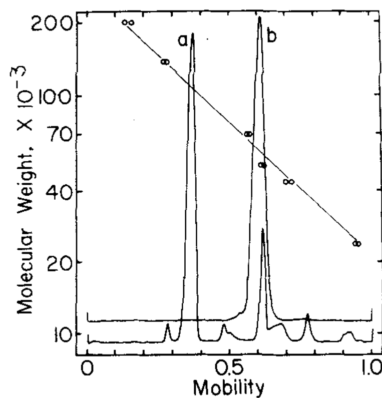

Figure 3. Molecular weight of protein as a function of electrophoretic mobility in 5% acrylamide, 0.14% methylenebisacrylamide sodium dodecyl sulfate, mercaptoethanol gels (Weber and Osborn, 1969). Protein standards include rabbit myosin (200,000), bovine serum albumin (136,000 and 68,000), ovalbumin (43,000) and immunoglobulin G (50,000 and 23,500). Absorbance scans of (a) undegraded oat phytochrome of specific absorbance ratio = 0.33 obtained from R. Hunt and of (b) degraded oat phytochrome of specific absorbance ratio = 0.77 are superimposed. With a specific absorbance ratio of 0.33 and with 64% of the Coomassie blue-staining material under the *ca.* 120,000 dalton peak (a), homogeneous undegraded oat phytochrome may be estimated to have a specific absorbance ratio of 0.52. (Previously unpublished data)

columns. Except for the statement that phytochrome, prior to rather than after the isoelectric focusing step, elutes at the void volume of Sephadex G-150 and is therefore unlikely to be degraded, no information concerning molecular or subunit size is given. It is stated that the purest fraction gave only a single band on basic acrylamide gels. However, no gel is shown and details, such as amount of protein loaded onto the gel and the stain used, are not given. Conversion of photoreversibility units given by Balangé and Rollin to the unit defined above indicates a specific absorbance ratio of 0.29 for their purest fraction, which represents a very small proportion of the recovered phytochrome, and 0.060 for their pool fraction as compared to an expected value of 0.5 to 0.7 for completely pure phytochrome (Table 1). Thus, the fractions are estimated to be about 50 and 10% pure, respectively.

A second approach has utilized the preferential pelletability of P_{fr} (see section IX below) for an 'affinity' purification. In principle, phytochrome is associated with pelletable material as P_{fr}, the pellet containing P_{fr} is washed, and phytochrome is then released to the supernatant fraction by appropriately changing the characteristics of the resuspending buffer. Pelletable material is then removed by centrifugation. Marmé *et al.* (1973) reported purifying phytochrome from *Cucurbita* using *in vitro*-induced pelletability of P_{fr}. The product had a specific absorbance ratio of about 0.07 with a yield of about 45%. Subsequent filtration through Sephadex G-200 increased the ratio to 0.4 which is equivalent to about 65% purity (Table 1). A modification of the Marmé *et al.* approach suggested by Marmé (1974), however, does not yield a soluble form of phytochrome but leaves it attached to 31 S ribonucleoprotein particles (see section IX). Thus, it should not be as effective as that originally proposed. In addition, as discussed below (section IX), it has since been found that these protocols do not work with other plant sources.

Smith and Elliot (1975) have purified phytochrome from maize by a protocol which in principle is the same as that suggested by Marmé *et al.* (1973), except that pelletability was induced by irradiating tissue before extraction. While the resultant phytochrome was undegraded as judged by filtration through Sepharose 4B, phytochrome content was expressed in arbitrary units and no information was given concerning total protein levels. Thus, it is impossible to estimate either yield or extent of purification. However, purification of phytochrome from oats by a method similar to that described by Smith and Elliot indicated that while purity is comparable to that obtained following brushite chromatography, the yield is considerably less (Boeshore and Pratt, 1977).

An immunoaffinity purification of phytochrome, utilizing antiphytochrome immunoglobulins covalently bound to a solid support has yet to be attempted but should offer a rapid method of obtaining highly purified, undegraded phytochrome (Ternynck and Avrameas, 1972).

IV. BIOCHEMICAL CHARACTERIZATION

(a) *Protein moiety*

The most difficult problem to resolve has been that of the conflicting properties for phytochrome obtained by different investigators using different etiolated tissues as their starting material (Briggs and Rice, 1972). As pointed out above, the conflicts may be attributed largely, if not wholly, to the problems associated with proteolysis of phytochrome *in vitro*. As a consequence, emphasis here will be on those studies which have recognized and dealt with the possibility of proteolysis.

Native phytochrome is a multimer of 120,000 dalton subunits as determined by sodium dodecyl sulfate gel electrophoresis (Cundiff, 1973; Rice and Briggs, 1973b; Roux *et al.*, 1975; Smith and Correll, 1975; cf. Figs. 3a and 4d). This value represents a correction of the earlier value of 42,000 reported by Correll *et al.* (1968c) which has unfortunately been accepted by standard reference works (Darnall and Klotz, 1972; Sober, 1970). The undegraded molecule elutes from molecular sieve columns with an apparent molecular weight of about 400,000 daltons (Pratt, 1973; Rice and Briggs, 1973b) and has a sedimentation value of about 9 S indicative of a much smaller molecule of about 180,000 daltons (Gardner *et al.*, 1971; Smith and Correll, 1975). These data are consistent with a nonglobular protein which has a size of about 240,000 daltons leading to the assumption that the native molecule is probably a dimer (Briggs and Rice, 1972).

Table 2. Amino acid compositions reported for phytochrome. For rye phytochrome the number of amino acids (to the nearest integer) are given per 120,000 daltons. For oat phytochrome, per 60,000 daltons. Data sources are as indicated

	Rye phytochrome		Oat phytochrome		
Amino acid	Correll *et al.* (1968c)	Rice and Briggs (1973b)	Mumford and Jenner (1966)	Rice and Briggs 1973b)	Roux (1972)*
Lys	65	58	33	41	35
His	20	28	17	19	16
Arg	23	47	30	26	23
Asp	120	104	59	50	55
Thr	48	46	24	45	24
Ser	118	75	40	37	42
Glu	120	128	54	63	59
Pro	63	88	36	30	32
Gly	149	77	37	27	42
Ala	99	110	50	43	56
Half-cys	0	26	11	10	ND†
Val	80	89	36	42	36
Met	11	32	4	31	12
Ileu	67	54	25	27	25
Leu	100	111	54	57	58
Tyr	44	23	18	15	10
Phe	35	43	25	25	24
Trp	ND	ND	7	ND	ND
Total residues	1162	1139	560	588	549

*Data have been recalculated from those presented by Roux to compensate for the H_2O lost during formation of peptide bonds. †Value not determined.

However, while Gardner (personal communication cited by Rice and Briggs, 1973b) and Cundiff (1973) have both reported preliminary equilibrium centrifugation data consistent with this interpretation, a rigorous shape independent determination of the molecular weight of phytochrome remains to be performed. The partial specific volume of undegraded rye phytochrome has been calculated to be 0.728 cm^3/g (Rice and Briggs, 1973b). Optical activity of the protein moiety is consistent with a tertiary structure composed of 20% α-helix, 30% β-helix, and 50% random coil (Tobin and Briggs, 1973).

Two reports of the amino acid composition of rye phytochrome have appeared (Correll *et al.*, 1968c; Rice and Briggs, 1973b) and it is apparent that there is striking disagreement between the two (Table 2). Of the 17 amino acids reported, 7 differ by more than 25%. Except for the reported absence of cysteine by Correll *et al.*, which is now thought to be in error (Briggs and Rice, 1972; Rice and Briggs, 1973b), there is nothing unusual about its amino acid composition. Resolution of the discrepancies between the two reports will require further experimentation although, when necessary, it is perhaps more conservative to accept the values reported by Rice and Briggs since they were aware of the possibility of protease-induced artifacts.

As already pointed out, it is critical in future investigations to verify the status of phytochrome preparations both prior to and following each experiment. With sufficiently pure samples sodium dodecyl sulfate electrophoresis is the method of choice because of the large difference between undegraded (120,000 daltons) and degraded (60,000 daltons) phytochrome (Fig. 3). With less pure samples elution volume from a calibrated gel exclusion column is satisfactory (Pratt, 1973; Rice and Briggs, 1973b; Smith and Elliot, 1975) although the method is both slow and consumes a relatively large sample volume. Immunochemical methods are not only more rapid but also consume less phytochrome and are thus to be preferred for routine assay of crude samples. Immunoelectrophoresis is a convenient method since degraded and undegraded phytochrome electrophorese at markedly different rates (Cundiff and Pratt, 1973). Sodium dodecyl sulfate gel electrophoresis of immunoprecipitates may also be used as a criterion of size (Fig. 4). Beginning with a crude phytochrome preparation (Fig. 4c) and antiserum against phytochrome (Fig. 4a), an immunoprecipitate is obtained (Fig. 4d) which contains bands derived from the antiserum (primarily immunoglobulin heavy and light chains, Fig. 4b) and the immunoprecipitated phytochrome (here 120,000 daltons).

While it appears likely that the undegraded phytochrome molecule described above represents the native form *in situ*, there is nevertheless some indication that a still larger form may exist. Correll *et al.* (1968c) reported the presence of a 14 S species of phytochrome in addition to the predominant 9 S species described above. More recently, Smith and Correll (1975) have presented evidence which indicates that a contaminant which sediments more rapidly than phytochrome is present in their preparations. The contaminant is neither photoreversible (Smith and Correll, 1975) nor cross reactive with antiserum against undegraded or degraded phytochrome (W. O. Smith, personal commun.). Hence the 14 S

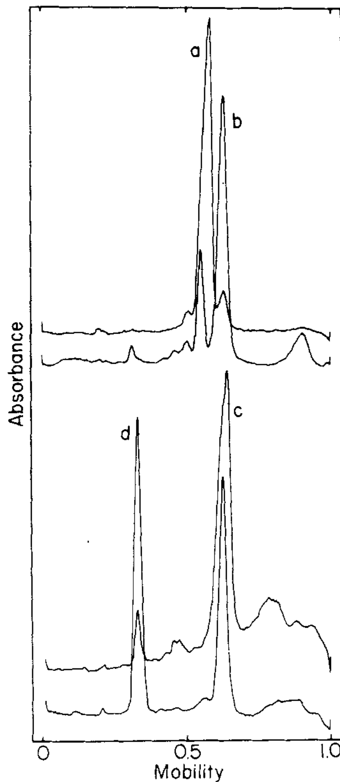

Figure 4. Absorbance scans of electrophoresis gels as in Fig. 3. (a) Whole rabbit serum, major band is albumin. (b) Rabbit serum enriched in immunoglobulin G by fractionation with 33% saturation ammonium sulfate. (c) Crude oat phytochrome preparation derived from pelletable phytochrome fraction as described in text. (d) Immunoprecipitate of phytochrome from (c) using a specific rabbit antiphytochrome serum. (Unpublished data of M. Boeshore and L. Pratt)

species reported earlier might not have been phytochrome. Nevertheless, a shoulder or small peak of photoreversibility preceding the 400,000 dalton species on molecular sieve columns has also been described (Pratt, 1973; Grombein and Rüdiger, 1976). While Grombein and Rüdiger associate this larger form with a previous association between phytochrome and particulate subcellular material (see section IX below), it is not yet clear whether this larger form represents aggregation of the native molecule only *in vitro* or a form which is also found *in situ*.

As pointed out above, recognition of the problems associated with endogeneous protease activity has permitted purification of undegraded phytochrome from a variety of plant sources other than rye including oats, peas and barley (Pratt, 1973; Roux *et al.*, 1975). While these phytochrome preparations have not been studied in as much detail as rye phytochrome, it is clear that they all appear to be comparable in molecular size, general biochemical properties (Cundiff, 1973; Pratt, 1973; Cundiff and Pratt, 1975b), and subunit size (Cundiff, 1973; Roux *et al.*, 1975; Pratt, unpublished). The differences among phytochromes from different sources noted in initial reports (Mumford and Jenner, 1966; Correll *et al.*, 1968c; Walker and Bailey, 1970a, b) are thus likely to be a consequence of different degrees of proteolysis rather than inherent differences in phytochrome itself.

Immunochemical assays have also been used for comparative studies of undegraded phytochrome isolated from different species. The grass phytochromes which have been tested (oat, barley, rye and maize) all cross react with identity in double diffusion assay using antiserum against both degraded (Pratt, 1973; Rice and Briggs, 1973a) and undegraded (Cundiff and Pratt, 1975b) oat phytochrome. While phytochromes from two oat cultivars (Garry and Newton) are identical by micro complement fixation assay using antiserum against degraded phytochrome, both rye and barley phytochrome exhibit reduced activity (Pratt, 1973). Pea phytochrome shows a spur upon double diffusion against oat phytochrome and antioat phytochrome sera leading to the suggestion that it is missing one or more of the determinants found in oat phytochrome (Pratt, 1973; Rice and Briggs, 1973a; Cundiff and Pratt, 1975b) and thus probably has relatively major differences in its primary structure. Micro complement fixation assays confirm this suggestion (Pratt, 1973). Immunoelectrophoretic comparison of the different phytochrome species tested (oat, rye, barley and pea) indicates that they all have similar electrophoretic mobilities and provides no evidence to indicate more than one molecular form of the chromoprotein in each instance (Pratt, 1973).

The relationship between undegraded and degraded forms of phytochrome (cf. Fig. 3) has also been examined by immunochemical techniques. Immunoelectrophoretic comparison of highly purified undegraded and degraded oat phytochrome has demonstrated that a specific antiundegraded oat phytochrome serum contains immunoglobulins specific for primary structure present in the native form which is absent from the 60,000 dalton, photoreversible degradation product (Cundiff and Pratt, 1973). This conclusion is supported by double diffusion (Fig. 5) and micro complement fixation assays using antiserum against the undegraded form of phytochrome (Cundiff and Pratt, 1975a). As might be anticipated, however, antiserum against degraded oat phytochrome is incapable of distinguishing between the undegraded and degraded forms, presumably because the primary structure of the degradation product must also be present in the native molecule (Pratt, 1973). "Common antigen" immunoelectrophoresis has verified that the degraded form of phytochrome obtained by added endoprotease (trypsin) is immunochemically indistinguishable from the *ca.* 60,000 dalton chromopeptide obtained from oats using an appropriate purification protocol (Fig. 3b; Mumford and Jenner, 1966; Hopkins and Butler, 1970).

Antiserum against the undegraded form of oat phytochrome is capable not only of recognizing the photoreversible chromopeptide derived from it but also other peptides which possess little or no visible

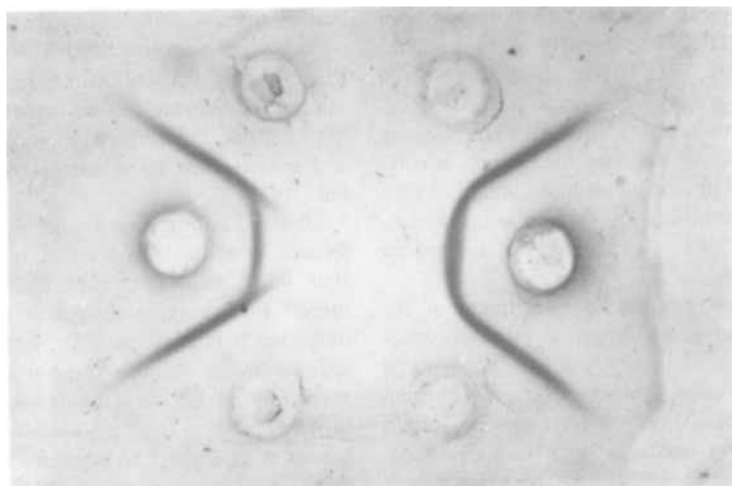

Figure 5. Ouchterlony double diffusion plate of antiundegraded oat phytochrome serum (left well) and antidegraded oat phytochrome serum (right well) against undegraded oat phytochrome (top and bottom wells) and degraded oat phytochrome (center well). (Unpublished data of R. Hunt and L. Pratt).

absorbance (Cundiff and Pratt, 1973, 1975a, 1975b) and are therefore otherwise undetectable. Thus, it has been possible to demonstrate that both endogeneous oat endoprotease as well as added endoproteases (trypsin, chymotrypsin, papain, *Streptomyces griseus* protease, and subtilisin) all yield similar degradation patterns by immunoelectrophoresis. These include at least two large peptides with little or no visible absorbance (Cundiff and Pratt, 1975a, 1975b). Separation of these degradation products by Bio-Gel P-200 chromatography followed by immunoelectrophoretic assay further indicates that the nature of the nonpigmented peptides obtained is a function of the form of phytochrome (P_r, P_{fr}, or cycling) present during proteolysis (Cundiff and Pratt, 1975a). Of particular interest is a large (*ca.* 90,000 daltons by gel filtration), apparently nonchromophore-containing peptide which is recognized by antiserum against the native molecule but not by antiserum against the 60,000 dalton chromopeptide. Thus, it is likely that the photoreversible degradation product which has been widely studied represents no more than about one-half of the original molecule.

Since the 60,000 dalton photoreversible phytochrome chromopeptide is relatively resistant to further proteolysis (Gardner *et al.*, 1971; Cundiff and Pratt, 1975b) the possibility arises that it may represent an active form of phytochrome *in situ*. However, attempts to determine whether significant levels of this chromopeptide are present in etiolated tissues either before or after irradiation with red light have so far indicated that it is not present (Pratt *et al.*, 1974; Grombein and Rüdiger, 1976).

A comparison of amino acid analyses of degraded oat phytochrome to those reported for undegraded rye phytochrome (Table 2) indicates that the degradation product has an amino acid distribution quite similar to that of the native molecule. Hence, the partial specific volume of degraded oat phytochrome of $0.736\ cm^3/g$, is quite close to that (0.728) reported for undegraded rye phytochrome (Rice and Briggs, 1973b).

The isoelectric point for rye phytochrome has not been reported, but it is between 5.9–6.2 for undegraded Garry oat phytochrome as compared to 5.6–5.9 for the degraded form from the same oat cultivar (Cundiff, 1973). In contrast to the isoelectric points reported by Cundiff, who used analytical 4% polyacrylamide gels, Balangé and Rollin (1973) reported a somewhat higher value of 6.25–6.45 using a different oat cultivar and preparative sucrose and glycerol gradients.

While it is apparent that the 120,000 dalton subunit of oat phytochrome contains carbohydrate as judged by a positive reaction to periodic acid–Schiff stain on sodium dodecyl sulfate gels (Roux *et al.*, 1975), no detailed analysis of the carbohydrate composition of the undegraded molecule is available. However, Roux *et al.* have partially determined the carbohydrate content of degraded oat phytochrome, finding about 3.5% carbohydrate by weight. They reported one glucosamine residue but no galactosamine, per 60,000 dalton peptide. Since the relationship between their purified phytochrome and the native molecule is not established it is not possible to extrapolate their observation to the larger presumably native phytochrome subunit.

(b) *Chromophore*

Because of the combined difficulties of being unable to readily obtain large quantities of phytochrome as well as being unable to separate the chromophore from the apoprotein with high yield (Siegelman *et al.*, 1966), characterization of the chromophore has been by comparison to known pigments (Grombein *et al.*, 1975a) or to model compounds (Scheer, 1976; Schoch and Rüdiger, 1976; Sugimoto *et al.*, 1976; Scheer and Krauss, 1977). Our present understanding of the

structure of the phytochrome chromophore has been well reviewed recently (Smith and Kendrick, 1976; Kendrick and Spruit, 1977). The major unresolved problem has been an exact identification of the structure of ring A of the linear tetrapyrrole chromophore of phytochrome (Fig. 6) and recent evidence, involving a comparison of phytochrome and C-phycocyanin spectra after denaturation with guanidinium chloride, indicates that this ring is hydrogenated (Grombein *et al.*, 1975a).

Neither the precise method of attachment of the chromophore to the apoprotein nor the number of chromophores per molecule is yet known (Schoch and Rüdiger, 1976). Investigation of products resulting from extensive degradation of phytochrome could provide useful information regarding both questions, but unfortunately this approach has not been employed since the report of Fry and Mumford (1971) which indicated that the chromophore of degraded oat phytochrome was attached to the peptide leu-arg-ala-pro-his-(ser, cys)-his-leu-glu-try. Comparable investigation of digestion products of undegraded phytochrome coupled with a more intensive search for possible different amino acid sequences associated with the chromophore is needed.

While it is tempting to assume that each 120,000 dalton subunit contains one chromophore, with two for the proposed native dimer, there is yet no concrete evidence to support this assumption. Correll *et al.* (1968a) proposed the existence of four chromophores in the intact molecule based upon analysis of visible absorption spectra. Pratt (1975a) has also presented spectral data which are consistent with the presence of at least two chromophores in the native molecule. Both sets of observations may also be interpreted as indicating that only one chromophore is attached to each molecule and that the anomalous spectral properties which have been described arise from the presence of different populations of phytochrome.

V. DIFFERENCES BETWEEN P_r AND P_{fr}

That P_r and P_{fr} are different is immediately obvious from consideration of their different absorption spectra and different morphogenic activities. Elucidation of these differences has been a much more difficult problem than recognition of their existence.

(a) *Protein moiety*

Differences between P_r and P_{fr} which were observed with what was almost certainly degraded phytochrome have been discussed in earlier reviews (Briggs and Rice, 1972; Smith and Kendrick, 1976). These differences include differential reactivity with 5 *M* urea, 5 m*M* *p*-chloromercuribenzoate, 5 m*M* *N*-ethylmaleimide, trypsin and pronase (Butler *et al.*, 1964b); differential reactivity with glutaraldehyde (Roux, 1972; Roux and Hillman, 1969); perturbations in the ultraviolet difference spectrum between P_r and P_{fr} indicative of changes in the distribution of aromatic amino acids exposed to the surrounding aqueous environment (Hopkins and Butler, 1970; Pratt and Butler, 1970a); differential activity in a micro complement fixation assay and differences in UV circular dichroism (Hopkins and Butler, 1970) and a difference in sedimentation coefficient (Hopkins, 1971).

Of equal importance are those attempts to find differences which failed since they lead to the conclusion that, while differences exist, they must be relatively minor. For example, Briggs *et al.* (1968) found no difference between P_r and P_{fr} with respect to velocity sedimentation (though their resolution was not as great as that obtained by Hopkins), electrophoretic mobility, elution from gel exclusion columns, and behavior with respect to brushite chromatography. In addition, Anderson *et al.* (1970) were not able to repeat the circular dichroism measurements of Hopkins and Butler (1970) in the far UV, while Pratt (1973) and Cundiff and Pratt (1975b) using antiserum against both degraded and undegraded phytochrome were unable to confirm the difference observed by micro complement fixation assay (Hopkins and Butler, 1970). Double diffusion and immunoelectrophoresis have also failed to demonstrate a difference between P_r and P_{fr} (Hopkins and Butler, 1970; Pratt, 1973; Rice and Briggs, 1973a; Cundiff and Pratt, 1975b).

Since the relationship between native and degraded forms of phytochrome was established, fewer attempts to find differences between P_r and P_{fr} have been reported. Tobin and Briggs (1973) found that undegraded rye phytochrome as P_r and P_{fr} exhibit identical CD spectra in the UV in contrast to the report with degraded oat phytochrome although they did repeat with undegraded rye phytochrome the UV absorbance differences reported for degraded oat phytochrome (Hopkins and Butler, 1970; Pratt and Butler, 1970a). Gardner *et al.* (1974) reported that undegraded rye phytochrome was more reactive with ^{14}C-*N*-ethylmaleimide as P_r than as P_{fr}.

In contrast to *N*-ethylmaleimide, both native and degraded phytochrome are more sensitive to multivalent cations as P_{fr} than as P_r (Lisansky and Galston, 1974, 1976; Pratt and Cundiff, 1975). P_{fr} is spectrally denatured (bleached) by cations such as Cu^{2+}, Co^{2+}, and Zn^{2+} at concentrations which only slightly alter the wavelength maximum for P_r absorption. These effects are largely reversed if the cation is chelated with an excess of ethylenediaminetetraacetate (Pratt and Cundiff, 1975).

(b) *Chromophore*

While one could comfortably assume that the protein moiety of phytochrome differed between the P_r and P_{fr} forms, it was not possible to make the same assumption regarding the chromophore. Since chromophore extinction of phytochrome is highly dependent upon its interaction with the protein moiety (Butler *et al.*, 1964b; Grombein *et al.*, 1975a) it is

possible that the absorption difference between P_r and P_{fr} might have arisen from a change in the degree of interaction with the apoprotein instead of, or in addition to, an actual change in the structure or chemistry of the chromophore. However, evidence cited below and discussed extensively elsewhere (Kendrick and Spruit, 1977) indicates that P_r and P_{fr} chromophores are different.

Low temperature and CD spectroscopy have led Burke *et al.* (1972) to conclude that P_r and P_{fr} chromophores are related by a *cis-trans* isomerization. However, more recent data indicate that the chromophore undergoes a chemical as well as structural isomerization (Grombein *et al.*, 1975a). Denaturation of P_r and P_{fr} by 6 *M* guanidinium chloride to decouple the interaction between protein and chromophore with respect to the absorption characteristics of the chromophore yield distinctly different products at an acidic pH. If the pH of denatured P_{fr} is increased, a form of phytochrome is obtained which is spectrally indistinguishable from denatured P_r. By comparison of the spectrum of denatured P_{fr} with that of mesobiliviolin and mesobilipurpurin, it is apparent that P_{fr} contains one *less* double bond in its resonating structure than P_r. Grombein *et al.* conclude that the lost double bond is in the bridge between rings A and B (Fig. 6). However, because of experimental difficulties, they are as yet unable to determine what other differences may exist, although they argue that rings B, C and D are the same as for P_r. Since the resonating system of the P_{fr} chromophore is thus smaller than that of P_r, it is reasonable to postulate that the spectrum of the P_{fr} chromophore is more strongly perturbed by the protein moiety than that of the P_r chromophore (Grombein *et al.*, 1975a).

Using model chromophores for phytochrome (Scheer, 1976), Scheer and Krauss (1977) have described a photochemical dimerization of a P_r model which leads to a product with the same spectral relationship to the P_r model as the actual P_{fr} chromophore has to the true P_r chromophore (Grombein *et al.*, 1975a). The dimer reverts thermally (but not photochemically) back to the original P_r model. Thus, it would appear that a useful model system is available which may lead to a better understanding of the phytochrome chromophore isomerizations.

VI. PHOTOCHEMISTRY

(a) *Phototransformations*

The initial observations of phytochrome phototransformation kinetics indicated that the opposing conversions were both first order with respect to phytochrome (Butler *et al.*, 1964a; Pratt and Briggs, 1966). However, later work indicated a more complex situation which could be explained by assuming the presence of two pools of phytochrome, each with a different rate constant for the phototransformation. This disagreement has since been resolved (see Smith and Kendrick, 1976, for review) in favor of simple, first order kinetics as originally reported (Everett *et al.*, 1970; Spruit and Kendrick, 1972; Schmidt *et al.*, 1973). The later work suffered in one case from an instrumentally-induced artifact (Purves and Briggs, 1968) and in the other case from the use of samples which were too opaque optically (Boisard *et al.*, 1971).

Intermediates in the pathways of the two photoconversions have been examined using three different approaches: (1) Pigment cycling by high fluence rate actinic light which results in the establishment of a steady-state level of phytochrome intermediates (Briggs and Fork, 1969; Kendrick and Spruit, 1973a); (2) flash kinetic spectrophotometry (Linschitz *et al.*, 1966; Linschitz and Kasche, 1967; Pratt and Butler, 1970b); and (3) irradiation at cryogenic temperatures followed by warming which results in the sequential appearance of intermediates (Cross *et al.*, 1968; Pratt and Butler, 1968). Details of the observations arising from the application of each of these methods have recently been reviewed (Kendrick and Smith, 1976; Kendrick and Spruit, 1976, 1977; Kendrick, 1977) and only a brief summary will be presented here. The two phototransformation pathways involve different intermediates, of which six have been identified in the P_r to P_{fr} path and two in the P_{fr} to P_r path. Of particular interest is the observation of Kendrick and

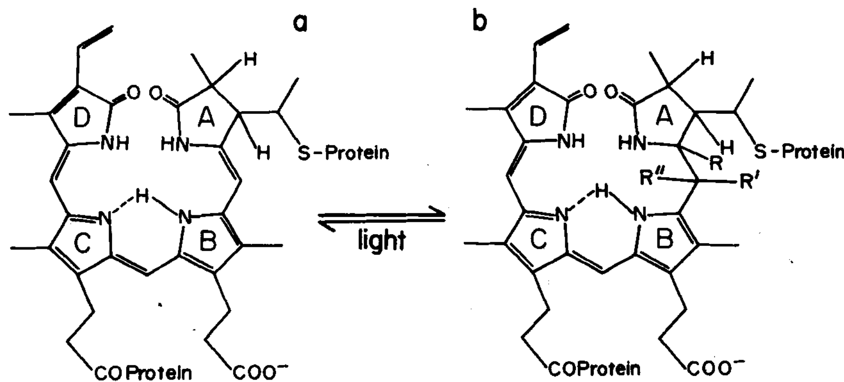

Figure 6. Proposed structures of the phytochrome chromophore in the P_r (a) and P_{fr} (b) forms. (After Grombein *et al.*, 1975a, and Scheer and Krauss, 1977).

Spruit (1973a) using their "quasi-continuous" dual wavelength spectrophotometer that, *in vivo*, close to 50% of the phytochrome may be present as intermediate forms if actinic fluence rates comparable to those found in nature are used. Thus, the possibility exists that one or more intermediate forms of phytochrome may have morphogenic activity. Unfortunately, all of the investigations *in vitro* utilized phytochrome either known to be degraded or of uncharacterized status and therefore remain to be repeated with the undegraded form. Since low temperature studies of phytochrome transformation *in situ* (Kendrick and Spruit, 1973b; Spruit and Kendrick, 1973) yield data which are in general agreement with the observations summarized above, it is likely that undegraded phytochrome behaves similarly to the degraded form.

(b) *Photostationary equilibria*

Since it is impossible to transform all P_r to P_{fr}, even by red light (Butler *et al.*, 1964a), it is necessary to determine the photoequilibrium level of P_{fr} at a defined wavelength ($P^{\lambda}_{fr_\infty}$) as a proportion of the total phytochrome pool. Given a value for $P^{red}_{fr_\infty}$ it is then possible to correct measured photoreversibility values for incomplete photoconversion by red actinic light in the spectrophotometer so that P_r and P_{fr} levels may be calculated (Kendrick and Smith, 1976). Since $P^{red}_{fr_\infty}$ may only be measured in the absence of all other pigments absorbing in the red and far red spectral regions (Butler *et al.*, 1964a) purified phytochrome preparations must be used. Thus, P_{fr_∞} values measured *in situ* (Pratt and Briggs, 1966; Schäfer *et al.*, 1972) are necessarily indirectly derived from an absolute value determined *in vitro*.

Derivation and application of the method used for determining $P^{red}_{fr_\infty}$ is presented by Butler *et al.* (1964a) and Butler (1972). Unfortunately, the original measurements by Butler *et al.* utilized phytochrome preparations which were almost certainly degraded. Repetition of these measurements with undegraded phytochrome, also from oats as originally used, indicated that undegraded phytochrome differs significantly from degraded phytochrome in its photochemical properties (Pratt, 1975b). $P^{red}_{fr_\infty}$ for undegraded phytochrome is 0.75 in contrast to the value of 0.81 reported for presumably degraded phytochrome. Since Pratt (1975b) repeated the measurement of 0.81 using phytochrome known to be degraded, it was concluded that the difference between 0.75 and 0.81 does not arise because of a difference in methodology or instrumentation. Thus, there is no longer any independent empirical justification for using 0.81 as a correction factor (Kendrick and Smith, 1976) for photoreversibility assays. Of course, it is always possible that $P^{red}_{fr_\infty}$ *in situ* is different from that measured *in vitro* but as already pointed out (Butler *et al.*, 1964a) there is no possibility of making the measurement *in vivo*. While the difference between 0.75 and 0.81 is often too small to be of significance because of the large variability generally encountered in phytochrome spectral assays, there are instances where a reevaluation of data would seem necessary (e.g. Oelze-Karow and Mohr, 1973).

Since $P^{\lambda}_{fr_\infty}$ is a function both of the extinction coefficients of P_r and P_{fr} at wavelength λ and of the quantum yields for the photoconversion of P_r to P_{fr} (ϕ_r) and P_{fr} to P_r (ϕ_{fr}) (Butler *et al.*, 1964a), undegraded phytochrome must therefore differ from the degraded form in one or both parameters. Since absorption spectra for P_r and P_{fr}, in the latter case corrected to eliminate absorption by the P_r which is present at photoequilibrium, are identical for degraded and undegraded phytochrome (Pratt, 1976), the ratio ϕ_r/ϕ_{fr} must differ. This conclusion is supported by the observation that this ratio is 1.0 for undegraded phytochrome (Pratt, 1975b) in contrast to the value of 1.5 which was determined for degraded phytochrome (Butler *et al.*, 1964a; Pratt, 1975b). A change in one or both of the quantum yields is reasonable, since much more protein is potentially involved in the phototransformation of the undegraded form.

Because corrected absorption spectra for the P_r and P_{fr} forms of degraded and undegraded phytochrome are superimposable, it follows that the isosbestic point in the P_r and P_{fr} spectra must be at the same wavelength for the two sizes of phytochrome as indicated in Fig. 1. However, this conclusion is in disagreement with spectra published by Pratt (1975b). Reexamination of the original data used for the latter presentation revealed that the spectrum indicated to be of undegraded oat phytochrome in the presence of ethylenediaminetetraacetic acid (Pratt and Cundiff, 1975) was actually of a preparation in the absence of the chelator. Thus, the spectrum published by Pratt (1975b) is in error and must be corrected here (Fig. 1).

Using the spectra for undegraded phytochrome in Fig. 1, $P^{665}_{fr_\infty}$ of 0.75 (Pratt, 1975b), and the extinction coefficients for undegraded phytochrome reported by Tobin and Briggs (1973), absolute extinction spectra for large phytochrome may be calculated for both forms (Fig. 7). By further assuming that the transformation quantum yields in the region of 600–730 nm are wavelength independent, P_{fr_∞} as a function of wavelength may also be calculated (Fig. 7). As observed for degraded phytochrome (Butler *et al.*, 1964a), P_{fr_∞} is relatively constant in the red region and decreases rapidly in the far red. Beyond about 720 nm absorption by P_r becomes so small that calculated P_{fr_∞} values exhibit a large percentage error and are thus not reliable.

Again using the extinction coefficients measured for undegraded phytochrome by Tobin and Briggs (1973) absolute values for ϕ_r and ϕ_{fr} may be calculated. Both were found to be 0.17 for undegraded oat phytochrome (Pratt, 1975b). While Gardner and Briggs (1974) reported different values ($\phi_r = 0.28$; $\phi_{fr} = 0.20$) for undegraded rye phytochrome, they incorporated three "errors" into their calculations as discussed else-

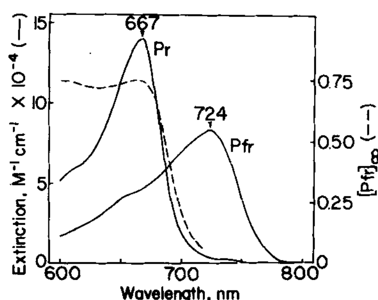

Figure 7. Extinction spectra for undegraded oat phytochrome of specific absorbance ratio = 0.13 in 0.1 *M* Tris, 0.1 m*M* EDTA, pH 7.8 (4°C). Relative P_r and P_{fr} extinction values as a function of wavelength (ϵ_r^λ and ϵ_{fr}^λ) are estimated as:

$$\epsilon_r^\lambda = [A_r^\lambda - ([P_{fr}]_\infty^{723})(\epsilon_{fr}^\lambda)]/[P_r]_\infty^{723}$$

$$\epsilon_{fr}^\lambda = [A_{fr}^\lambda - ([P_r]_\infty^{665})(A_r^\lambda)]/[P_{fr}]_\infty^{665}$$

where A_r^λ (A_{fr}^λ) are measured absorbance values after saturating far red (red) irradiation, $[P_r]_\infty^{665}$($[P_{fr}]_\infty^{665}$) is the proportion of phytochrome present as P_r (P_{fr}) at photoequilibrium under 665 nm light (taken as 0.25 and 0.75, respectively), and $[P_r]_\infty^{723}$($[P_{fr}]_\infty^{723}$) are corresponding values under 723 nm light (taken as 0.98 and 0.02, respectively). Relative values are converted to absolute values by normalizing the spectra to an extinction value of $14 \times 10^4 \, l \cdot mol^{-1} \cdot cm^{-1}$ for P_r at 667 nm (from Tobin and Briggs, 1973, for a molecular weight of 240,000 daltons). The proportion of phytochrome present as P_{fr} at photoequilibrium as a function of wavelength ($[P_{fr}]_\infty^\lambda$) are calculated from the extinction spectra shown based upon a value of 0.75 at 665 nm (Pratt, 1975b).

where (Pratt, 1976). Upon making the necessary corrections the data presented by Gardner and Briggs yield precisely the same quantum yield of 0.17.

VII. DESTRUCTION

Phytochrome destruction, also referred to as decay, is defined as the loss of photoreversible phytochrome *in vivo* following the photoconversion of P_r to P_{fr}. Although the process has been the subject of many investigations (see Frankland, 1972) neither its mechanism nor significance is understood.

Destruction is typically referred to as specific for the P_{fr} form, although evidence has been available which indicates that P_r derived from a red, far red irradiation sequence is also lost (Dooskin and Mancinelli, 1968; Chorney and Gordon, 1966). This apparent destruction of P_r has been reexamined by Mackenzie (1976) who found that about 30% of the phytochrome detectable by spectral assay in etiolated oat shoots is lost following a red, far red irradiation sequence in contrast to a loss of about 75% after a brief red irradiation. No loss was observed after far red irradiation alone. Whether this apparent destruction of P_r results from the same process which "destroys" P_{fr} is unknown. Because this apparent destruction of P_r occurs at a time when phytochrome is found by immunocytochemical assay to be associated with discrete regions within the cell (Mackenzie *et al.*, 1975), it is possible that destruction might be specific, not for the P_{fr} form of the chromoprotein as commonly assumed (Frankland, 1972), but rather for phytochrome which is associated with some as yet unidentified subcellular structure.

The latter possibility is seemingly supported by evidence which purportedly indicates that phytochrome destruction preferentially involves phytochrome which is associated with pelletable material in crude plant extracts (Boisard *et al.*, 1974; Boisard and Cordonnier, 1976). However, the enhanced pelletability of phytochrome was measured in *Cucurbita* extracts using conditions which, as discussed in section IX below, lead preferentially to an interaction between phytochrome and pelletable subcellular material *in vitro* but not *in vivo*. [As discussed further below, the observation that phytochrome pelletability was largely reversed immediately following a far red irradiation indicates that little "binding" had occurred *in vivo* (fig. 5 in Boisard *et al.*, 1974).] Thus, the association between pelletability and destruction must be coincidental rather than causal since destruction occurred prior to the condition leading to enhanced pelletability.

In order to study phytochrome destruction *in vitro* the products of the process, if any, must first be identified so that it will be possible to distinguish between destruction and spectral denaturation. Immunochemical evidence, using immunocytochemical, immunoelectrophoretic, radial immunodiffusion and micro complement fixation assays, indicates that the phytochrome apoprotein is largely, if not completely, degraded during destruction (Coleman and Pratt, 1974b; Pratt *et al.*, 1974). Attempts to detect products of destruction by immunoelectrophoresis failed. In addition, attempts to observe by micro complement fixation differences between photoreversibly detectable phytochrome isolated prior to and following extensive destruction have failed. Thus, the trivial hypothesis that phytochrome destruction may be no more than spectral denaturation, as well as other hypotheses (Spruit, 1972; Pratt *et al.*, 1974), may be eliminated. Purported attempts to study phytochrome destruction *in vitro* consequently offer no information about the destruction process since they almost certainly were only following spectral denaturation of the chromoprotein (Manabe and Furuya, 1971; Lisansky and Galston, 1974, 1976). Future attempts to monitor destruction *in vitro* should thus involve more than just a spectral assay to demonstrate that destruction has, in fact, occurred.

VIII. REVERSION

Reversion is the thermochemical conversion of P_{fr} to P_r occurring both *in vivo* and *in vitro* (see Smith and Kendrick, 1976, for review). However, reversion is not observed in all tissues, notably grasses, even though phytochrome isolated from these same plants undergoes reversion *in vitro*. Reversion observed *in vivo* is typically complete within an hour or less even

though P_{fr} is still present in the tissue. By contrast, reversion *in vitro* spans a time course of many hours and involves the total pool of P_{fr}.

Pike and Briggs (1972b), studying undegraded rye phytochrome, found that reversion exhibits complex kinetics which may be interpreted as indicating the existence of two independent pools of P_{fr} each reverting to P_r by a different first-order rate constant. They confirmed an earlier observation (Mumford and Jenner, 1971) that the rate of degraded oat phytochrome reversion is increased up to about 400-fold by reductants such as dithionite or reduced pyridine nucleotide. Mumford and Jenner had already demonstrated that the reductant functions in a catalytic manner, not being consumed during reversion of P_{fr} to P_r. Reversion of undegraded rye phytochrome is also accelerated by reductants, although not to as great an extent (Pike and Briggs, 1972b). In addition, reversion of undegraded rye phytochrome is accelerated by Mg^{2+} and Ca^{2+} (Negbi *et al.*, 1975). Detailed examination of the reversion kinetics indicated that the effect of the cations was to increase the proportion of P_{fr} which reverted by the faster of the two rate constants described by Pike and Briggs rather than altering either of the rate constants. Although no kinetic study was performed, Pratt and Cundiff (1975) observed that Fe^{3+} at 1 m*M* accelerated reversion of undegraded oat phytochrome with 20% of the P_{fr} originally present reverting to P_r within 5 min at 3°C. Reversion but not spectral denaturation of degraded oat phytochrome was also accelerated by Cu^{2+} at concentrations which spectrally denatured undegraded phytochrome.

Anderson *et al.* (1969) have described the existence in degraded oat phytochrome. of an apparently protonated form of P_{fr} which reverts more rapidly than the "normal" nonprotonated form. Since a decrease in temperature, as well as a decrease in pH, led to an increase in the proportion of P_{fr} present in the protonated form, phytochrome reversion could thus be temperature compensated. Anderson *et al.* calculated that at a pH of 6.2 the overall rate constant for reversion should be independent of temperature leading to a model reaction which has potential significance as a temperature independent time-keeping mechanism. Unfortunately, the analysis cannot be repeated with undegraded phytochrome since the latter is irreversibly denatured by the low pH's (Rice *et al.*, 1973) needed to study this phenomenon.

There is yet no answer to the question of why reversion occurs *in vitro* with phytochrome isolated from tissues which do not exhibit reversion *in situ*. A possible answer is provided by Manabe and Furuya (1971) and Shimazaki and Furuya (1975) who have described an apparent inhibitor of reversion isolated from etiolated pea shoots. The presumptive inhibitor is as yet uncharacterized except for the observations that it is of low molecular weight, resistant to boiling, and soluble in water-saturated *n*-butanol. Since Shimazaki and Furuya have only followed what they consider reversion at a single wavelength (730 nm) using crude and uncharacterized preparations of very low phytochrome concentration further investigation of this apparent inhibitor is warranted.

Since reversion results in the depletion of the active form of phytochrome from the cell and since the rate of reversion is greatly dependent upon a variety of factors over which the cell at least potentially has control, it is a process with obvious physiological implications. Additional characterization of reversion using demonstrably undegraded phytochrome is needed.

An "inverse reversion" of P_r to P_{fr} has also been described (Boisard *et al.*, 1968). While the observations leading to the suggestion that inverse reversion occurs are not in question, more recent information deriving from study of phytochrome transformations in dehydrated tissues has provided an alternate explanation of the observations which is more acceptable on thermodynamic grounds (Kendrick and Spruit, 1974). Kendrick and Spruit described a photoconversion of P_{fr} to an intermediate form in lyophilized pea tissue which apparently reverts thermally to P_{fr} thus leading, in darkness, to an absorbance increase in the far red spectral region as originally described by Boisard *et al.* Since "inverse reversion" is a phenomenon only observed in seeds during the initial stages of imbibition, it would appear that the process is probably a reversion of an intermediate in the P_{fr}-to-P_r phototransformation pathway as described by Kendrick and Spruit for lyophilized pea tissue.

IX. PELLETABILITY

Study of phytochrome-mediated morphogenesis at an organismal level has led to increasing support for the hypothesis that phytochrome modulates directly one or more membrane activities and is thus likely to be membrane-associated (see Satter and Galston, 1976, and Marmé, 1977, for discussions). This hypothesis has led in turn to a search for and characterization of associations between phytochrome and particulate, subcellular fractions. Two different types of association have been considered. First, a small proportion of the total phytochrome pool is always associated with particulate material (Rubinstein *et al.*, 1969; Evans and Smith, 1976; Furuya and Manabe, 1976). Second, some phytochrome associates with particulate material and thus becomes pelletable only following photoconversion to the active P_{fr} form (Quail *et al.*, 1973; Furuya and Manabe, 1976; Marmé *et al.*, 1976; Quail and Gressel, 1976).

The first type of association has so far received little attention because the small quantities of phytochrome which are inherently associated with particulate material are too near the detection limit of the spectral assay. As a consequence, meaningful interpretation of spectral data is exceedingly difficult. Evans and Smith (1976) have reported an association of

spectroscopically detectable phytochrome from nonirradiated barley with etioplast envelopes. However, two problems immediately arise in interpretation. Only about 0.4% of the extracted phytochrome is associated with the "envelope" fraction. The specific activity of phytochrome in this fraction is only about one-half that observed in the crude extract indicating that there is no enrichment for phytochrome. In addition, the method used to purify the etioplasts from which the envelopes are derived is one which does not dependably yield a pure preparation (Quail, 1977a). Thus, the fact that the purity of the etioplast preparations used for this particular report was not demonstrated leaves open the possibility that phytochrome associated with the "envelope" fraction may well have been present as a contaminant. Similarly, Furuya and Manabe (1976) have reported an association of spectroscopically detectable pea phytochrome with both 12,000 *g* ("mitochondrial") and 105,000 *g* pellets involving about 3% and 5%, repectively, of the initial phytochrome present. Again specific activity of both associations was significantly lower than that observed in the crude extract.

In contrast to the associations described above, phytochrome pelletability induced by irradiation may involve a large proportion of the phytochrome pool and is therefore relatively easy to study spectroscopically. In addition, the observation of light-enhanced pelletability appears to lend support to those hypotheses concerning the molecular mode of action of phytochrome which postulate the existence of a receptor for P_{fr} (Hartmann, 1966; Mancinelli and Rabino, 1975; Schäfer, 1975a). As a consequence, this latter association of phytochrome with particulate material has received considerable attention and represents a major area for review at this time.

As will be discussed below, it is now apparent that a majority of investigations of light-induced phytochrome pelletability suffer for at least three critical reasons. First, many investigations have employed *Cucurbita* and a protocol which led to data representing the sum of two different associations. Since it is not possible in most cases to quantitate the separate contributions of these two phenomena to observed results, a proper evaluation of these experiments becomes virtually impossible. Second, many experimental protocols suffered from methodological problems, again leading to potentially erroneous interpretation of data. Third, many reports which did not suffer from either of the above deficiencies have been shown to describe an artifactual association between phytochrome and what is probably degraded ribosomal material.

In an attempt to clarify this confusing status concerning light-induced phytochrome pelletability and its possible biological relevance, I shall initially emphasize selected data which permit a separate evaluation of the two different associations which have received attention. While Quail (1975a) has already reviewed the *in vitro*-induced association discussed below and concluded that it is an artifact, his review was written without the benefit of more recent evidence. Another discussion of this association is therefore desirable. Interpretation of other reports describing phytochrome pelletability will utilize this biased presentation of the two associations.

(a) In vitro-*induced association*

Enhanced pelletability of phytochrome as a consequence of irradiating crude extracts has been described for only four plants: *Cucurbita* (see Quail, 1975a, for review), maize (Quail, 1974b), *Sinapis* (Pratt and Marmé, 1976) and pea (Yamamoto and Furuya, 1975). Most attempts to observe *in vitro*-induced pelletability have failed (Pratt and Marmé, 1976). Attempts to confirm *in vitro*-induced pelletability with maize extracts have also failed (Marmé *et al.*, 1976; Pratt and Marmé, 1976) while only a small increase in pelletability (1.7-fold over control) is observed with pea extracts (Yamamoto and Furuya, 1975; Furuya and Manabe, 1976) compared to the enhancement observed with *Cucurbita* (about 10-fold over control). Thus, it is uncertain whether the phenomenon described with other plants is comparable to the one described using *Cucurbita*. Since *Cucurbita* provides the only *in vitro*-induced pelletability which has been thoroughly studied what follows will be a summary of that system.

A typical protocol for study of *in vitro*-induced pelletability (Fig. 8) utilizes centrifugation of crude extracts in the absence of added divalent cation followed by addition of a divalent cation, irradiation and final centrifugation. Red light-irradiation of a *Cucurbita* extract in optimal reaction medium results in a final pellet containing up to 80% of the extracted phytochrome (Marmé *et al.*, 1973) as compared to only 5–10% in an unirradiated control. In the absence of added divalent cation little or no increase in pelletable phytochrome over the dark control is observed (Marmé *et al.*, 1973). The optimal concentration of either $MgCl_2$ or $CaCl_2$ is about 10 m*M*. A further increase in ionic strength results in reversal of pelletability (Marmé *et al.*, 1973; Quail, 1975b; Pratt and Marmé, 1976). Light-induced pelletability is negligible at a pH of 7.5 or above and increases with decreasing pH down to about 6.5 below which phytochrome becomes unstable (Marmé *et al.*, 1973).

Do divalent cation, low pH and low ionic strength represent requirements for the association of phytochrome with subcellular material which is then pelleted during the final centrifugation (Fig. 8) or are these merely requirements for the aggregation of material with which P_{fr} is already associated? Both Marmé (1974) and Quail (1975c) have demonstrated by sedimentation velocity centrifugation that the divalent cation is required only to induce pelletability of phytochrome which is already associated with its putative binding partner. Quail (1975b, c) has further demonstrated that phytochrome cosediments in sucrose gradients under a wide variety of conditions

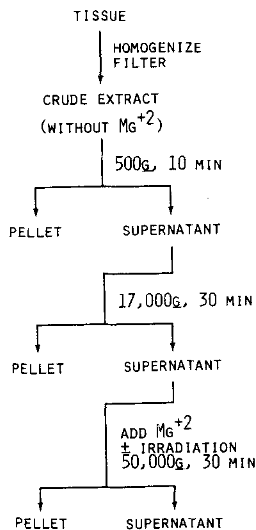

Figure 8. Protocol for assay of *in vitro*-induced phytochrome pelletability in *Cucurbita* extracts. (After Marmé *et al*., 1973)

with 31 S ribonucleoprotein material. The ribonucleoprotein presumably is derived from degraded ribosomal material (Quail and Gressel, 1976). As is the case for the divalent cation requirement, low pH is necessary for induction of pelletability of the 31 S particles and not for association between the particles and phytochrome (Quail, 1975c). Low ionic strength, by contrast, is required for maintenance of the association since the 31 S particles are readily sedimented in the presence of divalent cation at ionic strengths which release phytochrome to the supernatant (Quail, 1975b). For these and other reasons discussed more fully elsewhere (Quail, 1975a; Quail and Gressel, 1976) it is almost certain that this *in vitro*-induced association represents an electrostatic interaction between phytochrome and ribonucleoprotein which, although it may be specific for P_{fr} as opposed to P_r, is not specific for phytochrome.

The above discussion deals with unambiguous experiments which utilized *in vitro* irradiations. A major difficulty in dealing with the pelletability literature is that few experiments are this easy to interpret. The immediately following discussion will attempt to expand our understanding of *in vitro*-induced pelletability using data which, although generally not derived from *in vitro* irradiations, permit significant conclusions to be reached.

A significant limitation in the data summarized above is that heavier organelles and membrane fragments are discarded during the first two centrifugations of the crude extract (Fig. 8). While it is clear that phytochrome associates with 31 S ribonucleoprotein particles *in vitro*, it is not clear that this is the only possible *in vitro* association. Quail (1975b, 1975c) has analyzed sucrose gradients of resuspended phytochrome-containing pellets following irradiation of phytochrome *in vivo*. Because divalent cations aggregate organelles and membrane fragments rendering them impossible to separate by gradient centrifugation, the gradients were run in the absence of added divalent cations. Under these conditions an association of phytochrome not only with 31 S particles but also with larger particles which rapidly reach isopycnic equilibrium near 38% sucrose (w/w) is observed. It is not known whether this "heavy" phytochrome band represents 31 S particles associated with larger subcellular fragments or a unique association between phytochrome and some other subcellular component. This "heavy" band is distinct from commonly used marker enzyme bands and thus is not readily identified although Marmé (1974) suggested it coincides with plasma membrane markers. Since the irradiation was given prior to extraction it is also not clear whether the association yielding the "heavy" phytochrome band can occur *in vitro*, although irradiation of a 500 *g* supernatant may result in association of some phytochrome with "heavy" subcellular material (Quail, personal commun.). In any case, most other investigators have discarded material which yields this "heavy" band and thus reference to *in vitro*-induced pelletability unless otherwise qualified will refer only to the association with 31 S ribonucleoprotein which is obtained by protocols similar in principle to that given in Fig. 8.

Does the association with ribonucleoprotein described above occur *in vivo* as well as *in vitro*? Because extracts of red-irradiated tissue contain P_{fr}, it is not possible using *in vivo* red irradiation to distinguish between an association of P_{fr} which occurs *in vivo* and one which occurs *in vitro*. However, extraction of phytochrome as P_r after a red–far red irradiation cycle *in vivo*, should yield an association with the 31 S particles if it had occurred *in vivo* since such an irradiation sequence *in vitro* does so (Marmé *et al*., 1973). Centrifugation of a crude extract from such red–far red irradiated *Cucurbita* in the absence of added Mg^{2+} indicates that with these conditions 21% of the phytochrome and 28% of the RNA is pelletable (Table 1 in Quail, 1975b). Upon addition of Mg^{2+} to the crude extract any additional phytochrome which may be associated with 31 S particles should then pellet. However, while it is clear that the 31 S particles pellet as evidenced by the increase in RNA pelletability to 41%, little or no additional phytochrome is pelleted (24 vs 21%). By contrast, addition of Mg^{2+} to an extract of red-irradiated control tissue, which is expected to contain phytochrome associated with the 31 S particles, results in the same increase in pelletable RNA (from 29 to 42%) but a much larger increase in phytochrome pelletability (from 39 to 54%). Thus, since a small amount of phytochrome is normally found to nonspecifically coprecipitate with other material in the presence of added Mg^{2+} (Marmé *et al*., 1976), it is possible to conclude from this evidence that no detectable association occurs *in situ* between phytochrome and 31 S ribonucleoprotein particles or material giving rise to the 31 S particles. However, this question requires further consideration

since Quail (personal commun.) has preliminary data which lead to the opposite answer. Sucrose density gradient analysis at pH 7.6 and in the absence of divalent cations of extracts from red–far red irradiated *Cucurbita* indicates an association of phytochrome with what is probably 31 S ribonucleoprotein. The question thus remains unresolved.

Hence, it is clear that an association does occur *in vitro* between phytochrome and at least one other cellular component but that an understanding of this association is confused by the inappropriate protocols which have often been inadvertently employed for its study. The only such association which has been definitively characterized is between phytochrome and what is probably degraded ribosomal material. It is an association which is independent of the presence of added divalent cation and, over a limited range, pH; which is dependent upon low ionic strength; which does not occur *in vivo*; and which occurs with certainty only in *Cucurbita* extracts. It does not occur in most plant extracts tested. As discussed more fully by Quail and Gressel (1976), this association is thus almost certainly an artifact and cannot be implicated in the molecular mode of action of phytochrome.

In apparent contradiction to the conclusion just reached are observations with *Cucurbita* which imply that this *in vitro*-induced association is biologically significant. Jabben and Schäfer (1976) have noted a rhythmic variation in the proportion of phytochrome which is induced to pellet *in vitro*. Penel *et al.* (1976) have observed that pellets containing phytochrome associated with 31 S particles also contain peroxidase activity and have reported that this peroxidase activity may be reversibly modulated by red and far red light. Finally, Schäfer *et al.* (1976) have correlated the characteristics of *in vitro*-induced pelletability with phytochrome destruction and offered this correlation as evidence that a binding of P_{fr} with a receptor site is the first step leading to phytochrome destruction. All three reports described pelletability using protocols comparable to that in Fig. 8. Thus, only the almost certainly artifactual association with 31 S particles could have been expected, since the "heavier" subcellular fractions referred to above had been discarded prior to illumination (Quail, 1975a, c). One can only conclude that the correlations between pelletability and biological activities cited above are more likely to be coincidental than causal.

(b) In *vivo-induced association*

Beginning with the report of Marmé (1974) it has become apparent that there are at least two different associations which lead to enhanced phytochrome pelletability (Marmé *et al.*, 1976; Pratt and Marmé, 1976). In addition to the *in vitro*-induced association described above there is an association which is observed only following irradiation *in situ* (Grombein *et al.*, 1975b). However, as discussed below, it is still not clear in the latter case whether the actual association with pelletable material also occurs *in vivo*, since available evidence is also consistent with the possibility that the association, though induced *in vivo*, nevertheless occurs *in vitro*.

Two approaches are available for studying this latter association. The first is to use a protocol which always involves extraction of phytochrome as P_r (e.g. after a red–far red irradiation cycle) or which utilizes an extraction condition, such as high ionic strength, which prevents the *in vitro*-induced association. The second possibility is to use tissue which does not yield an *in vitro*-induced association under a wide variety of conditions. The second approach is certainly the most direct and the least equivocal.

Since red light-enhanced pelletability in oat extracts represents a 5- to 10-fold increase in the phytochrome content of a relatively low speed pellet (Pratt and Marmé, 1976; Fig. 9) and since irradiation of extracts under a variety of conditions, including those used to observe an enhancement following *in vivo* irradiation, does not result in any enhancement of phytochrome pelletability (Grombein *et al.*, 1975b; Pratt and Marmé, 1976), oat shoots represent a good system for the characterization of *in vivo*-induced pelletability.

An examination of the requirements for observing *in vivo*-induced pelletability (Grombein *et al.*, 1975b; Pratt and Marmé, 1976) indicates that a wide variety of buffers function equally well and that except for added divalent cation, with about 10 m*M* $MgCl_2$ or 10 m*M* $CaCl_2$ being optimal for most tissues, other reagents commonly used in pelletability studies such as sucrose and ethylenediaminetetraacetic acid, are superfluous. Thus a standard protocol (Fig. 9) involves irradiation of intact tissue followed by homogenization in a neutral or slightly basic buffer containing 10 m*M* $MgCl_2$, filtration through nylon or Miracloth, and a single centrifugation. Since organelles and membrane fragments aggregate in the presence of 10 m*M* $MgCl_2$ a relatively low speed centrifugation, such as 15 min at 20,000 *g*, is sufficient (Grombein *et al.*, 1975b). In particular, a low speed precentrifugation must be avoided. Grombein *et al.* reported that over 20% of that phytochrome which would ultimately pellet in response to a red irradiation of oat shoots was already present in a 500 *g*, 5 min pellet. The same observation was made using maize with as much as 50% of the pelletable phyto-

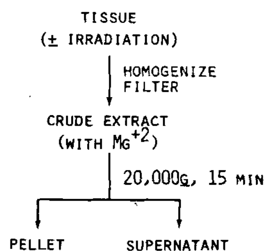

Figure 9. Protocol for assay of *in vivo*-induced phytochrome pelletability. (After Grombein *et al.*, 1975b)

chrome being found in a 1000 *g*, 10 min pellet (Pratt, unpublished). As will be discussed more fully below, most investigators discard such a low speed pellet thus making the resultant data more difficult to interpret.

In contrast to *in vitro*-induced pelletability, that induced *in vivo* is relatively insensitive to extraction conditions. Increasing pH up to about 8.5 has little effect on the level of observed pelletability and ionic strengths which completely eliminate the *in vitro* response have virtually no effect on the *in vivo* response (Pratt and Marmé, 1976). However, unlike the *in vitro* association, *in vivo*-induced pelletability is irreversibly lost if a divalent cation is not present *in vitro* (Pratt and Marmé, 1976; Quail, 1977b). The absence of a divalent cation prevents or reverses the association of phytochrome with its putative binding partner(s) as evidenced (1) by the further observations that readdition of Mg^{2+} does not lead to the repelletability of phytochrome (Pratt and Marmé, 1976) and (2) by the observations that in the absence of added divalent cation phytochrome in extracts of red-irradiated oat shoots sediments with the same velocity in a sucrose gradient (Pratt, unpublished) and is retarded by the same amount on molecular sieve gels (Quail, personal commun.) as phytochrome present in dark control extracts. Yet another difference between the two pelletability phenomena is that the *in vivo*-induced association is a general phenomenon having been observed in all of 11 plants tested, including *Cucurbita* (Pratt and Marmé, 1976).

While it is evident that *in vivo*-induced pelletability is distinct from the *in vitro*-induced association, there is no information in the former case which permits any conclusion concerning the cellular constituent(s) with which phytochrome is associated or any decision as to whether the association may initiate biological activity. Because of the divalent cation requirement a straightforward separation of phytochrome and its putative binding partner(s) from other subcellular debris has not been possible (Quail, personal commun.). Nevertheless, it has been possible to characterize the *in vivo*-induced association as one which has at least potential biological significance.

Is *in vivo*-induced pelletability a trivial Mg^{2+}-induced aggregation of phytochrome and/or coprecipitation with other components of the cell? Two pieces of evidence argue against this possibility. First, addition of Mg^{2+} after extraction of red-irradiated tissue aggregates subcellular debris but does not yield any enhanced pelletability of phytochrome (Pratt and Marmé, 1976). However, if Mg^{2+} is added within seconds after extraction in Mg^{2+}-free buffer pelletability is retained (Quail, 1977b). Second, extraction of red-irradiated tissue immediately following a 5 s saturating red irradiation near 0°C does not result in any enhanced pelletability even though both Mg^{2+} and P_{fr} are present (Pratt and Marmé, 1976; Quail, 1977b). Thus, if Mg^{2+} induces a coprecipitation of phytochrome with other subcellular material, this coprecipitation must occur at or near the moment of extraction in response to a P_{fr}-induced change in the tissue as discussed further below.

It is also possible to conclude that *in vivo*-induced pelletability in oats is not a function of extracting phytochrome as P_{fr} since pelletability after a red–far red cycle is equal to that obtained after a single red irradiation (Pratt and Marmé, 1976). However, this outcome is dependent both upon a brief period of incubation *in situ* (at 0.5°C) between the red and far red irradiations and upon extraction immediately subsequent to the far red irradiation. If a 5 s red exposure at 0.5°C, which yields maximal pelletability of about 60% of the phytochrome, is followed *immediately* by a far red irradiation, *in vivo*-induced pelletability is almost completely prevented (Pratt and Marmé, 1976). The time course for the process which leads to red-induced pelletability has been followed by controlling the time between a 5 s exposure and a 5 s extraction period, the latter stopping the process. The time course follows first order kinetics with a half-life of about 40 s at 0.5°C, it is temperature dependent, and it goes to completion within 5–10 s at 25°C (Pratt and Marmé, 1976). Quail (1977b) has made comparable observations also using oat shoots and has further demonstrated that the process leading to enhanced pelletability may also be stopped by a far red irradiation giving results similar to those obtained when the process is stopped by extraction. Since the process leading to enhanced pelletability is very rapid, attempts to investigate the induction of this phenomenon must utilize brief actinic exposures. The common use of longer exposure times (1–5 min) leads to difficulties in interpretation as discussed below.

Pelletability once induced by an *in vivo* irradiation and therefore not immediately photoreversible is nevertheless slowly reversed during incubation of red–far red irradiated tissue in darkness (Quail *et al.*, 1973). This far red reversal of pelletability (to be contrasted to the far red reversal of the *induction* of pelletability referred to above) is slow with a half-life of about 25 min at 25°C in oat shoots (Pratt and Marmé, 1976) as compared to 50 min originally reported for maize by Quail *et al.* This reversal is temperature dependent with a half-life for oats of about 100 min at 13°C. Below this temperature the process appears temperature independent since the half-life is 100 min at 3°C as well (Pratt and Marmé, 1976). Reversal occurs at a significant rate only *in situ*. *In vitro* incubation of pelletable phytochrome as P_r does not yield any measurable decrease in pelletability over a 2-h period. Incubation of pelletable phytochrome as P_{fr} either *in situ* or *in vitro* also does not result in any decrease in pelletability.

The amount of phytochrome which is induced to pellet in oat shoots is a linear function of the amount of P_{fr} produced during the initial light exposure up to about half-maximal levels of P_{fr} (Pratt and Marmé, 1976). No evidence for cooperativity with respect to

P_{fr} is observed as had been reported by Schäfer (1975b) using both *in vivo* and *in vitro* irradiations with a variety of tissues including oats. The reason for this apparent discrepancy is unknown but Quail (1974a) has also reported an inability to obtain co-operativity in pelletability using maize and *in vitro* irradiations.

Does *in vivo*-induced pelletability represent binding *in vivo* between phytochrome and a receptor as has been postulated (e.g. Boisard *et al.*, 1974; Marmé, 1974; Schäfer, 1975c)? While this is one possible interpretation, available data are consistent with at least two other possibilities. First, phytochrome may always be loosely associated with pelletable cellular constituents. Photoconversion of P_r to P_{fr} may then result in stabilization of this association during a brief incubation *in vivo*. Second, P_{fr} may induce a change in one or more biochemical parameters of the tissue, including as one possibility a change in phytochrome itself, which leads to its association with other cellular constituents during the moment of extraction or shortly thereafter. That formation of an association is limited to the moment of extraction is supported by the observation that if pelletability is prevented at this time (for example, by the absence of added divalent cation) then, it cannot be induced later *in vitro* except with rapidly declining effectiveness during the first 1–2 min after extraction (Quail, personal commun.). Thus, while is is tempting to speculate that pelletability is a measure of an *in situ* phytochrome–receptor interaction (e.g. Fuad and Yu, 1977b), there is yet no definitive evidence which permits this conclusion. As presented in more detail below, attempts to reach this conclusion have been based upon questionable interpretation of data and/or inadequate experimental designs. Interpretation of published data is limited to an operational description of the phenomenon (Fig. 10). It is possible only to conclude that (1) the process leading to the induction of pelletability represents the fastest intracellular response to P_{fr} yet described since it occurs within 5–10 s at 25°C and (2) the characteristics of this pelletability phenomenon are consistent with the possibility that it is of biological significance.

(c) *Interpretation*

As already acknowledged, the above discussion is a biased selection of data designed to present whenever possible only those experiments which represent unambiguously only one of the two types of association which have been characterized. With reference to the above characteristics of *in vitro*- and *in vivo*-induced pelletability, it will be possible to evaluate more critically the larger number of reports dealing with particulate forms of phytochrome which have appeared within the last few years.

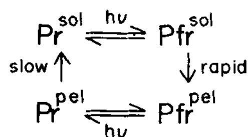

Figure 10. Summary of *in vivo*-induced phytochrome pelletability observations. P_r^{sol} (P_{fr}^{sol}) represents P_r (P_{fr}) which behaves as a soluble protein while P_r^{pel} (P_{fr}^{pel}) represents P_r (P_{fr}) which behaves as a particulate protein following extraction.

The overriding problem which has arisen with respect to investigations of phytochrome pelletability is the realization that more than one phenomenon is involved. Because both *in vitro*- and *in vivo*-induced pelletability are observed with *Cucurbita* (Marmé *et al.*, 1976; Pratt and Marmé, 1976), most experiments which utilize an *in vivo* red irradiation result in an unknown contribution of each of the two phenomena to observed results (Quail *et al.*, 1973, 1976; Boisard *et al.*, 1974; Marmé, 1974; Marmé *et al.*, 1974; Quail, 1975b, c; Schäfer, 1975b; Gressel and Quail, 1976; Quail and Gressel, 1976).

Unless one is interested in the apparently artifactual, *in vitro*-induced association with 31 S particles, there is no justification for continued use of *Cucurbita*. Since *in vitro*-induced pelletability has also been observed with maize (Quail, 1974b; Schäfer, 1975b), it is also important with this tissue to run appropriate controls utilizing *in vitro* irradiations to demonstrate that the latter is not a problem with the protocol being used.

A second difficulty in interpretation of pelletability data derives from the common practice of precentrifuging crude extracts of irradiated tissue in the presence of 10 m*M* $MgCl_2$ (Quail *et al.*, 1973; Boisard *et al.*, 1974; Marmé, 1974; Marmé *et al.*, 1974; Quail, 1975b, c; Schäfer, 1975b; Yu and Carter, 1976b, c, d; Yu *et al.*, 1976a; Fuad and Yu, 1977a, b, c). Although the absence of red light-induced pelletable phytochrome in low speed pellets has been demonstrated (Yu, 1975b), this control has been done in the *absence* of added divalent cation thereby eliminating the problem of coaggregation of organelles and membrane fragments induced by the cation. This control is therefore not applicable to the larger body of data cited. As has been previously mentioned, a low speed pellet already contains a large proportion of that phytochrome which has been induced to pellet by irradiation *in vivo*. Therefore, published experiments which utilize precentrifugation in the absence of any control become suspect. Unfortunately, most reports do not include sufficient data to estimate the contribution of this methodological problem to the results. However, a recent report by Yu and Carter (1976b), in which a 500 ***g***, 10 min pellet is discarded, does have enough information to serve as an example of this potential artifact. Since Yu and Carter have studied pelletability in part as a function of added $MgCl_2$ they have observed that phytochrome recovery from red-irradiated maize in the presence of $MgCl_2$ is only about 72% of that in the absence of $MgCl_2$ (Fig. 1 in Yu and Carter) or in the absence of red irradiation (Table 1 in Yu and Carter). The "missing" 28% is about what would be expected in the discarded 500 ***g***

pellet (Grombein *et al.*, 1975*b*). If one accounts for this presumably discarded, pelletable phytochrome, reported pelletability under optimal conditions increases from about 55% to almost 70%. If the distribution of phytochrome varies between this discarded pellet and the higher speed pellet as a function of the experimental protocol (e.g. varying cation concentration or incubation time) the resulting data would include this artifact and result in misleading conclusions.

A third problem in interpretation arises from the common use of irradiation times beyond that needed to photoconvert P_r to P_{fr} (e.g. Boisard *et al.*, 1974; Quail, 1974a; Quail and Schäfer, 1974; Yu *et al.*, 1976a; Fuad and Yu, 1977a, c). Since the induction of pelletability as a consequence of *in vivo* irradiations is very rapid, going to completion within about 10 s at 25°C (Pratt and Marmé, 1976), irradiation times which exceed a few seconds eliminate the possibility of discriminating between an effect of light on the induction of the phenomenon and on its expression. As a consequence of such a methodological artifact, Yu and co-workers have reached three conclusions. (1) Phytochrome once it has absorbed sufficient energy associates with pelletable material regardless of its form. This conclusion leads to the further suggestion that P_{fr} may not be the only physiologically active form of phytochrome (Yu *et al.*, 1976a). (2) The presumed binding reaction leading to enhanced pelletability is a "primary photoreaction" (Fuad and Yu, 1977c). (3) Red light-induced pelletability is not immediately reversible by far red light (Fuad and Yu, 1977b). Obviously, if the initial actinic red light exposure is longer than the time required for the induction of a response (such as pelletability) to go to completion, then one should not expect it to be reversed by far red light. Appropriately short actinic irradiations result in virtually complete far red reversal of pelletability (Pratt and Marmé, 1976). Also, since light establishes a photoequilibrium mixture between P_r and P_{fr} in both soluble and particulate fractions at *any* wavelength which is absorbed by both forms (Fig. 7; Butler *et al.*, 1964*a*), and since the photoequilibrium is dynamic (Fig. 10), any irradiation, if sufficiently long to result in the presence of each phytochrome molecule as P_{fr} for a long enough period to induce pelletability, should be expected to result in a near maximal response. Thus, even though P_{fr} may be the only form of phytochrome which leads to the induction of pelletability, blue and far red light should still be expected to result eventually in high levels of pelletability as observed by Yu *et al.* (1976a) and Fuad and Yu (1977c). This is especially true since the reversal of light-induced pelletability is very slow compared to its induction. That P_r is observed in the pelletable fraction (Fig. 10) does not mean that it got there as P_r (or as some hypothetical "activated" form of P_r) as has been argued (Yu *et al.*, 1976a; Fuad and Yu, 1977c).

Yet a fourth problem, described by Fuad and Yu (1977a), leads potentially to a new interpretation of earlier reports by Quail *et al.* (1973) and Manabe and Furuya (1975). Fuad and Yu reported that incubation at 0°C of crude maize extracts containing pelletable phytochrome results in a slow *in vitro* dissociation of phytochrome from pelletable material whether phytochrome is present as P_r or P_{fr}. Hence, any experimental design which systematically incorporates a variable incubation time prior to centrifugation would be expected to include this artifact and thus yield a misleading interpretation. Since Pratt and Marmé (1976) included a control for this possible artifact in their study of *in vivo*-induced pelletability in oats and found that phytochrome as either P_r or P_{fr} did not dissociate from pelletable material *in vitro*, it is not clear whether this artifact is of general concern. Thus, it cannot be concluded that the work of Quail *et al.* and Manabe and Furuya include this artifact without further data. Another cause for concern regarding this potential artifact is the observation that the green "safelights" used by Fuad and Yu (1977a) had an actinic effect on phytochrome leading to potentially spurious data. Extracts kept in total darkness did not demonstrate measurable reversion of P_{fr} to P_r while extracts kept under "safelights" demonstrated a significant rate of P_{fr} to P_r conversion. While Fuad and Yu misleadingly refer to this conversion as "reversion" (i.e. a nonphotochemical transformation), it is a clear indication that their "safelight" is not safe.

In addition to the direct attempts to demonstrate phytochrome pelletability discussed above, Yu and co-workers (Yu, 1975a, b, c; Yu *et al.*, 1976b; Yu and Carter, 1976a) have also described enhanced pelletability assayed in the absence of added divalent cation by *in vivo* incubation with glutaraldehyde or an imidoester which is intended to stabilize any association which may have occurred between phytochrome and another cellular constituent prior to extraction. They have demonstrated that conversion of P_r to P_{fr} in maize changes the tissue such that following brief "fixation" an approximately 7- to 8-fold increase in phytochrome pelletability is observed. As for any procedure involving *in situ* crosslinking of protein, the possibility that the association which is thus observed is a preparative artifact may not rigorously be excluded. However, since this association was observed in the absence of added divalent cation, they were able to separate subcellular fractions on sucrose gradients. An initial report (Yu, 1975b) concluded that there was an association of phytochrome with plasma membrane because of a correlation with vesicles which stain with phosphotungstate-chromic acid. A later report (Yu *et al.*, 1976b) indicated that phytochrome did not associate with plasma membrane based upon the absence of any correlation with lactoperoxidase-mediated ^{125}I-labelled material in sucrose gradients. As Quail and Hughes (1977) and Quail and Browning (1977) have recently demonstrated, both of these techniques

can yield misleading data and, in the absence of appropriate controls, firm conclusions regarding the significance of data obtained by these methods are not possible. It is also not yet clear whether the *in vivo*-induced association described by Yu and coworkers is the same as that observed with oats although the time course of the reversal of this light-enhanced pelletability following a far red exposure (Yu, 1975a) is comparable to that observed using a divalent cation as the stabilizing agent (Pratt and Marmé, 1976). Quail (personal commun.) has repeated with oats experiments described above which utilized glutaraldehyde and was unable to obtain an association comparable to that obtained with divalent cation.

One possible approach for an evaluation of potential biological significance of light-enhanced pelletability is to attempt to determine whether phytochrome is changed as a consequence of having become pelletable. Yu and Carter (1976b, c) have reported that maize phytochrome which has once been pelletable becomes as a consequence larger in size as evidenced by more rapid elution from molecular sieve columns. However, these data must be considered preliminary because no precautions concerning proteolysis were taken, no internal standards were cochromatographed with the phytochrome samples, relatively few fractions were collected resulting in poor resolution, and elution volumes of control phytochrome were in disagreement with published values (Pratt, 1973; Rice and Briggs, 1973b; Quail, 1977b).

Grombein and Rüdiger (1976), utilizing gel exclusion chromatography, have also reported an enrichment in the proportion of oat phytochrome present in a "very large" molecular weight form as a consequence of pelletability. Phytochrome shifted from an elution volume near that of ferritin to one closer to the void volume, with enhancement of this shift with "ageing" up to 4 days. They recognized that they had serious difficulties with high protease levels, the latter apparently degrading selectively the phytochrome which eluted at the volume of ferritin. In addition, they were working with very small quantities of phytochrome (no more than 0.003 units/ml in peak fractions) indicating that they may have been assaying only a selected fraction of the phytochrome which had actually been pelletable.

Quail (personal commun.) has also compared, by permeation through agarose, dark control oat phytochrome to that which has been pelleted and then released from the pellet by the removal of divalent cation. In contrast to the above reports, Quail observed no difference between the two in elution volume. Additional comparison of two such oat phytochrome preparations further indicated that no additional band was detectable upon electrophoresis in sodium dodecyl sulfate acrylamide gels (Fig. 4), although phytochrome mobility was decreased by about 4% (Boeshore and Pratt, 1977). In addition, no difference was observed by Ouchterlony double diffusion, immunoelectrophoresis or sedimentation velocity centrifugation assays (Boeshore and Pratt, 1977). The existence of such apparently contradictory reports indicates the need for further examination of this question.

Jose (1977) has recently described the use of gel filtration as an alternate method for isolating and characterizing pelletable phytochrome. She offers further evidence for two distinct kinds of pelletability in *Cucurbita* extracts and confirms by this different method many of the conclusions derived from differential and sucrose gradient centrifugation experiments.

MODE OF ACTION?

A large body of data derived from experiments utilizing intact plant tissues has led to two hypotheses (Mancinelli and Rabino, 1975; Schäfer, 1975a; Satter and Galston, 1976; Marmé, 1977) concerning the mode of action of phytochrome: (1) phytochrome modulates directly one or more membrane functions and (2) phytochrome, as P_{fr}, must bind with a receptor as an initial step in its function. Thus, one is led immediately to the speculation that a membrane-bound receptor for phytochrome exists (Boisard *et al.*, 1974; Schäfer, 1975c; Marmé, 1974; Pratt, 1977).

While phytochrome, at least as P_{fr}, may eventually be shown to be membrane-bound, it is clear from a consideration of its biochemical characteristics that it is not an integral membrane protein (Singer, 1974). The latter is normally insoluble in the absence of detergents while phytochrome is highly water soluble. A peripheral protein, on the other hand, is generally loosely bound, easily dissociated by mild treatments such as high ionic strength or addition of metal ion chelators, and often requires a divalent cation to maintain its association with the membrane *in vitro* (Singer, 1974). While *in vivo*-induced pelletable phytochrome satisfies these criteria, artifactual associations could also meet these same criteria since they are not very rigorous. Thus, phytochrome may behave as though it were a peripheral membrane protein under some circumstances but more definitive evidence is needed before this possibility may be accepted. There is yet no evidence which warrants the conclusion some have already reached that phytochrome is associated directly with membranes when it is pelletable (Marmé, 1974; Schäfer, 1975c) as opposed to being associated with some other cellular constituent which becomes associated with the particulate fraction in the presence of divalent cation.

If it is not yet possible to conclude that phytochrome is a peripheral membrane protein, is it possible at least to conclude that a receptor for P_{fr} exists? Again in contrast to the affirmative conclusion already reached by some (e.g. Schäfer, 1975a; Steinitz *et al.*, 1976), available biochemical evidence requires that this quesion also remain open. As in the argument for a membrane association, physiological data

derived from organismal studies which originally led to these hypotheses still provide the best evidence in their favor. In order to conclude that a receptor for P_{fr} exists, three criteria in common use (Cuatrecasas, 1974; Kende and Gardner, 1976) must first be satisfied. First, the interaction must be specific, not just for P_{fr} vs P_r but also for phytochrome vs all other proteins. Second, the binding affinity must be consistent with known biological activity which would normally be satisfied by a high affinity constant. Third, interaction of P_{fr} with a binding partner must be shown to be related to the expression of its morphogenic activity. As yet, not one of these three criteria have been satisfied with respect to phytochrome. We must conclude that any speculation concerning the existence or identity of a putative receptor is premature.

While the above analysis of our present knowledge concerning the molecular mode of action of phytochrome may seem overly pessimistic, our present understanding of the molecular properties of phytochrome should nevertheless serve as a good background for future work. In particular, the *in vivo*-induced pelletability phenomenon, which remains to be characterized in detail, is consistent so far with a primary step in the chain of events leading to the expression of P_{fr}-mediated morphogenesis. Continued study of this latter phenomenon should establish whether it has such biological relevance.

Acknowledgements—I thank the following for providing me with information of importance to the subject of this review prior to its publication: Drs. J. Boisard, M. Furuya, D. Marmé, P. Quail, W. Rüdiger, R. Satter, H. Scheer, W. Smith and R. Yu. Work from my own laboratory which has been included in this review has been supported by grants from the National Science Foundation (GB-17057 and PCM75-19125).

REFERENCES

Anderson, G. R., E. L. Jenner and F. E. Mumford (1969) *Biochemistry* **8,** 1182–1187.
Anderson, G. R., E. L. Jenner and F. E. Mumford (1970) *Biochim. Biophys. Acta* **221,** 69–73.
Balangé, A. P. (1974) *Physiol. Vég.* **12,** 95–105.
Balangé, A. P. and P. Rollin (1973) *Plant Sci. Lett.* **1,** 59–64.
Boeshore, M. and L. Pratt (1977) *Plant Physiol.* Suppl. **59,** 101.
Boisard, J. and M. Cordonnier (1976) *Physiol. Vég.* **14,** 411–421.
Boisard, J., D. Marmé and W. R. Briggs (1974) *Plant Physiol.* **54,** 272–276.
Boisard, J., D. Marmé and E. Schäfer (1971) *Planta* **99,** 302–310.
Boisard, J., C. J. P. Spruit and P. Rollin (1968) *Mededelingen Landbouwhogeschool Wageningen* 68–17.
Boisard, J., B. Gabriac-Marchal and P. Rollin (1974) *Physiol. Vég.* **12,** 601–616.
Borthwick, H. A., S. B. Hendricks, M. J. Schneider, R. B. Taylorson and V. K. Toole (1969) *Proc. Natl. Acad. Sci. U.S.* **64,** 479–486.
Briggs, W. R. and D. C. Fork (1969) *Plant Physiol.* **44,** 1081–1088.
Briggs, W. R. and H. V. Rice (1972) *Ann. Rev. Plant Physiol.* **23,** 293–334.
Briggs, W. R., W. D. Zollinger and B. B. Platz (1968) *Plant Physiol.* **43,** 1239–1243.
Briggs, W. R., H. V. Rice, G. Gardner and C. S. Pike (1972) In *Recent Advances in Phytochemistry* (Edited by V. C. Runeckles and T. C. Tso), Vol. 5, pp. 35–50. Academic Press, New York.
Burke, M. J., D. C. Pratt and A. Moscowitz (1972) *Biochemistry* **11,** 4025–4031.
Butler, W. L. (1964) *Ann. Rev. Plant Physiol.* **15,** 451–470.
Butler, W. L. (1972) In *Phytochrome* (Edited by K. Mitrakos and W. Shropshire, Jr.), pp. 185–192. Academic Press, London.
Butler, W. L. and D. W. Hopkins (1970) *Photochem. Photobiol.* **12,** 439–450.
Butler, W. L., S. B. Hendricks and H. W. Siegelman (1964a) *Photochem. Photobiol.* **3,** 521–528.
Butler, W. L., H. W. Siegelman aand C. O. Miller (1964b) *Biochemistry* **3,** 851–857.
Butler, W. L., K. H. Norris, H. W. Siegelman and S. B. Hendricks (1959) *Proc. Natl. Acad. Sci. U.S.* **45,** 1703–1708.
Chorney, W. and S. A. Gordon (1966) *Plant Physiol.* **41,** 891–896.
Clayton, R. K. (1970) *Light and Living Matter*, Vol. 1, 148 pp. McGraw-Hill, New York.
Coleman, R. A. and L. H. Pratt (1974a) *J. Histochem. Cytochem.* **22,** 1039–1047.
Coleman, R. A. and L. H. Pratt (1974b) *Planta* **119,** 221–231.
Correll, D. L., J. L. Edwards and W. Shropshire, Jr. (1968a) *Photochem. Photobiol.* **8,** 465–475.
Correll, D. L., J. L. Edwards, W. H. Klein and W. Shropshire, Jr. (1968b) *Biochim. Biophys. Acta* **168,** 36–45.
Correll, D. L., E. Steers, Jr., K. M. Towe and W. Shropshire, Jr. (1968c) *Biochim. Biophys. Acta* **168,** 46–57.
Cross, D. R., H. Linschitz, V. Kasche and J. Tenenbaum (1968) *Proc. Natl. Acad. Sci. U.S.* **61,** 1095–1101.
Cuatrecasas, P. (1974) *Ann. Rev. Biochem.* **43,** 169–214.
Cundiff, S. C. (1973) Ph.D. dissertation, Vanderbilt University, Nashville, TN.
Cundiff, S. C. and L. H. Pratt (1973) *Plant Physiol.* **51,** 210–213.
Cundiff, S. C. and L. H. Pratt (1975a) *Plant Physiol.* **55,** 212–217.
Cundiff, S. C. and L. H. Pratt (1975b) *Plant Physiol.* **55,** 207–211.
Darnall, D. W. and I. M. Klotz (1972) *Arch. Biochem. Biophys.* **149,** 1–14.
Dooskin, R. H. and A. L. Mancinelli (1968) *Bull. Torrey Botan. Club* **95,** 474–487.
Evans, A. and H. Smith (1976) *Nature* **259,** 323–325.
Everett, M. S., W. R. Briggs and W. K. Purves (1970) *Plant Physiol.* **45,** 805–806.

Frankland, B. (1972) In *Phytochrome* (Edited by K. Mitrakos and W. Shropshire, Jr.), pp. 195–225. Academic Press, London.
Fry, K. T. and F. E. Mumford (1971) *Biochem. Biophys. Res. Commun.* **45,** 1466–1473.
Fuad, N. and R. Yu (1977a) *Plant Cell Physiol.* **18,** 35–43.
Fuad, N. and R. Yu (1977b) *Photochem. Photobiol.* **25,** 491–496.
Fuad, N. and R. Yu (1977c) *Z. Pflanzenphysiol.* **81,** 304–307.
Furuya, M. and K. Manabe (1976) In *Light and Plant Development* (Edited by H. Smith), pp. 143–155. Butterworth, London.
Gardner, G. and W. R. Briggs (1974) *Photochem. Photobiol.* **19,** 367–377.
Gardner, G., W. F. Thompson and W. R. Briggs (1974) *Planta* **117,** 367–372.
Gardner, G., C. S. Pike, H. V. Rice and W. R. Briggs (1971) *Plant Physiol.* **48,** 686–693.
Govindjee and R. Govindjee (1975) In *Bioenergetics of Photosynthesis* (Edited by Govindjee), pp. 1–50. Academic Press, New York.
Govindjee and G. Papageorgiou (1971) In *Photophysiology* (Edited by A. C. Giese), Vol. 6, pp. 1–46. Academic Press, New York.
Gressel, J. and P. H. Quail (1976) *Plant Cell Physiol.* **17,** 771–786.
Grill, R. (1972) *Planta* **108,** 185–202.
Grombein, S. and W. Rüdiger (1976) *Hoppe-Seylers Z. Physiol. Chem.* **357,** 1015–1018.
Grombein, S., W. Rüdiger and H. Zimmerman (1975a) *Hoppe-Seylers Z. Physiol. Chem.* **356,** 1709–1714.
Grombein, S., W. Rüdiger, L. Pratt and D. Marmé (1975b) *Plant Sci. Lett.* **5,** 275–280.
Hendricks, S. B. (1974) In *Photophysiology* (Edited by A. C. Giese), Vol. 1, pp. 305–331. Academic Press, New York.
Hartmann, K. M. (1966) *Photochem. Photobiol.* **5,** 349–366.
Hopkins, D. W. (1971) Ph.D. dissertation, University of California, San Diego.
Hopkins, D. W. and W. L. Butler (1970) *Plant Physiol.* **45,** 567–570.
Hunt, R. E. and L. H. Pratt (1977) *Plant Physiol.* Suppl. **59,** 100.
Jabben, M. and E. Schäfer (1976) *Nature* **259,** 114–115.
Jose, A. (1977) *Planta* **134,** 287–293.
Kende, H. and G. Gardner (1976) *Ann. Rev. Plant Physiol.* **27,** 267–290.
Kendrick, R. E. (1977) In *Research in Photobiology* (Edited by A. Castellani), in press. Plenum, New York.
Kendrick, R. E. and H. Smith (1976) In *Chemistry and Biochemistry of Plant Pigments* (Edited by T. W. Goodwin), Vol. 2, 2nd ed., pp. 334–364. Academic Press, London.
Kendrick, R. E. and C. J. P. Spruit (1972) *Nature (New Biol.)* **237,** 281–282.
Kendrick, R. E. and C. J. P. Spruit (1973a) *Photochem. Photobiol.* **18,** 139–144.
Kendrick, R. E. and C. J. P. Spruit (1973b) *Photochem. Photobiol.* **18,** 153–159.
Kendrick, R. E. and C. J. P. Spruit (1973c) *Plant Physiol.* **52,** 327–331.
Kendrick, R. E. and C. J. P. Spruit (1974) *Planta* **120,** 265–272.
Kendrick, R. E. and C. J. P. Spruit (1976) In *Light and Plant Development* (Edited by H. Smith), pp. 31–43. Butterworth, London.
Kendrick, R. E. and C. J. P. Spruit (1977) *Photochem. Photobiol.* **26,** 201–214.
Kidd, G. H. and L. H. Pratt (1973) *Plant Physiol.* **52,** 309–311.
Linschitz, H. and V. Kasche (1967) *Proc. Natl. Acad. Sci. U.S.* **58,** 1059–1064.
Linschitz, H., V. Kasche, W. L. Butler and H. W. Siegelman (1966) *J. Biol. Chem.* **241,** 3395–3403.
Lisansky, S. G. and A. W. Galston (1974) *Plant Physiol.* **53,** 352–359.
Lisansky, S. G. and A. W. Galston (1976) *Plant Physiol.* **57,** 188–191.
Mackenzie, J. M., Jr. (1976) Ph.D. Dissertation, Harvard University, Cambridge, MA.
Mackenzie, J. M., Jr., R. A. Coleman, W. R. Briggs and L. H. Pratt (1975) *Proc. Natl. Acad. Sci. U.S.* **72,** 799–803.
Manabe, K. and M. Furuya (1971) *Plant Cell Physiol.* **12,** 95–101.
Manabe, K. and M. Furuya (1975) *Planta* **123,** 207–215.
Mancinelli, L. and I. Rabino (1975) *Plant Physiol.* **56,** 351–355.
Marmé, D. (1974) *J. Supramol. Struct.* **2,** 751–768.
Marmé, D. (1977) *Ann. Rev. Plant Physiol.* **28,** 173–198.
Marmé, D., J. Boisard and W. R. Briggs (1973) *Proc. Natl. Acad. Sci. U.S.* **70,** 3861–3865.
Marmé, D., J. Bianco and J. Gross (1976) In *Light and Plant Development* (Edited by H. Smith), pp. 95–110. Butterworth, London.
Marmé, D., J. M. Mackenzie, Jr., J. Boisard and W. R. Briggs (1974) *Plant Physiol.* **54,** 263–271.
Mitrakos, K. and W. Shropshire, Jr. (Editors) (1972) *Phytochrome.* Academic Press, New York. 646 pp.
Mohr, H. (1966) *Photochem. Photobiol.* **5,** 469–483.
Mohr, H. (1972) *Lectures on Photomorphogenesis.* Springer-Verlag, Berlin. 237 pp.
Mohr, H. (1974) *Photochem. Photobiol.* **20,** 539–542.
Mumford, F. E. and E. L. Jenner (1966) *Biochemistry* **5,** 3657–3662.
Mumford, F. E. and E. L. Jenner (1971) *Biochemistry* **10,** 98–101.
Negbi, M., D. W. Hopkins and W. R. Briggs (1975) *Plant Physiol.* **56,** 157–159.
Oelze-Karow, H. and H. Mohr (1973) *Photochem. Photobiol.* **18,** 319–330.
Papageorgiou, G. (1975) In *Bioenergetics of Photosynthesis* (Edited by Govindjee), pp. 319–371. Academic Press, New York.
Parker, C. W. (1976) *Radioimmunoassay of Biologically Active Compounds.* 239 pp. Prentice-Hall, Englewood Cliffs, NJ.
Penel, C., H. Greppin and J. Boisard (1976) *Plant Sci. Lett.* **6,** 117–121.

Pike, C. S. and W. R. Briggs (1972a) *Plant Physiol.* **49**, 521–530.
Pike, C. S. and W. R. Briggs (1972b) *Plant Physiol.* **49**, 514–520.
Pratt, L. H. (1973) *Plant Physiol.* **51**, 203–209.
Pratt, L. H. (1975a) *Photochem. Photobiol.* **21**, 99–103.
Pratt, L. H. (1975b) *Photochem. Photobiol.* **22**, 33–36.
Pratt, L. H. (1976) In *Light and Plant Development* (Edited by H. Smith), pp. 19–30. Butterworth, London.
Pratt, L. H. (1977) In *Research in Photobiology* (Edited by A. Castellani), in press. Plenum, New York.
Pratt, L. H. and W. R. Briggs (1966) *Plant Physiol.* **41**, 467–474.
Pratt, L. H. and W. L. Butler (1968) *Photochem. Photobiol.* **8**, 477–485.
Pratt, L. H. and W. L. Butler (1970a) *Photochem. Photobiol.* **11**, 503–509.
Pratt, L. H. and W. L. Butler (1970b) *Photochem. Photobiol.* **11**, 361–369.
Pratt, L. H. and R. A. Coleman (1971) *Proc. Natl. Acad. Sci. U.S.* **68**, 2431–2435.
Pratt, L. H. and R. A. Coleman (1974) *Am. J. Bot.* **61**, 195–202.
Pratt, L. H. and S. C. Cundiff (1975) *Photochem. Photobiol.* **21**, 91–97.
Pratt, L. H. and D. Marmé (1976) *Plant Physiol.* **58**, 686–692.
Pratt, L. H., R. A Coleman and J. M. Mackenzie, Jr. (1976) In *Light and Plant Development* (Edited by H. Smith), pp. 75–94. Butterworth, London.
Pratt, L. H., G. H. Kidd and R. A. Coleman (1974) *Biochim. Biophys. Acta* **365**, 93–107.
Purves, W. K. and W. R. Briggs (1968) *Plant Physiol.* **43**, 1259–1263.
Quail, P. H. (1974a) *Planta* **118**, 357–360.
Quail, P. H. (1974b) *Planta* **118**, 345–355.
Quail, P. H. (1975a) *Photochem. Photobiol.* **22**, 299–301.
Quail, P. H. (1975b) *Planta* **123**, 223–234.
Quail, P. H. (1975c) *Planta* **123**, 235–246.
Quail, P. H. (1976) In *Plant Biochemistry*, (Edited by J. Bonner and J. E. Varner), 3rd ed. pp. 683–711. Academic Press, New York.
Quail, P. H. (1977a) *Proc. Annual European Symposium on Photomorphogenesis*, p. 78, Bet Dagan, Israel.
Quail, P. H. (1977b) *Photochem. Photobiol.*, in press.
Quail, P. H. and A. Browning (1977) *Plant Physiol.* **59**, 759–766.
Quail, P. H. and J. Gressel (1976) In *Light and Plant Development* (Edited by H. Smith), pp. 111–128. Butterworth, London.
Quail, P. H. and J. E. Hughes (1977) *Planta* **133**, 169–177.
Quail, P. H. and E. Schäfer (1974) *J. Membrane Biol.* **15**, 393–404.
Quail, P. H., E. A. Gallagher and A. R. Wellburn (1976) *Photochem. Photobiol.* **24**, 495–498.
Quail, P. H., D. Marmé and E. Schäfer (1973) *Nature* (*New Biol.*) **245**, 189–190.
Rice, H. V. and W. R. Briggs (1973a) *Plant Physiol.* **51**, 939–945.
Rice, H. V. and W. R. Briggs (1973b) *Plant Physiol.* **51**, 927–938.
Rice, H. V., W. R. Briggs, and C. J. Jackson-White (1973) *Plant Physiol.* **51**, 917–926.
Roux, S. J. (1972) *Biochemistry* **11**, 1930–1936.
Roux, S. J. and W. S. Hillman (1969) *Arch. Biochem. Biophys.* **131**, 423–429.
Roux, S. J., S. G. Lisansky and B. M. Stoker (1975) *Physiol. Plant* **35**, 85–90.
Rubinstein, B., K. S. Drury and R. B. Park (1969) *Plant Physiol.* **44**, 105–109.
Satter, R. L. and A. W. Galston (1976) In *Chemistry and Biochemistry of Plant Pigments*, 2nd ed. (Edited by T. W. Goodwin), Vol. 1, pp. 680–735. Academic Press, London.
Schäfer, E. (1975a) *J. Math. Biol.* **2**, 41–56.
Schäfer, E. (1975b) *Photochem. Photobiol.* **21**, 189–191.
Schäfer, E. (1975c) In *Membrane Transport in Plants* (Edited by U. Zimmermann and J. Dainty) pp. 435–440. Springer-Verlag, Berlin.
Schäfer, E., T. U. Lassig and P. Schopfer (1976) *Photochem. Photobiol.* **24**, 567–572.
Schäfer, E., B. Marchal and D. Marmé (1972) *Photochem. Photobiol.* **15**, 457–464.
Scheer, H. (1976) *Z. Naturforsch.* **31c**, 413–417.
Scheer, H. and C. Krauss (1977) *Photochem. Photobiol.* **25**, 311–314.
Schmidt, W., D. Marmé, P. Quail and E. Schäfer (1973) *Planta* **111**, 329–336.
Schoch, S. and W. Rüdiger (1976) *Liebigs Ann. Chem.* **1976**, 559–565.
Shimazaki, Y. and M. Furuya (1975) *Plant Cell Physiol.* **16**, 623–630.
Siegelman, H. W. and E. M. Firer (1964) *Biochemistry* **3**, 418–423.
Siegelman, H. W., B. C. Turner and S. B. Hendricks (1966) *Plant Physiol.* **41**, 1289–1292.
Singer, S. J. (1974) *Ann. Rev. Biochem.* **43**, 805–833.
Smith, H. (1975) *Phytochrome and Photomorphogenesis*, 235 pp. McGraw-Hill, Maidenhead, England.
Smith, H. (Editor) (1976) *Light and Plant Development*, 516 pp. Butterworth, London.
Smith, H. and J. Elliot (1975) *Plant Sci. Lett.* **5**, 1–6.
Smith, H. and R. E. Kendrick (1976) In *Chemistry and Biochemistry of Plant Pigments*, 2nd ed. (Edited by T. W. Goodwin), Vol. 1, pp. 377–424. Academic Press, London.
Smith, W. O. Jr. and D. L. Correll (1975) *Plant Physiol.* **56**, 340–343.
Sober, H. A. (Editor) (1970) *Handbook of Biochemistry. Selected Data for Molecular Biology*, 2nd ed. CRC Press, Cleveland OH.
Song, P. S., Q. Chae and W. R. Briggs (1975) *Photochem. Photobiol.* **22**, 75–76.
Spruit, C. J. P. (1967) *Biochim. Biophys. Acta* **143**, 260–262.
Spruit, C. J. P. (1970) *Mededelingen Landbouwhogeschool Wageningen* 70–14.
Spruit, C. J. P. (1971) *Mededelingen Landbouwhogeschool Wageningen* 71–21.
Spruit, C. J. P. (1972) In *Phytochrome* (Edited by K. Mitrakos and W. Shropshire, Jr.), pp. 77–104. Academic Press, London.

Spruit, C. J. P. and R. E. Kendrick (1972) *Planta* **103**, 319–326.
Spruit, C. J. P. and R. E. Kendrick (1973) *Photochem. Photobiol.* **18**, 145–152.
Spruit, C. J. P. and H. C. Spruit (1972) *Biochim. Biophys. Acta* **275**, 401–413.
Steinitz, B., H. Drumm and H. Mohr (1976) *Planta* **130**, 23–31.
Sugimoto, T., K. Ishikawa and H. Suzuki Z1976) *J. Phys. Soc. Jpn.* **40**, 258–266.
Ternynck, T. and S. Avrameas (1972) *FEBS Lett.* **23**, 24–28.
Tobin, E. M. and W. R. Briggs (1973) *Photochem. Photobiol.* **18**, 487–495.
Tobin, E. M., W. R. Briggs and P. K. Brown (1973) *Photochem. Photobiol.* **18**, 497–503.
Walker, T. S. and J. L. Bailey (1970a) *Biochem. J.* **120**, 613–622.
Walker, T. S. and J. L. Bailey (1970b) *Biochem. J.* **120**, 607–612.
Weber, K. and M. Osborn (1969) *J. Biol. Chem.* **244**, 4406–4412.
Yamamoto, K. T. and M. Furuya (1975) *Planta* **127**, 177–186.
Yu, R. (1975a) *Aust. J. Plant Physiol.* **2**, 281–289.
Yu, R. (1975b) *J. Exp. Bot.* **26**, 808–822.
Yu, R. (1975c) *Aust. J. Plant Physiol.* **2**, 273–279.
Yu, R. and J. Carter (1976a) *J. Exp. Bot.* **27**, 283–293.
Yu, R. and J. Carter (1976b) *Ann. Bot.* **40**, 647–649.
Yu, R. and J. Carter (1976c) *Plant Cell Physiol.* **17**, 1321–1328.
Yu, R. and J. Carter (1976d) *Plant Cell Physiol.* **17**, 1309–1319.
Yu, R., N. Fuad and D. J. Carr (1976a) *Plant Cell Physiol.* **17**, 1131–1139.
Yu, R., J. Carter and T. Osawa (1976b) *J. Exp. Bot.* **27**, 294–302.