SPECTRAL PROPERTIES, MEMBRANE ASSOCIATION,

AND STABILITY OF OAT PHYTOCHROME.

Thesis submitted to the
University of Leicester
for the
degree of Doctor of Philosophy.

BY

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The nature of the association of etiolated oat seedling phytochrome with a crude membranous fraction was investigated and correlated with the parallel changes in a phytochrome-modulated physiological response.

Firstly, the spectrophotometric assay of phytochrome in clear and turbid solutions was examined. Within given limits, in neither sample type was there an artifactual component of the phytochrome reading. The addition of CaCO₃ particles was found to be capable of giving rise to artifacts and was therefore rejected.

A strongly hydrophobic association of around 5% of total phytochrome with a pelletable fraction was observed using etiolated tissue. Red light (R) in vivo increased this association to around 9%, an effect totally reversible by far-red light (FR) given immediately. The association appeared rather stronger for phytochrome pelleted after R than for "dark" or "R + FR" pelleted phytochrome.

Supernatant and resuspended pellet phytochrome were found not to be significantly different in terms of their spectral characteristics or stability in vitro.

Pfr disappeared in vivo more quickly from the pelletable fraction than from the supernatent fraction of seedlings given an increasing incubation in darkness after R. The amount of pelletable Pr remained relatively stable while supernatant Pr was lost. These observations suggested that supernatant Pfr was lost during the incubation, while pelletable Pfr was subject to both dark reversion and loss, although it was possible that supernatant Pr became pelletable.

During the incubation, the percentage of pelletable phytochrome rose, and FR reversibility of the R-induced increase in phytochrome pelletability was lost, as was FR reversibility of the inhibition of etiolated oat coleoptile elongation induced by R. Both these losses in FR reversibility correlated with the change in total Pfr in supernatant, pellet and tissue samples, but with the change in the proportion of Pfr, only in the pellet sample.

The significance of the observations is discussed with reference to the mode of action of phytochrome and a model is put forward to explain the observed characteristics of phytochrome association with the membranous pelletable fraction.
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| g            | a: gravitational force  
b: gram |
| h            | Planck's constant ($6.6 \times 10^{-34}$ Joule sec) |
| hr           | hour |
| in vitro     | in extracts from tissue |
| in vivo      | in live whole tissue |
| λ            | wavelength |
| ml           | millilitre |
| mm           | millimetre |
| mM           | millimolar |
| ν             | frequency of light energy |
| nm           | nanometre |
| p            | resuspended pellet |
| P            | phytochrome |
| PFR          | phytochrome in the Far-red light |
| P(p)         | total phytochrome in the resuspended pellet fraction. |
| Pr           | phytochrome in the red light-absorbing form. |
| P(sn)        | total phytochrome in the supernatant fraction. |
| Ptot         | total phytochrome in a sample |
| R            | red irradiation |
| R + FR       | red, followed by Far-red irradiations |
| r.p.m        | revolutions per minute |
| { }          | concentration. |
| MOPS         | Morpholinopropane sulfonic acid |
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GENERAL INTRODUCTION
In 1952, Borthwick, Hendricks and co-workers first demonstrated red/far-red photoreversibility, using the light-induced seed germination system (Borthwick, Hendricks, Parker, Toole and Toole, 1952). This discovery led to predictions concerning the nature of the pigment responsible for such photocontrol, and thus to the isolation and quantification of the pigment (Butler, Norris, Siegelman and Hendricks, 1959). The name phytochrome was adopted for the pigment and subsequently, many physiological plant responses have been shown to be modulated by phytochrome (see Smith, 1975, for general summary). The molecular properties of phytochrome and the basis of photoreversibility of the molecule have also been greatly elucidated in recent years (see Pratt, 1978, for review).

Throughout the years of investigation into phytochrome, a major objective has been the clarification of the primary mode of action of phytochrome. Despite a large volume of research, this objective has still not been achieved, largely because of the universal difficulties encountered in attempting to correlate specific molecular events with observed whole plant physiological responses. However, three main theories to explain the primary action of phytochrome have been established. These are briefly:

a) phytochrome controls gene activation by direct interaction with nuclear material (Mohr, 1966).

b) phytochrome controls enzyme activity by direct combination with enzyme molecules (eg: Tezuka and Yamamoto, 1974).
c) phytochrome interacts with membranes, either directly, or via a receptor molecule (Hendricks and Borthwick, 1967).

The evidence relating to the three theories has been reviewed recently by Mohr, (1972), (a); Schopfer, (1977), (b); and Marmè, (1977), (c).

Evidence suggests that these three possible modes of action of phytochrome may not be mutually incompatible; however, the primary (i.e; the first in time) action of phytochrome after photoconversion must occur almost instantaneously as phytochrome has been implicated in reactions occurring within seconds of irradiation, such as root tip adhesion (Yunghans and Jaffé, 1970), coleoptile surface electropotential changes (Newman and Briggs, 1972), and ATP content changes (White and Pike, 1974); and even within milliseconds of irradiation in the example of chloroplast orientation induction in Mougeotia (Haupt and Bretz, 1976). Such observations are consistent with a phytochrome interaction with a cell membrane or membranes as proposed by Hendricks and Borthwick (1967). There are numerous reports in the literature implicating phytochrome interaction with various cell membranes, based on physiological observations (eg: Haupt and Weisenseel, 1976), microspectrophotometric measurements (Galston, 1968), immunocytochemical localisation (Coleman and Pratt, 1974,a), and various in vitro fractionation studies, which are discussed in detail in Section II, and reviewed by Marmè, (1977). From the information available, it appears possible that phytochrome may be associated with many or all cell
membranes, acting upon each one to elicit different responses.

The major deficiency in all these studies has been the lack of correlation of changes in measurable phytochrome in an organ or membrane with the extent of a physiological response elicited in that system. Such combined measurements are often impossible, and, in cases where attempts to correlate physiological responses with changes in phytochrome content have been made, they have usually been done utilizing measurements made on whole seedlings. Such experiments have tended to yield variable results. Where a correlation between a response and the amount of Pfr in whole plants has been shown, nothing can be said about the intracellular location of the phytochrome responsible for modulation of the responses. However, in a number of cases, phytochrome modulation of a response has been demonstrated by the presence of red/far-red reversibility, while changes in detectable phytochrome could not be observed. For example, Hillman, (1968), has shown that far-red light is still capable of reversing red-induced inhibition of Pisum stem segment elongation, after spectrophotometrically detectable Pfr has disappeared. Briggs and Chon (1966) showed that red light sensitivity of the phototropic system was saturated at an irradiation level two orders of magnitude lower than that which gave rise to a measurable photoconversion of phytochrome. Also, this saturated phototropic sensitivity response was reversible by far-red light which itself gave rise to a minimum of 4% Pfr. Thus, far-red reversibility in this example was accompanied
by an increase in measurable Pfr. On the basis of these observations, both Hillman (1965, 1967) and Briggs and Chon (1966) suggested the possible existence of at least two different populations of phytochrome; a large, physiologically inactive pool of phytochrome which contributes to the majority of the spectrophotometric reading, and a small, physiologically active pool of phytochrome whose spectrophotometric measurement is masked by, and which exhibits different kinetics to the "bulk" fraction.

Some evidence has been found which supports the concept of the existence of kinetically distinguishable phytochrome populations. Butler and Lane (1965) showed that corn Pfr was subject to loss (destruction) only, in darkness in vivo, while cauliflower Pfr reverted to Pr in darkness in vivo. In vitro preparations from both etiolated rye and oat seedlings incubated as Pfr in darkness exhibited two or more kinetically distinguishable dark reverting phytochrome populations (Correll, Edwards and Shropshire, 1968; Pike and Briggs, 1972). However, these observations do not provide direct proof of the in vivo existence of two distinct populations of phytochrome within the same tissue.

The findings of Rubenstein, Drury and Park (1969), that etiolated oat 20,000 g supernatant and pelletable phytochrome exhibited strikingly different stabilities when incubated in white light in vitro gave renewed support to both the theory of "bulk" and "active" phytochrome, and that of membranes being the site of phytochrome primary action; as the 20,000 g pellet consisted of a crude membrane preparation, while the 20,000 g supernatant was representative of the cytosol.
In 1973, an increase in the amount of phytochrome associated with a 20,000 g pellet from Cucurbita, induced by either in vivo red irradiation (Quail, Marmé and Schafer, 1973), or in vitro red irradiation (Marmé, Boisard and Briggs, 1973) was reported. The in vitro association was later shown to be artifactual (Quail, 1975, a and c), but the in vivo association has been thoroughly investigated and characterized for a number of species (Pratt and Marmé, 1976), and shown to be irreversibly induced within 5 seconds of red irradiation (Pratt and Marmé, 1976; Quail, 1978,b). Such rapid kinetics have given rise to the proposal that this in vivo induced increase in phytochrome pelletability is a manifestation of the primary action of phytochrome upon photoconversion to Pfr (see Pratt, 1978 for review).

However, there are a number of serious drawbacks to such a proposal. Despite a great deal of effort, the increase in pelletability has not been proved to be a true in vivo event; association of phytochrome with pelletable material is maintained in vitro only in the presence of 10 mM divalent cations; the amount of phytochrome induced to pellet by red light (up to 60% of Ptot) does not correspond to a small "active" fraction; and the amounts of phytochrome pelletability induced by different irradiations have not yet been positively correlated with the levels of any physiological response induced by the same irradiations.

Close examination of the literature reveals that the supernatant and pelletable fractions studied by Rubenstein, Drury and Park (1969) were extracted without divalent
cations, and their pelletable fraction exhibited only around 5% of the total phytochrome present in the original filtrate before centrifugation. The reactions of this fraction have largely been ignored in the recent work upon in vivo phytochrome pelletability, because the amount of phytochrome pelleted shows little difference between treatments compared with the differences observed for "+ Mg\(^{2+}\)" extracts, and is so low as to make accurate spectrophoto-
metric measurement very difficult. However, it seemed reasonable to assume that a candidate for a small "active" phytochrome fraction would exhibit such properties, and the findings of Rubenstein, Drury and Park (1969) were judged to warrant further detailed investigation.

This thesis reports on the experiments undertaken to elucidate the properties of supernatant and pelletable phytochrome (prepared without divalent cations) including any differences between the two fractions, and to characterise the association of phytochrome with pelletable material. A crucial part of the work was an attempt to correlate changes in the phytochrome fractions - where differences between them developed - with changes in a physiological response, in order to investigate the possible significance of any such observed differences. The work is split into four distinct Sections. Section I deals with methodological problems encountered when performing spectrophotometric measurements, which had to be overcome to allow the direct comparison of supernatant and resuspended pellet phytochrome readings and the calculation of phytochrome pelletability to be carried out. Section II
investigates the nature of the association of phytochrome with pelletable material and the effect of \textit{in vivo} and \textit{in vitro} irradiations upon this association. Section III compares extracted supernatant and resuspended pellet phytochrome under various conditions \textit{in vitro} to see whether any differences between the two fractions could be observed which might contribute to the \textit{in vivo} properties of phytochrome. Section IV attempts to correlate changes in supernatant and pelletable phytochrome occurring \textit{in vivo} as a result of \textit{in vivo} irradiations, with the corresponding changes in a physiological response. The results of each Section are considered both specifically within that Section and also in the General Discussion at the end of the thesis.
SECTION I

1: **Introduction**

During the course of the work reported here, it was necessary to make quantitatively accurate spectrophotometric assays of phytochrome in qualitatively different extracted samples. The samples were of two different types; clear supernatants from a 30 000 g centrifugation, which had low total absorbance and insignificant light scattering activity; and turbid resuspended pellets of particulate material derived from the same centrifugation conditions, which had high apparent absorbance and significant scattering activity. In order to calculate pelletability values (see Appendix I), phytochrome measurements from supernatant and resuspended pellet samples had to be combined, thus it was essential for these measurements to be directly comparable. To this end, an examination of the relevant literature was made, followed by an evaluation of the effect on phytochrome readings of altering the significant parameters of the spectrophotometric measuring system. A suitable set of conditions was then defined under which combinations of the readings was valid.

2: **Literature Survey**

The major problem with turbid suspensions is that the scattering of incident light by particles in suspension tends to result in an artifactual increase in the path-length of the measuring beam through the sample (Butler, 1962). This increased pathlength results in an
intensification of the apparent sample absorbance as the light has more opportunities to strike, and thus be absorbed by a pigment molecule. Direct comparison of measurements in clear and turbid samples would therefore seem unwise, without firstly determining the effect of particles in suspension upon absorbance measurements. Many workers have attempted to overcome the problem of turbid samples by adding calcium carbonate (CaCO₃) powder to their samples (see Table I.1). The theory behind this modification of the assay procedure is again attributable to Butler (Butler, 1962). CaCO₃ particles are non-absorbing, and a dense slurry of CaCO₃ in suspension is highly light-scattering and produces a very large increase in the pathlength of the measuring beam resulting in absorbance intensifications of twenty times or more. The rationale of adding CaCO₃ to supernatant and pellet samples is that the increase in pathlength of the measuring beam due to the presence of CaCO₃ will far outweigh any increase due to particles in suspension in the turbid pellet sample, so that the effective pathlengths of both samples will be almost identical, and measurements under these conditions can be compared (Marmè et al, 1974; Quail, 1974; Schafer, 1974; Pratt and Marmè, 1976). This is a simple approach to a complex problem. However, detailed results to back up the underlying assumption have not been presented anywhere in the literature.

A close study of the original theory of spectroscopy of turbid materials (Butler, 1962) reveals that the addition
of CaCO₃ was used only for the study of absorbance characteristics in single samples, and not for quantitative comparisons between qualitatively different samples. The equation relating to the intensification of absorbance due to the addition of CaCO₃ is shown below (Equation I.1).

$$\beta = 2 \left[ - \frac{4 R_\infty^3}{(1-R_\infty^2)^2 S X} + \frac{1+R_\infty^2}{1-R_\infty^2} \right]$$  \text{... Equation I.1}  
(from Butler, 1962).

where,

- $\beta$ = the intensification factor due to the scattering component of the sample,
- $R_\infty$ = sample reflectance at infinite thickness,
- $S$ = scattering coefficient of the sample,
- $X$ = sample thickness (cm).

Certain conditions must be met for quantitative comparisons of supernatant and resuspended pellet samples to be made, based on Butler's theory. These conditions are summarised as follows:

1) The sample cuvette must have sidewall(s) which are internally reflecting.
2) The reflectances of the samples must be identical.
3) The scattering coefficients of the samples must be identical.

A theoretical analysis of the spectrophotometric measurement of supernatant and resuspended pellet samples shows that, in fact, these conditions are not usually met. Section I.3 explains this analysis and the conclusions drawn from it in more detail.
Table I.1
Representative Values for Specified Parameters of the Spectrophotometric Method used for Phytochrome Assay.

Cuvette S.I.R.; cuvette sidewalls are internally reflecting so that light can only pass through the ends of the cuvette.

Pellet Conc'n; the concentration of the resuspended pellet, expressed as gram (fresh weight of tissue equivalent). ml⁻¹ of resuspension buffer.

Thickness; the thickness in mm. of the resuspended pellet sample, through which the measuring beam passes.

CaCO₃; "+" indicates the addition of CaCO₃ powder to the resuspended pellet sample; "-" indicates the absence of CaCO₃.

<table>
<thead>
<tr>
<th>Cuvette S.I.R.</th>
<th>Pellet Conc'n</th>
<th>Thickness (mm)</th>
<th>CaCO₃</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>-</td>
<td>6.0</td>
<td>+</td>
<td>Butler, (1962).</td>
</tr>
<tr>
<td>No</td>
<td>?</td>
<td>10.0</td>
<td>+</td>
<td>Quail (1974,a &amp; b).</td>
</tr>
<tr>
<td>No</td>
<td>1.5</td>
<td>3.0</td>
<td>+</td>
<td>Grombein et al, (1975).</td>
</tr>
<tr>
<td>Yes</td>
<td>0.75-1.5</td>
<td>3.0</td>
<td>+</td>
<td>Pratt &amp; Marmé, (1976).</td>
</tr>
<tr>
<td>No</td>
<td>5.0</td>
<td>3.0</td>
<td>+</td>
<td>Jabben &amp; Deitzer, (1978,a&amp;b)</td>
</tr>
<tr>
<td>?</td>
<td>5.0</td>
<td>3.0</td>
<td>+</td>
<td>Roth-Bejerano &amp; Kendrick (1979).</td>
</tr>
</tbody>
</table>
3: A Theoretical Analysis of the Spectrophotometric Measurement of Supernatant and Resuspended Pellet Samples.

3:1 The effects of specific parameters on the intensification of absorbance by CaCO₃ particles.

3:1:1 The sample cuvette

Table I.1, column 1 shows that the characteristics of the cuvette used do not always satisfy Condition 1 above in the cases where the relevant information is available. Where side windows for actinic illumination are utilised, or horizontal measuring beams are used with open-topped cuvettes, scattered light may be lost. This loss of light cannot be taken into account in Equation I.1, thus the equation cannot be properly applied to samples assayed using these cuvettes.

3:1:2 Sample reflectance

The reflectance of a sample is a measure of the ability of the scattering particles in it to reflect back incident light. It is a function both of the purity of the CaCO₃ particles added, and also of the reflecting ability of the sample itself. A suspension of totally reflecting particles would have a reflectance at infinite thickness of 1.0; in practice, this situation does not exist as some absorption by the reflecting particles always occurs. Any impurities in the suspension which have high absorbance and low reflectance will lower the overall reflectance of the
sample slightly. It has been shown that even a small drop in reflectance can result in a large fall in the intensification of sample absorbance (Table I.2). In fact, the absorbance of a pure pigment solution at a high enough concentration can significantly reduce intensification due to CaCO₃ (Butler and Norris, 1960; Butler, 1962 and 1964).

A comparison of typical absorbances of supernatant and resuspended pellet samples at the concentrations routinely used (Table I.3), shows that the resuspended pellet has a much greater apparent absorbance than the supernatant. This difference is due to a combination of both the absorbing and the light scattering properties of the particulate resuspended pellet. Addition of CaCO₃ to these samples would thus, according to Butler's theory, lead to a situation where the reflectance of the resuspended pellet sample is lower than that of the supernatant sample, the magnitude of the difference depending on the concentration of particulate material in the resuspended pellet, and the actual degree of absorption of light by the resuspended pellet particles.

**3:1:3 Sample scattering coefficient**

The scattering coefficient of a sample is a measure of the ability of the scattering particles in the sample to deviate the incident light from a straight line. It is difficult to assess the effect of resuspended particles on the overall scattering coefficient of a sample also
Table I.2
The maximum intensifications of absorbance obtainable with CaCO$_3$ particle suspensions of different reflectances. (From Butler, 1964).

<table>
<thead>
<tr>
<th>Reflectance</th>
<th>Maximum Intensification</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.90</td>
<td>19</td>
</tr>
<tr>
<td>0.95</td>
<td>38</td>
</tr>
<tr>
<td>0.98</td>
<td>98</td>
</tr>
</tbody>
</table>

Table I.3
The mean apparent total absorbances of supernatant and resuspended pellet samples.

Mean apparent total absorbances at 700 nm. 3 samples each of supernatants and resuspended pellets from 30 000 g centrifugations were measured using an Aminco DW-2a spectrophotometer and clear plastic cuvettes.

Supernatant concentration was 0.3 g (tissue) m$^{-1}$. Resuspended pellet concentration was 2.0 g (tissue) m$^{-1}$.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean Apparent Absorbance(A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>0.08</td>
</tr>
<tr>
<td>Resuspended Pellet</td>
<td>0.80</td>
</tr>
</tbody>
</table>
containing CaCO₃ in quantitative terms; however, it is probable that the presence of resuspended pellet material in a CaCO₃ suspension will result in a lower sample scattering coefficient than would exist in supernatant samples with added CaCO₃. This conclusion is based on the observation that particulate material has a high apparent absorbance (Table I.3), at least part of which is probably due to actual absorption of light by the particles; thus, particulate material will probably reflect less of the light incident upon it than will a suspension of CaCO₃ particles, and will therefore probably give rise to less scatter than will a suspension of CaCO₃ particles. Hence, because the pelletable material will absorb some of the light that would otherwise have been scattered by the CaCO₃ particles, the sample will have a lower scattering coefficient than will a sample without absorbing particulate material; i.e., supernatant.

3:2 A comparison of the spectroscopy of supernatant and resuspended pellet samples; both in the presence and absence of CaCO₃.

3:2:1 In the presence of CaCO₃

Section I.3.1 has indicated that many workers have used CaCO₃ in cuvettes which do not allow the application of Butler's theory due to uncontrolled light losses from the samples, and also that the presence of highly absorbing particulate material from resuspended pellets in
suspensions of CaCO₃ particles will probably lead to lower values of reflectance and scattering coefficient in samples of this type compared with suspensions of CaCO₃ particles in supernatant samples. Equation I.1 predicts that a lower value of either or both of these parameters results in a lowering of the intensification factor, 8. It therefore follows that addition of CaCO₃ particles to supernatant and resuspended pellet samples will probably not eliminate inherent differences in absorbance and scattering between the samples due to the nature of the particulate material as routinely assumed by those who use CaCO₃, but will, on the contrary, be likely to reverse these supposed differences, thereby giving a much lower intensification of absorbance in resuspended pellet samples than in supernatant samples. The absorbance intensification by CaCO₃ of the resuspended pellet sample will also tend to decrease as the concentration of particulate material rises. Thus, the use of CaCO₃ in attempts to overcome differences between the optical properties of supernatant and resuspended pellet samples would seem, from this theoretical consideration to be likely to result in even greater discrepancies in phytochrome measurements than might already occur.

3:2:2 In the absence of CaCO₃

Even if CaCO₃ is not used, the problem of comparison of supernatant and resuspended pellet samples still remains. The assumption employed to justify the use of CaCO₃ was that resuspended pellets, due to their particulate nature,
were more light-scattering than supernatants, and would thus give artifactual high phytochrome readings (Marmé et al., 1974; Quail, 1974, 1 and 2; Schafer, 1974; Pratt and Marmé, 1976). In fact, this has been shown not to be the case (Rubenstein, Drury and Park, 1969). Mixing of a supernatant and resuspended pellet caused only a 15-20% increase in measurable phytochrome over the sum of the two samples in isolation, in the absence of CaCO₃ or any other light-scattering agent. Evidently, any effect of light scattering by the particulate material in the resuspended pellet was negated by light absorption by the same material, so that the comparison of supernatant, resuspended pellet and mixed samples showed little effect of particulate material on phytochrome readings. Yamamoto and Furuya (1975) also measured phytochrome in supernatant and resuspended pellet samples without using CaCO₃; however, they did not perform mixing tests comparable to those of Rubenstein et al.; and Table I.1 also shows that the characteristics of the sample cuvette and the concentrations of resuspended pellet samples used were not given in their report, so that it is difficult to draw meaningful conclusions from their results.

3:3 Course of action decided upon

The examination of the approaches to the problem of sample comparison taken by different workers, along with Butler's theory of the absorption of turbid materials, showed the necessity for a thorough study to establish an optimum set of parameters allowing the comparison of
supernatant and resuspended pellet samples. The limitations of the measuring instrument used (see Appendix I) meant that reflectance and scattering coefficients could not be calculated. However, \( \beta \) itself could be arrived at simply by comparison of sample readings both with and without CaCO\(_3\), and of samples with different amounts of particulate material. Equation I.1 shows that sample thickness is also important in the determination of \( \beta \), so the study undertaken investigated the effects of varying supernatant and resuspended pellet sample concentrations and thickness on phytochrome measurements, both in the presence and absence of CaCO\(_3\).

4: **Materials and Methods**

4:1 **General**

Measurements were made using supernatant and resuspended pellet samples from etiolated oat tissue. Supernatant samples were extracted and prepared as described in Appendix I.2. Resuspended pellet samples were prepared using tissue which had been given ten minutes standard red irradiation *in vivo*, and extracted in buffer containing 10 mM Mg\(^{2+}\). The presence of Mg\(^{2+}\) induced a large proportion of the total phytochrome to associate with pelletable material (Pratt and Marmè, 1976) allowing optimal accuracy in the reading. Pellets were resuspended in buffer without Mg\(^{2+}\), as the presence of Mg\(^{2+}\) leads to an artifactual turbidity of the sample (Marmè et al, 1974).
4:2 **Definitions**

Sample concentrations are expressed as:—
grams (fresh weight equivalent of tissue) per ml. of
sample; i.e., g (tissue) . ml⁻¹.

Phytochrome concentration is expressed as:—

\[ \text{Ptot}\{\Delta \Delta A \cdot 10^{-3} \text{ (660-730 nm)}\}. \text{ g}^{-1} \text{ (tissue)}. \]

These definitions are fully explained in Appendix I.3.

4:3 **Sample dilution**

Where necessary, dilution was achieved by the addition
of standard extraction buffer, without Mg²⁺, to give the
required concentration.

4:4 **The alteration of sample thickness**

The maximum sample thickness possible was 10.5 mm, the
internal length of the cuvettes used. Shorter sample
thicknesses were obtained by inserting tight-fitting clear
perspex blocks of varying thicknesses into the cuvette.
These were inserted into the side of the cuvette farthest
from the photo-multiplier, so that the sample distance from
the photo-multiplier was unaltered. The blocks did not
restrict, absorb or deflect the measuring beams, and
allowed examination of sample thicknesses from 0.5 mm to
10.5 mm. In practice, thicknesses under 2.5 mm were not
used because the signals from these samples were too small
to be accurately measured.
The addition of CaCO$_3$

CaCO$_3$ was of Analar grade, guaranteed 99.5% pure. CaCO$_3$ was added to samples until the sample surface had a silvery white sheen when settled, about five minutes after addition of the CaCO$_3$. This sheen indicated that the CaCO$_3$ and sample surfaces were then at identical positions. To achieve a sample height of 2.5 cm, only 2.0 ml of sample was needed in the presence of CaCO$_3$, compared to 2.6 ml in the absence of CaCO$_3$. This difference in volumes was taken into account when calculating phytochrome concentrations.

Results and Discussion

The effect of sample concentration on phytochrome measurements.

Figure I.1 shows the effect of a wide range of sample concentrations, including those generally used, on the phytochrome reading (Table I.1). Supernatant concentrations used in the references quoted in this Table are from 0.2 - 0.5 g . ml$^{-1}$.

In the absence of CaCO$_3$

In the absence of CaCO$_3$, there was very little effect of concentration on the phytochrome measurements calculated from the readings. Ptot . g$^{-1}$, calculated for supernatant samples remained constant over the concentration range studied. This result was expected because there was no scattering component present. In resuspended pellet samples,
FIGURE I.1
The Effect of Sample Concentration On The
Measurement of Phytochrome.

a) Supernatant Samples

Five-and-a-half day old etiolated oat coleoptiles were used, and extracted (see Appendix I) to give a concentration of \(0.5 \text{ g (tissue)} \cdot \text{ml}^{-1}\). This was diluted with extraction buffer to give the concentrations shown.

b) Resuspended Pellet Samples

Five-and-a-half day old etiolated oat coleoptiles were used, and extracted in the presence of 10 mM \(\text{Mg}^{2+}\), after 10 minutes Red irradiation in vivo, to give a resuspended pellet concentration of \(8 \text{ g (tissue)} \cdot \text{ml}^{-1}\). This suspension was diluted with \(-\text{Mg}^{2+}\) buffer to give the concentrations shown.

Resuspended pellet concentration is shown on a logarithmic scale for clarity.
a

Concentration of supernatant, g (tissue) ml⁻¹

b

Concentration of resuspended pellet, g (tissue) ml⁻¹
the calculated $P_{tot \cdot g^{-1}}$ was constant up to a concentration of around $2 \text{ g \cdot m}^{-1}$; but was lower at higher concentrations. This result indicates that, up to a concentration of $2 \text{ g \cdot m}^{-1}$, any light scattering due to the particulate nature of the sample which would lead to a higher reading was balanced by the opposite effect of actual light absorption by the particles; while, above this concentration, any increase in the scattering effect was exceeded by the opposite effect of the increase in light absorption by the sample, giving a lower apparent phytochrome reading.

5:1:2 In the presence of CaCO$_3$

In the presence of CaCO$_3$, the situation was very different. CaCO$_3$ intensified the supernatant reading over the whole concentration range, but this intensification was less at higher concentrations; $\beta = 5.6$ at $0.59 \text{ g \cdot m}^{-1}$, compared to $\beta = 6.4$ at $0.06 \text{ g \cdot m}^{-1}$. This result shows that, at the higher concentrations, the absorbance of the sample itself appears to lower the reflectance of the CaCO$_3$ suspension, thus reducing the absorbance intensification which CaCO$_3$ caused, as predicted by the theory of Butler (1962; 1964; see also Table 1.2), and the experimental results of Butler and Norris (1960). The effect of CaCO$_3$ on the phytochrome reading in resuspended pellet samples was more pronounced. At the lowest concentration used, $0.16 \text{ g \cdot ml}^{-1}$, the intensification factor was comparable to that obtained with supernatant samples ($\beta = 4.6$). However, as the concentration was increased, the intensification
dropped rapidly; beyond a concentration of 0.8g \cdot ml^{-1}, attenuation of the phytochrome signal occurred (\beta = 0.15 at 8.0g. m^{-1}). The results show that, if the concentration of pelletable material was low enough (less than 0.16g \cdot ml^{-1}), the presence of this material did not lower the intensification factor of the CaCO_3 suspension significantly compared to a supernatant sample, hence neither the reflectance nor the scattering coefficient were significantly altered. However, as the concentration of the resuspended pellet increased, the intensification factor dropped; therefore, the increase in particulate material must have caused the reflectance and/or the scattering coefficient of the CaCO_3 suspension to drop, agreeing with the qualitative conclusion reached earlier in this Section (I.3.1) from the theoretical application of Butler's theory of light scattering to supernatant and resuspended pellet samples.

However, one thing not taken into account in this explanation is the fact that, as sample concentration increases, both phytochrome concentration and particulate material concentration increase, therefore the changes in calculated values of P_{tot} \cdot g^{-1} could be partly or wholly due to an interaction between phytochrome and CaCO_3, and not particulate material and CaCO_3 as has been suggested. The next experiment investigated the effect of changes in particulate material concentration while maintaining a constant phytochrome concentration.
5:1:3 The effect of particulate material on absorbance intensification by CaCO$_3$

Pellets prepared from unirradiated etiolated tissue without Mg$^{2+}$ in the buffer have only around 5% of the total extractable phytochrome associated with them, the remaining 95% being in the supernatant (Pratt and Marmé, 1976). Thus, the addition of varying amounts of pelletable material to a constant volume of supernatant (both of which have been prepared from etiolated tissue without Mg$^{2+}$ in the buffer) will give a range of concentrations of particulate material while not significantly altering the amount of phytochrome present. A correction can be made for the small amount of phytochrome present in the particulate material (see legend to Figure 1.2).

The results of the investigation are shown in Figure 1.2. In the absence of added particulate material, the CaCO$_3$ intensification factor, $\beta$, was 6.5. This result agreed with that observed in Figure 1.1.a. In the absence of CaCO$_3$, as the concentration of pelletable material increased, the phytochrome reading remained constant; conclusive proof that the presence of resuspended pellet particulate material alone in a sample does not cause an intensification of the phytochrome signal. In the presence of CaCO$_3$, the intensification of the phytochrome reading due to the CaCO$_3$ suspension decreased as the concentration of pelletable material increased; conclusive proof that an increase in the concentration of pelletable material reduces the intensification of absorbance by CaCO$_3$ particles.
FIGURE 1.2
The Effect of The Concentration Of Particulate Material From A Resuspended Pellet On The Measurement Of Phytochrome In A Supernatant Sample

Samples were prepared from five-and-a-half day old etiolated oat coleoptiles. The final supernatant concentration was $0.5 \, \text{g (tissue)} \cdot \text{ml}^{-1}$. The resuspended pellet concentration was $10 \, \text{g (tissue)} \cdot \text{ml}^{-1}$. Samples were prepared for measurement by mixing together:

- $1.3 \, \text{ml}$ of supernatant,
- $0-1.1 \, \text{ml}$ of resuspended pellet,
- $1.3-0.2 \, \text{ml}$ of buffer.
- $2.6 \, \text{ml}$ final volume.

Correction for phytochrome in the resuspended pellet material was made by measuring a sample of:

- $0.6 \, \text{ml}$ of resuspended pellet,
- $2.0 \, \text{ml}$ of buffer.
- $2.6 \, \text{ml}$ final volume;

in the presence and absence of CaCO$_3$ and calculating, using these readings and Figure I.1.b the Ptot measurable due to resuspended pellet phytochrome at each of the concentrations used in this experiment, and subtracting that value from the measured Ptot to give the corrected Ptot value.

Sample thickness was 10.5 mm.
However, the effect was not nearly so pronounced as was the case for the measurement of phytochrome associated with pelletable material itself (see Figure I.1.b). At a concentration of $0.8 \text{g} \cdot \text{ml}^{-1}$, for instance, when $\beta$ was 1 for the resuspended pellet phytochrome, $\beta$ was 4.5 in the mixture of supernatant and pellet. The reason for the observed difference between $\beta$ values probably lies in the fact that, in resuspended pellet samples (see Figure I.1.b) the phytochrome was actually part of the particulate material in suspension, while in the mixture of supernatant and resuspended pellet, the phytochrome originated from the supernatant and therefore was in solution and not associated with particulate material. In the presence of CaCO$_3$, and at high concentrations of pelletable material in the absence of CaCO$_3$, soluble phytochrome is probably more readily accessible to the measuring beam than is the phytochrome intimately associated with the particulate material, due both to the disperse nature of supernatant phytochrome, and to the masking of pelleted phytochrome by the highly absorbing particles. Thus, the intensification factor due to CaCO$_3$ was higher in the mixture than in resuspended pellet samples alone at the same concentration of particulate material; and also, in the absence of CaCO$_3$, the reading in the mixture was not diminished at concentrations above $2 \text{g} \cdot \text{ml}^{-1}$ of particulate material.

5:1:4 The effect of the loss of scattered light from the cuvette.

That Butler's theory of absorption of turbid materials,
expressed in Equation I.1, appears to be applicable to samples which were measured in a cuvette which does not have internally reflecting walls parallel to the measuring beam seems rather incongruous; however, this situation can be explained by the observations that the sample volume was constant, and that the presence of particulate material alone did not cause absorbance intensification (see Figures I.1.b and I.2). Thus, the amount of light lost from the cuvette in the absence of CaCO₃ was not significantly different at different concentrations of pelletable material. In the presence of CaCO₃, light scattering does of course occur, thus more light was lost from samples containing CaCO₃ than from identical samples without CaCO₃. The effect of this difference was to give a lower absorbance intensification by CaCO₃ than expected; a prediction borne out by two observations. Firstly, the maximum intensification obtained using non-turbid supernatant samples was only 6.4, compared with Butler's (1962) values of 20 or over. Secondly, intensification values of less than 1 were observed at high particulate material concentrations (see Figure I.1.b), although Equation I.1 predicts a minimum value of 2.0 for \( \beta \). Thus, Butler's theory (1962) cannot be directly applied to the absorbance induced by CaCO₃ observed in particulate samples. However, because resuspended pelletable material has a much lower light scattering activity than CaCO₃ particles, the difference in the loss of scattered light over the range of concentrations of particulate material studied will be
insignificant compared with the losses due to the CaCO₃ scattering itself. Therefore, the effect of different sample concentrations on the apparent phytochrome reading in the presence of CaCO₃ can be interpreted using Butler's theory (1962).

5:2 The effect of sample thickness on phytochrome measurements.

Figure 1.3 shows the effect of the range of sample thicknesses available on phytochrome measurements in supernatant and resuspended pellet samples in the presence or absence of CaCO₃.

5:2:1 Results in the absence of CaCO₃

In the absence of CaCO₃, sample thickness, as would be expected, did not affect phytochrome measurements in supernatant samples (Figure I.3.a). In resuspended pellet samples however, the Ptot . g⁻¹ value calculated from phytochrome measurements was somewhat reduced at lower sample thicknesses (Figure I.3.b). This result probably indicates that; as sample thickness becomes less, the reduction in scattering by the particulate material - which is due to the reduced chances of incident light being deviated before it reaches the photomultiplier - was greater than any reduction in absorbance which would lead to increased scattering. This combination of these two effects led to a drop in scattering ability, and thus in
FIGURE I.3
The Effect Of Sample Thickness On
Phytochrome Measurement.

a) **Supernatant Samples**

Three and-a-half day old etiolated oat coleoptiles were used, and extracted giving a concentration of 0.25 g (tissue) . ml⁻¹.

b) **Resuspended Pellet Samples**

Three and-a-half day old etiolated oat coleoptiles were used, having been extracted and resuspended to give a concentration of 1.0 g (tissue) . ml⁻¹.

Ptot . g⁻¹ (tissue) values were calculated taking into account sample volume differences at different sample thicknesses.
the measured phytochrome reading. Application of Equation I.1 to this situation predicts the same result, as explained below.

The sample concentration is the same at all thicknesses, therefore the reflectance, $R^a$, and the scattering coefficient $S$, are constant. The only variable in Equation I.1 is $X$, the sample thickness. Equation I.1 predicts that, as $X$ decreases, so Term 1 of the Equation I.1 increases, and, because it is a negative term in Equation I.1, $\beta$ decreases from a maximum. Thus, absorbance intensification is reduced.

5:2:2 Results in the presence of CaCO$_3$

In the presence of CaCO$_3$, increasing the sample thickness resulted in a lower calculated Ptot $\cdot g^{-1}$ value, and therefore a lower intensification factor due to CaCO$_3$, in both supernatant and resuspended pellet samples. According to Equation I.1, the intensification factor should have increased with sample thickness in the same way as occurred with resuspended pellet samples in the absence of CaCO$_3$. This discrepancy from the predicted result cannot be explained by Equation I.1. The probable explanation is that, as sample thickness increased, so the amount of scattered light lost increased. The loss was not significant in the absence of CaCO$_3$ because there was little scattering in either supernatant or resuspended pellet samples alone, as already discussed. However, in the presence of CaCO$_3$, scattering is greatly increased,
the loss of scattered light is significant, and, because it varies with sample thickness, the loss must be considered. One cannot quantify the loss of scattered light, but, in simple qualitative terms, the reduction in intensification due to the greater loss of light at a sample thickness of 10.5 mm, compared to that at 2.5 mm, was probably enough to overcome the rise in intensification predicted by Equation I.1 for a greater sample thickness, thus leading to an overall drop in intensification. This theory would explain the result for supernatant samples seen in Figure I.3.a.

In resuspended pellets in the presence of CaC\textsubscript{3}, intensification fell dramatically as sample thickness increased, and attenuation of the reading occurred above a thickness of 6.5 mm, a different result to that observed in supernatant samples. Once again, the reason for the difference between supernatant and resuspended pellets lies in the effect of particulate material on the reflectance and scattering coefficient of a CaC\textsubscript{3} suspension. In a resuspended pellet sample these parameters are lower than in a supernatant sample; thus, as sample thickness increases, a lower increase in absorbance intensification would be expected than was predicted for supernatant samples (see Equation I.1), while the increased loss of light would be very similar to that occurring in supernatant samples (see Section 5.1.4). The overall predicted effect of increasing sample thickness would therefore be to cause a greater drop in absorbance intensification than was
observed for supernatant samples. Although only two results were obtained for supernatant samples, a difference between Figures I.3.a. and b can be seen which agrees with the prediction.

6: Conclusions and Recommendations

6:1 Conclusions

The results reported here show that the assumptions commonly made concerning the effects of CaCO₃ on samples with different spectral characteristics are untenable. Under certain conditions, the intensification due to CaCO₃ in both supernatant and resuspended pellet samples could be made equal, but this involved using either a very dilute pellet, or a very short sample thickness, both situations resulting in a small actual phytochrome reading, similar to the original sample before CaCO₃ addition.

Due to differences between the spectrophotometric measuring systems used, the results reported here cannot be compared directly with other workers' readings. The reason for this is that light losses may be different, cuvette geometry may be different, the photomultiplier may be a different distance from the sample - there are many possible variables. However, the overall trends in the results are applicable to all systems, and they indicate that the use of CaCO₃ is undesirable for three reasons. Firstly, the addition of CaCO₃ is unnecessary, because the artifact which it is supposed to eliminate - the scattering effect of resuspended pellets - does not exist at
concentrations less than or equal to 2 g (tissue equivalent) per ml. Secondly, CaCO$_3$ addition leads to artifacts in phytochrome readings in the presence of light-absorbing particulate material. Thirdly, avoidance of these artifacts requires dilution of the sample such that the readings become so small that there is no improvement in the signal to noise ratio compared to samples without CaCO$_3$.

6:2 Recommendations for the use of CaCO$_3$

As a consequence of the results reported here, it is recommended that, wherever supernatant and resuspended pellet samples have to be compared, the effect on phytochrome readings of resuspended particulate material alone should be checked first by performing dilution series of the samples. If no effect exists, then readings can be compared directly; if the resuspended pelletable material is found to be altering phytochrome readings, sample dilution should remove this effect. If CaCO$_3$ is added, its effect should be assessed by performing dilution series on both types of sample to confirm that particulate material is not artifactually reducing the absorbance intensification induced by CaCO$_3$.

Examination of the literature shows that on occasions where CaCO$_3$ has been utilized, the effect of its addition has not been assessed in this way in any report, thus the validity of all pelletability values calculated using readings of samples containing CaCO$_3$ must be questioned. In some cases, notably those utilizing low sample thicknesses
(3 mm or less), low resuspended pellet concentrations (2 g (tissue) m -1 or less), or cuvettes with internally reflecting sidewalls, it is possible that absorbance intensification by CaCO 3 gives non-artifactual results. However, this assumption cannot be made without firstly checking its validity by performing dilution series of samples as outlined above. Where greater values of sample thickness and concentration than those specified above are used, it seems highly likely that artifactually low resuspended pellet readings will be obtained, which would lead to underestimation of phytochrome pelletability.

Table 1.1 shows that sample thicknesses greater than 3 mm, and resuspended pellet sample concentrations greater than 2 g m -1 are used in the presence of CaCO 3; therefore, in these, and other reports utilizing similar parameters, the phytochrome readings must be considered erroneous unless characterization of the CaCO 3 effect to verify the validity of the results has been carried out.

Table 1.1 also shows that, in many reports, not all the necessary information is given to enable one to assess whether there is likely to be an effect on phytochrome measurements of resuspended pellet particulate material or CaCO 3 particles. This lack of necessary detail occurs in many reports dealing with the spectrophotometric measurement of phytochrome, and indicates a serious omission on the part of the authors of these reports. It cannot be too strongly stressed that proper assessment of the effect of CaCO 3 and/or resuspended particulate material should be carried out for the range of samples assayed
before comparison of phytochrome measurements is made. If this has not been done, at the very least, values for the parameters indicated in Table I.1 should be included in the report to enable readers to draw their own conclusions as to the validity of the results.

6:3 The parameters of the spectrophotometric assay adopted for use.

As a result of the findings reported here, it was decided to utilize a spectrophotometric measuring system avoiding the use of CaCO$_3$. A supernatant concentration of 0.25 g . ml$^{-1}$ or 0.33 g . ml$^{-1}$ was used. The use of these values gave a minimum signal to noise ratio of around 4 : 1. With resuspended pellets the signal was generally low therefore the maximum concentration possible was necessary. Figure I.1.b showed this to be 2 g . ml$^{-1}$: above this value, particulate material affected the reading and thus, a concentration of 2 g . ml$^{-1}$ was used for resuspended pellets unless otherwise stated. This value gave a minimum signal to noise ratio of around 2 : 1. A sample thickness of 10.5 mm was adopted. This thickness was used for convenience and accuracy as it was the standard cuvette pathlength and could therefore be reproduced routinely.

Using these values for the sample parameters relevant to the measurement of phytochrome, accurate comparisons of phytochrome readings from supernatant and resuspended pellets could be made, and combinations of measurements from the samples could be carried out with confidence using the
Perkin Elmer 156, without the presence in the results of artifacts due to the spectroscopic properties of the samples.
SECTION II
The Characterization And Red/Far-red Reversibility Of Phytochrome Association With A Crude Particulate Fraction From Dark-grown Oat Coleoptiles.
1: Introduction

It is considered by many workers that phytochrome association with a membrane or membranes in the plant cell is a likely prerequisite for phytochrome action, ultimately leading to the modulation of physiological responses (Marmé, 1977).

There have been a number of reports claiming phytochrome association with purified or enriched membrane fractions such as etioplasts (Smith, Evans and Hilton, 1978), mitochondria (Furuya and Manabe, 1976; Georgevich, Cedel and Roux, 1977), endoplasmic reticulum, (Williamson, Morré and Jaffe, 1975; Marmé, Bianco and Gross, 1976), and plasma membrane (Marmé, Bianco and Gross 1976); there have been many more reports dealing with phytochrome association with various crude particulate pellets consisting of mixtures of different membranes (e.g.: Rubenstein, Drury and Park, 1969; Marmé, Boisard and Briggs, 1973; Boisard, Marmé and Briggs, 1974; Quail, 1974 (a), (b); Grombeim, Rudiger, Pratt and Marmé, 1975).

Most of these reports have been confined to quantitative assessments of the association between phytochrome and the membrane fraction, while few have considered the qualitative aspects of the association; i.e.: the actual nature of the interaction between phytochrome and membranous material. Those which have given a confused picture of the situation, some suggesting that phytochrome is associated on the periphery of the membrane by weak electrostatic bonds, perhaps to a membrane protein (Gressel and Quail, 1976;
Quail, 1978 (a)), while others claim that phytochrome is integrally associated with a steroid component of the membrane (Roth-Bejerano and Kendrick, 1979; Brownlee, Roth-Bejerano and Kendrick, 1979).

Supporting evidence for the former proposal comes from much of the data on phytochrome association with crude membrane fractions (Boisard, Marmé and Briggs, 1974; Quail, 1974 (b), Pratt and Marmé, 1976), while the latter suggestion is in good agreement with results obtained using model membrane systems (Roux and Yguerabide, 1973; Georgevitch, Krauland and Roux, 1976) as well as one report dealing with phytochrome association with a crude membrane fraction (Rubenstein, Drury and Park, 1969). It was with this contradictory background that an attempt was made to characterize the association of phytochrome with particulate material from a 30 000 g centrifugation of crude extracts from dark-grown oat coleoptiles, as a basis for the further study of the significance of pelletable phytochrome.

The 30 000 g pellet is considered to be a crude membrane fraction (Marmé, Bianco and Gross, 1976; Pratt, 1978), thus data obtained using this fraction was judged to be pertinent to the problem of clarifying the association of phytochrome with membranes. The nature of this association was studied by performing a series of pellet washes, and by incubating the pellet in solutions of a number of different membrane-modifying reagents in standard extraction buffer, each known to have different specific effects on linkages between proteins and membranes (Maddy
and Dunn, 1976), and subsequently observing the fate of the pelletable phytochrome upon re-centrifugation. Also, a comparison of pelleted phytochrome from etiolated, ("Dark"), red-irradiated ("Red"), and red-followed-by-far-red-irradiated ("Red+Far-Red") tissue was made and a speculative proposal to explain the observed differences put forward.

2: Materials and Methods

2:1 Sample Preparation

The preparation of pellets for incubation studies and pelletability assays was performed as described in Appendix I.2. Magnesium ions (Mg$^{2+}$) were not included in the extraction or resuspension buffer unless indicated; where present, the Mg$^{2+}$ concentration was 10 mM.

2:2 Irradiation times

Ten minute irradiations were used, as described in Appendix I.2.1. Figure II.1 shows that the pelletability response detectable on extraction with Mg$^{2+}$ was saturated with five minutes in vivo irradiation, thus ten minute irradiations were ample for both photoconversion (see Appendix I.4.6) and induction of maximum pelletability.

2:3 Pellet washing

Pellet washes were performed by thoroughly resuspending pellets in the appropriate buffer solution at 4°C, to a concentration of 1 g (tissue) ml$^{-1}$. This suspension was
FIGURE II.1

The effect of increasing time of red irradiation in vivo on the pelletability of phytochrome extracted with 10 mM Mg$^{2+}$ in the buffer solution.

\[
\% \text{ pelleted phytochrome} = \frac{P_{\text{tot}} \ (\text{pellet})}{P_{\text{tot}} \ (\text{pellet}) \ + \ P_{\text{tot}} \ (\text{supernatant})} \times 100
\]
Time of in vivo irradiation, minutes

% pelleted phytochrome

0  10  20  30  40  50  60  70

0  1  2  3  4  5
then re-centrifuged and the subsequent supernatant and pellet separated; the pellet was resuspended a second time to a concentration of 2 g (tissue) \cdot m^{-1}, and the two fractions assayed for photoreversible phytochrome. Ideally, a larger volume of buffer would have been preferable for the wash; however, in that case, accurate measurement of the small amounts of phytochrome released to the supernatant would have been impossible.

Multiple washes were performed in the same way, with successive resuspensions giving a concentration of 1 g (tissue) \cdot m^{-1}, the final pellet resuspension after the last wash giving a concentration of 2 g (tissue) \cdot m^{-1}. Phytochrome was only assayed once in each individual sample.

2:4 Incubation of Resuspended Pellets

For each experiment, a large scale extraction of tissue was made, and equal aliquots of crude homogenate, each equivalent to 5 g fresh weight of tissue, were pipetted into separate tubes and centrifuged. The supernatants were discarded and the pellets resuspended in the appropriate solution in the centrifuge tube, to a volume of 5 ml, thus giving a resuspended pellet concentration of 1 g (tissue) \cdot m^{-1}. The resuspended pellets were then incubated in the dark at 4 C for 2 hours; the tubes were covered but O₂ was not excluded. After the incubation, the tubes were immediately centrifuged (30 000 g for fifteen minutes at 4 C), the subsequent supernatant and pellet separated, the pellets resuspended in standard extraction buffer to a
concentration of 2 g (tissue) \cdot ml^{-1}, and the samples assayed for total photoreversible phytochrome present.

The various solutions used were prepared by making up the appropriate concentrations in standard extraction buffer. The pH remained unchanged for all solutions except for 10 mM EDTA, in which the pH was raised to 7.8 again by the addition of a small volume of 1 M Tris solution, before pellet resuspension in the solution was performed.

3: Results and Observations

3:1 The effect of in vivo irradiation on phytochrome pelletability, both in the presence and absence of 10 mM magnesium ions (Mg^{2+}).

Before attempting to investigate the biochemical nature of the phytochrome association with the crude membrane pellet, it was necessary to quantify the relative amounts of phytochrome involved under different irradiation conditions. Figure II.2.a shows the percentage of phytochrome pelletable after different irradiations given to whole seedlings immediately prior to homogenisation in buffer with no Mg^{2+} present. Figure II.2.b shows the result for the identical experiment, but with 10 mM Mg^{2+} in the buffer.

A number of interesting observations can be made from these results. Firstly, the previous reports of Mg^{2+}-stimulated pelletability in oats (Grombein, Rudiger, Pratt and Marmé, 1975; Pratt and Marmé, 1976) have been
confirmed, with a roughly seven-fold increase in pelletability, from 9% using "Dark" tissue, to over 60% using "Red" tissue in the presence of Mg$^{2+}$. The dark levels of pelletability of around 5% without Mg$^{2+}$, and 8-9% with 10 mM Mg$^{2+}$, are also in good agreement with previous reports (Rubenstein, Drury and Park 1969; Grombein, Rudiger, Pratt and Marmé, 1975; Pratt and Marmé, 1976). It can be seen that far-red irradiation after red irradiation does not reverse the red-induced pelletability increase in the presence of Mg$^{2+}$, but completely reverses the red-induced increase in the absence of Mg$^{2+}$. The red-induced increase and far-red reversal of pelletability can be repeated by a second "red followed by far-red" set of irradiations. Similar effects may be occurring in the presence of Mg$^{2+}$ but would be too small to be seen in conjunction with the massive Mg$^{2+}$-induced increase in phytochrome pelletability. Far-red reversibility of red-induced pelletability has not previously been reported for any tissue using in vivo irradiations when extracted without divalent cations present. The significance of what is an extremely important result is considered in Section IV, with relevance to physiological responses, and also in detail in the General Discussion.

Having established the levels of pelletability obtained with different in vivo irradiations, the nature of pelletability was then investigated, firstly by washing the pellets in extraction buffer and observing the fate of pelletable phytochrome.
The effect of various in vivo irradiations on the pelletability of phytochrome when subsequently extracted.

All irradiations were of ten minutes duration.

D: no irradiation.
(R): red irradiation.
(R+FR): red, followed by far-red irradiation.
(R+FR+R): red, followed by far-red, followed by red irradiation.
(R+FR+R+FR): red, followed by far-red, followed by red, followed by far-red irradiation.

a: the extraction was performed without Mg$^{2+}$ in the buffer.
b: the extraction was performed with 10 mM Mg$^{2+}$ in the buffer.

Results shown in this and subsequent Figures with error bars indicate the mean value of at least three replicate experiments. Error bars represent the 95% confidence limits of the calculated means.
a without Mg$^{2+}$:

% pelletability of phytochrome

<table>
<thead>
<tr>
<th></th>
<th>D</th>
<th>(R)</th>
<th>(R+FR)</th>
<th>(R+FR+R)</th>
<th>(R+FR+R+FR)</th>
</tr>
</thead>
</table>

b with Mg$^{2+}$:

% pelletability of phytochrome

<table>
<thead>
<tr>
<th></th>
<th>D</th>
<th>(R)</th>
<th>(R+FR)</th>
<th>(R+FR+R)</th>
<th>(R+FR+R+FR)</th>
</tr>
</thead>
</table>
The effect of buffer washes on pelletable phytochrome

Pellets extracted from "Dark" or "Red" tissue, either with or without the addition of 10 mM Mg$^{2+}$ to the extraction buffer, were washed with extraction buffer either with or without added 10 mM Mg$^{2+}$, and the supernatants and resuspended pellets from the resulting centrifugation assayed for phytochrome. Figure II.3 shows the results obtained. Figure II.3.a. indicates that the majority of phytochrome pelletable in the absence of Mg$^{2+}$ was not removed by a simple buffer wash. The only difference between the reactions of pellets from "Dark" or "Red" tissue was that; around 15% of the total phytochrome signal was lost from the ("Red" pellet) while about the same amount was released to the supernatant; compared to a release of 25% of the total phytochrome signal to the supernatant from the "Dark" pellet, while no loss of phytochrome signal occurred.

A different result was obtained for phytochrome pelleted in the presence of Mg$^{2+}$. Washing in buffer with 10 mM Mg$^{2+}$ (Figure II.3.b.i) resulted in very little release of pelleted phytochrome for both "Dark" and "Red" samples. However, washing in buffer without Mg$^{2+}$ (Figure II.3.b.ii) showed that around 80% of the "Dark" pelleted phytochrome remained pelletable, while only around 25% of the measured phytochrome remained pelletable in the sample from "Red" tissue. Other workers have obtained even less retention of "Red" phytochrome, pelleted in the presence of Mg$^{2+}$, and then washed in the absence of Mg$^{2+}$. 15% has been quoted for zucchini (Marmé, Mackenzie, Boisard and Briggs, 1974), and
The effect of pellet washing on phytochrome pelletability.

The readings in separate fractions are expressed as a percentage of the total phytochrome in the original pellet before washing.

D: no irradiation.
(R): ten minutes red irradiation.

sn: supernatant fraction.
p: pelletable fraction.

a: pelletable phytochrome extracted without Mg$^{2+}$ in the buffer.
b: pelletable phytochrome extracted with 10 mM Mg$^{2+}$ in the extraction buffer.
i: pellet washed with buffer containing 10 mM Mg$^{2+}$.
ii: pellet washed with buffer without Mg$^{2+}$. 
a) extracted without Mg$^{2+}$

b) extracted with Mg$^{2+}$

i) washed with $^{+}$Mg$^{2+}$ buffer

ii) washed with $^{-}$Mg$^{2+}$ buffer
8% for oats (Pratt and Marmé, 1976), so the higher than expected result reported in Figure II.3.b.ii may have been due to some co-precipitation of released phytochrome with the pellet due to the low volume of wash buffer used.

The apparent increase in the total phytochrome signal after washing visible in Figure II.3.b. could have been due to a "sieve effect" (Mackenzie, Briggs and Pratt, 1978) in the initial pellet if the phytochrome was present in densely-packed discrete regions, thus giving an artificially low phytochrome reading. The release of pelleted phytochrome would have removed the effects and the correct reading would then have been made.

Figure II.3.b therefore shows that the magnesium-induced pelletability of "Red" phytochrome was a weak association of the pigment with the crude membrane pellet which was disrupted if divalent cations were removed. The 3.5% increase in pelletable phytochrome from "Dark" samples which was induced in the presence of 10 mm Mg\(^{2+}\) was not lost after the removal of Mg\(^{2+}\). This result suggests that such an association of phytochrome with membranes material may be different from the majority of the phytochrome/membrane association induced by Mg\(^{2+}\) in pellets from "Red" tissue, and may instead be broadly similar to that seen in pellets originally extracted without Mg\(^{2+}\).

Having confirmed that the large increase in pelletability of phytochrome from "Red" tissue in the presence of 10 mm Mg\(^{2+}\) was due to a very weak interaction between the phytochrome and the pellet (this will be fully
considered in the General Discussion) attention was focussed on the phytochrome pelletable without Mg\textsuperscript{2+} in the buffer.

Figure II.4 shows the effect of a series of three washes in extraction buffer of pellets derived from "Dark" or "Red" tissue extracted without Mg\textsuperscript{2+} in the buffer. It can be seen that there was little difference in the effectiveness of the washes in "Dark" and "Red" pellets. In both cases, after three washes, around 40\% of the originally pelleted phytochrome could still be measured in the pelletable fraction. However, not all the 60\% lost from the pellet appeared in the supernatant; only around 30\% was seen in this fraction, and the rest of the phytochrome signal was lost. This loss could have been due to actual degradation of phytochrome as a result of the successive centrifugations, or the progressive action of some destructive agent in the solution. Evidence in Section III tends to support the former possibility.

A previous report showed a 30\% release of phytochrome to the supernatant after three washes of the pellet (Rubenstein, Drury and Park, 1969), but 80\% of the original phytochrome signal was destroyed by the washes, so the phytochrome studied here is more stable to washes than that reported by Rubenstein et al.

Thus, the majority of phytochrome pelleted in the absence of Mg\textsuperscript{2+} is certainly not trivially co-precipitated or tenuously associated with the pelletable material. This is true of both "Dark" and "Red" pelletable phytochrome. The following series of experiments was designed to
FIGURE II.4

The effect of a series of three washes with standard extraction buffer (no Mg$^{2+}$) on phytochrome pelletability in extracts from:-

a: unirradiated tissue; Dark.
b: tissue given ten minutes red irradiation in vivo; Red in vivo.
p: pelletable phytochrome after successive washes.
sn: phytochrome in the supernatant appearing after each wash (cumulative total).

signal loss: phytochrome signal not accounted for in either the pellet or the supernatant fractions (cumulative total).

The readings in separate fractions are expressed as a percentage of the total phytochrome in the original pellet.
a  Dark

- Graph showing % of Ptot in original pellet for successive pellet washes.
- Three curves: p, sn, signal loss.

b  Red in vivo

- Graph showing % of Ptot in original pellet for successive pellet washes.
- Three curves: p, sn, signal loss.

- Original pellet vs. successive pellet washes.
investigate the nature of this wash-resistant phytochrome association with pelletable material.

3:3 The effect of in vitro irradiations on pelletable phytochrome.

An in vitro photoconverting irradiation, whether it was red, converting Pr to Pfr, or far-red, converting Pfr to Pr, caused a weakening in the association of phytochrome with pelletable material. The weakened association can be seen more clearly in Table II.1 which shows the actual amounts of phytochrome per gram of tissue, calculated for etiolated or red-irradiated tissue, both with and without an in vitro irradiation. A comparison of these results shows that the effect of the in vitro irradiation was almost to halve the amount of phytochrome recovered in the pellet after the incubation, while not significantly increasing the amount recovered in the supernatant, thereby implicating a loss of about 50% of the pelletable phytochrome in both cases. This loss could have occurred immediately upon in vitro irradiation, during the incubation or during the centrifugation - it is impossible to say from these data. However, two other sets of results show where this loss occurs.

Firstly, Figure II.5.b. shows the effect of in vitro irradiation of a crude extract from etiolated tissue on the subsequent pelletability of phytochrome (in the absence of divalent cations). It can be seen that irradiation in vitro caused a drop of around 40% in the level of
Table II.1

The Effect of in vitro Irradiation On Pelletable Phytochrome

Phytochrome was measured in the supernatant (sn) and pellet (p) resulting from the incubation and subsequent re-centrifugation of a resuspended pellet in extraction buffer (see Methods - 2.4 - for details).

"Dark": original pellet from etiolated tissue.
"Red": original pellet from red-irradiated tissue.

In vitro irradiations were of ten minutes duration, and were red for "Dark" samples, and far-red for "Red" samples, given to the original samples immediately after pellet resuspension, and before the two-hour incubation.

Units are phytochrome; \( \Delta \Delta A \cdot 10^{-3} \cdot g^{-1} \) (tissue).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>&quot;Dark&quot;</th>
<th></th>
<th>&quot;Red&quot;</th>
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<tbody>
<tr>
<td></td>
<td>sn</td>
<td>p</td>
<td>sn</td>
<td>p</td>
</tr>
<tr>
<td>without in vitro irradiation.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>0.35</td>
<td>0.42</td>
<td>0.62</td>
</tr>
<tr>
<td>with in vitro irradiation.</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>0.25</td>
<td>0.17</td>
<td>0.50</td>
<td>0.33</td>
</tr>
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around 40% in the level of pelletability from that seen in 'dark' extracts. This was not reversed by a further far-red irradiation in vitro. As Figure II.5.a. shows, there was no loss in the total phytochrome recovered from the centrifuged sample after in vitro irradiation; but, of course, the percentage of pelletable phytochrome in the crude extract was so small that the loss of 40% of this fraction without being released into the supernatant fraction would have been impossible to detect. What is clear is that in vitro irradiations had no effect on the recovery of supernatant phytochrome. Therefore, if the effect of the in vitro irradiation of a crude extract of etiolated tissue was similar to that on the resuspended pellets, it indicates that the loss occurred either immediately after irradiation, or as a result of centrifugation step, as no incubation occurred.

Secondly, Table II.2 summarises relevant data drawn from Section III; and it can be clearly seen that in vitro irradiations of resuspended pelleted phytochrome, from both etiolated and red-irradiated tissue did not lead to a significant drop in photoreversible phytochrome in the samples, measured either immediately or within six hours of the irradiation.

Taking these three sets of data together, it can be seen that the effect of an in vitro irradiation was only seen after a centrifugation, suggesting that an in vitro irradiation somehow destabilised pelleted phytochrome, making it more labile, which caused it to break down during the centrifugation step.
FIGURE II.5

The effect of in vitro irradiations on the pelletability of phytochrome in an extract from etiolated (un-irradiated) tissue.

a: total phytochrome measured in the supernatant and pellet fractions after centrifugation.

b: the percentage pelletability calculated from "a" for each treatment.

D: unirradiated extract.

R: extract given ten minutes in vitro red irradiation.

R+FR: extract given ten minutes in vitro irradiation followed by ten minutes in vitro far-red irradiation.

p: pelleted phytochrome.

sn: supernatant phytochrome.
Table II.2
The Effect of Incubation Following in vitro Irradiation, Without Centriguation, On Phytochrome Pelletability.

Phytochrome was measured in pellets prepared from either "Dark" or "Red" tissue, resuspended in extraction buffer, then given ten minutes in vitro irradiation; red for "Dark" samples, and far-red for "Red" samples. Separate aliquots from each sample were assayed for phytochrome at specific stages in the procedure. Results for three separate samples are shown (1, 2, 3).

"Dark": unirradiated etiolated tissue.
"Red": red irradiated tissue.

(-): reading before in vitro irradiation.
(+, T₀): reading immediately after in vitro irradiation.
(+, T₆): reading six hours after in vitro irradiation.

Units are phytochrome: $\text{Delta A} \times 10^{-3} \text{ g}^{-1} \text{ (tissue)}$.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>&quot;Dark&quot;</th>
<th>&quot;Red&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-)</td>
<td>(+, T₀)</td>
</tr>
<tr>
<td>1</td>
<td>1.10</td>
<td>1.10</td>
</tr>
<tr>
<td>2</td>
<td>1.10</td>
<td>1.00</td>
</tr>
<tr>
<td>3</td>
<td>0.95</td>
<td>0.95</td>
</tr>
</tbody>
</table>
3:4 The effects of various membrane-modifying reagents on pelletable phytochrome.

Pellets prepared from either "Dark", "Red" or "Red + far-red" tissue were treated with the appropriate reagent as described in Methods, 2:4, and the proportions of the original pelletable phytochrome recovered in the supernatant and pellet were calculated.

3:4:1 The effect of the experimental protocol on phytochrome stability.

A major problem in this series of experiments was the fact that a proportion of the original pelletable phytochrome was lost as a result of the two-hour incubation and the re-centrifugation. (See Table II.3). It can be seen that around 40% of the original phytochrome could not be detected in the pellet or supernatant obtained after resuspension in standard extraction buffer, (Table II.3.c.) Table II.3 also shows that this loss was not due to the two treatments themselves, but was a function of the combination of the two treatments. A similar effect has been reported previously (Rubenstein, Drury and Park, 1969), which indicates that phytochrome, after a two hour incubation in solution, although completely photoreversible, had become more labile to the stress of a further centrifugation than if the sample had been centrifuged immediately upon resuspension. The effect is similar for phytochrome from both "Dark" and "Red" tissue.
In order to study the effects of the membrane-modifying reagents on phytochrome pelletability, the incubation was necessary, as mere resuspension of the pellet followed by an immediate centrifugation would not have given the reagent time to act on the membranes in the resuspended pellet (Maddy and Dunn, 1976). Therefore, in order to be able to see the effects of the reagents on the solubilization or otherwise of pelletable phytochrome, and not simply the non-specific effect of the procedure on phytochrome breakdown, resuspension of pellets in extraction buffer only was performed as a control. Thus, the total of supernatant plus pelleted phytochrome measured in the buffer control was taken as 100%, and the amount measured in other treatments was expressed as a percentage of this buffer control. This explains why, in the presence of some reagents, greater than 100% of the total phytochrome recovered in the buffer control could be seen. These reagents stabilised the phytochrome making it more resistant to the incubation and re-centrifugation, thus leading to a greater recovery of the total phytochrome originally pelleted.

3:4:2 The specific effects on pelletable phytochrome of the membrane-modifying reagents used

A complication in the interpretation of the effects of the reagents comes from the observation in a number of samples of significantly greater losses of photoreversibly detectable phytochrome than seen in the buffer control.
TABLE II.3
The Effect of Different Treatments Upon The Pelletability of Phytochrome.

Pellets containing phytochrome prepared from "Dark" and "Red" tissue were resuspended in extraction buffer and subjected to different treatments as shown, and then assayed again for phytochrome.

Treatments
a) The resuspended pellet was left in darkness at 4 °C for two hours and then assayed.
b) The resuspended pellet was centrifuged and a second pellet and supernatant obtained which were then assayed.
c) The resuspended pellet, after dark incubation, was centrifuged and a second pellet and supernatant obtained and assayed.

All figures are quoted as a percentage of the total phytochrome measured in the original resuspended pellet; this was around $1.05 \times 10^{-3} \text{A}$ for "Dark" samples, and $1.52 \times 10^{-3} \text{A}$ for "Red".

"loss" refers to the percentage of the phytochrome signal not recovered in either the resuspended pellet (p) or the supernatant (sn).

"Dark": unirradiated etiolated tissue.
"Red": red irradiated etiolated tissue.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>&quot;Dark&quot;</th>
<th></th>
<th></th>
<th>&quot;Red&quot;</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p</td>
<td>sn</td>
<td>loss</td>
<td>p</td>
<td>sn</td>
<td>loss</td>
</tr>
<tr>
<td>original pellet</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>a) incubation</td>
<td>98</td>
<td>-</td>
<td>2</td>
<td>99</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>b) washing</td>
<td>77</td>
<td>27</td>
<td>-4</td>
<td>71</td>
<td>19</td>
<td>10</td>
</tr>
<tr>
<td>c) incubation +</td>
<td>30</td>
<td>28</td>
<td>42</td>
<td>33</td>
<td>29</td>
<td>38</td>
</tr>
</tbody>
</table>

TABLE II.3
Such losses could have been due to destruction of the phytochrome molecule, or to spectral bleaching of the molecule due to its environment (Butler, Siegelman and Miller, 1964; Pratt and Cundiff, 1975). The source of the lost phytochrome cannot be determined; therefore, the only results which can give positive information on the nature of the phytochrome association with membranous material are those where a significant increase in phytochrome in the supernatant was measured in conjunction with a significant decrease in phytochrome measured in the pellet.

Figures II.6 and II.7 show that this condition was met only with 1M KI, 20 mM Na cholate, 1% Triton X-100 and 10 mM EDTA (for "Red" and "Red + Far-Red" pelletable phytochrome). In all other cases, any loss of phytochrome from the pellet was accompanied by a loss of photoreversible signal, without an increase in phytochrome measured in the supernatant.

An analysis of the actions of these effective reagents indicates the nature of the phytochrome association with membranous material. Potassium iodide (KI) is a chaotropic agent, which disrupts hydrophobic linkages; sodium cholate is an ionic detergent and Triton X-100 a non-ionic detergent, both of which solubilise membranes by breaking down hydrophobic internal linkages within the phospholipid layer (Maddy and Dunn, 1976). Therefore, the dissociation of pelletable phytochrome by these reagents is indicative of a hydrophobic linkage of phytochrome to the membrane, probably to the internal phospholipid non-polar region.
FIGURE II.6

The specific effects of various membrane-modifying reagents on phytochrome pelletability. Pellets were resuspended in the appropriate solution and incubated for two hours at 4°C then re-centrifuged to give supernatant and pellet samples as shown (see Methods, 2:4 for full details).

Readings in individual fractions are expressed as a percentage of total phytochrome recovered after a buffer wash, thus %sn + %p = 100 for buffer samples.

- sn: supernatant phytochrome.
- p: pelleted phytochrome.

a): no in vitro irradiation given to extracts.

D: no in vivo irradiation given to seedlings.

(R): red irradiation given to seedlings before homogenisation.

b): ten minutes in vitro irradiation was given to extracts prior to incubation of the resuspended pellet.

R: red irradiation given in vitro to extracts from totally etiolated seedlings.

(R)FR: far-red irradiation given in vitro to extracts from red-irradiated seedlings.
FIGURE II.7

The specific effects of various membrane-modifying reagents on phytochrome pelletability. Pellets were resuspended in the appropriate solution and incubated for two hours at 4 C then re-centrifuged to give supernatant and pellet samples as shown (see Methods, 2:4 for full details).

Readings in individual fractions are expressed as a percentage of total phytochrome recovered after a buffer wash, thus % sn + % p = 100 for buffer samples.

- sn: supernatant phytochrome.
- p: pelleted phytochrome.

D: no irradiation.
(R): red irradiation given in vivo to seedlings before homogenisation.
(R+FR): red, followed by far-red irradiation given in vivo to seedlings before homogenisation.

Individual irradiations were of ten minutes duration each.
10mM Ca\(^{2+}\)

10mM EDTA

1\% Triton X-100

Buffer
This interpretation is supported by the observation that potassium chloride, \((\text{KC}1)\), a high ionic strength reagent, effective in the removal of peripheral, electrostatically-bound proteins from membranes (Maddy and Dunn, 1976), did not cause an increase in the recovery of soluble phytochrome in the supernatant. Figure II.6 shows that 10 mM EDTA induced the release of a small amount of pelletable phytochrome from both the "Red" and the "Red + Far-Red" pellets. EDTA (ethylene diamino tetra-acetic acid) is a chelating agent and its effect would implicate divalent cations in the association of "Red" and "Red + Far-Red" phytochrome with membranes. In the same context, 10 mM Ca\(^{2+}\) was seen to "protect" pelletable phytochrome from breakdown or release to the supernatant for all three treatments, but its effect was most pronounced for "Dark" phytochrome, followed by "Red" then "Red + Far-Red" phytochrome. One possible explanation for such a differential effect would be that divalent cations were already involved in the association of "Red + Far-Red" phytochrome, and, to a lesser extent, "Red" phytochrome with pelletable material, thus the addition of exogenous calcium ions to "Red" or "Red + Far-Red" pellets had less effect than did Ca\(^{2+}\) addition to "Dark" pelletable phytochrome. Such an explanation of the effect of Ca\(^{2+}\) agrees with the deductions already made concerning EDTA. Figure II.2.b. shows that divalent cations were certainly effective in causing phytochrome association with pelletable material, and, although this association was shown to be a weak surface attachment, divalent cations
may also be involved in the stronger phytochrome association with membranes.

3:4:3 General observations

As well as being able to make specific observations on the effects of the membrane-modifying reagents used, several general observations can be made concerning the effects of the treatments on the pelletable phytochrome from "Dark", "Red" and "Red + Far-Red" tissue.

3:4:3:1 The distribution of total phytochrome recovered in buffer controls.

Figures II.6 and II.7 show that the effects of an incubation in extraction buffer, followed by recentrifugation, were similar for "Dark" and "Red" pellet ed phytochrome; hence, the stability of the phytochrome association with membranes was similar for the two treatments. However, Figure II.6 shows that, after the incubation and re-centrifugation of a "Red+Far-Red" pellet, about 15% less phytochrome was pelleted than was observed in "Dark" or "Red" samples, which suggests that the "Red + Far-Red" pellet ed phytochrome was not quite so stably associated with membranes as either "Dark" or "Red" pelleted phytochrome.

3:4:3:2 A general comparison of "Dark" and "Red" pelleted phytochrome.

Given that "Dark" and "Red" buffer controls gave
similar results following incubation and re-centrifugation (see 3:4:3:1), a general comparison of the behaviour of "Dark" and "Red" pelletable phytochrome from Figures II.6.a. and II.7 would appear to indicate that "Red" pelleted phytochrome was more resistant to membrane-modifying reagents than was "Dark" pelleted phytochrome (with the exception of EDTA and Ca$^{2+}$, the effects of which have already been discussed). More "Dark" pelleted phytochrome than "Red" pelleted phytochrome was either released to the supernatant or lost its photoreversibility. The possibility exists however, that these results were due, not to differences between the strengths of types of association of "Dark" and "Red" pelleted phytochrome with membranes, but to differences between the reactivities of Pr and Pfr to the reagents used. In order to test this possibility, samples from "Dark" and "Red" tissue were prepared identically to those used for Figure II.6.a. the only difference being a photoconverting irradiation in vitro given immediately after resuspension of the pellet in the reagent. Therefore, dark samples were given a red irradiation, and incubated as Pfr, and "Red" samples were given a Far-red irradiation, and incubated as Pr. The results from these samples are shown in Figure II.6.b.

Comparison of Figure II.6.a with b shows clearly that the differences in pelletability between "Dark" and "Red" phytochrome are as evident in Figure II.6.b. as they were in Figure II.6.a.; therefore these differences are not due to differences between the reactivity of Pr and Pfr, but to the overall effect of an in vivo Red irradiation, not
only by photoconverting Pr to Pfr, but also by inducing both an increase in, and a change in the characteristics of phytochrome association with membranous material.

4: Conclusions and Discussion

The main conclusion which can be drawn from the results presented in this section is that there are two major types of phytochrome association with pelletable membranous material, namely; the association of over 60% of the total extractable phytochrome with pelletable material in the presence of divalent cations, when present as Pfr or immediately cycled Pr; and the association of a maximum of approximately 10% of total extractable phytochrome with pelletable material in the absence of divalent cations, whether in the Pr or the Pfr forms (see Figure II.2). These results confirm the previous findings of others (Rubenstein, Drury and Park, 1969; Grombein, Rudiger, Pratt and Marmè, 1975; Pratt and Marmè, 1976). The extra association of Pfr with pelletable material seen in the presence of divalent cations appears to be entirely dependent on the presence of these cations; washing the fraction in buffer without added divalent cations releases the majority of the associated phytochrome to the supernatant. This also confirms previous reports (Marmè, Mackenzie, Boisard and Briggs, 1974; Pratt and Marmè, 1976), and indicates that the association of this phytochrome with pellets is weak and electrostatic in nature. This divalent cation-induced
association of Pfr and membranous material has been studied many times in detail in a number of species (Marmë, Boisard and Briggs, 1973; Grombein, Rudiger, Pratt and Marmê, 1975; Pratt and Marmê, 1976; Quail (1976.b)), and it is this fraction to which reports claiming both an electrostatic association of phytochrome with membranes (Quail, 1978a), and phytochrome association with a membrane protein (Gressel and Quail, 1976) refer.

Uncycled Pr, which was associated with pelletable material in the presence of divalent cations was much more strongly bound to the pelletable material than was the large excess of Pfr whose association was induced by red light. Uncycled Pr did not show a significantly greater release to the supernatant fraction during a wash in buffer without divalent cations as compared to a wash with divalent cations in the buffer. Although the association of uncycled Pr with pelletable material was not studied further, characteristics of this association along with the observed percentage pelletability of uncycled Pr in the presence of Mg²⁺. (Figure II.2.b), suggest that the association is similar to that of uncycled Pr observed in the absence of divalent cations.

The greater part of the association between phytochrome and pelletable material extracted from etiolated and red-irradiated tissue in the absence of divalent cations was shown by means of several pellet washes, not be a spurious co-precipitation of soluble phytochrome with pelletable material as has previously been suggested (Marmê, Boisard
Incubation of resuspended pellets containing phytochrome in a number of membrane-modifying reagents showed that, despite non-specific losses due to the experimental protocol, a significant release of phytochrome from the pelletable material occurred in reagents which disrupt hydrophobic linkages, characteristic of the internal phospholipid layer of the membranes (see Figures II.6 and 7). These results suggest that the small percentage of phytochrome pelletable in the absence of added divalent cations is a strongly-bound intrinsic membrane protein. Phytochrome from red-irradiated tissue appeared to be more strongly bound than phytochrome from etiolated tissue; this difference in binding was shown not to be due to any variation in the reactivities of Pr and Pfr. The binding of "Red" phytochrome appeared to show some properties indicating the association of divalent cations with the bound phytochrome, e.g.; the significant release of phytochrome by EDTA into the supernatant.

Pellets extracted from "Red + Far-Red" tissue showed an increased non-specific release of phytochrome to the supernatant, a less pronounced effect of Triton X-100 and Ca\(^{2+}\), and a greater effect of EDTA than was seen from "Dark" or "Red" pellets. (see Figure II.7). This suggests that phytochrome in "Red + Far-Red" tissue was rather less intrinsically bound, and more peripherally associated electrostatically to the membrane.
The general characteristics of the phytochrome which was pelletable in the absence of divalent cations, suggest a strong hydrophobic interaction with the inner phospholipid component of membranes, agreeing with the findings of Roux and Yguerabide (1973), Georgevich, Krauland and Roux (1976), and Roth-Bejerano and Kendrick (1979). It can be seen that these workers studied phytochrome and membrane association in the absence of divalent cations (with the exception of Roth-Bejerano and Kendrick who used 1 mM Mg$^{2+}$ but achieved pelletability results with barley comparable to those found with no divalent cations in oats). Thus, it would seem that the question of the nature of phytochrome association with membranes can be resolved as follows: a small proportion of the total recoverable phytochrome is intrinsically associated with membrane lipids, and pellets in the absence of divalent cations. This proportion is nearly doubled by the effect of red light in vivo. In contrast, the majority of recoverable phytochrome when present as Pfr or cycled Pr, associates electrostatically onto the membrane periphery in the presence of divalent cations.

The significance of the observed differences in both the nature and the content of phytochrome association with membranous material will be assessed in the General Discussion in conjunction with results from physiological experiments reported in Section IV.
SECTION III

The Stability And Spectral Properties in vitro

Of Supernatant And Pelletable Phytochrome
1: **Introduction**

Supernatant and pelletable phytochrome were first suggested as candidates for the "bulk" and "active" fractions (see General Introduction) respectively by Rubenstein, Drury and Park (1969) on the basis of observed differences in the stability of the two fractions. They found that supernatant phytochrome was stable during incubation in white light for up to 70 hours while phytochrome in a resuspended pellet sample lost its photoreversibility with a half-life of around 20 hours. When incubated in darkness, pelletable phytochrome was more stable, having a half-life of around 50 hours while again supernatant phytochrome showed no significant loss of photoreversibility.

Recent studies have claimed to show differences between the molecular properties of supernatant and pelletable phytochrome (Boeshore and Pratt, 1977; 1978). However, these studies, as is true of the majority of investigations of "pelletable" phytochrome, used Mg\(^2+\) during extraction, so that the "pelletable" phytochrome consists mostly of cytosolic (supernatant) phytochrome weakly associated with pelletable material, and only a small proportion of true membrane-bound phytochrome.

Thus, there has been very little comparison of the in vitro properties of true pelletable (membrane-bound) and supernatant (cytosolic) phytochrome up until the present, and it was with this background that the experiments reported in this Section were carried out.
Having established in Section II that a non-artifactual binding of phytochrome to a membranous fraction does exist, the question then arises as to whether supernatant and pelletable phytochrome differ significantly in their properties, and, if so, whether such differences could help to explain the phenomenon of phytochrome binding, or provide supporting evidence for the "bulk" and "active" fractions theory. In order to investigate the stability and spectral properties of supernatant and pelletable phytochrome, the behaviour of the pigment was measured under various conditions of incubation and irradiation.

2: Methods

2.1 The preparation and incubation of supernatant and pelletable phytochrome.

Supernatant and pelletable phytochrome were prepared as described in Appendix I.2, from either etiolated, red-irradiated or red-followed-by-far-red-irradiated tissue. The supernatant concentration was 0.5 g (tissue) . ml⁻¹, and the resuspended pellet concentration was 2.0 g (tissue) . ml⁻¹. In vitro irradiations were performed immediately after resuspension of the pellet in buffer. Supernatant and resuspended pellet samples were then incubated in darkness at 4 °C in covered vials for up to 48 hours. Oxygen was not excluded from the solutions or vials.
2:2  **Heating of samples for degradation assay.**

Samples in test-tubes were heated to the specified temperature in a water bath; the tubes were then immediately removed from the water bath and quickly cooled in ice. They were assayed spectrophotometrically as soon as they had reached a temperature of 4°C. All operations were carried out under a dim green safe-light.

2:3  **Operation of the Aminco DW-2a Spectrophotometer and the Midan-T accessory**

2:3:1  **For detection of phytochrome decay**

The machine was operated in the dual beam scanning mode using the Midan-T for baseline correction. The scan speed was 2 nm s⁻¹ using the medium response time, and a slit width of 3 nm. This enabled an expanded full scale of 0.01 Å to be used, without excessive machine noise.

Two 2.5 ml aliquots of unirradiated sample were pipetted into clear plastic cuvettes and these placed in the sample and reference positions in the spectrophotometer. The measuring beams were equalised and a scan from 600-800 nm performed and recorded in the Midan-T. This was subsequently used as the baseline, "0", and was subtracted from all other scans, so that inherent differences between the two samples would be eliminated. Using the baseline correction facility of the Midan-T the corrected baseline was plotted as it was scanned, and is indicated as "0-0" in the Figures (i.e. the original difference spectrum between the sample
and reference - the baseline "0" - subtracted from itself, to give the corrected baseline). The sample was then given one minute actinic red irradiation horizontally using a projector and a 660 nm interference filter (see Appendix I.4.8 for specification), taking care not to irradiate the reference. After the actinic irradiation was complete and the projector switched off, a second scan was made and simultaneously plotted with baseline correction; this scan is indicated as "R - 0" in the Figures. When it was complete the sample was then given one minute actinic far-red irradiation using the projector as before, with a 730 nm filter. After the far-red irradiation, a third scan was made and simultaneously plotted with baseline correction; this scan is indicated as "R/FR - 0" in the Figures. The non-photoreversible drop in absorbance in the red region of the spectrum is defined as the ΔA, (R/FR - 0), at the wavelength which is the peak height of this non-photoreversible absorbance change.

2:3:2 Analysis of photoreversible phytochrome

The machine was operated in the same mode as for 2:3:1. Two 2.5 ml aliquots of the sample were pipetted into clear plastic cuvettes and simultaneously irradiated with actinic red irradiation as previously described for two minutes. The two cuvettes were placed in the sample and reference holders and a scan from 600-800 nm performed. This scan represented the baseline and was stored in the Midan-T. The sample was then given one minute of actinic far-red
irradiation as previously described and a second scan performed and stored in the Midan-T in a separate memory. The first scan was then subtracted from the second scan in the Midan-T and the resultant difference spectrum stored in the machine's memory. This different spectrum represented photoconversion of phytochrome from Pfr to Pr by far-red irradiation. Recording difference spectra in this way avoided problems with red-induced non-photo-reversible changes such as spectral alterations of phytochrome, or protochlorophyll photoconversion. A single difference spectrum had a low signal to noise ratio, so that peak heights were difficult to judge; therefore, multiple difference spectra were obtained and added for each sample using the Midan-T, and a sum of these spectra was obtained and stored. Two spectra were used for supernatant samples, because these had a low noise level; but for pellet samples, four spectra were added because the signal to noise ratio was about half that of supernatant samples. Curve smoothing of the summated difference spectra was performed in the Midan-T and the smoothed difference spectra plotted and used to obtain the spectral characteristics quoted in Table III.1.

2:4 Construction of the action spectrum for phytochrome decay

Samples of supernatant phytochrome heated to 50 C were used, so as to obtain the maximal response (see 3:3:2:1).
The samples were actinically irradiated with varying doses of different wavelengths of light; any photoreversible phytochrome was then converted back to Pr by a subsequent one minute actinic far-red irradiation so that the non-photoreversible absorbance could be seen. The differing actinic wavelengths employed were obtained by using interference filters. The total fluence transmitted by each filter per second was measured using a spectroradiometer (see Appendix I.4) and the total fluence given to the samples varied by altering the time of irradiation.

The response was measured using the spectrophotometer in the dual beam mode with an unirradiated aliquot of heated sample as the reference. Absorbance changes were measured at a single wavelength, 662 nm, the peak of the non-photoreversible absorbance change (see Figure III.8). Several different total fluence values were used for each wavelength and the results plotted out in a semi-log graph form, as shown in Figure III.9.

3: **Results and Observations**

3:1 **Stability of photoreversible phytochrome in vitro**

3:1:1 **Stability of Ptot after various irradiation treatments**

Phytochrome was extracted from etiolated, red-irradiated, and red-followed-by-far-red-irradiated tissue, and separated into the supernatant and resuspended pellet fractions as described in Appendix I. The samples were split up and either given no further irradiation, or given photoconverting
in vitro irradiations. They were then incubated for 48 hours in the dark, or under continuous white light, the photoreversible phytochrome being measured at intervals throughout the incubation. The results are shown in Figures III.1-3.

A number of observations can be made from these data. Firstly, there is little difference between the stabilities of supernatant and pelletable phytochrome. The only significant differences can be seen in Figures III.2.i, and 2.iii, at the 24 hour point, at which stage significantly greater loss of supernatant phytochrome than pelletable phytochrome had taken place. These would indicate that soluble phytochrome, extracted as PPr and then photoconverted to Pr in vitro, is subsequently less stable than membrane-bound phytochrome treated in the same way. It is impossible to say from these results whether this difference has any biological significance, but it is interesting to note that this observation parallels that which shows soluble phytochrome to be less stable than membrane-bound phytochrome in vivo after the tissue had received a red irradiation. (see Section IV).

Secondly, if phytochrome was incubated as Pr, irrespective of its source or environment, a loss of around 50% of the photoreversible phytochrome was observed over a 48 hour dark incubation at 4 C. A similar loss was observed in samples incubated under continuous white light; again, irrespective of the source or environment of the phytochrome. On the other hand, if phytochrome was incubated after a final red irradiation, with the majority of the pigment
FIGURES III.1-3

The effect of a 48 hour incubation at 4 C on the amount of photoreversible phytochrome detectable in supernatant (sn) and resuspended pellet (p) samples irradiated in various ways.

Key to Irradiations:

D: no irradiations
(R): red irradiation, \textit{in vivo}
(R+FR): red irradiation, followed by far-red irradiation, \textit{in vivo}
R: red irradiation, \textit{in vitro}
R+FR: red irradiation, followed by far-red irradiation, \textit{in vitro}
Continuous W: continuous white light incubation \textit{in vitro}.

Various combinations of \textit{in vivo} and \textit{in vitro} irradiations were given to samples as indicated in the legends to individual graphs. All irradiations were of ten minutes duration.

Total phytochrome Ptot detected at a particular time is expressed as a percentage of that present in the same sample at the beginning of the incubation, "T₀".
FIGURE III.1

Samples extracted from etiolated tissue.
Figure III.1

i: D

ii: R + FR

iii: R

iv: continuous W

% of Plot at T₀

Incubation (hr)
FIGURE III.2

Samples extracted from tissue which had been given a red irradiation immediately before extraction.
Figure III.2

(i) (R) FR

% of Plot at T₀

Incubation (hr)

6 24 48

0 20 40 60 80 100

(ii) (R)

% of Plot at T₀

Incubation (hr)

6 24 48

0 20 40 60 80 100

(iii) (R) continuous W

% of Plot at T₀

Incubation (hr)

6 24 48

0 20 40 60 80 100
FIGURE III.3

Samples extracted from tissue given red, followed by far-red irradiations, immediately before extraction.
Figure III.3

i: \((R+FR)\)

\[\begin{array}{l}
\text{Incubation (hr)} \\
6 \quad 24 \quad 48
\end{array}\]

\[\begin{array}{l}
\% \text{ of Plot at } T_0 \\
100 \quad 80 \quad 60 \quad 40 \quad 20 \quad 0
\end{array}\]

\[\begin{array}{l}
\text{i: (R+FR)} \\
p \quad sn
\end{array}\]

ii: \((R+FR)R\)

iii: \((R+FR)\) continuous

\[\begin{array}{l}
\text{Incubation (hr)} \\
6 \quad 24 \quad 48
\end{array}\]

\[\begin{array}{l}
\% \text{ of Plot at } T_0 \\
100 \quad 80 \quad 60 \quad 40 \quad 20 \quad 0
\end{array}\]

\[\begin{array}{l}
\text{ii: (R+FR) R} \\
p \quad sn
\end{array}\]

\[\begin{array}{l}
\text{iii: (R+FR) continuous} \\
p \quad sn
\end{array}\]
present in the PPr form, a maximum loss of only 20% was observed, again, irrespective of the pre-treatments.

These observations can be seen more clearly in Figure III.4, where the results of Figures III.1-3 for 48 hours have been pooled:– a: according to the form in which the phytochrome was incubated; and b: according to the form in which the phytochrome was extracted from the tissue.

Figure III.4.b shows that in vivo irradiations have no significant effect on the subsequent stability of phytochrome in vitro, despite the fact that there is around 75% more phytochrome in the "extracted as PPr" pellet than the others (see Figure III.2). The difference between the "extracted as Pr" and the "extracted as cycled Pr" pelletable phytochrome may have some biological significance as it corresponds with the weaker binding of cycled Pr compared to non-cycled Pr, shown in Section II.

Figure III.4.a, clearly indicates that there is significantly less loss of photoreversible phytochrome when it is incubated as PPr, compared to Pr or to cycling Pr + PPr. Similar observations have been made using pea phytochrome (Shimazaki and Furuya, 1980). It is tempting to account for the loss from the "incubated as PPr" samples as being due to the 25% or so of Pr necessarily present in these samples. If the Pr were to act as it does in the "incubated as Pr" samples, then 50% of it would lose its photoreversibility, giving a total loss from the sample of 12-13%, which is not significantly less than the actual observed loss. If Pr loss were occurring, the amount of
FIGURE III.4

Pooled results for $T_{48}$, taken from Figures III.1-3, showing the measured percentage loss of phytochrome during the 48 hour incubation.

a): results pooled according to the form of incubation of phytochrome.

- Incubated as Pr: pooled data from:
  - Figures III.1.i.
  - 1.ii.
  - 2.i.
  - 3.i.

- Incubated as PPr: pooled data from:
  - Figures III.1.iii.
  - 2.ii.
  - 3.ii.

- Incubated as cycling P: pooled data from:
  - Figures III.1.iv.
  - 2.iii.
  - 3.iii.

b): results pooled according to the form of phytochrome upon extraction.

- Extracted as Pr: pooled data from:
  - Figure III.1.i-iv.

- Extracted as PPr: pooled data from:
  - Figure III.1.i-iii.

- Extracted as cycled P: pooled data from:
  - Figure III.3.i-iii.

sn: supernatant phytochrome
p: pelleted phytochrome
incubated as Pr

incubated as Pfr

incubated as cycling Pr \rightleftharpoons Pfr

extracted as Pr

extracted as Pfr

extracted as cycled Pr
Pr should go down with time while the amount of Pfr should remain constant. In order to investigate this question, the amounts of Pr and Pfr were measured in samples incubated after a final red irradiation, and the results are shown for individual treatments in Figure III.5, and discussed in the following sub-Section.

3:1:2 The stability of Pr and Pfr in samples incubated after a final red irradiation.

Figure III.5 shows that different samples varied in their behaviour. A disturbing observation can be seen in Figure III.5.iii.b. The resuspended pellet phytochrome appeared not to have been fully photoconverted by the in vitro red irradiation, giving an apparent ratio of 49% Pfr : 51% Pr at \( T_o \), rather than the expected 75% Pfr : 25% Pr. Subsequently, Pr appeared to decay while Pfr remained stable. However, Pr decay is probably not the explanation of the results in Figure III.5.iii.b, because the measured apparent Pfr : Pr ratio in samples from the same extracts maintained under continuous white light is also approximately 1 : 1 at \( T_o \), and after 48 hours illumination. The white light used is known to set up a measured photoequilibrium of 69% Pfr : 31% Pr (see Appendix I), therefore it appears highly unlikely that the lower-than-expected apparent % Pfr observed both in samples in Figure III.5.iii.b, and under continuous white light is actually due to phytochrome absorbance changes.

What was actually observed in the \( T_o \) readings shown in Figure III.5.iii.b, was that the far-red irradiation given
first to the sample during phytochrome measurement - which
photoconverted Pfr to Pr and was the basis for the
calculation of the relative amounts of Pr and Pfr -
appeared also to cause a significant non-photoreversible
absorbance change due to Pfr to Pr photoconversion, so that
the amount of Pfr was underestimated. The change was non-
photoreversible so that Ptot, calculated from subsequent
red and far-red irradiations, is measured correctly. The
change only occurred in extracts from tissue given red-
followed by-far-red irradiations in vivo, and appeared to
decay with time after the in vitro red irradiation (see
Figure III.5.iii.b) so that the T48 reading resembled
that observed in supernatant samples (see Figure III.5.iii.a).
What gives rise to the non-photoreversible change is not
known, but it is probably another pigment in the membranous
pelletable material.

Apart from the observation in Figure III.5.iii.b,
described above, there was no significant difference
between supernatant and pelletable phytochrome. Phytochrome
photo-converted to Pfr in vivo remained at a constant level
but the Pr present showed loss during the dark incubation
(see Figure III.5.ii). Phytochrome photoconverted to Pfr
in vitro appeared to be lost, but Pr remained at a constant
level (see Figure III.5.ii and iii). A possible
explanation of the results in Figure III.5 is that Pfr
formed by an in vitro photoconverting irradiation is less
stable than Pfr formed by an in vivo irradiation and
reverts in the dark to Pr which is then subject to loss
FIGURE III.5

An analysis of the changes in Pr and Pfr in samples incubated in darkness at 4°C after a final red irradiation.

Pr and Pfr are shown as a percentage of the Ptot value at $T_0$ for each sample.

a: supernatant samples
b: resuspended pellet samples.

i: analysis of Figure III.1.iii.

R: red irradiation in vitro.

ii: analysis of Figure III.2.ii.

(R): red irradiation in vivo.

iii: analysis of Figure III.3.ii.

(R+FR)R: red irradiation, followed by far-red irradiation in vivo,

followed by red irradiation in vitro.
a supernatant

Incubation (hr) Incubation (hr)

% of Ptot at T0

Pfr  Pr

i : R

Pfr  Pr

ii : (R)

Pfr  Pr

iii : (R + FR) R

Pfr  Pr

6  24  48

b pellet

Incubation (hr)

Pfr  Pr

i : R

Pfr  Pr

ii : (R)

Pfr  Pr

iii : (R + FR) R

Pfr  Pr

6  24  48
at roughly the same rate as the reversion. This explanation is supported by the data of Shimazaki and Furuya (1980), which show significant Pfr reversion in pea extracts if the Pfr is formed in vitro. The incubation which they used was 25°C for one hour. This temperature would be expected to induce higher rates of reversion and loss than were observed at 4°C, thus, comparison of the results reported here with those of Shimazaki and Furuya (1980) indicate that, either Pr loss is not stimulated as much as Pfr reversion at higher temperatures or, there are significant differences between the rates of reversion and decay in oat and pea tissue.

The result for cycling phytochrome (Figure III.4.a) can be explained using the same proposals. During cycling, the Pr present at any time would be subject to decay and would then be replaced by new Pr molecules to maintain a constant percentage of Pr in photoequilibrium; thus Pr would always be available for decay.

Therefore, these results indicate that photoreversible Pr is less stable in vitro than Pfr; Pfr formed in vitro probably being subject to dark reversion to Pr in vitro, while Pfr formed in vivo is completely stable in vitro over the time period studied.

3:2 The spectral properties of photoreversible phytochrome in vitro

Difference spectra of fully photoreversible phytochrome in supernatant and resuspended pellet samples from etiolated, red irradiated and "red-followed by far-red" irradiated
The Spectral Properties of Photoreversible Phytochrome.

The spectral properties of photoreversible phytochrome were measured from difference spectra of identical samples to those used in Figures III.1-3. For an explanation of the construction of difference spectra see Methods, 2.3.2.

a: supernatant samples; the accuracy of wavelength readings was ± 1 nm.
b: resuspended pellet samples; the accuracy of wavelength readings was ± 2 nm.

T₀: measurements were made immediately after sample preparation.
T₂₄: measurements were made on samples after they had been incubated for 24 hours at 4°C.

Irradiations:

R: ten minutes red light.
R+FR: ten minutes red, followed by ten minutes far-red light.
cont'W: continuous white light.

Absorbance characteristics:

R peak: the peak in the red region of the difference spectrum.
FR peak: the peak in the far-red region of the difference spectrum.
I.B.point: the isosbestic point of the difference spectrum.
P:FR rat*: the ratio of the heights of the red and far-red peak
P'chrom' inc* as:cycling P:Pr→Pfr.
### a  supernatant phytochrome

<table>
<thead>
<tr>
<th>Irradiation(s)</th>
<th>Absorbance characteristics(nm)</th>
<th>P' chrom' inc' as:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vivo</td>
<td>In vitro</td>
</tr>
<tr>
<td>T₀⁻</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>R</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>R + FR</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>T₂₄</td>
<td>—</td>
<td>R + FR</td>
</tr>
<tr>
<td>—</td>
<td>R</td>
<td>—</td>
</tr>
<tr>
<td>R + FR</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>R</td>
<td>—</td>
<td>cont' W</td>
</tr>
<tr>
<td>R + FR</td>
<td>—</td>
<td>cont' W</td>
</tr>
</tbody>
</table>

### b  pelletable phytochrome

<table>
<thead>
<tr>
<th>Irradiation(s)</th>
<th>Absorbance characteristics(nm)</th>
<th>P' chrom' inc' as:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vivo</td>
<td>In vitro</td>
</tr>
<tr>
<td>T₀⁻</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>R</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>R + FR</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>T₂₄</td>
<td>—</td>
<td>R + FR</td>
</tr>
<tr>
<td>—</td>
<td>R</td>
<td>—</td>
</tr>
<tr>
<td>R + FR</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>R</td>
<td>—</td>
<td>cont' W</td>
</tr>
<tr>
<td>R + FR</td>
<td>—</td>
<td>cont' W</td>
</tr>
<tr>
<td>—</td>
<td>R</td>
<td>—</td>
</tr>
<tr>
<td>R + FR</td>
<td>—</td>
<td>cont' W</td>
</tr>
<tr>
<td>R</td>
<td>—</td>
<td>cont' W</td>
</tr>
<tr>
<td>R + FR</td>
<td>—</td>
<td>cont' W</td>
</tr>
</tbody>
</table>

### Cycling

- Pr
- Pfr
- P
tissue were recorded both immediately after various in vitro photoconverting irradiations following extraction (i.e., $T_0$), and again, after a 24 hour dark incubation (i.e., $T_{24}$). The results are shown in Table III.1. A number of observations can be made from these results.

Firstly, there are no significant differences between the spectral properties of supernatant and pelletable phytochrome from the same treatments. An earlier study (Quail, 1974.b) had reached the same conclusion, although in that case, using Cucurbita and Zea phytochrome, differences between the same different samples were up to 20 nm, and the accuracy of the assignment of pelletable phytochrome peaks appeared to be in the order of ± 10 nm. Quail's study also used Mg$^{++}$ when extracting pelletable phytochrome so it was not truly representative of membrane-associated phytochrome.

Secondly, there are not significant differences between the spectral properties of phytochrome at $T_0$, in vitro after the different in vivo irradiations. In fact the values of these parameters are almost identical to those measured for phytochrome in vivo (see Table III.2), indicating that extraction has not significantly altered the phytochrome.

Thirdly, there are significant differences after 24 hours between phytochrome incubated as Pr and phytochrome incubated as Pfr, irrespective of the source of the phytochrome. The trends are seen in both supernatant and pelletable samples of phytochrome, but variability between samples of pelletable phytochrome within groups (due to
Table III.2

The Parameters of The Phytochrome Difference Spectrum in vivo.

2.5 g of etiolated coleoptiles were chopped and crushed into a cuvette to a height of 2.5 cm and measured in the spectrophotometer, and a difference spectrum recorded. The values shown are the means of three difference spectra.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red Peak Wavelength:</td>
<td>660 nm.</td>
</tr>
<tr>
<td>Far-red Peak Wavelength:</td>
<td>730 nm.</td>
</tr>
<tr>
<td>Isosbestic Point:</td>
<td>687 nm.</td>
</tr>
<tr>
<td>Peak Height Ratio:</td>
<td>0.88.</td>
</tr>
</tbody>
</table>
the low signal to noise ratio) is too large to allow significance to be attached to these results. After a 24 hour incubation, phytochrome incubated as Pfr in supernatant samples showed the same characteristics as did phytochrome at T_o, whereas phytochrome incubated as Pr showed a shift upwards of 4 nm in the red peak and 2.5 nm in the isosbestic point, along with a shift downwards of 7 nm in the far-red peak. Phytochrome incubated under white light showed the upwards shift of the red peak seen in Pr samples, but only a partial shift down of the far-red peak, of around 3 nm. This probably indicates the presence of a mixture of altered Pr and unaltered Pfr in the samples due to the photoequilibrium between Pr and Pfr molecules which exists. Continuous cycling may also contribute to a change in the properties of phytochrome.

The changes in spectral properties of Pr during its incubation could well be an indication of an early stage of the process which leads eventually to the loss of spectral competence of the phytochrome molecule observed in Figure III.4.

3:3 Assessment of the loss of photoreversible phytochrome in vitro

3:3:1 The characterization of absorbance changes in incubated Pr.

Phytochrome photoreversible signal loss could indicate either: total loss of the molecule by destruction of the
protein and the chromophore, and/or a decay or bleaching of the chromophore group while the protein component remains intact. In order to detect the loss of the protein moiety, antigenic methods must be employed (Coleman and Pratt, 1974b; Pratt, Kidd and Coleman, 1974), as the phytochrome protein is a very small proportion of the total protein present in the sample. Assay of protein in this way was not feasible so spectral assay of phytochrome was employed. This method cannot detect loss of the protein group but it can detect partial spectral decay of phytochrome by disclosing absorbance losses in particular regions of the spectrum. A limitation of the method is that difference spectra must be used because the contribution of phytochrome to the overall absorbance spectrum is so small. A second limitation is that changes can only be observed once per sample, as a result of the first red and/or far-red irradiation(s), thus it is impractical to use resuspended pellet samples because of their low signal to noise ratio, so that the majority of measurements in this section of the Results were made on supernatant samples. Also, the greatest loss of photoreversibility was seen in samples incubated as Pr so that supernatant samples incubated as Pr were studied in more detail.

Figure III.6.a, shows a representative series of scans of a supernatant sample of Pr obtained immediately after preparation (i.e., at $T_0$) according to Methods (see 2:3:1). It can be seen that red irradiation produces a
typical phytochrome difference spectrum due to Pfr formation (Quail, 1974.a), which is almost totally reversed by far-red light, the difference between "0 - 0" and "R/FR - 0" being due to a small proportion of Pfr not photoreversed by the far-red irradiation.

Figure III.6.b shows a similar series of scans for an identical sample which had been incubated for 24 hours in the dark at 4°C. It can be seen that the first red irradiation produces a different difference spectrum to that seen in "a", with the far-red peak shifted down by around 10 nm and the red peak shifted up by the same amount. The peak shapes and heights are also different. In fact, the far-red peak is significantly reduced in height while the red peak appears to be slightly greater in amplitude. The isosbestic point has also shifted up by several nm. A far-red irradiation only partially reverses the spectral changes produced by the red irradiation ("R/FR - 0"). The characteristics of the difference spectrum of the photoreversible phytochrome ("R - 0" - "R/FR - 0") were shown in Table III.1 and these showed differences from the original photoreversible phytochrome. However, "R/FR - 0" also shows that a non-photoreversible spectral change in the sample occurred in the red region of the spectrum. It seemed probable that this non-photoreversible absorbance drop was the result of partial Pr alteration during incubation leading to spectral alterations upon actinic red irradiation, as its value roughly equalled the loss of fully photoreversible phytochrome in the red region of
FIGURE III.6

Difference spectra of supernatant phytochrome incubated in the Pr form. Samples were extracted from red-irradiated tissue and then given a far-red irradiation in vitro and incubated in darkness at 4°C for 24 hours.

i: $T_0$ spectra: samples were measured immediately after far-red irradiation.

ii: $T_{24}$ spectra: samples were measured after 24 hours incubation in darkness, following far-red irradiation.

The sequence of reading of difference spectra:

1: O-O: corrected baseline.
2: R-0: difference spectrum after red irradiation of the sample.
3: R/FR-0: difference spectrum after a red irradiation followed by a far-red irradiation of the sample.

The method of construction of the difference spectra is explained in Methods 2:3:1.
the spectrum. However, further investigation of the change was necessary to establish whether this was indeed the case.

3:3:2 Investigation of the characteristics of the non-photoreversible absorbance drop in the red region.

3:3:2:1 The temperature effect on absorbance changes.

Having observed the development of the non-photoreversible absorbance change in samples of supernatant Pr incubated in the dark at 4°C for 24 hours it was thought that the change might be temperature-dependent, and thus accelerated at higher temperatures. Thus, samples were heated to a range of temperatures in the dark, immediately cooled, and then scanned and irradiated in sequence (see Methods 2:3:1) in order to measure the absorbance changes occurring. The results are shown in Figure III.7.i. It can be seen that, as the non-photoreversible absorbance drop increased, it was paralleled by a fall in the photoreversible absorbance change due to phytochrome, up to a maximum at 50°C, above which temperature protein denaturation destroyed the pigment. This is clearly shown in Figure III.8 and by the comparison of Figure III.8 with Figure III.6.a.

Samples of Pfr, prepared identically to the Pr samples, apart from a photoconverting red irradiation in vitro were treated in the same way and the results are shown in Figure III.7.ii. Only at 50°C was any non-
FIGURE III.7

The effect of temperature on the non-photoreversible absorbance drop seen in the red spectral region in supernatant samples extracted from etiolated tissue, then immediately heated and assayed.

\[ \Delta A_{672 \text{ nm}} \times 10^{-4} \text{ : the change in absorbance at } 672 \text{ nm.} \]

i): Samples heated as Pr: no irradiation was given before heating.

ii): samples heated as Pfr: samples were given one minute photoconverting red irradiation \textit{in vitro} before heating. After cooling, samples were given one minute far-red irradiation to photoconvert Pfr back to Pr. This produced no non-photoreversible drop in absorbance at 672 nm.

a: absorbance drop seen after the one minute actinic red irradiation.

b: absorbance regained after the one minute far-red irradiation which followed the red irradiation. This represents the photoreversible absorbance change due to undecayed phytochrome.

c: "a" - "b": i.e.: the non-photoreversible absorbance drop.

For an explanation of the irradiation sequence of samples and the construction of graphs, see Methods 2:2 and 2:3:1.
i: Heated as Pr

\[ \Delta A_{672\text{nm} \cdot 10^{-4}} \]

\[ \text{Temperature (C)} \]

ii: Heated as Pfr

\[ \Delta A_{672\text{nm} \cdot 10^{-4}} \]

\[ \text{Temperature (C)} \]
Difference spectra of supernatant phytochrome after heating to 50°C as Pr, followed by immediate cooling of the sample. Samples were extracted from etiolated tissue.

The sequence of reading of difference spectra:

1: 0-0: corrected baseline.
2: R-0: difference spectrum after red-irradiation of the sample.
3: R/FR-0: difference spectrum after a red irradiation followed by a far-red irradiation of the sample.

The method of construction of the difference spectra is explained in Methods 2:3:1.
photoreversible change seen and this can practically all be accounted for by the 25% Pr present in the sample. Therefore Pfr does not show any partial spectral decay prior to complete destruction.

3:3:2:2 Action spectrum of the loss of photoreversibility

A final piece of evidence in this investigation comes from an action spectrum for the induction of the response shown in Figure III.10. The procedure for obtaining measurements to construct the action spectrum is described in Methods 2.4. The measurements are shown in Figure III.9. In order to obtain a valid action spectrum the fluence value necessary to achieve a specific response at each wavelength had to be calculated. The values obtained for 654 nm show that the response is log [fluence] dependent at this wavelength, and a similar relationship is assumed for all other wavelengths, so that the gradient for each wavelength is identical; but the threshold fluence value which elicits a positive response varies. Extrapolation of the dose/response lines for each wavelength gives a series of calculated fluences which induce the same response; in this case aΔA 662 nm of $1.0 \times 10^{-3}$. The calculated fluence values were plotted against wavelength to give the action spectrum of the response shown in Figure III.10. Although rather crude, it clearly shows the characteristics of the Pr photoconversion spectrum (Smith, 1975).

Thus, it can be concluded from these various pieces of evidence that the non-photoreversible drop in absorbance in the red region of the spectrum is the result of a
partial decay of Pr during dark incubation. Similar observations have been made in pea phytochrome incubated as Pr, but only in pelletable fractions and not in supernatant fractions (Shimazaki and Furuya, 1980). Also, using purified phytochrome preparations, absolute absorbance changes in Pfr corresponding to these observations have been made (Butler, Siegelman and Miller, 1964; Pratt and Cundiff, 1975).

3:3:3 The relative contributions of partial and total decay to the loss of photoreversible phytochrome.

Having ascertained that, at least in supernatant samples incubated as Pr, some of the loss of photoreversibility was due to partial decay of Pr, the relative proportions of the observed loss of photoreversibility due to this partial decay, and to total loss of photoreversibility during incubation were calculated for all samples incubated for 48 hours shown in Figures III.1-3. The results of these calculations are shown in Figure III.11. As for Figure III.4, it can be seen that the form of phytochrome during incubation does have an effect upon phytochrome loss. There is no significant development of partially decayed phytochrome when supernatant phytochrome is incubated as Pfr, while around 14% of the original ΔΔA can be accounted for as partially decayed phytochrome if the sample is incubated with the phytochrome as Pr.

A surprising observation is that of large amounts of apparent phytochrome loss in pelleted samples when incubated
The dose/response lines at various wavelengths for the non-photoreversible absorbance drop measured at 662 nm.

Dose is expressed as log \{total fluence\} (\mu\text{Mol} \cdot \text{m}^{-2}).

Response is expressed as \Delta A 662 nm \cdot 10^{-3}.

For the explanation of the method of construction of the lines see Methods 2.4, and Results 3.3.2.2.
FIGURE III.10

The action spectrum for the induction of the non-photoreversible absorbance drop in the red spectral region (measured as \( \Delta A_{662\text{ nm}} \cdot 10^{-3} \))

Each point indicates the fluence required to induce a response of a non-photoreversible absorbance drop of \( 1.0 \cdot 10^{-3} \) \( A \) at each individual wavelength.

Individual wavelengths were obtained using a series of interference filters described in Appendix I.

The procedure for the construction of the action spectrum is outlined in Methods 2.4, and Results 3:3:2:2.
total fluence ($\mu$Mol.m$^{-2}$) required for standard response
as Pr. Phytochrome loss cannot, in fact, be the explanation, because, in a number of individual samples, a greater non-photoreversible absorbance drop was seen after the first red irradiation than could be accounted for by known photoreversible phytochrome loss (see Figure III.4). Changes in other pigments which are also present in the pellet such as those associated with etioplast membranes could contribute to the non-photoreversible absorbance change observed, but it is not practical to attempt to confirm this explanation by means of spectral analysis of resuspended pellet samples due to their low signal-to-noise ratio. Therefore, only in supernatant samples can the relative contributions of partial and total decay of phytochrome be calculated.

Using Figure III.11 and Figure III.4.a, these proportions were calculated and the results are shown in Table III.3. It can be seen that roughly the same amount of signal loss is due to complete spectral decay in samples incubated both as Pr and as Pfr, but there is virtually no partial decay in Pfr samples, compared to 28% in Pr samples. As already discussed in Results 3:1:2 the total loss in Pfr samples is probably due to decay of Pr formed by dark reversion in samples where the Pfr was formed in vitro. In samples incubated under continuous white light all the loss was due to complete decay. This is explained by the fact that any partially degraded Pr which develops would quickly be photoconverted by white light and thus lose its photoreversibility totally.
FIGURE III.11

The non-photoreversible drop in absorbance in the red spectral region.

Measurements were made after a 48 hour dark incubation on the same samples used in Figures III.1-3. The non-photoreversible absorbance drop was measured as the non-photoreversible ΔΔA (660 nm - 730 nm) caused by the first actinic irradiation of samples. Because there was no absorbance change at 730 nm due to the non-photoreversible red drop (Figure III.8), this was equivalent to ΔA 660 nm.

The non-photoreversible absorbance change was expressed as a percentage of the original phytochrome reading at T₀.

See the Legend to Figure III.4.a for a breakdown of the individual sample readings used for each histogram.

sn: supernatant phytochrome.
p: pelleted phytochrome.
% drop of original phytochrome reading

incubated as Pr

incubated as Pfr

sn p

sn p
Table III.3
The Contribution of Partial and Complete Phytochrome Decay
To The Loss Of Photoreversible Phytochrome In Supernatant
Samples Incubated For 24 Hours In Darkness At 4°C.

Explanation of terms:
1: this value is taken from Figure III.4 and represents the percentage loss of completely photoreversible phytochrome.
2: this value is taken from Figure III.11 and represents the percentage of the original photoreversible phytochrome signal which is induced to photoconvert by red light, but is not photoreversed by subsequent far-red light.
3: this value is double that of 2, because it represents the percentage of original photoreversible phytochrome which gave rise to the non-photoreversible signal, by including the loss of the far-red peak in the observed difference spectrum (see Figure III.6.ii.).
4: this value is "1-3", and represents the percentage phytochrome which has lost all photochemical activity.

<table>
<thead>
<tr>
<th>Proportion of original phytochrome signal, after 24 hours.</th>
<th>Phytochrome incubated as:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pr</td>
</tr>
<tr>
<td>1: total loss of signal, %</td>
<td>48</td>
</tr>
<tr>
<td>2: non-reversible signal, %</td>
<td>14</td>
</tr>
<tr>
<td>3: partially decayed Ptot, %</td>
<td>28</td>
</tr>
<tr>
<td>4: totally decayed Ptot, %</td>
<td>20</td>
</tr>
</tbody>
</table>
As discussed in Results, 3:3:1, it is impossible to say from the results presented here whether the totally decayed phytochrome has been completely destroyed, including its protein component, or has only suffered chromophore degradation. However, the results in this Sub-section show that Pr in vitro undergoes a non-photochemical decay which is manifested upon red irradiation as a non-photoreversible absorbance drop in the red spectral region, while Pfr appears not to undergo such a change.

4: Conclusions and Discussion

The results presented in this Section show that there are no significant differences between supernatant and pelletable phytochrome, either in their stability or their spectral properties. The only difference between the two fractions existed in the extent of the development of a non-photoreversible drop in absorbance in the red region of the spectrum, induced by actinic red irradiation. This was due to partial decay of Pr in supernatant samples, but either included, or was entirely due to, another component in resuspended pellet samples. If this component was, as suggested, etioplast membrane-associated pigments in the pellet, then it is entirely possible that pelletable phytochrome acted in the same way as supernatant phytochrome in all respects.

These results do not agree with previous observations (Rubenstein, Drury and Park, 1969); however, it is difficult to make direct comparisons because Rubenstein
et al used a supernatant from a 105 000 g centrifugation, and a washed pellet from a 40 000 g centrifugation; after extraction of the phytochrome under fluorescent white light and a 1 500 g pre-centrifugation of the crude homogenate. A balance sheet for phytochrome was not presented so that the losses up to and during the 1 500 g centrifugation cannot be assessed. However, around 25% of the total phytochrome present in the 1 500 g supernatant (which was used for further centrifugations) was not accounted for in the 40 000 g pellet or the 105 000 g supernatant. From the results presented in Section II of this thesis concerning the effect of further centrifugations on previously centrifuged phytochrome it seems likely that this loss was from the supernatant fraction; thus, the preparations studied by Rubenstein et al (1969) do not appear to have been truly representative of the total extractable supernatant and pelletable phytochrome fractions, whereas the preparations used in the present study were so representative (see balance sheet in Appendix I).

As previously mentioned, the report of Quail (1974.a) is also unrepresentative of true membrane-associated and cytosolic phytochrome, and only the report of Shimazaki and Furuya (1980) referred to earlier deals with the appropriate phytochrome populations. However, other reports dealing with in vitro properties of phytochrome can provide information on supernatant phytochrome as they use purified or partially-purified phytochrome from a supernatant fraction.

It appears to be a common observation that Pr is more
stable than Pfr in vitro, with Pfr subject to dark reversion as suggested in Results 3:1:2 (Correll, Edwards and Shropshire, 1968; Anderson, Jenner and Mumford, 1969; Pike and Briggs, 1972), or shifts in absorption peaks (Briggs, Zollinger and Platz, 1968). The lower stability of Pfr than Pr has been confirmed in this thesis for supernatant phytochrome in which Pfr was formed by an in vitro red irradiation; but not when an in vivo photoconverting red irradiation was used. Pfr formed in vivo was found to be as stable as Pr in vitro (see Figure III.5.ii). The different effects of in vivo and in vitro irradiations on phytochrome phototransformations would suggest that phytochrome is in different forms in vivo and in vitro, a view put forward by Briggs and Fork (1969), based on phytochrome intermediate studies. Such an explanation would explain why Pr has always appeared to be more stable than Pfr in vitro, because extractions of phytochrome have always been performed with phytochrome in the Pr form, Pfr then being formed by in vitro red irradiation in order to avoid Pfr breakdown in vivo by endoproteases (Correll, Edwards and Shropshire, 1968; Anderson, Jenner and Mumford, 1969; Pike and Briggs, 1972). It is feasible that in vitro photoconversion of phytochrome does not give rise to Pfr with the same characteristics as Pfr formed in vivo, and thus studies comparing the stability of Pr and Pfr in vitro may not be truly representative of the two forms of phytochrome. The extraction of Pfr formed in vivo would seem to be a better method to employ for comparison of Pr and Pfr in vitro.
The different red absorption peaks for Pr and Pfr reported by Briggs, Zollinger and Platz (1968) have been confirmed here to within 2 nm using difference spectra (see Table III.1.a) but, rather than seeing a shift down of the peak in Pfr as reported by Briggs et al (1968), a change in the red peak of Pr upon extraction was observed here while the Pfr peak remained the same as that observed in vivo. As Briggs et al (1968) did not indicate the in vivo absorption or difference spectrum peaks, it is impossible to say which of their in vitro forms, Pr or Pfr, differed from the in vivo form.

Thus, the studies of some in vitro properties of supernatant and pelletable phytochrome reported here, while indicating differences between the stabilities of Pr and Pfr, give no support to the theory that supernatant and pelletable phytochrome exhibit different properties.
SECTION IV

In vivo Changes In Supernatant And Pelletable Phytochrome
And Their Possible Significance In Phytochrome-modulated
Responses.
1: Introduction

In Section III it was shown that the in vitro properties of supernatant and pelletable phytochrome were very similar, giving no support to the idea that the supernatant and pelletable fractions might represent "bulk" and "active" phytochrome, as previously suggested (Rubenstein, Drury and Park, 1969). One can interpret the findings of Section III as showing that there are not two distinct types of phytochrome molecule, but only one, which is either soluble (supernatant) or membrane-bound (pelletable).

It had already been shown in Section II that red light in vivo caused a significant increase in pelletable phytochrome and it seemed possible that other changes in supernatant and pelletable phytochrome might be elicited in vivo using different irradiation conditions, these changes being a function of the phytochrome molecule's environment in vivo and thus being irreproducible in vitro.

The aim of the experiments in this Section was to see if any such differences could be induced, and, if so, could they in any way give support to the proposals that supernatant and pelletable phytochrome might function in a way suggested by the "bulk" and "active" hypothesis. In order to do this, it was necessary to create a situation in the tissue which could lead to differences between supernatant and pelletable phytochrome. Consideration of the mechanism of phytochrome destruction proposed in Section II gave rise to the idea that a difference between supernatant and pelletable phytochrome might be observed during the course of phytochrome destruction in vivo. To investigate
this possibility, a number of different in vivo irradiations were used which led to phytochrome destruction, and the supernatant and pelletable phytochrome subsequently studied by preparing samples at increasing time intervals after the irradiation. Observed changes were then compared to changes in a suitable physiological response measured in identically irradiated tissue.

2: Methods

2:1 Pelletability assay

The procedure used was the standard pelletability assay (see Appendix I.2) without Mg$^{2+}$ present in the buffer.

2:2 Measurement of seedling elongation

Dry seeds were planted individually, twenty per 20 x 15 cm tray, and grown in standard conditions (see Appendix I.1). Seedlings were used at the same age at which they were harvested for pelletability assays. In order to eliminate variability between batches, individual seedling elongation was calculated by measuring seedling length immediately before irradiation, and then again 24 hours later.

Each sample was taken from two trays of seedlings, and at least 30 individual seedlings from 3 - 6 cm long were measured per sample. Each treatment was carried out on three separate occasions so that the final mean elongations calculated for each treatment represent the
results from at least 90 seedlings.

When performing the experiments on far-red reversibility of the red effect on elongation, in order to reduce variability even more, the seeds were pre-selected. Seeds were soaked in tap water, then germinated for two days on three layers of moist "Kimwipe" paper in an enclosed container. After this time, seedlings showing an emerged radicle were selected and individually planted on three layers of moist "Kimwipe" in the inverted lids of plastic, 20 x 10 cm sandwich boxes, 20 seedlings per lid. The lids were then covered with the inverted box and the seedlings incubated in standard conditions. This operation, carried out under dim green safe-light, resulted in more uniform germination; seedlings with coleoptiles from 1.5 - 3.5 cm long were selected and measured as described above.

3: Results and Observations

3:1 The effect on phytochrome of various irradiations followed by a five hour incubation in vivo.

Four and-a-half day old etiolated oat seedlings were given either a red irradiation, a red irradiation followed by a far-red irradiation, or white light irradiation. P_tot, P_f, and P_r were immediately measured in the seedlings, and in supernatant and pelleted fractions extracted from seedling tissue at the same time, T_o. The treated seedlings were then incubated in darkness - or, in the case of the white-light-treated seedlings, in continuous white light -
and the same measurements performed again in tissue and extracts taken after five hours, $T_5$. The results are shown in Figure IV.1. For ease of comparison of the effect of the incubation on phytochrome in whole tissue, supernatant and pelletable phytochrome fractions, the total phytochrome measured in each of these three samples at $T_0$ was expressed as 100%, made up of Pfr and Pr as shown; the $T_5$ values of Ptot, Pfr and Pr were therefore expressed as a percentage of the Ptot in that fraction at $T_0$.

Figure IV.1 is perhaps most easily assessed by firstly comparing the behaviour of tissue, supernatant and pelletable phytochrome within a treatment, and then comparing the behaviour of the same fraction in different treatments, where appropriate.

Figure IV.1.a. shows the behaviour of phytochrome subsequent to a single red irradiation. Red irradiation initially sets up a Pfr : Pr ratio of around 70% : 30% in all three populations, which agrees well with the calculated ratio of 73% : 27% (see Appendix I.4.1). After the five hour incubation Ptot had been reduced due to phytochrome destruction; down to 32% of the original value in whole tissue samples, and 25% in supernatant samples, but only down to 55% in pelletable phytochrome samples. What is possibly more striking is the observation that all the phytochrome remaining in the pelletable fraction appeared as Pr, while the supernatant fraction contained Pfr and Pr in roughly the same ratio as was initially present. The tissue sample at $T_5$ gave a Pfr to Pr ratio of 45% : 55%, intermediate between that observed in the supernatant and
The effect of various irradiations, followed by a five hour incubation, on phytochrome in vivo in etiolated oat seedlings.

- **a:** Red. Seedlings were given ten minutes red irradiation, followed by five hours of darkness.
- **b:** White. Seedlings were given five hours of continuous white light.
- **c:** Red + Far-red. Seedlings were given ten minutes red, followed by ten minutes far-red irradiation, then five hours of darkness.

$P_{tot}$, $P_{r}$ and $P_{fr}$ were measured:

- **i:** whole tissue (packed 0.5 - 1.0 cm lengths of seedlings).
- **ii:** a 30 000 g supernatant extracted from seedlings.
- **iii:** a resuspended 30 000 g pellet extracted from seedlings.

$T_O$: measurements were made immediately after the ten minute irradiation. (The $T_O$ reading for continuous white light, "c", was taken after ten minutes of white light).

$T_{25}$: measurements were made after the five hour incubation.

Phytochrome in each sample was expressed as a percentage of the total phytochrome measured in that sample at $T_O$. 
a Red

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<th>T_0</th>
<th>T_5</th>
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<tr>
<td>iii</td>
<td>Pr</td>
<td>Pr</td>
</tr>
<tr>
<td>ii</td>
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<td>i</td>
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b White

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C Red + far-red

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<td>i</td>
<td>Pr</td>
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pelletable fractions at that time.

Figure IV.1.b indicates the situation for a five hour white light incubation. Initially, around 70% Pfr was set up in tissue and the two fractions measured, again agreeing with the calculated photoequilibrium which was 0.68 for the white light source (see Appendix I.4.3). After the five hour incubation, Ptot had been reduced to around 20% of the original value in all three samples. The Pfr to Pr ratio had not altered in tissue, but appeared slightly higher - though not significantly so - in the supernatant fraction. Unfortunately, it was impossible to measure Pfr accurately in resuspended pellet samples because of absorbance changes due to photosynthetic pigments, which had increased greatly over the five hours, so that the resuspended pellet samples - which contained a high concentration of etioplast membranes - were distinctly green in colour. The absorbance changes did not affect the accuracy of Ptot calculations, but interfered only with the first red and far-red irradiations, suggesting that one or more pigment intermediates were present as a result of the extraction, which were photoconverted by either the first red or far-red actinic irradiation and thenceforth did not affect further photoconversions. The absorbance changes were similar to those observed and described in Section III (Figure III.5.b.iii), except that the pigments or intermediates involved may be different. This observation illustrates the problems involved when attempting to measure Pfr and Pr in resuspended pellet samples. As a precaution, the accuracy of the readings in resuspended pellet samples in Figure IV.1.a was verified by taking
measurements on different samples given either red or far-red irradiation first. The results obtained were identical, both indicating that no Pfr was present.

Figure IV.I.c shows the effect of a five hour dark incubation on tissue given red followed by far-red irradiation. All the phytochrome is in the Pr form, as expected, in all samples. There is a loss of around 25% of original Ptot measured in both tissue and supernatant samples, but an apparent increase of around 20% in Ptot in the resuspended pellet samples. The difference between the supernatant and pelletable phytochrome is barely significant at the 5% level, but it is interesting to note that it corresponds to the observation in "a" that pelletable phytochrome in the Pr form is less prone to destruction than is supernatant phytochrome.

The behaviour of phytochrome in tissue samples was fairly closely paralleled by that in supernatant samples, and similar to that previously reported for tissue, both for red light only, and for red light followed by far-red light (Chorney and Gordon, 1966; Dooskin and Mancinelli, 1968; Jabben, 1980; Stone and Pratt, 1979), and also for continuous white light (Clarkson and Hillman, 1968; Jabben and Deitzer, 1978.b). However, the behaviour of pelletable phytochrome in red irradiated tissue, subsequently incubated in darkness, was very different from that of the supernatant or whole tissue phytochrome, contrasting with the only previously recorded investigation of red-irradiated tissue (Rubenstein, Drury and Park, 1969), which concluded that
FIGURE IV.2

The effect of phytochrome pelletability of various irradiations followed by a five hour incubation.

a: seedlings given ten minutes red irradiation followed by five hours darkness.

b: seedlings given ten minutes red irradiation, followed by ten minutes far-red irradiation, then five hours darkness.

c: seedlings given five hours continuous white light.

\[ T_0: \text{phytochrome pelletability in extracts taken immediately after the irradiation (for "c", after ten minutes of white light).} \]

\[ T_5: \text{phytochrome pelletability in extracts taken after the five hour incubation.} \]

The percentage of phytochrome pelletability equals:

\[ \frac{\text{P}_{\text{tot in } 30\,000\,g\,\text{pellet}}}{\text{P}_{\text{tot in } 30\,000\,g\,\text{supernatant}}} \times 100 \]
% pelletability of phytochrome

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<td>c</td>
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Red

Red + Far-Red

Continuous W
there was no change in the relative amounts of total supernatant and pelletable phytochrome during the time between extraction immediately after red irradiation, and a further extraction six hours later. Pfr and Pr were not measured. The relative amounts of supernatant and pelletable phytochrome measured in the present investigation can most easily be seen in the pelletability data shown in Figure IV.2. There is an increase in pelletability seen in all three treatments, although this increase is only significant in the case of red-irradiated tissue, because of the lower rate of destruction of pelletable phytochrome.

The investigations showed that, in the case of tissue given a red irradiation followed by a dark incubation, significant differences between supernatant and pelletable phytochrome developed. The next experiment studied the development of these differences in more detail.

3:2  **The in vivo changes in supernatant and pelletable phytochrome during a five hour dark incubation of etiolated seedlings following a red irradiation.**

Figure IV.3 shows the changes in whole tissue, supernatant and pelletable phytochrome during the five hour dark incubation in vivo following red irradiation of the tissue. Once again, the amount of phytochrome in each fraction is expressed as 100%, immediately after the red irradiation.

"a" shows that the reduction of tissue Ptot in darkness after red irradiation - which was due to phytochrome
FIGURE IV.3

The effect of ten minutes red irradiation, followed by up to five hours of darkness, on phytochrome in vivo in etiolated oat seedlings. Samples were taken immediately after irradiation, and after one, three, and five hours of darkness.

a: samples of whole tissue.
b: samples of a 30 000 g supernatant extracted from seedlings.
c: samples of a resuspended 30 000 g pellet, extracted from seedlings.

$P_{tot}$, $P_{fr}$ and $P_{r}$ in each sample were expressed as a percentage of the total phytochrome measured in that sample at $T_0$. 
destruction (Coleman and Pratt, 1974b) was almost entirely accounted for by Pfr loss, though a small amount of Pr also disappeared. The results of "a" agree with previous reports (Chorney and Gordon, 1966; Dooskin and Mancinelli, 1968).

"b" shows that the situation in supernatant samples closely followed that observed in tissue, though not exactly, the most specific difference being the significantly greater final percentage loss of Pr, which occurred mainly in the early stages of the dark period.

"c" shows clearly that the situation in the pelletable phytochrome fraction was very different from that in the supernatant phytochrome. Firstly, the loss of pelletable Ptot proceeded much more slowly than did the loss of supernatant Ptot, as already observed in Figure IV.1, the difference being due to the high stability of Pr throughout the dark period while Pfr disappeared more rapidly than in the supernatant fraction; after three hours of darkness it has virtually all gone. It appears possible that some Pfr reverted to Pr, as the amount of Pr increased by an amount similar to the greater loss of Pfr seen in the resuspended pellet fraction compared to the supernatant fraction. These observations are shown in Figure IV.4 which superimposes not only the graphs from Figure IV.3 showing the change in % Pfr in both resuspended pellet and supernatant samples, but also the graphs showing the observed change in pelletable Pr from Figure IV.3 and the predicted change in pelletable Pr if dark reversion of pelletable Pfr had taken place equivalent to the observed differences between supernatant and resuspended pellet % Pfr values. The Pr (measured)
and Pr (predicted) graphs closely resemble one another. This evidence agrees with the idea that Pfr dark reversion occurs in the pelleted phytochrome fraction, but does not, of course, provide solid proof of the theory. The results could also be explained by supernatant Pr becoming pelletable during the incubation. The various possible explanations for the results presented in Figures IV.3 and 4 are discussed more fully in the Discussion at the end of this Section.

The changes in the relative amounts of Pfr and Pr in tissue, supernatant and pelletable phytochrome can be seen more clearly in Figure IV.5, which shows the Pfr . \( P_{tot}^{-1} \) value at each time point studied. Figure IV.5.a shows Pfr . \( P_{tot}^{-1} \) values for supernatant and pelletable phytochrome. The percentage of Pfr remained steady over the time period studied in the supernatant fraction while it dropped quickly in the pelletable phytochrome fraction, being virtually zero after five hours darkness.

Figure IV.5.b shows that the measured Pfr . \( P_{tot}^{-1} \) in tissue dropped gradually over the five hour incubation. It certainly did not follow the observed trend in the supernatant phytochrome fraction. In fact, a combination of the values for supernatant and pelletable phytochrome - taking into account the changing percentage of pelletable phytochrome - would give predicted Pfr . \( P_{tot}^{-1} \) values closely matching those actually measured in the tissue samples (see Figure IV.5.b). The close agreement between measured and predicted Pfr . \( P_{tot}^{-1} \) values for tissue further supports the validity of the observed differences
A comparison of measured and predicted Pr levels in pelletable phytochrome from etiolated oat seedlings given ten minutes red irradiation followed by an increasing period of darkness.

Pr (measured) and Pfr (measured):
these graphs are taken from Figure IV.3.c.

Pfr (destruction only):
this graph is taken from Figure IV.3.b and represents the predicted change in Pfr in the pelletable phytochrome if the Pfr destruction rate were the same as that observed in supernatant samples.

Pr (predicted):
this graph represents the predicted Pr present in pelletable phytochrome samples if the Pr present at $T_0$ remained stable, and the difference between Pfr (measured) and Pfr (predicted) was due to dark reversion of Pfr to Pr. The graph is constructed from the equation:

$$\text{Pr (predicted)} = \text{Pr at } T_0 + \{ \text{Pfr (measured)} - \text{Pfr (predicted)} \} \text{ at } T_n.$$
Pr (measured)
Pr (predicted)
Pfr (destruction only)
Pfr (measured)
FIGURE IV.5

Comparisons of calculated and measured $P_{fr} \cdot P_{tot}^{-1}$ values for etiolated oat seedlings given ten minutes red light, followed by an increasing period of darkness.

a: supernatant (sn) and pelleted (p) phytochrome from a 30 000 g centrifugation of tissue.

b: calculated values in tissue:

measured: calculated from measurements of whole tissue.

predicted: calculated from the (sn) and (p) values at each point in "a".
Values were obtained using the following calculation:

\[
P_{fr} \cdot P_{tot}^{-1} \text{ (predicted)} =
\]

\[
P_{fr}(sn) \cdot P^{(sn)^{-1}} x \frac{P(sn)}{P(sn) + P(p)}
\]

\[
+ P_{fr}(p) \cdot P^{(p)^{-1}} x \frac{P(p)}{P(sn) + P(p)}
\]
between supernatant and pelletable phytochrome.

The remainder of the experiments in this Section attempted to investigate the question of whether the observed differences between supernatant and pelletable phytochrome have any detectable biological significance.

3:3 The effect of in vivo far-red irradiation on red-induced pelletability.

In order to test for a possible biological significance of the observed differences between pelletable and supernatant phytochrome, the response of phytochrome pelletability itself was studied first, as it was considered to be the most likely phenomenon to be influenced by these differences. The results in Section II showed that the red-induced increase in pelletability of phytochrome was totally reversible by far-red light given immediately (see Figure IV.6, a repeat of Figure II.2.a). According to Figure IV.3.c, the amount of Pfr in the pellet was almost zero by 3 - 5 hours after the red irradiation; therefore, it would be reasonable to suppose that if there was virtually no Pfr in the pelletable fraction one would expect virtually no effect of far-red light, upon the level of pelletability of the phytochrome present. Therefore, the effect on pelletability of a far-red irradiation at increasing times after a red irradiation was studied.

Identical paired samples from the same batch of tissue were given a red irradiation and then incubated in darkness. At the times indicated, one sample only of each pair was given a far-red irradiation; both samples were then
FIGURE IV.6

The effect of *in vivo* irradiations on phytochrome pelletability. (This is a repeat of Figure II.2.a).

Tissue samples were homogenized in buffer which did not contain Mg$^{2+}$.

D: no irradiation

(R): red irradiation *in vivo*.

(R+FR): red, followed by far-red irradiation *in vivo*.

(R+FR+R): red, followed by far-red, followed by red irradiation *in vivo*.

(R+FR+R+FR): red, followed by far-red, followed by red, followed by far-red irradiation *in vivo*.

All irradiations were of ten minutes duration.

\[
\text{% pelletability of phytochrome} = \frac{P(p)}{P(sn) + P(p)} \times 100.
\]

p: pelletable phytochrome.

sn: supernatant phytochrome.
without $\text{Mg}^{2+}$:

% peltability of phytochrome

- D
- (R)
- (R+FR)
- (R+FR+R)
- (R+FR+R+FR)
FIGURE IV.7

The changes in Ptot in etiolated oat seedlings during a dark interval after ten minutes red light.

a: in a 30 000 g supernatant.
b: in a resuspended 30 000 g pellet.

(R)-(D): seedlings given a ten minute red irradiation in vivo, followed by an increasing period in darkness before sample extraction.

(R)-(D)-(FR): seedlings given a ten minute red irradiation in vivo, followed by an increasing period in darkness, followed by a ten minute far-red irradiation in vivo before sample extraction.

D: unirradiated seedlings.
a supernatant

b pellet

Dark interval after R (hr)

\[ \text{Ptot [} \Delta \text{A}(660-730) \cdot 10^{-3} \cdot \text{g}^{-1} \text{(tissue)}] \]
processed identically to obtain supernatant and pelletable fractions. The total amount of phytochrome in each sample was measured and the results are shown in Figure IV.7.

As expected there were no significant differences between the amounts of supernatant phytochrome measured in the two treatments throughout the five hour time period (Figure IV.7.a.). However, there were significant differences at $T_0$ between the "Red" and "Red-plus-far-red" samples of pelletable phytochrome (Figure IV.7.b). These differences became less with time, until, after five hours, the two treatments gave virtually the same amounts of pelletable phytochrome. In other words, by five hours after the red irradiation there was practically no effect of far-red light in reducing the amount of pelletable phytochrome observed in the "red only" sample.

The changes in supernatant and pelletable phytochrome are combined in Figure IV.8 which shows the changes in pelletability of phytochrome with time. Far-red reversibility of pelletability became progressively less as the time between red and far-red irradiations increased. Unfortunately, this change cannot be clearly seen due to the fact that relative phytochrome pelletability increases in both treatments as time progresses. This apparent increase is due to the slower rate of destruction of pelletable phytochrome as compared to that of supernatant phytochrome. However, far-red reversibility of the red-induced increase in phytochrome pelletability can be calculated (see Legend to Figure IV.9), and follows a similar time course to that of the loss of Pfr from the pelletable phytochrome fraction (see Figure IV.9).
In order to compare the two phenomena more easily, each was converted into a percentage value. Pelletable Pfr was expressed as a percentage of pelletable Ptot at T₀, when the red irradiation was given. Far-red reversibility of pelletability was expressed as a percentage of the red-induced increase in pelletability at each time point studied. The results are shown again in Figure IV.10 in a correlation diagram, which shows that, when there is no Pfr in the pelletable phytochrome fraction, the predicted far-red reversibility of the red-induced pelletability increase is almost zero. The correlation coefficient of 0.938 is not significant at the 5% level, indicating either the existence of a non-linear relationship between the parameters, or a fairly large amount of experimental error; it is impossible to conclude which from the data available. However, it can be concluded that, as the amount of Pfr in the pelletable phytochrome fraction falls, so also does the potential for the red-induced increase in phytochrome pelletability to be reversed by far-red light, such that, when no Pfr is detectable in the pelletable fraction, no measurable response of the pelletable phytochrome to far-red light can be observed.

The last part of the Results Section attempts to determine whether the observed behaviour of pelletable phytochrome might have some physiological significance.

3:4 The effect of red and far-red irradiations on etiolated oat seedling elongation.

A suitable physiological response had to be selected
FIGURE IV.8

The changes in phytochrome pelletability during a dark period after ten minutes red light, and the effect of a far-red irradiation on pelletability, given after the dark period but before extraction of samples.

Calculations were made using the data of Figure IV.7.

(R) - (D): seedlings given a ten minute red irradiation in vivo, followed by an increasing period in darkness before sample extraction.

(R) - (D) -(FR): seedlings given a ten minute red irradiation in vivo, followed by an increasing period in darkness, followed by ten minutes far-red irradiation in vivo, before sample extraction.

(D): unirradiated tissue.

\[
\text{% phytochrome pelletability} = \frac{P}{P_{(sn)} + P_{(p)}}
\]

p: pelletable phytochrome.

sn: supernatant phytochrome.
Dark interval after R (hr)

% pelletability of phytochrome

(R) - D

(R) - D - (FR)
Twin plot of the changes with time of:

a: the far-red reversibility of the red-induced increase in pelletability; calculated from the data of Figure IV.8.

Pelletability changed with time in the \((R) - D\) samples, therefore, to calculate reversibility at each time, a predicted dark value was needed. This was found as follows:

\[
\text{Predicted } D \text{ pelletability at } T_n = \frac{D \text{ pelletability at } T_0 \times (R) - D \text{ pelletability at } T_n}{(R) - D \text{ pelletability at } T_0}
\]

From this:

percentage far-red reversibility of red-induced increase in pelletability equals:

\[
\frac{100 \times \left[ (R) - D \text{ pelletability at } T_n - (R) - D - (\text{FR}) \text{ pelletability at } T_n \right]}{(R) - D \text{ pelletability at } T_n - \text{predicted } D \text{ pelletability at } T_n}
\]

b: the amount of Pfr in a 30 000 g pellet, \(- Pfr (p) -\) expressed as a percentage of the Ptot in a 30 000 g pellet sample \(- P (p) -\) extracted from seedlings immediately after they had been given a ten minute red irradiation.

\[
\% Pfr (p) \text{ at } T_n = \frac{Pfr (p) \text{ at } T_n}{P (p) \text{ at } T_n} \times 100
\]
The far-red reversibility of the red-induced increase in phytochrome pelletability (x-axis), correlated with the amount of Pfr in the pellet at $T_n$, expressed as a percentage of the Ptot at $T_o$ (y-axis).

Data taken from Figure IV.9.

$r = $ correlation coefficient.
Far-red reversibility of red-induced pelletability increase (%)
to investigate the effects of red and far-red irradiations. Whole etiolated oat seedling elongation was chosen as the response to be studied for several reasons. Elongation could be easily quantified; it could be measured in whole seedlings without breakage or excision of the tissue; it is a response known to be modulated by phytochrome; and it could be measured in tissue identical to that used for the previous investigations on pelletability.

Firstly, the elongation after various irradiation sequences was measured; the results are displayed in Figure IV.11. Red light inhibits elongation in both the mesocotyl and the coleoptile of whole seedlings. Previous investigations have shown inhibition of mesocotyl elongation by red light (Loercher, 1966; Blaauw, Blaauw-Jansen and van Leeuwen, 1968; Jabben and Deitzer, 1979; Vanderhoef, Quail and Briggs, 1979; Mandoli and Briggs, 1980), but coleoptile elongation has generally been shown to be stimulated by red light (Aghion, Jouglard and Lourtioûx, 1962; Hillman, 1966; Hopkins and Hillman, 1966; Dooskin and Mancinelli, 1968). However, these studies have been performed on isolated segments. In whole seedlings, inhibition of elongation by red light has been observed (Steward and Wiegand, 1968; Wiegand and Stewart, 1974), and also, elongation inhibition by white light after four to five days growth has been shown (Thomson and Miller, 1961). In a recent study (Mandoli and Briggs, 1980) the effect of red light on coleoptile elongation in whole seedlings was studied over a range of ages, and compared to green "safelight". For seedlings over
FIGURE IV.11

The mean elongation (cm) per seedling, which took place during a 24 hour dark incubation of the seedlings after various *in vivo* irradiations.

a: in coleoptiles

b: in mesocotyls

D: no irradiation.

(R): red irradiation.

(R)+(FR): red, followed by far-red irradiations.

(R)+(FR)+(R): red, followed by far-red, followed by red irradiations.

(R):(FR):(R):(FR): red, followed by far-red, followed by red, followed by far-red irradiations.

All irradiations were of ten minutes duration.
a coleoptile

b mesocotyl

mean elongation per seedling (cm.)

D R R+FR R+FR+R R+FR+R +FR
FIGURE IV.12

The mean elongation per coleoptile which took place during the 24 hour period after the red irradiation, calculated for samples given red followed by far-red irradiations with an increasing intervening dark period.

D: no irradiation.
(R): red irradiation.
(R)+(FR): red, followed immediately by far-red irradiation.
(R) + 1 hr D +(FR): red, followed by 1 hour darkness, followed by far-red irradiation.
(R) + 3 hr D +(FR): red, followed by 3 hours darkness, followed by far-red irradiation.
(R) + 5 hr D +(FR): red, followed by 5 hours darkness, followed by far-red irradiation.
mean elongation per coleoptile (cm)

D | R | R+FR | R | R | R  
---|---|------|---|---|---  
+1hrD | +3hrD | +5hr D  
+FR | +FR | +FR
88 hours old, the green "safelight" was found to stimulate elongation more than did the red irradiation. In the experiment reported here, seedlings were 110 hours old, and the "dark" treatment required seedling measurement under green "safelight" before the 24 hour incubation, thus the apparent red inhibition of coleoptile elongation can be accounted for by the observations of Mandoli and Briggs.

The effect of red light on both mesocotyls and coleoptiles was partially reversed by far-red irradiation, agreeing with previous reports (Hopkins and Hillman, 1966; Dooskin and Mancinelli, 1968; Jabben and Deitzer, 1979). A further "red plus far-red" irradiation combination repeated the effects of the first cycle almost completely in mesocotyls, but to a much lesser extent in coleoptiles, indicating the presence of a cumulative, non-reversible effect of the irradiations.

The elongation response is extremely complex, containing several distinct phases (Wiegand and Stewart, 1974; Vanderhœf, Quail and Briggs, 1979). However, the fact that significant far-red reversibility of the inhibition of seedling elongation can be observed, means that a comparison can be made between this observation and the far-red reversibility of the red-induced increase in phytochrome pelletability. Because the mesocotyl and coleoptile are different organs and may therefore act differently in their response, and because mesocotyl elongation is difficult to measure accurately, it was decided only to study the far-red reversibility of the
inhibition of coleoptile elongation in detail.

A red irradiation was given to seedlings, followed by a dark incubation of up to five hours, followed by a far-red irradiation at the appropriate times, followed by up to 24 hours further dark incubation. The mean coleoptile elongation in the 24 hours following the red irradiation was calculated for each treatment. The results are shown in Figure IV.12. It is clear that 100% far-red reversibility was not achieved, but that the amount of reversibility caused by far-red light fell off over the time period studied, and was not significantly greater than zero after five hours. These results are similar to those for far-red reversibility of red-induced phytochrome pelletability and the two sets of data are compared in Figure IV.13. The two responses are in close agreement apart from the first point at $T_o$.

The correlation diagram in Figure IV.14 compares the two sets of results directly, giving a correlation coefficient of 0.983 which is significant at the 2% level. A potential far-red reversibility of elongation inhibition of around 8% is predicted when the reversibility of pelletability is zero. This difference is within the limits of experimental variation. However, the relationship may only be partially linear; a curve of best fit would predict that far-red reversibility is completely lost for both responses at the same time. From the data available, it is impossible to conclude which situation exists and, for the present study, linear correlation of data was
FIGURE IV.13

Twin plot of:-

a: the far-red reversibility of the red-induced increase in phytochrome pelletability (repeat of Figure IV.9.a.).

b: the far-red reversibility of the red-induced inhibition of coleoptile elongation, calculated for each time point from Figure IV.12.

% far-red reversibility of coleoptile elongation inhibition equals:-

\[
\frac{\text{elongation for (R + n hours D + FR)} - \text{elongation for R}}{\text{elongation for D} - \text{elongation for R}} \times 100
\]

where "n" equals 0, 1, 3 or 5 hours, and elongation values are taken from Figure IV.12.
increase in pelletability

inhibition of elongation

Far-red reversibility of red-induced effect (%)
The far-red reversibility of the red-induced phytochrome pelletability increase (x-axis), correlated with the far-red reversibility of red-induced coleoptile growth inhibition (y-axis).

$r$ - correlation coefficient.
Far-red reversibility of red-induced coleoptile growth inhibition (%) vs. far-red reversibility of red-induced pelletability increase (%). The correlation coefficient is $r = 0.983$. 
considered to be the best means of comparing the changes in two parameters in order to investigate the possibility of a direct relationship between them.

Figure IV.15 correlates the far-red reversibility of red-induced elongation inhibition with both (a), the changes in the amount of Pfr with time, and (b), the changes in the proportion of Pfr with $\frac{\text{Pfr}}{\text{Ptot}}$ at $T_n$ with time in each tissue, supernatant and pelletable phytochrome. "a" shows that there is a reasonable correlation of total Pfr present with the far-red reversibility in all three phytochromes populations. "b" shows that, only in the pelletable phytochrome fraction is there a meaningful correlation between $\frac{\text{Pfr}}{\text{Ptot}}$ (at $T_n$) and far-red reversibility. In all four cases where a reasonable correlation occurs, the calculated regression lines are not significantly displaced from a 1:1 relationship.

4: Conclusions and Discussion

The major aim of the experiments documented in this Section was to investigate the behaviour of supernatant and pelletable phytochrome in vivo after various combinations of irradiations, and to see whether any differences between the two fractions developed. The results shown in Figures IV.1-4 show conclusively that differences between the supernatant and pelletable phytochrome fractions developed in vivo during a five
FIGURE IV.15

Far-red reversibility of red-induced inhibition of coleoptile elongation (x-axis), correlated with, on the y-axis:

a: the amount of Pfr at $T_n$, expressed as a percentage of the Ptot at $T_0$.
b: the amount of Pfr at $T_n$, expressed as a percentage of the Ptot at $T_n$.

i: $t =$ whole tissue samples.
ii: $sn = 30\ 000$ g supernatant samples.
iii: $p =$ resuspended $30\ 000$ g pellet samples.

$r: = correlation coefficient.$
Far-red reversibility of red-induced inhibition of coleoptile elongation (%)
hour dark incubation following a red irradiation.

Pfr and Pr were lost at equal rates from the supernatant fraction, maintaining a constant value of Pfr(sn) • P(sn)^{-1}. In the pelletable phytochrome fraction, Pfr appeared both to decay and to dark-revert, while Pr appeared to be relatively stable (Figure IV.3). As already pointed out, the observed rise in Pr in the pelletable fraction by no means proves dark reversion; it is also possible that supernatant Pr became pelletable during the dark incubation. From these data it is impossible to make a conclusive distinction between these two possibilities. However, it seems more likely that the observation in Figure IV.3.c. represents dark reversion of pelletable Pfr for two reasons. Firstly, an increase in pelletable phytochrome has always been observed to require light energy, and has only previously been seen when phytochrome is in the Pfr form, not the Pr form; it therefore seems unlikely that dark binding of Pr could occur. Secondly, as already mentioned, the increased rate of loss of Pfr in the pelletable fraction as compared to the supernatant fraction closely parallels the corresponding increase in pelletable Pr (see Figure IV.4).

Dark reversion has been reported not to occur in vivo in oats or any other monocotyledon investigated (Hopkins and Hillman, 1965), but has been observed in vivo in a number of dicotyledons (eg., Butler, Lane and Siegelman, 1963; Hillman, 1964; Kendrick and Hillman, 1970; Frankland, 1972), and in vitro in both purified oat and rye phytochrome preparations (Correll, Edwards and
Shropshire, 1968; Pike and Briggs, 1972.a). A possible explanation of the apparent lack of in vivo reversion in oats comes from Figure IV.4.a. Readings of whole tissue, "a", showed no increase in Pr because they incorporate the breakdown of Pr in the large proportion of cytosolic (supernatant) phytochrome, 90% of the total, so that any possible reversion of membrane-bound (pelletable) Pfr leading to a rise in Pr would not have been observed. Thus, the breakdown of results in Figure IV.3.a. into b and c may well have revealed the existence of dark reversion of Pfr in pelletable phytochrome, but not in supernatant phytochrome in etiolated oat seedlings. However, as previously noted, these results do not prove the existence of dark reversion, and further tests would be necessary to do this.

Whatever the phenomenon responsible, the observed differences between phytochrome in the supernatant and pelletable fractions are highly important. The loss of Pfr from the pellet correlated well with the loss of far-red reversibility of pelletability (Figure IV.9), which enhances the credibility of the observed differences between pelletable and supernatant phytochrome, which are completely unprecedented.

In the preliminary experiment reported here, far-red reversibility of red-induced inhibition of coleoptile elongation also correlated well with far-red reversibility of the red-induced increase in pelletability, an observation which agrees with the concept that pelletable phytochrome might be a candidate for the "active" phytochrome fraction, as proposed by Rubenstein et al (1969).
The whole concept of "bulk" and "active" phytochrome will be considered in a broad sense in the General Discussion along with the question in general of the connection between spectrophotometric measurements of phytochrome and physiological responses.

As far as the response studied here - the phytochrome control of coleoptile elongation - is considered, it is not possible to conclude whether the change in the total amount of Pfr, or the change in the proportion of Pfr in the total phytochrome was the important parameter in controlling the response. The changes in total Pfr in tissue, supernatant and pelletable phytochrome samples all correlated well with far-red reversibility of red-induced elongation inhibition; thus, if the change in total Pfr present was the significant parameter, either cytosolic (supernatant) or membrane-bound (pelletable) phytochrome could have been responsible for the response. However, if it was the change in the proportion of Pfr in the total phytochrome which was the significant parameter, then Figure IV.15.b shows conclusively that, for the elongation response, it was the pelletable phytochrome fraction which limited the potential far-red reversibility, as the proportion of Pfr in the total supernatant phytochrome remained relatively constant while the potential for far-red reversibility was lost. It is thus evident that further experimental work is highly desirable, in order to determine whether phytochrome control of physiological responses is determined by the total amount of Pfr, or the proportion of Pfr in the total phytochrome in etiolated tissue.
GENERAL DISCUSSION
As outlined in the General Introduction, the aim of the investigations undertaken for this thesis was to elucidate the properties of, and the differences between supernatant and pelletable phytochrome prepared without divalent cations; to characterise the association of phytochrome with membranous particulate material, and to correlate changes in supernatant and pelletable phytochrome — where differences between them developed — with changes in a physiological response in order to investigate the possible significance of any such observed differences.

During the course of the work, three widely-accepted assumptions concerning various aspects of the nature of phytochrome had to be considered. These assumptions were:

1: that the inherent light-scattering properties of resuspended pellet samples lead to artifactually distorted phytochrome readings which cannot be combined with those from supernatant samples; and that the differences between the two sample types can be overcome by the addition of a suspension of CaCO$_3$ particles to the phytochrome-containing samples. (Marmé et al, 1974; Schafer, 1974; Pratt and Marmé, 1976).

2: that the *in vivo* phytochrome reading of a tissue sample is representative of the phytochrome in all the plant cell constituents within that sample. (eg., Steinitz, Schafer, Drumm and Mohr, 1979; Mohr, 1972).
3: that the small percentage of phytochrome pelletable from tissue extracts during a low speed (10,000 - 20,000 r.p.m.) centrifugation without divalent cations present in the extraction buffer is the result of contamination of the pellet by co-precipitation of soluble phytochrome, and is biologically insignificant. (Marmè, Mackenzie, Boisard and Briggs, 1974; Quail, 1980).

The first assumption was examined in detail in Section I where the methodological problem of combining supernatant and resuspended pellet phytochrome readings was investigated. The literature survey, the theoretical analysis of phytochrome spectroscopy, and the results present all support the belief that Assumption "1" is untenable for the majority of measuring systems routinely used in phytochrome research. Further experiments however, are desirable in order to clarify the situation. Dilution and sample thickness series experiments similar to those reported in Section I should be carried out using a cuvette with characteristics which conform to the original criteria stipulated by Butler (1960). In this way, the effect of phytochrome and resuspended pellet particulate material upon the reflectance and scattering coefficient of a CaCO₃ suspension can be properly quantified, so that the intensification effect of the CaCO₃ suspension on the phytochrome reading can be accurately predicted and investigated without interference from the problems introduced by the loss of scattered light through clear cuvette sidewalls.
The second assumption was considered in Section IV where the in vivo changes in supernatant and pelletable phytochrome during a dark incubation after an in vivo red irradiation were investigated. The results of Section IV.3.2. prove that Assumption "2" is also incorrect. Initially, these results were treated with caution as they were totally unexpected; however, they were extremely reproducible and, as Figure IV.4 and 5 show, the combination of readings from different fractions exhibits good mathematical agreement, confirming the validity of the results.

The repercussions of these results are immense, raising doubts about the validity of all phytochrome readings made on tissue samples during dark incubation after irradiation. Many reports correlate changes in phytochrome readings in whole tissue with the extent of various responses.

The original assumption that the in vivo phytochrome reading of a tissue sample is representative of the phytochrome in all the tissue cells and organelles of cells within that sample was questioned by Hillman (1965; 1967), and Briggs and Chon (1966), when the concept of "bulk" and "active" phytochrome populations having different kinetics was first proposed. However, no direct evidence was found which gave credence to the "bulk" and "active" phytochrome hypothesis, and, although not discounted, the possibility of such populations existing has largely been conveniently ignored in experiments which link measured phytochrome content with a measured system response. Experiments
of this sort have been used in attempts to explain how phytochrome functions at whatever site(s) it is active.

It is important to note that the accurate measurement of the changing amounts of Pfr and Pr is crucial to all models of phytochrome action. If Pfr only is the active component then it is the change in the total amount or concentration of Pfr which is the important parameter; however, if both Pfr and Pr function in phytochrome action then it is the changes in both Pfr and Pr, and therefore the ratio of Pfr to Pr, which are the important parameters. A number of workers have correlated the level of response to the measured Pfr . Ptot\(^{-1}\) set up by an initial irradiation (eg: Hopkins and Hillman, 1966; Dooskin and Mancinelli, 1968); and linear relationships between response level and Pfr . Ptot\(^{-1}\). (Steinitz, Schafer, Drumm and Mohr, 1979), and between response level and log \(\{\text{Pfr}\}\) (Loercher, 1966) have been claimed. Such linear relationships only exist between certain arbitrary limits of Pfr . Ptot\(^{-1}\), and these limits vary between reports. Mohr and co-workers have based their theory of phytochrome action on the contention that Pfr is the only active molecule. The main reason for this is that changes in \(\{\text{Pfr}\}\) of tissue samples parallel changes in phytochrome-modulated responses (eg., Drumm and Mohr, 1974), therefore the postulation of a second parameter in the control mechanism of phytochrome is considered unnecessary (Mohr, 1972). However, as mentioned above, linearity of the correlation between \(\{\text{Pfr}\}\) and response is limited; and in fact, in the reference cited above (Drumm and Mohr, 1974),
the Pfr level established by the initial irradiation was not measured but calculated using an assay which predicts Pfr levels, assuming a Pfr breakdown rate with a half-life of 45 minutes (Oelze-Karow and Mohr, 1973). It is clear that if differential rates of Pfr loss exist within the tissue such as those reported in Section IV then the predicted Pfr levels may well be incorrect, and thus the relationship described between Pfr and response might not exist. This example indicates the kind of assumptions often made which would not be tenable if the concept of "bulk" and "active" phytochrome were seriously considered. Because correlations of \(\{\text{Pfr}\}\) and response were achieved using Pfr readings from whole tissue samples, Mohr and co-workers conclude that the "bulk" and "active" phytochrome hypothesis is not necessary to explain phytochrome action (Steinitz, Schafer, Drumm and Mohr, 1979).

It is obvious that experiments based on measurements of initial Pfr . Ptot\(^{-1}\) set up by various irradiations can give no information on the nature of the active phytochrome component because, a: the amount of Pfr formed is exactly paralleled by the amount of Pr lost, therefore any correlation of response with Pfr can also be made in the negative form with Pr; and b: the initial Pfr . Ptot\(^{-1}\) set up in all parts of tissue samples will probably be the same (Section III showed that no significant differences between supernatant and resuspended pellet phytochrome Pfr . Ptot\(^{-1}\) values existed when the fractions were extracted immediately after irradiation), therefore any "bulk" and "active" fractions which might exist will
exhibit the same initial levels of Pfr. It is the changes in phytochrome content occurring after the initial irradiation which are important when considering the action of phytochrome, which is why experiments investigating far-red reversibility of responses at increasing times after the initial irradiation were employed in this work. As the Discussion in Section IV points out, it is impossible to say from the results in Section IV whether it is the observed change in Pfr or the Pfr : Ptot ratio which is the important parameter in the response studied here, but, if pelletable phytochrome is the "active" phytochrome controlling the response, then it is the change in Pfr : Ptot and not in the amount of Pfr which is the crucial factor. Obviously, further experiments are needed to attempt to differentiate between the two possibilities. One way to do this might be to utilize a system where Ptot remains constant and Pfr : Ptot changes with time after an irradiation. If, as was suggested in Section IV, Pfr in the pelletable fraction is capable of dark-reverting, such a system might be achieved by in vivo treatment of tissue with EDTA, Na azide or 2-mercaptoethanol to prevent phytochrome loss, while not inhibiting Pfr reversion (Furuya, Hopkins and Hillman, 1965; Stone and Pratt, 1979).

A change in the Pfr : Ptot ratio rather than in the total Pfr content of tissue has been implicated in the phytochrome control of segment elongation (Hopkins, 1970), and has been proposed as being the critical parameter in
phytochrome modulation of physiological responses by Smith (1975; 1980). By showing that the assumption that phytochrome readings of whole tissue represent the phytochrome in all parts of that tissue is incorrect, the results of Section IV have both given the first solid support to the concept of "bulk" and "active" phytochrome fractions, and added to the evidence suggesting that Pr, as well as Pfr may be involved in the action of phytochrome.

The third assumption considered throughout the thesis was that concerning the significance of the small percentage of phytochrome pelletable in the absence of Mg^{++}. Rubenstein, Drury and Park (1969) first suggested that such phytochrome may be significant, representing an "active" phytochrome fraction, but since their work, the vast majority of research has concentrated on the in vivo irradiation-induced, Mg^{2+} - stabilised pelletability of phytochrome as being a possible candidate for the primary action of phytochrome upon photoconversion.

In order to attempt to assess the biological significance of phytochrome association with particulate pelletable material, both in the presence and absence of Mg^{2+}, it is necessary to decide whether one considers either or both of these two very different types of association to be capable of being the basis for phytochrome-modulated responses.

The weak, electrostatic association of Pfr or cycled Pr with membranes, induced by in vivo irradiation is standardly referred to as in vivo red-light induced
pelletability, and has been studied extensively (Grombein, Rudiger, Pratt and Marmé, 1975; Pratt and Marmé, 1976; Quail, 1978a). Despite arduous efforts, it is still unknown as to whether this association actually takes place in the plant cells in situ, or is an artifactual association induced in vitro by the presence of divalent cations in the homogenization buffer. This problem results from the necessity of an in vitro separation of cytosolic and membrane fractions in order to assay phytochrome pelletability. Thus, opinion must be guided by circumstantial evidence. The various relevant data have been reviewed recently by Quail (1980). The most significant observation made is that the kinetics of four totally different observed processes involving phytochrome are in extremely close agreement, suggesting that they are representative of the same phenomenon. These processes are:

1: The sequestering, and loss of sequestering, of phytochrome in the cell, visualised immunocytochemically. (Kass and Pratt, 1978; Mackenzie, Coleman, Briggs and Pratt, 1975).

2: The development, and loss, of "the potential to pellet" of phytochrome in vivo as cycled Pr (Pratt and Marmé, 1977; Quail, 1976b).


These processes all develop with a half-life of around five seconds, and relax or lose their effectiveness with a half-life of around thirty minutes at 25°C.

The close agreement of the kinetics of these different processes strongly imply, as briefly alluded to by Quail, (1980), that the majority of phytochrome in the cell, soluble as unirradiated Pr, upon photoconversion to Pfr, rapidly self-aggregates in random areas in the cytoplasm (visualised as sequestering). A far-red photoconversion immediately (within five seconds) after the red irradiation prevents the aggregation occurring, but if given subsequent to this, after aggregation has occurred, merely photoconverts Pfr to Pr without causing any immediate disaggregation. Disaggregation does, however, occur slowly, as cycled Pr gradually returns to the cytosol. While phytochrome is aggregated, either as Pfr or as cycled Pr, destruction of phytochrome occurs, and extraction in the presence of divalent cations will preserve the aggregates which, due to their size, will pellet during a 20 000 g centrifugation. Extraction without divalent cations will result in a rapid disaggregation during the disruptive homogenisation and dilution step resulting in soluble Pr or Pfr being seen in pelletability assays. This phytochrome is not subsequently pelletable upon the addition of divalent cations due to the large dilution of the phytochrome.

Several other seemingly disparate results also support this theory. These are:

1: Intracellular concentrations of 5 mM Ca^{2+} have been found in *Nitella* (Weisenseel and Ruppert, 1977) and *Mougeotia* (Wagner and Klein, 1978), and, if comparable to
intracellular concentrations in seedlings, indicate that the intracellular divalent cation concentration is probably high enough to induce self-aggregation of Pfr along the lines shown to occur in pure phytochrome solutions (Yamamoto, Smith and Furuya, 1980).

2: The pelleting of red-irradiated tissue with 10 mM Mg$^{2+}$ using different dark incubation times after the red irradiation shows Pfr breakdown to have occurred in the pelletable fraction only, and not in the supernatant. (Boisard, Marmè and Briggs, 1974). This result agrees with the proposal that only aggregated Pfr is both pelletable and subject to destruction, while any soluble (i.e., non-aggregated) Pfr is not destroyed; thus extraction and centrifugation following red irradiation both immediately, and after several hours dark incubation will show up this difference between aggregated and soluble Pfr.

3: It has been shown that Pfr destruction in vivo can be inhibited by incubating coleoptiles in a buffer containing 2 mM EDTA (Furuya, Hopkins and Hillman, 1965). EDTA did not inhibit O$_2$ uptake, and thus it was suggested that the Pfr destruction process was dependent on the presence of metal ions. It should be noted that another similar experiment gave inconclusive results (Stone and Pratt, 1979).

4: In oat seedlings, the half-life of Pfr destruction is roughly sixty minutes at 25 C. Taking this first order rate together with that of the loss of sequestering (30 minutes), one can predict the in vivo loss of cycled Pr before complete disaggregation of the molecules. Assuming
that all cycled Pr is aggregated, around 67% of the molecules will return to the soluble form while 33% are destroyed. This prediction agrees with published results (Dooskin and Mancinelli, 1968; Stone and Pratt, 1979).

Thus it is believed that current data available support the conclusion that the apparent weak electrostatic association with pelletable material of Pfr or cycled Pr photoconverted in vivo is actually the in vitro expression of in vivo aggregation of Pfr molecules in conjunction with endogenous divalent cations. The question remains however, is this type of phytochrome association capable of being the basis of a phytochrome-modulated response? As has already been said (Quail, 1980), there is no direct evidence that the association is indicative of membrane binding. The apparent weak electrostatic association with pelletable material could well be a simple co-precipitation of the aggregated phytochrome with membranous material, the electrostatic forces dependent on divalent cations merely holding the aggregate together.

None of the many studies on this type of pelletability have correlated or linked it in any way to a physiological response (Quail, 1980); thus, in the light of these cumulative observations, it seems unlikely that this type of association is the basis of phytochrome-mediated physiological responses.

2: The strong hydrophobic association of phytochrome with membranes

The nature of the association of this fraction of
phytochrome does not, in itself, necessarily imply that the association is found in vivo and maintained in vitro; however, it is strongly suggestive of this possibility. No special conditions are necessary to recover the pelleted fraction, merely a pH which ensures that phytochrome does not denature, pH > 6.5. On the basis of this observation, and the hydrophobic nature of the association - a type of bonding which is unlikely to be induced trivially during sample preparation, it was concluded that the association between phytochrome and membranes is representative of the binding which exists in vivo.

Is such an association capable of being the basis of a phytochrome-mediated response? A permanent association of at least a significant proportion of actively functioning phytochrome with the membrane would seem a better basis for light to exert control over membrane properties than a larger-scale light-induced binding of soluble phytochrome to a receptor, especially since the level of total phytochrome is so much lower in light-grown tissue than in etiolated tissue. An intrinsic membrane protein would also be well able to modulate such parameters as membrane permeability and membrane potential. Therefore, this type of phytochrome association with membranes is certainly capable of being the basis of the modulation of the different membrane properties reported (Haupt and Weisenseel, 1976; Marmè, 1977; Smith, Evans and Hilton, 1978; Hale and Roux, 1980). As noted in the Conclusions (4:1) phytochrome from both "Red" and "Red + Far-Red" tissue exhibits
rather different binding characteristics from that of etiolated tissue. Also, nearly twice as much phytochrome is bound to membranes in "Red" extracts as in "Dark" or "Red + Far-Red" tissue extracts. This fact could well be the clue to the differences in the properties of the bound phytochrome after different *in vivo* irradiations. The simplest explanation of the increase in bound phytochrome induced by red light is that there is an association of a soluble Pfr molecule with every previously membrane-bound phytochrome molecule, present in etiolated tissue as Pr, but now Pfr. This idea has previously been suggested (Roth-Bejerano and Kendrick, 1979; Smith, Evans and Hilton, 1978). Because the photostationary state set up in red light is 75% Pfr : 25% Pr (Pratt, 1975), one would expect only a 75% increase in pelletability with red light, which is what was found (see Figure II.2.a). Other workers have noted a similar response when working with membrane-associated phytochrome in crude membrane or organelle preparations. (Georgevich, Cedel and Roux, 1977; Pratt and Marmé, 1976; Roth-Bejerano and Kendrick, 1979; Smith, Evans and Hilton, 1978). In order to explain the equally strong or stronger binding of phytochrome and membranes from red-irradiated tissue, one must postulate that the soluble Pfr molecule, once bound, becomes embedded in the membrane as deeply as is the natively bound molecule.

What could be the nature of this putative primary association between soluble and bound Pfr? A possible answer to this question may come from a comparison of the strengths of binding of the phytochrome from "Dark" and
"Red" tissue. Apart from the overall stronger binding of phytochrome from "Red" tissue the only differences are in their responses to Ca$^{2+}$ and EDTA. The opposite effects of the two additions were explained in "Results 3:3:2" as indicating a possible involvement of divalent cations in "Red" phytochrome binding to membranes. Such an involvement could be most simply achieved by the formation of a divalent cation bridge (possibly Ca$^{2+}$) between the two Pfr molecules as the first stage of the binding of the soluble molecule. Upon subsequent absorption of the soluble molecule into the membrane, the divalent cation bridge would remain. It would thus be a point of attack for EDTA, its breakage leading to a loosening of the binding of the molecules. The effect of divalent cations has already been reported in the phenomenon of Pfr aggregation, thus this idea is plausible. The formation of native phytochrome dimers has also been proposed (Briggs and Rice, 1972), and, just as interestingly, so has the dimerisation of a model Pr chromophore; the change into the dimeric form giving rise to characteristics similar to those of the Pfr chromophore (Scheer and Krauss, 1977). The phytochrome chromophore is known to have acidic COO$^-$ groups and is thought to be in the linear tetrapyrrole form in the native molecule (Rudiger, 1972; Song, Chae and Gardner, 1979). A 90$^\circ$ rotational change of the chromophore with respect to the protein group of the molecule has long been implicated in the Pr to Pfr photoconversion (Haupt and Weisenseel, 1976), on the basis of these data, a tentative explanation of the increased binding seen with an in vivo red irradiation
would involve the exposure out of the membrane of the chromophore of the native phytochrome molecule upon photoconversion to Pfr. The chromophore could then bind to the soluble Pfr chromophore by a divalent cation bridge between exposed COO\(^{-}\) groups, the molecular dimer so formed reaching a stable situation when the second Pfr molecule binds into the membrane beside the first molecule. This arrangement could then create a selectively permeable channel in the membrane and could lead to increased membrane conductivity, a phenomenon observed using purified phytochrome and model membranes (Roux and Yguerabide, 1973; Georgevich, Cedel and Roux, 1977). A somewhat similar idea has been proposed (Brownle\(\text{e}\), Roth-Bejerano and Kendrick, 1979), but without an explanation of the observed increase in membrane-bound phytochrome or any suggestion as to the means of linkage of the phytochrome molecules involved.

This proposal can also incorporate the observed binding characteristics of phytochrome from "Red + Far-red" tissue. The fact that the percentage of pelletable phytochrome from "Red + Far-red" tissue is the same as that from "Dark" tissue suggests that there is simply a reversal of the red light effect, and a dissociation of red-induced bound phytochrome, leaving the original "Dark" bound phytochrome. However, several pieces of evidence point to a more complex situation. Firstly, the characteristics of the binding of "Red + Far-red" pelleted phytochrome (Figure II.6) suggest that it is generally
more labile than "Dark" bound phytochrome. Secondly, Figure II.6 also suggests that there is a more significant contribution of electrostatic binding involving divalent cations than of hydrophobic binding to the "Red + Far-red" pelleted phytochrome association with membranes. These two results taken together would suggest that the bound phytochrome from "Red + Far-red" tissue is more peripheral than that of "Dark" tissue, and involves divalent cation associations similar to those in bound phytochrome from "Red" tissue. The proposal of interconverting membrane-bound phytochrome as peripheral Pr and intrinsic Pfr has previously been proposed by Smith (1976), and such an arrangement is compatible with the current concepts of membranes being dynamic, fluid-like entities.

It can be concluded, that this discussion of pelletable phytochrome provides a strong argument against the assumption that the small percentage of phytochrome recovered in low-speed centrifugation pellets is simply the result of non-specific co-precipitation of soluble phytochrome and is biologically insignificant. It does not prove that the assumption is wrong, but raises serious doubts as to whether it can be taken for granted as has previously been done.

In summary then, the work reported in this thesis has provided evidence for a strong association of a small percentage of total phytochrome in etiolated oat seedlings with a particulate material fraction from the same tissue. An increased level of the association is red/far-red
reversible, and the kinetics of the loss of far-red reversibility of this increase closely parallel those of the loss of pelletable Pfr but not supernatant Pfr, and far-red reversibility of red-induced coleoptile elongation inhibition. It is suggested that these results provide evidence for the theory that membrane-associated phytochrome is responsible for phytochrome-modulated physiological responses, and has different kinetics to those of soluble phytochrome under particular circumstances.
APPENDIX I

General Materials and Methods.
1: **Preparation And Growing Of Plant Material**

Seeds of *Avena sativa* L., (var' Mostyn) were purchased from the National Institute of Agricultural Botany, Cambridge, in 1977. The seeds had been organomercury treated.

100 g of dry, unhusked seeds were sprinkled onto dry vermiculite at a depth of 3 cm in 35 cm x 20 cm seed trays. The seeds were covered with 1 cm of dry vermiculite and the vermiculite was then soaked with tap water. The trays were placed in a growth room maintained in complete darkness apart from when plants were deposited in or removed from the room, at which times a green safelight torch was used for illumination (see Appendix I.4 for specification). The growth room temperature was maintained at 25 C ± 1 C, and humidity at 80% R.H. The trays were watered once a day with tap water. Planting was performed at around 5 p.m. on Day 0, and harvesting at around 9 a.m. on Day 5 for seedlings used routinely. These seedlings are referred to as Four-and-a-half day old seedlings. Three-and-a-half and five-and-a-half day old seedlings (see Section I) were harvested a day earlier and a day later respectively. Four-and-a-half day old seedlings varied from 3 - 6 cm in length, and one tray yielded, on average, 35 - 45 g of seedlings.

2: **Preparation of Tissue, Supernatant And Resuspended Pellet Samples**

All operations were carried out under dim green safelight.
2:1 **In vivo irradiation(s)**

Where required, *in vivo* irradiations were performed by transferring trays of seedlings to the appropriate light box for the irradiation, which was of ten minutes duration (or continuous white light) and then replacing the trays in the dark growth room.

2:2 **Harvesting**

Seedlings at room temperature were cut with scissors just above the surface of the vermiculite. The cut seedlings included mesocotyl, coleoptile and the enclosed leaf. Seedlings were then chopped into approximately 1 cm lengths and used in this form for tissue samples for phytochrome measurements, or homogenised to give supernatant and resuspended pellet samples.

2:3 **Tissue Samples**

2.5 g of chopped seedlings (from 2.2) were gently loaded into a standard plastic cuvette using a glass rod. They were then compacted to a depth of exactly 2.5 cm using a specially made plunger. The sample was allowed to equilibrate for five minutes before measuring phytochrome with the spectrophotometer.

2:4 **Supernatant and resuspended pellet samples**

Preparation of supernatant and resuspended pellet samples was carried out at 4 C in a cold room under dim
green safelight. Chopped seedlings (from 2.1) were homogenised in cold (4°C) 25 mM Tris/MOPS buffer, pH 7.9 - at a tissue to buffer ratio of 1 g tissue : 2 ml buffer - using a "Polytron" blender with a 2 cm diameter head running at half speed for 2 - 5 seconds. The homogenate was squeezed through four layers of 1 mm mesh muslin giving a filtrate with a pH of 7.6 - 7.8. The filtrate was then centrifuged in an MSE 18 centrifuge at 16 000 r.p.m. (30 000 g) for 15 minutes at 5°C. This centrifugation gave a similar phytochrome pelletability value to the commonly-used centrifugation of 11 000 r.p.m. (20 000 g) for 30 minutes. The quicker centrifugation was preferred in order to give the minimal time between sample homogenisation and measurement. After centrifugation, the supernatant was decanted off the pellet and made up with homogenising buffer to a volume of 1.25 x the volume of buffer originally used in the homogenisation, to give the supernatant fraction, which thus had a concentration of 0.4 g (tissue) ml⁻¹. The pellet was resuspended in homogenising buffer to give a concentration of 2.0 g (tissue originally homogenised to give the pellet) : 1 ml of sample solution. This was the resuspended pellet fraction. In vitro irradiations of ten minutes duration, and/or incubation, if required, were given to these fractions before phytochrome measurement. The sample preparation procedure is summarised in the flow chart overleaf. Also, Table A.I.1 gives three examples of balance sheets for the procedure, showing that there was an average loss of less than 10% of measurable phytochrome during the centrifugation step.
4.5 day old etiolated seedlings.

(in vivo irradiations) →

Homogenised in 25 mM Tris/MOPS buffer, pH 7.9 : 2 ml per gram (tissue).

Squeezed through 4 layers of muslin.

Filtrate centrifuged for 15 minutes at 16 000 r.p.m. (30 000 g).

supernatant → resuspended pellet

(in vitro irradiations) →

incubation → phytochrome measurement.
Table A.1.1

Representative Balance Sheets For The Standard Phytochrome Extraction.

In each case, phytochrome was extracted from 4.5 day old etiolated tissue by the method described in 2.4 to give a filtrate which was centrifuged to give supernatant and resuspended pellet samples. (Phytochrome readings in the tissue before homogenisation cannot be compared with the readings here because of the scattering effect of tissue, which results in an artifactually high apparent phytochrome reading).

Phytochrome is expressed as units,

\[ \Delta \Delta A (660 - 730 \text{ nm}) \cdot g^{-1} \text{ (tissue)}. \]

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Filtrate</th>
<th>sn</th>
<th>p</th>
<th>% loss.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.40</td>
<td>7.40</td>
<td>1.00</td>
<td>10.6</td>
</tr>
<tr>
<td>2</td>
<td>8.52</td>
<td>6.65</td>
<td>0.73</td>
<td>13.4</td>
</tr>
<tr>
<td>3</td>
<td>8.24</td>
<td>7.40</td>
<td>0.98</td>
<td>1.7</td>
</tr>
</tbody>
</table>

The average loss of phytochrome = 7.4 %.

sn = supernatant.
p = resuspended pellet.
3: **Phytochrome Concentration**

Samples for phytochrome measurement had a volume of 2.5 ml, which filled the cuvette so that the sample just obscured the measuring beam slit. Thus, it was assumed that all the phytochrome in a sample was being intercepted by the measuring beam. The measured phytochrome per sample had then to be converted to a calculated phytochrome per gram of tissue originally homogenised, taking into account the volume of the sample (2.5 ml), and the concentration of the sample (0.4 g . ml\(^{-1}\) for supernatants; 2.0 g . ml\(^{-1}\) for resuspended pellets). The final phytochrome concentration was given as:

$$P_{tot} (\Delta \Delta A 660 - 730 \text{ nm}) . g^{-1} (\text{tissue}).$$

(or Pr or Pfr)

4: **Specification of Light Sources Used**

The total fluence rate in the 400 - 800 nm band was measured with a spectroradiometer (Gamma Scientific Inc', San Diego). The measurement was broken down to give the fluence rate in each 10 nm band from 400 - 800 nm. These values were then combined to give a calculated phytochrome photoequilibrium for each source using the calculation of Tasker (1977). The time required for phytochrome to arrive at photoequilibrium under continuous illumination was found by measuring the amounts of Pfr and Pr after different irradiation times.

N.B: the photoequilibrium value equals:
the amount of Pfr at photoequilibrium
the amount of Ptot at photoequilibrium

4:1  The red light source

Lights:

4 x 20 watt 2 foot deluxe natural fluorescent tubes (Thorn Electric). The first 4 cms of each end was masked with black insulation tape to remove the far-red output of the tubes.

Filters:

Below the lights was a 2 cm thick clear perspex envelope containing 1.5 % copper sulphate solution. Below the envelope was one layer of No.14 (Ruby) Cinemoid (Rank Strand) sandwiched between two sheets of glass.

Total fluence rate, 400 - 800 nm = 2.28 \( \mu \text{M.m}^{-2}.\text{s}^{-1} \).

Calculated phytochrome photoequilibrium (\( \phi_c \)) = 0.73.

Time required to attain \( \phi_c \) = 5 minutes.

4:2  The far-red source

Lights:

10 x 150 watt clear envelope single coil tungsten lamps, semi-immersed in running water.
Filters:

Below the water bath was one layer (3.2 mm) of red "400" perspex (Röhm and Haas) and one layer (3.2 mm) of "deep blue" Cinemoid sandwiched between two sheets of glass.

Total fluence rate, 400 - 800 nm = 26.18 μM.m⁻².s⁻¹.

\[ \phi_C = 0.0003. \]

Time required to attain \( \phi_C \) = 10 minutes.

4:3 The white light source

(25 C; used for in vivo irradiations).

Lights:

20 x 20 watt 2 foot deluxe natural fluorescent tubes.

Filters:

None.

Total fluence rate, 400 - 800 nm = 57.2 μM.m⁻².s⁻¹.

\[ \phi_C = 0.68. \]

Time required to attain \( \phi_C \) = 2 minutes.

4:4 The white light source.

(4 C; used for in vitro irradiations).

Lights:

2 x 65 watt 8 foot fluorescent tubes.
Filters:

None.

Total fluence rate, 400 - 800 nm = 11.51 \mu M.m^{-2}.s^{-1}.

\[ \phi_c = 0.69. \]

Time required to attain \( \phi_c \) = 5 minutes.

4:5 Green safe lights

Lights:

a: laboratory. 1 x 20 watt 5 foot or 1 x 10 watt 2 foot green fluorescent tube with the first 4 cm of each end masked with black insulation tape.

b: torch. 6 watt Daylight Colourtube fluorescent tube with first 1 cm of each end masked.

Filters:

2 layers of No.39 (green) Cinemoid between which was sandwiched thin gauge black plastic to reduce the light intensity.

Total fluence rate, 400 - 800 nm = 0.0042 \mu M.m^{-2}.s^{-1}.

Cut-off wavelength = 575 nm.

\[ \phi_c = 0.64. \]

\( \phi_c \) would take an extremely long time to be established, due
to the very low fluence rate. After two hours irradiation of tissue, supernatant and resuspended pellet samples;

\[ \phi \text{ estimated} = 0.01. \]

Normally, samples received less than 30 minutes irradiation during preparation.

4:6 **Perkin-Elmer 156 Red source**

**Light:**

Passively cooled 120 watt tungsten projector lamp with integral mirror (Thorn Electric Reference No. A1/24).

**Filters:**

1 inch square 660 nm interference filter (Ealing Beck) with 20 nm half bandwidth.

Total fluence rate, \(400 - 800 \text{ nm} = 245 \text{ \(\mu\text{M.m}^{-2}\text{s}^{-1}\).} \)

\[ \phi_c = 0.74. \]

Time required to attain \(\phi_c = 30 \text{ seconds.} \)

4:7 **Perkin-Elmer 156 far-red source.**

**Light:**

As for red source.

**Filter:**

1 inch square 730 nm interference filter (Ealing Beck) with 30 nm half band width.
Total fluence rate, 400 - 800 nm = 255 \mu\text{M.m}^{-2}\text{s}^{-1}.

\[ \phi_C = 0.0051. \]

Time required to attain \( \phi_C \) = 3 minutes.

4:8 Projector source

(used for action spectrum construction).

**Light:**


**Filters:**

Balzer intereference filters with half band widths of 10 nm were used for each wavelength shown on the action spectrum in Figure III.10.

Total fluence rate was different for each filter. The values were used to calculate the fluence delivered during the irradiation times employed.

\( \phi_C \) was not applicable to the use of these filters.

5: The Perkin-Elmer 156. Spectrophotometer

The Perkin-Elmer 156 is a dual wavelength spectrophotometer and was used in conjunction with a custom-built side illumination accessory for actinic irradiation. The measuring beam was horizontal, as was the actinic beam,
which was at $90^\circ$ to the measuring beam. This meant that totally internally reflecting cuvettes could not be used; instead, clear plastic cuvettes were used. These had internal dimensions of 1.0 cm x 3.5 cm. The cuvette holder was a custom-built water-cooled block with 1.0 cm x 2.5 cm windows for both measuring and actinic beams. The measuring beam wavelengths were 660 nm and 730 nm. Their intensities were not high enough to cause any measurable sample photoconversion during reading.

Typical noise levels were $0.5 \times 10^{-3}$ A for resuspended pellets, and $0.1 \times 10^{-3}$ A for supernatants.

6: The Measurement of $P_{tot}$, $P_{fr}$ And $P_{r}$

A detailed analysis of the theory of the measurement of phytochrome can be found in Smith's book (Smith, 1975).

The relative amount of phytochrome in a sample is measured as:

\[
\{A_{660} - A_{730} \text{ after saturating far-red light} \} - \{A_{660} - A_{730} \text{ after saturating red light.} \}
\]

This is usually abbreviated to:

\[
\Delta A_{fr} - \Delta A_{r},
\]

or simply:

\[
\Delta \Delta A.
\]
However, the term "ΔΔA" can be confusing as phytochrome can be measured using other wavelengths, giving different readings, thus the term "ΔΔA (660 - 730 nm) is used in Figure legends in this thesis.

Each sample is photoconverted several times and an average of ΔΔA values taken. This ΔΔA value must be corrected for two phenomena:

1: Under saturating red light, not all Pr is converted to Pfr, because of the overlapping absorption spectra of the two forms. The photoequilibrium set up is 75% Pfr : 25% Pr (Pratt, 1975). Thus, all ΔΔA readings must be multiplied by a factor of 1.33 to give the total amount of phytochrome present.

2: Because of the same absorbance characteristics of Pr and Pfr discussed in "1", the time to completion of the maximum photoconversion possible by far-red light is lower than that by red light (compare Appendix I.4.6 and 7). 60 second irradiations with the actinic red source set up the 75% Pfr : 25% Pr photoequilibrium, but 60 seconds far-red light only photoconverts 80% of the original Pfr back to Pr : 3 minutes irradiation is needed for the setting-up of 100% Pr. This length of irradiation is not practical for making a large number of measurements quickly, so 1 minute red and far-red actinic irradiations were used. This meant that ΔΔA readings had to be multiplied by a
factor of 1.25 to take account of incomplete photoconversion by far-red light.

Combining the two corrections from "1" and "2", it can be seen that the term obtained for total phytochrome is $\Delta \Delta A \times 1.67$.

This is not an absolute measurement of phytochrome, but a relative estimate, thus the quantification of phytochrome is done using an arbitrary scale. On this basis one unit of phytochrome is taken as a corrected $\Delta \Delta A$ of $10^{-3}$. Thus, phytochrome is expressed as units, $\Delta \Delta A.10^{-3}$ (660 - 730 nm). Expressions for Pr and Pfr can also be given, taking into account the corrections described.

$$\text{Pr} = \Delta \Delta A \text{ after red light} + (0.25 \times \text{Ptot}).$$

$$\text{Pfr} = \Delta \Delta A \text{ after far-red light} \times 1.25.$$
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