PHYTOCHROME AND THE UNROLLING OF THE FIRST LEAF OF BARLEY (HORDEUM VULGARE L.)

This thesis is a report of work done by myself in the Department of Developmental Biology, Research School of Biological Sciences, Australian National University. Where the results of other workers have been used to further the arguments presented in this thesis, they have been acknowledged.

A thesis offered for the Degree of Master of Science in the Australian National University by DAVID JOHN CROSSLEY, B.A. (York).

December, 1970
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David J. Crowley.
CONTENTS

List of Tables iii
List of Figures v
List of Plates vii
Abbreviations viii
Acknowledgements x
Summary xi

PART 1: SURVEY OF THE LITERATURE ON PHYTOCHROME

I The Discovery of Phytochrome 2
II Assay of Phytochrome 4
III Isolation of Phytochrome 8
IV In Vitro Phytochrome 11
V In Vivo Phytochrome 17
VI Correlations between Physical Assays of Phytochrome in Tissues and the Physiological Responses of these Tissues 28
VII Phytochrome-mediated Responses 33
VIII Theories on the Mechanism and Mode of Phytochrome Action 41

PART 2: EXPERIMENTAL

IX The Unrolling of the Barley Leaf 49
X Materials and Methods 51
XI The Effects of Various Light Treatments on Barley Leaf Unrolling 62
XII The Effects on Barley Leaf Unrolling of Treatment with Growth Substances 72
XIII Early Biochemical Events During Barley Leaf Unrolling 78
XIV Discussion 90
Bibliography 99
LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>34</td>
</tr>
<tr>
<td>II</td>
<td>35</td>
</tr>
<tr>
<td>III</td>
<td>36</td>
</tr>
<tr>
<td>IV</td>
<td>38</td>
</tr>
<tr>
<td>V</td>
<td>51</td>
</tr>
<tr>
<td>VI</td>
<td>54</td>
</tr>
<tr>
<td>VII</td>
<td>55</td>
</tr>
<tr>
<td>VIII</td>
<td>64</td>
</tr>
<tr>
<td>IX</td>
<td>69</td>
</tr>
<tr>
<td>X</td>
<td>81</td>
</tr>
<tr>
<td>XI</td>
<td>83</td>
</tr>
<tr>
<td>XII</td>
<td>84</td>
</tr>
</tbody>
</table>

I Slow Red, Far-red Light Reversible Photomorphogenetic Responses of Plants.
II Slow Red, Far-red Light Reversible Metabolic Responses of Plants.
III Fast Red, Far-red Light Reversible Responses of Plants.
IV Escape Rates for Various Red, Far-red Light Reversible Responses of Plants.
V Chemicals used in the Experimental Investigations.
VI Manufacturers of the Chemicals used in the Experimental Investigations.
VII Sources of the Barley Seed used in the Experimental Investigations.
VIII Size Classes of Barley Leaf Segments 24 h after Irradiation with Red Light.
IX The Effect of Theoretical Variations in the Recycling of Phytochrome on the Percentages of PFR remaining in Barley Leaf Segments after Irradiation with Red and Far-red Light.
X Solutions used in the Extraction of RNA from Barley Leaf Segments.
XI Total RNA Extracted per Barley Leaf Segment.
XII Results of Preliminary Experiments on the Synthesis of RNA in Barley Leaf Segments after Irradiation with Red Light.
<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>XIII</td>
<td>87</td>
</tr>
<tr>
<td>XIV</td>
<td>88</td>
</tr>
</tbody>
</table>

**TABLE** XIII
Total Protein Extracted per Barley Leaf Segment.

**TABLE** XIV
Results of Preliminary Experiment on the Synthesis of Protein in Barley Leaf Segments after Irradiation with Red Light.
<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14A</td>
</tr>
<tr>
<td>2</td>
<td>42A</td>
</tr>
<tr>
<td>3</td>
<td>55A</td>
</tr>
<tr>
<td>4</td>
<td>56A</td>
</tr>
<tr>
<td>5</td>
<td>56B</td>
</tr>
<tr>
<td>6</td>
<td>62A</td>
</tr>
<tr>
<td>7</td>
<td>62B</td>
</tr>
<tr>
<td>8</td>
<td>63A</td>
</tr>
<tr>
<td>9</td>
<td>68A</td>
</tr>
<tr>
<td>10</td>
<td>68B</td>
</tr>
<tr>
<td>11</td>
<td>73A</td>
</tr>
<tr>
<td>12</td>
<td>73B</td>
</tr>
<tr>
<td>13</td>
<td>74A</td>
</tr>
</tbody>
</table>

A proposed scheme for the transformation PR to PFR in vitro.

A proposed scheme for the control of plant growth and development by phytochrome.

Energy distribution of the safelight at the level of the bench top.

Energy distribution of the red source at a distance of approximately 20 cm.

Energy distribution of the far-red source with various filters at the level of the specimens.

The effect of the safelight on the unrolling of barley leaf segments.

Red light-induced unrolling of barley leaf segments.

Time course of red light-induced unrolling of barley leaf segments.

Reversal by far-red light of the red light-induced unrolling of barley leaf segments.

Reversal by far-red light of the red light-induced unrolling of barley leaf segments.

The effects of gibberellic acid and kinetin on the unrolling of barley leaf segments from the variety Pallas.

The effects of gibberellic acid and kinetin on the unrolling of barley leaf segments from the variety Pallas.

The effects of gibberellic acid and kinetin on the unrolling of barley leaf segments from the variety Parkland.
<table>
<thead>
<tr>
<th>FIGURE</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>Flow-sheet for the experiments on the synthesis of RNA in barley leaf segments after irradiation with red light.</td>
</tr>
<tr>
<td>15</td>
<td>Procedure for the estimation of total RNA by the orcinol reaction.</td>
</tr>
<tr>
<td>16</td>
<td>Results of an experiment on the synthesis of RNA in barley leaf segments after irradiation with red light.</td>
</tr>
<tr>
<td>17</td>
<td>Flow-sheet for the experiments on the synthesis of protein in barley leaf segments after irradiation with red light.</td>
</tr>
<tr>
<td>18</td>
<td>Procedure for the estimation of total protein by the Lowry Test.</td>
</tr>
<tr>
<td>19</td>
<td>Results of an experiment on the synthesis of protein in barley leaf segments after irradiation with red light.</td>
</tr>
<tr>
<td>20</td>
<td>Hypothetical scheme for the control of barley leaf unrolling by phytochrome.</td>
</tr>
</tbody>
</table>
LIST OF PLATES

PLATE

1

Seven day-old seedlings of barley, var. Pallas, grown under the experimental conditions.

57A

2

The cutter used to cut segments from the barley seedlings.

58A

3

Barley seedlings, showing how the segments were cut.

58B
ABBREVIATIONS

The following abbreviations are used in this thesis:

Act D  actinomycin D
Amo 1618 2-isopropyl-4'-((trimethylammonium chloride)
-5- methylphenylpiperidine-1-carboxylate
ATP  adenosine triphosphate
cm  centimeter(s)
cm²  square centimeter(s)
CPM  counts per minute (radioactivity)
DNA  deoxyribonucleic acid
EDTA  ethylenediaminetetraacetic acid
Fig.  Figure
f.c.  foot candle(s)
g  gram
GA₃  gibberellic acid
h  hour(s)
HER  high energy reaction
IAA  indole acetic acid
l  litre
M  molar
mg  milligram(s) (10⁻³ g)
min  minute(s)
ml  millilitre(s) (10⁻³ l)
mM  millimolar (10⁻³ M)
N  normal
nm  nanometer(s) (10⁻⁶ cm)
P₉₀₂R  far-red light absorbing form of phytochrome
PR  red light absorbing form of phytochrome
ppm  part(s) per million
RNA  ribonucleic acid
sec  second(s)
ABBREVIATIONS (Cont.)

soln  solution
TCA  trichloracetic acid
var  variety
v/v  volume to volume
w/v  weight to volume
µ    micron (10⁻³ cm)
µg   microgram (10⁻⁶ g)

To Miss Margaret Gordon I give thanks for her permanently cheerful demeanour in the laboratory and her freely-given help and advice on many matters.

Mr lan Norris steadfastly gave up the best part of a Long Weekend in order to produce computer programs which performed in microseconds statistical calculations which would have taken me several weeks.

Dr Suzanne Poulsen kindly allowed me to see pre-print copies of the paper cited as (158), (159) and (160) in the bibliography.

I also wish to acknowledge the gift of barley seed from Dr D.M. Reid, Department of Biology, University of Calgary, Alberta, Canada, and the receipt of an A.M.P. Research Scholarship from the Australian National University.
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SUMMARY

Experiments were performed on segments cut from the first leaf of dark-grown barley seedlings. The segments were found to unroll during a 24 h dark period after an irradiation with red light. This effect was reversible by an irradiation with far-red light immediately following the red-irradiation, thus implicating phytochrome in the control of the response to red light. Unrolling to the level achieved after red-irradiation was not observed after an exogenous application of GA$_3$ to segments maintained in the dark, but this was observed after an application of kinetin. There was no large increase in the bulk synthesis of RNA within 6h after an irradiation with red light which resulted in unrolling; a similar increase in the bulk synthesis of protein could also not be observed, though due to technical difficulties, this result cannot be considered to be conclusive. A hypothetical scheme for the control of barley leaf unrolling by phytochrome is presented.
PART 1: SURVEY OF THE LITERATURE ON PHYTOCHROME

The effect of visible radiant energy in regulating plant growth and development has been known for over a century (32), but not until the 1940's were quantitative action spectra measured for morphogenetic effects of light. The results of these measurements on such phases of plant development as inhibition of elongation of the hypocotyl of barley, stimulation of extension growth of the Avens coleoptile, and stimulation of leaf growth in pea were then suggested that the spectral region of maximal effectiveness in promoting the responses was in the red between 600 and 700 nm (33) or in the far-red between 700 and 800 nm and also that chlorophyll a was found to inhibit the response.

One of the systems in which an inhibitory as well as a stimulatory effect of irradiation was found was the germination of seeds of certain varieties of lettuce, particularly the variety Grand Rapids. This was investigated further by Berthwick et al (20, 31). They found that red light in the region 640-670 nm was most effective in stimulating the seed germination. Radiation in the far-red region 720-750 nm was ineffective in inhibiting germination. When alternate irradiations of red and far-red light were given, the physiological response expressed depended on the quality of the radiation given last in the series (31).

On the basis of these results, Berthwick and his co-workers suggested that the germination of lettuce seeds was controlled by two pigments which had maximal absorptions at 650 and 750 nm. The pigments could undergo the following reactions (31):

\[
\text{Pigment 1} + 2 \text{Red Light} \rightarrow \text{Pigment X} + \text{H} \\
\text{Absorption maximum: 650 nm} \\
\text{Absorption maximum: 750 nm} \\
\text{Pigment 2} + \text{Far-Red Light} \rightarrow \text{Pigment + HX} \\
\text{GROWTH.}
\]
I. The Discovery of Phytochrome

The effect of visible radiant energy in regulating plant growth and development has been known for over a century (72), but not until the 1940's were quantitative action spectra measured for morphogenetic effects of light. The results of these measurements on such phases of plant development as inhibition of elongation of the mesocotyl of grasses, stimulation of extension growth of the Avena coleoptile, and stimulation of leaf growth in pea and bean suggested that the spectral region of maximal effectiveness in promoting the responses was in the red between 600 and 700 nm (72). In some systems, radiation between 700 and 800 nm and also that below 500 nm was found to inhibit the response.

One of the systems in which an inhibitory as well as a stimulatory effect of irradiation was found was the germination of seeds of certain varieties of lettuce, particularly the variety Grand Rapids. This was investigated further by Borthwick et al (20, 21). They found that red light in the region 640-670 nm was most effective in stimulating the seed germination, radiation in the far-red region 720-750 nm was effective in inhibiting germination. When alternate irradiations of red and far-red light were given, the physiological response expressed depended on the quality of the radiation given last in the series (21).

On the basis of these results, Borthwick and his co-workers suggested that the germination of lettuce seeds was controlled by two pigments which had maximal absorptions at 650 and 730 nm. The pigments could undergo the following reactions (21):

\[
\begin{align*}
\text{Pigment + RX} & \xrightarrow{\text{Red light}} \text{Pigment X + R} \\
\text{Absorption} & \text{max} \text{imum 650 nm} & \text{Absorption} & \text{max} \text{imum 730 nm} \\
\text{Pigment X + R} & \xrightarrow{\text{Far-red light, darkness}} \text{Pigment + RX}
\end{align*}
\]
The photoreaction was found to be reversible as rapidly as radiant energy was supplied and to be independent of temperature; because of these characteristics, it was suggested in a later paper (20) that there was only one pigment existing in two interconvertible forms:

\[
P \quad \xrightarrow{\text{Absorption maximum } 660 \text{ nm}} \quad P^* \quad \xrightarrow{\text{Absorption maximum } 735 \text{ nm}}\]

Subsequent work has demonstrated the existence of such a pigment in living plant tissues, and it has been possible to prepare extracts containing it. The properties of the pigment, subsequently called "phytochrome" (17), have been found to be extremely similar to those postulated on the basis of results of physiological experiments. It is now generally accepted that phytochrome is a photoreceptor pigment involved in the control of the many physiological responses of plants initiated by red radiation and subsequently reversible by far-red radiation.
II Assay of Phytochrome

The existence of the pigment phytochrome was originally postulated on the basis of results of physiological experiments which suggested the occurrence in plant tissues of a pigment which was photoreversible between two forms, absorbing radiation at about 660 and 730 nm. It was found possible to design an assay method based solely on these, as yet undemonstrated, radiation-absorbing properties (38). To this day, this purely physical technique is the only one available for assaying phytochrome.

In 1959, Butler and co-workers (38) published the first report of a pigment in plant cells that existed in two interconvertible forms. Using a single-beam recording spectrophotometer designed to measure small optical density changes in dense light-scattering material, they were able to show that irradiation with red and far-red light caused changes in the optical density of tissue from dark-grown oat seedlings. Far-red radiation caused the optical density to decrease at 735 nm and to increase at 660 nm; red radiation had the opposite effect.

The assay method developed from this work was a double beam differential spectrophotometer (37, 38). This instrument irradiates the sample with alternating flashes of (non-actinic) light at two fixed wavelengths; the measuring wavelength and the reference wavelength. The optical density of the sample at the reference wavelength is then electronically subtracted from that at the measuring wavelength, and the difference between the two optical densities (ΔOD) is displayed on the instrument's meter. The ΔOD scale has an arbitrary zero adjustment so that the values are not absolute (37).

The amount of total photoreversible phytochrome present in the sample is proportional to the change in the ΔOD reading following irradiation of the sample with actinic red and far-red light. This value is referred to
Δ(ΔOD) is proportional to the total photoreversible phytochrome present; to find the amount of the form of phytochrome absorbing red light (PR) or far-red light (PFR) present, the ΔOD is measured before any actinic irradiation, and then this value is subtracted from that obtained after the first actinic irradiation (37).

\[
\text{eg } P_{\text{FR}} \propto (\Delta OD_{\text{FR irradiation}} - \Delta OD_{\text{INITIAL}})
\]

The values of Δ(ΔOD) are not arbitrary since the subtraction eliminates the system response, and the instrument is calibrated with neutral screens of known density (37, 96). Difficulties are experienced, though, because the relationship between phytochrome concentration and Δ(ΔOD) value is not simple. The relationship to the concentration of total photoreversible phytochrome can be expressed as (37):

\[
\Delta (\Delta OD) = 2\beta ac l
\]

where \(c\) is the concentration of phytochrome, \(l\) is the sample thickness, \(\beta\) represents the extinction coefficients of the two forms of phytochrome, \(a\) is the factor of enhancement due to light scatter in the sample, and the factor of 2 is present because the term Δ(ΔOD) measures the total amount of reversible PR plus the total amount of reversible PFR.

Of these factors, the extinction coefficients can be calculated reasonably easily (37). The factor of enhancement due to light scatter, however, is much more difficult to calculate and this makes comparison of readings between different samples very difficult (23, 79, 96). Some attempt can be made to orient sample particles similarly in different samples of the same material, but where the different samples are of different material (eg stem and leaves) this is nearly impossible (29, 73, 96). Varying sample thicknesses also create problems in the comparison of measurements from different samples (29). If the thickness of a light-scattering
sample is doubled, the average optical path length is more than doubled since light is not only scattered additionally within the added sample material but also back and forth between the original and the added material.

There are two further complications which affect the assay of phytochrome by this method. These are the conversion of protochlorophyll to chlorophyll by actinic red light, and the overlap of the absorption spectra of $P_R$ and $P_{FR}$ at 660 nm.

Irradiation of the sample with actinic red light transforms $P_{FR}$ to $P_R$ and also converts protochlorophyll to chlorophyll (37). This latter conversion, which is irreversible, affects the absorbancy of the sample at 660 nm but not at 730 nm. In maize, after the initial conversion there is a period of two hours during which there is no further synthesis of protochlorophyll, but after this time the absorbancy of the sample at 660 nm is again affected (37). In the original measurements with the differential spectrophotometer (37, 38), the wavelength of the measuring beam was set at 660 nm and that of the reference beam at 730 nm. Therefore, values of $\Delta OD$ and hence of $\Delta (\Delta OD)$ were affected by the changes in absorbance at 660 nm caused by protochlorophyll conversions. It was necessary to irradiate the sample with actinic red light at least twice before a reading could be taken, and because of this the initial amount of $P_R$ present could never be measured directly. This was eventually overcome (35, 120) by setting the wavelength of the measuring beam at 730 nm and that of the reference beam at 800 nm. Since both forms of phytochrome are essentially transparent to radiation at 800 nm, the values of $\Delta OD$ will be smaller (120) but the value of $\Delta (\Delta OD)$ will be the same.

The overlap at 660 nm in the absorption curves of $P_R$ and $P_{FR}$ caused similar problems when the measuring wavelength was set at 660 nm (120). Some of the absorbance measured at 660 nm before actinic irradiation
was due to $P_{FR}'$ and the necessary correction to be applied was difficult to calculate. This was easily overcome by using 730 and 800 nm as the wavelength pair, and after 1965, an increasing number of workers began to use this technique (47, 115, 117, 118, 189). The only correction it is necessary to apply is for the incomplete conversion of $P_R$ to $P_{FR}$ by red radiation, which converts only about 80% of the $P_R$ (35):

$$P_{TOTAL} = 1.25 (\Delta OD_R \text{ irradiation} - \Delta OD_{FR} \text{ irradiation})$$

This incomplete conversion is also due to the overlap in absorbance of $P_R$ and $P_{FR}$ at 660 nm.

1. The extraction of the pigment from the plant tissue.

2. The purification of the crude extract.

Extraction techniques are still based on straightforward methods of protein chemistry, but recently the purification stages have been greatly improved.

III. I. Extraction of Phytochrome

Phytochrome is normally extracted from dark-grown tissues since these contain no chlorophyll, which interferes with the assay technique, and they contain more phytochrome than light-grown tissues, in which phytochrome decay takes place. The phytochrome/protein ratio and phytochrome per g fresh weight varies greatly from species to species of plant (72); there are also differences between varieties of the same species, and in the photoactivity per mg of phytochrome isolated from plants grown for the same batch of seed stored for increasing lengths of time (111). Phytochrome has been extracted from etiolated seedlings of Arabidopsis, Avena and Secale, and from dark-grown Pisum epinasty tissues; of these Avena seedlings appear to be the best source for phytochrome extraction at present (72). Phytochrome has also been extracted from the algae Mastocarpus, the liverwort Selaginella (122), and the moss Physcomitrella (117).
III. Isolation of Phytochrome

The existence of phytochrome was postulated in 1952, but it was not until seven years later that the pigment was successfully isolated. In 1959, Butler et al (38) published the technique for the first successful isolation. The methods used were quite straightforward techniques of protein chemistry; the only innovation was the spectrophotometric method used to assay the pigment, but it was the development of this assay technique which had caused the delay.

The isolation of phytochrome takes place in two stages:

1) The extraction of the pigment from the plant tissue.
2) The purification of the crude extract.

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Phytochrome has been extracted from etiolated seedlings of Zea, Hordeum, Avena and Secale, and from dark-grown Pisum epicotyl tissue; of these Avena seedlings appear to be the best source for phytochrome extraction at present (72). Phytochrome has also been extracted from the alga Mesotaenium, the liverwort Sphaerocarpus (202) and the moss Mnium (17).
In the original extraction procedure (38), etiolated maize shoot tissue was homogenized in phosphate buffer containing 0.01 M ascorbate and 0.01 M cysteine. The homogenized material was passed through cheesecloth, and the filtrate centrifuged at low speed; the supernatant was then again centrifuged at high speed. The resulting supernatant was brought to 0.33% saturation with ammonium sulphate and the precipitate redissolved in 0.25 of the original volume. This procedure resulted in a crude preparation of phytochrome with a purification factor of about 6 compared with the initial extract.

Later workers used essentially the same extraction technique, though the purification stages were often modified. All work is carried out in a coldroom at 2-3°C and normally under a weak green safelight in order to maintain the phytochrome in the PR form - this last precaution is essential when using Pisum tissue as a source, since extracts from this material contain a substance, "P FR killer", which immediately destroys phytochrome in the P FR form but not in the PR form (74). Plant material is also often chilled to 2-3°C several hours before extraction to eliminate possible non-photochemical transformations. Some workers freeze the tissue before extraction, and grind or break up the frozen material in various ways (48, 130). The extraction medium consists of an appropriate buffer such as phosphate (38,182) or Tris (51,128), a metal-complexing agent such as ethylenediaminetetraacetic acid (EDTA), and a sulphhydryl compound such as 2-mercaptoethanol or cysteine. These additions to the buffer ensure maximal stability of the phytochrome molecule, particularly by inhibiting phytochrome decay when the extract is exposed to light (77) (see also sections IV.2.a) and V.2.a) below). It is also important that the pH of the medium remains between 7.5 and 8.5 during extraction and purification (48, 72).

A rather different technique was employed in the extraction of phytochrome from the alga Mesotaenium, which had been grown in the light and which therefore contained large amounts of chlorophyll (202). The cells were
disintegrated in a French press in a buffered medium; the extract was then lyophilized, and the lyophilized material ground to a powder. This powder was extracted with acetone and ether. The powder was then dissolved in buffer and normal purification techniques were employed. This method resulted in the removal of large amounts of chlorophyll in the acetone and ether extractions, and it may be applicable to extractions of phytochrome from tissue of light-grown higher plants. However, phytochrome has been successfully extracted from a number of species of green higher plants by techniques similar to those outlined earlier (130).

III.2. Purification of Extracted Phytochrome

In 1964, Siegelman and Firer (182) achieved a 60-fold purification of phytochrome from etiolated Avena using a combination of centrifugation, calcium phosphate column chromatography, gel filtration on Sephadex G-50 and G-200 and DEAE-cellulose column chromatography. Between each of the stages of purification, protein was precipitated with ammonium sulphate and then redissolved. This method has since formed the basis of most phytochrome extractions.

Mumford and Jenner (153) achieved a 750-fold purification in 1966 by further purification of the extract derived from Siegelman and Firer's procedure. This technique involved electrophoresis on support-free flowing films, and exclusion chromatography on Bio-Gel P-150. Correll et al (51) in 1968 purified phytochrome approximately 1200 times with a method involving ammonium sulphate fractionation, dialysis, calcium phosphate chromatography and DEAE-cellulose column chromatography; in this method the pH was constantly adjusted to 7.6-8.0 by the addition of KOH. Recently, Correll and Edwards (48) have refined this procedure even further by replacing the dialysis stages with Sephadex G-50 chromatography during which it was possible to maintain a constant pH of 7.8.
IV. In Vitro Phytochrome

IV.1. In Vitro Properties of Phytochrome

Phytochrome was first isolated in 1959 (38) but it was not until 1964 that the first estimate of its molecular weight was published. Siegelman and Firer (182) used analytical centrifugation techniques to estimate the molecular weight of phytochrome to be between 90,000 and 150,000. In 1966, Mumford and Jenner (153) found a molecular weight of between 55,000 and 62,000 by exclusion chromatography. Briggs et al (30) discovered two components in freshly-isolated phytochrome; one with a molecular weight of approximately 180,000 and the other with an approximate molecular weight of 80,000.

All the studies so far discussed were performed on phytochrome isolated from etiolated oat seedlings. Correll and his co-workers (52) worked on phytochrome from etiolated annual rye. They found that rye phytochrome existed as 14-S hexamers and 9-S tetramers made up of 2-5 monomer units. These aggregations were observed directly in electron micrographs. The monomer units existed as single polypeptide chains, each with a molecular weight of approximately 42,000. The monomer units appeared to be associated through noncovalent bonds.

The molecular weight of isolated phytochrome seems to depend on the extraction method employed. Briggs et al (30) found that with increasing time after isolation and increase in handling, a greater percentage of isolated phytochrome was found to be composed of the smaller component. Correll and Edwards (48) modified the previous extraction procedures to ensure that the pH was at all times maintained as close to 7.8 as possible, and that the extracts received as little handling as possible. They discovered two aggregates of phytochrome in their extracts from both oat and rye seedlings. One fraction sedimented at almost twice the rate of the other fraction. With fresh extracts most of the phytochrome was found to consist
of the larger aggregate; when dialysis steps were used to remove residual buffers or salts, a shift towards the smaller aggregate occurred.

These results would all suggest that most phytochrome in the living plant exists in a highly aggregated state. Aggregation and disaggregation of subunits of the phytochrome molecule might provide a mechanism for the action of light on the pigment. Briggs et al (30) could find no difference between $P_R$ and $P_{FR}$ in binding properties on calcium phosphate, sedimentation velocity in sucrose density gradients, elution volume from Sephadex G-200 columns or electrophoretic mobility. But in these experiments, only those fractions containing the low molecular weight form were used, and the conversion from the $P_R$ form to the $P_{FR}$ form was done by irradiating these fractions with red light in vitro. Thus, the highly aggregated forms of phytochrome had already been lost in the extraction procedure.

In recent studies, an antiserum to phytochrome has been prepared by injecting preparations of the pigment into rabbits (105). The results of immunochemical investigations of $P_R$ and $P_{FR}$ backed up by spectroscopic evidence, suggested that there are conformational differences between the protein moieties of the two forms, but these differences appeared to be rather slight from the standpoint of gross structure.

Siegelman et al (184) were successful in cleaving the chromophore from the associated protein in isolated oat phytochrome. From chromatographic and spectral properties, they concluded that the chromophore is a bilitriene closely similar but distinct from the chromophore of the algal pigments c-phycocyanin and allophycocyanin.

IV.2. In Vitro Transformations of Phytochrome

Investigations of the in vitro transformations of phytochrome have concentrated on:
1. The dark reversion of P_{FR} to P_{R}

and 2. The light-stimulated transformations of P_{R}
    to P_{FR} and P_{FR} to P_{R}.

IV.2. a). Dark reversion of phytochrome in vitro

In an early study, Mumford (152) found that in phytochrome preparations from etiolated oat seedlings, dark reversion of P_{FR} to P_{R} occurred without loss of photoreversible phytochrome. This was later found to be a normal occurrence in in vitro preparation and is presumably due to the inhibition of the disappearance of phytochrome which takes place in the dark in vivo (see section V.2.a) below) by the inclusion of metal-complexing agents in the extraction medium (77).

Taylor (201) demonstrated the occurrence of reversion of P_{FR} to P_{R} in phytochrome extracts prepared from oat, pea, and parsnip tissues. His results indicated that particularly in pea phytochrome the reversion process was not first order and that there might be intermediates formed on the P_{FR} to P_{R} pathway.

Anderson et al (2) studied the formation of intermediates in the reversion process more fully. They investigated reversion in phytochrome preparations from etiolated oat seedlings over a temperature range from -50 to +15°C and in aqueous and mixed aqueous-buffered solutions of pHs 4.7 to 8.6. They discovered that phytochrome in vitro behaves as a thermochromic as well as a photochromic protein, especially at lower temperatures and below pH 6.0. The rate at which dark reversion took place was strongly dependent on temperature and pH. Anderson et al suggested that their results could be explained by postulating the formation of a third form of phytochrome as below:

\[ R \rightarrow FR + H^+ \rightarrow FRH \]
where R, FR and FRH are forms of phytochrome with absorption maxima at wavelengths of 663, 727 and approximately 650 nm respectively.

IV.2.b) Light-stimulated transformations of phytochrome in vitro

The pre-eminent work in this field is that by Linschitz, Kasche and their co-workers (53, 132, 133). They studied phytochrome isolated from etiolated oat seedlings using sophisticated techniques involving extremely short flashes (duration measured in microseconds) of light of various wavelengths. They also performed some low temperature studies. Their final conclusions from all this work were that there are at least four stages on the PR to PFR pathway. Two of the intermediates have been characterized; they are a P710 form with an absorption maximum at 710 nm, and PBL which is a low absorbance form discovered when PR was irradiated for a long time at -35°C. PBL appeared to give rise to both PR and PFR on warming to -10°C. Two further stages on the PR to PFR pathway are known from the formation of absorption maxima at 692 and 695 nm. The various stages postulated for the transformation are shown in Fig.1. The configuration of R1 in these schemes probably corresponds to a single substance, but R2 is probably composed of several intermediate forms of phytochrome (133). It can be seen that in the hypothetical scheme (Fig.1), there are two parallel pathways in the PR to PFR conversion. The fact that the first two stages of the reaction are photoreversible at low temperatures suggests that the primary reaction is an isomerization of the chromophore (53).

The PFR to PR phototransformation appeared to occur in two stages, though none of the intermediates were characterized (132). There was a strong indication that the two reactions occurred in parallel:
FIG. 1. A proposed scheme for the transformation $P_R$ to $P_{FR}$ in vitro. After Cross et al (53).
Correll et al (50) studied the kinetics of the phototransformations of phytochrome in a preparation from etiolated annual rye. The kinetics of the disappearance of $P_{FR}$ after irradiation at 660 nm indicated that two populations of $P_{FR}$ existed initially; the kinetics of the appearance of $P_R$ indicated that this was appearing in two forms. Chemical treatments, such as alteration of the concentration of sodium chloride in the medium, addition of sodium dodecyl sulphate and glutaraldehyde and replacement of air with nitrogen and hydrogen altered the properties of the various forms of phytochrome present. It was suggested that at least four species of chromophore exist in rye phytochrome, with absorption maxima at 580, 660, 670 and 730 nm.

Pratt and Butler (163) also worked with phytochrome from etiolated annual rye. They studied the photoconversions at low temperatures. Actinic irradiation of $P_R$ below $-150^\circ$ produced an intermediate form of phytochrome with maximum absorbance near 695 nm. Actinic irradiation of this intermediate converts it back to $P_R$; when it is warmed above $-150^\circ$, it decays to a low absorbance form which decays to $P_{FR}$ on further warming. These results are very reminiscent of those of Linschitz, Kasche and their co-workers (53, 132, 133) discussed above. Pratt and Butler's intermediate absorbing at 695 nm presumably corresponds to the $R_{695}$ of Linschitz and Kasche; the low absorbance form may possibly correspond to Linschitz and Kasche's $P_{BL}$, though in this case, only $P_{FR}$ appeared on warming the intermediate. Pratt and Butler (163) also discovered that the phototransformation $P_{FR}$ to $P_R$ below $-150^\circ$ was very similar to that observed by Linschitz and Kasche (132) by flash technique.

It seems clear that in vitro phototransformations of phytochrome are not first order reactions, but that both the $P_R$ to $P_{FR}$ and the $P_{FR}$ to $P_R$ conversions involve the formation and subsequent decay of several intermediates. But great care should be taken when comparing the in vitro with the in vivo situation. The fact that $P_{FR}$ decay (see
Differences in extraction procedures and in the amount of handling of the extracted material have been shown to produce changes, particularly in the state of aggregation of the phytochrome molecule (30, 48). There has been only one study of phototransformations of phytochrome in *in vitro* and *in vivo* systems derived from similar batches of plants. Briggs and Fork found evidence for two intermediates on the $P_R \rightarrow P_{FR}$ pathway in phytochrome extracted from etiolated oat seedlings. In *in vivo* studies on similar plants, they found evidence for the existence of two intermediates very similar to those found in the *in vitro* system (28). The main difference between the two systems was that much higher steady state levels of intermediates were found in the living plants than in the *in vitro* system.
V. In Vivo Phytochrome

V. 1. In Vivo Distribution of Phytochrome.

Investigations of the distribution of phytochrome in the living plant have invariably used spectrophotometric methods to assay the pigment. Such investigations are therefore subject to all the criticisms raised in section II above during the discussion of the assay methods for phytochrome. Unfortunately, since phytochrome is present in plant tissues only in extremely low concentrations, it seems likely that spectrophotometry will continue to be the only method available for investigating the distribution of the pigment.

Three aspects of the distribution of phytochrome in vivo have been investigated:

1). Comparisons of the distribution between different organs of the same plant.

2). Investigation of the distribution within one particular organ.

3). Investigations of the intracellular location of the pigment.

V.1.a). Distribution in the whole plant.

Correll and Shropshire (47) studied the distribution of phytochrome during the growth of coleoptiles and primary leaves of etiolated Secale cereale L. They found that the concentration of phytochrome per organ and per mg dry weight at any one time was very similar in the two organs. However, phytochrome per cell (measured as phytochrome per µg DNA) was much higher in the coleoptile compared with the leaf, whereas phytochrome per g fresh weight was generally lower in the coleoptile. Briggs and Siegelman (29) carried out similar studies on the coleoptiles and primary leaves of etiolated seedlings of Hordeum, Zea and Avena. In Hordeum and Avena, phytochrome values, expressed per g fresh weight were
generally higher in the coleoptile than the leaf, but in \textit{Zea}, the values were very similar. In \textit{Pisum} and \textit{Phaseolus}, phytochrome content per gram fresh weight in the bud and the leaves was generally greater than in the remainder of the shoot. Furuya and Hillman (73) also found that in \textit{Pisum}, phytochrome was concentrated mainly in the bud and especially in the epicotyl hook.

\textbf{V.1.b). Distribution within particular plant organs}

Briggs and Siegelman (29) also investigated the variation of phytochrome content within particular plant organs. In the seedlings of monocotyledons, they found that phytochrome content per fresh weight was greater at the bases of cotyledons and leaves as compared with other parts of these organs. The authors claimed that generally the highest concentrations of phytochrome were found in tissues that were either meristematic or which had recently been meristematic. Koukkari and Hillman (125) found a similar association between high phytochrome content and actively growing or developing regions of modified underground stems and storage roots. Correll et al (49) could not confirm this correlation. In coleoptiles of \textit{Secale}, the phytochrome content increased towards the apex rather than towards the base. The phytochrome content per cell in the coleoptiles was found to be the same at any given age (length). In leaf tissues, the best correlation that could be made was between phytochrome content and cell expansion; the phytochrome content of all leaf cells was found to increase in direct proportion to their expansion (increase in dry weight).

All the above studies have necessarily been made on samples of tissue taken from several different plants. Recently, it has become possible to measure the phytochrome content of different areas of a single organ. Using these methods, Boisard and Malcoste (13) investigated the phytochrome content of different areas of single embryos of \textit{Cucurbita pepo}. They found that there was a decreasing concentration gradient from the cotyledons to
the vegetative axis. This was confirmed by using larger samples made up of pieces cut off several different embryos.

V.1.c). Intracellular distribution

The first attempt to investigate the intracellular distribution of phytochrome in higher plants was made by Gordon (80). He fractionated cells from etiolated maize seedlings and analyzed each of the intracellular fractions spectrophotometrically. Phytochrome could be demonstrated spectrophotometrically only in the "mitochondrial" and the "soluble protein" fractions. The phytochrome concentration in the "mitochondria" was approximately double that of the "soluble fraction". However, evidence has been presented against this work (78). Galston (78) investigated thin sections of etiolated seedlings of oat and pea with a microspectrophotometer which had a beam which could be stopped down to 1.0 and 0.5 µ diameter. The analysis beam could also be used to produce actinic radiation by disengaging the wavelength and slit drives and opening the shutter for 2-5 min. Phytochrome detection was based on one of two criteria: a) reversible absorption peak shifts in living cells in the 750-650 nm region resulting from alternating exposures to actinic 650 and 750 nm light and b) reversible percentage absorption changes in the 500-600 nm region in frozen, dried and glycerol mounted sections resulting from alternating exposures to 650 and 750 nm light. Using these criteria, Galston was able to demonstrate the presence of phytochrome only in the nuclei of the cell. Scans of other regions of the cell failed to reveal any phytochrome. Using the beam 0.5 µ in diameter he was able to show that the phytochrome appeared to be located in the region of the nuclear membrane.

The recent successful preparation of an antiserum to phytochrome (105) opens up the possibility of using immunochemistry as a very precise method for determining the intracellular location of phytochrome molecules.
Studies on fungi, algae, mosses and ferns have indicated that in these lower plants, phytochrome may be located near the surface of the cell in discrete photoreceptor bodies. In early studies (12, 88), Haupt and his co-worker Bock observed the movements of the chloroplast of the alga Mougeotia after irradiating parts of the cell with micro-beams of polarized red and far-red light. They concluded that the phytochrome molecules of Mougeotia are oriented in a spiral pattern round the cell surface. Spores of many plant species have also been found to respond preferentially to the plane of polarization of the irradiating light rather than to its direction of propagation (63, 109, 155). In the case of the moss Funaria, germination of the spores is red, far-red light reversible; when the spores are irradiated with polarized light, the chloronemal axis develops preferentially in a direction perpendicular to the plane of polarization (109). This led the authors to conclude that germination in the intensity range used is controlled by rod-shaped dichroic photoreceptors (in this case probably containing phytochrome) located in or near the cell surface and oriented tangentially to it. Since this initial hypothesis, several modifications have been proposed to explain particular results with different systems. Etzold (63) suggested that the axis of maximum absorption of phytochrome turns by 90° during the transformation from PR to P_{FR}. In the P_{FR} form the (supposedly rod-shaped) molecules are oriented tangentially to the wall; in the P_{FR} form the molecules are arranged perpendicularly to the wall. Experimental evidence which supports this hypothesis was obtained by Haupt et al (89) in later experiments with the alga Mougeotia similar to those described above. In contrast, Nebel (155) interpreted his data on the growth of the protonemata of the fungus Physcomitrium after various irradiations with polarized red and far-red light in terms of the cycling of phytochrome due to some absorption of red light by P_{FR}. He suggested that the photoreceptors are disk-shaped and that the orientation does not alter between the red
and far-red absorbing states.

V.2. In Vivo Transformation of Phytochrome

The phytochrome present in dark grown plants was found to be in the $P_R$ form (37). After a brief actinic irradiation with red light, $P_{FR}$ was found to be unstable in the tissue and the rate of its disappearance in the dark was followed. In dark grown maize mesocotyls at room temperature, $P_{FR}$ had totally disappeared in about 3 hr. During this period, total photoreversible phytochrome also declined until about 80% of the phytochrome initially present had disappeared. The remaining 20% was present as $P_R$. At first it was thought that, in the dark, 20% of the $P_{FR}$ had reverted back to $P_R$ (37), but it was later shown (36) that red light converted only 80% of $P_R$ to $P_{FR}$ in vitro. Therefore, it was possible to explain the $P_R$ remaining in the mesocotyl tissue at the end of the dark period as resulting not from dark reversion of $P_{FR}$ to $P_R$ but from incomplete conversion of $P_R$ to $P_{FR}$ by red light (36, 56, 162). $P_{FR}$, therefore, appeared to decay completely to a non-photoreversible form during the dark period after irradiation of the tissue with red light.

Following these preliminary studies, further investigations revealed that in vivo transformations of phytochrome were rather more complicated than first thought and also differed between different species of plants. These investigations were concerned with the effects of various biochemical and environmental factors on phytochrome decay, the process of apparent synthesis of phytochrome, reversion of phytochrome in the dark, and the discovery of intermediate forms of phytochrome formed during the phototransformations.

V.2.a). Factors affecting phytochrome decay

In their original work (37), Butler et al found that decay was inhibited by low temperatures, and by
replacing the atmosphere in the incubation vessel with nitrogen. In an extension of this work, Butler and Lane (35) claimed to have established a correlation between rate of decay of $P_{FR}$ and respiration (measured as oxygen uptake). Decay was inhibited by lowering the oxygen concentration and by respiratory inhibitors such as carbon monoxide, cyanide and azide. Furuya et al (17) were unable to confirm the correlation with respiration; EDTA and mercaptoethanol (both of which form complexes with metals) inhibited decay without inhibiting respiration. They concluded that $P_{FR}$ decay is a metal-dependent process, probably oxidative but not necessarily dependent on respiration. Further confirmation of the lack of dependence of decay on respiration came when Bradley and Hillman (25) showed that concentrations of 2,4-dinitrophenol which uncoupled the respiratory chain had no effect on $P_{FR}$ decay.

In cauliflower floret tissue there appears to be no decay of $P_{FR}$ after irradiation (35, 37, 126); the total phytochrome content remains at the same level throughout the dark period after irradiation. This was originally also thought to be the case in Pastinaca root tissue (95) but it has now been shown that decay does occur but at a very slow rate (126). In all other tissue so far tested decay of $P_{FR}$ has been found to occur.

The rate of decay of $P_{FR}$ differs between monocots and dicots (107). In Zea the rate of decay saturates when only about 10% of the total phytochrome content is in the $P_{FR}$ form (37); it is still measurable when the percentage drops to 1%. In monocots generally the rate of decay is more or less constant (107). In dicots, $P_{FR}$ decays rapidly at first, and then more slowly - in the tissues studied the first phase of rapid decay lasted about an hour. In the dicot Amaranthus the rate of decay was found to be directly proportional to the fraction of total phytochrome which was in the $P_{FR}$ form (117).

All the work so far discussed has assumed that the
PR form of phytochrome is stable and that only the PFR form undergoes light-activated decay. Chorney and Gordon (41) obtained evidence that PR in etiolated Avena seedlings also decays during the dark period after a brief irradiation. Since few experimental details are given of the methods used for preparing the specimens for spectrophotometry, it is difficult to judge whether the apparent decay of PR is real or an artefact of the experimental procedure. Chorney and Gordon also measured an action spectrum for the light-activation of the decay process. There are two broad maxima, one between 380 and 440 nm and the other between 600 and 700 nm, peaking at about 660 nm. This suggests that phytochrome itself could act as the photoreceptor for the light-activation of its own decay, whether this be the decay of the PFR form only or of the PR form as well.

V.2.b). Phytochrome synthesis

In most plant tissues, the PFR form of phytochrome is unstable and undergoes decay to a non-photoreversible form. A brief irradiation is sufficient to transform a large proportion of the phytochrome content of tissues to the PFR form. The end result of this brief irradiation, therefore, is the loss of a large proportion of the phytochrome present. It would be expected that the decay process would reduce the phytochrome content of plant tissues under constant illumination to a very low level (45). But there is much evidence that the phytochrome system is active in light-grown plants; therefore it is reasonable to suggest that some process or sequence of processes acts to maintain a significant level of phytochrome in light-grown plants. One of these processes is apparent phytochrome synthesis.

Early work (29, 35) demonstrated that there was some increase in total phytochrome during growth of etiolated seedlings. Clarkson and Hillman (43) gave etiolated pea epicotyl hooks repeated irradiations with red light to reduce total phytochrome to a low level. The
tissue was then returned to darkness and total phytochrome measured at intervals. After an initial decay, the phytochrome content did not decrease further as would be expected due to the decay process, but was actually found to increase. This "apparent phytochrome synthesis" took place only when the total phytochrome had been reduced to a critical value between 16 and 22% of the original content. All the phytochrome synthesised was in the $P_R$ form. The amount synthesised seemed to be related to the amount by which total phytochrome content fell below the critical concentration; therefore it resulted in the equalization of the phytochrome content of tissues which had received different light treatments. Later work on the same system (44) showed that the synthesis was irreversibly inhibited by cycloheximide, actinomycin D and was reversibly inhibited by auxins. It was not inhibited when growth of the hook segments was inhibited by mannitol. There was some indication that the capacity for phytochrome synthesis decreases as the cells approach maturity.

When pea plants were placed in continuous red light, the phytochrome content at first decreased at constant rate, presumably due to decay (45). Then the rate declined until a steady concentration of phytochrome was reached. The stabilization of this concentration was presumably due to an equilibrium between $P_{FR}$ decay and apparent $P_R$ synthesis.

Apparent phytochrome synthesis has also been observed in etiolated seedlings of Amaranthus (117).

V.2.c). Reversion of phytochrome

Though it is possible to explain most of the apparent reversion of $P_{FR}$ to $P_R$ on the basis of incomplete conversion of $P_R$ to $P_{FR}$ by red-irradiation (35), several cases of true reversion have been established. Several workers (35, 37, 126) have established that $P_{FR}$ in cauliflower floret tissue does not decay following irradiation but apparently reverts quantitatively to the $P_R$ form. True reversion occurs when the amount of $P_R$ reappearing during the dark period.
after irradiation is greater than can be accounted for by incomplete conversion of \( P_R \) to \( P_{FR} \) by the irradiation. True reversion appears to occur in many dicots, though not in monocots (107). It does not occur, though, in the dicot Amaranthus (115, 117). Since this reversion is also accompanied by \( P_{FR} \) decay (except in cauliflower) the reversion is never quantitative.

Reversion in Pisum was not inhibited by concentrations of azide which inhibited decay and respiration (77). In parsnip root tissue reversion was reduced by low temperature, but not by low oxygen levels (126). Reversion, therefore, seems to be a non-oxidative process.

An apparent "inverse reversion" process in seeds has now been observed by three different groups of workers (14, 118, 189). After a saturating dose of far-red light which converted the \( P_{FR} \) present in dry cucumber seeds (apparently synthesised in this form) to \( P_R \), there was a gradual re-appearance of \( P_{FR} \) (measured spectrophotically) in the dark (189). This process was very slow, the \( P_{FR} \) content increasing slowly over a period of several days. There was no change in total phytochrome during this period. The process was much faster in imbibed Amaranthus seeds; it seemed to be complete 15 min after the initial irradiation when the samples were held at 0°C (118).

This reappearance of \( P_{FR} \) in the dark after a saturating far-red irradiation has generally been interpreted as an "inverse reversion" of \( P_R \) to \( P_{FR} \) in the dark (14, 118, 189). It is possible, though, that the initial irradiation could have caused the formation of a new form of phytochrome which then became converted to \( P_{FR} \) in the dark (22). Evidence against this interpretation, however, is that in one case (118) the effect was observed not only as an increase in absorption at 735 nm but also as a decrease at 635 nm, although at this wavelength the change was of smaller magnitude.
V.2.d. Intermediates formed during the phototransformations of phytochrome

Spruit (186, 187, 188) investigated the photochemical reactions of phytochrome in etiolated pea plumules and the action spectra for the phototransformations of the in vivo pigment at very low temperatures (-79 and -196°). The effect of the low temperatures was to improve the separation of minor absorption bands in the difference spectra and to stabilize the intermediate forms of phytochrome which caused these absorption bands. This allowed the determination of action spectra for the phototransformations between these forms. Spruit concluded that the phytochrome system in pea consists of at least four components with absorption maxima at 650, 670, 698 and 744 nm. These can be designated $P_{R}^{A}$ (650 nm), $P_{R}^{FR}$ (744 nm), $P_{B}^{R}$ (670 nm), and $P_{B}^{FR}$ (698 nm). Bleaching of either component by light is a separate reaction, not necessarily accompanied by the formation of absorption bands of the other components. At room temperature there is energy transfer between the four forms that may be partly of a physical and partly a chemical nature. The chemical reactions can be largely suppressed by cooling.

It is probable that the forms absorbing at 670 and 744 nm are the $P_{R}$ and $P_{FR}$ forms observed at room temperatures; the forms absorbing at 650 and 698 nm are probably photoproducts of $P_{R}$ and $P_{FR}$ which act as transient intermediates in the transformations at room temperature.

Purves and Briggs (166) obtained evidence for the existence of two forms of phytochrome in tissues of oat, pea and maize. The tissues were irradiated with various doses of red and far-red radiation at the same intensity, and the results plotted as log 10 percentage phytochrome remaining against dose in seconds. It was found that the curves produced were not straight lines but could be resolved into two components, one completing the transformation very rapidly and the other completing it more slowly. In cauliflower tissue there were at least three components, one of these (possibly a complex of
Briggs and Fork (28) irradiated oat coleoptile tissue with a high intensity mixed red and far-red light source and showed that this caused measurable absorbancy increases at 543 nm. In the dark period after irradiation, the signal caused by the increase in absorbancy decayed back to its original level. When the exposure time was kept constant and the intensity of the source was increased, the signal height was increased. Alternate irradiations with red and far-red light demonstrated that the signal decayed only after red-irradiation.

These results were interpreted on the basis that during the rapid cycling from $P_R$ to $P_{FR}$ and back, induced by the mixed light source, there was an accumulation of other forms of phytochrome which were intermediates in the phototransformations (28). This accumulation caused the changes in the absorbancy of the tissue at 543 nm. High light intensities caused a faster rate of cycling, resulting in a larger percentage of the pigment being in the intermediate forms. This explains the higher signal levels at the high light intensity. The fact that the signal decayed only after red-irradiation suggests that the intermediates were on the pathway $P_R$ to $P_{FR}$.

Precise analysis of the in vivo data was impossible due to signal noise (28). However, many of the results were very similar to those obtained with phytochrome extracts from the same tissue. These results were discussed in section IV.2.b) above. Analysis of the in vitro data suggested the presence of two intermediates on the $P_R$ to $P_{FR}$ pathway (27). Therefore, these two intermediates may also be present in vivo (28).
VI. Correlations between Physical Assays of Phytochrome in Tissues and the Physiological Responses of these Tissues

Hillman (96) used a series of light sources which, by means of different filter combinations, produced radiations containing various proportions of red and far-red light. Spectrophotometric measurements showed that irradiation of etiolated pea stem segments with the various light sources resulted in the production of photostationary states of phytochrome in the tissue. The higher the proportion of red light in the radiation, the higher the $\frac{P_{FR}}{P_{TOTAL}}$ ratio in the tissue.

All concentrations of $P_{FR}$ were shown to inhibit the elongation of the segments; maximum inhibition was achieved when the $P_{FR}$ content was about 40-60% of the total phytochrome (96). Below this concentration, the degree of inhibition could be directly correlated with the $P_{FR}$ content. Similar results were obtained for Avena coleoptiles (108); in this case, maximal promotion of the response (elongation of the coleoptiles) was achieved when the $P_{FR}$ content was in excess of about 50% of the total phytochrome.

Other work (136) on Avena mesocotyls showed that the inhibition of elongation caused by irradiation with red light could be partially reversed by subsequent exposure to far-red light. Increasing doses of far-red light progressively reduced the $P_{FR}$ concentration which had been set by the preceding red-irradiation. The degree of inhibition of mesocotyl elongation was proportional to the final $P_{FR}$ concentration set at the end of the two light exposures.

Pjon and Furuya (157) used various doses of pure red light to transform measurable percentages of the phytochrome content of etiolated rice coleoptiles to $P_{FR}$. The elongation of the coleoptiles was found to be directly proportional to the amount of spectrophotometrically
detectable Pₚₑₚ present in the tissue.

Briggs and Chon (26) investigated the effect of red light in altering the phototropic sensitivity of Zea coleoptiles. By using masks, they were able to investigate the distribution of sensitivity to light within the coleoptile, and by in vivo spectrophotometry they showed that the distribution of sensitivity was similar to the distribution of phytochrome. A similar correlation between the distribution of sensitivity and of phytochrome was found in Phaseolus hypocotyl hooks (20). The rate and angle (measured 20 hours after induction) of hook opening was found to be directly proportional to the initial amount of Pₚₑₚ present.

To discover the absolute amount of Pₚₑₚ required for subsequent expression of the photoresponse, various experiments (26, 67, 68, 120) were performed in which the tissue was pretreated with red light and Pₚₑₚ allowed to decay. One of the difficulties associated with this technique is that the pretreatment also causes de-etiolation of the tissues. In one series of experiments, (67, 68) one batch of etiolated pea plants were pretreated with a brief irradiation of pure red light once every two hours and a second batch received the same treatment interposed over continuous irradiation with far red light. It was found that these treatments produced plants with the same degree of etiolation but with four-fold differences in phytochrome content. Segments from plants which had received either pretreatment or which had received no pretreatment and had been held in the dark, all responded in the same way to various photostationary levels of Pₚₑₚ set up by irradiation with different light sources. This suggests that it is the Pₚₑₚ/Pₚ ratio rather than the absolute concentration of Pₚₑₚ which controls the subsequent expression of the photoresponse. In other experiments involving red pretreatments, it was found that reduction of the total phytochrome content of Zea coleoptiles by about 50% did not alter the subsequent red light sensitivity (26). In Phaseolus hooks the physiological response after a red
pretreatment was proportional to the amount of $P_R$ remaining in the tissue (120).

Some experimental results, however, cannot be explained by a simple correlation between phytochrome content or ratio and the physiological response. When segments of etiolated pea tissue were pretreated with red light 8-9 hours before the experiments, it was found spectrophotometrically that all the phytochrome present in the segments at the time of the experiments was in the $P_R$ form (96). However, light sources producing a $\frac{P_{FR}}{P_{TOTAL}}$ ratio of less than 20% promoted elongation, and only those sources which produced more than 20% $P_{FR}$ inhibited the response. Also in etiolated pea tissue, far-red light was still effective in reversing the effect of a red light pretreatment when there was no detectable $P_{FR}$ present (59). A similar result was obtained with etiolated rice coleoptiles (157); a loss of 50% of the $P_{FR}$ present was observed within 1.5 hours after a red exposure, whereas a 50% loss of the photoreversibility of the response was not observed until 9 hours after the red-irradiation. Finally, in Zea (26) it was found that dosages of red light sufficient to saturate the physiological system were two orders of magnitude too small to induce measurable phytochrome transformation.

Two theories were originally proposed to explain the non-correlation between phytochrome observed spectrophotometrically and some physiological responses (26, 67, 96, 97, 197).

One of the theories suggested that there were two types of phytochrome present in plant tissues; these were "active" and "bulk" phytochrome. "Active" phytochrome formed only a small fraction of the total phytochrome and this was the form which regulated photomorphogenic responses. "Bulk" phytochrome was physiologically inactive. To explain the anomalous results, it was suggested that "active" phytochrome had a much slower decay rate in darkness than "bulk" phytochrome (96), or that it was packaged in some
manner which facilitated its transformations in both directions (26). "Active" phytochrome was not easily detectable by spectrophotometry since the signal due to the "active" form was obscured by the signal emanating from the "bulk" phytochrome (26, 96).

The second theory proposed that $P_{FR}$ decayed in darkness to a physiologically active form which was not spectrophotometrically detectable (96).

Recently, two distinct types of phytochrome have been found in cucumber seeds (189). In dark-stored dry seeds 75% of the phytochrome present was found to be in the $P_{FR}$ form. This type of phytochrome seemed to be completely different spectrophotometrically from the phytochrome synthesised as $P_R$ after the seeds underwent inhibition. The "dry-seed phytochrome" appeared to have a non-standard difference spectrum and also appeared to undergo "inverse reversion" ($P_R$ to $P_{FR}$) in the dark. The $P_{FR}$ form of phytochrome was also shown to be present in non-irradiated seeds of *Amaranthus*, though its presence could not be demonstrated until the seeds had imbibed (118). "Inverse dark reversion" was also demonstrated in dark imbibed seeds. During inhibition, phytochrome appeared in two stages: one immediately, and one after about 8 hours. This suggested that there were two pools of phytochrome; one (possibly the type which underwent "inverse dark reversion") which was rehydrated immediately upon imbibition, and one which was synthesised at about eight hours after imbibition.

The presence of $P_{FR}$ in etiolated plant tissues has not yet been demonstrated; but this may be due to the fact that such tissue is normally irradiated first with actinic far-red light when spectrophotometric measurements are being made. If a small fraction of the phytochrome initially present in etiolated tissue was in the $P_{FR}$ form, and did undergo "inverse dark reversion", this could provide an adequate explanation for the non-correlations between physical measurements of phytochrome content and physiological...
photoresponses quoted above. All of these experiments were anomalous in that they required PpR to be present in the tissue when it was spectrophotometrically undetectable.

The time interval between the effective irradiation and the display of red, far-red light reversible responses varies enormously from less than a minute to days, or even weeks in the case of some of the flowering responses. For experimental convenience, the responses can be divided into two classes:

1. Slow responses - the response takes more than 60 min to be displayed.

2. Fast responses - the response is displayed in less than 60 min.

# VII.1. Slow Responses

The vast majority of red, far-red light reversible responses fall into this category. These responses are found throughout the plant kingdom, and they can be divided into two types, according to whether the response is displayed as a change in the development of the plant
VII. Phytochrome-mediated Responses

Following the initial proposal of the existence in plant tissues of a photoreversible pigment which absorbed red and far-red radiation (21), there was renewed interest in the effect on plants of radiation at these wavelengths. This interest has resulted in the discovery of a large number of plant responses which are elicited by red radiation the effect of red light being reversible by far-red radiation. Phytochrome has been demonstrated to be present in the tissues of most species of plants in which these red, far-red light reversible responses occur. It now seems to be generally accepted that phytochrome is involved in some way in the photo-control of all red, far-red light reversible responses of plants (98). There is some doubt, though, as to whether phytochrome is the only pigment involved in some of the responses, (31, 60, 139, 140, 173, 177, 178, 193, 204, 206, 211).

The time interval between the effective irradiation and the display of red, far-red light reversible responses varies enormously from less than a minute to days, or even weeks in the case of some of the flowering responses. For experimental convenience, the responses can be divided into two classes:

1). Slow responses - the response takes more than 60 min to be displayed.

2). Fast responses - the response is displayed in less than 60 min.

VII.1. Slow Responses

The vast majority of red, far-red light reversible responses fall into this category. These responses are found throughout the plant kingdom, and they can be divided into two types, according to whether the response is displayed as a change in the development of the plant
(photomorphogenesis) or in its metabolism. Tables I and II list some of the known red, far-red light reversible responses of plants. These responses are either inhibited or promoted by effective irradiation with red light; this effect of red light is reversed by far-red.

Table I. Slow Red, Far-red Light Reversible Photomorphogenic Responses of Plants

<table>
<thead>
<tr>
<th>Response</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seed germination.</td>
<td>15,19,20,21,116,138,177,196,203,211,218.</td>
</tr>
<tr>
<td>Spore germination.</td>
<td>32,144,193,194.</td>
</tr>
<tr>
<td>Photoperiodically controlled changes in whole-plant morphology.</td>
<td>175.</td>
</tr>
<tr>
<td>Ontogeny of specific plant organs</td>
<td>62,137.</td>
</tr>
<tr>
<td>Phototropic growth.</td>
<td>8,11,63,154,155.</td>
</tr>
<tr>
<td>Changes in the morphology of the thalli of liverworts and gametophytes of ferns.</td>
<td>55,70,71,141,148.</td>
</tr>
<tr>
<td>Auxin-induced lateral root initiation.</td>
<td>76.</td>
</tr>
<tr>
<td>Geotropic growth</td>
<td>10,11.</td>
</tr>
<tr>
<td>Nutatory growth</td>
<td>40,120,122,170,217.</td>
</tr>
<tr>
<td>Changes in the ultra-structural of chloroplasts.</td>
<td>142.</td>
</tr>
<tr>
<td>Elongation of stem internodes.</td>
<td>58, 67, 68, 94, 97, 114, 172, 197.</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>Elongation of hypocotyls.</td>
<td>57, 102, 145, 180, 204.</td>
</tr>
<tr>
<td>Elongation of mesocotyls.</td>
<td>174.</td>
</tr>
<tr>
<td>Expansion and unrolling of leaves.</td>
<td>119, 134, 147.</td>
</tr>
<tr>
<td>Formation of unicellular epidermal hairs.</td>
<td>146.</td>
</tr>
</tbody>
</table>

Table II. Slow Red, Far-red Light Reversible Metabolic Responses in Plants.

<table>
<thead>
<tr>
<th>Response</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthocyanin biosynthesis.</td>
<td>83, 84, 145, 183, 206.</td>
</tr>
<tr>
<td>Biosynthesis of phenols.</td>
<td>60.</td>
</tr>
<tr>
<td>Changes in the RNA content of tissues.</td>
<td>104, 111, 212.</td>
</tr>
<tr>
<td>Chlorophyll biosynthesis.</td>
<td>1, 143.</td>
</tr>
<tr>
<td>Biosynthesis of flavonoids.</td>
<td>24, 75, 171.</td>
</tr>
<tr>
<td>Carbohydrate metabolism.</td>
<td>81, 121, 164, 165, 190, 191.</td>
</tr>
<tr>
<td>Uptake of ions and nutrients by isolated tissues and whole plants.</td>
<td>81, 124, 191, 195.</td>
</tr>
<tr>
<td>Changes in the concentrations of specific enzymes in tissues.</td>
<td>23, 34, 61, 64, 82, 100, 213.</td>
</tr>
</tbody>
</table>
VII.2. Fast Responses

There are not many fast red, far-red light reversible plant responses known, and there does not seem to be a standard type of response which is always fast. Table III lists the fast responses which are known at present.

Table III. Fast Red, Far-red Light Reversible Responses of Plants

<table>
<thead>
<tr>
<th>Response</th>
<th>Time interval between effective irradiation and display of response.*</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adhesion of root tips of barley and mung bean to negatively-charged glass surfaces in the presence of IAA, ATP, ascorbic acid, Mn$$^{++}$$, Mg$$^{++}$$, K$$^+$$ and Ca$$^{++}$$.</td>
<td>30-150 sec</td>
<td>110, 199, 200.</td>
</tr>
<tr>
<td>Positive phototactic movement of the chloroplast of the alga Mougeotia.</td>
<td>10-45 min</td>
<td>86, 87, 89.</td>
</tr>
</tbody>
</table>
Induction of synthesis of the enzyme phenylalanine ammonia-lyase in mustard seedlings after pre-irradiation.

**Synergism between PFR and subthreshold concentrations of GA₃ in promoting the germination of lettuce seeds (measured after 24 h).

**Transmission of a red light-induced stimulus causing the unrolling of the barley leaf (measured after 24 h).

* The first figure in this column is the time interval at which the response is first displayed; the second figure is the time interval at which the maximal response is attained.

** These fast responses are, in fact, potentiations of later responses; they can only be observed when the later responses are displayed several hours, or days, later.
VII.3. The Escape Reaction.

The definition of red, far-red light reversible responses requires that the effect on the plant of irradiation with red light, should be reversible by subsequent irradiation with far-red light. However, separation of the two irradiations by a sufficient length of time allows the initial products of the red irradiation to undergo further reactions, until they attain a state beyond which the reactions are irreversible by far-red irradiation. This process is the escape reaction.

The length of time required for the escape reaction varies greatly. In some systems, also, increasing the time interval after red irradiation results in a decreasing degree of reversibility until at a specific time interval, full irreversibility is achieved. Table IV lists some of the escape rates for various red, far-red light reversible plant responses.

Table IV. Escape Rates for Various Red, Far-red Light Reversible Responses of Plants

<table>
<thead>
<tr>
<th>Response</th>
<th>Rate of escape (time for 50% loss of reversibility)</th>
<th>Temperature (°C)</th>
<th>Material</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effectiveness of interrupting dark period with red light in inhibiting flower initiation.</td>
<td>3 min</td>
<td>22</td>
<td>Pharbitis</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>15 min</td>
<td>20</td>
<td>Xanthium</td>
<td>208</td>
</tr>
<tr>
<td></td>
<td>25 min</td>
<td>20</td>
<td>Glycine</td>
<td>208</td>
</tr>
<tr>
<td>Induction of IAA oxidase inhibitor.</td>
<td>30 min</td>
<td>26</td>
<td>Pisum</td>
<td>100</td>
</tr>
</tbody>
</table>
Seed germination 3 h 25 Lactuca 8

Elongation of excised coleoptile segments 6 h 26 Avena 108

Elongation of intact coleoptiles 8 h 27 Oryza 156

Seed germination 9 h 20 Lactuca 20

VII.4. Slow Responses, Fast Responses, the Escape Reaction and the Mechanism of Action of Phytochrome

The ultimate aim of most workers in investigating red, far-red light reversible responses of plants is to determine how, at the biochemical level, the responses are controlled by phytochrome. The major discovery that still needs to be made is how the active PFR form of phytochrome is involved biochemically in the metabolism of the cell. The elucidation of this problem requires an experimental system in which the initial effect of PFR on cell metabolism can be observed by experimental techniques. The main difficulty is experienced in discerning the initial effect of PFR - conveniently termed the "mechanism" of action of phytochrome - from the processes by which this initial action is transformed into the observed plant response - here termed the "mode" of action of phytochrome. Obviously, systems involving slow responses are not particularly useful in this respect; the mode of action of phytochrome in these cases involves rather long and complicated processes. Fast responses may be more useful, but care should be taken in selection of the particular system to be used in experimental investigations. For example, the processes leading to the red light-induced positive phototactic
movement of the Mougeotia chloroplast are likely to be quite complicated and therefore must have rather fast reaction rates because the response is first displayed only ten minutes after the red-irradiation. More useful experimental systems are likely to be those in which the response is some biochemical or biophysical event (such as the induction of phenylalanine ammonia-lyase synthesis, or the attachment of root tips to glass) or the potentiation of another response which is displayed at some later time (such as the synergism between PFR and GA$_3$ in lettuce seed germination). Such responses are likely to be situated relatively early on the pathway followed subsequent to the initial effect of PFR on cell metabolism.

Probably even more useful for experimental investigations are systems which have a well-defined escape reaction. This criterion implies that the mechanism of action of phytochrome in the system is also well-defined and therefore more readily observable by experimental techniques. The rate of the escape reaction, however, should not be too fast, otherwise the products of the initial reaction with PFR will be too short-lived to be easily assayable. A more useful criterion is that the escape reaction should proceed from complete reversibility to complete irreversibility in a relatively short time.

These criteria will be referred to later in the introduction to the experimental part of this thesis (section IX below).
VIII. Theories on the Mechanism and Mode of Phytochrome Action.

VIII.1. The Mechanism of Action of Phytochrome

In an early paper on the red, far-red light reversibility of lettuce seed (20) it was suggested that under the influence of red-irradiation, the red, far-red photoreversible pigment combined with a reactant to give the far-red absorbing form and a changed reactant molecule. The far-red absorbing form was photodissociable by far-red light to give back the red absorbing form and the original reactant (see equations in section I). Subsequent work has indicated that it is the P$_{FR}$ form of phytochrome which is physiologically active (91,92,93), and that the phototransformations of phytochrome probably involve configurational changes in the molecule (see sections IV and V above). The original hypothesis was therefore modified to include these findings; it was then suggested that P$_{FR}$ was an active form of an enzyme, and that P$_R$ was inactive (16,17,90,91). This assertion has never been conclusively proved or disproved.

The discovery in vivo of intermediate forms of phytochrome other than P$_R$ and P$_{FR}$ (see section V.2.d) above) suggests that the phototransformations of P$_R$ to P$_{FR}$ may not result in the production of only one active substance which reacts enzymically with one substrate in the cell. The transformations of each of the intermediates on the pathway P$_R$ to P$_{FR}$ may involve interaction (though not necessarily enzymic reactions) with metabolic processes in the cell, especially as some of the transformations, in vitro at least, are not light-dependent (53,132,133,163). The recent discovery of what appears to be two distinct, long-lived forms of phytochrome in seeds (118,189) also supports the hypothesis that the mechanism of action of phytochrome involves interaction between several forms of the pigment and
metabolic processes.

A theoretical scheme for the control of plant growth and development by phytochrome, which postulates several different forms of the pigment, has recently been proposed by Borthwick et al. (22). The scheme was originally proposed to account for the so-called "high energy reaction" (HER) in terms of phytochrome. An HER occurs when plant responses controlled by phytochrome are negated or modified by irradiation for a long time in the 680-760 nm region.

The scheme is outlined in Fig. 2. According to Borthwick et al. (22) it accounts for:

1). The photoreversibility of phytochrome,
2). The dark reversion of PFR to PR,
3). The decay of PFR to a non-photoreversible form,
4). The logical necessity of phytochrome combining with something (symbolic X, irrespective of the nature of X) to lead to responses,
5). The HER - this is accounted for by suggesting:
   a) The formation of another form of phytochrome, PpRH from PFR by prolonged irradiation between 680 and 760 nm,
   and b) That PFRX is photodissociable by irradiation in the region of 720 nm and has a much lower absorbance in the region 600-690 nm than does PFR.

Borthwick et al. (22) assemble an impressive collection of physiological evidence to support their scheme, but are unable to present much evidence for the physical existence of PpRH and PFRX.
FIG. 2. A proposed scheme for the control of plant growth and development by phytochrome. Unless marked as such, all reactions are not light-dependent. After Borthwick et al. (22).
According to the proposed scheme (Fig. 2) the initial action of $P_{FR}$ in the plant cell would be the formation of $P_{FRX}$; this would eventually lead to the observed plant response. It seems probable, though, that this is a far too simplified concept. It would seem likely that the several intermediates formed on the $P_{R}$ to $P_{FR}$ pathway (and also, perhaps, those formed on the $P_{FR}$ to $P_{R}$ pathway) would each have an effect on cell metabolism. The HER would then operate through the effect of prolonged irradiation in the 680-760 nm region in changing the relative concentrations of these intermediates (which would obviously have different absorption spectra from $P_{R}$ and $P_{FR}$) in the cell.

VIII.2. Mode of Action of Phytochrome

The fact that the initial action of $P_{FR}$ in the cell may, in fact, involve several discrete reactions, complicates even further the task of determining the mode of phytochrome action in controlling the display of any specific plant response. The web of possible reaction pathways has started spreading even before the processes which transform the initial action into an observable response have been reached. The only way to approach the investigation of the mode of phytochrome action is to concentrate on one particular system which has a well-defined red, far-red light reversible response. Presumably, a specific response would have only a limited number of pathways leading to its display, and it might be possible to unravel the pathways in time. Unfortunately, though most investigators of phytochrome action have concentrated their work on one particular system, they have tended to claim that the mode of phytochrome action they propose for their system applies to all other systems as well; indeed these modes have actually been put forward as mechanisms of action of phytochrome.

There are, at present, two theories about the
action of phytochrome in controlling plant responses:

1). Differential gene activation under the control of phytochrome.

2). Phytochrome-controlled changes in the permeability of cells.

VIII.2.a). Differential gene activation

This theory (103,150) suggests that the genotype of a particular cell can be divided into three parts: active, inactive and potentially active. Potentially active genes are those whose activity can be started or increased in some way by PFR. Which genes are active, inactive or potentially active in a particular cell of the multicellular system is determined by the regulating factors which determine the pattern of primary differentiation in that system.

This theory accounts only for "positive" photoresponses - those in which metabolic or photomorphogenic responses are initiated by the presence of PFR. To account for "negative" responses - where the response is inhibited by PFR - it is necessary to postulate that PFR can reversibly repress certain genes. There is no experimental evidence for this.

However, there is some experimental evidence from the mustard seedling system, and also certain other systems, that the genome is involved in some way in the control of red, far-red light reversible "positive" responses in these systems. The most conclusive evidence for the involvement of the genome has come from experiments in which inhibitors of DNA-dependent RNA synthesis (such as actinomycin D) and ribosomal protein synthesis (such as puromycin) inhibit the display of red, far-red light reversible "positive" responses (40,131,179). There have also been a number of attempts to determine the level of RNA in dark grown tissues after red, far-red
and red followed by far-red irradiation. Two of these investigations (104,212) have demonstrated a slight red, far-red light reversible increase in RNA content. However, since the RNA was measured as total RNA, it is difficult to draw conclusions about the synthesis of specific RNAs under genetic control in these systems. In one investigation of the RNA content of terminal buds of dark-grown pea plants (111), an increase in the ribosomal fraction was observed in RNA extracted from plants which had received red-irradiation, as compared with RNA extracted from plants maintained in darkness; this effect was reversible by far-red light (see also section XIII below).

It appears likely that the genome is involved in some way in the control by phytochrome of plant responses when display is inhibited by RNA and protein synthesis inhibitors such as actinomycin D and puromycin. However, it has not been conclusively proved that the control is exerted through differential gene activation, or that the genome is involved in the control of all red, far-red light reversible plant responses. Indeed, actinomycin D has no effect on the display of certain phytochrome-mediated responses (112). The only conclusion it is possible to draw from the results of experiments claimed to support the gene activation theory is that the pathway for the mode of action of phytochrome in certain plant responses passes, at some stage, through the genome of the cells involved.

VIII.2.b). Changes in the permeability of cells

This theory was proposed to account for the very rapidly-displayed red, far-red light reversible plant responses (92), particularly nyctinastic leaf movements (66,101,112) and the adhesion of root tips to glass (110, 199,200). The theory states that an early consequence of phytochrome action is a change in permeability of the cells involved, and possibly of intracellular components.
(92). Some proponents have even gone so far as to state that permeability changes might be the initial action of PFR if the phytochrome molecule were positioned in the cell membrane (110,199,200).

In the case of nyctinastic leaf movements, it seems to be indisputable that changes in membrane permeability are involved in the phytochrome control of the response. For some time before the discovery of phytochrome involvement, it was suggested that changes in the permeability of pulvinar cells were the direct causal factors of the leaf movements (66). The pulvini have now been shown to be the photoreceptors for phytochrome control (127) and changes in the rate of electrolyte efflux from the cut bases of pinnae subjected to various light treatments has been shown to be red, far-red light reversible (112).

The red, far-red light reversible adhesion of root tips to a negatively-charged glass surface has been shown to be due to the development of a positive bioelectric potential at the tip in red light, and a negative potential in far-red light (110). The curves of the changes in potential with time regularly show a plateau about the time of completion of the adhesion or release of the root tips. It is suggested that this plateau could be a manifestation of the termination of the photoconversion of one form of phytochrome and the beginning of that of another form. It is further suggested that the development of the bioelectric potentials is due to changes in the permeability of the plasmalemma which could be caused by changes in the configuration of the phytochrome molecule if the phytochrome holochrome were structurally part of the plasmalemma.

It seems likely that changes in the permeability of membranes, possibly also causing changes in bioelectric potentials, are involved in the pathway of the mode of phytochrome action in the control of nyctinastic leaf
movement and the adhesion of root tips to glass. The recent discovery that the phytochrome control of the germination of lettuce seeds is sensitive to artificial electrostatic fields (181) suggests that the development of bioelectric potentials as part of the mode of phytochrome action may be relatively widespread throughout red, far-red light reversible plant responses.
II. The Unrolling of the Barley Leaf

The first leaf of the grass seedling is tightly rolled while it remains inside the coleoptile (33). In the light, the leaf unrolls as soon as it breaks through the coleoptile, in dark-grown plants, however, the leaf remains tightly rolled throughout its development except for a slight tendency to unroll at the tip. If the dark-grown leaf is illuminated at this stage, it unrolls slowly; this unrolling is caused by a greater growth of the cells of the upper mesophyll as compared with the cells in the remaining mesophyll (33).

PART 2: EXPERIMENTAL

Further experiments showed that the light-induced unrolling of dark-grown wheat leaves was a red, far-red light reversible process and it was suggested that unrolling is controlled by phytochrome. Virgin's observations were confirmed by Klein et al. (121) for maize leaves and by Wagner (208, 209) for barley leaves.

The dark-grown barley leaf is a very suitable system for investigations into the mechanism and mode of phytochrome action. The red, far-red light reversible response is shown by segments of the leaf only less in length (40). This makes experimental treatment of replicates with irradiations or test solutions relatively easy. The following characteristics also favour barley leaf unrolling as an experimental system:

1). The response is evident and measurable 16-24 h after the experimental treatment (49, 120, 127).

2). The pronounced effects of various plant growth substances and metabolic inhibitors (40, 120, 127 and A. Neumann, personal communication) suggest that the initial effect of irradiation may be a general biochemical or biophysical event. This
IX. The Unrolling of the Barley Leaf

The first leaf of the grass seedling is tightly rolled while it remains inside the coleoptile (33). In the light, the leaf unrolls as soon as it breaks through the coleoptile; in dark-grown plants, however, the leaf remains tightly rolled throughout its development except for a slight tendency to unroll at the tip. If the dark-grown leaf is illuminated at this stage, it unrolls slowly; this unrolling is caused by a greater growth of the cells of the upper mesophyll as compared with the cells in the remaining mesophyll (33).

Virgin (207) showed that the light-induced unrolling of dark-grown wheat leaves was a red, far-red light reversible process and he suggested that unrolling is controlled by phytochrome. Virgin's observations were confirmed by Klein et al (121) for maize leaves and by Wagné (208, 209) for barley leaves.

The dark-grown barley leaf is a very suitable system for investigations into the mechanism and mode of phytochrome action. The red, far-red light reversible response is shown by segments of the leaf only 1cm in length (40). This makes experimental treatment of replicates with irradiations or test solutions relatively easy. The following characteristics also favour barley leaf unrolling as an experimental system:

1). The response is evident and measurable 15-24 h after the experimental treatment (40,158,167).

2). The pronounced effects of various plant growth substances and metabolic inhibitors (40,158,160,167 and R.Menhenett, personal communication) suggest that the initial effect of irradiation may be a precise biochemical or biophysical event. This
conclusion is supported by the reported effects of red light in stimulating certain biosyntheses (159,168).

3). The escape reaction appears to be completed within 5-6 h (R. Menhenett, personal communication).

These factors will be considered more fully in the experimental sections of this thesis which follow.
X. Materials and Methods

X.1. Materials

X.1.a). Chemicals

The chemicals used in the experimental investigations reported in this thesis are listed in Table V; the manufacturers of these chemicals are listed in Table VI:

**Table V. Chemicals used in the Experimental Investigations**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Grade*</th>
<th>Storage**</th>
<th>Source***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>AR</td>
<td>4°C</td>
<td>Ajax and Merck</td>
</tr>
<tr>
<td>p-Amino salicylic acid</td>
<td>LR</td>
<td>RT</td>
<td>BDH</td>
</tr>
<tr>
<td>Benzene</td>
<td>AR</td>
<td>4°C</td>
<td>Ajax</td>
</tr>
<tr>
<td>&quot;Calgon&quot;</td>
<td>-</td>
<td>RT</td>
<td>Calgon Corp.</td>
</tr>
<tr>
<td>m-Cresol+</td>
<td>LR</td>
<td>-20°C</td>
<td>Merck</td>
</tr>
<tr>
<td>Cupric sulphate</td>
<td>AR</td>
<td>RT</td>
<td>BDH</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>AR</td>
<td>4°C</td>
<td>BDH</td>
</tr>
<tr>
<td>p-Dioxan</td>
<td>Scintillation</td>
<td>4°C</td>
<td>M.C.+ B.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>Glass-distilled</td>
<td>40litre poly--thene vessel at</td>
<td>Aquator 60E still</td>
</tr>
<tr>
<td>Ethanol</td>
<td>AR</td>
<td>4°C</td>
<td>Selby and Merck</td>
</tr>
<tr>
<td>Ferric chloride (hydrated)</td>
<td>AR</td>
<td>RT</td>
<td>H + W</td>
</tr>
<tr>
<td>Chemical</td>
<td>Grade*</td>
<td>Storage**</td>
<td>Source***</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>--------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td>Folin and Ciocalteu's reagent</td>
<td>AR</td>
<td>4°</td>
<td>Ajax</td>
</tr>
<tr>
<td>Gibberellic acid</td>
<td>Purum</td>
<td>-20°; D</td>
<td>Fluka</td>
</tr>
<tr>
<td>&quot;Haemo-sol&quot;</td>
<td>-</td>
<td>RT</td>
<td>Meinecke</td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td>AR</td>
<td>RT</td>
<td>BDH</td>
</tr>
<tr>
<td>Kinetin</td>
<td>-</td>
<td>-20°; D</td>
<td>Sigma</td>
</tr>
<tr>
<td>L-Leucine-4, 5-T</td>
<td>-</td>
<td>-20°; D</td>
<td>Radiochem</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>Scintillation</td>
<td>RT</td>
<td>M.C.+ B.</td>
</tr>
<tr>
<td>Naphthalene 1:5 disulfonic acid, sodium salt</td>
<td>Technical</td>
<td>RT</td>
<td>Fluka</td>
</tr>
<tr>
<td>Orcinol++</td>
<td>LR</td>
<td>RT; PL</td>
<td>BDH</td>
</tr>
<tr>
<td>Phenol</td>
<td>AR</td>
<td>-20°</td>
<td>Merck</td>
</tr>
<tr>
<td>PPO(2,5 diphenyl-oxazole)</td>
<td>Scintillation</td>
<td>4°</td>
<td>Packard</td>
</tr>
<tr>
<td>&quot;Sno-wite&quot; (household bleach)</td>
<td>-</td>
<td>RT</td>
<td>Purex</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>AR</td>
<td>RT</td>
<td>BDH</td>
</tr>
<tr>
<td>Sodium carbonate</td>
<td>AR</td>
<td>RT</td>
<td>Ajax</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>AR</td>
<td>RT</td>
<td>BDH</td>
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<tr>
<td>Chemical</td>
<td>Grade *</td>
<td>Storage **</td>
<td>Source ***</td>
</tr>
<tr>
<td>------------------------</td>
<td>---------</td>
<td>------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
<td>LR</td>
<td>RT</td>
<td>BDH</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>AR</td>
<td>RT</td>
<td>Merck</td>
</tr>
<tr>
<td>Sodium potassium tartrate</td>
<td>AR</td>
<td>RT</td>
<td>BDH</td>
</tr>
<tr>
<td>Trichloracetic acid</td>
<td>AR</td>
<td>RT; D</td>
<td>BDH and Merck</td>
</tr>
<tr>
<td>Tri-isopropynaphthalenesulphonic acid, sodium salt</td>
<td>Technical</td>
<td>RT</td>
<td>Eastman</td>
</tr>
<tr>
<td>&quot;Trizma&quot; base</td>
<td>AR</td>
<td>RT; D</td>
<td>Sigma</td>
</tr>
<tr>
<td>&quot;Trizma&quot; HCl</td>
<td>AR</td>
<td>RT; D</td>
<td>Sigma</td>
</tr>
<tr>
<td>Uracil-6-T</td>
<td>-</td>
<td>-20°; D</td>
<td>Radiochem.</td>
</tr>
<tr>
<td>Uridine-6-T</td>
<td>-</td>
<td>-20°; D</td>
<td>Radiochem.</td>
</tr>
</tbody>
</table>

* AR = analytical reagent grade or equivalent; LR = laboratory reagent grade or equivalent.
** RT = stored at room temperature; D = desiccated; PL = protected from light.
*** See Table VI for full details of chemical manufacturers.
+ Phenol and m-cresol were further purified by distillation with an air condenser before use.
++ Orcinol was purified by recrystallization from benzene before use.
<table>
<thead>
<tr>
<th>Abbreviation used in Table V</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ajax</td>
<td>Ajax Chemicals Ltd, Sydney, N.S.W. Australia.</td>
</tr>
<tr>
<td>BDH</td>
<td>British Drug Houses, Poole, Dorset. England.</td>
</tr>
<tr>
<td>Calgon Corp.</td>
<td>Calgon Corporation, St. Louis, Missouri, U.S.A.</td>
</tr>
<tr>
<td>Eastman</td>
<td>Eastman Organic Chemicals, Rochester, New York, U.S.A.</td>
</tr>
<tr>
<td>Fluka</td>
<td>Fluka, A.G., Buchs, Switzerland.</td>
</tr>
<tr>
<td>M.C. + B.</td>
<td>Matheson, Coleman and Bell, Norwood (Cincinatti), Ohio, U.S.A.</td>
</tr>
<tr>
<td>Meinecke</td>
<td>Meinecke and Co. Inc., Baltimore, Maryland, U.S.A.</td>
</tr>
<tr>
<td>Merck</td>
<td>E. Merck, A.G., Darmstadt, W. Germany.</td>
</tr>
<tr>
<td>Purex</td>
<td>Purex Australia Pty Ltd., Sydney, N.S.W., Australia.</td>
</tr>
<tr>
<td>Selby</td>
<td>H.B. Selby and Co. Pty. Ltd., Sydney, N.S.W., Australia.</td>
</tr>
<tr>
<td>Sigma</td>
<td>Sigma Chemical Co., St. Louis, Missouri, U.S.A.</td>
</tr>
</tbody>
</table>
X.1.b). Plant material

Seed of three varieties of barley was used; these varieties and the sources of the seed are listed in Table VII.

Table VII. Sources of the Barley Seed used in the Experimental Investigations

<table>
<thead>
<tr>
<th>Variety</th>
<th>Harvest</th>
<th>Source</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pallas</td>
<td>1968 British</td>
<td>Cundy and Sons Ltd., Bury St.Edmunds, Suffolk, England.</td>
<td>4°C</td>
</tr>
<tr>
<td>Cape</td>
<td>1968 Australian</td>
<td>D.and D.Products Griffith, N.S.W., Australia.</td>
<td>4°C</td>
</tr>
<tr>
<td>Parkland</td>
<td>Canadian</td>
<td>Dr.D.M.Reid, Dept. of Biology, University of Calgary, Alberta, Canada.</td>
<td>Room temperature</td>
</tr>
<tr>
<td></td>
<td>(Date unknown)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

X.1.c). Light sources

X.1.c). (i) The safelight.

This consisted of one Philips TL40W/17 122 cm 40 watt green fluorescent tube with a filter of four sheets of primary green Cinemoid No. 39 and one sheet of primary deep blue Cinemoid No. 20 (Strand Electric, Melbourne). The safelight was fixed 48 cm (measured from the centre of the cross-section of the fluorescent tube) above the working top of the bench; the energy distribution of the radiation emitted by the safelight at the level of the bench top is presented in Fig. 3.

X.1.c). (ii) Red source

This consisted of two Philips TL20W/15 61cm 20 watt red fluorescent tubes with a filter of one sheet
FIG. 3. Energy distribution of the safelight at the level of the bench top.
Measurements were made with a specially-constructed spectroradiometer employing a Schott Veril S200 continuous line interference filter and an RCA 1P28 photomultiplier with an S-5 response. The instrument was calibrated with a Gamma Model 220 Calibrated Optical Source System. The slit width used was 1.6 mm which gave a bandwidth for 50% transmission of about 12 nm.
of Plexiglas 501 (red) 3mm thick (supplied by Plastral Trading, Northcote, Victoria; manufactured by Röhm and Haas, Darmstadt, W.Germany). Irradiation was commenced and stopped by removing and replacing a sheet of cardboard which fitted into a light-tight slide arrangement beneath the filter. The source was fixed either 48 or 124 cm (measured from the centre of the cross-section of the fluorescent tubes) above the specimens to be irradiated; the energy distribution of the radiation emitted by the source at a distance of approximately 20 cm is presented in Fig. 4.

X.I.c). (iii) Far-red source.

This consisted of one Philips PF318 500 watt 250 volt Argaphoto BM lamp with an internal mirror, surrounded by a cylindrical bath of flowing water 2 cm deep at the sides and 3 cm deep at the base, through which the light beam emerged. The water used was cold tap water filtered through a Nocol Model P 10 Purafil water filter (Nocol Industries, Prospect, South Australia) fitted with X250 filter paper to remove particles down to 2 microns; the flow rate of the water was regulated to 8.0 ± 0.5 litres per minute. The lamp and water bath assembly was mounted in a light-tight box above a Corning CS 1-69 glass filter (Corning Glass Works, Corning, New York). Below this was a light-tight slide arrangement into which various filters mounted in holders could be fitted. Irradiation was commenced and stopped by removing and replacing a piece of plywood backed with black velvet cloth which fitted into a further light-tight slide arrangement beneath the filter slide. The source was fixed 24 cm (measured from the lamp filament) above the specimens to be irradiated; the energy distribution of the radiation emitted by the source with various filter combinations at the level of the specimens is presented in Fig. 5.

The red and far-red light sources were both run from standard mains power stabilized to 240 volts with
FIG. 4. Energy distribution of the red source measured at a distance of approximately 20 cm.

Measurements above 600nm: Isco Spectroradiometer Model SR.
Measurements below 600nm: the instrument specified in Fig. 3.
FIG. 5. Energy distribution of the far-red source with various filters at the level of the specimens.

Measurements above 600nm: Isco Spectroradiometer Model SR.
Measurements below 600nm: the instrument specified in Fig. 3.
- With one Kodak Wratten gelatin filter No. 87 (W87).
- With one Kodak Wratten gelatin filter No. 88A (W88A).
an Electronic AC Voltage Stabilizer, type SP 5000 (Stabilac Pty Ltd., Sydney, N.S.W.).

X.2. Manipulation of Material

X.2.a). Growth of the barley seedlings.

The barley seed was sown approximately 1 cm deep in river sand which had been previously washed with tap water and then autoclaved for 1 h. 25 g of seed per plastic seed tray (29 x 35 x 6 cm) were used. The sand was saturated with distilled water after sowing and excess water was poured off; no further watering was given. The seed trays were placed in drawers or on shelves of steel filing cabinets in a constant temperature darkroom set at 25 ± 1°.

In occasional experiments, the seeds were sterilized before sowing by vigorously shaking in a one quarter strength solution of "Sno-wite" household bleach (equivalent to 1% sodium hypochlorite), for 5 min. The seeds were then left in the solution for 15 min, washed three times with distilled water and dried with filter paper. This procedure appeared to have no effect on the experimental results.

X.2. b). Cutting of segments from the dark-grown seedlings

After the specified time interval from sowing (5-8 days), the seed trays were moved to a bench in the constant temperature darkroom. At this stage the barley shoots consisted of the first leaf only (Plate 1). All subsequent manipulations were carried out under the dim green safelight and the trays of seedlings were covered up when not being directly used. Samples of 10-20 seedlings were cut approximately 1 cm above the level of the sand and the leaves were then laid in a single layer on a square wax block so that the tips were all level with the edges of the block. Segments 1.0 cm long and a specified distance from the tips of the leaves (1 or 2 cm)
PLATE 1. Seven day-old seedlings of barley, var. Pallas, grown under the experimental conditions
were cut using a specially-constructed cutter made from two razor blades supported by blocks of perspex (see Plates 2 and 3). The segments were removed from the cutter and handled subsequently with a camel hair brush. Immediately after removal from the cutter, they were floated on distilled water in a 9cm glass petri dish. The petri dish containing the segments was placed in darkness when not in use.

X.2.c). Measurement of the widths of the segments

The unrolling of a grass leaf results in an increase in the width of the leaf. The relationship between the amount of unrolling (i.e., internal circumference) and the width as measured is not linear, but weakly sigmoidal. However, the measured width can be taken as a practical determination of the amount by which the leaf has unrolled (207).

The widths of the segments were measured at specified times after the experimental treatments (20-40 h). The segments were placed on the glass of a negative carrier of a photographic enlarger. The enlarger was adjusted to throw an image of the segments magnified five times on to a piece of graph paper. The width of the image of each segment was then measured at the exact mid-point of its length. Normally, these were at least 10 segments in any one treatment; the average width per segment was calculated.

X.3. Light Treatments

After a sufficient number of segments had been cut, lots of approximately 15 segments were selected at random and transferred to 5cm plastic petri dishes containing two layers of Whatman No. 1 filter papers previously moistened with distilled water. The segments were arranged on the filter paper so that no segment was touching any other. The petri dishes were then placed in darkness and
PLATE 2. The cutter used to cut segments from the barley seedlings.

Upper: set up to cut segments 1 cm from the tips of the leaves.

Lower: set up to cut segments 2 cm from the tips of the leaves.
PLATE 3. Barley seedlings, showing how the segments were cut.  
Upper: Segments cut 1 cm from the tips of the leaves.  
Lower: Segments cut 2 cm from the tips of the leaves.
removed at random to receive the specified light treatments. After the irradiation, each petri dish was returned to darkness. After the specified time interval (normally 24 h), the petri dishes were removed from darkness and the measurement of the widths of each segment was taken. All the operations apart from the measurement of segment widths was carried out in the constant temperature darkroom.

X.4. Chemical Treatments.

X.4.a). Treatment with plant growth substances

Barley leaf segments were treated with solutions of plant growth substances either by floating them on the solution for a specified period, or by vacuum infiltration.

If the treatment was to be given by floating, random lots of approximately 15 segments were transferred from distilled water to 5 cm plastic petri dishes containing the required solutions. The petri dishes were left in the dark for the specified period, then removed and the measurement of segment widths carried out.

The process of vacuum infiltration involved the repeated creation and breaking of a vacuum. Randomly-selected lots of segments were transferred from distilled water to 25 ml beakers containing 10 ml of the required solution. The segments were held below the surface of the liquid by tightly-fitting plungers of plastic mesh. The beakers were placed in a desiccator (10 inches internal diameter) which was exhausted by an Edwards vacuum pump, model EB 3; the desiccator was then covered with a black cloth. The pump was switched on, allowed to run for 90 sec, and was then switched off. The vacuum was slowly released over the following 30 sec. period until atmospheric pressure was reached. The pump was then switched on again and the procedure was repeated for a total of 30 min (15 cycles). The pump
had been previously set to create a vacuum of 24 inches of mercury; this value was reached about 30 sec after the pump was switched on. After vacuum infiltration, the solution from each beaker was discarded and the segments were washed several times in distilled water. They were then transferred to 5cm petri dishes containing moistened filter paper and held in the dark until measurements were taken (normally 24 h after treatment).

X.4.b). Treatment with radioactive solutions

Treatment with radioactive solutions was always carried out by floating the segments on the required solution for the specified time in the dark. The segments were cut as usual and then were either all transferred to a 9 cm glass petri dish containing the radioactive solution, or an aliquot of the solution was added at a specified time to each 5 cm plastic petri dish containing a randomly-selected lot of segments.

X.5. Cleaning of the Glassware

The following procedure was used to clean the glassware used in the experimental investigations reported in this thesis.

1) Preliminary rinse in hot tapwater.
2) Machine wash in hot tapwater containing "Calgon".
3) Three machine rinses in hot tapwater.
4) Final rinse in running distilled water.
5) Dry by hot air.

In addition, glassware which had contained radioactive material was left overnight in tapwater containing "Haemolsol" and was rinsed in hot tapwater before going through the above procedure.
X.6. Statistical Treatment of the Results

X.6.a). Results from experiments involving light treatments

In the experiments on the effect of increasing the time of irradiation or the time between irradiation and measurement, the normal Student's t-test for comparing the mean of a single small sample with a known standard, assuming a normal population, was used (5). Confidence limits for the true population mean at the p=0.05 level were calculated for each point on the graphs of results (Fig 6, Fig 7, Fig 8).

In the red, far-red light reversal experiments, Bailey's (5) modified t-test for comparing the means of two small samples from normal population was used. This test can be used to calculate the confidence limits for the difference between the true means of two samples. In these experiments, results from treatments receiving red and far-red light were compared with the corresponding controls which had received the same amount of far-red, but no red light. Confidence limits for p=0.05 were calculated for the means of each pair of results and these limits were plotted for each pair of points on the graph. Where a pair of points does not lie within the marked limits, the difference between these two points is significant at the p=0.05 level.

X.6.b). Results from experiments involving treatment with growth substances and incorporation of radioactive substances

Bailey's (5) modified t-test was again applied, in this case to determine whether there were differences at the p=0.05 level between the mean of the results from each treatment and the mean of the results from each of the other treatments. In the graphs, histograms bearing letters with subscripts (e.g. a₁ ,b₂ ,c₃ ) are significantly different from the histogram bearing the corresponding simple letter (e.g a,b,c) at the p=0.05 level (Fig 11, Fig 12, Fig 13, Fig 16, Fig 19).
XI. The Effects of Various Light Treatments on Barley Leaf Unrolling

XI.1. The Safelight

In Fig. 6, the effects of various durations of the safelight on barley leaf segments are shown. The segments were cut and placed on moist filter paper in petri dishes with the lids removed; the dishes were left on the workbench under the safelight for the specified times. The lids were then replaced and the segments were held in the dark for 24 h before measurement.

Irradiation with the safelight does result in some unrolling of the segments as compared with the dark controls, especially with irradiations longer than 1.5 h. However, the experimental procedure used in later experiments resulted in the segments remaining exposed to the safelight for no longer than 30 min; with an irradiation of this duration, unrolling was minimal.

XI.2. Red Light

In Fig. 7, the effects of various durations of red light at two intensities on barley leaf segments are shown. Two varieties, Pallas and Cape, were used in the experiments illustrated and the widths of the segments were measured 24 h after irradiation. Irradiation with red light resulted in pronounced unrolling of the leaf segments from both varieties compared with the control segments maintained in the dark.

VI.2.a). Unrolling of the dark control segments

When the widths of segments were measured directly after cutting, the mean values were about 1.4 mm for Cape and about 1.2 mm for Pallas. The mean widths of the control segments in two of the experiments illustrated in Fig. 7 were greater than these values. The cutting and handling procedure, therefore, caused some
FIG. 6. The effect of the safelight on the unrolling of barley leaf segments.
FIG. 7. Red light-induced unrolling of barley leaf segments.
unrolling of the control segments in these experiments but this was not large compared with the unrolling response to red light. In general, the amount of unrolling of the dark controls varied rather greatly from experiment to experiment, but it was never more than 30% of the maximal unrolling and was usually much less.


The two lower graphs in Fig. 7 illustrate the results of two experiments performed in exactly the same way, on two consecutive days on the same variety of barley. In one case the maximal response (average width after red light minus width of dark control) is about 2.2mm whereas in the other case it is about 1.7mm. The response to red light, generally, was quite variable, and there seemed to be no logical explanation for the variability. Within one experiment performed on one day, the results were usually quite consistent in that similar red light treatments produced similar responses. The responses obtained in experiments performed on different days, however, were usually quantitatively quite different. In occasional experiments, red light treatments which normally induced maximal unrolling failed to produce unrolling of the treated segments greater than that of the dark controls.

It was thought that the variability in the maximal response to red light might be caused by measurements being taken while unrolling was still being completed. Fig. 8 illustrates the results of an experiment in which the measurements of segment widths were taken at various times after red irradiation. The amount of unrolling shown by the segments seems to bear no simple relationship to the time of measurement. (It should be noted in this context that the statistical limits marked on the graph are confidence limits for the value of the mean when p=0.05. They should not be taken as an estimate of the statistical significance of the difference between points. This latter estimate would involve the calculation of a modified Student's t-test (5) for each
<table>
<thead>
<tr>
<th>Barley variety</th>
<th>Cape</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of seedlings (days)</td>
<td>7</td>
</tr>
<tr>
<td>Distance from tips of leaves at which segments were cut (cm)</td>
<td>2</td>
</tr>
<tr>
<td>Height of red source (cm)</td>
<td>48</td>
</tr>
<tr>
<td>Maximum number of segments per mean width value</td>
<td>15</td>
</tr>
<tr>
<td>Minimum number of segments per mean width value</td>
<td>13</td>
</tr>
</tbody>
</table>

FIG. 8. Time course of red light-induced unrolling of barley leaf segments.
pair of points under consideration).

Poulson and Beevers (158) have recently published time courses of unrolling for barley leaf segments irradiated with white light (660 f.c mixed incandescent and fluorescent - R. Poulson, personal communication) and floated on a 0.001 M sodium acetate buffer. These time courses run from 0-20 h; for the first 4-6 h following irradiation, unrolling is slow, then there is a period of rapid expansion lasting approximately 6 h followed by a period of less rapid unrolling. Unrolling is complete about 24 h after irradiation (158).

It seems likely, therefore, that the variability in the response of the leaf segments to red light was not due to measurements being taken while unrolling was still being completed. Statistically, the variability of the means was due to the fact that the segments were not all of one, or even two, size classes when measured. Table VII presents the data from the experiment illustrated in the bottom graph of Fig. 7 in terms of the number of segments in each of the specified size classes.

Table VIII. Size Classes of Barley Leaf Segments

<table>
<thead>
<tr>
<th>Time in red light (sec)</th>
<th>No. of sample</th>
<th>Mean width of segments (mm)</th>
<th>Size class (width in mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15</td>
<td>1.38</td>
<td>Less than 1.5</td>
</tr>
<tr>
<td>15</td>
<td>15</td>
<td>2.86</td>
<td>1.5 2.0 2.5 3.0 3.5 4.0 4.5</td>
</tr>
<tr>
<td>30</td>
<td>15</td>
<td>3.11</td>
<td>4.0 4.5</td>
</tr>
<tr>
<td>45</td>
<td>15</td>
<td>2.99</td>
<td>4.0 4.5</td>
</tr>
<tr>
<td>60</td>
<td>15</td>
<td>3.07</td>
<td>4.0 4.5</td>
</tr>
<tr>
<td>75</td>
<td>15</td>
<td>3.31</td>
<td>4.0 4.5</td>
</tr>
<tr>
<td>90</td>
<td>15</td>
<td>3.06</td>
<td>4.0 4.5</td>
</tr>
<tr>
<td>105</td>
<td>15</td>
<td>3.05</td>
<td>4.0 4.5</td>
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<tr>
<td>120</td>
<td>15</td>
<td>3.18</td>
<td>4.0 4.5</td>
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<tr>
<td>135</td>
<td>15</td>
<td>3.47</td>
<td>4.0 4.5</td>
</tr>
<tr>
<td>150</td>
<td>15</td>
<td>2.64</td>
<td>4.0 4.5</td>
</tr>
<tr>
<td>165</td>
<td>15</td>
<td>3.41</td>
<td>4.0 4.5</td>
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<tr>
<td>180</td>
<td>15</td>
<td>2.89</td>
<td>4.0 4.5</td>
</tr>
<tr>
<td>195</td>
<td>15</td>
<td>3.13</td>
<td>4.0 4.5</td>
</tr>
<tr>
<td>210</td>
<td>15</td>
<td>3.21</td>
<td>4.0 4.5</td>
</tr>
<tr>
<td>225</td>
<td>15</td>
<td>3.47</td>
<td>4.0 4.5</td>
</tr>
<tr>
<td>240</td>
<td>15</td>
<td>3.16</td>
<td>4.0 4.5</td>
</tr>
<tr>
<td>255</td>
<td>15</td>
<td>2.93</td>
<td>4.0 4.5</td>
</tr>
<tr>
<td>270</td>
<td>15</td>
<td>2.90</td>
<td>4.0 4.5</td>
</tr>
<tr>
<td>285</td>
<td>15</td>
<td>2.31</td>
<td>4.0 4.5</td>
</tr>
<tr>
<td>300</td>
<td>15</td>
<td>3.20</td>
<td>4.0 4.5</td>
</tr>
</tbody>
</table>
The large number of size classes into which the data fall is also reflected in the great width of the confidence limits marked on the graphs in Fig. 7 and Fig. 8. Poulson and Beevers apparently did not find this great diversity of size classes (R. Poulson, personal communication); their segments were either completely unrolled or completely rolled. Comparison with their experiments is complicated, however, by the different light source used, and also the fact that they floated their segments on acetate buffer. The acetate could well have been metabolized by the segments to produce, for instance, an effective amount of ethylene (161) in the closed petri dishes which they used to contain the segments in the dark (R. Poulson, personal communication). That the acetate buffer did have some effect on the system is shown by the fact that the lag phase in unrolling following irradiation could be abolished by pre-incubation of the segments in the buffer in the dark (158).

The system used in the experiments reported in this thesis would seem to be uncomplicated by factors such as the production of ethylene from an external substrate. The great variability, in these experiments, in the widths of individual leaf segments from a sample irradiated with red light can perhaps be explained by postulating different characteristics for the recycling of phytochrome in each segment. It has been known for some time that even the purest red light source converts only about 80% of $P_R$ to $P_{FR}$ and that $P_{FR}$ has a slight but definite absorption maximum in the red region (see sections II and V.2 above). If the local environments within each segment were slightly different, this could well affect the rate of the recycling between $P_R$ and $P_{FR}$ induced by the red-irradiation. The final percentage of $P_R$ converted to $P_{FR}$ would therefore be different in each segment, resulting in different degrees of unrolling. The local environments within the segments could well be affected
by the differing lengths of time the segments were necessarily floated on distilled water, or the differing amounts of handling received by different batches of seedlings, the segments from which were subsequently randomized.

Part of the variability in the unrolling response to red light may also be explained by differences between segments in the amounts of an inhibitor leached out during the initial flotation on distilled water (see section XIV.2 below).

To explain the gross differences in the response of samples of segments to red-irradiation in experiments performed on different days, it is necessary to postulate factors having the same effect on all the segments used in a particular experiment. The nature of such factors is unknown.

XI.3. Far-red Light

XI.3.a). Preliminary experiments

A large number of experiments were performed in which samples of segments were irradiated with red light sufficient to cause maximal unrolling and this irradiation was immediately followed (within 15 sec) with an irradiation by far-red light. The duration of this second irradiation was varied from sample to sample of segments. A second series of samples was run as a control; these received the same duration of far-red light as the first series, but were not irradiated with red light.
Most of these experiments were carried out with far-red light sources which were non-standardized since the equipment necessary to measure the energy distribution of the radiation produced by the sources was not available. Using the manufacturers' published transmission curves for the filters employed in the various light sources, it was possible to draw the following conclusions from these experiments.

1). Irradiation of the segments with far-red light alone always resulted in unrolling of the segments to give a mean width greater than that of the dark control. This response to far-red light (mean segment width after far-red light minus width of dark control) was normally 10-60% of the maximal red response.

2). Reversal of the red light-induced unrolling by far-red light occurred only down to the level of the opening induced by far-red alone.

3). Reversal was always achieved with radiation from sources in which the filter had a 1% transmission cut-on point between about 720 and 780 nm. Reversal did not occur when the segments were irradiated with light which had passed through filters with cut-on points at higher wavelengths than 780 nm.
XI.3.b). Experiments with the standardized far-red light source

Fig.9 and Fig.10 illustrate the results of red, far-red light reversal experiments using the standardized far-red light source described in section XI.1.c) and the filters specified in Fig.5. The widths of the segments were measured 24 h after the irradiation. It can be seen that reversibility of the red light-induced unrolling of the segments down to the level of the opening induced by far-red light alone, was generally achieved.

XI.3.c). The variability of the reversibility by far-red light

Examination of Fig.9 and Fig.10 reveals that in the experiments illustrated there, reversibility by far-red light of the red light-induced unrolling of the barley leaf segments was highly variable. In several cases, reversal was not achieved by durations of far-red light longer than the minimum duration necessary to achieve reversal, but reversal was achieved by irradiations with far-red light of even longer duration. Reversibility, like the maximal response to red light, was also variable between experiments performed in the same way on similar material (Fig.9). This variability is to be expected if the effects of minor variations of procedure on the recycling of phytochrome postulated in section XI.2.c) above really do occur.

After the initial irradiation with red light, the phytochrome in each of the segments making up a sample would tend to be in a different photostationary state from that in each of the other segments. Since a small amount of recycling between the two forms of phytochrome also takes place under irradiation with far-red light (42), the effect of the second irradiation of the segments would be to magnify the differences in the phytochrome states. For example, suppose the first irradiation leads to photostationary states being set up in the segments in which the percentage of $P_{FR}$ varied from 60 to 90%. If in the
second irradiation, the percentage conversion of $P_{FR}$ to $P_{R}$ varied from 80 to 90%, the final percentages of $P_{FR}$ remaining in the tissues would be as set out in Table IX.

Table IX. The Effect of Theoretical Variations in the Recycling of Phytochrome on the Percentages of $P_{FR}$ remaining in Barley Leaf Segments after Irradiation with Red and Far-red Light

<table>
<thead>
<tr>
<th>% conversion of $P_{R}$ to $P_{FR}$ by the initial irradiation with red light</th>
<th>% $P_{FR}$ remaining after irradiation with far-red light resulting in 80% conversion of $P_{FR}$ to $P_{R}$</th>
<th>% $P_{FR}$ remaining after irradiation with far-red light resulting in 90% conversion of $P_{FR}$ to $P_{R}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>70</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>80</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>90</td>
<td>18</td>
<td>9</td>
</tr>
</tbody>
</table>

It can be seen that in the two most extreme cases (initial conversion 90% with far-red conversion of 80%; and initial conversion 60% with far-red conversion 90%) there would be a three-fold difference in the final content of $P_{FR}$ in the segments. This would result in differences in the final widths of each segment after the 24 h dark period. This explains the large confidence limits for the differences between the means in Fig.9 and Fig.10. In cases where the variations in the recycling of the pigment were large, there would also be large difference in the mean width values for each sample of segments. This would explain the variability in reversal by far-red light within the same experiment.

Part of this variability may also be explained by differences in leaching-out of an inhibitor between segments as mentioned in section X.2.b) above. Further details of the inhibitor hypothesis are presented in
Presumably, the factors causing gross differences between the maximal responses to red light in different experiments would also be operating in these reversal experiments. The variability in reversibility between different experiments can, therefore, also be explained on this basis.

XI.3.d). Far-red light-induced unrolling

The recycling of phytochrome under irradiation with far-red light also explains far-red light-induced unrolling of the barley leaf segments. Presumably, under irradiation with far-red light, a small amount of PFR (approximately 5% (96) ) will be produced in the dark-grown segments and this will result in a small amount of unrolling of the segments. Indeed Mohr and his co-workers regularly use continuous irradiation with low intensity far-red light for kinetic analysis of responses controlled by PFR (eg 149,150,151,210). The irradiation with far-red light sets up a photostationary state in which a low but constant level of PFR is maintained. The response to this constant level of PFR is similar to the response obtained if the material is irradiated with red light for a short period (213). The effectiveness of the given photostationary state is a function of the intensity of the radiation (151).

In the experiments reported here, the maximum duration of far-red light which the segments received was 10 min, whereas Mohr and his co-workers regularly used at least 6 h of continuous far-red light in their experiments. Nevertheless, Fig.9 and Fig.10 show that with increasing duration of far-red light there was a general trend towards greater unrolling of the barley leaf segments after the 24 h dark period, though the level of unrolling caused by treatment with red light alone was never reached. The intensity of the far-red source used in these experiments was lower than that normally used
by Mohr and his co-workers (eg in 151 the irradiance (ie total radiant energy from the far-red source used) was 3,500 ergs/cm² sec ± 5%). Therefore, it is likely that by increasing the duration of the far-red light treatment to periods greater than 10 min, unrolling of the leaf segments to the level of the red light-induced opening would be achieved.

The low-level radiation below 500 nm emitted by the far-red light sources (Fig. 5) could also have had an effect on the recycling of phytochrome, since the absorbancies of \( P_R \) and \( P_{\text{FR}} \) in this wavelength region are different (36, 182). This effect might also have been a contributory factor to the unrolling of the segments after irradiation with far-red light.

Both inhibited by the infiltration of the growth retardants (2-chloroethyl) trimethylammonium chloride (CCC) and 3-isopropyl-4′-(trimethylammonium chloride)-3-methylphenyl piperidine-1-carboxylate (Apo [168][167,168]).

Poulsen and Bevers [158] also found an effect of \( \text{GA}_3 \) on barley leaf unrolling. At the optimum concentration of \( \text{GA}_3 \) of 1.5 x 10⁻⁶ M (approx. 5 mg/l) the \( \text{GA}_3 \)-induced response in segments maintained in the dark was only about 40% of the response induced by continuous irradiation with white light. Flooding the segments in a \( \text{GA}_3 \) solution in acetate buffer under continuous white light for 20 h increased the light-induced response by about 30%. However, increases in the light-induced response of between 50 and 100% were achieved either by adding \( \text{GA}_3 \) to the flotation medium within 5 h of a short irradiation of 2 h white light, or by flooding the segments in \( \text{GA}_3 \) solution for 2-6 h before a light treatment of 12 h duration without the hormone in the flotation medium. The main effect of \( \text{GA}_3 \) was to shorten the lag phase between the irradiation and the onset of the period of rapid unrolling. However, it must be remembered that these results may be complicated by the possibility of metabolites of the acetate buffer on which the segments were flooding away possible metabolic products, ethylene cannot be excluded (151).
XII. The Effects on Barley Leaf Unrolling of Treatment with Growth Substances

XII.1. Introduction

Reid (167) found that infiltration of 10 mg/l of gibberellic acid (GA$_3$) into dark-grown barley leaf segments maintained in the dark resulted in the unrolling of the segments to similar mean width values to those found following irradiation with red light. He found that there was a dramatic increase in gibberellin-like substances extracted from the leaf segments for a short period immediately after red-irradiation; this increase and the unrolling of intact leaf segments were both inhibited by the infiltration of the growth retardants (2-chloroethyl) trimethylammonium chloride (CCC) and 2-isopropyl-4"-(trimethylammonium chloride)-5-methylphenylpiperidine-1-carboxylate (Amo 1618) (167,168).

Poulson and Beevers (158) also found an effect of GA$_3$ on barley leaf unrolling. At the optimum concentration of GA$_3$ of $1.5 \times 10^{-5}$ M (approx. 5 mg/l) the GA$_3$-induced response in segments maintained in the dark was only about 40% of the response induced by continuous irradiation with white light. Floating the segments on a GA$_3$ solution in acetate buffer under continuous white light for 20 h increased the light-induced response by about 30%. However, increases in the light-induced response of between 50 and 100% were achieved either by adding GA$_3$ to the flotation medium within 5 h of a short irradiation of 2 h white light, or by floating the segments on GA$_3$ solution for 2-8 h before a light treatment of 12 h duration without the hormone in the flotation medium. The main effect of GA$_3$ was to shorten the lag phase between the irradiation and the onset of the period of rapid unrolling. However, it must be remembered that these results may be complicated by the possibility of metabolism of the acetate buffer on which the segments were floating, among possible metabolic products, ethylene cannot be excluded (161).
Beevers et al. (7) worked on a similar leaf unrolling system using segments of wheat leaves rather than barley. They found that both GA$_3$ (optimum concentration 5ppm) and kinetin (optimum concentration 10 ppm) caused unrolling of wheat leaves when the segments were floated on solutions of the hormones in the dark. The authors state that the extent of unrolling induced by the hormones was not as great as that induced by red light, though this statement is contradicted by their published data on unrolling in red light. Beevers et al. also found a red light-induced increase of gibberellins in wheat leaves very similar to that found by Reid (167,168) in barley leaves. This increase did not occur if the red-irradiation was immediately followed by far-red light.

Kang and Zeevart (113) also worked on wheat leaves. They reported that kinetin strongly promoted unrolling in red light as well as in the dark. Low concentrations of indoleacetic acid (IAA) were said to inhibit the response to red light.

XII.2. The Effect of GA$_3$ and Kinetin on Barley Leaf Unrolling

Leaf segments of the variety Pallas were vacuum infiltrated with GA$_3$ at concentrations of 10 and 100mg/l; kinetin at a concentration of 20mg/l; or distilled water. All solutions were made up in distilled water. The segments which had been infiltrated with distilled water alone either received 5 or 10 min of red light or were maintained in the dark. All samples of segments were maintained in the dark after treatment, in petri dishes containing moist filter paper, for 24 h before measurement. This experiment was carried out with segments from 5-, 6-, 7- and 8-day old seedlings. The results are presented in Fig.11 and Fig.12.

Leaf segments of 7 day-old seedlings of the variety Parkland were floated on solutions of 100 mg/l GA$_3$; 20 mg/l kinetin; and 100 mg/l GA$_3$ plus 20 mg/l kinetin; or
FIG. 11. The effects of gibberellic acid and kinetin on the unrolling of barley leaf segments from the variety Pallas.

For explanation of symbols see page 61.
FIG. 12. The effects of gibberellic acid and kinetin on the unrolling of barley leaf segments from the variety Pallas.

For explanation of symbols see page 61.
on distilled water. The segments floated on distilled water received 5 min of red light or dark. All samples of segments were maintained floating on the solutions in petri dishes in the dark for 24 h before measurement. The results of two similar experiments are presented in Fig.13.

XI.2.a). The response to GA$_3$

In the experiments with the Pallas variety (Fig.11 and Fig.12), only a few replicates showed unrolling caused by GA$_3$ to be significantly different (at the p=0.05 level) from the unrolling of the dark control segments. The response to GA$_3$ was generally less than 20% of the red light-induced response. This is in direct contradiction to the results of Reid (167) who worked with exactly the same variety of barley.

Since performing the experiments with the Pallas variety, Dr Reid has had some difficulty in obtaining similar results with other barley varieties (D.M.Reid - personal communication to D.J.Carr). His recent results which are most similar to those previously obtained with the Pallas variety have been obtained with the Canadian variety Parkland, though even these results could not be repeated consistently. Dr Reid very kindly donated some of the seed of the Parkland variety which he was using, and the experiments performed with this seed are reported in Fig.13.

The response to GA$_3$ by leaf segments of the Parkland variety was generally greater than the response obtained in experiments with the Pallas variety. In all experiments, the GA$_3$-induced unrolling was significantly greater (at the p=0.05 level) from the unrolling of the dark control. In the lower graph of Fig.13 is illustrated the largest GA$_3$-induced response obtained in all the experiments with plant hormones. Even this is only 40% of the red light-induced response, and the GA$_3$-induced unrolling is still significantly different from the unrolling
FIG. 13. The effects of gibberellic acid and kinetin on the unrolling of barley leaf segments from the variety Parkland.

For explanation of symbols see page 61.
after red light at the p=0.05 level.

The results demonstrate that with the seed and the techniques used, the exogenous application of GA$_3$ to dark-grown barley leaf segments maintained in the dark did not result in the unrolling of the segments to a similar extent to the unrolling induced by red light. This would suggest that gibberellins play only a minor role in the unrolling of barley leaf segments.

Such a hypothesis does not conform with Reid's results on barley leaf segments (167) or the results of Beevers et al (7) on wheat leaf segments. However, in his original work, Reid (167) found that far-red light could reverse the response to exogenous GA$_3$ in some segments of a sample but not in others from the same sample. This suggests that there was a variation in the type of response expressed by segments which had received the same GA$_3$ treatment. This variation together with the fact that Dr Reid has had difficulty, more recently, in obtaining similar results, suggests that the induction, by exogenously supplied GA$_3$, of unrolling to the level induced by red-irradiation may be limited to certain batches of seed only, or may be influenced by pretreatments such as the storage conditions of the seed or stray light during germination.

This conclusion does not conform with the conclusions of Reid (167,168) and Beevers et al (7) that an increase in endogenous gibberellins is involved in the unrolling response to red light. There could either be a great difference between the action of the (uncharacterized) endogenous gibberellins and exogenous GA$_3$ or the endogenous gibberellins could be simply not involved in the unrolling response to red light (see section XIV.2 below). In support of the latter hypothesis, there is some evidence that the growth retardant CCC, which was found by Reid (167, 168) to inhibit the red light-induced increase in endogenous gibberellins, inhibits the unrolling response to red light by
only 50% in experiments on barley leaf segments performed similarly to those reported in this thesis. The growth retardant Amo 1618, which was also found to inhibit the increase in gibberellin activity (168), had little effect on red light-induced unrolling in these experiments (R. Menhennett, personal communication).

The results of Poulson and Beevers (158) are not in conflict with the results reported above since they found a significant effect of GA\textsubscript{3} only in a synergism with red light.

XII.2.b). The response to kinetin

In both the experiments with the Pallas variety (Fig.11 and Fig.12) and the experiments with the Parkland variety (Fig.13), barley leaf segments maintained in the dark unrolled when treated with kinetin. Frequently the response to kinetin was significantly greater than the response to red light. In the experiments with Pallas, there is slight evidence that the kinetin response developed between the fifth and sixth days of growth of the seedlings (Fig.11).

These results are in line with those of Beevers et al (7) and Kang and Zeevaart (113); both these groups of workers used segments from wheat leaves. An unrolling response to kinetin in barley leaf segments has not been reported previously. The significance of these results will be discussed in section XIV.2 below.

XII.2.d). Interaction between GA\textsubscript{3} and kinetin

In the experiments with the Parkland variety (Fig.13) the mean width values for the leaf segments obtained when kinetin and gibberellin were applied together were no different from those obtained when kinetin was applied alone. Therefore, there was no interaction between the effects of the hormones on the unrolling of leaf segments from the Parkland variety.
This is further evidence in support of the hypothesis that gibberellins are relatively inactive in the unrolling of barley leaf segments.

Paulsen and Keeney [158, 159] found that floating barley leaf segments on solutions of certain protein and RNA synthesis inhibitors under continuous illumination with white light resulted in the inhibition of the unrolling response. Similar inhibition was obtained by Held [167] and Carr and Said [40] by infiltrating the inhibitor solutions into barley leaf segments immediately before or immediately after an irradiation with red light; the segments were then maintained in the dark for 15 h before measurement. These results suggest that RNA and protein synthesis are involved in the leaf unrolling response to red light.

There are various reports of red, far-red light reversible changes in the RNA levels of dark-grown plants. Reversible increases in the total RNA content of rye seedlings (104) have been reported, and Jaffe (151) found a similar red, far-red light reversible increase in the ribosomal fraction of RNA isolated from the terminal buds of dark-grown pea plants. Weidner et al [212] found a similarly reversible increase in the RNA content of whole mustard seedlings. In further work, Weidner and Mohr [211A] found a 21% increase in the specific activity of total RNA in mustard seedlings only 3 h after the onset of a continuous low intensity irradiation with far-red light. They claimed that this increase was induced by the constant low level of Pfr set up by the far-red light. Fractionation of the total RNA showed that all fractions were homogeneously labelled [211A]. Poulsen and Bevers [159, 160] demonstrated increased levels of RNA in barley leaf segments maintained in continuous white light as compared with segments maintained in the dark; this increase was mainly in cytoplasmic ribosomal RNAs [150].
XIII. Early Biochemical Events During Barley Leaf Unrolling

XIII.1. Introduction

Poulson and Beevers (158, 159) found that floating barley leaf segments on solutions of certain protein and RNA synthesis inhibitors under continuous illumination with white light resulted in the inhibition of the unrolling response. Similar inhibition was obtained by Reid (167) and Carr and Reid (40) by infiltrating the inhibitor solutions into barley leaf segments immediately before or immediately after an irradiation with red light; the segments were then maintained in the dark for 15 h before measurement. These results suggest that RNA and protein synthesis are involved in the leaf unrolling response to red light.

These are various reports of red, far-red light reversible changes in the RNA levels of dark-grown plants. Reversible increases in the total RNA content of rye seedlings (104) have been reported, and Jaffe (111) found a similar red, far-red light reversible increase in the ribosomal fraction of RNA isolated from the terminal buds of dark-grown pea plants. Weidner et al (212) found a similarly reversible increase in the RNA content of whole mustard seedlings. In further work, Weidner and Mohr (211A) found a 31% increase in the specific activity of total RNA in mustard seedlings only 3 h after the onset of a continuous low intensity irradiation with far-red light. They claimed that this increase was induced by the constant low level of P<sub>FR</sub> set up by the far-red light. Fractionation of the total RNA showed that all fractions were homogenously labelled (211A). Poulson and Beevers (159, 160) demonstrated increased levels of RNA in barley leaf segments maintained in continuous white light as compared with segments maintained in the dark; this increase was mainly in cytoplasmic ribosomal RNAs (159).
The levels of the enzyme responsible for the synthesis of mRNA (DNA-dependent RNA polymerase) in the nuclei of dark-grown pea seedlings were found to rise after a brief irradiation of the seedlings with red light; this effect was largely reversible by far-red light (23). The level of RNA polymerase was also found to rise after illumination of dark-grown maize seedlings (192); and the level of the enzymes was greater in extracts from barley leaf segments which had been irradiated with white light than in extracts prepared from segments maintained in the dark (159).

Using continuous irradiation with far-red light Weidner et al (212) showed that the total protein content of whole mustard seedlings increases under the influence of PFR. Kang and Zeevaart (113) reported that the incorporation of leucine-C\(^{14}\) into protein in vivo was greater in wheat leaf segments which had received red light than in segments which had been maintained in the dark. In in vitro experiments, the incorporation of leucine-C\(^{14}\) into protein by ribosomal preparations from barley segments irradiated with white light was greater than the incorporation by preparations from segments which had been held in the dark (159). Amino acid incorporation by ribosomes prepared from dark-grown maize and bean plants was stimulated up to 200% by giving the plants a brief irradiation with red light (216). The percentage of polysomes in the maize and bean preparations was also increased by the irradiation (216). The level of polysomes in dark-grown barley leaf segments was similarly increased when the seedlings were given an irradiation with white light (159).

There is, therefore, quite a large amount of evidence that RNA and protein synthesis in dark-grown plants is stimulated by irradiation of the plants, particularly with red light. In some cases, the red light-induced increase in RNA synthesis has been shown to be reversible by far-red light. However, if RNA and protein synthesis are involved in controlling the leaf unrolling
response to red light, the increases in synthesis must take place soon after the irradiation with red light and certainly before the escape reaction for far-red reversibility has been completed.

Poulson and Beevers (158) floated barley leaf segments on solutions of the antibiotic actinomycin D (act D) at various times after the beginning of an irradiation with continuous white light. Act D is said to inhibit DNA-dependent RNA synthesis by complexing with two-stranded DNA at guanine residues. They found that unrolling of the leaf segments was inhibited by act D applied two hours after the beginning of the light period. However, when the applications were made 4 and 8 h after the beginning of the light period, act D failed to inhibit the unrolling of the segments. Cycloheximide, which inhibits protein synthesis by cytoplasmic ribosomes, inhibited the unrolling of the leaf segments regardless of the time it was applied. Similar experiments with act D were carried out by Carr and Reid (40) and Reid (167). In these experiments, the antibiotic was infiltrated into the segments at increasing times after a relatively short irradiation with red light. Act D was most effective at inhibiting unrolling 80 min after the irradiation; when applied 185 min after, some release from the inhibition was achieved. Further similar experiments on barley leaf segments have now been performed using several inhibitors of RNA and protein synthesis (R. Menhenett, personal communication). When certain of the inhibitors were infiltrated within 4 h after the irradiation, maximal inhibition of unrolling was obtained; infiltration after this period caused a decline in the effectiveness of inhibition. These results suggest that light-stimulated increases in RNA and protein synthesis which affect leaf unrolling take place within a few hours after the irradiation. This is consistent with the results of experiments on the escape reaction in barley leaf unrolling which indicate that the reaction is completed 5-6 h after the irradiation (R. Menhenett, personal communication).
The experiments described below were designed to discover whether light-stimulated increases in RNA and protein synthesis do take place in barley leaf segments within 4-6 h after irradiation with red light.

XIII.2. RNA Synthesis after Red-Irradiation

XIII.2.a. Experimental procedure

In these experiments, leaf segments of the barley variety Pallas were given an irradiation with red light and were then left in dark for 2, 4 or 6 h. Control segments received no light treatment. 2 h before the end of the dark period, a solution of uracil-6-T in distilled water was added to each petri dish in which segments were held on moist filter paper. At the end of the dark period the segments were homogenized; all steps up to and including the homogenization were carried out in the darkroom. The homogenate was then transferred to the light and RNA was extracted. Samples were taken, an estimate was made of total RNA, and the radioactivity of the RNA was counted.

The RNA extraction procedure was essentially that of Loening and Ingle (135). It is presented in more detail in Fig. 14 and Table X.

| Table X. Solutions used in the Extraction of RNA from Barley Leaf Segments |
|---------------------------------------------|-----------------------------------------------|
| **Abbreviation in Fig. 14** | **Composition of Solution** |
| Homogenization medium | 10mM Trizma buffer pH. 7.6 containing 50mM sodium chloride and 0.05% (w/v) naphthalene 1:5 disulphonate. |
| TPNS/PAS soln. | Homogenization medium containing 1% (w/v) tri-isopropynaphthalene sulphonate and 10% p-amino salicylic acid. |
| Phenol mixture | Solution in the following proportions: 50g phenol, 7.0 ml m-cresol, 5.5ml distilled water, 0.05g 8-hydroxyquinoline |
FIG. 14. Flow-sheet for the experiments on the synthesis of RNA in barley leaf segments after irradiation with red light.
0.5ml of supernatant from extraction

↓

Add 0.5ml 5% TCA

↓

Heat at 90° for 20 min

↓

Add 2.5ml water, 0.3ml orcinol reagent and 3.0ml ferric chloride reagent. Mix.

↓

Heat in boiling water bath in stoppered tubes for 45 min

↓

Allow to cool

↓

Read extinctions at 660nm against a blank standard in Hitachi Model 101-0006 UV-VIS Spectrophotometer

↓

Read extinctions at 540nm

Reagents

Orcinol

10% orcinol in absolute ethanol

Ferric chloride

0.1% FeCl₃·6H₂O in concentrated HCl

TCA

5% trichloracetic acid in distilled water

FIG. 15. Procedure for the estimation of total RNA by the orcinol reaction
Homogenization medium plus TPNS and PAS

Homogenization medium containing 0.5% (w/v) tri-isopropyl naphthalene sulphonate and 5% (w/v) p-amino salicylic acid.

Ethyl alcohol

95% (v/v) ethyl alcohol

Sodium acetate solution

0.15M sodium acetate, pH 6.0

Total RNA was estimated by the orcinol reaction (39) (Fig. 15) except that the following formula was used:

\[
\text{O.D.}_{660}^{\text{RIBOSE}} = \frac{4.1 \times \text{O.D.}_{660} - \text{O.D.}_{540}}{3.9}
\]

This formula had been developed experimentally and its use resulted in the production of a straight-line calibration curve (R. Yu, personal communication). Radioactivity was counted in a Beckman LS-250 scintillation counter set to "Automatic Quench Compensation". The following scintillation fluid was used (6):

- PPO (2,5-diphenyloxazole) 5g/l
- Napthalene 100g/l
- p-Dioxan Balance of litre

The efficiency of counting was about 20%.

XIII.2.b). Results

The results of one of these experiments are presented in Fig. 16 and Table XI.
FIG. 16. Results of an experiment on the synthesis of RNA in barley leaf segments after irradiation with red light.

For explanation of symbols see page 61.
Table XI. Total RNA Extracted per Barley Leaf Segment

<table>
<thead>
<tr>
<th>Time after treatment at which segments were homogenized (h)</th>
<th>Treatment</th>
<th>Total RNA per segment (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Dark</td>
<td>0.67</td>
</tr>
<tr>
<td>5 min red light</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Dark</td>
<td>0.37</td>
</tr>
<tr>
<td>5 min red light</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Dark</td>
<td>0.28</td>
</tr>
<tr>
<td>5 min red light</td>
<td>0.39</td>
<td></td>
</tr>
</tbody>
</table>

From the top graph in Fig. 16, it can be seen that there were rather large differences between treatments in the amount of radioactive label which penetrated into the segments. This is unlikely to be an effect of the treatments, since the variation appears random, except that the incorporation into segments between 2 and 4 h after treatment is significantly greater for both treatments than the incorporation between 0 and 2 h and 4 and 6 h.

From the bottom graph in Fig. 16 a general upward trend with increasing time after treatment can be seen in the incorporation of activity into RNA. In the segments homogenized 6 h after treatment significantly more counts were incorporated into the RNA of dark control segments than into that of the irradiated segments. This is the only difference between the dark and the light treatments in the amount of radioactivity incorporated into RNA.

In Table XI approximately twice as much total RNA per segment was extracted from the segments homogenized 2 h after treatment as compared with the segments homogenized...
about one quarter more RNA was extracted from irradiated segments homogenized 6 h after treatment than from dark control segments homogenized at the same time; this is the only significant difference between the light and dark treatments in the amount of RNA extracted.

The sample of segments measured 24 h after treatment gave the maximal response to red-irradiation.

Previous to the experiment illustrated in Fig. 16, two similar preliminary experiments were carried out using a slightly modified technique. In these experiments, all segments were given a standard incubation of 1 h in the radioactive tracer (in this case uridine-5-T) and were then either given red irradiation followed by a dark period of 2, 4 or 6 h, or were held in the dark throughout. The results of these experiments are summarized in Table XII.

Table XII. Results of Preliminary Experiments on the Synthesis of RNA in Barley Leaf Segments after Irradiation with Red Light

<table>
<thead>
<tr>
<th>Time after treatment at which segments were homogenized (h)</th>
<th>Treatment</th>
<th>First experiment</th>
<th>Second experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Dark</td>
<td>3.8</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>5 min red light</td>
<td>3.6</td>
<td>5.8</td>
</tr>
<tr>
<td>4</td>
<td>Dark</td>
<td>4.3</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>5 min red light</td>
<td>2.9</td>
<td>5.2</td>
</tr>
<tr>
<td>6</td>
<td>Dark</td>
<td>2.9</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>5 min red light</td>
<td>4.1</td>
<td>10.5</td>
</tr>
</tbody>
</table>

Discussion of the results

The use of a radioactive tracer in these experiments enables all RNA synthesized after the time of.
addition of the tracer to be so identified; total RNA can be measured by the orcinol reaction. If there is an increase in RNA synthesis after irradiation with red light, it would be expected that the RNA extracted from red-irradiated leaf segments would have a higher specific activity (measured as CPM/µg total RNA) than that extracted from the corresponding dark control segments. The time of commencement of this increased synthesis could be found by comparing the specific activities of RNA extracted from red-irradiated leaf segments at various times after the irradiation.

The bottom graph in Fig.16 demonstrates that in the experiment illustrated there, no RNA extracted from red-irradiated leaf segments had a significantly higher specific activity than that extracted from the corresponding dark control segments. Table XII shows that in the preliminary experiments there was a slight increase in the specific activity of RNA extracted 6 h after red-irradiation.

Poulson and Beevers (159) are the only workers who have carried out similar experiments. They found similar increases in the specific activity of RNA extracted from irradiated barley leaf segments after 6 and 16 h of continuous white light, as compared with the RNA extracted from corresponding dark control segments. Poulson and Beevers' experiments, however, are subject to several fundamental criticisms. Their irradiated segments were incubated with the radioactive label (P\(^{32}\) - orthophosphate) for a period of 6 or 16 h under continuous illumination, whereas the control segments were incubated with the label in the dark (159). It is possible, therefore, that the differences in the specific activities of the RNAs could be accounted for by an increased uptake of label in the light as compared with the uptake in the dark. This light treatment also initiated greening of the segments (159), so the increased RNA synthesis found may be the result of greening processes rather than processes leading to the unrolling of the leaf segments.
In the experiments reported here, these problems were avoided by incubating all segments with the label in the dark either before or after some of the segments received the irradiation with red light, and by keeping the duration of the irradiation short enough to prevent the initiation of greening. The results of these experiments, however, are subject to the criticism that the specific activities of RNA finally obtained are very low. This could be due either to the label not penetrating the leaf segments or to the fact that very little synthesis of RNA was actually taking place. The top graph in Fig. 16 shows a rather random variation in the amount of radioactivity which actually penetrated into the segments; it is not possible to say, however, whether all, or only a fraction, of the radioactive label actually reached the physiological pools from which RNA was being synthesized.

The technique used in these experiments measures only the specific activity of total RNA. It is possible that there is an increased synthesis, after irradiation of the leaf segments with red light, of specific RNAs and that this increase is quantitatively too small to be measured by these experiments. The increase in the specific activities of these RNAs could well be masked by the bulk radioactivity of the total RNA (150A, 150B). In order to discover whether this was the case, it would be necessary to fractionate the total extracted RNA and measure the specific activity of each fraction.

With the methods and techniques used in the experiments reported here, it was not possible to demonstrate a great increase in the synthesis of RNA in barley leaf segments within 6 h of red-irradiation, as compared with the synthesis in dark control segments. A slight increase 6 h after red-irradiation was found in preliminary experiments, but this could not be confirmed in a later experiment.

XIII.3.a). Experimental procedure

The experimental procedure (Fig.17) was very similar to that used in the experiments on RNA synthesis, except that leucine-4,5-T was used as the radioactive label. The protein was extracted by precipitation with trichloracetic acid (TCA) and hydrolysis with sodium hydroxide (Fig.17). Total protein was estimated by the Lowry Test (39) (Fig.18). Radioactivity was counted by the same method used in the experiments on RNA synthesis. The efficiency of counting was about 18%.

XIII.3.b). Results

The results of these experiments are presented in Fig.19 and Table XIII.

<table>
<thead>
<tr>
<th>Time after treatment at which segments were homogenized (h)</th>
<th>Treatment</th>
<th>Total protein per segment (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Dark</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>5 min red</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>light</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Dark</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>5 min red</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>light</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Dark</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>5 min red</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>light</td>
<td></td>
</tr>
</tbody>
</table>

From the top graph in Fig.19, it can be seen that more label penetrated the segments between 2 and 4 h and between 4 and 6 h after treatment than between 0 and 2 h after treatment. In the two periods of highest penetration, more label penetrated into the dark treatments than into the red-irradiated ones.

From the bottom graph in Fig.19, the protein extracted from the barley leaf segments 4 h after treatment had higher specific activities than that extracted 2 h and
Cut segments
Float on distilled water in 9 cm petri dish.
Lots of 20 to damp filter paper in separate 5 cm petri dishes.
5 min red light Dark
Dark for specified time.
2 h before homogenization add 10 ml leucine-4,5-T solution (specific activity 57.6 Ci/mM; conc. of soln. 10 µCi/ml) to petri dishes in dark.
Wash with distilled water in filter funnel.
3 ml ice cold 5% TCA
Homogenize at top speed for 5 min in micro-attachment of Sorvall Omni-Mixer homogenizer, vessel cooled in ice bath.
Transfer to 30 ml Corex glass centrifuge tube in ice.
Add 7 ml 5% cold TCA. Mix thoroughly.
Leave overnight in ice in cold room.
Centrifuge at 14,000 rpm (20,000 g) for 1 h at 0°C in SS34 head of Sorvall RC2-B centrifuge.
Remove supernatant.
Centrifuge at 14,000 rpm for 30 min in Sorvall.
Heat at 65° for 15 min. Allow to cool.
Centrifuge at 14,000 rpm for 30 min in Sorvall.
Add 10 ml 5% TCA.
Heat at 85-90° for 15 min. Allow to cool.
Centrifuge at 14,000 rpm for 30 min in Sorvall.
Grind precipitate in teflon in glass homogenizer in 10 ml 0.1 N NaOH.
Warm gently for several hours.
Centrifuge at 14,000 rpm for 30 min in Sorvall.
Supernatant upon centrifugation dialyze against distilled water.
Grind precipitate in teflon in glass homogenizer in 10 ml 0.1 N NaOH.
Warm gently for several hours.
Centrifuge at 14,000 rpm for 30 min in Sorvall.
Take 3 x 0.5 ml samples of supernatant and add to scintillation vials containing 10 ml scintillation fluid.
Take 2 x 0.5 ml samples of supernatant and estimate protein by Lowry reaction.
FIG. 17. Flow-sheet for the experiments on the synthesis of protein in barley leaf segments after irradiation with red light.
0.5ml of supernatant from extraction

Add 0.1ml sodium deoxycholate reagent, 0.3ml sodium hydroxide reagent and 0.1ml distilled water

Add 4.0ml reagent C.

Allow to stand for 10 min

Add 0.5ml diluted Folin-Ciocalteu reagent with instantaneous and vigorous mixing

Allow to stand for 30 min

Read extinctions at 660nm against a blank standard in Hitachi Model 101-0006 UV-VIS Spectrophotometer

Reagents

Sodium deoxycholate
5% sodium deoxycholate in distilled water

Sodium hydroxide
1.0 N sodium hydroxide in distilled water

Reagent A
2% sodium carbonate in distilled water

Reagent B
1% cupric sulphate mixed with an equal volume of 2% sodium potassium tartrate immediately before use.

Reagent C
50ml reagent A mixed with 1ml of reagent B immediately before use

Diluted Folin-Ciocalteu Reagent
100ml of the commercial reagent mixed with 136ml distilled water to give a solution N in acid.

FIG. 18. Procedure for the estimation of total protein by the Lowry Test
FIG. 19. Results of an experiment on the synthesis of protein in barley leaf segments after irradiation with red light.

For explanation of symbols see page 61.
6 h after treatment. In the segments homogenized 6 h after treatment significantly more radioactivity was incorporated into the protein of dark control segments than into that of red-irradiated segments.

Table XIII shows that significantly less protein was extracted from the segments homogenized 4 h after treatment than from the segments homogenized 2 h and 6 h after treatment. About one third less protein was extracted from the dark control segments homogenized 6 h after treatment as compared with the red-irradiated segments extracted at the same time.

The sample of segments measured 24 h after treatment gave the maximal response to red-irradiation.

One preliminary experiment was performed in a similar way to the preliminary experiments performed on RNA synthesis. The results of this experiment are summarized in Table XIV.

Table XIV. Results of Preliminary Experiment on the Synthesis of Protein in Barley Leaf Segments after Irradiation with Red Light

<table>
<thead>
<tr>
<th>Time after treatment at which segments were homogenized (h)</th>
<th>Treatment</th>
<th>CPM/µg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Dark 5 min red light</td>
<td>25.8 39.3</td>
</tr>
<tr>
<td>4</td>
<td>Dark 5 min red light</td>
<td>19.7 24.9</td>
</tr>
<tr>
<td>6</td>
<td>Dark 5 min red light</td>
<td>21.2 17.4</td>
</tr>
</tbody>
</table>

XIII.3.c). Discussion of the results

Similarly to the experiments on RNA synthesis, if there is an increase in protein synthesis in the barley leaf segments after irradiation with red light, the protein extracted from red-irradiated segments would have a higher
specific activity than that extracted from the corresponding dark control segments. It can be seen from the bottom graph in Fig. 19 that no protein extracted from red-irradiated segments has a specific activity significantly higher than that of the protein from the corresponding dark control segments. Table XIV shows that in the preliminary experiment slight increases in the specific activity of protein from red-irradiated segments as compared with that from the corresponding dark control segments were found in the extractions performed 2 h after treatment.

Poulson and Beevers (159) found increased levels of polysomes in ribosome preparations prepared from segments which had received 6 and 16 h of white light as compared with preparations from dark control segments. They also found increased levels of the enzyme RNA polymerase.

Since barley leaf segments would contain far more protein than RNA, it is more likely in these experiments that increased synthesis of specific proteins (such as RNA polymerase) after red light has been masked by the radioactive labelling of total protein. Also, very low amounts of protein were actually extracted; this was due to the great difficulty experienced in hydrolysing the TCA precipitate with sodium hydroxide. This was necessary to bring the protein into solution for radioactive counting in scintillation fluid. Because of this technical difficulty the results of these experiments are probably not particularly meaningful. The radioactive labelling technique remains viable, however, provided that some reliable method can be found for fractionating the protein preparation, and for the counting of the radioactivity of the protein fractions.
XIV. Discussion

XIV.1. Summary of Results and Possible Conclusions

The results of the experiments reported in this thesis and the conclusions which it is possible to draw from these results are summarized below.

1). Irradiation of dark-grown barley leaf segments with short durations of red light results in the unrolling of the segments; this is completed and measurable after a 24 h dark period. The effect of red light is reversible by far-red light given immediately after the red-irradiation. This demonstrates that the unrolling of the leaf segments in response to a short irradiation with red light is mediated by the pigment phytochrome.

2). The unrolling of the leaf segments to the level achieved after red-irradiation is not induced by application of exogenous GA$_3$ under the conditions specified above, but it is induced by the application of exogenous cytokinins. The fact that kinetin can act as a substitute for red light in initiating the unrolling of the leaf segments suggests that cytokinins may be involved also in the unrolling response of the segments to red light.

3). Increased bulk synthesis of RNA could not be demonstrated (with the methods and under the conditions specified above) to be one of the early biochemical events occurring after irradiation of the leaf segments with red light; a similar synthesis of bulk protein could also not be demonstrated, though this could possibly be due to technical difficulties. It is likely that bulk synthesis of RNA,
and possibly protein, is, therefore, not directly involved in the early pathway between the perception of the light stimulus by phytochrome and the processes leading to the unrolling of the leaf segments.

XIV.2. Phytochrome Action in Barley Leaf Unrolling

Both Beevers and his co-workers (7) working with wheat leaf segments, and Reid and his co-workers (167,168) using barley, were able to demonstrate an increase in gibberellin activity within minutes of the cessation of an irradiation of the leaf segments with red light. Beevers et al (7) found that this red light-induced increase in activity was reversible by far-red light, but Reid (167) could not demonstrate such a reversibility. Both groups of workers also showed that unrolling of the leaf segments to the level achieved by red-irradiation could be initiated by treatment of the segments with exogenous GA₃. Therefore, they concluded that the control of leaf unrolling by phytochrome might be mediated partly through the induction of increased activity of endogenous gibberellins, either by increased biosynthesis (167) or by the release of free gibberellins from an inactive or bound form (7).

From their experiments with inhibitors of RNA and protein synthesis Reid and his co-workers (40,167) concluded that RNA and protein synthesis were intimately involved in the control of barley leaf unrolling by phytochrome. Indeed, Carr and Reid (40) even go so far as to suggest that PₚR and DNA are associated in a complex in which the PₚR molecule physically represses the expression of certain genes. The complex was thought to be dissociable by red light. Later this hypothesis was modified so that the initial mechanism of PₚR action was the derepression of genes resulting in the synthesis of gibberellins; these gibberellins then caused the
derepression of further genes and this resulted in the processes leading to unrolling (167). This hypothesis was supported by work which indicated that certain inhibitors of RNA and protein synthesis inhibited both the red light-induced synthesis of endogenous gibberellins (167A) and unrolling (167).

Poulson and Beevers (158,159,160) also implicate RNA and protein synthesis in the control of barley leaf unrolling. They suggest, however, that unrolling takes place in two stages. The leaf segments are postulated to contain a limited amount of precursor molecules whose functions are inhibited by endogenous inhibitors. This inhibition can be overcome by illumination, treatment by GA₃, and by leaching-out of the inhibitors during flotation of the segments on acetate buffer. The information contained in the precursor molecules is sufficient to sustain only limited unrolling of the leaf segments; during later stages of light-induced unrolling, RNA and protein synthesis take place, and the information necessary for complete unrolling to occur is provided (159).

The results of the experiments reported here do not provide supporting evidence for any of these theories on the mechanism and mode of phytochrome action in the unrolling of barley leaf segments.

The failure to induce unrolling, to the level attained in red-irradiated segments, by the application of exogenous GA₃ was repeated many times and seemed to be a perfectly valid result. This result, coupled with the difficulties encountered by Dr Reid (personal communication to D.J. Carr) in obtaining results with other varieties of barley similar to those he earlier obtained with Pallas (167), suggest that gibberellin-induced barley leaf unrolling may be affected by several complicated and unknown factors such as varietal differences; conditions of storage of the seed; age of the seed; and conditions during germination. It seems likely, therefore, that the barley leaf unrolling response to red light is not controlled simply by changes in the levels of endogenous gibberellins.
Further evidence which supports this hypothesis had been obtained by Reid (167,167A) in his original experiments. He found that infiltration of chloramphenicol into barley leaf segments had little effect on the red light-induced unrolling, but it did inhibit the increase in endogenous gibberellin activity after red-irradiation. This would suggest that the increase in endogenous gibberellins is not connected with the control of red light-induced unrolling of the leaf segments. Chloramphenicol is said to selectively inhibit protein synthesis in plant chloroplasts while having no effect on protein synthesis by cytoplasmic ribosomes (167A). Reid (167) also obtained evidence which suggested that much of the endogenous gibberellin activity in red-irradiated barley leaf segments was associated with the chloroplasts. It is likely, therefore, the red light-induced increase in endogenous gibberellin activity is concerned more with the processes leading to greening of the segments than with processes leading to unrolling. However, it must be noted that in these experiments, the growth retardants CCC and Amo 1618 inhibited both the increase in activity of endogenous gibberellins after red-irradiation, and the red light-induced unrolling (167,168).

The role of RNA and protein synthesis in the phytochrome control of barley leaf unrolling remains unclear. The results of the experiments reported here suggest that there is no bulk synthesis of either RNA or protein in the 6 h period after red-irradiation of the segments, but due to technical difficulties these results cannot be taken as conclusive. Poulson and Beevers' results (159) showing increased specific activity of cytoplasmic ribosomal RNAs and soluble RNA after 6 h of continuous illumination must be regarded as suspect for reasons already given. However, their results showing an increase in DNA-dependent soluble RNA polymerase activity and in the levels of polysomes after irradiation are valid. These results suggest the effect of illumination was to increase the synthesis of mRNAs; such an increase in the synthesis of specific RNA species would not necessarily be shown up by
the experiments reported here (150A, 150B). The criticism remains, however, that in Poulson and Beevers' experiments, the suggested increase in the synthesis of mRNAs after illumination might be connected with the process of greening rather than that of leaf unrolling. This conclusion is supported by the effects of chloramphenicol in Poulson and Beevers' experiments (159). Chloramphenicol, in low concentration, had little effect on unrolling, but did inhibit greening; it also inhibited generally the incorporation of radioactivity into RNA under continuous illumination. This inhibition of radioactive incorporation was similar to the effects on radioactive incorporation of other inhibitors of RNA and protein synthesis which did inhibit leaf unrolling.

At some stage during the processes initiated by irradiation of the leaf segments and resulting in the unrolling response, reference must be made to the genetic material in the leaf cells, since this will presumably be the only repository of the information necessary for the successful completion of unrolling. The point at issue here, is whether this reference to the genetic material, which according to the accepted dogma must involve RNA and protein synthesis, is connected with the phytochrome control of leaf unrolling, or whether it takes place after the control by phytochrome has been relinquished. The results of some of the experiments with inhibitors of RNA and protein synthesis suggest that syntheses of RNA and possibly protein which are essential to the expression of the leaf-unrolling response do take place before the escape reaction for far-red light reversibility has been completed; that is while the system is still under phytochrome control. These results suggest that this phytochrome control is mediated through RNA and protein synthesis, but do not conclusively prove that this is so. In order to provide this conclusive proof, it would be necessary to demonstrate that specific syntheses of RNA and protein do take place in red-irradiated segments which eventually unroll, whereas similar syntheses do not take place either in segments
maintained in the dark or in segments irradiated with red followed by far-red light, the segments receiving either of these treatments remaining rolled. This has not yet been demonstrated.

The discovery in the experiments reported here that maximal unrolling of leaf segments from two different varieties of barley, can be induced by application of exogenous kinetin, may be relevant to the question of the involvement of RNA and protein synthesis in the phytochrome control of leaf unrolling. It appears that exogenously-applied kinetin may substitute for red light in inducing the maximal unrolling in the leaf segments. There is also some evidence which indicates that there may be an increase in endogenous cytokinin activity after irradiation of the segments with red light (R. Menhenett, personal communication). It has been known for some time that cytokinins appear to control the levels of RNA and protein in various plant tissues (185) and there is some indication that they might exert this control through being constituents of various tRNA species (185). In detached barley leaves floated on kinetin solution (10 mg/1) in the dark, there was a significantly increased incorporation of Cl4-alanine into protein 6 h after detachment as compared with the incorporation in control leaves floated on distilled water (3). A similar result was found in experiments on the effect of kinetin on the incorporation of P32 into RNA, though first measurements were not taken until 48 h after detachment of the barley leaves (4).

It is possible, therefore, that phytochrome may exert its control of barley leaf unrolling by the stimulation by PFR of an increase in the activity of endogenous cytokinins. These cytokinins could then control the, so far undemonstrated, synthesis of specific RNAs and proteins involved in the processes leading to the unrolling response.
If Poulson and Beevers' concept (158) of inhibitors of the initial stage of unrolling, capable of being overcome by GA\textsubscript{3}, were incorporated into the hypothesis of cytokinin action in unrolling, the experimental results on the effects of exogenous GA\textsubscript{3} could be explained. The inhibitors would inhibit the action of endogenous cytokinins present in the segments in darkness, before the irradiation with red light. The inhibition would be overcome by exogenous GA\textsubscript{3}, leaving the endogenous cytokinins responsible for the minimal unrolling induced by the application of GA\textsubscript{3} (Fig. 20). (In Reid's barley leaf system (167) and that of Beevers et al. (7) using wheat leaves, the release from inhibition of the endogenous cytokinins would result in sub-maximal rather than minimal unrolling.) It is suggested that the inhibition would not be overcome by the red-light induced increase in endogenous gibberellin activity, which may not be connected with leaf unrolling at all, though in Reid's system (167) the inhibition of red light-induced unrolling by CCC and Amo 1618 makes the application of this hypothesis rather doubtful (Fig. 20). If the endogenous gibberellins really are synthesized in the chloroplast, as suggested above, their postulated failure to release the inhibition of endogenous cytokinins could be due to their isolation from the cytoplasm by the chloroplast membrane (191A). Alternatively, the gibberellins activated by red light might be of different types from GA\textsubscript{3} and they might be unable to act in a similar way to GA\textsubscript{3} in the cell.

In Poulson and Beevers' original concept (158), the inhibition of unrolling was to be reversible by application of red light. However, it is now postulated that treatment with red light does not overcome the inhibition of the endogenous cytokinins already present in the segments, either by the induction of an increase in the activity of endogenous gibberellins or by other means. If the effect of red light was to increase the activity of a cytokinin which was not affected by the postulated inhibitors, this would explain several experimental results. It could be suggested (Fig. 20), that complete maximal
FIG. 20. Hypothetical scheme for the control of barley leaf unrolling by phytochrome. The events connected by dashed arrows occur only in certain barley leaf unrolling systems.
unrolling required the active presence of both the cytokinins present in the segments in the dark, and the cytokinins which appeared in an active form after red-irradiation. If only the cytokinins present in the dark were active, minimal unrolling would occur; if only the red light-induced cytokinin were active, nearly, but not quite maximal unrolling would result. This hypothesis would explain Poulson and Beevers' (158) results in which an additive effect on unrolling of light and GA$_3$ treatment given together was found. Part of the variability in the responses to various light treatments found in the experiments reported here could be explained on the basis of differences between segments in the amount of the inhibitors leached out of the segments by the initial flotation on distilled water.

It should also be stated that expansion growth need take place in only a small percentage of the cells of the lower mesophyll of the leaf for the unrolling of the leaf segments to occur (33). The synthesis of cytokinins and of the specific RNAs and proteins which is suggested above need take place only in the cells which do undergo this expansion. Analyses of samples of whole leaf segments for changes in cytokinin, RNA and protein levels after various light treatments may therefore result in the swamping of the large changes in these levels in a relatively small number of cells by the inclusion of a large number of cells in which the changes do not occur.

XIV.3. The Mechanism of Phytochrome Action

The hypothetical scheme outline in Fig. 20 deals only with the mode of action of phytochrome in barley leaf unrolling. P$_{FR}$ is seen as stimulating an increase in the level of an endogenous cytokinin, but there is no
attempt to suggest how this might be done. None of the experiments performed on barley leaf unrolling have ever thrown any light on the mechanism of phytochrome action in this system. Barley leaf unrolling falls into the class of slow red, far-red light reversible responses, and this means that the processes leading from the initial conversion of $P_R$ to $P_{FR}$ in the cell to the eventually-displayed response must take a relatively long time to be completed. The suggestion that the rate of the escape reaction for far-red reversibility is relatively slow (reaction completed in 5-6 h) suggests that the presence of $P_{FR}$ in the cells may be required for a relatively long time for the unrolling response to occur. It is likely, therefore, that in this system, rapid changes in cellular permeability, as have been suggested to explain some of the fast red, far-red light reversible responses (66, 92, 110, 112, 199, 200), are unlikely to be involved in the initial action of $P_{FR}$ in the cell. Because of this apparent indication that the initial action of $P_{FR}$ is relatively slow, it is possible that an elaboration of experiments similar to those reported in this thesis performed, perhaps, on a suitable micro-scale, may eventually elucidate the mechanism of phytochrome action in barley leaf unrolling. It is likely, however, that this mechanism of action would not be universally applicable to all phytochrome-mediated responses because of the possibility (outlined in section VIII.1 above) that the intermediates formed during the $P_R$ to $P_{FR}$ transformation are involved in the mechanism of phytochrome action. It could be postulated that the reactions initiated by these intermediates would be different for each phytochrome-mediated response; these differences would then explain the wide range and the diversity of responses which are mediated by phytochrome.
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