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A STUDY OF PHYTOCHROME, ITS ISOLATION, STRUCTURE AND PHOTOCHEMICAL TRANSFORMATIONS

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CONTENTS

1. INTRODUCTION

1.1. EFFECT OF LIGHT ON PLANT DEVELOPMENT

As an introduction to this study of the structure of the plant pigment phytochrome and the mechanism of its photoreversibility, a brief account will be given of its discovery and its role in the regulation of physiological reactions.

The appearance of a plant is determined both genetically and physiologically. Genetic properties fix the basic characteristics of the plant, while its growth and development are influenced by physiological or environmental factors. Of the environmental factors, light is particularly important. Not only is light the energy source for photosynthesis, but it also stimulates morphogenesis. This morphogenetic action differs from photosynthesis in that, in most cases, the amount of light energy required is small. It is as though light triggers off reactions by which the metabolism of the plant is driven in a definite direction. The energy for the subsequent reactions, leading to the physiological effects, is ultimately derived from the photosynthetic process. Plant physiologists have known for a long time that the wavelength of the light was important in the induction of morphogenetic responses.

Photomorphogenetic reactions may be divided into several – at first sight unrelated – groups. The most important of these are the regulation of formative effects, flowering, seed germination, and the development of seedlings. The last group includes the effect of light on etiolated seedlings and excised parts of these such as stem and coleoptile segments.

The study of photomorphogenesis was greatly stimulated by the experiments of GARNER and ALLARD[1] in 1920 on the photoperiodic control of flowering. They were among the first to study the effect of the photoperiod quantitatively and observed that in some plants flowering is regulated by the relative duration of day and night in a 24 hour cycle. They called this effect 'photoperiodism'. Their work was followed up between 1945 and 1950 by a research group in Beltsville, USA, under the direction of H. A. BORTHWICK and S. B. HENDRICKS. These workers used a large spectrograph to study the effect of monochromatic light on flower induction and were able to compose action spectra for this photoperiodic effect in several plant species ([2,3]). These action spectra were all very similar, showing a pronounced maximum of activity in the red part of the spectrum between 600 and 700 nm. Speculations about the pigment responsible for light absorption focused attention on phycocyanin, the pigment in blue-green algae, a chromoprotein with similar absorption properties[4]. The work during this period has been reviewed by PARKER and BORTHWICK[5].

From 1948 onwards another approach was followed by E. C. WASSINK and coworkers at Wageningen University in The Netherlands. They studied the effect of light of restricted wavelength ranges on plant development[6]. Of particular relevance to further developments was their discovery that far-red light definitely influenced flowering and internode elongation in *Brassica*([7,8]). More-

over their results suggested that the effect of far-red light might be antagonistic to that of red light[7]. This work has been reviewed by WASSINK and STOLWIJK[9]

In 1935, FLINT and MCALISTER[10] had found that the light dependent germination of lettuce seed was promoted by red and inhibited by far-red irradiation. Maximum inhibition was observed in the region about 730 nm. These experiments were repeated by BORTHWICK et al.[11] in 1952. Using the spectrograph, they were able to measure the action spectrum for the induction of lettuce seed (var. Grand Rapids) germination. They confirmed, that the effect of a weak irradiation with far-red light between 700 and 750 nm counteracted that of red light. Moreover, by irradiating the seeds alternately with red and far-red light, they showed that the effect was photoreversible: the last irradiation always determined the overall effect, in this case either germination or no germination. Subsequently it was found that, under certain conditions, the induction of flowering was also red/far-red reversible[12, 13]. The same was found for certain other photoresponses in developing seedlings, for instance by DOWNS[14] for leaf and hypocotyl elongation in dark-grown bean seedlings and by WITHROW et al.[15] for the opening of the plumular hook of bean seedlings. The work of WITHROW's group deserves special mention, because they designed a series of interference-filter monochromator units with which more detailed action spectra could be determined. The spectra had maxima at about 660 and 730 nm. The results at this stage could be summarized as follows:

Many different responses are characterized by very similar action spectra.
The response to incident energy is logarithmic.
The threshold intensity for induction of the response is low.
The induction by red light can be reversed by far-red.

These observations led to the general conclusion that all these photomorphogenetic responses are controlled by the same mechanism and presumably involve the same photoreceptor pigment.

In addition to these low-energy photoresponses, there are others which are stimulated at a higher energy level. WASSINK and STOLWIJK[16] found that a photoperiod of high-energy monochromatic irradiation had an effect opposite to that of a photoperiod of white light followed by a short low-energy treatment with the same monochromatic light. SIEGELMAN and HENDRICKS[17] and MOHR[18] assumed at first that this so-called 'high-energy reaction', which showed maximum response in the blue and far-red regions, was due to other receptor pigments. MOHR[19] believed that it was due to the activation of a metal-flavoprotein enzyme by visible radiation. HENDRICKS and BORTHWICK[20], on the other hand, thought that the high-energy reaction was triggered off by the red/far-red pigment system. Recently, the work of HARTMANN[21] has suggested that at least those high energy reactions which show maximum response in the far-red region can be attributed to the red/far-red photoreversible pigment. This review, however, will be confined to low-energy reactions which are directly red/far-red reversible.

1.2. Kinetics of physiological responses induced by red and far-red light

The hypothesis that the physiological reactions, induced in plants by low-energy red and far-red light, were due to the presence of a photoreversible receptor pigment was at this stage gaining more and more support. Borthwick and Hendricks([20]) deduced values for the molecular absorption coefficient of the hypothetical pigment and gave it the name phytochrome. Its photoreversibility may be represented schematically as follows:

$$P_R \underset{\text{far-red light}}{\overset{\text{red light}}{\rightleftharpoons}} P_{FR}$$

where P_R and P_{FR} represent the two interconvertible forms of the pigment.

Further studies indicated that a relation could be established between the degree of conversion of the pigment with light and the corresponding physiological response and that the relation could be formulated in terms of reaction kinetics. Thus, quantum efficiencies could be determined for the interconversion of the two pigment forms in vivo with red or far-red irradiation, without knowledge of the chemical nature of the pigment and its concentration([22]). Conversion in either direction is initially first-order with respect to incident radiation energy([22, 23, 24]). For a reversible first-order reaction, the fraction (F) of pigment conversion corresponding to a given radiation energy (E) can be calculated from the following equation([25]):

$$d[P] / dE = -k[P_o](1-F)$$

Here, $[P_o]$ is the initial amount of one of the pigment forms in a unit cross section of the test object, and $d[P]/dE$ represents the change in the amount of this pigment form with respect to the incident energy, E. A similar first order differential equation expresses the reverse change, and the two equations can be resolved to give the degree of conversion that corresponds to a given physiological response.

1.3. Discovery of phytochrome and its detection by differential spectrophotometry

At this stage the objectives of isolating the pigment and of elucidating its mode of action seemed within reach. First, however, a method of assay had to be developed. The probability that the pigment was a protein made reintroduction of extracts into living plant tissue unpromising. An attempt was therefore made to design a method to detect the pigment spectrophotometrically([24]). A search for blue or blue-green albino or etiolated tissue, which would indicate high concentration of the pigment([26]) was, however, unsuccessful.

The existence of a pigment with photoreversible properties could be demonstrated in vivo after the development of a sensitive differential spectrophoto-

meter by BUTLER et al.[26] in 1959. With this instrument, OD differences could be recorded directly in scattering media such as etiolated plant parts. Subsequently, measurements were made in extracts of etiolated maize seedlings with a dual-wavelength spectrophotometer[27]. The latter worked on the same principle as the double-beam instrument designed by CHANCE for measurement of the kinetics of the enzymes peroxidase and cytochrome c[28]. This double-beam spectrophotometer automatically recorded differences in optical density (OD) between 660 and 730 nm. The amount of pigment can be estimated from the change in the OD difference after irradiation of the sample with red (660 nm) and far-red light (730 nm) alternately:

$$P_{total} = \Delta(\Delta OD) = [\Delta OD_{far-red} - \Delta OD_{red}]$$
$$\Delta OD_{red} = [OD_{660} - OD_{730}] \text{ after red radiation}$$
$$\Delta OD_{far-red} = [OD_{660} - OD_{730}] \text{ after far-red radiation}$$

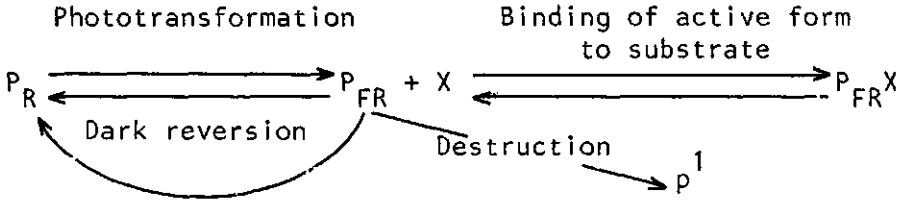
BUTLER's group determined the concentration of the pigment to be in the order of 10^{-6} M in etiolated material and probably about 10^{-7} M in normal green tissue[26, 29].

Etiolated seedlings have been used preferentially since they contain more phytochrome and much less chlorophyll than plants grown in the light. High levels of chlorophyll interfere with measurement of phytochrome, because the absorption spectra of the two pigments overlap between 640 and 700 nm. The small amount of protochlorophyll, present in etiolated seedlings, is transformed into chlorophyll by red irradiation. This causes an increase in the absorbance at 675–680 nm (the maximum of the chlorophyll band), while the simultaneous transformation of P_R into P_{FR} results in a decrease in absorbance at 660 nm. The net result in the spectrum of seedlings or extracts is that the decrease in optical density at 660 nm after red irradiation is lower than it would be if phytochrome were the only pigment in the extract. Since the protochlorophyll → chlorophyll transformation is irreversible, subsequent irradiation with far-red only transforms P_{FR} back into P_R. For this reason, preparations in which phytochrome is to be estimated are usually irradiated first with red light.

The assumption of BUTLER et al.[26] that phytochrome contains a protein moiety proved to be correct. Isolation of the pigment from dark grown maize shoots by methods of protein chemistry resulted in a purification factor of 6.0, according to the $\Delta(\Delta OD)$ values. The pigment was found in the 140,000 × *g* supernatant and could be precipitated with $(NH_4)_2SO_4$ at 33% saturation. It was retained upon dialysis against a buffer of pH 8.4 at 2 °C. Photoreversibility was maintained for a period of at least two weeks when solutions were stored at −15 °C, but was lost when the solutions were heated to 50 °C[26]. BUTLER's group developed a method for the isolation of the pigment from green leaves of several plants, including spinach[29] and demonstrated that the concentration of the pigment in etiolated seedlings was strongly reduced when they are placed in light.

1.4. Photochemical and non-photochemical transformations of phytochrome

Other experiments showed that the pigment can undergo non-photochemical changes both in vivo and in vitro. A spontaneous transformation of P_{FR} in the dark could be demonstrated in maize seedlings[29, 30] as well as with isolated phytochrome[31]. In contrast to the photochemical transformations, dark reversion of P_{FR} was found to be strongly temperature-dependent in vitro in the absence of oxygen. In the presence of oxygen on the other hand dark reversion is masked by another reaction resulting in loss of P_{FR} either by destruction or bleaching[31, 32] ('phytochrome decay'). These dark reactions are believed to be responsible for some of the physiological effects of phytochrome transformations. The reactions taking place in the phytochrome system in plants have been discussed by several authors. Hartmann[21] formulated them as follows:



A complication in the study of phytochrome is that it is impossible to convert more than 80 % of the pigment into the P_{FR} form with light of 660 nm[21, 32, 33], because the absorption spectra of P_R and P_{FR} overlap in the red region.

Destruction of phytochrome in the dark seems more pronounced in monocotyl seedlings than in dicotyl seedlings. This may be due to stimulation of resynthesis of P_{FR} by red light in dicotyls[34]. Although a form of phytochrome which was not subject to destruction was detected in the florets of cauliflower, the pigment could not be isolated from this material[35]. A close relationship was found between destruction of phytochrome and respiration in maize, the destruction being reduced by lowering the partial pressure of oxygen[36]. Measurements in primary leaves and internodes of dark-grown pea seedlings showed that, in the presence of oxygen, P_{FR} disappears in the dark without P_R being formed, while under anaerobic conditions P_R is formed immediately[37]. These results agree with those of the above-mentioned in vitro experiments[31]. Hillman et al.[38] showed that destruction of P_{FR} in *Avena* is inhibited by EDTA. It is worth mentioning that the term destruction in this context has been chosen on practical grounds and does not imply more than a loss in detectability as a result of the P_{FR} being bleached[39].

1.5. Relation between phytochrome concentration and physiological response

Work on the primary function of phytochrome, which was recently reviewed by Hillman[40], now centres on the problem of how to correlate the content,

state, and transformations of phytochrome with physiological responses. Experiments along these lines have been made possible by the application of differential spectrophotometry to measure differences in optical density in intact plant material. With a very sensitive instrument it was even possible to measure the phytochrome content of dry seeds([41]). As mentioned already, differential spectrophotometry has been applied preferentially to etiolated plant seedlings in which the phytochrome concentration can be measured easily. Responses to phytochrome transformation have been studied on physiological phenomena such as stem growth in *Pisum* (pea) and *Avena* (oat), phototropic curvature in *Zea* (maize), and germination of light-sensitive seeds. In vivo measurements suggest that phytochrome concentrations are highest in meristematic tissue, and that there is a correlation between phytochrome concentration and light sensitivity in different parts of *Zea* and *Avena* seedlings([42]). Often, however, no positive correlation is found. For instance, no phytochrome has yet been detected in the leaves of the classical photoperiodic plant, *Xanthium*.

The most straightforward experiments involve the establishment of various levels of P_{FR} in the seedlings or excised parts by irradiation either with pure red light or with mixtures of red and far-red. The tissues are then put back in the dark and the responses to these P_{FR} levels are allowed to develop and subsequently assayed. Such experiments demonstrated that this type of response depends only upon the relative amount of P_{FR} rather than upon the ratio P_{FR}/P_R, and is usually the same whether this level is attained by energy dosage (red light) or by adjustments to a photostationary state (mixtures of red and far-red). This proves that this physiological response is not energy-dependent([43, 44]). It was found that the response is roughly proportional to the logarithm of phytochrome conversion, up to a maximum near 50% P_{FR}. A different explanation is required for the high-energy reactions, which are energy dependent and are characterised by a more complicated dose-response relation([21]).

Several observations raise some doubt about the general validity of this picture for low-energy reactions. One is the '*Zea* paradox', discovered in 1966 by BRIGGS and CHON([45]). The phototropic curvature of coleoptile tips is sensitive to irradiation with red or far-red light, and the red sensitization is fully reversible with far-red. Curvature reaches a maximum, however, before any phytochrome transformation can be detected spectrophotometrically. Another anomaly, the '*Pisum* paradox', was discovered in 1965 by HILLMAN([43]). Inhibition of the elongation of stem segments, excised from material grown in the dark, was compared with that in segments from seedlings which had been pretreated with red light for 15 min to reduce their phytochrome content and then put back in the dark for 8 hours to ensure that the remaining phytochrome was in P_R from. Both dark-grown and pretreated segments were irradiated with steady-state mixtures of red and far-red light to give various degrees of conversion to P_{FR}, and stem elongation was recorded during a subsequent period of 20 hours. With the dark-grown samples, all levels of P_{FR} inhibited stem elongation, maximum inhibition being attained with 40 to 60% P_{FR}. In the pretreated

samples, absolute stem elongation was, as expected, lower. Most striking, however, was that steady-state irradiation establishing levels of up to 20% P_{FR} promoted elongation (relative to pretreated controls). It was as though at least 20% P_{FR} was present in the pretreated controls, although no P_{FR} could be detected spectrophotometrically. Here again, therefore, a P_{FR} response is found in the apparent absence of this pigment form.

Since the relatively high phytochrome concentration in etiolated seedlings is rapidly reduced to a low level (like that in green leaves) upon irradiation, these paradoxes suggest that the bulk of the phytochrome, spectrophotometrically detectable in etiolated seedlings, is inactive and that a minor non-detectable amount is physiologically active. In the case of the '*Zea* paradox', this active fraction should be far more sensitive to conversion with low-energy red light. This, however, is difficult to understand, since the quantum efficiency for phototransformation of the bulk phytochrome is itself very high. The '*Pisum* paradox' can be explained if it is assumed that the rate of reversion from P_{FR} to P_R is lower for the physiologically active phytochrome fraction. Another controversial situation was described in 1969 by KENDRICK, SPRUIT and FRANKLAND([46]). They measured phytochrome levels during the germination of *Amaranthus* seeds. During imbibition phytochrome appears in two phases, the first immediately after sowing and the second after about 8 hours. The second phase probably represents phytochrome synthesis. The seeds germinate both in the dark and in red but not in far-red and blue light. Phytochrome accumulation, on the other hand, takes place in the dark and in far-red but is inhibited by red and blue light. Here again, therefore, no relationship between phytochrome concentration and the physiological effect could be demonstrated.

1.6. PHYTOCHROME PROPERTIES AND BIOLOGICAL ACTIVITY

Speculations about the nature of the physiologically active form of phytochrome have stimulated the search for different phytochrome species. PURVES and BRIGGS([47]) believed that they had demonstrated the existence of kinetically separable pigment forms in the phytochrome extracted from oat seedlings. They determined dose-response curves for the phototransformation of partially purified phytochrome in both directions. When the logarithm of pigment conversion was plotted against the dose of red light, the result in both cases was a curve, which could be resolved ('peeled') into two straight lines. They assumed this to demonstrate the presence of two populations of phytochrome molecules, a fast- and a slow-transforming species. This turned out to be due to an instrumental error. They have recently shown that there was in fact a linear relationship between light dose and the logarithm of pigment conversion, which is what one would expect for first-order photochemical reactions. A non-linear relationship was also reported by CORRELL et al.([48]) for the dark reversion of phytochrome isolated from annual rye. Plots of the OD changes at 660 and 730 nm were comparable with those originally recorded by PURVES and BRIGGS, but are presumbly also incorrect since the same instrument was used. As it is, the absence of a

biological test for phytochrome activity prevents any distinction in physiological activity being made between possible fast and slow populations or between active and bulk species.

Different phytochrome species have also been reported in vivo in other biological material. SPRUIT[49] recorded a difference in absorption between the phytochrome in the leaves and that in the stems of etiolated pea seedlings. The absorption maximum of the pigment in the leaves was at 650 nm, as opposed to 665 nm for the stems. TAYLOR and BONNER[50] found a difference between the phytochrome species isolated from the alga *Mesotaenium* and from the liverwort *Sphaerocarpus.* Absorption maxima in the two organisms were at 649 nm (P_R), 713 nm (P_{FR}) and 655 nm (P_R), 720 nm (P_{FR}) respectively. In an extract from the moss *Mnium*, values of 658 nm (P_R) and 721 nm (P_{FR}) were measured[51].

1.7. SCOPE OF THE INVESTIGATION

The purpose of the foregoing literature survey was to highlight the important function of phytochrome as a regulator of photomorphogenetic reactions, and at the same time to underline how difficult it is to relate quantitatively the transformations of the pigment with those physiological effects in which its role seems undeniable.

Neither the experiments in vitro nor those in vivo have, so far, demonstrated conclusively the existence of different phytochrome species. In the experiments with isolated phytochrome, the reported effects may be caused by the presence of the pigment in several states of denaturation, while the position of the absorption maxima in vivo may be affected by light scattering in the samples or by association of the pigment with particular cell fractions. The absorption characteristics of the pigment depend to a great extent on the way the chromophore is associated to the protein moiety, and discrepancies in absorption might be attributable to the existence in plants and other organisms of phytochrome species differing in their protein structure.

Consequently, this study is primarily devoted to the molecular properties of the pigment. In order to have enough purified phytochrome available for the experiments, a reliable large-scale isolation procedure had to be developed. The experiments were designed to supply information about the interaction between the chromophore and the protein moiety of the pigment, especially in connection with its unique photoreversible properties. The following aspects were in fact studied:

The effect of denaturation on the photoreversibility of the pigment.

The changes in optical activity of the pigment during its phototransformations.

The phototransformations of the pigment at low temperature, in relation to subsequent dark reactions at normal temperature.

In these studies we have repeatedly made use of the algal pigment C-phycocyanin as a model substance for phytochrome, because it could be easily ob-

tained in relatively large quantities and since its chromophore is probably chemically related to that of phytochrome. The work was concluded with a study of methods for cleaving the phytochrome chromophore from the protein and a preliminary analysis of the molecular structure of the separated chromophore.

2. LARGE-SCALE ISOLATION AND PURIFICATION OF PHYTOCHROME FROM OAT SEEDLINGS

2.1. Introduction

Investigations into the mechanism of phytochrome photoreversibility have been hampered by the laborious and time-consuming methods of isolating the pigment([52, 53, 54]). The most serious problem with the current procedures is that the phytochrome readily loses activity during handling of large volumes of extract.

A batch adsorption method was therefore designed which enables the phytochrome in an extract of oat seedlings to be concentrated more rapidly. The method is suitable for amounts of starting material up to 25 kg. The adsorbent was calcium phosphate gel, which has also been used by others([52, 53, 54]) in the purification of phytochrome extracts by column chromatography.

The development of a suitable isolation method necessitated a study of the extraction efficiency and of the interaction between the calcium phosphate gel and phytochrome. Particular attention was focused on the method of grinding the seedlings, the quantity and the molarity of the extraction buffer, the adsorptive capacity of the gel, and the pH and ionic strength of both the phytochrome extract and the eluting buffer. The isolation method finally developed comprises grinding and extraction of the seedlings, separation of the extract from the cell debris, high speed centrifugation of the extract, adsorption on calcium phosphate gel, desorption, precipitation with $(NH_4)_2SO_4$, gel filtration on Sephadex G-50 followed by a second batch adsorption on calcium phosphate gel. Concentrates prepared in this way contained no coloured components other than phytochrome. Further purification was effected by chromatography on DEAE-Sephadex A-50 and gel filtration on Sephadex G-200.

2.2. Methods

2.2.1. *Cultivation and extraction of seedlings*

Oat seeds (variety Zonne II ex Svalöv, Sweden) were sown on cellulose padding in plastic trays covered with a sheet of perforated plastic. Seedlings were grown on tap water at an air temperature of 25 °C and a relative humidity of 85% in a growth room in the dark. After 6 days, the seedlings were harvested under a green safelight by cutting them off above the perforated covers with a slide knife. In this way 25 kilograms could be harvested within one hour.

In preliminary experiments, 25 g lots of seedlings were crushed with a pestle and mortar in 50 ml of 0.015 M Tris (pH 10.35). This yielded an extract with a pH of 7.8. Quantities of up to 10 kg of seedlings were extracted with a solution of 0.015 M Tris, 0.001 M EDTA and 0.1 M β-mercaptoethanol in a volume to weight ratio of 1:1, while 25 kg lots were extracted with a solution containing 0.15 M Tris, 0.01 EDTA and 1.0 M β-mercaptoethanol in a volume-to-weight ratio of 1:10. In the latter case the pH of the extract was about 7.0. Quantities of

10–25 kg of seedlings were ground in an electric stainless-steel mincer (Bizerba, Germany) within one hour after harvesting. The grinding and all subsequent isolation steps were performed in a cold room at 2–4°C under a green safelight. The safelight consisted of a Philips TL 40 W green fluorescent tube behind a 3 mm layer of Röhm and Haas plexiglass yellow no. 303.

Large volumes of extract could be successfully centrifuged with the Szent-Gyorgyi and Blum continuous-flow system on the Servall RC-2 centrifuge. The residence time of the extract in the rotor of the centrifuge was chosen as short as possible at a flow rate of about 60–80 ml/min. During centrifugation a yellow slimy precipitate of small cell fragments and mitochondria is removed from the extract.

2.2.2. *Concentration and desalting of extracts*

Large amounts of extracts were concentrated by batch adsorption on calcium phosphate gel (See 2.2.4 and 2.3.3). Initially, attempts were made to concentrate small volumes (10–100 ml) of phytochrome extract with Carbowax 20 M gel. A gel of 50% high molecular weight Carbowax 20 M in water was prepared. Visking cellulose tubings containing the phytochrome solutions were immersed in the viscous gel. Dehydration of the phytochrome solution was allowed to proceed until nearly all the liquid had been transferred to the gel. It is supposed that water as well as salt is withdrawn from the phytochrome solutions during this concentration procedure. The contents of a tubing were then dissolved in a volume of buffer suitable for subsequent chromatography. The resulting solution was centrifuged for 10 minutes at 10,000 rev/min to sediment undissolved denatured protein.

Apparently, the Carbowax contained impurities which diffused into the dialysis tubings causing phytochrome inactivation. Concentration of extracts was therefore attempted with Sephadex G-50 powder. The powder was added to the extracts and allowed to swell for 1 hour. The swollen gel particles were filtered off over a Büchner funnel with a filter-paper. One gram of dry gel removes about 10 ml of liquid. These methods were, however, not practicable to be used in the large scale isolation procedure.

Extracts could also be concentrated by precipitation with $(NH_4)_2SO_4$ at 50% saturation. This was achieved by adding to an extract an equal volume of saturated $(NH_4)_2SO_4$ solution, the pH of which had been adjusted before use from 6.5 to 8.0 with concentrated ammonia. When phytochrome was to be precipitated from purified fractions, the $(NH_4)_2SO_4$ was recrystallized from distilled water before use.

Crude extracts and redissolved $(NH_4)_2SO_4$ precipitates were desalted by filtering them over columns of Sephadex G-50. This procedure, originally developed for 6 × 40 cm columns, was scaled up for 10 × 50 cm columns, on which quantities up to 300 ml could be desalted. The columns were washed with a solution of 0.01 M Tris buffer (pH 7.8), 0.001 M EDTA, and 0.01 M β-mercaptoethanol. Optical density at 280 nm (OD_{280}) was automatically recorded with a Uvicord II photometer (LKB, Sweden).

2.2.3. *Determination of phytochrome by differential spectrophotometry*

Since there is as yet no biological assay for extracted phytochrome, its concentration could be estimated only from the specific absorption maxima at 660 and 730 nm. As mentioned earlier, phytochrome can exist in two forms, which are interconvertible with light of 660 or 730 nm:

$$P_R \underset{730\text{ nm}}{\overset{660\text{ nm}}{\rightleftharpoons}} P_{FR}$$

The phytochrome concentration can be calculated by measuring ΔOD(= OD_{660}-OD_{730}) after irradiation of the sample with red light, and again after irradiation with far-red light. The difference [$\Delta(\Delta OD)$] between these two readings, i.e. $(\Delta OD)_{far\text{-}red} - (\Delta OD)_{red}$, is a measure of phytochrome activity and is proportional to the concentration of phytochrome([52]). Differences in OD may be measured with a differential spectrophotometer. When phytochrome concentration is sufficiently high, however, the differences can be measured with a normal spectrophotometer.

A Leitz slide-projector (Pradovit N-24), in combination with I.L.-type interference filters from Schott Mainz, was used for the actinic irradiation of samples. The interconversion of P_R and P_{FR} is very fast when the light intensity is sufficiently high([26]). A Philips low-voltage projection lamp (No. 6289 C, 24V, 150W, 4250 Lumen) was used as a high intensity light source. Light from this lamp passes the condenser system of the slide-projector and is filtered through either the 660 nm or the 730 nm interference filter, which is placed in front of the cuvette. According to the manufacturer the filters had the following properties:

660 nm filter, I.L. No. 319118 λ_m = 665 nm
T_m = 38.5%
HW = 10.5 nm

730 nm filter, I.L. No. 184805 λ_m 725.5 nm
T_m = 26.5%
HW = 13 nm

λ_m = Wavelength for maximum transmission.
T_m = Maximum transmission at λ_m.
HW = Halfwidth at λ_m.

A housing for a standard cuvette holder was built in the open space between the light shutter and the tube of the slide-projector (Fig. 1). The projection lens itself was not used. If the interference filter/cuvette combination is placed immediately behind the light shutter, the cuvette is irradiated with equal light intensity over the whole surface. The cuvettes used for routine analyses had inner dimensions of 5 × 25 × 25 mm and normally contained 3 ml of solution. The cuvette was situated with its largest sides perpendicular to the direction of irradiation. The OD values of irradiated samples were measured in the Zeiss PMQ II spectrophotometer; in this case the cuvettes were turned so that the pathlength

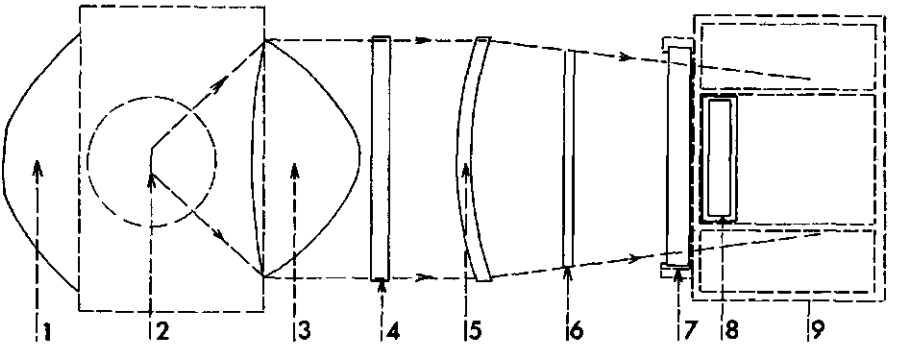


FIG. 1. Optical lay-out of the monochromatic interference-filter unit. 1. Reflector; 2. Lamp filament; 3. Condenser lens; 4. Heat filter; 5. Second condenser; 6. Light shutter; 7. Interference filter; 8. Cuvette; 9. Cuvette holder.

of light was 25 mm. The red-sensitive photocell of the spectrophotometer was used for the whole wavelength range between 600 and 800 nm. The slit-width in this range varied between 0.02 and 0.03 mm. The intensity of the measuring beam was so low that no photoconversion of phytochrome could be observed in the spectrophotometer during measurement. The samples were irradiated alternately with red and far-red light in the interference-filter unit. It was shown experimentally that irradiation times of 1 minute are normally sufficient for the interconversion reactions between P_R and P_{FR} to proceed to saturation. As mentioned earlier, conversion of protochlorophyll to chlorophyll is most rapid in the red region of the spectrum. As our phytochrome preparations contained varying amounts of protochlorophyll, their spectra might be expected to change during red irradiation. This is illustrated in Fig. 2, which represents schematically the changes in the absorption spectrum of an extract containing both phytochrome and protochlorophyll.

When the extract is first irradiated with red, protochlorophyll is converted to chlorophyll, while P_R changes to P_{FR}. The decrease in OD_{660} due to conversion of P_R is partially offset by an increase in OD_{660} due to conversion of protochlorophyll into chlorophyll. After a subsequent irradiation with far-red, OD_{660} is higher than in the non-irradiated extract. The phytochrome activity is therefore found from $[\Delta(\Delta OD)] = \Delta OD_{far\text{-}red} - \Delta OD_{red}$. On the other hand, one can determine the amount of P_{FR} initially present by first irradiating the sample with far-red light. This does not transform the protochlorophyll, but converts only the P_{FR} initially present in the extract into P_R. Concentration of P_{FR} in the extract is given by $\Delta OD_{far\text{-}red} - \Delta OD_{initial}$.

During the measurements, extracts often became cloudy as a result of protein denaturation, causing an increase of OD over the whole range between 600 and 800 nm (see Fig. 3), so that an effect of protochlorophyll conversion could not be shown in their spectra.

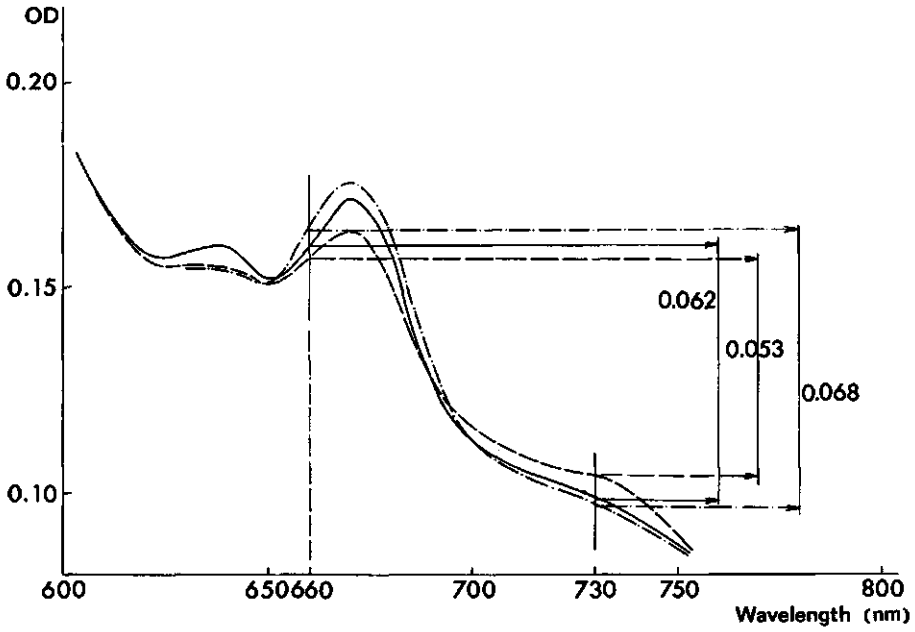


Fig. 2. Schematical representation of the effect of protochlorophyll transformation on phytochrome spectra. Spectrum of non – irradiated extract (———), spectrum after red irradiation (- - - -), spectrum after far-red irradiation (– . – . –). $\Delta OD_{initial} - \Delta OD_{red} = 0.009$, $\Delta OD_{far-red} - \Delta OD_{red} = 0.015 \equiv P_R + P_{FR}$, $\Delta OD_{far-red} - \Delta OD_{initial} = 0.006 \equiv P_{FR}$.

2.2.4. *Preparation of calcium phosphate gel*

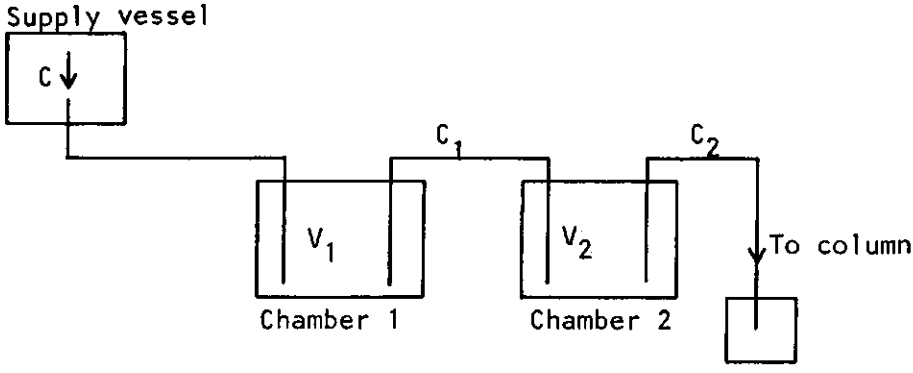
Calcium phosphate gel was used in its neutral hydroxylapatite form. The gel was prepared on pilot-plant scale by the method of TISELIUS, HJERTÉN and LEVIN[55]. Fifty litres each of 0.5 M $CaCl_2$ and 0.5 M Na_2HPO_4 were poured simultaneously into a plastic vessel, each at a rate of 60 ml/min. The slurry was stirred continuously at 30 rev/min. After the gel had settled, it was washed three times with 20 l of demineralized water and then transferred to a 75-litre steam-jacketed jam boiler. Here the gel was gently boiled for 1 hour in an alkaline solution (25 equiv. NaOH) to transform it from the brushite form to the basic hydroxylapatite form. It was neutralized by repeated washings with 120-litre portions of 0.01 M sodium phosphate buffer (pH 6.8). Each time the suspension was heated just to boiling and allowed to settle again, and the liquid was decanted. The procedure yielded 10 l of settled gel slurry which was diluted 1:1 v/v before use with 0.001 M sodium phosphate buffer (pH 6.8). Dry matter content of the diluted slurry was 120 mg/ml. The adsorption properties of the gel remained constant when stored at 2–4 °C.

2.2.5. *Purification of phytochrome concentrates by DEAE-Sephadex column chromatography*

From DEAE-Sephadex A-50, particle size 40–120 μ, the 75–120 μ fraction

was used for column packings, while the 40–75 μ fraction was used for batch adsorption. Particles of the large size-fraction were stirred for one hour in a 0.01 M H_3PO_4 solution. The swollen gel was filtered on a Büchner funnel and washed with 0.01 M potassium phosphate buffer (pH 7.8) until the pH of the filtrate was 7.8. In preliminary experiments it was shown that phytochrome can be adsorbed on the gel from a 0.01 M potassium phosphate buffer (pH 7.8) and can be eluted with the same buffer solutions containing in between 0.25 and 0.30 M NaCl. Columns (2.5 × 40 cm) were packed by hand from an evacuated slurry. Flow rates were between 50 and 100 ml/h. The columns were loaded with 2–10 ml of phytochrome concentrate and eluted with an NaCl gradient (0–0.5 M). The gradient system comprises a supply vessel containing 200 ml of 1.0 M NaCl in buffer, and two mixing chambers each containing 100 ml of buffer (0.01 M potassium phosphate, pH 6.6).

A flow sheet of the gradient system is given below:



$V_1 + V_2 = V$ = total volume of mixing chambers
dv = volume of liquid flowing through the system in time dt
$C_1°, C_2°$ = initial salt concentration in chambers 1 and 2
C_1, C_2 = actual salt concentration in chambers 1 and 2
C = salt concentration in supply vessel
Δ_1 = $C-C_1°$
Δ_2 = $C-C_2°$

The increase in NaCl molarity in the system can be calculated from equation (2) below. This equation is derived from the differential equation (1), relating molarity and flow in the system:

$$V\,dC_2 = (C_1-C_2)\,dv \quad (1)$$

$$C_2 = C-(\Delta_2 + \Delta_1\, v/V)e^{-v/V} \quad (2)$$

A theoretical curve for the salt concentration in the column eluate, calculated from equation (2), is given in Figure 13. The effluent was collected with a fraction collector (L.K.B., Sweden) fitted with a 3 ml siphon, and the protein concentration was determined by measuring the absorbance at 280 nm on a Uvicord II photometer (L.K.B., Sweden).

2.2.6. *Molecular weight estimation by gel filtration on Sephadex G-200*

The different types of Sephadex gel, namely G-75, G-100 and G-200, are each characterized by the range in the size of molecules which are retarded by internal diffusion into the gel particles. The upper limit of this range corresponds to molecules which are so large that they are fully excluded from the gel. Below this limit, the relationship between molecular weight and elution volume is determined by the fact that large molecules can diffuse less readily into the gel than smaller ones and therefore move faster through the gel. This effect can be expressed by the following formula which relates the elution volume V_e to the distribution coefficient K_d:

$$V_e = V_o + K_d V_i, \text{ where } K_d = V_e - V_o / V_i$$

V_o = the void volume

V_i = the volume of buffer bound by the particles.

The value of K_d for a given substance is determined by the size and shape of its molecules. It has been found that for a number of comparable proteins the ratio V_e/V_o is proportional to the logarithm of their molecular weight M:

$$M = C\, e^{Ve/Vo} \text{ or } V_e/V_o = 1/C_1 \log M$$

where C and C_1 are constants. This was found to hold for proteins in the molecular weight range 3,500–50,000 on Sephadex G-50 and 3,500–150,000 on Sephadex G-100([56]). Sephadex G-200 gel could separate proteins with molecular weights up to 225,000([57]), and has been used for the determination of the molecular weight of lactate dehydrogenase([58]). Gel filtration on Bio-Gel P-100 was used by MUMFORD and JENNER([53]) to estimate the molecular weight of phytochrome. They found a value of about 60,000. SIEGELMAN and FIRER([52]) determined a molecular weight range of 90,000–150,000 for phytochrome by ultracentrifugation of their purest fractions.

Sephadex G-200, particle size 40–120 μ, was sieved in two batches. The batch with particle size 75–120 μ was used for our experiments. The gel was allowed to swell for 8 hours at 80 °C in the same buffer as used for the experiments. The gel particles were then suspended in an amount of buffer sufficient to ensure that the resulting suspension was still sufficiently fluid to allow air bubbles to escape readily. The gel suspensions were evacuated with a water pump. The column was prepared by pouring the suspension of gel in buffer into a 2.5 × 40 cm column (Pharmacia, Sweden), already filled with buffer. The gel suspension was poured on to the top of the column from a vessel which was constantly stirred. The addition of gel was continued until the bed height was about 42 cm and the vessel was then replaced by a buffer reservoir. After 1 day the bed had settled to a constant height and the flow rate had become constant. A sample applicator (Pharmacia, Sweden) was placed on top of the gel and the packing was checked by passing 1.5 ml of blue dextran solution through it. All experiments were performed at 4 °C in the cold room. Columns were equilibrated with 0.1 M potassium phosphate buffer (pH 6.6) with a flow rate of about 40 ml/h. For analytical gel filtration, proteins of known molecular weight were dissolved in 1 ml of 0.1

M buffer, to which 0.5 ml of blue dextran solution (10 mg blue dextran in 0.6 ml glycerol and 0.4 ml buffer) was added, and the solution was applied to the top of the column. Glycerol was added to the protein solutions to enable strict comparison with the phytochrome solutions which contain 20% v/v glycerol. Phytochrome solutions were placed on the column without blue dextran, because a heavy phytochrome agglomerate is eluted together with the blue dextran peak (see 2.3.6). After the solution had sunk into the gel, the buffer reservoir and a drop-counting fraction collector (L.K.B., Sweden) were connected, and fractions of approximately 1.6 ml were collected. As soon as all the material placed on the column had been eluted, the column was ready for another run. Runs with proteins of known molecular weight and with phytochrome were performed on the same column. Concentrations of proteins and blue dextran in the eluates were estimated by measuring the absorbance at 280 nm on a Uvicord II. The phytochrome activity was determined as described in 2.2.3. After the number of fractions with activity had been counted on the recorded paper, the elution volume and the void volume were determined with a burette.

2.3. Results

2.3.1. *Influence of the extraction method on phytochrome activity*

In preliminary experiments, the oat seedlings were crushed in a precooled mortar and pestle with extraction buffer (0.1 M $Na_4P_2O_7.10\ H_2O$, 0.002 M EDTA, 0.05 M β-mercaptoethanol, pH 9.2) at a volume-to-weight ratio of 1:1. To begin with, fresh seedlings were compared with deep frozen seedlings. The results are summarized in Table 1.

More inactive protein is extracted from frozen seedlings than from fresh ones so that the specific activity in an extract of fresh seedlings is 2.5 times higher. It was therefore decided to start from fresh seedlings, despite the fact that frozen seedlings are easier to crush.

An absorption spectrum of an initial extract is shown in Figure 3. The only prominent peak in the spectrum is that of chlorophyll with a maximum at 675 nm. Phytochrome could hardly be detected in these initial extracts. A concentration procedure therefore had to be developed and evaluated.

Table 1. Influence of starting material on the phytochrome activity in the supernatant of the initial extract. Extracts centrifuged at 100,000 × *g*

Starting material	Phytochrome activity [Δ(ΔOD)/2.5 cm]	Protein conc. (mg/ml)	Specific activity [Δ(ΔOD)/cm.mg protein]
Deep-frozen seedlings	0.0075	3.7	0.0020
Fresh seedlings	0.0130	2.4	0.0054

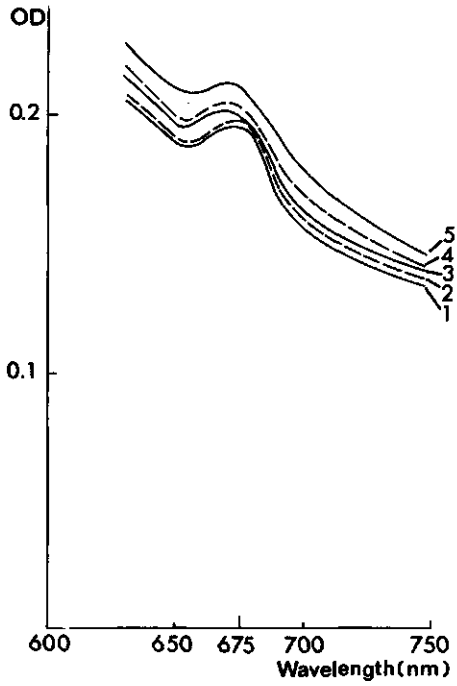


FIG. 3. Absorption spectra of a 100,000 × *g* supernatant of the initial extract. Radiation sequence: 1. non-irradiated 2. red (1 min) 3. far-red (2 min) 4. red (1 min) 5. far-red (2 min). Δ(ΔOD) for 1st sequence = 0.006; Δ(ΔOD) for 2nd sequence = 0.003.

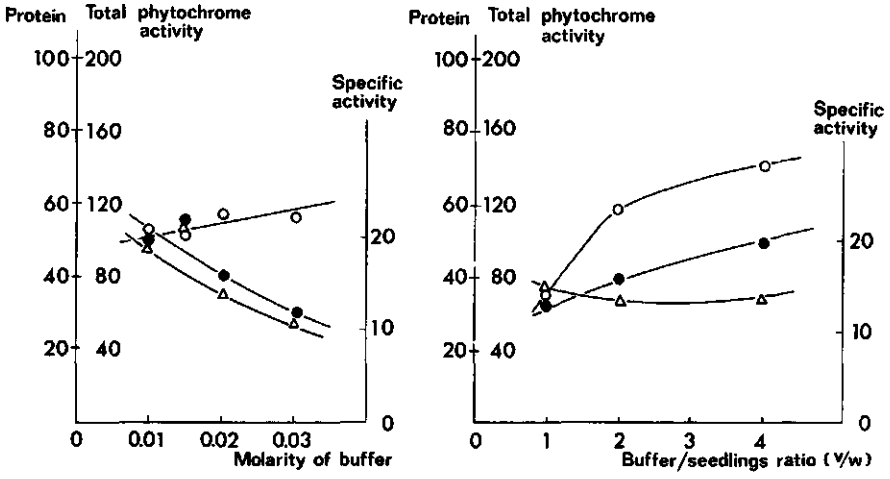


FIG. 4. Influence of the molarity of the extraction buffer on the efficiency of phytochrome extraction. Oat seedlings (25 g) were extracted with 50 ml Tris buffer (pH 8.0), the molarity of which was varied from 0.01 to 0.03 M. Directly after grinding the pulp was brought to pH 7.6 with 0.01 N NH_4OH and stirred for 1 hour. (left)

FIG. 5. Effect of the buffer/seedlings ratio (v/w) on the efficiency of phytochrome extraction. Oat seedlings (25 g) were extracted with increasing amounts of 0.015 M Tris buffer (pH 8.0) in volume to weight ratios of 1, 2 and 4:1. Total phytochrome activity, $10^3 \times$ [Δ(ΔOD)/cm]. volume (ml) (●—●), protein (mg) (○—○), specific activity, $10^3 \times$ Δ(ΔOD)/cm.mg protein (△—△). (right)

Firstly, the effect of varying the molarity of the extraction buffer (Tris) was studied. The amount of phytochrome extracted decreased with increasing molarity, while the protein concentration of the extract increased (Fig. 4). In other words, more selective extractions are obtained with buffers of low molarity. Tris buffer of molarity 0.01 and pH 8.0 had insufficient capacity to maintain the pH of the extract above 7.5. For practical purposes, therefore, we used 0.015 M Tris (pH 10.3) which was sufficient to give a final pH between 7.5 and 8.0 when the volume-to-weight ratio of buffer and seedlings was 1:1. When portions of seedlings were extracted with increasing amounts of buffer, it was found that the total amount of phytochrome extracted increased slightly (Fig. 5). The specific activity, however, did not increase.

2.3.2. *Concentration and desalting of extracts*

As mentioned in 2.2.2, the initial extracts could be concentrated with Carbowax 20 M, but phytochrome was inactivated. Batchwise concentration with Sephadex G-50 powder did not inactivate the phytochrome.

The pigment is reported to have a molecular weight of about 55,500([59]). Low molecular weight proteins in the extracts will, therefore, diffuse more readily than phytochrome into the Sephadex particles, and the phytochrome should be purified. When none of the phytochrome diffuses into the gel particles, the concentration factor based on the increase in phytochrome activity should be equal to that based on the decrease in volume. The concentration factors found for activity are in fact lower, because protein is lost in the void volume of the gel (see 2.2.6). To estimate how much protein is lost, a model experiment was performed with bovine serum albumin. The theoretical loss can be calculated from the known properties of the Sephadex G-50 and, for a fourfold concentration in two steps, comes out at 43.7%. In the model experiment, losses of 50.5 and 51.6% were found for fresh and regenerated powders respectively (see Table 2).

The differences between these losses and the theoretical loss of 43.7% (in fact the theoretical loss should be even lower since concentration was less than fourfold) are possibly due to the diffusion of some serum albumin into the gel particles themselves.

TABLE 2. Concentration of bovine serum albumin with Sephadex G-50

Fraction	Volume (ml)	Protein conc. (mg/ml)	Loss of total protein (%)	Concentration factors	
				Volume	Protein conc.
Solution 1*	100	2.95			
1st Concentrate	58	3.70		1.73	1.25
2nd Concentrate	34.5	4.00	50.5	2.90	1.36
Solution 2**	100	2.95			
1st Concentrate	52	3.95		1.93	1.34
2nd Concentrate	29.5	4.50	51.6	3.40	1.53

* Fresh Sephadex G-50 powder ** Powder used several times

TABLE 3. Concentration of phytochrome extracts with Sephadex G-50

Isolation No.	Fraction	Volume (ml)	Protein conc. (mg/ml)	Loss of total protein (%)	Activity [Δ(ΔOD)/ 2.5 cm]	Specific activity [Δ(ΔOD)/ cm. mg. protein]	Concentration factors			
							Volume	Protein conc.	Activity	Specific activity
18	Supernatant*	630	2.40		0.006	0.0025				
	Concentrate	160	3.45	63.5	0.0155	0.0045	3.31	1.44	2.58	1.80
19a	Supernatant	350	3.35		0.010	0.0030				
	Concentrate	76	4.10	74.2	0.030	0.0073	4.60	1.23	3.00	2.43
19b	Supernatant	350	3.05		0.010	0.0033				
	Concentrate	65	3.90	76.2	0.022	0.0056	5.40	1.28	2.20	1.70
20a	Supernatant	300	2.50		0.011	0.0044				
	Concentrate	90	3.15	62.3	0.016	0.0051	3.23	1.26	1.46	1.16
20b	Supernatant	300	2.50		0.014	0.0048				
	Concentrate	72	3.40	67.4	0.018	0.0053	4.17	1.36	1.28	1.07

* In all cases, the supernatant (100,000 × *g*) of the initial extract was concentrated

The results of the concentration of phytochrome extracts with Sephadex G-50 are given in Table 3.

The concentration factors show that the specific activity of phytochrome is increased with this method, while the loss of protein is even higher than in the model experiment. Both effects must be due to the presence of low molecular weight proteins in the phytochrome extracts, which diffuse into the gel particles.

The protein in the phytochrome concentrates is separated into two bands by gel filtration over Sephadex G-50. Gel filtration was performed in a solution of 0.01 M Tris-buffer (pH 7.8), 0.001 M EDTA, and 0.01 M β-mercaptoethanol. Phytochrome was present in the first band, which contains all the protein. The curves for OD_{280} values of gel filtrates from columns of 2.5 $\times$ 30 cm and 5.5 $\times$ 30 cm are given in Figures 6 and 7. For those fractions in which it was measured, the phytochrome concentration is represented by the shaded blocks in the Figures. Most of the phytochrome was normally present in the first half of the protein band. In some isolations the protein band was divided into a first and second half (I and II) (see Table 4) and the purification was continued with

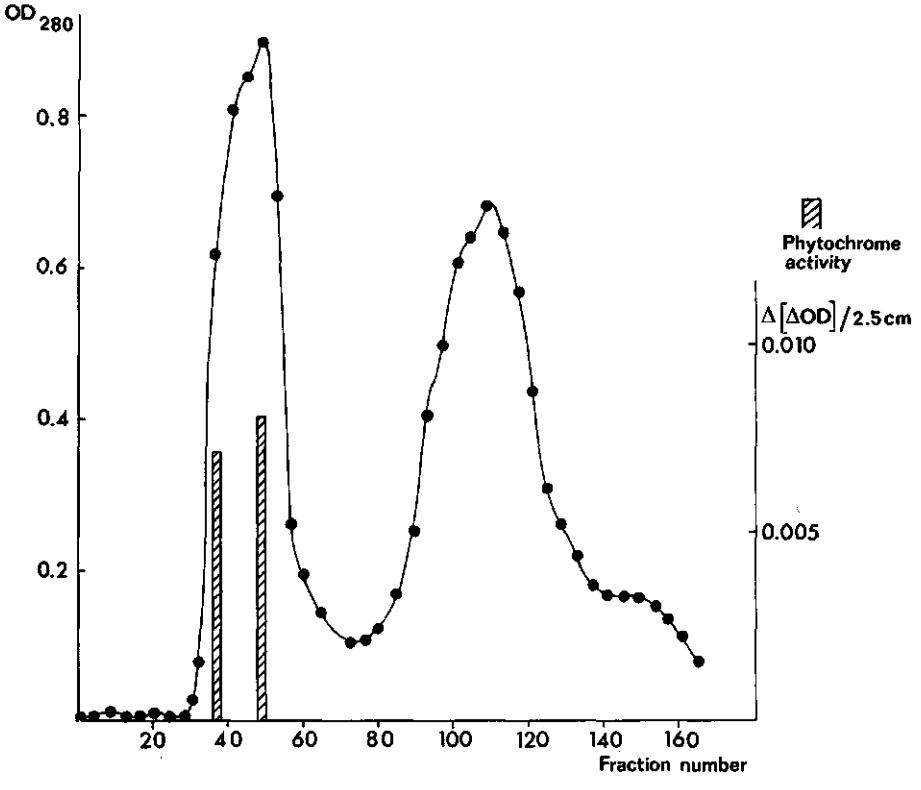


FIG. 6. Gel filtration of concentrated phytochrome extract over Sephadex G-50 (isolation 12; column 2.5 $\times$ 30 cm), OD_{280} (•—•).

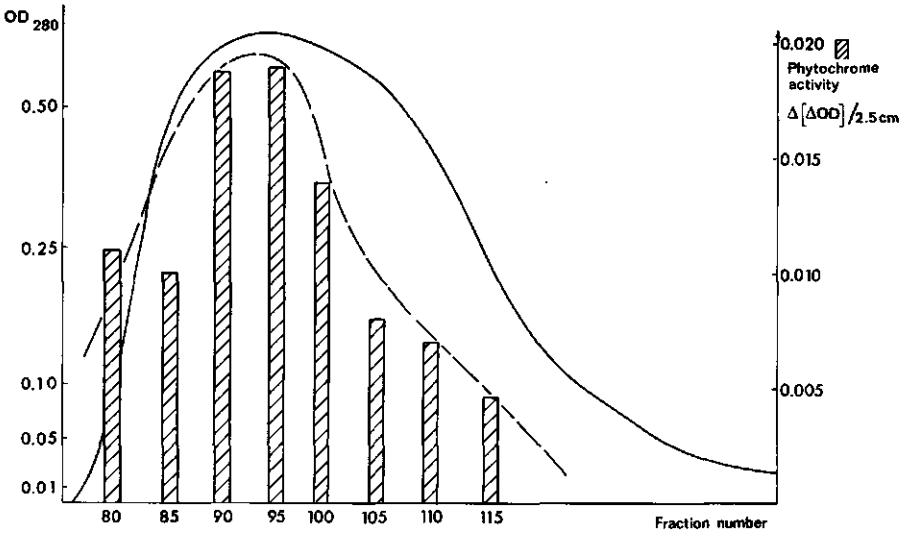


FIG. 7. Gel filtration of concentrated phytochrome extract over Sephadex G-50 (isolation 20; column 5.5 × 30 cm, first band); recorded OD_{280} (———), measured activity of fractions (- - - - -).

TABLE 4. Degree of purification of phytochrome after various concentration steps (Isolation 18)

Fraction		Volume (ml)	Protein conc. (mg/ml)	Phytochrome activity [Δ(ΔOD)/2.5 cm]	Recovery of total activity (%)	Specific activity [Δ(ΔOD)/cm. mg protein]	Purification factor
Supernatant (100,000 × *g*)		630	2.40	0.006	(100)	0.0025	(1.0)
Concentrate*		160	3.45	0.0155	61.9	0.0042	1.8
Gel filtration							
	I	60	1.70	0.009		0.0053	2.1
	II	74	1.25	0.011		0.0088	3.5
	I + II	134			35.8		
Concentrate*	I	17	3.20	0.0245		0.0077	3.1
Concentrate*	II	20.5	2.40	0.016		0.0067	2.7
	I + II	37.5			19.7		
Precipitation with $(NH_4)_2SO_4$	I	6	1.80	0.033		0.0183	7.3
	II	6	1.80	0.0165		0.0092	3.7
	I + II	12			7.8		

* Concentration with Sephadex G-50 powder

these separate fractions. Separation on the 2.5 × 30 cm column was much sharper than on the 5.5 × 30 cm one.

The result of the gel filtrations of one isolation, No. 18, and of the subsequent concentration of the active fraction with $(NH_4)_2SO_4$, is presented in Table 4. During gel filtration, no volume concentration was achieved. The loss of phytochrome during several gel filtrations depends on the sharpness of the eluted protein band. Purification was continued only with fractions of high activity. Some degree of purification is obtained by gel filtration. The most important result of

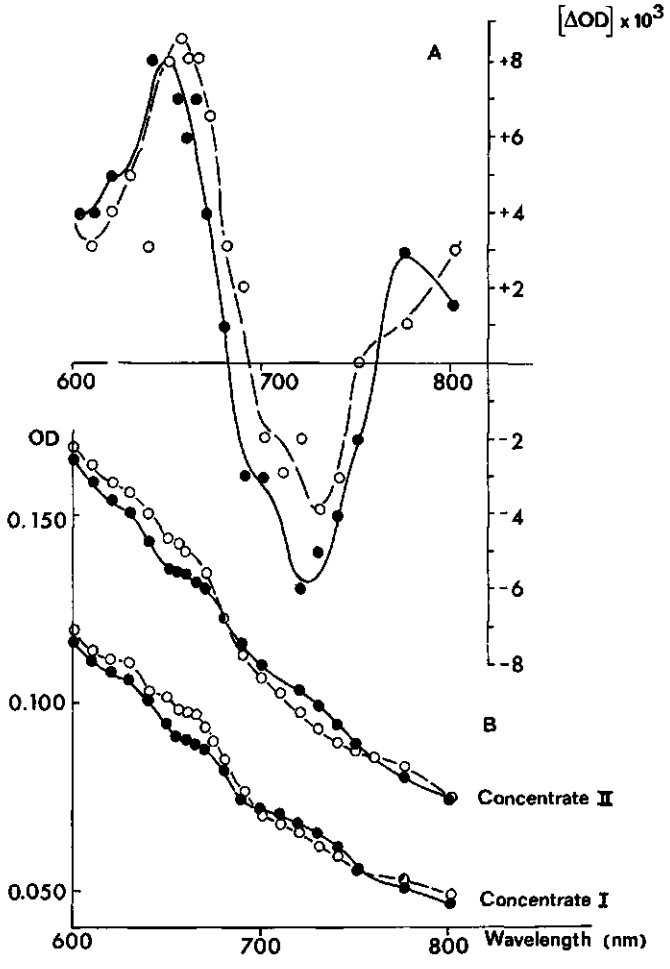


FIG. 8. Absorption and difference spectra of phytochrome concentrates I and II (isolation 18, table 4). A. Difference spectrum between far-red and red irradiated concentrate I (○—○) and concentrate II (●——●). B. Absorption spectra after red irradiation (●——●) and far-red irradiation (○—○).

this step, however, is the removal of low molecular weight components (peptides and pigments), which are found in the second band. Purification is accompanied by a change in the buffer from high to low ionic strength (0.1 M potassium phosphate to 0.01 M Tris).

Phytochrome could be detected in the concentrated extracts by difference spectrophotometry. The absorption spectra of concentrates I and II of isolation 18 (see Table 4) and the difference spectra deduced from them are shown in Fig. 8.

2.3.3. *Batch adsorption of phytochrome on calcium phosphate gel*

Because the concentration techniques previously described in the literature were not suitable for large volumes of extracts, a method was designed which involved the adsorption of phytochrome on calcium phosphate gel. Preliminary experiments demonstrated that the calcium phosphate gel should have a stable configuration in order to ensure a constant adsorptive capacity. The brushite form is not stable and, moreover, phytochrome was adsorbed too strongly on brushite. We therefore used the neutral hydroxylapatite form. The adsorptive capacity of the hydroxylapatite gel was greater for bulky gels with a low dry weight value. Slow formation of the gel gives rise to large gel particles, although these tend to be partially broken down by mechanical shear during boiling. The gels should therefore be boiled very gently. The preparation method adopted yielded a gel with a dry weight of 95 mg/ml when tried out on a small scale.

The adsorptive capacity of the gel was determined by adding increasing amounts of gel to a phytochrome solution. The results are shown in Figure 9. Almost complete adsorption of phytochrome was achieved at a gel concentration of 7% v/v. For gels with higher dry weights (120 mg/ml), as much as 20% v/v had to be added. The effect of pH on the adsorptive capacity of the gel was studied at pH 6, 7 and 8. It was confirmed that calcium phosphate gels adsorb more protein at low pH, as is shown in Figure 10. Adsorption of phytochrome is most selective at pH 6. Since the pigment is unstable below this pH, the extracts were acidified to pH 6.2 with 1 N HCl after addition of the gel in the large scale preparations. The effect of pH on the elution of phytochrome from the gel is illustrated in Fig. 11. It was found that desorption increased with increasing. pH. The specific activity of the eluate showed little variation. Because phytochrome is unstable above pH 8.0, pH 7.8 was chosen as the most suitable compromise (see 3.2.1). Finally, by varying the molarity of the potassium phosphate elution buffer (pH 7.8) between 0.1 and 0.4 it could be shown that, within this range, ionic strength has no great influence on the elution of phytochrome from the gel.

2.3.4. *Yields of large-scale phytochrome isolations*

These experiments led to the design of a large-scale isolation method, which is summarized step by step in Table 5.

Although more phytochrome is extracted from the seedlings with dilute buffer (Fig. 4), we used a concentrated buffer in the large-scale extraction, since the

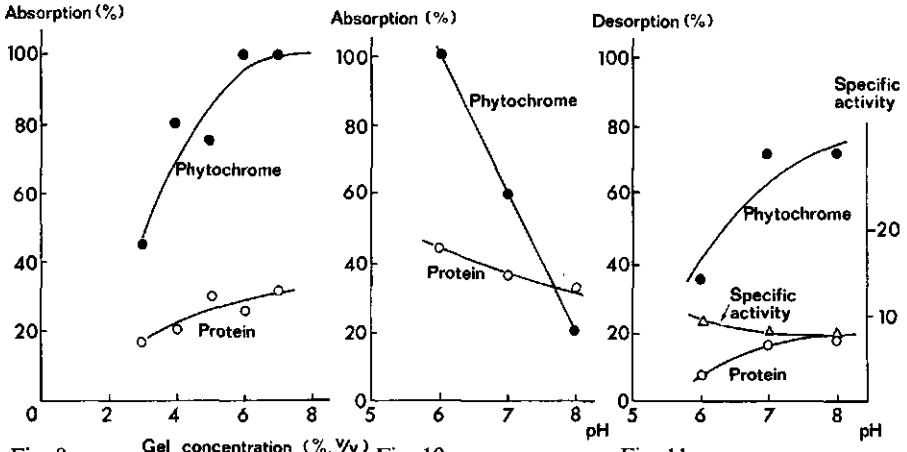


Fig. 9 Fig. 10 Fig. 11

FIG. 9. Effect of the amount of calcium phosphate gel on the adsorption of phytochrome. Increasing amounts (3–7%, v/v) of gel with a dry weight of 95 mg/ml were added to 20 ml portions of phytochrome extract (activity = 0.002 Δ(ΔOD)/cm).

FIG. 10. Effect of pH on the adsorption of phytochrome. Gel (6%, v/v) was added to 20 ml phytochrome extract, adjusted to pH 6, 7, or 8.

FIG. 11. Effect of pH on the desorption of phytochrome. Gel (6%, v/v) was added to 20 ml phytochrome extract at pH 6.2. Gels were sedimented by centrifuging and eluted with 5 ml of 0.1 M potassium phosphate buffers at pH 6, 7, or 8. Specific activity $10^3 \times \Delta(\Delta OD)/cm.mg$ protein (△—△).

TABLE 5. Procedure for large-scale extraction and purification of phytochrome from oat seedlings

Operation	Method	Resulting fractions
GRINDING	25 kg oat seedlings are ground with 2.5 l of a solution of 0.15 M Tris (pH 10.3), 0.01 M EDTA and 1.0 M β-mercaptoethanol in a stainless steel mincer.	
EXTRACTION	The slurry is stirred for 1 hour. Cell debris is separated by centrifugation at 1400 rev/min in a spin dryer fitted with a cloth filter.	
CENTRIFUGING	17.5 l of extract is centrifuged in the continuous flow system of the Servall RC-2 at 30,000 × *g* with a flow rate of 120 ml/min.	——→ *Centrifuged extract I*
CALCIUM PHOSPHATE ADSORPTION	To the extract (pH 7.0) 0.2 vol. of calcium phosphate gel is added and the pH is adjusted to 6.2 with 1 N HCl. The suspension is stirred for 30 min and centrifuged in the spin dryer at 1400 rev/min.	

Table continued p. 26

Table 5 (continued).

Operation	Method	Resulting fractions
DESORPTION	The calcium phosphate cake is resuspended in 1.5 vol. of buffer (0.1 M potassium phosphate pH 7.8, 0.001 M EDTA, 0.02 M β-mercaptoethanol) and stirred for 30 min. The extracted gel is sedimented by centrifugation for 10 min at 14,600 $\times$ g in the Servall RC-2. Desorption is repeated with 1 l of buffer and the eluates are combined.	——→ *Combined eluates II*
PRECIPITATION WITH $(NH_4)_2SO_4$	To the combined eluates an equal volume of saturated $(NH_4)_2SO_4$ solution (pH 7.3) is carefully added and the suspension is stirred for 30 min and allowed to settle for 1 hour. The settled slurry is centrifuged for 10 min at 14,600 $\times$ g. The sediment is redissolved in 300 ml of 0.1 M potassium phosphate buffer, pH 7.8.	——→ *$(NH_4)_2SO_4$ concentrate III*
SEPHADEX G-50 GEL FILTRATION	The $(NH_4)_2SO_4$ concentrate is filtered on a 50 $\times$ 10 cm column and eluted with three times its volume of buffer (0.01 M Tris, pH 7.8, 0.001 M EDTA, 0.01 M β-mercaptoethanol).	——→ *Eluate IV*
2ND CALCIUM PHOSPHATE ADSORPTION	900 ml of eluate are treated with 0.2 volume of calcium phosphate gel in the same way as described above. The gel is eluted successively with 125 and 50 ml of 0.1 M potassium phosphate buffer, pH 7.8.	——→ *Eluate A V* *Eluate B VI*
STORAGE	Storage at –20 °C is possible at this stage if 0.2 volume of glycerol is added.	
PRECIPITATION WITH $(NH_4)_2SO_4$	To each of the eluates an equal volume of $(NH_4)_2SO_4$ is added. The sediment is redissolved in a minimal amount of 0.1 M potassium phosphate buffer (pH 6.6) containing 0.2 volume of glycerol.	——→ *$(NH_4)_2SO_4$ concentrate VII*
DEAE-SEPHADEX CHROMATOGRAPHY	10–20 ml concentrate is chromatographed on a 2.5 $\times$ 40 cm column with a 0–0.5 M NaCl gradient in 0.01 M potassium phosphate buffer, pH 6.6.	——→ *1st peak fractions VIII* *2nd peak fractions IX*
PRECIPITATION WITH $(NH_4)_2SO_4$	Phytochrome in each peak fraction is precipitated by addition of $(NH_4)_2SO_4$ and redissolved as before	——→ *1st peak fractions X* *2nd peak fractions XI*

TABLE 6. Influence of the amount of extraction buffer on the yield of phytochrome

Weight of seedlings (kg)	Extraction buffer/seedlings weight ratio (v/w)	Total volume of extract (l)	Total phytochrome activity [Δ(ΔOD)/cm]. volume (ml)	Yield of phytochrome (Activity per kg seedlings)	Specific actitivty [Δ(ΔOD)/cm. mg protein]
25	2	65	160	6.4	0.0018
25	1	40	125	5.0	0.0018
25	0.1	17.5	130	5.3	0.0022

TABLE 7. Recovery and purification of phytochrome throughout the various stages of the large-scale isolation procedure*

Operation	Fraction	Volume (ml)	Protein conc. (mg/ml)	Phytochrome activity [Δ(ΔOD)/cm]	Specific activity [Δ(ΔOD)/cm.mg protein]	Recovery of total activity (%)	Purification factor
Centrifuging	I	17,000	3.3	0.006	0.002	(100)	(1)
Calcium phosphate adsorption	II	3.100	4.1	0.021	0.005	63.1	2.5
Precipitation with $(NH_4)_2SO_4$	III	280	13.0	0.190	0.015	52.2	7.5
Sephadex G-50 gel filtration	IV	1,050	3.65	0.042	0.012	43.1	6
2nd Calcium phosphate adsorption	V	114	7.6	0.180	0.024	20.1	12
	VI	121	5.8	0.117	0.020	13.9	10
Calcium phosphate adsorption**	V	105	7.6	0.180	0.024	100	1 (12)***
Precipitation with $(NH_4)_2SO_4$	VII	20	25.7	0.760	0.030	80.4 (27.3)	1.2 (15)
DEAE-Sephadex chromatography	VIII	105	0.6	0.044	0.072		3 (36)
	IX	102	1.4	0.026	0.019	38.5 (13.1)	0.8 (9.5)
Precipitation with $(NH_4)_2SO_4$	X	5	10.7	1.090	0.102		4.2 (51)
	XI	6	25.8	0.680	0.026	50.4 (17.1)	1.1 (13)

* The names of the fractions correspond with those in the last column of Table 5. The results are those of a representative run in which 26 kg of seedlings were extracted with 2.5 l of extraction buffer. Protein was determined by LOWRY's method after precipitation with trichloroacetic acid

** Of the first eluate from the second calcium phosphate adsorption, 105 ml were taken for the remaining purification stages

*** Values between brackets are based on the total activity and specific activity of the initial extract

handling of large volumes of extract constitutes a serious drawback in the isolation procedure. Another advantage of using a more concentrated extraction buffer is that the specific activity of phytochrome in the extract is higher. With 0.15 M Tris solution, a pH of about 7.0 is reached in the inital extract. The phytochrome yields with different amounts of extraction buffer are compared in Table 6.

The specific activity of the initial extract is strongly dependent on the way in which the seedlings are ground. In general, favourable results were obtained by gently crushing them with a pestle and mortar in order to extract as little bulk protein as possible. The slurry produced with the Bizerba mincer is rather coarse which explains why this instrument could be used successfully in large-scale extractions.

Batch adsorption was only successful with a clear extract. High speed centrifugation was, therefore, an essential step. The complete procedure (Table 5) could be performed by two workers in $3\frac{1}{2}$ days. Specific activity, recovery and degree of purification of phytochrome after each successive step are summarized in Table 7. After two batch adsorptions on calcium phosphate, the purification factor was 10–12, recovery of activity was 34%, while the volume of the phytochrome extract had been reduced to about one hundredth of its initial value. Subsequent purification by chromatography on DEAE-Sephadex A-50 gave a main fraction with a specific activity of 0.102, i.e. about 50 times purer than the initial extract. Total recovery after this step was 17%. The absorption and difference spectra of the phytochrome solution after the second batch adsorption on calcium phosphate gel (Fig. 12) show that, even at this stage, a marked purification had been achieved.

2.3.5. *Chromatography on DEAE-Sephadex A-50*

Columns (2.5 × 40 cm) were prepared and packed as described in 2.2.5. Phytochrome extracts which had been purified by two batch adsorptions on calcium phosphate gel and precipitation with $(NH_4)_2SO_4$ (fraction VII, Table 5) were chromatographed. After gradient elution, fractions with phytochrome activity were pooled and again precipitated with $(NH_4)_2SO_4$. Sediments were dissolved and concentrates stored with 20% v/v glycerol in the deep-freeze.

A typical elution curve (Fig. 13) shows that phytochrome activity is divided into two peaks. In this case phytochrome activity is measured as OD_{660}, which is not fully equivalent to activity expressed as $\Delta(\Delta OD)$. The protein elution curve is characterized by a sharp peak and a second broader peak with a pronounced shoulder. The first-peak fractions have high phytochrome activity, and the same holds for fractions corresponding to the shoulder of the second peak, although their specific activity is lower. The active fractions are eluted at NaCl molarities of 0.2–0.3 (first peak) and 0.45–0.6 (shoulder of second peak). The existence of two discrete phytochrome peaks might be due to a difference between the behaviour of P_R and P_{FR} on the column. It was found, however, that both peak fractions contained mainly P_R and only little P_{FR}. The absorption spectra of concentrates of the two peak fractions, and their specific activities are given in Figu-

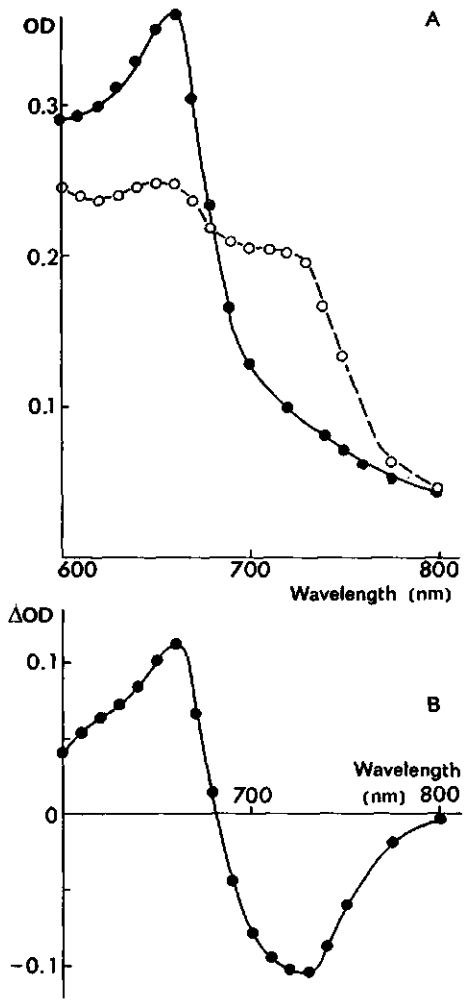


FIG. 12. A. Absorption spectra of phytochrome extract after adsorption on calcium phosphate gel; after irradiation with far-red (●—●), after irradiation with red light (○—○). B. Corresponding difference spectrum (ΔOD).

re 14. As can beseen, there is no essential difference between the two absorption spectra. That the specific activity of the first-peak fraction is highest shows up most of all in the 372 nm absorption band. The lower the level of protein impurities, the sharper this band becomes. One can readily demonstrate that the 372 nm band is due to phytochrome: it shifts to 392 nm when the pigment is converted to the P_{FR} form with red light. Figure 15 shows the ultraviolet part of the absorption spectrum of the phytochrome solution, purified up to and including the DEAE-Sephadex chromatography stage. The absorption maximum at 285 nm shows that the pigment is a protein. Recovery of activity for the chromatography step is usually about 60 % and may be as high as 85 %. The purification

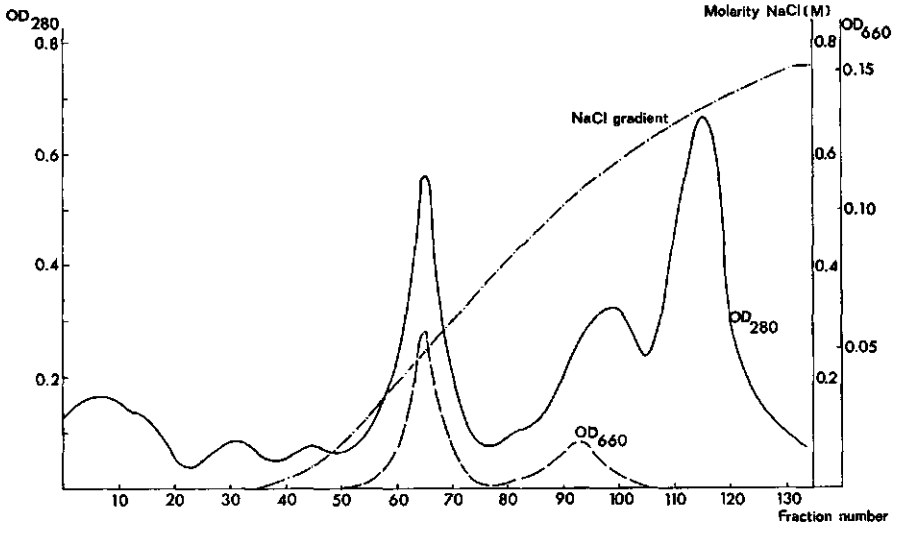


FIG. 13. Chromatography of phytochrome concentrate on DEAE-Sephadex A-50 (isolation 37).

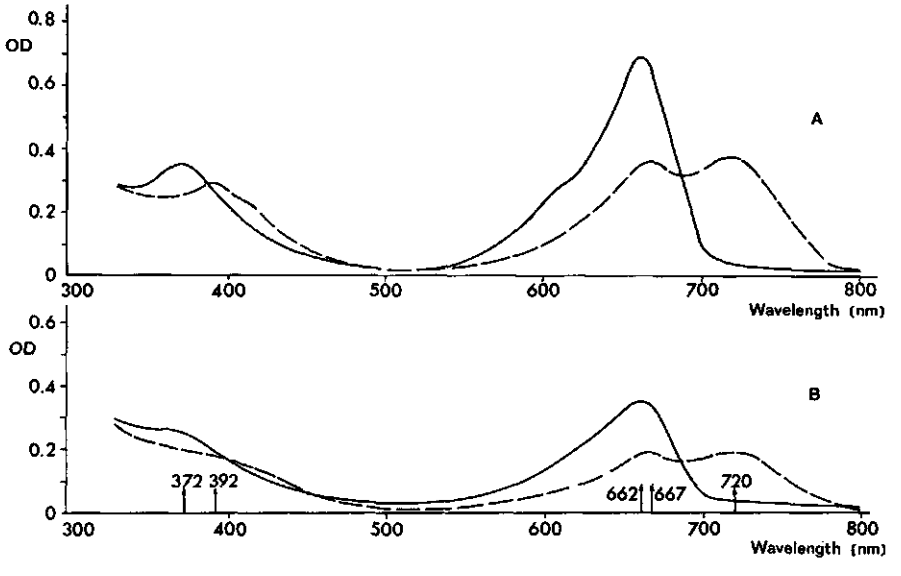


FIG. 14. Absorption spectra of phytochrome fractions after DEAE-Sephadex chromatography (isolation 37). P_R (———), P_{FR} (- - - -). A. 1st peak fraction; protein concentration 3.3 mg/ml, specific activity, 0.240 Δ(ΔOD)/cm.mg protein. B. 2nd peak fraction; protein concentration 3.7 mg/ml, specific activity 0.109 Δ(ΔOD)/cm.mg protein.

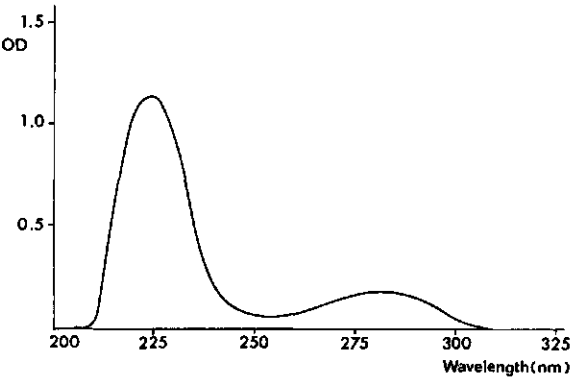


FIG. 15. Ultraviolet absorption spectrum of the 1st peak fraction after DEAE-Sephadex chromatography (isolation 37). Protein concentration 1.46 mg/ml, specific activity, 0.205 Δ(ΔOD)/cm. mg protein. Spectrum measured in 1:10 dilution.

factor achieved for the first-peak fraction is between 5 and 10. The results of the experiment are shown in Figure 13 and summarized in Table 8.

In the next experiment, first-peak fractions were rechromatographed to see whether they behaved as stable protein fractions. For this purpose phytochrome solutions of about 100 ml were concentrated, before chromatography, by precipitation with an equal volume of 100% saturated $(NH_4)_2SO_4$ solution (pH 7.25).

TABLE 8. Total activity and purity of phytochrome fractions after DEAE-Sephadex chromatography (Isolation 37)

Fractions	Volume (ml)	Protein conc. (mg/ml)	Phytochrome activity [Δ(ΔOD)/cm]	Recovery of activity (%)	Specific activity [Δ(ΔOD)/cm.mg protein]	Purification factor	Recovery per step (%)	
							Protein	Activity
Concentrate	41	4.96	0.116	(100)	0.023	(1.0)		
Thawed concentrate* (After storage)	60	2.36	0.078	97.7	0.033		69.6	97.7
Precipitation with $(NH_4)_2SO_4$	5.7		0.700					
DEAE-Sephadex chromatography								
1st peak fraction	48	0.16	0.040		0.250	10.6		
2nd peak fraction	65.5	0.10	0.016		0.164	7.0		
Total				62.7			10.1	64.0

* Contains 30% v/v glycerol

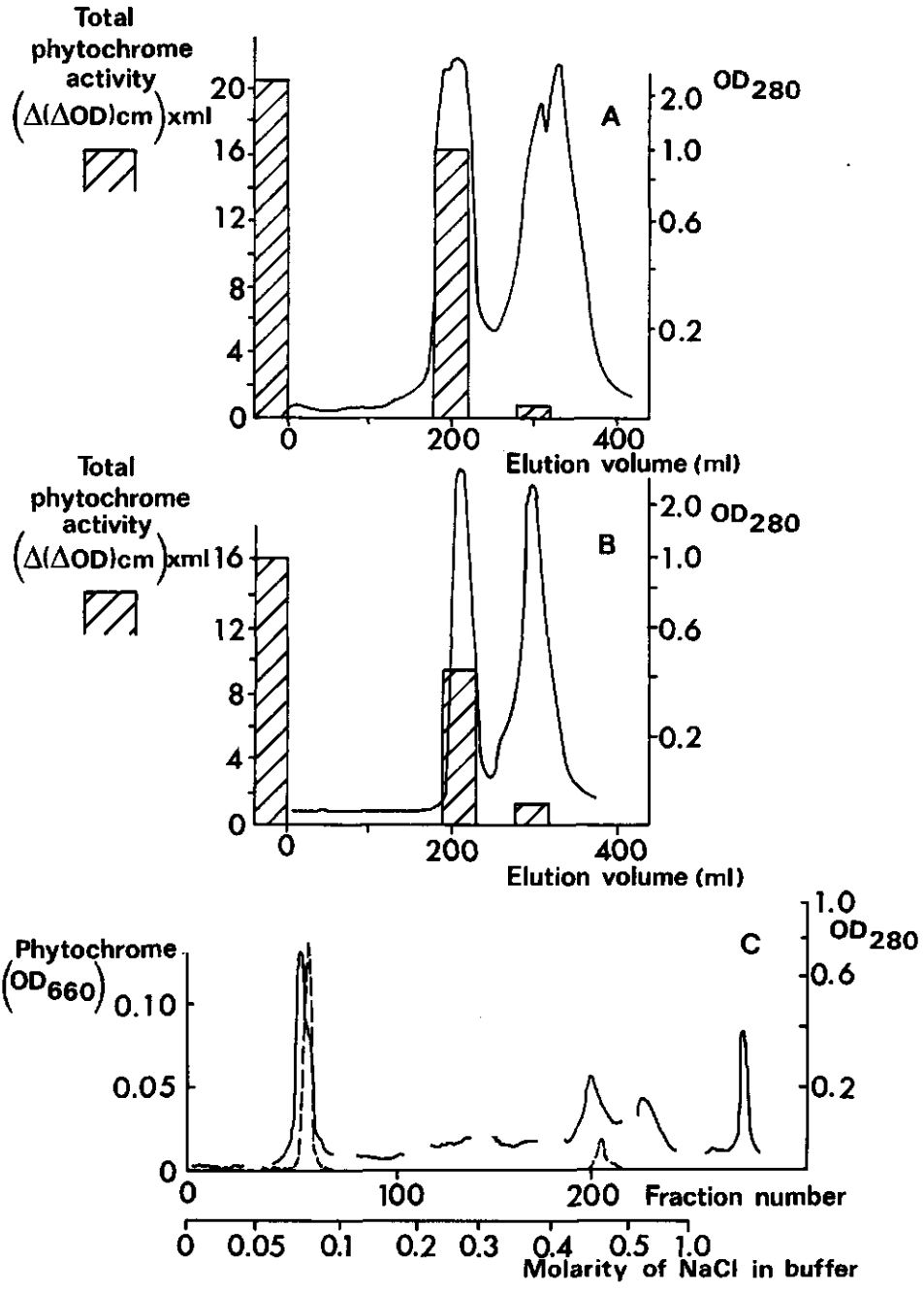


FIG. 16. Chromatography and rechromatography of a phytochrome concentrate on DEAE-Sephadex A-50 (isolation 55). A. First run. B. Rechromatography of first peak. C. Stepwise elution of column. (See Table 9). Shaded blocks represent total phytochrome activity of combined peak fractions. Blocks on the left represent initial phytochrome activity.

The precipitates were dissolved in 10 ml of 0.1 M potassium phosphate buffer (pH 6.6) to a final volume of about 15 ml. After the solution had been put onto the column and had penetrated into the DEAE-Sephadex gel, the gradient system (see 2.2.5) was connected to the column. Figure 16A shows an elution curve of a representative run. Again, there were two protein peaks with phytochrome activity. Total phytochrome activity of the combined fractions of each of the two peaks, which is represented by the shaded blocks in the Figure, was much higher for the first peak than for the second. The block on the left corresponds to the initial activity of the phytochrome solution. The first-peak fractions were concentrated by precipitation with $(NH_4)_2SO_4$ and the concentrate was rechromatographed on a new DEAE-Sephadex column. The column was eluted with the same gradient system. Again, two protein peaks with phytochrome activity were obtained (Fig. 16B) which were eluted at the same NaCl molarities as the first and the second peak of the initial run. Phytochrome activity is again represented by shaded blocks, which express total activity of combined peak fractions. Most phytochrome activity was again found in the first peak, the second peak containing only 13% of that of the first. Moreover, rechromatography effected a further purification, with an increase in specific activity of the first-peak fraction from 0.066 to 0.090. Data on the recovery and purification of the phytochrome fractions during the successive column elutions are given in Table 9. The second protein peak was also rechromatographed and

TABLE 9. Chromatography and rechromatography of phytochrome solution on DEAE-Sephadex A-50 (Isolation 55)

Fraction	Volume (ml)	Phytochrome activity [Δ(ΔOD)/cm]	Recovery of activity (%)	Specific activity [Δ(ΔOD)/cm.mg protein]	Purification factor
Calcium phosphate batch adsorption:					
1st eluate	110	0.230	(100.0)	0.028	(1.00)
Precipitation with $(NH_4)_2SO_4$	19.5	1.050	80.9	0.037	1.32
DEAE-Sephadex A-50 chromatography:					
1st peak fraction	54	0.300	64.0	0.066	2.36
2nd peak fraction	60	0.012	2.8	0.046	1.64
Rechromatography of 1st peak fraction on DEAE-Sephadex A-50					
1st peak fraction	30	0.320	37.9	0.090	3.21
2nd peak fraction	120	0.010	4.7	0.010	0.36

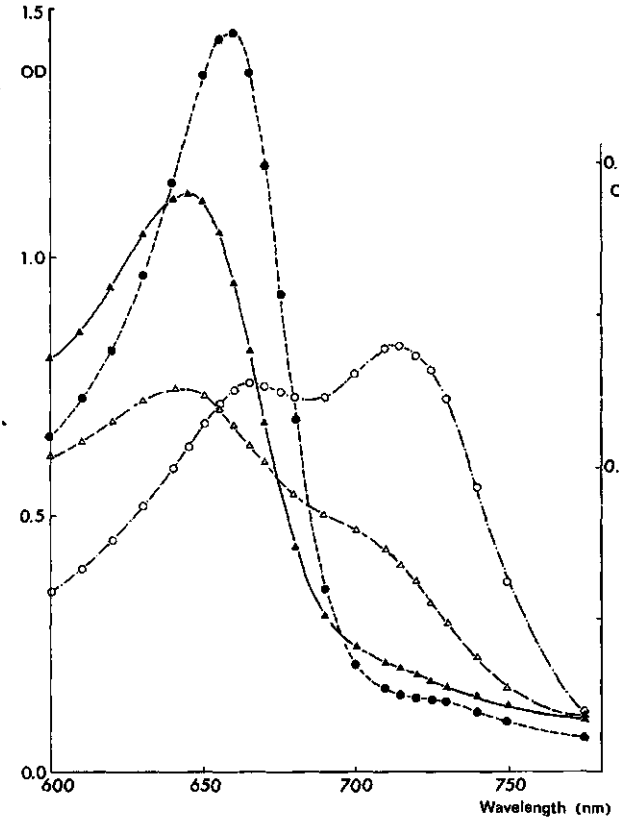


FIG. 17. Comparison between the absorption spectra of first and second peak fractions after chromatography ← on DEAE-Sephadex A-50. First peak fraction P_R (●———●), P_{FR} (○—○) second peak fraction P_R (▲—▲), P_{FR} (△- -△).

eluted at 0.4 M NaCl. The absorption spectrum of this material was different from that of the first-peak fraction, suggesting that the phytochrome in the second fraction had been partly denatured (see Figure 17). Stepwise elution (Fig. 16C) showed that the phytochrome in the first peak is hardly bound to the ion-exchanger, being eluted at an NaCl molarity of 0.05, whereas the second protein peak was eluted at the same molarity as before. From Figure 16C it can be seen that phytochrome activity coincides with the right hand shoulder of the first protein peak. It might be possible to achieve a more efficient separation between the phytochrome and other material in the peak with a very gradually increasing salt gradient.

2.3.6. *Gel filtration on Sephadex G-200*

The two phytochrome fractions obtained by DEAE-Sephadex chromatography were purified by gel filtration on Sephadex G-200; the column was equilibrated with elution buffer (0.1 M potassium phosphate, pH 6.6) before use. The OD_{280} and OD_{660} of the fractions were measured with a Zeiss PMQ II spectro-

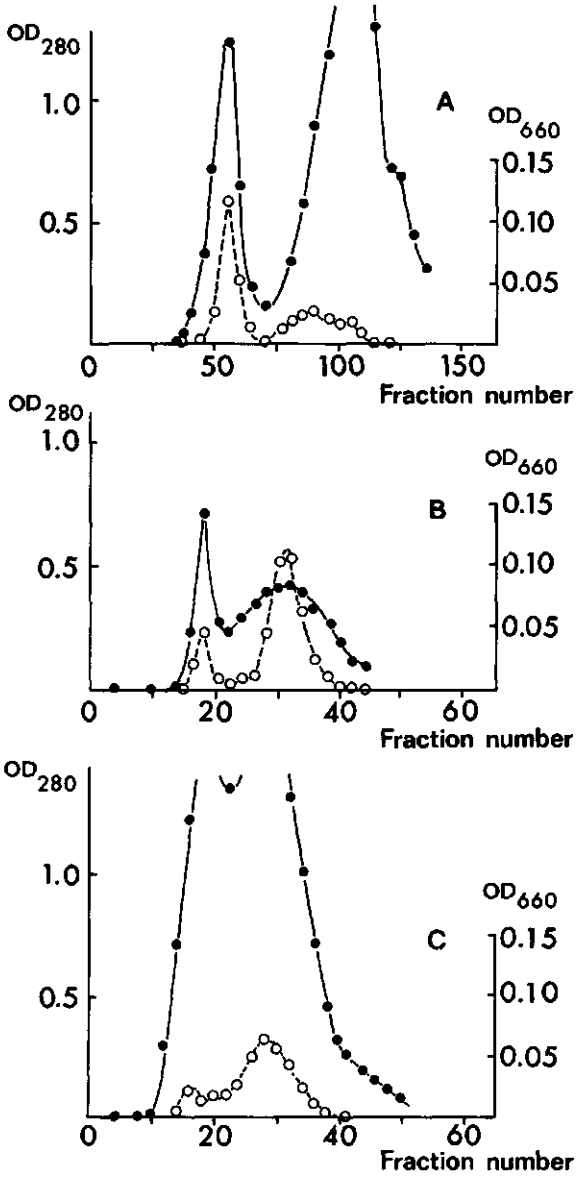


FIG. 18. Preparative gel filtration on Sephadex G-200 of phytochrome fractions obtained by DEAE-Sephadex chromatography. A. Two protein peaks obtained by DEAE-Sephadex chromatography. B. Gel filtration of first peak. C. Gel filtration of second peak (See Table 10). OD_{280} (●—●), OD_{660} (○—○).

photometer. The results are shown in Figure 18. Since the pigment is almost completely in the P_R form during the isolation procedure, OD_{660} may again be taken as a measure of phytochrome activity. The phytochrome solution used for this experiment gave the usual elution curve (Fig. 18A) upon DEAE-Sephadex chromatography. Gel filtration of the first-peak fraction split it into a peak con-

TABLE 10. Sephadex G-200 gel filtration of phytochrome fractions separated on DEAE-Sephadex A-50 (Isolation 46)

Fraction	Volume (ml)	Phytochrome activity [Δ(ΔOD)/cm]	Recovery of activity (%)	Specific activity [Δ(ΔOD)/cm. mg protein]	Purification factor
Calcium phosphate batch adsorption:					
1st eluate	62	0.196	(100.0)	0.050	(1.00)
Precipitation with $(NH_4)_2SO_4$	8	1.093	72.0	0.044	0.88
DEAE-Sephadex A-50 chromatography:					
1st peak fraction	60	0.074	36.5	0.125	2.50
2nd peak fraction	105	0.028	24.2	0.025	0.50
Precipitation with $(NH_4)_2SO_4$					
1st peak fraction	5	0.650	26.7	0.110	2.20
Sephadex G-200 gel filtration					
1st peak fraction					
HMW* fraction	18	–	–	–	–
Main fraction	35.5	0.076	22.2	0.690	13.82
Precipitation with $(NH_4)_2SO_4$					
2nd peak fraction	9.5	0.280	21.8	0.020	0.40
Sephadex G-200 gel filtration					
2nd peak fraction		–	–	–	–
HMW* fraction	12	0.060	16.3	0.070	1.42
Main fraction	33				

* High molecular weight fraction

taining high molecular weight* material with only little phytochrome activity, and a smooth second peak, the main phytochrome fraction, with high activity (Fig. 18B). Surprisingly, gel filtration of the second-peak fraction followed a similar pattern (Fig. 18C), although the specific activities of the two resultant peak fractions were much lower. Quantitative results are recorded in Table 10.

The main phytochrome fractions after gel filtration were respectively six and three times purer than the 1st and 2nd DEAE-Sephadex peak fractions. According to these results, both DEAE-Sephadex peak fractions give rise to high molecular weight material upon Sephadex G-200 gel filtration.

To find out more about the behaviour of phytochrome fractions on the G-200

* The term high molecular weight fraction is used in analogy with the terminology of analytical gel filtration. (see 2.3.7)

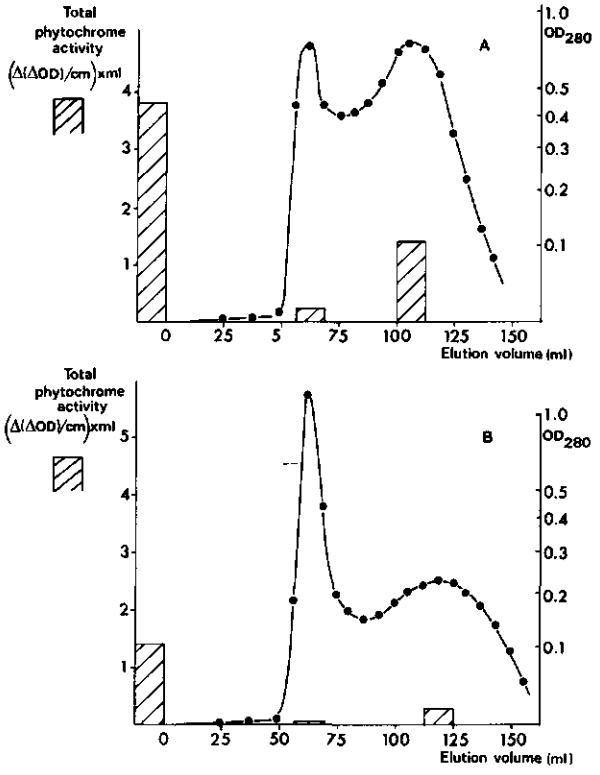


FIG. 19. Separation of high molecular weight phytochrome from the main fraction by gel filtration on Sephadex G-200. A. Gel filtration of first peak fraction obtained by DEAE-Sephadex chromatography. B. Refiltration of main phytochrome fraction.

column, gel filtration of the main phytochrome fraction was repeated. This experiment is illustrated in Figure 19 and the quantitative results are given in Table 11.

The elution curve of Figure 19B shows that the relative amount of high molecular weight material is even higher in the second gel-filtration run. For both the first and the second run, the activity of the first-peak fraction is low but measurable. Why these high molecular weight fractions showed such low activity cannot yet be decided. During all gel-filtration runs inactivation of phytochrome was appreciable. One may assume that inactivated molecules (which must be partly denatured) associate more readily than active ones. The phytochrome molecules in the first-peak fractions must be very large because they are fully excluded by the Sephadex G-200 gel. The blue dextran peak and the high molecular weight phytochrome peak overlapped each other fully. Figure 20 shows this more clearly. In this case the phytochrome preparation was loaded onto the column just 10 fractions behind the blue dextran. The elution curve showed that the heavy phytochrome peak, was eluted exactly 10 fractions after the blue dextran and, therefore, was not retarded at all on the column.

TABLE 11. Repeated Sephadex G-200 gel filtration of phytochrome preparation purified by DEAE-Sephadex chromotagraphy (Isolation 53)

Fraction	Volume (ml)	Phytochrome activity [Δ(ΔOD)/cm]	Recovery of activity (%)	Specific activity [Δ(ΔOD)/cm. mg protein]	Purification factor
Calcium phosphate batch adsorption:					
1st eluate	54	0.136	(100.0)	0.022	(1.00)
Precipitation with $(NH_4)_2SO_4$	6.8	0.832	77.0	0.036	1.64
DEAE-Sephadex A-50 chromatography:					
1st peak fraction	135	0.031	57.0	0.062	2.82
2nd peak fraction	60	0.013	10.6	0.019	0.86
Precipitation with $(NH_4)_2SO_4$					
1st peak fraction	5	0.758	51.6	0.066	3.00
Sephadex G-200 gel filtration					
HMW* fraction	15	0.012	2.5	0.052	2.36
Main fraction	33	0.043	19.2	0.044	2.00
Precipitation with $(NH_4)_2SO_4$					
Main fraction	4.2	0.336	19.2	0.044	2.00
Refiltration on Sephadex G-200					
HMW* fraction	21	0.002	0.6	-	-
Main fraction	45	0.006	3.7	-	-

* High molecular weight fraction

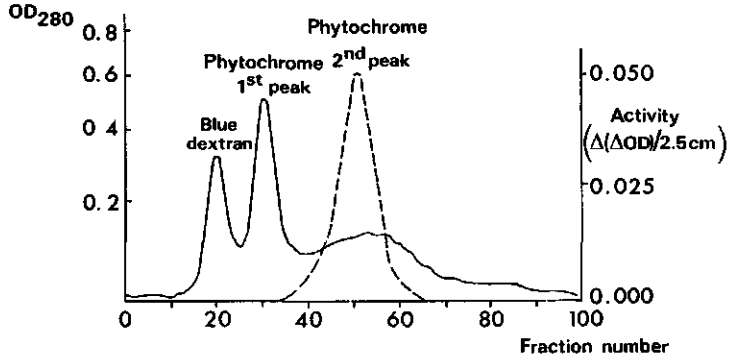


FIG. 20. Comparison of the elution volumes of blue dextran and the high molecular weight fraction of phytochrome on Sephadex G-200. Phytochrome solution was loaded onto the column 10 fractions behind blue dextran. OD_{280} (———), phytochrome activity Δ(ΔOD)/2.5 cm (- - - -).

2.3.7. *Molecular weight estimation by gel filtration on Sephadex G-200*

The molecular weight of phytochrome in a purified fraction was estimated by filtering the fraction and solutions of proteins of known molecular weight on the same column (see 2.2.6). Blue dextran (Pharmacia) was used for each run to determine the void volume. This material, which has a molecular weight of about 2,000,000, is fully excluded from the gel and hence its elution volume is equal to the void volume. Each calibration run was carried out with one or two proteins in combination with blue dextran according to the following scheme:

γ-globulin – β-lactoglobulin – blue dextran
γ-globulin – albumin (egg) – blue dextran
albumin (bovine) – blue dextran
albumin (egg) – blue dextran

Such a set of calibration runs was made both before and after a phytochrome filtration, because the elution properties of the column change slightly during use. In Figure 21, the results of the runs with proteins have been combined into one elution pattern for comparison with that of phytochrome. The first phytochrome peak again coincides with the blue dextran peak which means that it is fully excluded by the gel. In the phytochrome runs, therefore, the first peak was used to determine the void volume. The experimentally determined elution volumes of the model proteins are given in Table 12.

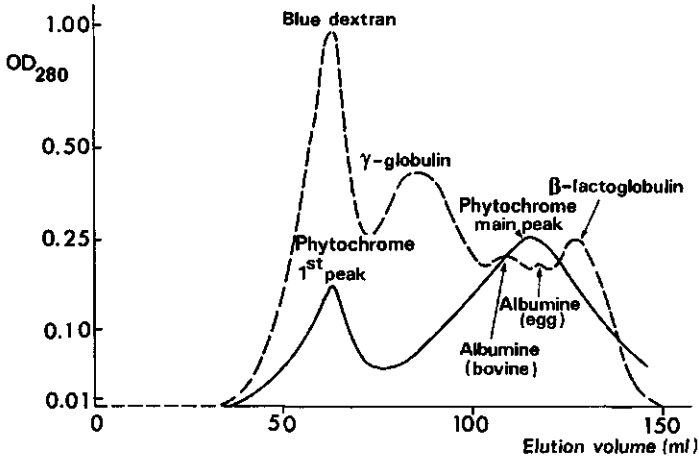


FIG. 21. Analytical gel filtration on Sephadex G-200. Comparison of the elution volumes of phytochrome and proteins of known molecular weight. OD_{280} phytochrome (———), OD_{280} proteins, cumulative (- - - - -).

TABLE 12. Relative elution volumes of phytochrome and model proteins on a Sephadex G-200 column

Protein	Void volume, V_o (ml)	Elution volume, V_e (ml)	V_e/V_o	Molecular weight
γ-Globulin	62.9	85.9	1.365	160,000*
Albumin (bovine)	62.1	110.5	1.78	67,000*
Albumin (egg)	62.1	125.3	2.02	45,000*
β-Lactoglobulin	63.0	130.2	2.07	39,000*
Phytochrome:				
High molecular weight fraction	62.1	62.1	1.00	⩾ 225,000**
Main fraction	62.1	117.1	1.89	56,700***
	62.1	118.7	1.91	54,300***

* Taken from ANDREWS[56]
** Taken from LEACH and O'SHEA[57]
*** Calculated from Figure 21

The elution volumes of γ-globulin, albumin (bovine) and albumin (egg) were determined in duplicate. A calibration graph was then constructed by plotting V_e/V_o against the logarithm of molecular weight for each of the proteins (Fig. 22); molecular weights of the purified proteins were taken from the literature[56, 57].

A phytochrome preparation, purified by DEAE-Sephadex chromatography and preparative Sephadex G-200 gel filtration (see Table 10) was used for the molecular weight determination. The activity and specific activity of the main

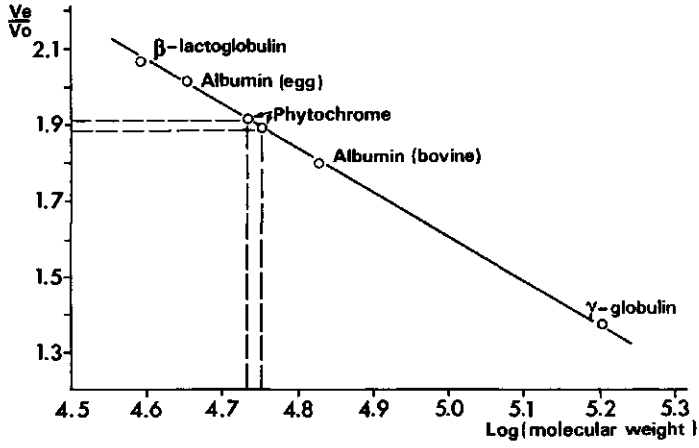


FIG. 22. Calibration graph for the determination of the molecular weight of phytochrome by analytical gel filtration on Sephadex G-200 (see Table 12).

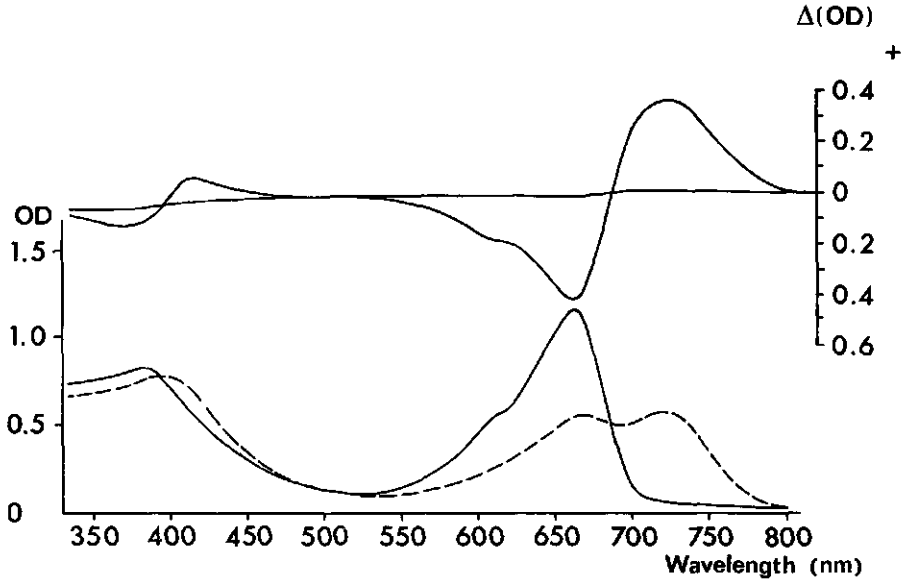


FIG. 23. Absorption spectra of the phytochrome preparation used for molecular weight determination. P_R (———), P_{FR} (- - - -), and the corresponding difference spectrum (———)(top).

phytochrome fraction after gel filtration and precipitation with $(NH_4)_2SO_4$ were 1.090 and 0.100 respectively; the absorption spectra of this fraction in the P_R and P_{FR} forms are shown in Figure 23. Two determinations of the elution volume of the main phytochrome fraction gave values of 1.886 and 1.910 for V_e/V_o. From the calibration graph (Fig. 22), we thus determined molecular weights of 54,300 and 56,700 respectively for phytochrome, with a mean value of 55,500. The phytochrome in the high molecular weight fraction, with an elution volume equal to the void volume of the column, must be an association product with a molecular weight above 200,000.

The spectra in the region 300–800 nm of the main fraction after gel filtration on Sephadex G-200 were measured on a Cary-14 double beam spectrophotometer. The spectra which are shown in Figure 24 are representative for the purest phytochrome fractions we have obtained.

2.3.8. *Equilibrium concentration of P_{FR} upon saturation with red light*

When phytochrome is irradiated with a saturating dose of far-red light, the resulting pigment form has no absorption above 710 nm. One may conclude that the pigment is present exclusively in the P_R form. When, however, P_R is irradiated with a saturating dose of red light it is not completely converted to P_{FR}. The resulting absorption spectrum has a shoulder at 665 nm (Fig. 24), which may indicate that not all P_R has been transformed into P_{FR}. This may be due to an overlap of the absorption bands of P_{FR} and P_R in the red region of the spectrum between 600 and 660 nm. Thus, a saturating dose of red light should establish a

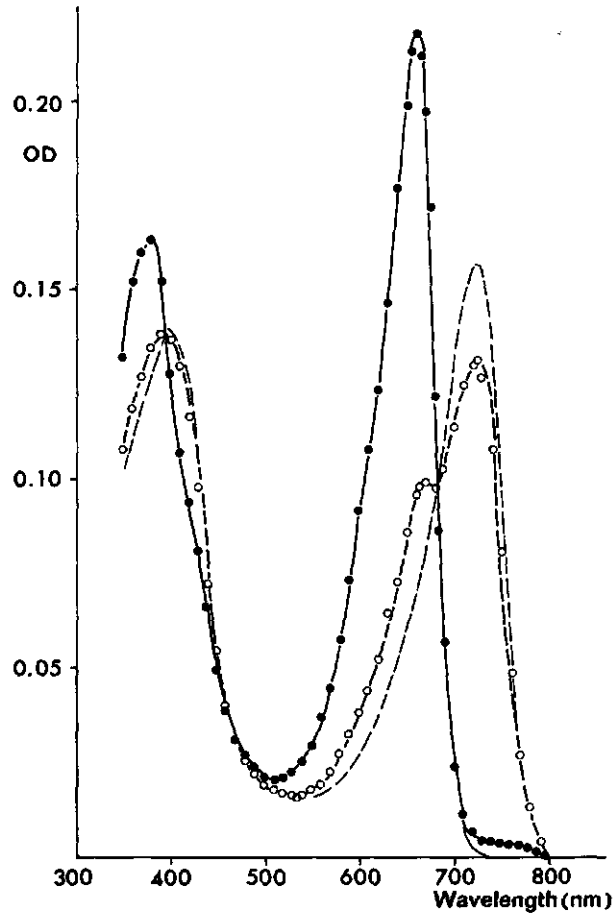


FIG. 24. Absorption spectra of a typical phytochrome preparation after Sephadex G-200 gel filtration. Activity 0.180 Δ(ΔOD)/cm, specific activity 0.085 Δ(ΔOD)/cm. mg protein. Preparation irradiated for 3 min with far-red light of 737 nm, (●—●), preparation irradiated for 3 min with red light of 658 nm, (○- -○). Calculated spectra pure P_R (———) and pure P_{FR} (- - - -).

photostationary state, for which the equilibrium concentrations of the two pigment forms can be calculated from the absorption spectra of purified phytochrome according to a method of BUTLER et al.[33] in the following way:

Conditions for a steady state were derived from the kinetics for the photoconversion of phytochrome with red ($\lambda = 665$ nm) and far-red ($\lambda = 725$ nm) light. These reactions are both first order. Consequently, the overall conversion rate may be represented by:

$$dx/dt = [\Phi_R(1-x)A_R - \Phi_{FR} \times A_{FR}]E_\lambda \qquad (3)$$

and the absorbance A_λ of a mixture of P_R and P_{FR} by:

$$A_\lambda = (1-x)A_{R_\lambda} + x\, A_{FR_\lambda} \qquad (4)$$

where x = mole fraction of P_{FR} (1–x = mole fraction of P_R), A_{R_λ} and A_{FR_λ} = absorbance at wavelength λ of P_R and P_{FR}, Φ_R and Φ_{FR} = quantum yields for

conversion of P_R and P_{FR}, and E_λ = intensity of monochromatic irradiation of wavelength λ.

At $\lambda = 725$ nm, $A_{R_{725}} = 0$ and, according to equation 4, the absorbance at 725 nm is entirely due to P_{FR}, i.e. $A_{725} = x\, A_{FR_{725}}$. Thus,

$$d\, A_{725}/dt = A_{FR_{725}} \cdot dx/dt = [\Phi_R(1-x)A_{R_\lambda} - \Phi_{FR}\, x\, A_{FR_\lambda}]E_\lambda\, A_{R_{725}} \quad (5)$$

which gives the rate of change in absorbance at 725 nm due to irradiation at any wavelength. When suitable boundary conditions (i.e. irradiation of P_R with light of $\lambda = 665$ nm, $x = o$ at $t = o$ and irradiation of P_{FR} with light of $\lambda = 725$ nm, $x = x_{max}$ at $t = o$) are applied, the following equations may be derived from equation 5 for the steady state.

$$1-x_{max} = (1 + \Phi_R/\Phi_{FR})^{-1} \cdot (A_{660})_{min}/(A_{660})_{max} \quad (6)$$

$$\text{and } \Phi_R/\Phi_{FR} = E_{725} \cdot k_{660}/E_{660} \cdot k_{725} \cdot (A_{725})_{max}/(A_{660})_{max} \quad (7)$$

where k_{660} and k_{725} are the rate constants for the first order conversion of P_R and P_{FR}. These rate constants can be determined at equal quantum flux and, according to the measurements of BUTLER et al.([33]), $k_{658} \cdot E_{737}/k_{737} \cdot E_{658} = 2.6$ for our experiment in which the pigment was irradiated with light of 658 nm and 737 nm (Fig. 24).

The absorbance values needed for solution of equations 6 and 7 were taken from the peak values of Figure 24; namely $(A_{660})_{max} = 0.218$, $(A_{660})_{min} = 0.096$, $(A_{725})_{max} = 0.131$. Thus, in equation 7, Φ_R/Φ_{FR} is 1.56 and, by substitution in equation 6, $(1-x)_{max} = 0.17$. This means that 17% of the pigment is present as P_R in the photostationary state.

The value for $(1-x)_{max}$ derived from the absorption spectra in Figure 24 differs slightly from that calculated by BUTLER et al.([33]), who found 19–20% P_R in the photostationary state. This difference may be due to the fact that they determined their rate constants (k_λ/E_λ) with phytochrome preparations which were presumably partly denatured.

The amounts of each pigment form in the photostationary states after irradiation with red light ($\lambda = 658$ nm) and with far-red light ($\lambda = 737$ nm) were also calculated by a method suggested to us by Dr. K. M. HARTMANN (University of Freiburg, Germany). This calculation is based solely upon the absorbance of the pigment mixture in the two stationary states, the contributions of P_R and P_{FR} to the measured absorbances A_{737} and A_{658} being defined by equation 4. The contributions to the absorbance at 730 nm can be written as follows:

$$A_{737/730} = (1-x)_{737}\, A_{R_{730}} + x_{737}\, A_{FR_{730}} \quad (8)$$

$$A_{658/730} = (1-x)_{658}\, A_{R_{730}} + x_{658}\, A_{FR_{730}} \quad (9)$$

$A_{R_{730}}$ and $A_{FR_{730}}$ can be eliminated from equations 8 and 9 and numerical values for $A_{737/730}$ and $A_{658/730}$ can be calculated from Figure 24. This gives: $0.030 \leqslant x_{737}/x_{658} \leqslant 0.037$.

According to BUTLER et al.([33]) $x_{658} \approx 0.8$, so that $x_{737} = 0.03$ and $(1-x)_{737}$

$= 0.97$. This means that 3% P_{FR} is present in the stationary state reached after irradiation with far-red light ($\lambda = 737$ nm). This correlates with the absorption measured above 700 nm for this case (Fig. 24).

Similar equations can be written for the absorbance at 658 nm:

$$A_{737/658} = (1-x)_{737} A_{R_{658}} + x_{737} A_{FR_{658}} \quad (10)$$

$$A_{658/658} = (1-x)_{658} A_{R_{658}} + x_{658} A_{FR_{658}} \quad (11)$$

$A_{R_{658}}$ and $A_{FR_{658}}$ can be eliminated from equations 10 and 11, since for the steady state, the following conditions must apply (see equation 5):

$$A_{R_{658}} \Phi_R / x_{658} = A_{FR_{658}} \Phi_{FR} / (1-x)_{658}$$

in which $\Phi_R/\Phi_{FR} \approx 1.5$. The values of $(1-x)_{737}$ and x_{737} have already been calculated to be 0.97 and 0.03 respectively; numerical values for $A_{737/658}$ and $A_{658/658}$ can be derived from Figure 24. Equations 10 and 11 can now be solved and the values of x_{658} and $(1-x)_{658}$, i.e. the mole fractions of P_R and P_{FR} in the stationary state after irradiation with red light ($\lambda = 658$ nm) are found to be 0.83 and 0.17 respectively.

This value of 0.17 for $(1-x)_{658}$ calculated with the method of HARTMANN agrees with that estimated by the method of BUTLER et al.([33]). Now that mole fractions of P_R and P_{FR} present in both stationary states are known, equations 8–11 can be used to calculate the absorbance of P_R and P_{FR} at each wavelength of the spectrum. Plots of these absorbances (A_{R_λ} and A_{FR_λ}) against wavelength result in theoretical spectra for pure P_R and P_{FR}. These are represented in Figure 24, in as far as they deviate from those of the steady-state mixtures. Deviation is most prominent between the spectrum of pure P_{FR} and that of the pigment in the stationary state after irradiation with red light ($\lambda = 658$ nm). The shoulder at 660–665 nm in the latter spectrum is largely due to the amount of P_R (17%) present in the steady-state mixture. The calculated spectrum of pure P_R above 700 nm is in agreement with the observation that, after irradiation with far-red light ($\lambda = 737$ nm), the pigment contains a low percentage (3%) of P_{FR}.

In addition to the rate constants, BUTLER's team determined the steady-state levels of P_R and P_{FR} after irradiation at various wavelengths, from which the quantum efficiencies ($\varepsilon_\lambda \Phi_\lambda$) for the $P_R \rightarrow P_{FR}$ and $P_{FR} \rightarrow P_R$ transformations were calculated. A plot of ($\varepsilon_\lambda \Phi_\lambda$) against wavelength gives the action spectrum for the transformations of phytochrome in vitro. In the action spectrum for the $P_{FR} \rightarrow P_R$ conversion, there is no shoulder present in the red region. This correlates with the theoretical spectrum that we worked out for P_{FR} (Fig. 24), indicating that the absorption spectrum of P_{FR} corresponds to a mixture of about 20% P_R and 80% P_{FR}. The calculations of BUTLER et al.([33]) showed that the ratio $\varepsilon_{R_\lambda}/\varepsilon_{FR_\lambda}$ is constant between 546 and 665 nm. Thus, irradiation at these wavelengths should always lead to the same steady state. We found, however, that when a saturating red irradiation is followed by a subsequent irradiation with light of 573 nm, the equilibrium shifted slightly towards P_{FR}, the shift corresponding to an increase of 2% in the level of P_{FR} (Fig. 25). The assumption

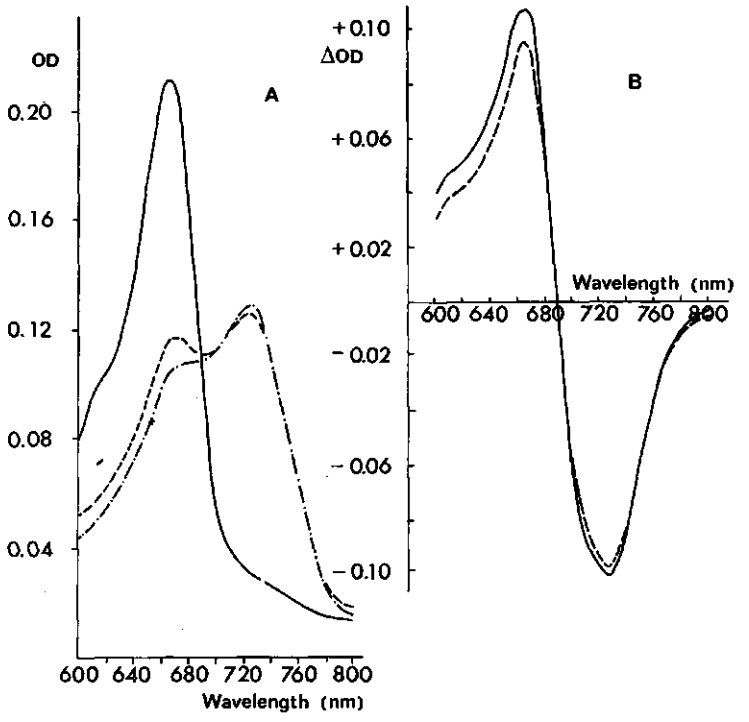


FIG. 25. Absorption and difference spectra showing the displacement of the photostationary state towards P_{FR} by a supplementary irradiation with light of 573 nm. A. P_R, after 2 min irradiation 725 nm (———), P_{FR}, after 2 min irradiation 665 nm (- - - -), P_{FR}, after 8 min additional irradiation 573 nm (- . - . -). B. Difference spectrum $\Delta OD[P_R\text{-}P_{FR_{(660)}}]$ (- - - -), difference spectrum $\Delta OD[P_R\text{-}P_{FR_{(573)}}]$ (———).

that $\varepsilon_{R\lambda}/\varepsilon_{FR\lambda}$ is constant in the red region is therefore only an approximation. Especially at wavelengths shorter than that of the red maximum, deviations from the steady state at 665 nm may be found when the rate constants are determined with phytochrome preparations which are fully reversible, like the ones in Figures 24 and 25.

The fact that P_R cannot be fully transformed into P_{FR} has important implications for the interpretation of the experiments in Chapter 2.

2.4. DISCUSSION

The choice of batch adsorption on calcium phosphate gel for the large-scale isolation of phytochrome rested on two considerations. Firstly, the method designed by SIEGELMAN and HENDRICKS([60]) for 10 kg amounts of seedlings is very time-consuming, chiefly because of the ultra-filtration step and the necessity of chromatographing large volumes. These preliminary isolation steps were also applied by MUMFORD and JENNER ([53]). Secondly, we were unable to repro-

duce the results of these two groups with the chromatography of phytochrome on columns of calcium phosphate gel in the brushite form. We found that the gel which was prepared by the method of SIEGELMAN, WIECZOREK and TURNER[61] adsorbed phytochrome too strongly and that its properties did not remain constant when the brushite gel was stored for longer periods.

The first steps of the procedure described, up to the second calcium phosphate adsorption, are much quicker than those of the other methods[52, 53, 60], and they are easy to carry out. Moreover, the specific activity of 0.022 at this stage compared favourable with that after calcium phosphate chromatography with the other methods (see Table 13). So did the recovery of 35–40%. It is impossible to compare the extraction efficiencies because the initial amount of phytochrome present in the seedlings was not known. The very large amount of phytochrome extracted by MUMFORD and JENNER[53] is striking. With a buffer to seedlings ratio of 2 v/w (Table 6), we extracted as much phytochrome as SIEGELMAN and FIRER[52]. Although the extraction efficiency is somewhat lower with a ratio of 0.1 v/w, concentrated Tris solution has the advantage of yielding an extract of one third the volume, with a higher specific activity (Table 6). The other steps in our phytochrome isolation are based on the pioneering studies of SIEGELMAN and FIRER[52]. The problem of storing phytochrome concentrates can be solved to a large extent by adding 20% (v/v) or more glycerol to the solutions[62].

Purification of phytochrome fractions by chromatography on DEAE-Sephadex and by gel filtration on Sephadex always caused loss of activity. Experience with some fifty isolations indicated that the stability of phytochrome solutions increases with the degree of purification. Loss of photoreversibility does not necessarily involve complete bleaching. The partial inactivation of the phytochrome in the second DEAE-Sephadex peak showed up most clearly in the spectrum of the P_R form, the absorption maximum being shifted to lower

TABLE 13. Comparison of the batch adsorption procedure with methods involving chromatography on calcium phosphate

Purification Method	Crude extract		Purified extract	
	Total activity per 25 kg seedlings	Specific activity	Recovery of activity (%)	Specific activity
Chromatography on calcium phosphate:				
Siegelman and Firer[52]	160	0.002	41	0.017
Mumford and Jenner[53]	480	0.001	20	0.020
Batch adsorption on calcium phosphate:	130–160	0.002	35–40	0.022

In all cases activity is expressed as [Δ(ΔOD)/cm]. Data cited by other authors have been converted accordingly.

wavelengths. Irradiation of the preparations with red light transferred 83% of P_R into the P_{FR} form (Fig. 24). As a routine we used the $\Delta OD_{730-660}$ value of the P_{FR} form as a measure of quality. This is as high as 0.100 for active preparations (activity, $\Delta(\Delta OD)/cm = 1.0$), while it is negative for preparations which have lost some of their reversibility.

The results of the Sephadex G-200 gel filtration experiments indicate that the high molecular weight fraction may be formed by association of non-active phytochrome molecules. This pigment form, which retained some colour but no photoreversibility, is perhaps comparable with the so-called P* form obtained by BUTLER et al.([63]) upon denaturation of active phytochrome. We determined the molecular weight of phytochrome to be about 55,500, which agrees with the estimate of MUMFORD and JENNER([53]). It is intriguing that the phytochrome isolated from rye seedlings under similar conditions by CORRELL et al.([64]) had a molecular weight of between 150,000 and 190,000. Apparently this is a tetramer, because the molecular weight of the corresponding monomer was 42,000. Unfortunately, the relative activities of monomer and tetramer were not measured.

Changes in optical activity, accompanying phototransformation of the pigment, and its difference spectra at low temperature as described in Chapter 3, were studied with phytochrome preparations purified by the method described above.

3. ASPECTS OF THE CHEMISTRY AND THE PHOTOCHEMISTRY OF PHYTOCHROME IN VITRO

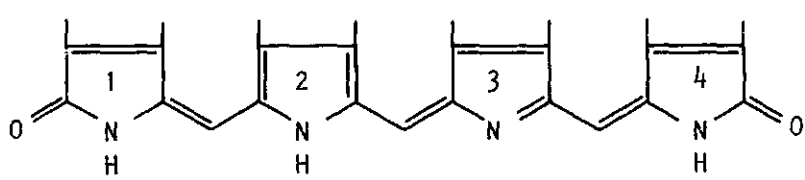
3.1. Scope of the experiments

The chemistry and photochemistry of phytochrome were studied by a number of techniques, which yielded information about how the chromophore is associated to the protein moiety.

Denaturation experiments showed that the protein part of the pigment plays an essential role in the phototransformation by interacting specifically with the chromophore. When denaturing agents cause the collapse of the tertiary protein structure, the colour usually disappears. The P_{FR} form is always more susceptible to such action than P_R([63]). Mild denaturation destroys the photoreversibility, the absorption spectrum of the phytochrome solution becoming comparable to that of the free chromophore. A study of the optical activity of the pigment indicated that the conformation of the complex changes during phototransformation. Changes in the optical rotatory dispersion and circular dichroic spectra were found to correspond with the shifts in the absorption spectra([65]). More detailed information about the transformation reactions was obtained from low-temperature difference spectra. At low temperatures, light-induced changes in the chromophore can be studied independently of conformational changes in the protein part.

One may describe the photochemical transformation of phytochrome as a combination of isomerization of the chromophore with a change in conformation of the protein part. For each of the two chromophore isomers, a protein conformation exists which gives a stable complex of the pigment – in one case P_R and in the other case P_{FR}.

Investigation of the properties of the blue algal pigments, C-phycocyanin and allophycocyanin, which are chemically related to phytochrome, gave some insight into the detailed structure of phytochrome. The phycocyanins also consist of a chromophore (phycocyanobilin), bound to a protein moiety by a covalent bond. Phycocyanobilin is a tetrapyrrole and belongs to a group of compounds known as bilitrienes:



bilitriene

Because of their close resemblance to phytochrome, and because large amounts of them were more readily available, the phycocyanins were often used as model substances. Although the absorption spectra of C-phycocyanin and especially allophycocyanin are very similar to those of the P_R form of phytochrome, no photoreversibility has been reported for the algal pigments.

3.2. Denaturation

3.2.1. *Effect of pH*

It was confirmed that phytochrome is stable only between pH 6.5 and 7.5 and that the reversibility of the pigment is progressively lost as the pH deviates considerably from 7.0. The effect of increasing the pH is shown in Figure 26; 0.1 M NaOH was added to a phytochrome solution in 0.01 M potassium phosphate buffer of pH 7.1 until a pH of 8.1, or 8.6, was reached. The absorption spectra show that the value of (OD_{730}–OD_{660}) for the P_{FR} form changes from positive to negative as the pH increases from 7.1 to 8.1. From the difference spectra it can be seen that $\Delta(\Delta OD)$, a measure of activity or photoreversibility, falls from 0.098 at pH 7.1 to 0.042 at pH 8.1 and 0.038 at pH 8.6. When a phytochrome solution at pH 7.3 was acidified with 0.1 M HCl to pH 6.5, a comparable effect was found, as is illustrated in Figure 27. In this case the decrease in photoreversibility is smaller. Above pH 8.0 and below pH 6.0, the pigment becomes insoluble and precipitates, although below pH 6.0 the precipitated pigment initially remains photoreversible.

These experiments gave the first indication that the protein moiety plays an essential role in the phototransformation of phytochrome.

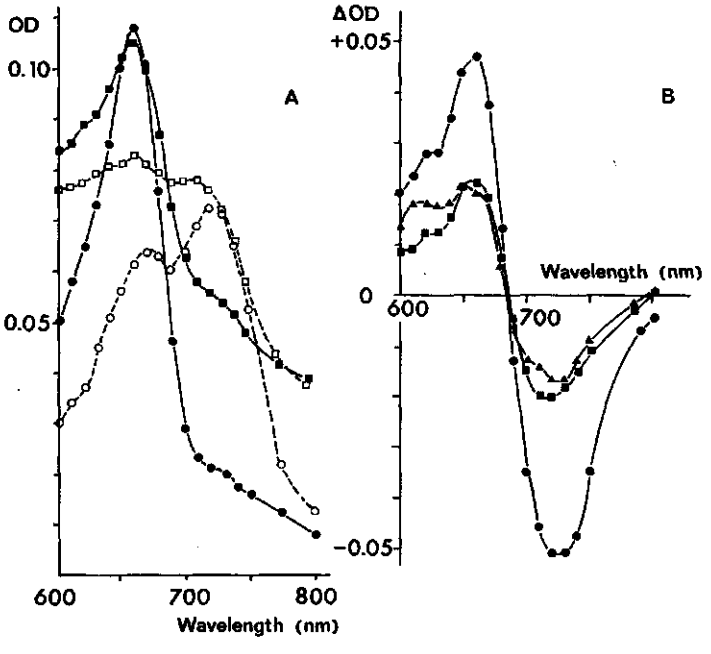


FIG. 26. Changes in the absorption of phytochrome with increasing pH. A. Absorption spectra at pH 7.1 (●—● P_R, ○- -○ P_{FR}) and pH 8.1 (■— ■ P_R, □- □ P_{FR}). B. Difference spectra, ΔOD (P_R-P_{FR}), at pH 7.1 (●—●), 8.1 (▲—▲), and 8.6 (■—■).

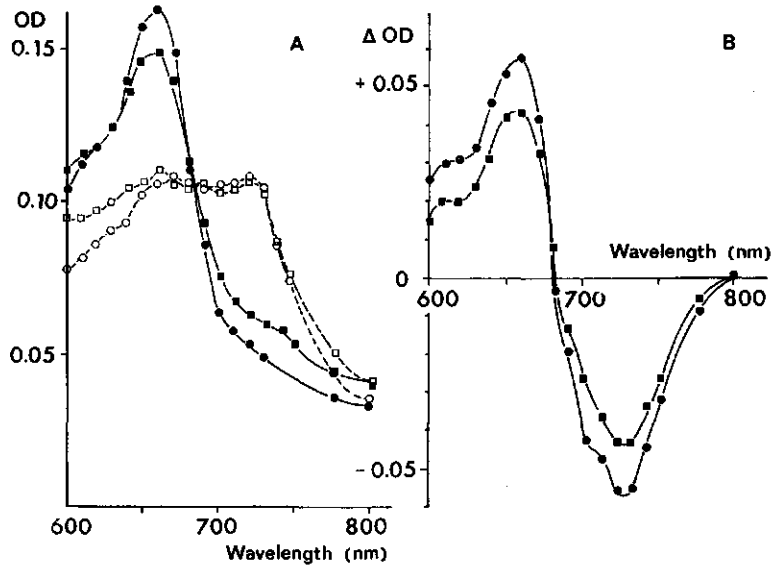


FIG. 27. Changes in the absorption of phytochrome with decreasing pH. A. Absorption spectra at pH 7.3 (●—● P_R, ○—○ P_{FR}) and pH 6.5 (■— ■ P_R, □- -□ P_{FR}). B. Difference spectra, $\Delta OD(P_R\text{-}P_{FR})$, at pH 7.3 (●—●) and 6.5 (■— ■).

3.2.2. *Effects of organic solvents and detergents*

When phytochrome and other biliproteins, such as C-phycocyanin, are dissolved in trifluoroacetic acid (TFA), the relative intensity of their long-wavelength absorption band decreases. The absorption spectrum eventually becomes comparable to that of the free chromophore. This means that the protein has a synergistic effect on the intensity of the red absorption band of the chromophore. This is shown for phytochrome in Figure 28. Freeze-dried P_R and P_{FR}, the absorption spectra of which are shown, were dissolved in pure TFA. After about 30 min, both solutions have the same absorption spectrum. The effect of TFA on C-phycocyanin and allophycocyanin is similar (see Fig. 29). These pigments were isolated from the blue-green alga, *Plectonema boryanum* (see Section 4.2). Another argument in favour of a specific interaction between chromophore and protein is that, although C-phycocyanin and allophycocyanin have the same chromophore, their absorption spectra differ greatly at long wavelengths.

Whereas the phytochrome chromophore is relatively stable in TFA solution, strong denaturants, particularly combinations of urea with binders of SH groups (e.g. p-chloromercuric benzoate, N-ethylmaleimide), bleach the pigment. This was shown by BUTLER, SIEGELMAN and MILLER([63]) who found that P_{FR} was always bleached faster than P_R. They suggested that the P_R form of phytochrome is more stable to denaturation and that conformational changes might be involved in the phototransformations of the pigment.

In contrast to the above-mentioned conventional denaturing agents that al-

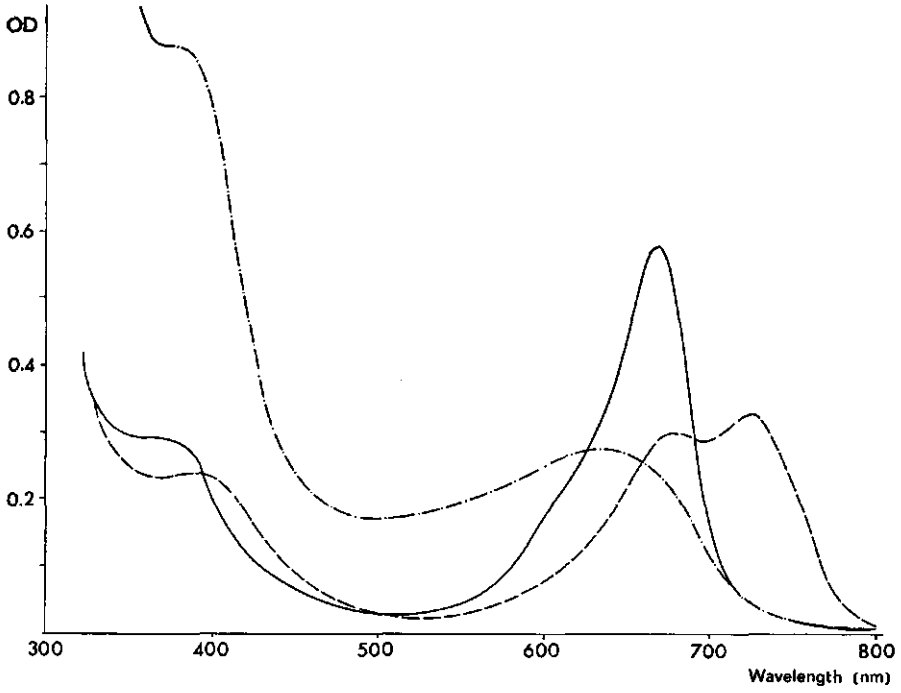


FIG. 28. Effect of trifluoroacetic acid (TFA) on the absorption of phytochrome. P_R (———) P_{FR} (- - - - -) Phytochrome in pure TFA (-.-.-.-.-).

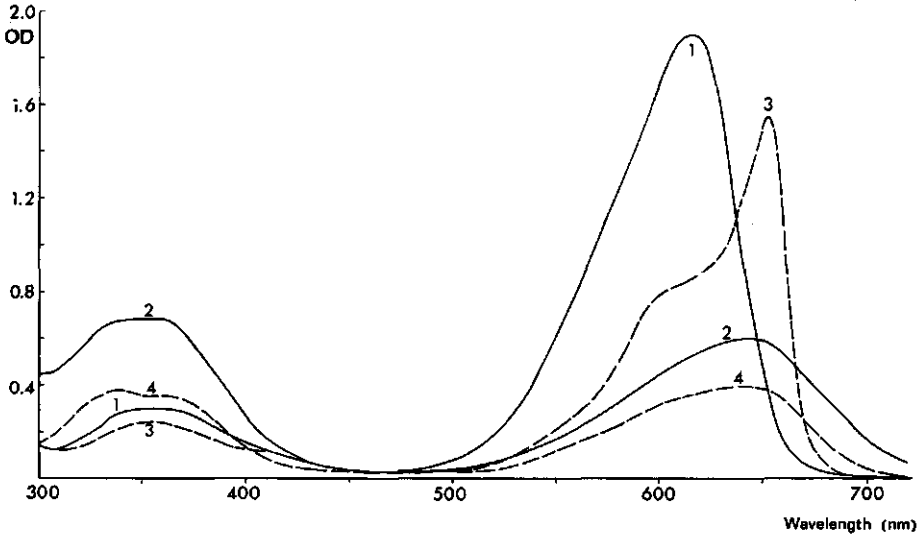


FIG. 29. Effect of trifluoroacetic acid (TFA) on the absorption of C-phycocyanin and allophycocyanin. 1. C-Phycocyanin 2. C-Phycocyanin in pure TFA 3. Allophycocyanin 4. Allophycocyanin in pure TFA.

most entirely disrupt the ordered structure of the native protein, less polar solvents, which are miscible with water, might be expected to induce very mild denaturation. 2-Chloroethanol (2-CE) and dimethylsulfoxide (DMSO) were chosen to test this hypothesis. It is known that the helix content in haemoglobin, as estimated from optical rotatory dispersion measurements, does not decrease upon addition of up to 20% v/v of 2-CE. Only when the proportion of added 2-CE was increased to 50%, did haemoglobin lose part of its helical structure([66]). DMSO forms stronger hydrogen bonds than water and should therefore be able to disrupt the tertiary structure of a protein molecule([67]).

Each of the two solvents was added in increasing proportions to solutions of C-phycocyanin and phytochrome. Effects of 2-CE and DMSO on the long-wavelength absorption bands showed up above a concentration of 10% v/v. (Figs. 30 and 31). For C-phycocyanin, the 620 nm absorption maximum shifts

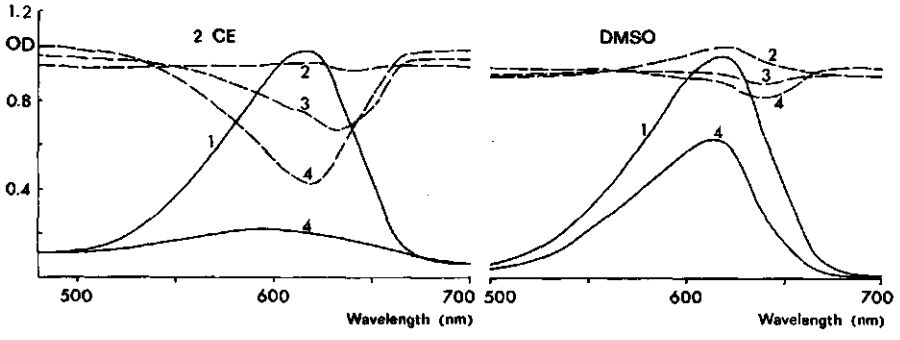


FIG. 30. Effect of 2-Chloroethanol (2-CE) and dimethylsulfoxide (DMSO) on the absorption of C-phycocyanin. Absorption spectra (———) and difference spectra (- - - -) for 0.33 mg/ml C-phycocyanin solution (1) and same solution containing by volume 1% (2), 10% (3), or 25% (4) 2-CE or DMSO.

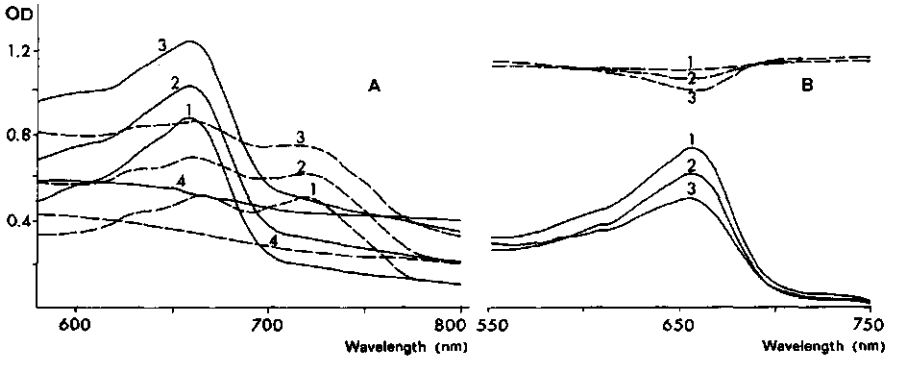


FIG. 31. Effect of 2-Chloroethanol (2-CE) and dimethylsulfoxide (DMSO) on the absorption of phytochrome. A. Absorption spectra of P_R (———) and P_{FR} (- - - -) without 2-CE (1) and containing by volume 1% (2), 2% (3) or 50% (4) 2-CE. B. Absorption (———) and difference (- - - -) spectra for P_R without DMSO (1) or with 10% (2) or 20% (3) DMSO.

to a shorter wavelength (600 nm) and its relative intensity decreases (Fig. 30), indicating disruption of the interaction between chromophore and protein. From the corresponding difference spectra it is clear that 2-CE has a stronger denaturing effect than DMSO. Addition of 1 and 2% 2-CE to solutions of P_R and P_{FR} caused gradual bleaching of both pigment forms, while the pigment lost its colour completely when 50% 2-CE was added (Fig. 31A). The same effect was found for 10–20% DMSO (Fig. 31B). The experiments with both solvents did not suggest that at the concentrations used, P_{FR} was less stable than P_R.

Effects of sodium lauryl sulfate (SLS) on the absorption spectra of phytochrome are illustrated in Figure 32. In this experiment SLS (5×10^{-4}M) was added to solutions of either P_R or P_{FR} and changes in OD_{660} and OD_{730} were measured for about one hour thereafter. The decrease in OD_{660} of the P_R form is slower than the decrease in OD_{730} of the P_{FR} form, which suggest that P_R is more stable to denaturation by SLS than P_{FR}. Ultimately, OD_{660} of both solutions decreases to the same value. The absorption spec-

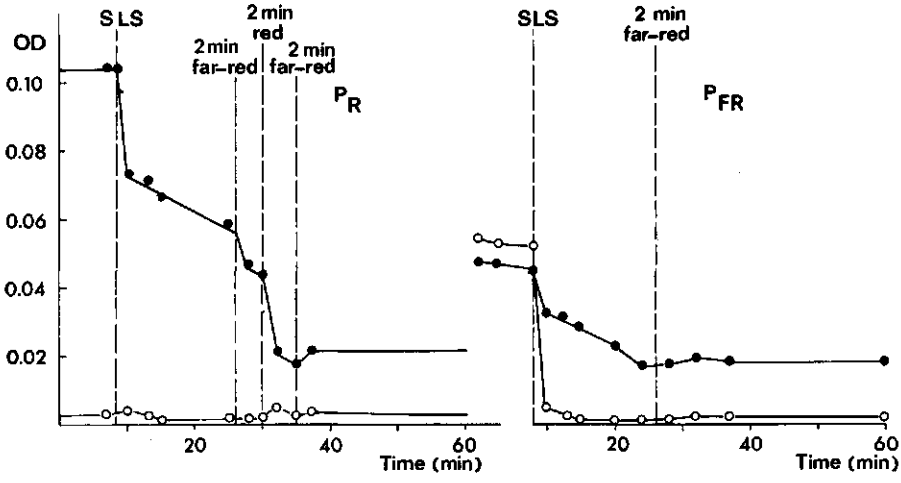
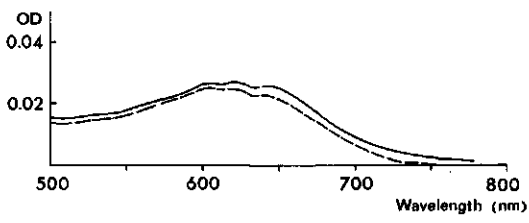


FIG. 32. Denaturation of P_R and P_{FR} with sodium lauryl sulfate (SLS). Concentration of added SLS, 5×10^{-4} M; ●—● OD_{660}, ○—○ OD_{730}.

FIG. 33. Absorption spectra of phytochrome 25 min after addition of sodium lauryl sulfate (SLS). SLS concentration, 5×10^{-4}M; P_R (——), P_{FR} (- - - -).



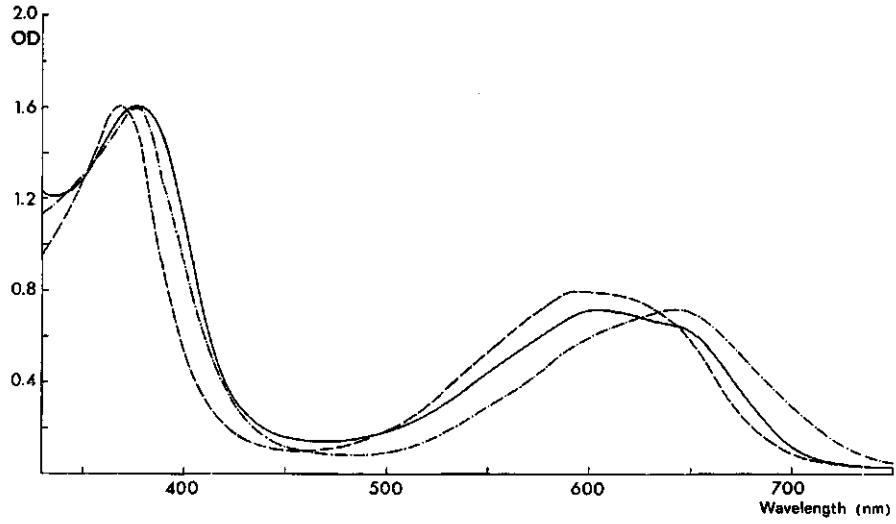


FIG. 34. Absorption spectra of dimethyl esters of bilitrienes. Phytochrome chromophore (———), phycocyanobilin (- - - -), mesobiliverdin (-.-.-.-.-.-).

tra, recorded 25 minutes after addition of SLS, were identical to that of the neutral chromophore (compare Figs. 33 and 34), and reversibility was lost (Fig. 32).

One may conclude that denaturation of the protein destroys the photoreversibility of the pigment, the absorption spectrum becoming like that of the dissociated chromophore.

3.2.3. *Effect of glutaraldehyde*

We also studied the effect of glutaraldehyde (GA), which is commonly used as a cross-linking agent for the stabilization of crystalline proteins. The idea was that cross-links formed between peptide chains should prevent the protein from changing its conformation. The effect of adding 2% v/v of GA to phytochrome solutions in both the P_R and P_{FR} forms was studied as a function of time, while the reversibility of the reaction was also checked at several stages (Fig. 35). When GA was added to a solution of P_R, the absorption maximum shifted immediately from 662 to 655 nm. No further change occurred during a period of 10 min at 15°C in the dark. Irradition of the sample with far-red for 30 min to keep the pigment in the P_R form bleached the pigment, causing a uniform reduction in relative intensity over the width of the 655 nm absorption band. When at this stage the sample was irradiated with red light for 4 min, a further reduction in the intensity of the 655 nm band occurred without an increase in absorption at 730 nm. Irradiation with far-red for 8 min partly reconverted the pigment to the P_{655} form. After dialysis overnight to remove GA, the sample became turbid, but the intensity of the 655 nm band was again reduced upon irradiation with red light.

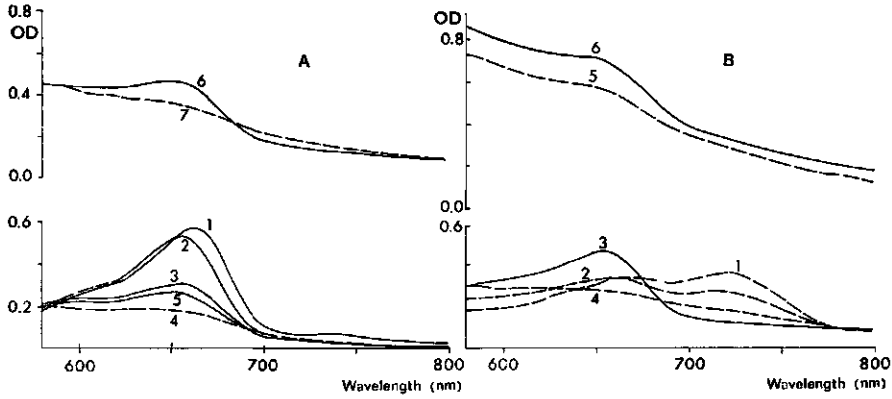


FIG. 35. Effect of glutaraldehyde (GA) on the absorption of P_R and P_{FR}. A. Absorption spectra of P_R without GA (1), and with 2% GA (2–7) in the dark (2), after 30 min far-red (3), 4 min red (4), 8 min far-red (5) and after dialysis (6), and 8 min red (7). B. Absorption spectra of P_{FR} without GA (1), and with 2% GA (2–6), in the dark (2), after 8 min far-red (3), 8 min red (4) and after dialysis (5) and 8 min far-red (6).

A parallel experiment with P_{FR} gave similar results in that, after addition of GA, a decrease of the absorption at 655 nm upon irradiation with red light is no longer accompanied by an increase at 730 nm. The results of these experiments agree with those published recently by CORRELL et al.[48] and suggest that addition of GA prevents P_R from undergoing the conformational change which accompanies its phototransformation to P_{FR}.

3.2.4. *Discussion*

The denaturation experiments indicate that the light absorption of phytochrome and C-phycocyanin is not due to the chromophore alone (Figs. 28 and 29). The red absorption bands of the chromophores are intensified and shifted to longer wavelengths by interaction of the chromophores with their proteins. Presumably the chromophores of phytochrome and C-phycocyanin are coupled to the polypeptide chain by a covalent peptide or ester bond. Since the polypeptide is coiled, other amino acid side chains should be able to approach the chromophores and interact with them. This could explain the observed effects on the absorption of the chromophore. The results of CARRION et al.[68], who studied inter- and intramolecular complexes in polypeptides, suggest that weak charge transfer[69] could be established between the chromophores and the aromatic amino acid side chains of the polypeptide and that this may cause the shift of absorption to longer wavelengths. On the other hand, the increase in absorption intensity and the sharpening of the absorption band may be due to a reduction in the freedom of the electrons in the chromophore when the latter is complexed with the protein.

3.3. Optical rotatory dispersion and circular dichroism

3.3.1. *Principles*

Circular dichroism (CD) and optical rotatory dispersion (ORD) are closely related to ordinary absorption and dispersion. Circular dichroism is a measure of the difference in absorption coefficients of an optically active medium for left and right circularly polarized light. Optical rotation is a measure of the difference in the refractive indices of the medium for the two circularly polarized components. A plot of optical rotation against wavelength gives an optical rotatory dispersion curve. Variations of CD and optical rotation with wavelength give more structural information than ordinary absorption or dispersion about dissymmetric molecules.

A beam of plane-polarized light can be resolved into a right and left circularly polarized component, E_R and E_L, in phase with each other and with equal amplitude (Fig. 36A). When the beam passes through an optically active medium, E_R and E_L will travel with unequal speeds and be unequally absorbed on account of the difference between the refractive indices, n_R and n_L, and between the absorption coefficients, ε_R and ε_L, of the medium for the two components. Thus, a difference is established between both the amplitude and the phase of the transmitted components and the resultant light beam is elliptically polarized (Fig. 36B). The angle α, through which the major axis of elliptical vibration is rotated, is called the optical rotation. The ratio of the minor to the major axis of elliptical vibration, which is given by tangent Θ, is a measure of circular dichroism. The abnormal optical rotation in the vicinity of an absorption band of an optically active material is known as the Cotton effect. Unlike absorption, CD and optical rotation may assume positive or negative values (Figs. 37A and 37B).

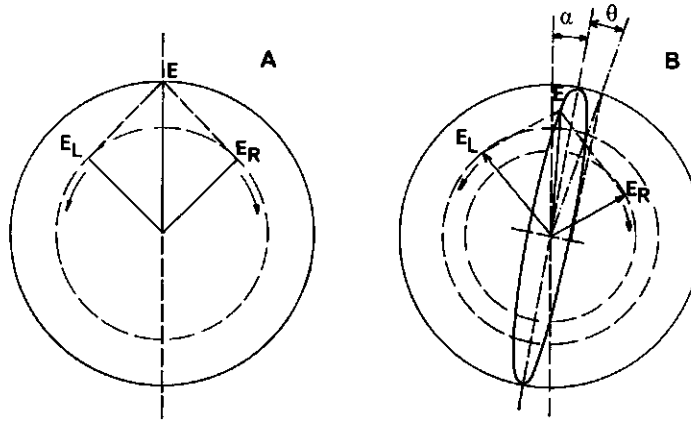


FIG. 36. Effect of an optically active medium on a light beam. A. Incident light, plane polarized. B. Transmitted light, elliptically polarized; α = optical rotation, Θ = ellepticity.

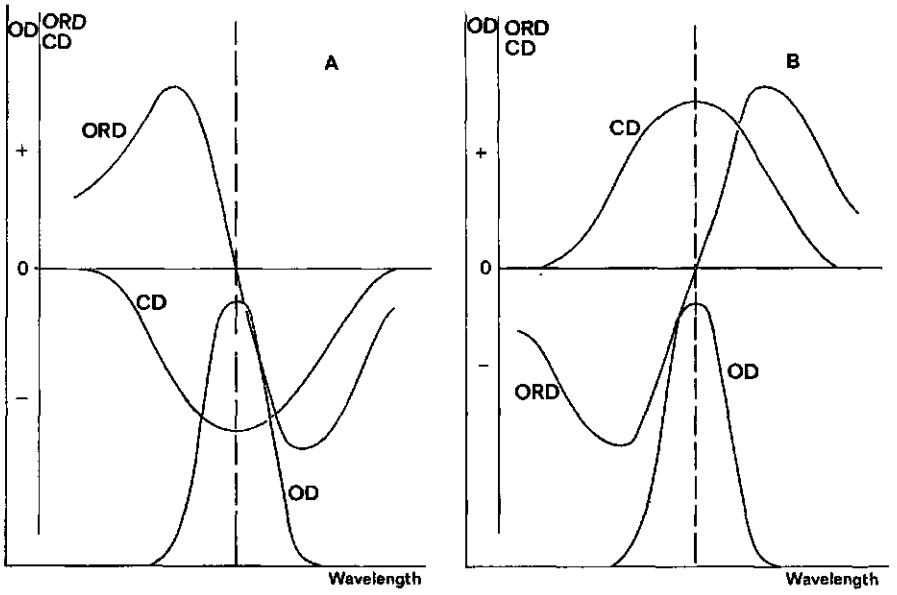


FIG. 37. Behaviour of the circular dichroism (CD) and optical rotation (ORD) near an absorption band of an optically active material. A. Negative Cotton effect. B. Positive Cotton effect.

For structural and stereochemical investigations, ORD and CD are equally useful and, to a large extent, complementary. Background effects, due to optical rotation of the protein part, however, often show up in the ORD of visible absorption bands but not in CD curves. The precise location of the Cotton effects in CD curves is particularly useful in identifying overlapping or hidden optically active transitions.

Cotton effects in proteins may be due to several phenomena([70]):

a. Cotton effects in the peptide absorption band in the far-ultraviolet are due to the secondary structure of the peptide chain. These effects are conformation-dependent and vanish in the random coil. The percentage of α-helix in a polypeptide chain, for instance, can be calculated from the Cotton effect at 233 nm([71]).
b. Binding of dyes or chromophores to a helical structure can induce a Cotton effect in their absorption bands([72]). When the dyes are not themselves optically active, it is assumed that they are assymmetrically bound to the peptide helix.
c. Likewise, prosthetic groups are often asymmetrically bound and display Cotton effects at the wavelengths of their most prominent absorption bands, e.g. haemocyanin([73]), cytochrome c([74]), and ferredoxin([75]).
d. The binding of co-enzymes containing chromophores to their apo-enzymes can cause a Cotton effect. This effect was observed when flavine adenine dinucleotide is bound to D-amino acid oxidase([76]).

Particularly relevant to the phytochrome problem are the experiments of STRYER and BLOUT[72]. They showed that when symmetrical (optically inactive) dye molecules are bound to synthetic helical polypeptides, a Cotton effect is induced in the absorption band of the dyes. No Cotton effect was observed in complexes with randomly coiled polypeptides. The molar rotations of these induced Cotton effects may be exceptionally large. For a particular absorption band, the sign of the induced Cotton effect for L-polypeptides is opposite to that for D-polypeptides.

MOSCOWITZ et al.[77] measured the optical rotation of some urobilins. These tetrapyrroles are called bilidienes because they consist of two dipyrrylmethenes connected by a saturated bridge. An extremely high Cotton effect was found in the 490 nm absorption band when the urobilins were dissolved in solvents, such as chloroform, which promote intramolecular hydrogen bonding. According to the authors, this is due to the inherent dissymmetry of the dipyrrylmethene configuration. In the cyclic configuration of the molecule, dissymmetry is accentuated because steric hindrance prevents the keto groups of the end rings from being coplanar. In accordance with this hypothesis, it could be shown that the destruction of internal hydrogen bonding, due to external competitition from CH_3OH or CCl_3COOH, reduced the size of the Cotton effects. Metal ions, such as Zn^{++}, brought about the same result.

BACON KE[78] reported the enhancement of the intrinsic Cotton effects of chlorophylls and carotenoids when they were complexed with lipoproteins in chloroplasts. This suggests a strong interaction either between the pigment molecules themselves or between the pigment molecules and the attached macromolecules. The former explanation is supported by SAUER[79] who found that the optical rotation of chlorophylls was enhanced at high concentrations in chloroform solution. He attributed this effect to chlorophyll aggregation and felt that a similar pigment-pigment interaction could also account for the enhanced optical rotation observed in quantasomes (chloroplast fragments).

3.3.2. *Experimental*

Optical rotatory dispersion and circular dichroism up to 700 nm of solutions of phytochrome and C-phycocyanin were measured by the author with a Durrum-Jasco ORD recorder at the laboratory of Molecular Biology of the E.T.H. Zürich. These measurements were only partly successful, because the absorption band of P_{FR} is beyond the 700 nm limit of the instrument. Moreover the noise level of the instrument, even in the 600–700 nm region, was very high. We were able to measure the ORD of both the P_R and P_{FR} forms of the pigment up to 800 nm in a very sensitive spectropolarimeter built by Dr. EMEIS of the Department of Theoretical Organic Chemistry of Leyden University[80]. The CD spectra of phytochrome and C-phycocyanin over the range of 300–800 nm were recorded with a Roussel-Jouan dichrograph of the same laboratory.

In the experiments with the Durrum-Jasco recorder red and far-red light for irradiating the samples was provided by a low intensity slide projector and line filters of 665 and 725 nm, as described in 2.2.3, while in the experiments with

the Roussel-Jouan dichrograph band filters (type AL, Schott, Mainz) were used. Maximum transmittance of the two band filters was 57% at 660 nm and 52% at 720 nm and the bandwidth of the resultant light beams was 20 nm.

ORD and CD of phytochrome were recorded in a solution with specific activity of about 0.25 $\Delta(\Delta OD)$/cm.mg protein and a protein concentration of 2 mg/ml. The specific activities used for the measurement of ORD in the spectropolarimeter and CD in the Roussel-Jouan dichrograph were 0.075 and 0.792 $\Delta(\Delta OD)$/cm.mg protein respectively. C-phycocyanin was isolated from freeze-dried blue-green algae (*Plectonema boryanum*) by ammonium sulfate fractionation and calcium phosphate chromatography. The C-phycocyanin fractions were purified until an OD_{620}/OD_{280} value of 4–5 (see 4.2). For the calculation of phytochrome activity, optical densities at 665 and 725 nm were measured with a Zeiss PMQ II spectrophotometer. Absorption spectra were measured in a Unicam SP 800 recording spectrophotometer.

3.3.3. *Optical rotatory dispersion*

The ORD curves for P_R (Fig. 38) show a negative Cotton effect in the red absorption band, with a point of inflection at 664 nm. The higher the absorption at 662 nm, the greater the Cotton effect (curve 3). When P_R is converted to P_{FR},

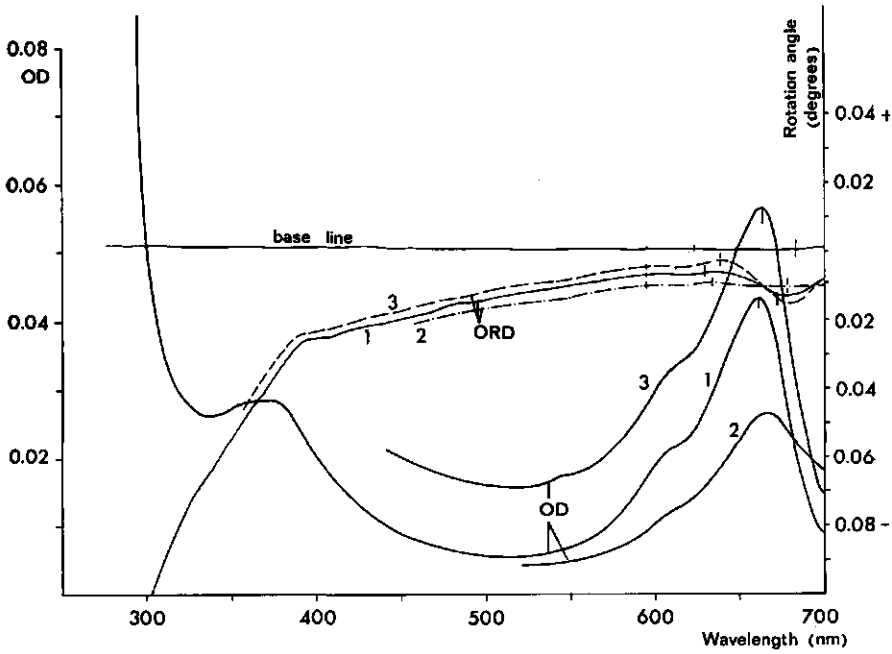


FIG. 38. Optical rotatory dispersion (ORD) curves and absorption spectra of phytochrome. After 2 min far-red irradiation, $P_R + P_{FR}$ (1), after 4 min red irradiation, $P_{FR} + P_R$ (2). After 4 min far-red irradiation, P_R (3). N.B. The short vertical lines on the ORD and CD curves in Figures 38, 39, 40 and 41 are a measure of the noise of the signal.

the Cotton effect at 664 nm disappears (curve 2), while it is assumed that a second Cotton effect will be found at the maximum of the far-red band. As previously mentioned, however, the Durrum-Jasco apparatus can only measure up to 700 nm. In the 600–700 nm region the noise level is fairly high and may, moreover, be increased by fluorescence, as BERNS et al.([81]) assumed to be the case with the Cotton effect in the 620 nm band of C-phycocyanin. Repeated scanning showed the Cotton effects to be reproducible. A second Cotton effect at the blue absorption of P_R can be seen in curves 1 and 3. This effect is superimposed upon that of the protein and is, therefore, not very clear. The ORD of the protein is due to the inherent asymmetry of its secondary structure, e.g. α-helix or β-conformation.

The CD curve of P_R (Fig. 39) shows that the Cotton effect at 370 nm is positive. The curve confirms that the Cotton effect of P_R at 665 nm is negative, while another negative effect is evident at 610 nm corresponding to the shoulder in the absorption peak. We did no try to record the ORD and CD of phytochrome solutions in the ultraviolet; the high OD at 280 nm indicated that the level of proteinaceous impurity was too high for good resolution in this wavelength range.

Figure 40 shows the long-wavelength ORD curves of P_R and P_{FR}, measured in the spectromolarimeter of EMEIS. The curve for P_R shows a negative Cotton effect with a point of inflection somewhat below 660 nm. The curve for P_{FR} is distinctly different form the P_R curve and shows a positive Cotton effect with a point of inflection around 710 nm. The ORD curve for P_{FR} is more uncertain

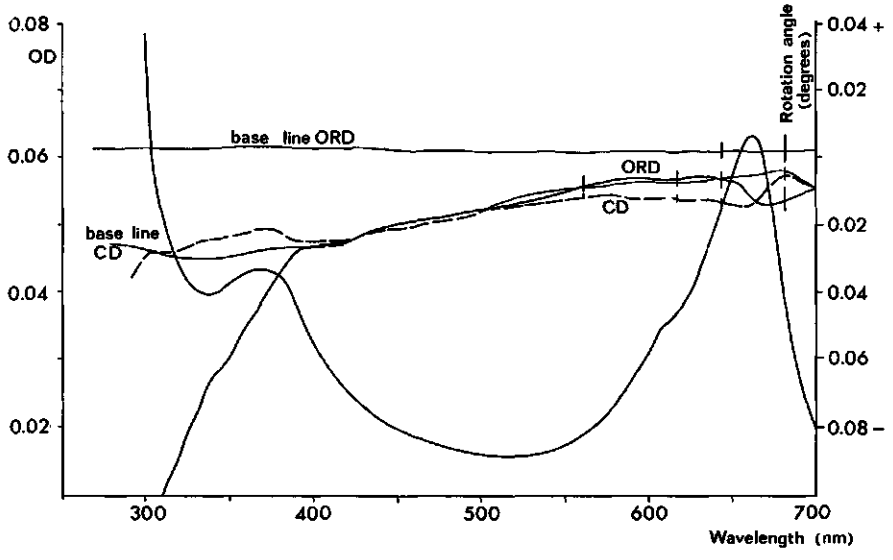


FIG. 39. Optical rotatory dispersion, circular dichroism, and absorption of P_R. Preparation irradiated for 4 min with far-red light. CD scale: 0.1 = ΔOD 0.005.

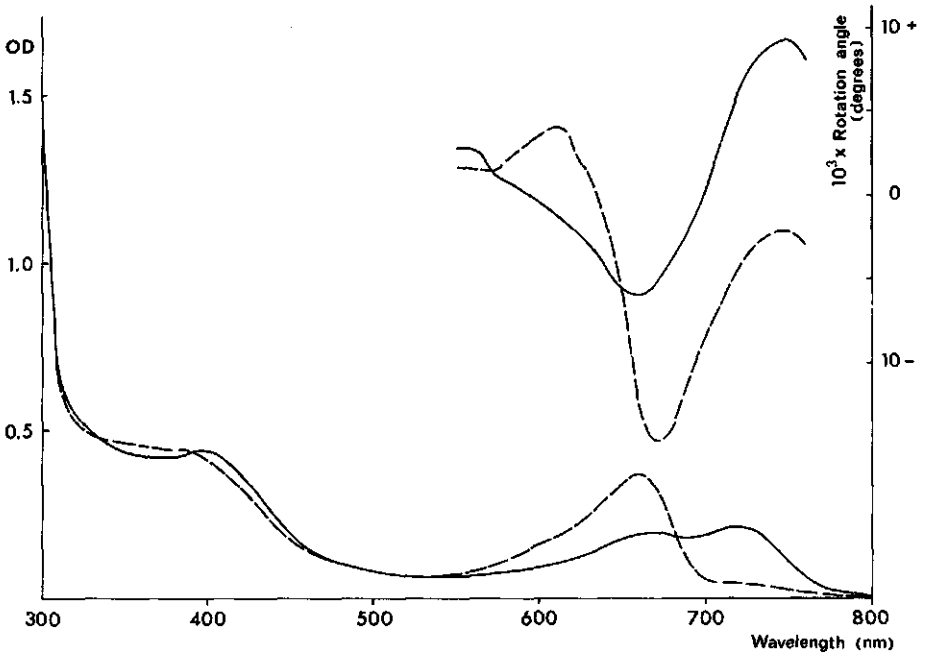


FIG. 40. Absorption spectra and long-wavelength ORD curves of phytochrome. P_R (- - - -), P_{FR} (———).

than that for P_R, because the pigment cannot be completely converted into the P_{FR} form (see 2.3.8). The absolute value of the rotation in this experiment is unknown since the optical rotation is superimposed upon that of the protein. This problem does not arise with the CD measurements.

ORD of *Plectonema* phycocyanin was measured at two concentrations, corresponding to OD_{618}/cm values of 0.850 and 2.830. The ORD curves (Fig. 41) show a positive Cotton effect in the red absorption band with a point of inflection at 626–628 nm and a negative Cotton effect in the blue absorption band with a point of inflection at about 340 nm. Compared with the maximum of the red absorption band of C-phycocyanin at 618 nm, the point of inflection of the positive Cotton effect is shifted by 8–10 nm to the long-wavelength end of the spectrum.

3.3.4. *Circular dichroic spectra*

Tracings of the original CD spectra of P_R and P_{FR} are reproduced in Figure 42. A negative circular dichroic effect for P_R is found around 660 nm, while there is no optical activity in the 725 nm region. As already mentioned, irradiation with red light for 4 minutes converts the complex into a photostationary state with about 80% P_{FR} (see 2.3.8). The presence of non-transformed P_R is

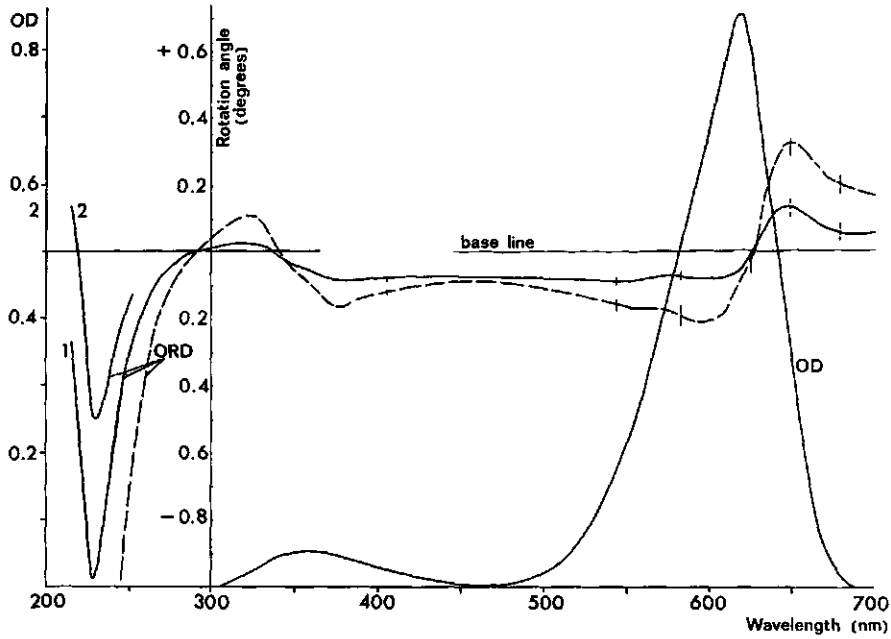


FIG. 41. Absorption spectrum and optical rotatory dispersion curves of *Plectonema* C-phycocyanin. Absorption and ORD (———) curve for solution with $OD_{618}/cm = 0.850$, and ORD (- - - -) curve for solution with $OD_{618}/cm = 2.830$. ORD scale = 1.0 = 0.1° (1) or 0.2° (2).

evident from the absorption spectra of P_{FR} in Figure 43. Accordingly, the circular dichroic spectrum of P_{FR} displays a residual negative value at 660 nm, while the 725 nm absorption band of P_{FR} is associated with a positive circular dichroic effect (Fig. 42). By irradiating phytochrome in the P_{FR} form with far-red light it could be shown that the reverse reaction from P_{FR} to P_R is accompanied by a change in circular dichroism from a positive effect above 700 nm to a negative effect near 660 nm. No circular dichroism remains above 700 nm after the transition, in agreement with the assumption that P_{FR} is completely converted to P_R. The blue absorption band of P_R shifts to the red by about 15 nm (375 to 390 nm) upon conversion to P_{FR}. The blue absorption band of P_R exhibits positive circular dichroism, which diminishes upon irradiation with red light (Fig. 42). The effect, however, does not change sign, but remains positive and finite. The circular dichroic tracings have been redrawn in Figure 43, to enable comparison with the absorption spectra of P_R and P_{FR}.

We also measured the CD of C-phycocyanin and that of its chromophore phycocyanobilin. The results are shown in Figure 44. In contrast to P_R, the long-wavelength absorption band of C-phycocyanin is associated with a positive Cotton effect. The effect remains positive when the pigment is dissolved in trifluoroacetic acid. The completely dissociated chromophore, dissolved in chloroform, however, has a negative Cotton effect of lower intensity. The blue absorption band of C-phycocyanin was associated with a negative Cotton

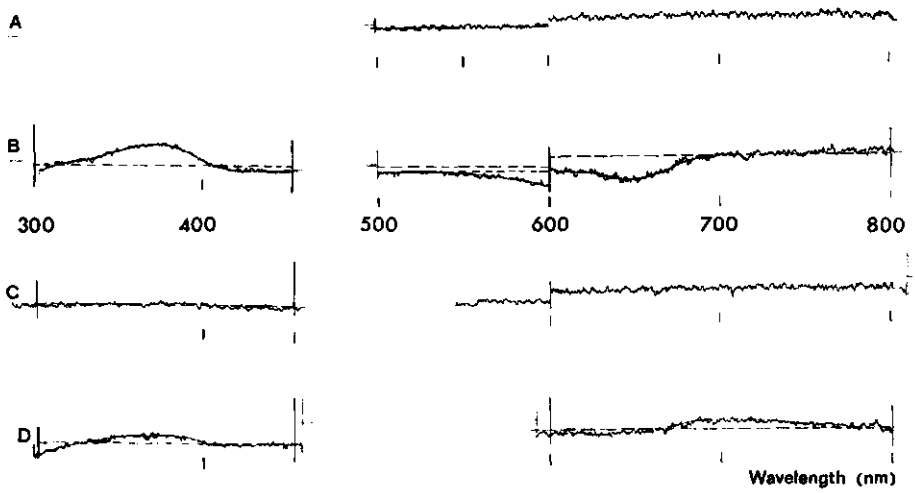


FIG. 42. Circular dichroic tracings for phytochrome. A. Base line. B. P_R, after 4 min far-red irradiation. C. Base line. D. P_{FR}, after 4 min red irradiation.

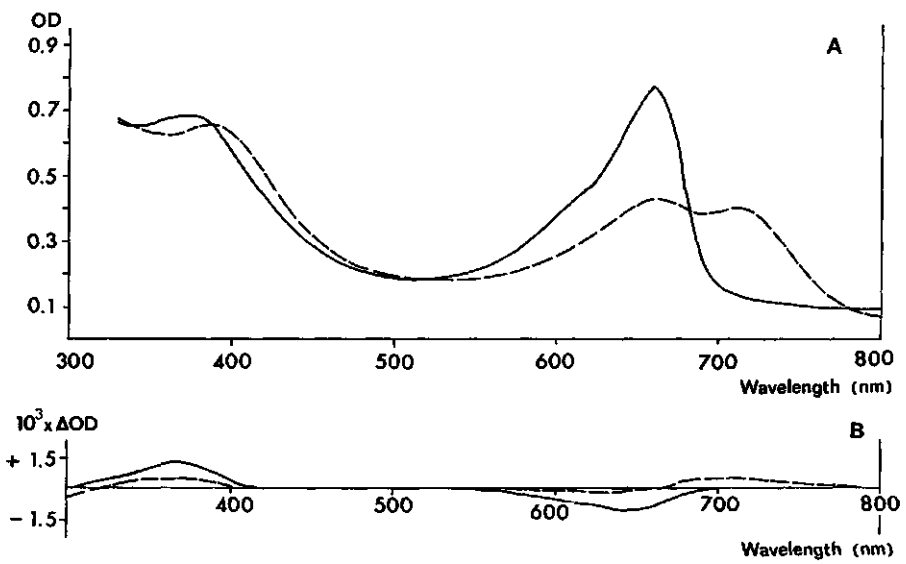


FIG. 43. Absorption spectra and circular dichroic spectra of phytochrome. A. Absorption B. Circular dichroism; P_R (———), P_{FR} (- - - - -).

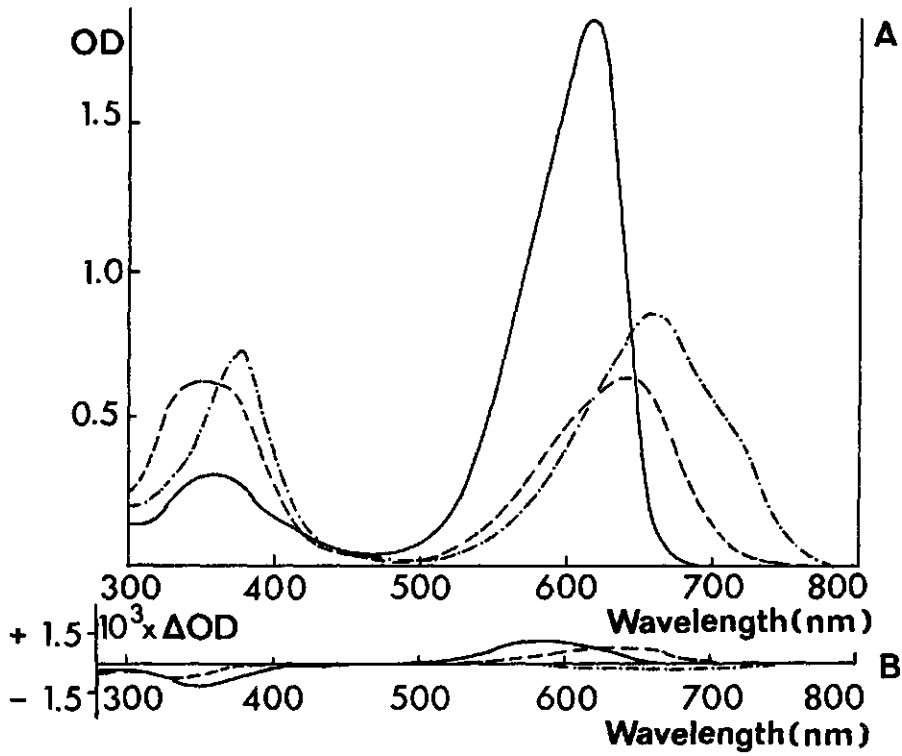


FIG. 44. Absorption spectra and circular dichroic spectra of *Plectonema* C-phycocyanin and its chromophore, phycocyanobilin. A. Absorption B. Circular dichroism. C-phycocyanin in potassium phosphate buffer (———) and in trifluoroacetic acid (- - - -); phycocyanobilin in acid chloroform (-.-.-.-.-).

effect, again of a sign opposite to that of P_R. No Cotton effect could be detected for the free chromophore in the blue absorption band.

3.3.5. *Discussion*

The ORD and CD measurements with phytochrome show that P_R and P_{FR} are optically active in their red and blue absorption bands. Moreover, changes in the optical activity occur when P_R is transformed to P_{FR} or vice versa. The fact that the Cotton effects associated with the long-wavelength absorption bands of P_R and P_{FR} are opposite in sign means that the chromophore is attached to the protein part in a different way in the two pigment forms. The observed optical effects, however, are small. If we assume that the phytochrome chromophore has a bilitriene structure([82]), it is likely that the optical acitvity of the chromophore is enhanced by its association with the protein in a way similar to that envisaged by MOSCOWITZ([77]). The fact that the molecules of most bilitrienes are symmetrical also supports this argument.

The ORD and CD spectra of C-phycocyanin and its chromophore yielded important information about the origin of the optical activity. The intensities of the Cotton effects in C-phycocyanin are almost equal to those in phytochrome, although in C-phycocyanin each protein unit contains 2–3 chromophores[83, 84, 85], while phytochrome, judging from its specific absorbancy, contains no more than one chromophore per protein unit. The optical effects of C-phycocyanin are of a sign opposite to those of P_R throughout the whole visible spectrum. The Cotton effect at 660 nm of the free protonated chromophore of C-phycocyanin in chloroform is much less intense than, and of opposite sign to that of the intact pigment. The low optical activity of the free chromophore is in accordance with the structure of the phycocyanobilin diester, which was recently elucidated by SIEGELMAN[86] and RÜDIGER[87] (see Chapter 4). The molecule of phycocyanobilin contains only one asymmetric C-atom, situated in one of the outer rings. Thus, it is evident that the optical activity of the pigment in the red absorption band is largely induced by the association of the chromophore with the protein moiety. Recent experiments of BOUCHER, CRESPI and KATZ[88] confirm these conclusions. They found that the intensity of the Cotton effect of C-phycocyanin at 620 nm gradually decreases upon denaturation with increasing amounts of urea (2 to 8 M).

The parallel between the optical properties of C-phycocyanin and its chromophore and those of phytochrome tends to confirm the hypothesis that the optical activity of the latter is due to asymmetric bonding between the protein moiety and a chromophore which itself has little or no optical activity. In a subsequent experiment, we recorded the CD spectra of P_R and P_{FR} in the ultraviolet, because Cotton effects in this region of the spectrum are associated with the secondary structure (α-helix, β-conformation, or random coil) of the protein part. No significant difference between the spectra of P_R and P_{FR} in this region could be detected. HOPKINS and BUTLER[89] recently did the same experiment and reported that there was a slight shift of 2 nm in the Cotton effect associated with the protein band at 222 nm. This indicates that the overall change in the conformation of the protein part during the phototransformation of phytochrome is very small.

It is interesting to note that the optical activity of rhodopsin, the visual pigment of the retina, is also attributed to the association of the chromophore with the protein. Circular dichroic spectra of rhodopsin show that the optical activity is lost when the pigment is bleached[90, 91]. Certainly, there are some striking similarities between the behaviour of phytochrome and rhodopsin.

The experiments described so far have yielded only indirect information about conformational changes in the protein part of phytochrome. It appears that these changes are very small and may be compared with the allosteric transitions in other protein pigments and in certain enzyme systems. Recent evidence indicates that, with haemoglobin for instance, even small changes in the protein conformation are of essential significance in the biological functioning of the molecule[92].

3.4. TRANSFORMATIONS AT LOW TEMPERATURE

3.4.1. *Principles*

The absorption bands of protein-pigment complexes in the ultraviolet and visible regions often become sharper at very low temperature, e.g. the temperature of liquid nitrogen. The effect is most prominent with extensively-conjugated chromophores which absorb in the long-wavelength part of the visible spectrum. For this reason, low-temperature techniques have been employed, for instance to detect minute differences in the absorption spectra of haemoglobins and cytochromes([93, 94]). In addition, short-lived intermediates may be stabilized at low temperature([95, 96]) and rapid transitions may be slowed down([97]).

The measurements of the optical activity of phytochrome suggested an interaction between chromophore and protein. Spectral shifts caused by irradiation of the pigment may, therefore, be partly due to conformational changes in the protein part of the pigment complex. Low-temperature studies enable such changes to be distinguished from those of the chromophore alone, since the protein is prevented from assuming different conformations. This, together with the possibility of detecting short-lived intermediates in the chromophoric transition, prompted an analysis of low-temperature absorption spectra.

SPRUIT([98–101, 37, 62]) had already applied the low-temperature technique in his extensive study of etiolated plant parts and extracts containing phytochrome. We were extremely interested to know whether the spectra in vivo were identical with those of purified phytochrome at low temperature. This led to a close collaboration with Dr. SPRUIT for this phase of the study, in which his apparatus([98]) was used to record the spectra of our highly purified phytochrome solutions. The technique followed, was the one applied by SPRUIT([99, 102]), and is briefly described below.

3.4.2. *Experimental*

The instrument used was a Cary 14 double-beam recording spectrophotometer with which difference spectra arising from phototransformations of the pigment at low temperature could be directly recorded. To this end the instrument was fitted with a scattered transmission accessory and with an optical system for actinic irradiation of the samples. The sample cells were irradiated from the front with a 500-W slide projector through interference filters with maximum transmission at 658 and 737 nm. An EMI 9558 B photomultiplier was used to measure light absorption. For measurements at the temperature of liquid nitrogen, the sample holder was incorporated in a Dewar flask equipped with windows to enable the light beam to pass through (Fig. 45). The brass sample holder is filled with liquid nitrogen. The samples are not themselves immersed in the liquid nitrogen but are cooled by conduction through the metal of the holder. When necessary, the cells were warmed by blowing air through the liquid nitrogen in order to evaporate it. The temperature could be measured with a thermocouple, soldered to the lower edge of the sample compartment.

The phytochrome solutions used for the low-temperature measurements were

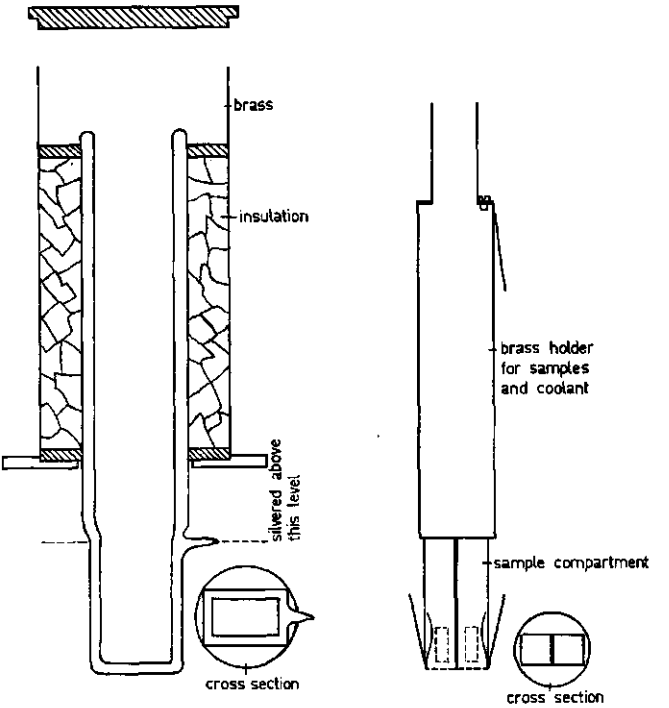


FIG. 45. Liquid nitrogen Dewar and sample holder for measurements of difference spectra at low temperature, according to SPRUIT[102].

made up with buffer containing about 80% v/v glycerol[99]. Such solutions do not crystallize when cooled in polyethylene cells. In several cases, however, an air channel was formed in the centre of the cell when the solutions were frozen to −196° for a second time, probably as a result of contraction of the glycerol solution. This caused a shift in the base line of the difference spectrum.

Phytochrome was purified by the procedure described in Section 2.2 and by subsequent gradient elution on DEAE-Sephadex. Two fractions were obtained: fraction 1, which was eluted at very low salt concentration, and fraction 2, which was eluted at a higher salt concentration. Then both fractions were gel filtrated over Sephadex G-200. Activities of the fractions before and after dilution with glycerol are given in Table 14. Two experiments were performed, each of them with both fraction 1 and fraction 2.

Experiment 1 dealt with the conversion of P_R into P_{FR}. The phytochrome solutions in both cells were brought into the P_R form by irradiating them for 3 min at room temperature with far-red light of wavelength 737 nm. They were then cooled to −196°C with liquid nitrogen and held at this temperature for 45 min to reach thermal equilibrium. The zero line was measured at this stage, since both cells then contain P_R. One cell was irradiated for 3 min with red light

TABLE 14. Activity of phytochrome fractions before and after dilution with glycerol

Experiment no.	Fraction no.	Dilution			Activity Δ(ΔOD)/cm		Specific activity [Δ(ΔOD)/cm.mg protein]
		Vol. of sample (ml)	Vol. of buffer (ml)	Conc. of glycerol in buffer (% v/v)	Before dilution	After dilution	
1	1	1.0	6.0	88	1.090	0.156	0.066
2	1	2.0	6.0	80	1.090	0.273	0.066
1	2	2.2	5.0	75.5	0.657	0.201	0.070
2	2						

of 653 nm and, with the other non-irradiated cell as a reference, the first difference spectrum was recorded. Both cells were then warmed to −15 °C and left for 30 min to attain equilibrium. They were cooled again to −196 °C and left for 45 min, after which the second difference spectrum was measured.

In experiment 2, the conversion of P_{FR} into P_R was studied. The procedure was exactly the same, except that the pigment was first brought into the P_{FR} form at room temperature, and that, after the cells had been cooled to −196 °C, one of them was irradiated with far-red light.

3.4.3. *Results*

The results of these experiments are shown in Figures 46–49. When interpreting the difference spectra, one must bear in mind that positive peaks are associated with pigment forms that appear during the reaction step, while negative peaks are associated with pigments forms that disappear. When P_R (fraction 1) is transformed with red light at −196 °C, a new pigment form appears with an absorption maximum at 693 nm (P_{693}); this form is then converted to normal P_{FR} in a sequence of dark reactions as the solution is warmed to −15 °C (Fig. 46). Such an intermediate, with a discrete absorption maximum in between those of P_{FR} and P_R, is not formed in the reverse reaction, $P_{FR} \rightarrow P_R$. Instead, irradiation with far-red light at −196 °C causes the direct formation of a broad absorption peak with a maximum at 663 nm. When the phytochrome solution is warmed to −15 °C in the dark, this broad absorption peak narrows and intensifies to give the normal P_R peak at 665 nm (Fig. 47).

Thus, there is an essential difference between the two reactions in that only the $P_R \rightarrow P_{FR}$ transformation proceeds by way of an unstable intermediate (P_{693}). In the reverse reaction it seems that the chromophore is transformed directly into the P_R form, and that subsequent warming merely brings about the stabilization of the chromophore by the protein part to yield the P_R complex. This is reflected by the gradual sharpening of the absorption band at 665 nm.

Fraction 2 behaved in exactly the same way as fraction 1 in the $P_R \rightarrow P_{FR}$ transformation (Fig. 48). In the reverse reaction, however, fraction 2 gave a different result. Although P_{663} was formed directly from P_{FR} as before, it was

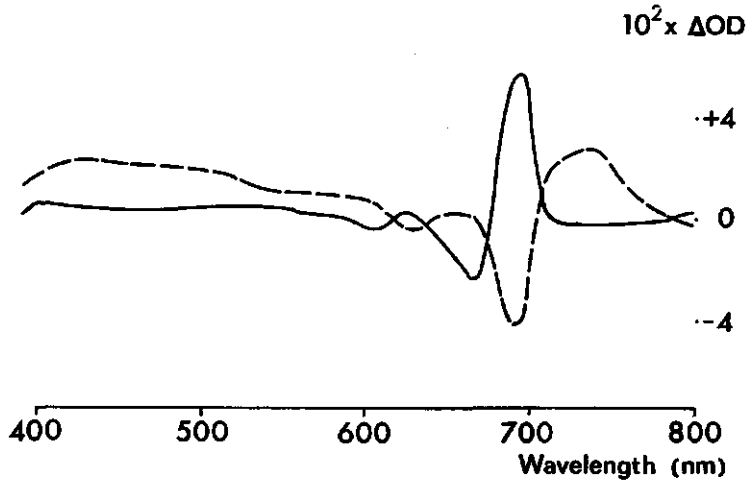


FIG. 46. Low-temperature transformation of P_R in DEAE-Sephadex fraction 1. Difference spectra recorded after irradiation for 3 min with red light at –196 °C, $P_R \rightarrow P_{693}$ (———), and after subsequent warming to –15 °C, $P_{693} \rightarrow P_{FR}$ (- - - -).

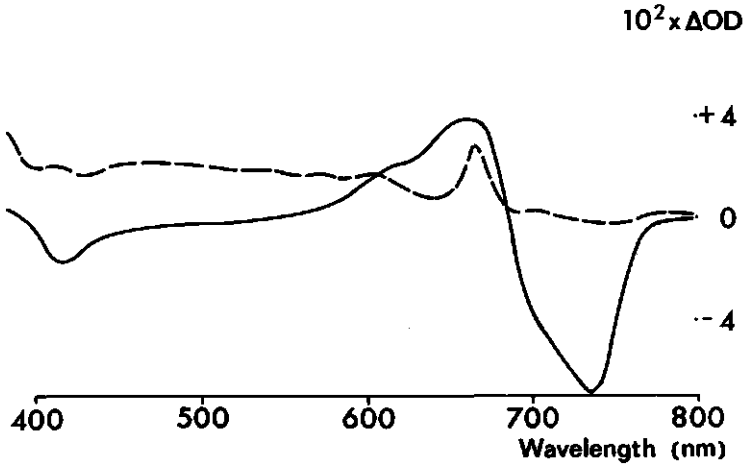


FIG. 47. Low-temperature transformation of P_{FR} in DEAE-Sephadex fraction 1. Difference spectra recorded after pretreatment for 3 min with red light, and irradiation for 3 min with far-red light at –196 °C, $P_{FR} \rightarrow P_{663}$ (———), and after subsequent warming to –15 °C, $P_{693} \rightarrow P_R$ (- - - -).

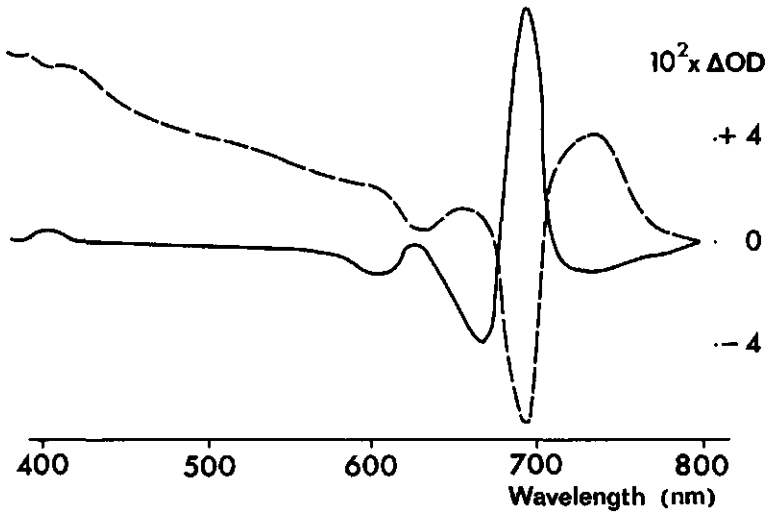


FIG. 48. Low-temperature transformation of P_R in DEAE-Sephadex fraction 2. Difference spectra recorded after irradiation for 3 min with red light at –196 °C, $P_R \rightarrow P_{693}$ (———), and after subsequent warming to –15 °C, $P_{693} \rightarrow P_{FR}$ (- - - -).

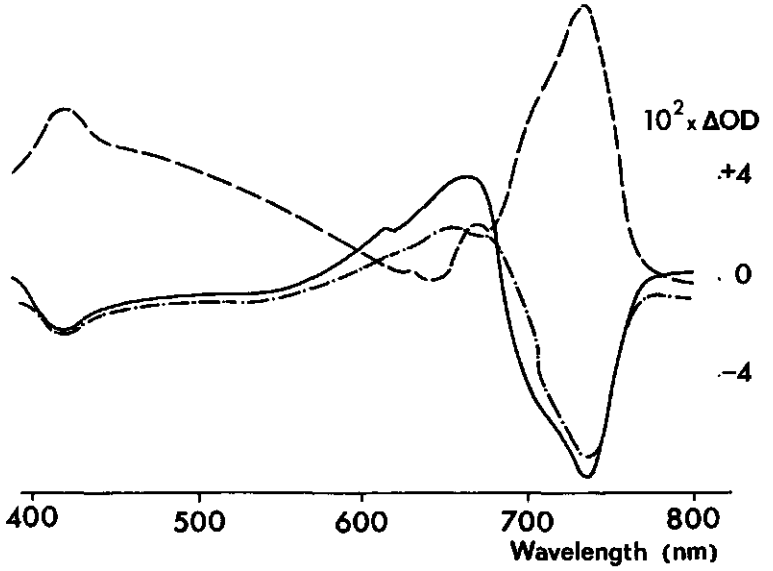


FIG. 49. Low-temperature transformation of P_{FR} in DEAE-Sephadex fraction 2. Difference spectra recorded after pretreatment for 3 min with red light, and irradiation for 3 min with far-red light at –196 °C, $P_{FR} \rightarrow P_{663}$ (———), and after subsequent warming to –15 °C, $P_{663} \rightarrow P_{FR}$ (- - - -), and after irradiation for 3 min with red light at –196 °C, $P_{FR} \rightarrow P_{663}$ (– . – .).

not completely converted into P_R upon being warmed: instead much of the P_{FR} was regenerated (Fig. 49). When this P_{FR} was irradiated with red light ($\lambda = 653$ nm) at -196 °C, it was transformed once again to P_{663}, so that under these conditions red and far-red light have the same effect (Fig. 49).

Additional experiments were performed with fractions 1 and 2 from another phytochrome isolation to see whether the abnormal behaviour of fraction 2 could be reproduced. Fraction 1 [activity after dilution, 0.165 $\Delta(\Delta OD)$/cm; specific activity, 0.024 $\Delta(\Delta OD)$/cm.mg protein] was converted into the P_{FR} form, irradiated at -196 °C with far-red light, warmed to -20 °C in the dark and cooled again to -196 °C. Fraction 2 (activity after dilution, 0.202; specific activity, 0.057) underwent the same treatment, except that it was warmed only to -64 °C. Difference spectra were recorded as before. In both cases P_{FR} was transformed directly into P_{663} as before. Upon warming, fraction 1 showed an increase in absorption at 673 nm, instead of at 665 nm, and in the far-red (Fig. 50). A similar result was obtained when fraction 2 was warmed, although regeneration of P_{FR} was more pronounced and the red band sharpened at 667 nm, the normal maximum of P_R (Fig. 51). P_{FR} is always partly bleached when irradiated with far-red at -196 °C and one may suppose that the resulting absorption spectrum is comparable to that of the dissociated chromophore.

The results of these additional experiments suggest that the regeneration of P_{FR} during the dark reaction is not peculiar to fraction 2, but possibly depends on the state of denaturation of the pigment fraction used for the measurement. The second DEAE-Sephadex fractions apparently contain an amount of altered pigment for which a conformational change in the dark during the $P_{FR} \rightarrow P_R$ transformation is more difficult.

3.4.4. *Discussion*

The results with purified phytochrome correlate with those in vivo. In his measurements with pea plumules, SPRUIT[99] showed that P_R is transformed into P_{FR} by way of an intermediate form (P_{698}), which is converted into P_{FR} upon warming to -20 °C (Fig. 52). In the reverse reaction, P_{FR} is transformed to a form with a broad absorption band around 650 nm and is partly bleached. Upon warming, P_{FR} is partly regenerated (Fig. 53). The spectra in vivo are not fully comparable with those of purified phytochrome, since the warming-up cycle began at -70 °C and not at -196 °C. The results nevertheless suggest that the intermediate P_{698} is stable up to -70 °C in vivo. SPRUIT concluded that the photoreversibility of phytochrome involves two pigment system: $P_R \leftrightharpoons P_{698}$ and $P_{650} \leftrightharpoons P_{FR}$. Conversions within each system are supposed to be possible below -70 °C, whereas interconversion between the two systems is only possible above -70 °C. This interpretation was principally chosen to explain why P_{FR} is regenerated when a phytochrome solution, irradiated with far-red light, is warmed up in the dark.

As already mentioned, one may tentatively conclude that the $P_R \rightarrow P_{FR}$ reaction involves the formation of an intermediate, in which the chromophore is isomerized; this is followed by the transformation of the protein part. There is

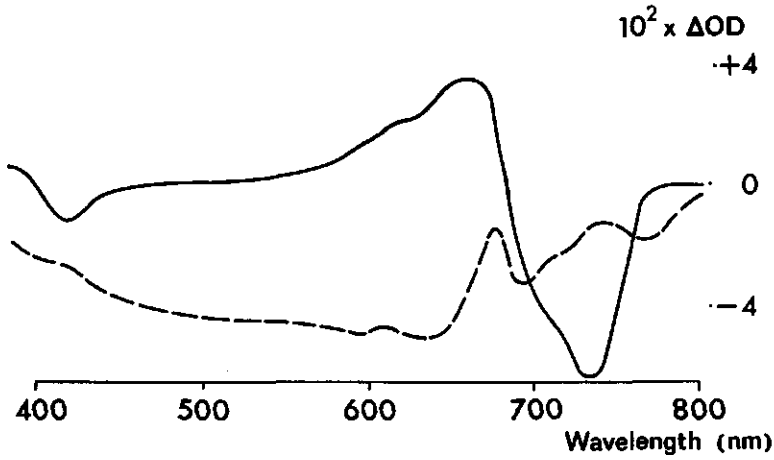


FIG. 50. Low-temperature transformation of P_{FR} in DEAE-Sephadex fraction 1. Difference spectra recorded after pretreatment for 3 min with red light, and irradiation for 3 min with far-red light at −196 °C, $P_{FR} \rightarrow P_{663}$ (———), and after subsequent warming to −20 °C $P_{663} \rightarrow P_{673}$ (- - - -).

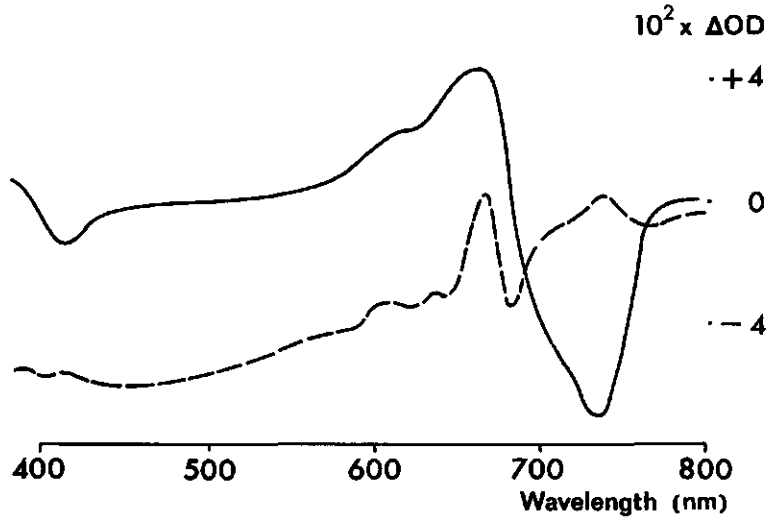


FIG. 51. Low-temperature transformation of P_{FR} in DEAE-Sephadex fraction 2. Difference spectra recorded after pretreatment for 3 min with red light, and irradiation for 3 min with far-red light at −196 °C, $P_{FR} \rightarrow P_{663}$ (———), and after subsequent warming to −64 °C, $P_{663} \rightarrow P_R$ (- - - -).

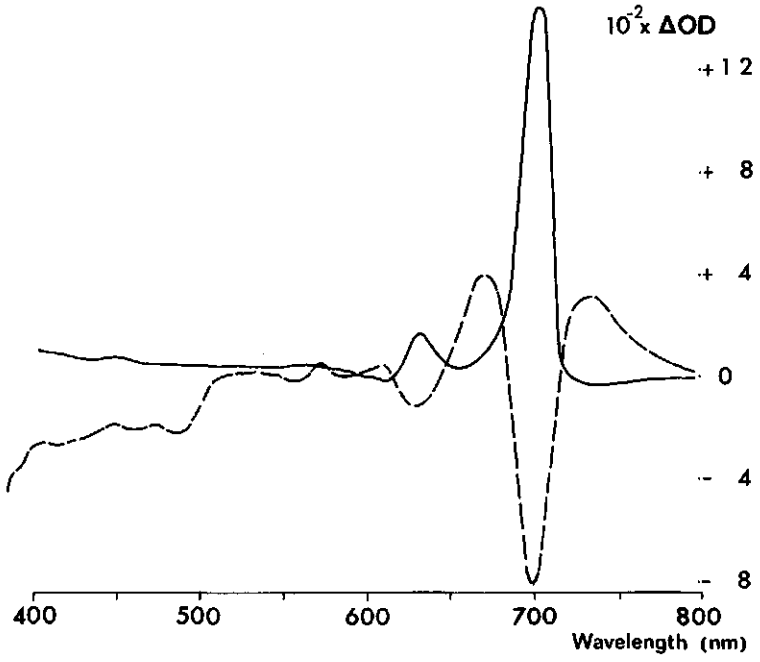


FIG. 52. Low-temperature transformation of P_R in pea plumules. Difference spectra for photoconversion at –196 °C (———), and subsequent warming from –70 ° to –20 °C (- - - -), after photoconversion at –70 °, as recorded by SPRUIT[99].

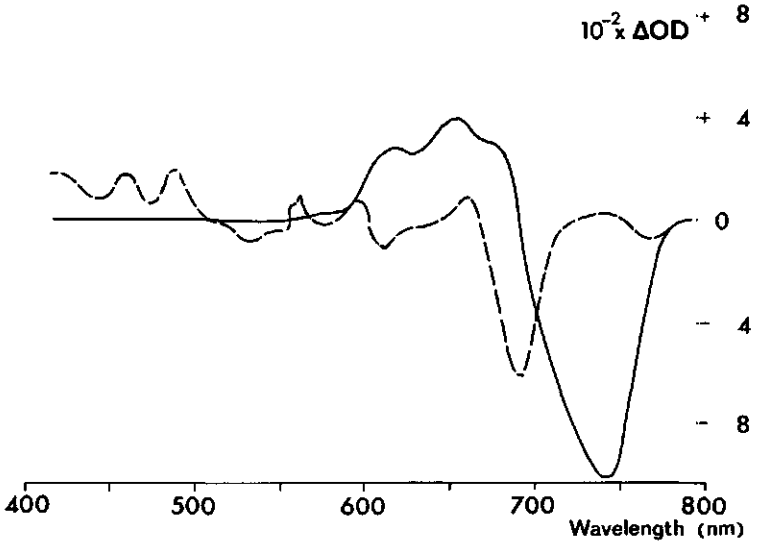
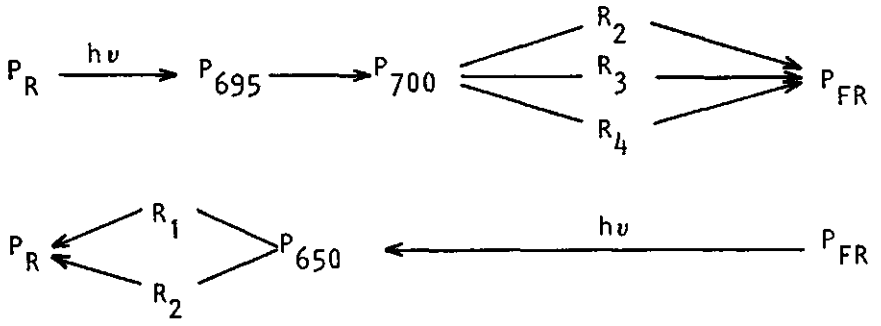


FIG. 53. Low-temperature transformation of P_{FR} in pea plumules. Difference spectra for photoconversion at –196 °C (———), and subsequent warming from –70 ° to –20 °C (- - - -), after photoconversion at –70 °, as recorded by SPRUIT[99].

no such well-defined intermediate in the reverse reaction. It is unlikely that the photochemical transformation involves two pigment systems, as suggested by SPRUIT[99]. A single pigment system, with different pathways for the forward and reverse reactions, seems more probable.

Later work by the team of LINSCHITZ[103] and by PRATT and BUTLER[104] supports these conclusions. LINSCHITZ'S team studied the forward reaction by flash photolysis at low temperature, and found a similar principal intermediate, P_{692}, for the $P_R \rightarrow P_{FR}$ transformation. This intermediate, which is formed in a fast reaction, reverts to a second intermediate, P_{695}, in the dark. The P_{695} form is stable up to $-70°C$. When the temperature rises above $-70°C$, P_{FR} is formed in several temperature-dependent steps. The last of these intermediates, which is formed at $-35°C$, is a bleached pigment form with a very broad absorption band of low intensity at 650 nm[103]. Its absorption spectrum is comparable with that of denatured phytochrome (see Fig. 28). The bleaching effect probably reflects dissociation of the chromophore and the protein moiety (see 3.2.4).

LINSCHITZ et al.[105] have also studied phytochrome transformations at room temperature by flash photolysis. Their results which agree well with those of our own low-temperature experiments, deserve mentioning in some detail. The apparatus used records the effect of a $5\mu s$ flash of irradiation on the absorption at a particular wavelength at very short intervals after the flash. This is achieved with an oscillograph, which is able to scan the signal at regular intervals of 0.2 ms, 5 ms, 100 ms, 1 s or 5 s. Difference spectra were compiled from the oscillograms recorded at many different wavelengths, for both the $P_R \rightarrow P_{FR}$ and $P_{FR} \rightarrow P_R$ transformations. The former appeared to have two short-lived intermediates. The first was formed after an immeasurably short time and was characterized by an absorption peak at 695 nm, which decayed after 0.2 ms. The second intermediate had an absorption maximum at 710 nm, which decayed after 0.5 ms. Three more steps followed before formation of P_{FR} was complete. The authors concluded that P_{695} does not simply correspond to an electronically excited state of the chromophore, because its lifetime is too long, but that it must be some sort of an isomer. As for the reverse transformation, the flash converts P_{FR} directly into a form with maximum absorption at 650 nm. The intensity of the absorption increases to its maximum value within 2 s. The slow dark reactions observed in these experiments are comparable with the dark reactions found at low temperatures, and may likewise be attributed to conformational changes of the protein part. After analysis of the oscillograms, LINSCHITZ et al. proposed the following pathways for the transformations of phytochrome: (see page 75) where R_1, R_2 etc. are reaction stages characterized by a definite lifetime. Although this overall picture of the phytochrome transformations seems plausible, it is questionable whether the proposed reaction scheme is correct in all details. Our experiments confirm that phytochrome is very unstable, so that the preparation used by LINSCHITZ et al., which was the same for all runs, presumably contains phytochrome in various stages of denaturation. This may explain the parallel pathways proposed for the dark reactions.



BRIGGS and FORK([106]) were able to detect one of the long-lived intermediates in the dark reaction sequence. They suggested that, if this intermediate, P^1, is indeed transformed slowly into P_{FR} in a sequence of light-independent reactions, continuous irradiation of phytochrome with a mixture of red and far-red light should cause the accumulation of a detectable level of P^1 by the time a steady state has been reached. Accumulation of the intermediate could be detected spectrophotometrically both in vitro and in vivo (*Avena* coleoptiles) by an increase in absorbance at 543 nm (where P^1 absorbs more strongly than P_R and P_{FR}) after the actinic beam had been turned on. Absorbance at 543 nm decreased again when the light was turned off. It was found that glycerol slowed down the decay of the intermediate, which made it possible to record a difference spectrum between P^1 and P_{FR} in the region between 365 and 580 nm.

3.5. INTERPRETATION OF RESULTS

The denaturation experiments indicated that the light absorption of intact phytochrome and other bile pigments is influenced by the association between chromophore and protein. In view of the shift in the maximum of the red ab-absorption band to a longer wavelength upon association, it seems that absorption is determined not so much by the covalent bond between chromophore and protein part, but more by the direct surroundings of the chromophore, i.e. the coiled peptide chain. This red shift may be due to energy transfer between the chromophore and the aromatic side chains of phenylalanine, tyrosine and tryptophane. Such energy transfer has been studied extensively by fluorescence spectrometry of complexes between a fluorescent probe and a protein([107]), for instance between the fluorescent dye, 1-anilino-8-naphtalene sulfonate (ANS), and apomyoglobin. In this case, apart from a shift in the emission spectrum, the absorption maximum of the complex in the visible region is shifted towards the red([108]). Further support for the argument is provided by the energy transfer in model compounds composing a polypeptide chain with an energy donor (α-naphthyl) at the carboxyl end and an energy acceptor (dansyl) at the imino end([109]). These compounds, the so-called 'spectrometric rulers', illustrate that energy transfer depends on the distance between electron donor and acceptor,

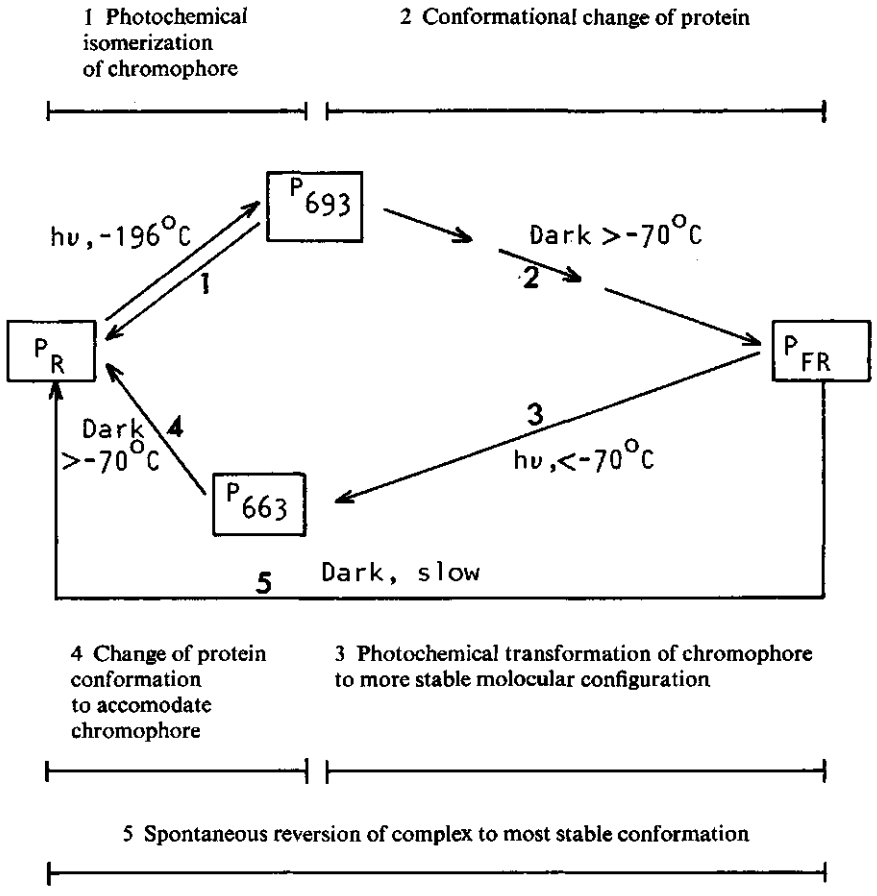
and on their mutual orientation, which is dependent on the conformation of the peptide chain.

The type of energy transfer mentioned above is characteristic for π^x–π transitions between plane-parallel aromatic systems. A transfer mechanism based on a π^x–n transition is, however, another possibility; for instance, tryptophan could induce a partial proton shift in the chromophore. This will be discussed more extensively in Chapter 4. In phytochrome we are confronted with a large red shift upon the phototransformation of P_R into P_{FR}. Such a shift can hardly be accounted for unless one assumes that the chromophore itself is altered.

The optical studies strongly suggest that both a change in the structure of the chromophore and a change in the conformation of the protein take place upon phototransformation of phytochrome. First of all, comparison of the optical activity of C-phycocyanin and that of its chromophore indicate that optical activity is primarily induced by the association of the chromophore with the protein. In the case of phytochrome, the change in the sign of the Cotton effect when P_R is transformed to P_{FR} suggests that the structure of the chromophore itself is changed. In both pigment forms, the chromophore is apparently stabilized by a particular conformation of the peptide chain around it.

The low temperature experiments, together with the flash photolysis studies, gave more information about this mechanism. It was possible to divide the phototransformations either into a light-dependent step and subsequent dark reactions or, with flash photolysis, into fast and slow reaction steps. For the $P_R \rightarrow P_{FR}$ transformation, a definite intermediate (P_{693}) was detected. The fact that this intermediate is detectable only at low temperature or by rapid scanning in flash photolysis suggests that it is formed by a reaction within the chromophore and that the protein is not directly involved. In the subsequent dark reactions at higher temperature, the conformation of the protein part changes so that a second stable complex, P_{FR}, is formed. There is no evidence that the transformation $P_{FR} \rightarrow P_R$ involves the same intermediate; P_{FR} is transformed directly into a pigment form (P_{663}), absorbing in the red region of he spectrum. This implies that in this case the conformational change of the protein is easier than the reverse change accompanying the formation of P_{FR}. This is further illustrated by the fact that P_{FR} reverts spontaneously to P_R by way of a very slow dark reaction([31]), the half-life of P_{FR} being 9 hours at 25 °C. The rate of this dark reaction is highly temperature-dependent, which means that thermal activation is sufficient to effect the conformational change in the reverse reaction of P_{FR} into P_R. The dark reversion of P_{FR} has been studied by ANDERSON et al.([110]) in the temperature range from −50 °C to 25 °C at pH values between 4.7 and 8.6. At −50 °C they also found an intermediate with a broad absorption band in the red, the formation of which was favoured at pH values below pH 6.0. This intermediate is comparable with that found in our low-temperature experiments. This led them to propose a protonation mechanism for the dark reaction, which will be discussed in Chapter 4.

On the basis of all the data discussed so far, we may postulate the following scheme for the phototransformations of phytochrome:



The transformation of P_R into P_{FR} can be described as the uptake of an amount of light energy which causes an isomerization of the chromophore and leads to an unstable transition state (P_{693}). The extra energy of this state induces a local conformational change in the protein and results in the formation of a new complex between chromophore and protein. Both the chromophore and the protein are, however, less stable in the P_{FR} complex and fall back to their most stable configurations, either by the uptake of a low-energy light quantum or by thermal excitation in the dark.

An attempt to describe how the chromophore is involved in such reactions will be considered in Chapter 4 on the basis of what is known about its molecular structure.

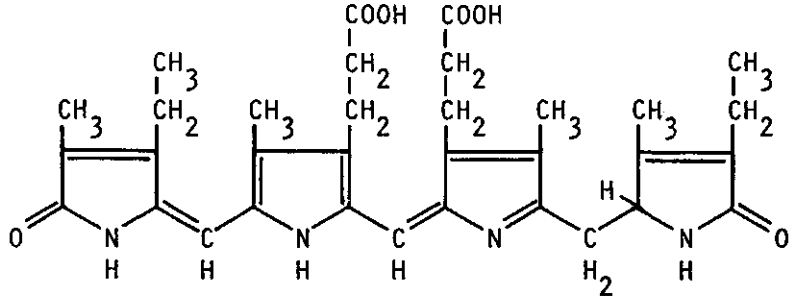
4. INVESTIGATIONS INTO THE STRUCTURE OF THE PHYTOCHROME CHROMOPHORE

4.1. INTRODUCTION

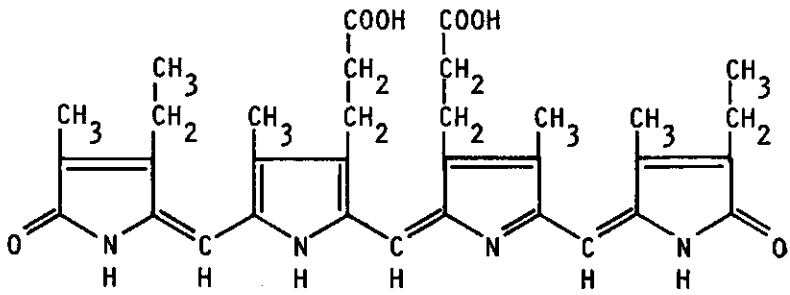
In Chapter 3, the photochemical transformations of phytochrome were attributed to an isomerization of the chromophore in combination with a change in the conformation of the protein part of the pigment. It is evident that knowledge of the structure of the phytochrome chromophore would help to elucidate the transformation mechanism on a molecular level.

In our study of the phytochrome chromophore we have followed two lines of approach. In both cases, C-phycocyanin served as a model compound. Firstly, because of its resemblance to phytochrome and because it could be prepared in larger amounts, C-phycocyanin was an excellent model for a study of the cleavage of the chromophore from its protein part. Just as phycocyanobilin, the phytochrome chromophore is linked to its protein part by covalent bonds. Secondly, phycocyanobilin was used to test whether mass spectrometry was an informative and reliable method for the analysis of the structure of bile pigments. Mass spectrometry was chosen because it was expected that only minor quantities of the phytochrome chromophore could be obtained. Moreover, the structure of phycocyanobilin was interesting in itself; renewed study of this pigment by O'HEOCHA[111] had suggested that the structure postulated for it by LEMBERG and BADER[112] in 1933 was incorrect.

O'HEOCHA[111] concluded that the absorption spectrum of phycocyanobilin in its native form, was intermediate between that of a bilidiene and that of a bilitriene, whereas LEMBERG and BADER[112] had originally proposed that it had a mesobiliviolin (I) structure. Mesobiliviolin is a bilidiene with two unsaturated bridges and is violet-coloured, while the bilitriene mesobiliverdin (II) has three unsaturated bridges and is blue-green. The difference in colour is readily understood from the difference in conjugation. The latter is greater in mesobiliverdin which consequently absorbs at longer wavelengths than mesobiliviolin.



I. mesobiliviolin



II. mesobiliverdin

By using strong hydrochloric acid solutions of increasing acidity to hydrolyse the pigment, O'HEOCHA[111] demonstrated that it was extremely difficult to isolate the phycocyanobilin without chemical modification. FUJITA and HATTORI[113] then introduced a milder isolation method, which consists of refluxing the C-phycocyanin with methanol. Using the latter method, two American research groups were able to isolate sufficient amounts of phycocyanobilin dimethyl ester for NMR spectrometric analysis. Their results were published in two preliminary reports[114, 115]. Meanwhile, our efforts were concentrated on a new semi-micro cleavage method, and on the purification of phycocyanobilin in the diacid form – something in which the other investigators had not been successful. This work will be described briefly in this Chapter. In cooperation with Dr. B. L. SCHRAM of the Unilever Research Laboratory Duiven, the structure of phycocyanobilin was analysed by mass spectrometry. A difference in the state of oxidation was observed between the diacid and diester forms of the chromophore, a discrepancy which remained a source of controversy between us and the other workers.

In 1966, SIEGELMAN was the first to use methanol extraction for the isolation of the phytochrome chromophore[82]. The very small amounts obtained were compared with other bile pigments by thin-layer chromatography. Unfortunately, our own efforts to design other methods of selectively cleaving the chromophore of phytochrome were unsuccessful. For analysis of the phytochrome chromophore, we therefore had to use the small quantities obtained by methanol extraction. The results will be compared with those of a method, recently introduced by RÜDIGER which involves degradation of the tetrapyrrolic chromophore and analysis of the individual pyrrole rings[116]. This chapter will be concluded with a discussion of possible mechanisms for the isomerization of the chromophore which was put forward in Chapter 3 as a principal step in the photoreversible transformation of the pigment.

4.2. Isolation of phycocyanobilin and other bilitrienes

C-phycocyanin and allophycocyanin were isolated from *Plectonema boryanum** by extraction with buffer, precipitation with ammonium sulfate and column chromatography on calcium phosphate gel in the brushite form([117]). The pigments thus obtained were purified until the values of the ratio OD_{620}: OD_{280} were 5.0 and 2.5 respectively. The absorption spectra of the protein pigments are shown in Figure 54.

The two methods described in the literature for the cleavage of phycocyanobilin each had a serious drawback; hydrolysis with strong hydrochloric acid gave too many breakdown products([111]), while methanol extraction gave a yield of only about 10–20%([115]). In order to increase the yield of the chromophore, a method used for the cleavage of benzyl ester bonds in solid-phase peptide synthesis([118]) was adapted for our purpose. This method involves treatment of the algal pigment with hydrogen bromide gas in a trifluoroacetic acid solution with the exclusion of oxygen. Cleavage of phycocyanobilin in this way was highly selective. With purified pigment fractions the recovery by weight of phycocyanobilin from C-phycocyanin and allophycocyanin was 5.1 and 3.3% respectively, calculated on the basis of the specific absorption coefficient of phycocyanobilin: $OD^{1\%}_{1cm} = 350$ at 365 nm([111]). For C-phycocyanin this amounts to a yield of chromophore of about 100%.

The routine isolation procedure was as follows. Amounts of 100 mg of protein pigment were dissolved in 30 ml of trifluoroacetic acid, after which the solu-

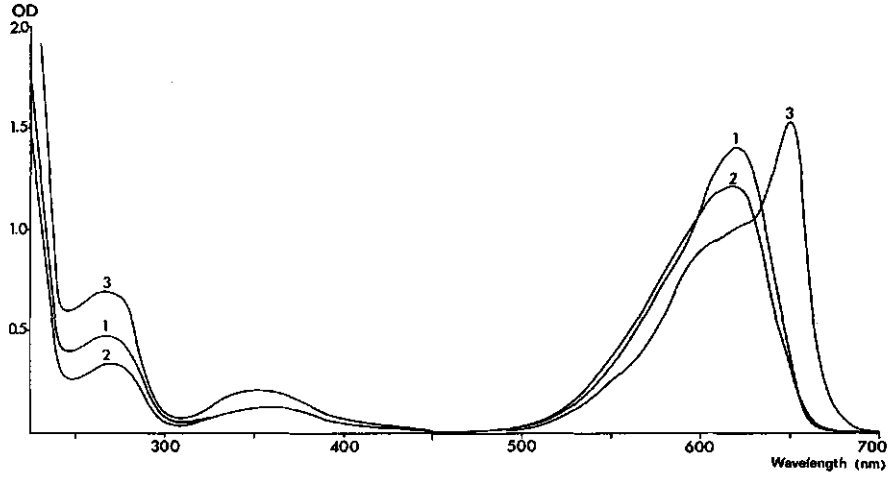


Fig. 54. Absorption spectra of phycocyanins isolated from *Plectonema boryanum* by precipitation with $(NH_4)_2SO_4$ and chromatography on calcium phosphate gel. 1,2 C-phycocyanin, 3 allophycocyanin.

* The algae were kindly supplied to us by Mrs. Beryl J. Hopkins of the Unilever Research Laboratory Welwyn, England.

tion was flushed for 30 min with hydrogen bromide gas. The solution was then refluxed at 75 °C for another 30 min, during which it was flushed with nitrogen. The solvent was evaporated under vacuum, and the residual protein film was redissolved in 20 ml of methanol to which 10 ml of chloroform were added. The solution was separated into two phases by the addition of 20 ml of water, the phycocyanobilin being recovered in the chloroform phase. The chloroform extract was then washed with an excess of double-distilled water. During the cleavage and purification no oxidative breakdown of phycocyanobilin was observed.

While the chromophore, recovered in this way, is in the diacid form, refluxing with acidic methanol after hydrolysis with hydrogen bromide gas gave a mixture of diacid, monoester and diester in proportions dependent on the acidity of the solution and on the refluxing time. For instrumental analysis the samples had to be very pure and free from traces of solvent impurities. The pigments were therefore purified by batch adsorption on G-HR silica gel (Machery-Nagel), dissolved in methanol and transferred once more to chloroform. The diacid form of the pigment was separated from the esters by chromatography on a silica gel column (Mallinckrodt; 2.5 × 5 cm) in chloroform and elution with a chloroform-methanol gradient. The components are eluted in the sequence: diester, monoester, diacid. The diacid is recovered at a methanol concentration of 3% by volume. The separation could be checked qualitatively by subjecting the eluted fractions to thin-layer chromatography on G-HR silica gel and eluting with methyl ethyl ketone/water/glacial acetic acid (95:5:0.3 v/v). The absorption spectra of the diacid, the monoester, and the diester forms of phycocyanobilin, which are essentially the same, are shown in Figure 55.

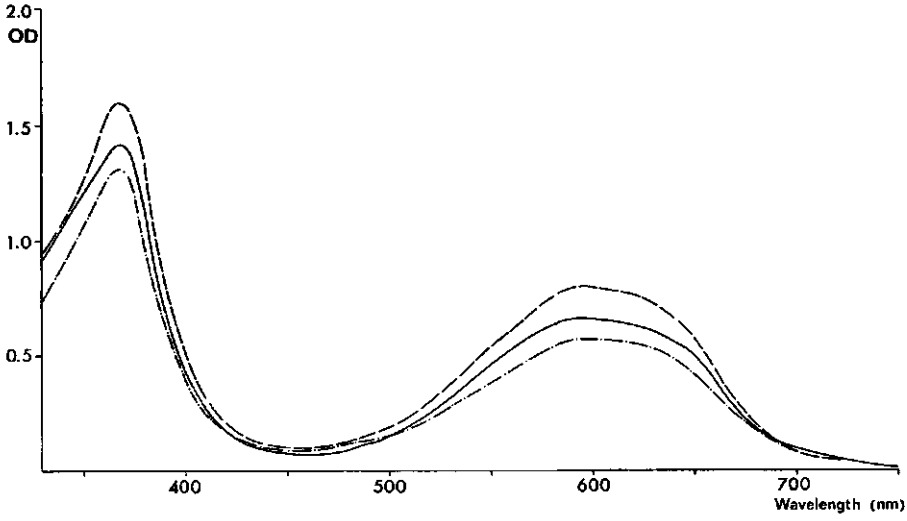


FIG. 55. Absorption spectra of phycocyanobilin in the acid and esterified forms. Diacid (———), monoester (- - - -) and diester (. – . –).

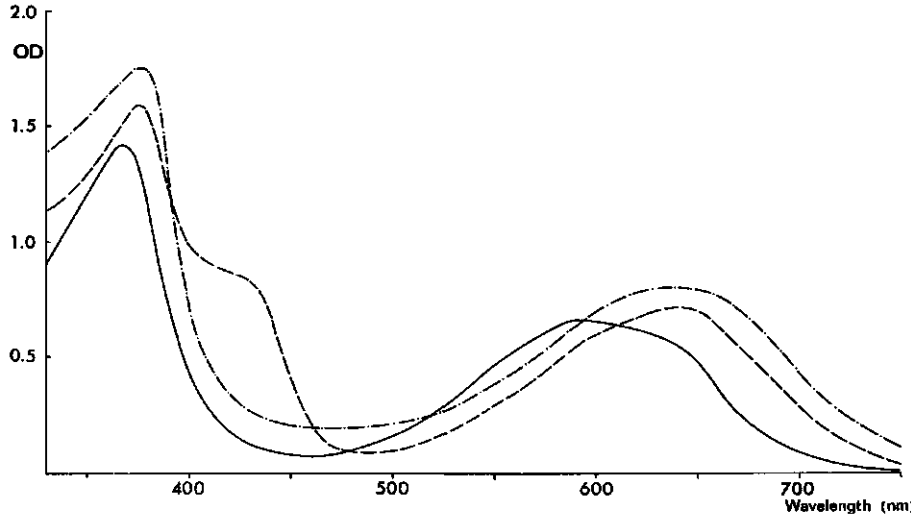


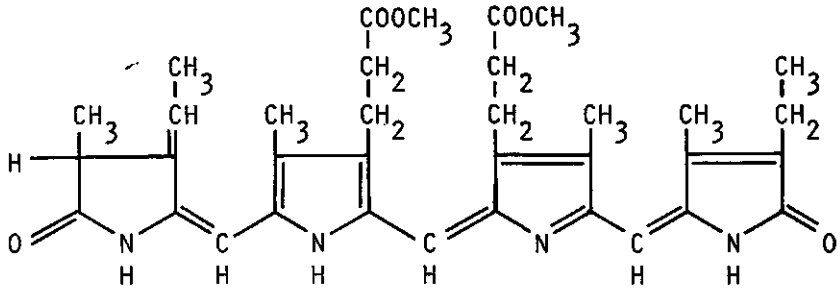
FIG. 56. Comparison between the absorption spectra of phycocyanobilin and mesobiliverdin; phycocyanobilin (———), mesobiliverdin (- - - - -). mesobiliverdin diester (– · – ·).

Mesobiliverdin was prepared by oxidation of mesobilirubin with ferric chloride, either in an acidic solution of methanol([119]) or in trifluoroacetic acid solution([120]). The solutions were refluxed at 70°C for 15 min under nitrogen. Mesobiliverdin was obtained in the diester and the diacid form respectively. Mesobilirubin was prepared first by the reduction of bilirubin (Merck) with a palladium/carbonblack catalyst in 0.1 N sodium hydroxide solution([121]). In this reaction a green ferrous complex is formed which can be decomposed with alkali. Mesobiliverdin diacid and diester were extracted with ether and purified by column chromatography in the same way as phycocyanobilin. Mesobiliverdin was also prepared by direct isomerisation of phycocyanobilin with ferric chloride in trifluoroacetic acid.

The absorption spectra of mesobiliverdin and its diester in neutral $CHCl_3$ are compared with that of phycocyanobilin in Figure 56. Mesobiliverdin absorbs at longer wavelengths than the deep blue phycocyanobilin, and is therefore greenish-blue. The spectrum of mesobiliverdin, prepared by oxidation of mesobilirubin, has a shoulder at 430 nm. This is due to a yellow component, which could not be fully separated by chromatography from the blue-green mesobiliverdin.

4.3. ANALYSIS OF THE STRUCTURE OF BILITRIENES

We could confirm the structure given by other investigators([85, 86]) for phycocyanobilin dimethyl ester by NMR analysis of a 40-mg sample on a Varian



III. phycocyanobilin dimethyl ester

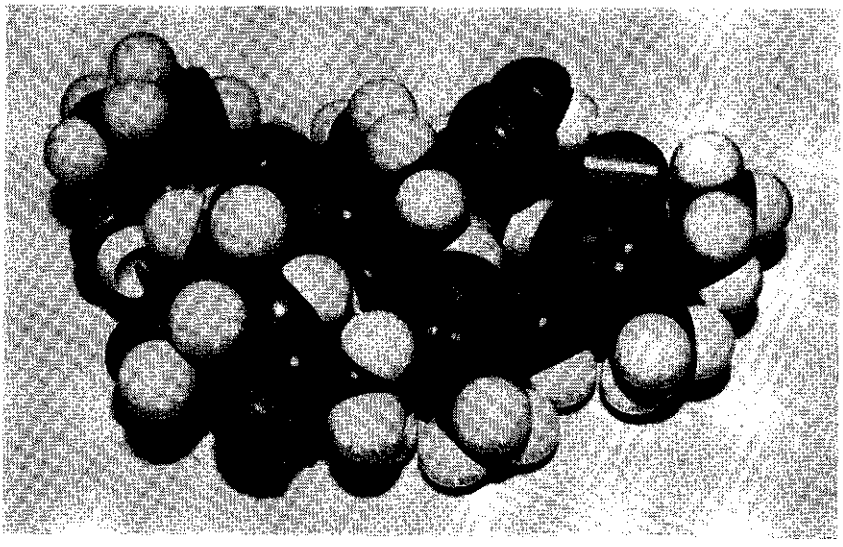
HA 100 Mc spectrometer*. The difference between phycocyanobilin diester (III) and mesobiliverdin (II) lies in a substituent group of one of the terminal rings, this being ethylidene in phycocyanobilin instead of ethyl in mesobiliverdin.

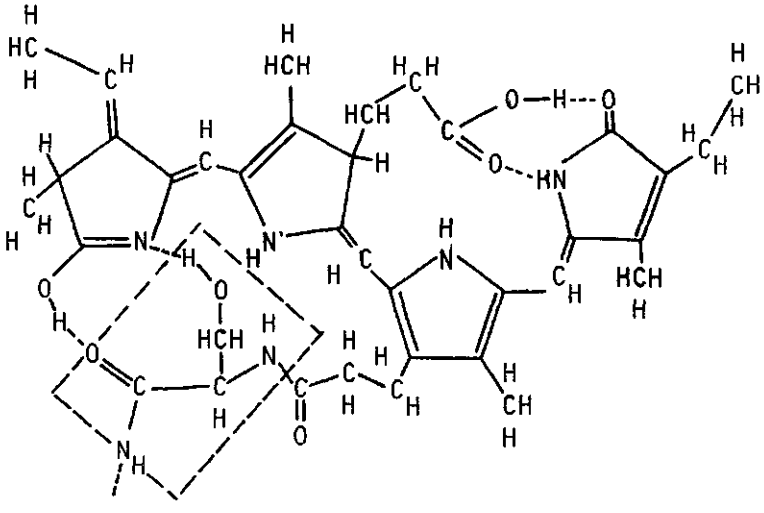
Mass spectrometric analysis of the pigments was carried out with a GEC-AEI MS-9 instrument. Two results of these measurements are of particular importance to the phytochrome problem. First of all, we found a discrepancy between the apparent molecular weights of the diacid and diester forms of phycocyanobilin. According to their mass spectra the diacid had a mol.wt. of 588, the diester form of 614 ($\equiv 586 + 2\,CH_2$), indicating a relative loss of two hydrogen atoms. The fragmentation patterns clearly showed that the diacid is broken at the central bridge of the molecule, the highest peaks being for the dipyrrole ions, while the diester is more stable, the highest peak being for the molecular ion. Secondly, when mesobiliverdin diacid was analysed, non-specific degradation (pyrolysis) occurred and neither the molecular ion nor dipyrrole fragments were found. This result suggested that mesobiliverdin in the diacid form is less volatile than phycocyanobilin and that it cannot be evaporated in the high-vacuum source of the instrument. The same was found for the diacid form of the phytochrome chromophore; this will be discussed further under 4.5.

The difference in volatility between phycocyanobilin and mesobiliverdin can be tentatively explained on the basis of molecular models which show that intramolecular hydrogen bonding is more probable in phycocyanobilin, because of the presence of two extra hydrogen substituents. With the aid of molecular models, Dr. B. L. SCHRAM and the author showed that, when hydrogen bonding in the molecule was optimal, the most constricted structure is that with the central rings (2 and 3) of the tetrapyrrole molecule in the *trans*-configuration. Such a configuration is illustrated below for phycocyanobilin (IV), coupled by a peptide bond to a serine residue of the surrounding peptide chain.

Such a configuration can have a large influence on the position of the molecule within its protein core. So far, most explanations of the photochemical transformations of phytochrome are based on the conventional linear arrange-

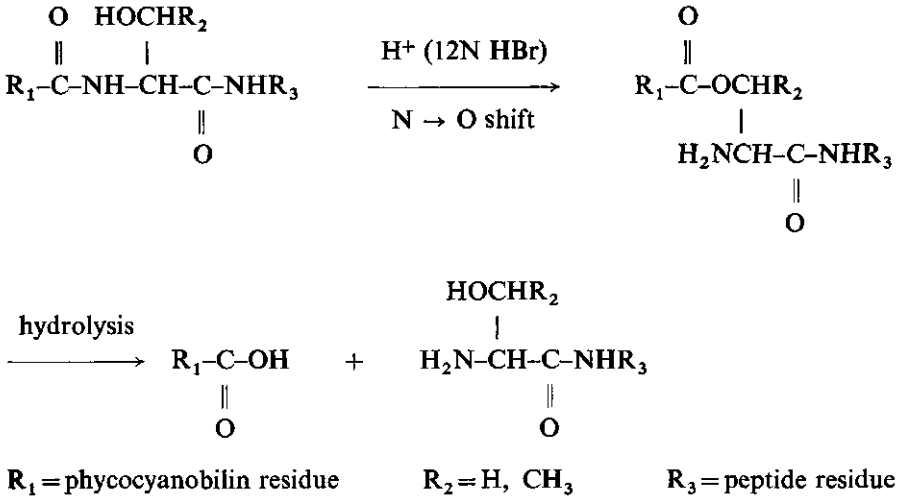
* The NMR spectrum was measured by MR. DAVID FROST of the Unilever Research Laboratory, Vlaardingen, the Netherlands.





IV. phycocyanobilin linked to serine residue of protein

ment of tetrapyrroles. The reason for coupling phycocyanobilin to the serine residue was provided by our cleavage experiments, since it is known that hydrolysis with hydrogen bromide proceeds specifically by way of intramolecular N → O shifts in peptide bonds involving serine and threonine. This mechanism can also explain the formation of the acid form of the chromophore, as is shown in the following scheme:



Very recently, CRESPI and SMITH([122]) reported the analysis of peptides formed by enzymatic hydrolysis of C-phycocyanin. They found that aspartic acid, serine, leucine, and cysteine were the most abundant amino acids in these peptides.

4.4. ISOLATION OF THE PHYTOCHROME CHROMOPHORE

The expectation that treatment of phytochrome with hydrogen bromide in trifluoroacetic acid solution would give a good yield of chromophore was not fulfilled. This suggested that the phytochrome chromophore is not coupled to its protein moiety by way of a serine or threonine residue, and forced us to return to the methanol extraction method. As already mentioned, this method gives a very low yield, estimated by SIEGELMAN et al.([82]) to be below 10%. In one of our successful experiments we started with a phytochrome solution of a total activity 26,000 $\Delta(\Delta OD)$/cm (300 mg protein). The pigment was carefully precipitated by addition of trifluoroacetic acid (to 4% v/v). The deep-blue precipitate was washed twice with methanol, upon which it became greenish blue and flocculent, and then refluxed with 90 ml of methanol for 4 hours at 67°C. The undissolved pale green protein was removed by centrifuging. The clear supernatant was diluted with one volume of water and extracted three times with 30 ml of chloroform. The pale blue chloroform phase was evaporated to a volume of 1–2 ml and washed five times with 2 ml of water. It was then evaporated in a stream of nitrogen gas and dissolved in 1 ml of a 5% solution of hydrochloric acid in methanol to esterify the chromophore. The blue pigment did not dissolve completely in this solvent, and 0.2 ml of chloroform had to be added to bring all of the pigment into solution. After being left overnight at 4°C under nitrogen, the solution was diluted with one volume of water and extracted with 0.5 ml of chloroform. This solution was chromatographed on a small column (0.5 × 3.5

cm) of silica gel (Mallinckrodt). The chromophore was eluted as a narrow band with the chloroform, which shows it to be in an esterified form. Some non-esterified material was left on top of the column. After a subsequent washing sequence we ended up with 0.4 ml of solution ($OD_{377} = 1.6$, $OD_{604} = 0.7$), which was analysed by ultraviolet spectrophotometry and mass spectrometry. If it is assumed that the specific absorption of the phytochrome chromophore is equal to that of phycocyanobilin ($OD^{1\,cm}_{1\,\%} = 350$ at 365 nm), the yield of chromophore in this case was 20 μg.

After extraction with methanol the protein residue is pale green. This indicates that the yield of free chromophore is low. In several cases, more chromophore could be isolated by a second extraction with methanol containing 0.01 M sodium methanolate. The latter appears to act as a transesterification catalyst. This agrees with the observation that this second extraction yielded mainly esterified chromophore, together with some apparently non-esterified compounds.

The acid and esterified chromophores were compared with other bilitrienes by thin-layer chromatography on G-HR silica gel (Fig. 57). The plate was eluted with chloroform/ethyl acetate (1:1 v/v). In this way only the ester forms are separated, while the acid forms remain at the base line. The esterified phytochrome chromophore, obtained from the initial methanol extraction (Fig. 57, spot no. 6), has the same R_f value as the dimethyl esters of phycocyanobilin and mesobiliverdin. The other two chromophore fractions originated from the second extraction with sodium methanolate. One of these (no. 5) contained some acid chromophore, together with a second component with the same R_f value as phycocyanobilin monomethyl ester. Thus, the phytochrome chromophore behaves very similarly to phycocyanobilin upon thin-layer chromatography. The presence of more than one ester form suggests that the phytochrome chromophore has the two acid substituents normally found in bile pigments.

The absorption spectrum of the esterified chromophore of phytochrome is compared with those of the dimethyl esters of phycocyanobilin and mesobili-

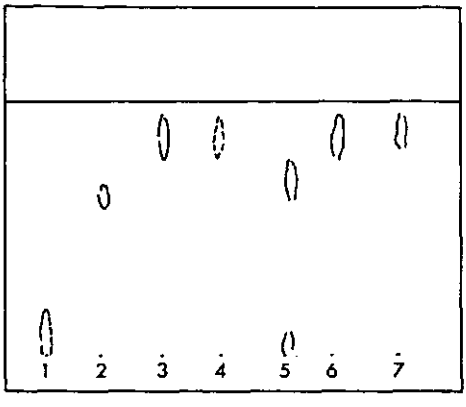


FIG. 57. Thin-layer chromatography of the phytochrome chromophore and other bilitrienes. 1. Phycocyanobilin 2. Phycocyanobilin monoester 3. Phycocyanobilin diester 4. Mesobiliverdin 5. Monoester and acid form of phytochrome chromophore 6,7. Diester of phytochrome chromophore.

TABLE 15. Absorption maxima of bilitriene diesters

Compound	Maxima (nm)		
Phytochrome	377	604	(650)
Phycocyanobilin	368	595	
Mesobiliverdin	378	(600)	640

verdin in Fig. 34. The close similarity between the spectra of the phytochrome chromophore and both phycocyanobilin and mesobiliverdin is evident. The absorption maxima are compared in Table 15. No difference was found between the spectra of the acid and esterified chromophore of phytochrome.

The suggestion of WALKER and BAILY[123] that there is a difference between the chromophores of the P_R and P_{FR} forms does not seem justified, since the raw methanol extracts contain impurities. After purification of the extracts on a silica gel column, we could detect no difference between the chromophore fractions of the P_R and P_{FR} forms of phytochrome.

4.5. COMPARISON OF THE STRUCTURE OF THE PHYTOCHROME CHROMOPHORE WITH THAT OF BILITRIENES

Even with batches of 100–200 mg phytochrome available from our large scale isolations, we were able to prepare only minute amounts of phytochrome chromophore. This is illustrated by the following calculation, in which it is assumed that one phytochrome molecule (mol.wt. about 50,000) contains one chromophore of mol.wt. about 600:

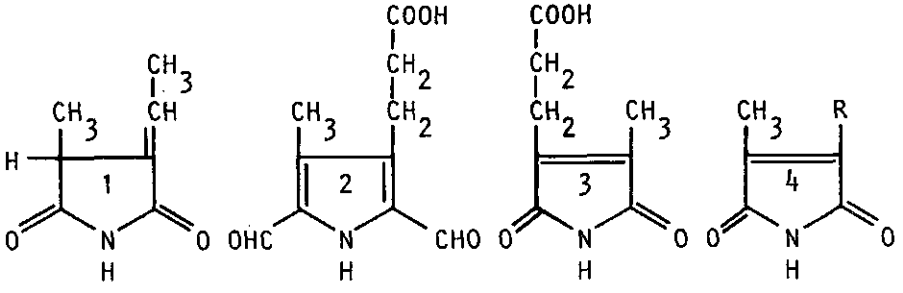
$$200 \text{ mg protein} \rightarrow 200 \times \frac{600}{50{,}000} = 2.4 \text{ mg chromophore.}$$

If the starting material is 30% pure, this figure becomes 0.72 mg, while if the efficiency of the methanol extraction is taken to be 10%, only 0.072 mg of chromophore would be obtained. Moreover, this chromophore has to be further purified by column chromatography. It was, therefore, evident that analysis by NMR spectrometry was out of the question. As mentioned in 4.3, the acid form of the phytochrome chromophore proved to be non-volatile in the electron source of the mass spectrometer. This suggests that the phytochrome chromophore differs from phycocyanobilin and that it is a typical bilitriene. Analysis of the dimethyl ester was unsuccessful because of a failure in the mass spectrometer. Unfortunately, the sample was lost and no more purified phytochrome was available at that time. The mass spectrometry experiments nevertheless suggested very strongly that the phytochrome chromophore was a bilitriene.

This was indeed proved by RÜDIGER in 1969[116] who entered this field after a study of other bile pigments[87, 124]. He adopted a degradation method, which had been developed for porphyrins and other tetrapyrroles and which could be used on a micro scale for the analysis of C-phycocyanin and other biliproteins.

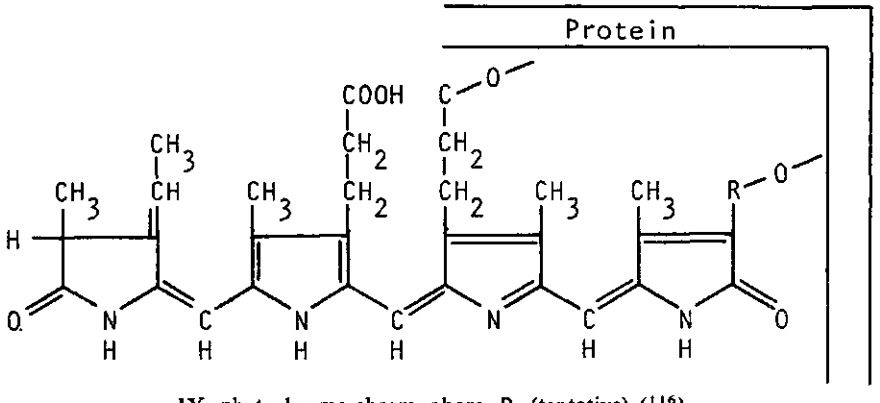
C-Phycocyanin was treated with chromium trioxide in acid solution. This resulted in the cleavage and breakdown of the chromophore without extensive degradation of the protein. The chromophore was oxidized to the following pyrrole derivatives: methylethylidenesuccinimide, haematinic acid and methylethylmaleimide. These compounds were identified by RÜDIGER by thin-layer chromatography. We were able to confirm these results by mass spectrometry and infrared analysis, using both model compounds kindly supplied by Dr. W. RÜDIGER and maleimides which we had prepared ourselves from C-phycocyanin and phycocyanobilin. These analyses showed that the formula given in 4.3 for the phycocyanobilin diester (III) is correct.

RÜDIGER has since applied the method to a phytochrome sample isolated by CORRELL[54] from rye seedlings. Degradation of the phytochrome with 1% sodium dichromate at pH 1.7 at 20°C gave compounds V and VI:



V. methylethylidenesuccinimide | VI. dialdehyde of haematinic acid | VII. haematinic acid | VIII. unknown imide

Degradation with 1% chromium trioxide in 2 N sulphuric acid at 20°C gave haematinic acid (VII) instead of the dialdehyde. The fact that under very mild conditions the dialdehyde of haematinic acid is formed while degradation with chromium trioxide gives the acid, indicates that the original covalent bond involves one of the acid substituents. Exhaustive oxidation with chromium tri-



IX. phytochrome chromophore, P_R (tentative) ([116])

oxide at 100 °C gave an additional unidentified imide (VIII). The formation of this imide suggests an additional covalent bond to the protein through ring 4. From these data RÜDIGER deduced formula IX for the phytochrome chromophore[116].

The presence of a second covalent bond with a different chemical configuration may explain our finding that the phytochrome chromophore is not split from the protein by hydrogen bromide gas in trifluoroacetic acid solution.

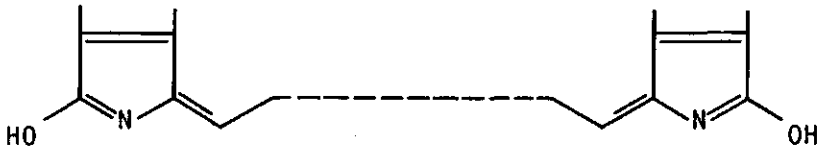
If RÜDIGER's deductions are correct, the only differences between the structure of the phytochrome chromophore and that of mesobiliverdin lie in the nature of one of the substituents on each terminal ring. With the phytochrome chromophore one of these is ethylidene while the other is unknown. The phytochrome sample analysed by RÜDIGER, however, was in a denatured state. The precise configuration of all substituents and of the covalent bonds with the protein will have to be analysed in an active pigment sample. A further complication is that the differences between the configuration of the chromophore in the P_R and P_{FR} forms will almost certainly not be maintained in their degradation products. Nevertheless RÜDIGER's method seems to bring the elucidation of the structure of the phytochrome chromophore within reach.

4.6. Possible mechanisms for the isomerization of the phytochrome chromophore

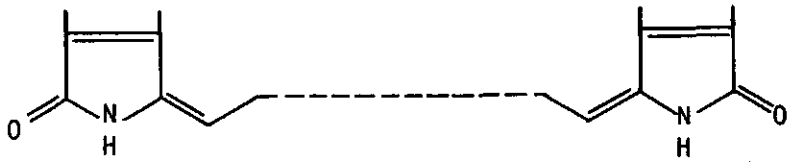
The strongest arguments in favour of an isomerization of the chromophore during the photochemical transformations of phytochrome arose from the measurements of optical activity (3.3) and from the low-temperature studies (3.4). In a bilitriene molecule, there are several possible isomerization mechanisms. The Cotton effects in the absorption bands of P_R and P_{FR} were attributed to an intrinsic asymmetry of the chromophore which is enhanced by asymmetric binding to the protein. The intrinsic asymmetry must be due to the presence of an asymmetric carbon atom in the ring with the ethylidene substituent. Further asymmetry may be the result of the chromophore adopting a specific spatial configuration, e.g. a cyclic configuration as suggested by MOSCOWITZ for urobilins[77], or a *trans*-configuration similar to that outlined for phycocyanobilin earlier in this Chapter (4.3). The photochemical transformation of rhodopsin into preluminorhodopsin is associated with a *cis-trans* isomerization of the chromophore retinal[90, 91, 127]. It is, however, unlikely that the reversible absorption shifts in phytochrome are caused by such a simple geometric isomerization of its chromophore.

Another way to explain the change in absorption is to assume a lactim-lactam tautomerization in the two terminal rings of the molecule, resulting in an extension of the conjugated system in the lactam form by two double bonds.

Interpretation of the NMR spectrum of phycocyanobilin[85, 86] and of the infrared spectra of phycocyanobilin and the phytochrome chromophore recorded at the Unilever Research Laboratory Duiven by Dr. H. COPIER suggests that, at least in chloroform, methanol, and trifluoroacetic acid, the lactam con-



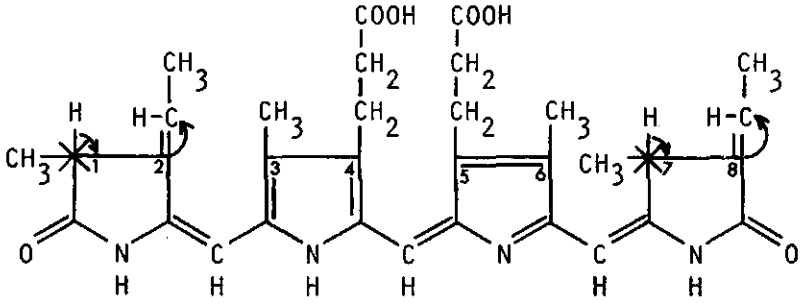
Lactim



Lactam

figuration is preferred. Furthermore, there was no evidence for the supposition that interconversion between the lactim and lactam configurations occurred readily.

SIEGELMAN has proposed a structure (X) for the P_R form of the phytochrome chromophore with ethylidene substituents at both ends (C_1 and C_7) of the tetrapyrrole ([125]):



X. chromophore of phytochrome, P_R (proposed) ([125])

He suggested that reversibility could then be explained by isomerizations in both terminal rings, by which the ethylidene groups are converted into ethyl groups and the double bond assumes a position in the ring. Consequently, the conjugated system of the molecule is extended by three double bonds. The results of RÜDIGER([116]), however, are incompatible with the presence of a second ethylidene substituent. Although this isomerization seems energetically possible for the $P_R \rightarrow P_{FR}$ phototransformation, reintroduction of double bonds into rings 1 and 4 as part of the conjugated system in the reverse transformation seems very unlikely, even for a photochemical reaction. Further objections to the scheme proposed by SIEGELMAN are that it does not take into account interactions between chromophore and protein and that it is primarily based on evidence from experiments with phycocyanobilin.

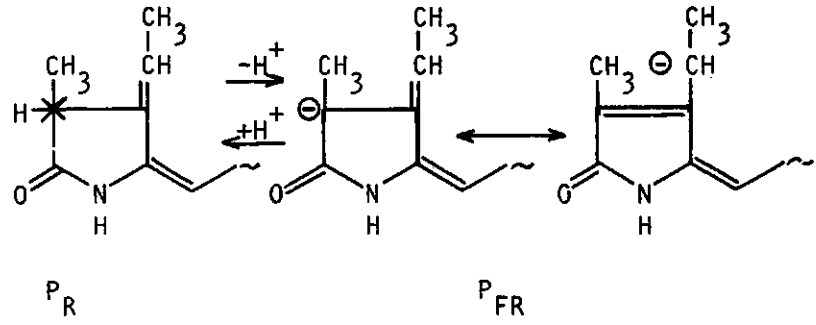
TABLE 16. Shift of the absorption maxima of phycocyanobilin dimethyl ester in strong acid solutions

Solvent	Wavelength of maxima (nm)	
$CHCl_3$	372	595
$CHCl_3$ 5% HCl	380	665
CH_3OH	368	620
CH_3OH 5% HCl	379	694

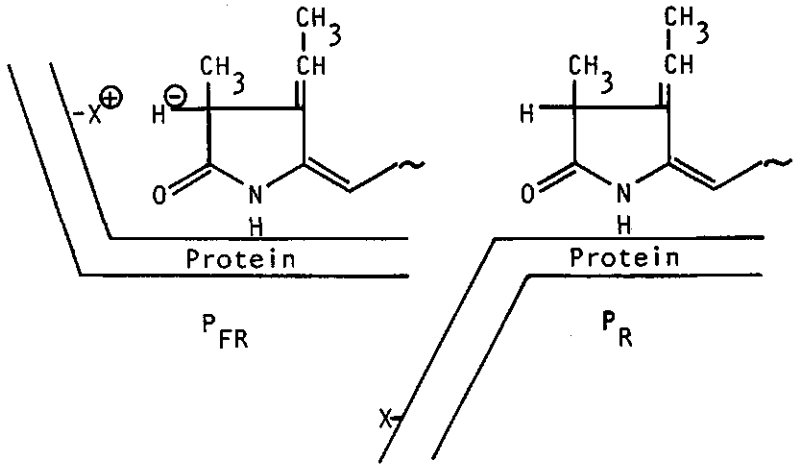
The structure of phycocyanobilin (III) and that proposed by RÜDIGER for the phytochrome chromophore (IX) both have a pyrrolenine group in one of the central pyrrole rings (2 or 3) of the molecule. When the pyrrolenine group of phycocyanobilin is protonated in strong acid solution, the red absorption band is shifted by about 70 nm to longer wavelengths, as shown in Table 16. This wavelength shift is comparable to that accompanying the interconversion of the P_R and P_{FR} forms of phytochrome. Another protonation reaction, causing comparable spectral changes, is found in phlorins and oxophlorins. JACKSON et al.([126]) showed that the blue oxophlorin turns green when converted into its mono-cation by protonation. These spectral shifts occur only in strongly acidic solutions. It therefore seems unlikely that such a reaction takes place within the protein core of the phytochrome molecule in neutral aqueous solution.

We believe that the scheme for the transformations of phytochrome (see 3.5) can be better explained by an intramolecular electron and/or proton shift, which is induced photochemically in the chromophore and stabilized by specific interactions with the protein part. The two mechanisms described below take into account such interactions of the chromophore with the protein. CRESPI et al.([85]) suggest that the phytochrome chromophore is bound to the protein by the ethylidene substituent instead of by a peptide bond. They suppose that the absorption of light might cause a change in the electron distribution in this terminal ring of the chromophore, and hence the observed photochromic shift. This would only be possible if the surrounding part of the peptide chain would adopt a particular configuration. Their scheme, however, is purely hypothetical and somewhat far-fetched and must await experimental evidence. A more plausible mechanism involving the configuration of the terminal ring with the ethylidene substituent is given by RÜDIGER([116]). He found that denatured phytochrome turned blue when treated with dilute trifluoroacetic acid solution. Subsequent degradation gave a higher yield of methylethylidene succinimide (V) than the untreated product, which instead gave methylethylmaleinimide. This indicates a shift of the double bond from the ethylidene substituent into the ring. A comparable shift is observed when phycocyanobilin is boiled with strong alkali. The transformation of P_R to P_{FR} is, therefore, explained by RÜDIGER as a proton exchange, as illustrated below (see p. 92).

This assumes the presence of a proton-acceptor group on the surface of the protein moiety in the vicinity of the ring which stabilizes one of the negatively



charged mesomeric forms. RÜDIGER's hypothesis seems very reasonable. A further requirement, however, is that the photochemically induced change in the electronic distribution of the ring system should enable proton transfer only when the proton-acceptor group is in the vicinity of the ring. This situation would be characteristic for the P_{FR} form, while in the P_R form the proton-acceptor group should move away from the ring by a conformational change in the protein. This is illustrated below:



Thus, it may be assumed that the change in electronic distribution occurs only when the chromophore is excited by light and when a proton acceptor or donor is very close to the chromophore. In this situation, a partial proton shift appears more probable than the complete exchange of a proton.

It is interesting to note that ANDERSON et al.([112]) proposed a similar reaction for the dark reversion $P_{FR} \rightarrow P_R$. They studied this non-photochemical reaction at pH values of 4.7 and 8.6 between $-50°$ and 25 °C. The dark transformation at -50 °C was greatly accelerated below pH 6, which was attributed to the uptake of a proton:

$$P_{FR} \xrightarrow[H^+]{-50\,^\circ C} HP_{FR}$$

The HP_{FR} form had an absorption maximum at 665 nm, which is very close to the maximum of the intermediate in the photochemical reaction at −196 °C (see 3.4). These experiments at least show that a proton transfer may occur in the dark reaction.

BUCKSER and HOOPER([127]) recently proposed an elegant mechanism, involving a similar rearrangement of the electron distribution, to explain the colour changes that take place during the bleaching of rhodopsin. The mechanism was based on the finding that conjugated polyenes (such as the chromophore, retinene) can form stable carbonium ions in acid solution, which absorb at much longer wavelengths than their parent molecules([128]). They also assumed that stabilized carbonium ions can be formed in retinene by interaction with an imidazole group of histidine in the protein moiety. At neutral pH the imidazole group acts as a proton donor. The absorption spectrum of the pigment then depends on the place at which in the retinene chain the carbonium ion is stabilized. The carbon atom of the retinene chain with which the proton donor is associated is determined by the geometrical configuration of the retinene (*cis* or *trans*) and by the conformation of the protein. In this case it is clear that the reversible photoisomerization is a result of reactions in the protein moiety as well as in the chromophore.

5. GENERAL DISCUSSION

Many difficulties had to be overcome during the development of a procedure for the large-scale isolation of phytochrome. Because the initial pigment concentration is very low (about 10^{-6} M), its estimation with a normal spectrophotometer gave rise to serious problems. Phytochrome could, however, be determined spectrophotometrically in enriched solutions without difficulty. Measurement of the phytochrome activity in initial extracts indicated the importance of the way in which the seedlings are crushed. When the material was disintegrated too severely, a large amount of inactive protein was extracted, which made subsequent purification of phytochrome more difficult. After much experimentation, a commercial mincer was found in which large amounts of seedlings could be crushed very gently. This mincer had about the same effect as a pestle and mortar on smaller portions. A suitable extraction buffer yielded an initial extract of low ionic strength. This was important for the next purification step, comprising batch adsorption of the pigment on calcium phosphate gel, since adsorption of proteins on this gel is most efficient when the ionic strength and the pH of the extract are low. Optimal conditions for phytochrome adsorption were determined by systematic variation of the ionic strength and the pH of the buffer. Under certain conditions, phytochrome was preferentially adsorbed to the gel, so that the pigment extract was concentrated as well as purified. During the subsequent purification steps, maintaining the stability of the pigment proved to be the most serious problem. Addition of saccharose or glycerol to pigment concentrates enabled storage of the solutions at low temperature without loss of activity.

The pigment was inactivated to a certain extent during column chromatography, as was evident from a loss of photoreversibility, although its stability increased upon purification. Inactivation is due to partial denaturation of the pigment molecule. Loss of photoreversibility is effected even by mild denaturation which illustrates the unstable nature of the pigment. More severe denaturation causes partial bleaching, accompanied by a shift of the absorption maximum of the red form to 650 nm which is characteristic for denatured phytochrome. In many cases, therefore, phytochrome preparations contained some inactivated pigment. For this reason, experiments on the molecular properties of phytochrome, have to be interpreted very cautiously. In the discussion of our work and that of others, attention was paid to this complication.

The purest phytochrome fractions obtained after gel filtration on Sephadex G-200 contained very little inactive pigment, but they were not fully free from other protein impurities. Electrophoretic separation of these impurities was unsuitable, because photoreversibility was lost even under the most gentle conditions. On the basis of their analytical ultracentrifugation studies, CORRELL et al.[54] have suggested the existence of phytochrome tetramers, composed of subunits with a molecular weight of 42,000. The phytochrome in this case was isolated from rye seedlings and differed in several other respects from oat phyto-

chrome. In our experiments, high molecular weight agglomerates were separated by gel filtration. No photoreversibility was left in the agglomerates, suggesting that they arise from the association of inactivated phytochrome molecules. The molecular weight of the active phytochrome was estimated by analytical gel filtration to be 55,500. This value is in agreement with that determined for phytochrome from oat seedlings by MUMFORD and JENNER([53]). Our batch adsorption procedure which is very simple was the first to combine extraction on a large scale with fast initial purification.

Measurements of the difference spectra both in vivo and in extracts of several plants suggest that different species of phytochrome exist. It is unlikely that the relatively minor differences in the absorption maxima are due to differences in chromophore structure; they are, more probably, due to differences in the structure of the protein moiety. The spectra of phytochrome, isolated from different plant species will have to be carefully compared before such structural differences can be confirmed.

The unique photochemical properties of phytochrome namely the stability of both molecular forms at room temperature and their photoreversible transformation with long-wavelength visible light made further study of its structure extremely intriguing. Our approach for studying the mechanism of the photoreversibility of the pigment was largely determined by the available experimental techniques. In cooperation with other laboratories the following aspects have been studied:

The effect of denaturation on the photoreversibility of the pigment.

The changes in optical activity of the pigment during its phototransformations.

The phototransformations of the pigment at low temperature in relation to subsequent dark reactions at normal temperature.

In a series of denaturation experiments it was shown that the adoption of a specific configuration by the protein part surrounding the chromophore is an absolute requirement for the photoreversibility of phytochrome. The pigment is only stable between pH 6 and pH 8. Activity is lost above pH 8, while below pH 6 the pigment becomes insoluble, initially without loss of reversibility. When the freeze-dried pigment is dissolved in pure trifluoroacetic acid, its photoreversibility is lost, but the chromophore itself remains stable. Gentle denaturation with low concentrations of water-miscible solvents (2-chloroethanol and dimethyl sulfoxide) caused a gradual loss of photoreversibility. At higher concentrations, the chromophore was bleached. The same is true for the denaturation of phytochrome with a combination of urea and mercapto group binders, as shown by BUTLER et al.([63]). Addition of glutaraldehyde appeared to obstruct the $P_R \rightarrow P_{FR}$ transformation, since irradiation with red light caused no increase in absorption at 730 nm although absorption at 660 nm decreased as expected. It seems that glutaraldehyde prevents the change in conformation which is needed for stabilization of the chromophore in the P_{FR} form by making cross-linkages between the peptide chains.

While these experiments provided the first indication that the conformation

of the protein part changes during phototransformation, the optical rotatory dispersion and circular dichroism have shown that the optical activity of the pigment is different in the two forms and that the photoreversible transformations of the pigment with red and far-red light are accompanied by corresponding changes in optical activity. A comparison of the optical activity of C-phycocyanin with that of its free chromophore showed that the asymmetry of the chromophore is enhanced, and that the optical effect changes its sign when it associates with the protein part. As we showed, the structure of the phytochrome chromophore is closely related to that of phycocyanobilin. Thus, the change in the sign of the optical effect from negative to positive upon phototransformation of the intact pigment from P_R into P_{FR} suggests that the association between chromophore and protein part is different in the two pigment forms. This provided a further indication that phototransformation involved not only isomerisation of the chromophore, but also a change in the protein conformation. By irradiation of *Mougeotia* cells with a microbeam of polarized light, HAUPT et al.[129] recently showed that phytochrome molecules have a dichroic character in vivo as well and that the axis of its dichroism changes during photoconversion, P_R absorbing light parallel to the surface of the cell and P_{FR} absorbing light perpendicular to the surface.

It is interesting to compare the optical properties of phytochrome with those of the visual pigment rhodopsin studied by others[90, 91]. Rhodopsin undergoes photochemical bleaching which ultimately results in the dissociation of the chromophore from the protein. Changes in optical activity during the photoreversible steps of the bleaching sequence could not be studied separately, because the intermediates are only stable at liquid nitrogen temperature. It could, however, be shown that after complete bleaching, the optical activity of the pigment had disappeared. This becomes plausible if one assumes that the chromophore – retinal – is asymmetrically bound to the opsin, and that it induces a helical conformation in part of the opsin. Apparently, both interdependent properties are lost upon dissociation of the retinal from the opsin[127].

Further information about the phototransformation mechanism of phytochrome was provided by the difference spectra for the photoconversion at liquid nitrogen temperature and for the changes in the pigment during subsequent warming in the dark. It is assumed that the low temperature substantially hinders the conformational changes of the protein part. In the $P_R \rightarrow P_{FR}$ transformation an unstable intermediate was formed with an absorption maximum at 693 nm. The formation of this intermediate can be best explained as a photoisomerisation of the chromophore. This reaction is photoreversible at low temperature. In subsequent dark reactions at higher temperature ($> -70°C$), the conformation of the protein moiety of the intermediate changes in a way, leading to stabilization of the chromophore isomer, thus yielding the P_{FR} form. The phototransformation of P_{FR} to P_R at liquid nitrogen temperature was not characterized by a similar intermediate, but a broad absorption band formed immediately in the red region. Subsequent warming of the sample in darkness was accompanied by a sharpening of the red absorption band and an increase

of its intensity, until the characteristic spectrum of the P_R form is attained. In partially denatured phytochrome preparations the dark reaction did not exclusively yield the P_R form. Instead, upon warming, the absorption in both the red and far-red regions increased, indicating that part of the pigment reverted to the P_{FR} form. This suggests that the conformational change involved in the $P_{FR} \rightarrow P_R$ transformation is more difficult in preparations of this type.

The spectra of phytochrome transformations in vitro at liquid nitrogen temperature were in good agreement with those reported by SPRUIT([99]) for the transformations in vivo. This indicates that the phototransformations of isolated phytochrome are fundamentally the same as those taking place in the plant. Our results also confirmed those obtained by LINSCHITZ et al.([105]) in flash photolysis studies of phytochrome transformations. In the $P_R \rightarrow P_{FR}$ transformation, these authors detected the formation of a short-lived intermediate within 0.2 ms after the flash. This was followed by a sequence of slower reactions, which were light-independent. No similar intermediate was formed in the reverse reaction, but within 0.2 ms, P_{FR} was converted to an intermediate, absorbing in the red region of the spectrum. The finding([29, 31]) that P_{FR} can be converted back to P_R by thermal energy in a dark reaction, demonstrates that it is less stable than P_R. ANDERSON et al.([110]), who studied this dark reversion at -50°C and pH values below 6, suggested that a protonation may be involved. This may indicate that proton transfer is also involved in the phototransformation.

The experiments with intact phytochrome gave an overall picture of the mechanism of its photoreversibility. The study of the chemical structure of its chromophore was initiated to enable a more detailed interpretation.

Our isolation experiments showed clearly that it would be an extremely laborious task to obtain sufficient amounts of purified phytochrome for chemical analysis of the chromophore. Originally the Beltsville group had suggested that, according to its spectral properties, phytochrome could be a biliprotein([4]) and subsequent work made this suggestion more convincing([82]). We decided, therefore, to use phycocyanobilin as a model compound in the study of the chromophore structure by high-resolution mass spectrometry, a method for which only microgram amounts are needed. This approach resulted in the design of a new method for the cleavage of phycocyanobilin from C-phycocyanin, by treatment of the latter with hydrogen bromide in trifluoroacetic acid solution. In this way, phycocyanobilin could be completely split off. After evaporation of the acid solvent, the free chromophore could be extracted by methanol, transferred to chloroform by phase separation, and purified by batch adsorption and gradient elution on silica gel. Analysis of microgram amounts of purified phycocyanobilin by mass spectrometry showed that it was easily evaporated in the high-vacuum source of the instrument and that it had a molecular weight of 588. During this analysis, the molecule was split at its central bridge, resulting in high peak values for the dipyrrole fragments in the mass spectrum. In contrast to this, the diester of phycocyanobilin had a molecular weight of 614, which corresponds to a molecular weight of 586 for the diacid. The diester was more stable than the acid on electron impact, so that the molecular ion peak was

the highest in the spectrum. The structure of the diester was elucidated by NMR spectrometry by other authors([85, 86]) and we were able to confirm it by the same technique, For this analysis, about 40 mg of purified diester was needed. Unfortunately, we did not succeed in making a reliable NMR spectrum of the chromophore in the diacid form, because its solubility in the available deuterated solvents was too low. The discrepancy between the properties of phycocyanobilin (e.g. easy evaporation and fragmentation) and those of its diester were explained by the presence of two 'extra' hydrogen atoms, situated near the central bridge of the non-esterified molecule. These lead to a high degree of intramolecular hydrogen bonding, causing the acid groups to be masked. Another argument for this reasoning was that neither mesobiliverdin nor the phytochrome chromophore in the diacid form gave a mass spectrum similar to that of phycocyanobilin. They were not evaporated and were presumably pyrolysed, yielding unspecific peaks in their spectra. We, therefore, concluded that the structure of the phytochrome chromophore is more of a bilitriene character than that of phycocyanobilin.

A further complication is that neither in the case of phycocyanobilin nor in that of the phytochrome chromophore it is known how these chromophores are coupled to their protein moieties, apart from the knowledge that covalent bonds are involved. A recent publication in which the analysis of phycocyanobilin peptides was described([122]) illustrates the difficulty of this problem. The fact that phycocyanobilin is completely split off with hydrogen bromide gas in acid solution may well imply that it is coupled through one of its propionic acid substituents to a serine residue in the protein by a peptide bond.

The mentioned cleavage method did not work with phytochrome, suggesting that its chromophore is coupled either by a different covalent bond or by more than one. Other authors([130, 82]) showed that the chromophores of C-phycocyanin and phytochrome can be cleaved by boiling with methanol, although in the case of the phytochrome chromophore the yield is very low. With this method we have been able to purify the phytochrome chromophore from the raw methanol extract by the same techniques as used for phycocyanobilin, but on a micro scale. The phytochrome chromophore had the properties of a tetrapyrrole with two acid substituents; a monoester and a diester form could be separated by thin-layer chromatography. As with phycocyanobilin, there were no detectable differences between the spectra of the diacid and diester forms. With the small amounts of purified phytochrome chromophore available, a reliable comparison of ultraviolet spectra with those of other bile pigments, particularly phycocyanobilin and mesobiliverdin, could be made. The spectrum of the chromophore in neutral chloroform is similar to the spectra of these two tetrapyrroles. Its absorption band in the blue region has a maximum at 377 nm, the same as for mesobiliverdin, while its red absorption band has a maximum at a wavelength 10 nm longer than that of phycocyanobilin. The red band maximum of mesobiliverdin, however, is at a wavelength of 40 to 50 nm longer than those of both phycocyanobilin and the phytochrome chromophore.

Summarizing, it can be said that mass spectrometry of the phytochrome chro-

mophore suggests a bilitriene structure, with the four pyrrole rings in full conjugation, whereas spectrometry in the visible region suggests that the chromophore closely resembles phycocyanobilin, in which the conjugation is interrupted in ring 1 by the presence of the ethylidene substituent.

The ultimate proof that the phytochrome chromophore has a tetrapyrrole structure, however, came not from instrumental analysis but from the application of a degradation technique from classical organic chemistry. RÜDIGER et al.([87]) adapted degradation with chromium trioxide, a method known for a long time in porphyrin chemistry, to the analysis of bile pigments. They showed that treating biliproteins with chromium trioxide in weakly acid solution cleaved the chromophores from their protein moieties and oxidized them to maleimides without causing much protein degradation. Moreover, this treatment did not affect the original substituents on the separate pyrrole rings and, provided pH and reaction temperature were carefully controlled, it indicated which rings were coupled to the protein by covalent bonds. The fact that the substituents on the pyrrole rings stayed intact, could be confirmed in our laboratory by infrared analysis and mass spectrometry of maleimides obtained from phycocyanobilin. by degradation with chromium trioxide. RÜDIGER([116]) has since analysed a small amount of a denatured phytochrome preparation from CORRELL et al.([54]). Microgram amounts of maleimides, originating from the phytochrome chromophore were compared with model compounds by a very sensitive detection method on thin layers of silica gel. The provisional results showed three pyrrole rings to be identical with those of phycocyanobilin, namely methylethylidenesuccinimide (ring 1), haematinic acid (ring 2), and haematinic acid coupled to the protein part (ring 3). The fourth ring was not identified but is presumably methylvinylmaleimide. Recently, RÜDIGER analysed active phytochrome obtained from MUMFORD and JENNER([53]). It was found that ring 1 was not detached from the protein during oxidation with chromium trioxide and that there was no difference between P_R and P_{FR} in this respect. In both cases, ring 4 was converted into methylvinylmaleimide. This led RÜDIGER to the conclusion that the phytochrome chromophore is coupled to its protein part by way of one acid substituent and by an unknown bond to ring 1. The conflicting results of his first analysis may then be ascribed to artifacts caused by denaturation.

In the light of the structural data obtained so far on the phytochrome chromophore, we took a further look at the photoreversibility mechanism of the pigment and attempted to evaluate several suggestions in the literature. It was concluded that mechanisms in which the surrounding protein plays no integral part, such as lactim-lactam tautomerism and the extension of the conjugation in the molecule by introduction of two ethylidene double bonds into the ring system([125]), are not compatible with our results. Since the spectrum of phycocyanobilin shows a shift of 70 nm to longer wavelength in acid solution owing to the presence of one pyrrolenine group, and since the dark transformation of P_{FR} is accelerated below pH 6.0, it seems more plausible that a proton transfer is involved. The suggestion of RÜDIGER and CORRELL([116]) that a proton is removed from ring 1 when P_R is converted into P_{FR} agrees with this view. We have

developed this line of thought further by assuming that what has been referred to as chromophore 'isomerisation' in our model may in fact be equivalent to a photochemical rearrangement of the charge distribution. This could result in the formation of the unstable intermediate P_{693}, with one of the pyrrole rings acquiring a proton-donating capacity. The assymetric carbon atom in ring 1 is tentatively considered as the most probable site for the charge localization. The localized charge could then be stabilized by the approach of a proton-acceptor group on the protein which may cause a further rearrangement in the ring, for instance the introduction of the double bond of the ethylidene substituent into the ring. The proton transfer is visualized not as a full exchange but as a partial transfer, which makes the reversibility of the reaction more plausible. This explanation does not exclude the possibility of the intermediate also being stabilized to a certain extent by interaction with the protein.

One may conclude from the foregoing discussion that phytochrome comprises a tetrapyrrole chromophore which is coupled to a protein moiety by one or more covalent bonds. The structure of the chromophore shows a close similarity to that of phycocyanobilin. Absorption of light by the pigment presumably causes a rearrangement of charge in the chromophore, causing the maximum of the absorption band to shift from 660 to 693 nm. The rearrangement of charge is stabilized by a specific interaction between the chromophore intermediate and the protein, once the conformation of the latter has undergone a slight alteration. This alteration can take place in the dark at temperatures above $-70\,^{\circ}C$ and results in a further shift of the absorption maximum to 730 nm, which is characteristic for P_{FR}. This model is based on evidence from denaturation studies, measurement of optical activity and spectral changes at low temperatures and on what is known about the structure of the chromophore.

Our results did not throw any light on the question of the biochemical point of attack of phytochrome in the plant metabolism. During the last few years research in this field has gradually shifted to the biochemical front. Evaluation of fast phytochrome responses, particularly chloroplast movement in *Mougotia* cells([129]) and closure of *Mimosa* and *Albizzia* leaves in the dark([131, 132, 133]), suggests that phytochrome is located in membranes and influences their permeability, which implies that it has a regulatory function([134]). Thus, phytochrome may only be physiologically active when it is bound to particulate cell fractions such as membranes. The bulk phytochrome present in etiolated seedlings would then be inactive. In a recent publication, RUBINSTEIN et al.([135]) described the existence of small amounts of bound phytochrome which could be separated from the cell homogenate of etiolated oat seedlings by centrifuging at 10,000 $\times g$.

The work on phytochrome-regulated enzyme synthesis is also important. This line of approach was initiated by several workers([136, 137, 138, 139]) who studied the induction by light of the synthesis of phenylalanine ammonialyase, a key enzyme in the biosynthesis of phenolic compounds in plants, and by MOHR and coworkers([140]) who investigated the light inhibition of the synthesis of the enzyme lipoxygenase. Whether phytochrome regulates enzyme synthesis

by repression or activation of genetic information, by affecting the transcription of the genetic information, or by direct action on the enzyme itself, has yet to be elucidated. The work by ERLANGER and coworkers[141, 142] on the photochemical regulation of enzyme activity is very interesting in this respect. They have shown that certain photochromic dyes can bind to the active site of the enzyme acetylcholine esterase. The *cis* isomers of these dyes, which are formed by exposure to light of 320 nm, are stronger inhibitors of the enzyme than the *trans* isomers, the latter are formed by exposure of the dyes to light of 420 nm. In this way, the activity of the enzyme can be regulated by light. The authors see this mechanism as a model for phytochrome action.

Study of the structure and the photochemical properties of phytochrome along the lines followed in this investigation, helps us to understand the role of the pigment in the perception by a plant of a photostimulus, generally of low light intensity. The photostimulus triggers a chain of reactions leading to many physiological reactions in plants. As yet, very little is known about the identity of the various steps in this reaction chain. More thorough knowledge of the primary photochemical reactions awaits a complete analysis of the complex structure of the pigment. Such knowledge seems essential for the elucidation of the reaction chain along which the photostimulus is amplified so as to influence ultimately the plant metabolism. It seems quite probable that effects on membrane permeability and on the regulation of enzyme synthesis play an important role in the regulatory function of phytochrome.

SAMENVATTING

Experimenten met phytochroom werden uitgevoerd met het doel nadere informatie te verkrijgen over de structuur van het pigment en het mechanisme van zijn photochemische omzettingen. De photoreversibele omzetting vormt de sleutel tot het begrip van de regulerende functie van het pigment bij photomorphogenetische reacties in planten. De physiologische experimenten over het effect van monochromatisch licht van lage intensiteit, die leidden tot de ontdekking van het pigment in planten, werden samengevat in *Hoofdstuk 1*. Dit overzicht behandelt voorts onderzoekingen over door phytochroom gecontroleerde reacties in geïsoleerde plantendelen, alsmede recent werk over het gedrag van het pigment in vitro.

Als eerste voorwaarde voor de experimenten gold de mogelijkheid om het pigment volgens een simpele methode te kunnen extraheren en concentreren op voldoend grote schaal. De uitwerking van een dergelijke methode wordt beschreven in *Hoofdstuk 2*. Voor verdere zuivering van het pigment werden de gebruikelijke technieken uit de eiwitchemie toegepast, zoals kolomchromatografie en gelfiltratie. Hierbij bleek, dat de photoreversibiliteit van het pigment gemakkelijk verloren gaat, hoewel de stabiliteit toeneemt naarmate de zuiverheid groter wordt. Het molecuulgewicht van het pigment werd geschat op 55,500 met behulp van analytische gelfiltratie op Sephadex G-200. Tijdens de zuivering werd een gedenatureerde pigmentfractie gevormd met een molecuulgewicht groter dan 200,000, die geen reversibiliteit meer vertoonde.

De nadere bestudering van de photoreversibele omzetting wordt in *Hoofdstuk 3* beschreven. Wanneer denaturatie optreedt, verliest het pigment onmiddellijk zijn photoreversibiliteit, hetgeen er op wijst, dat het eiwitgedeelte daarbij een belangrijke rol speelt. Metingen van optische rotatie en circulair dichroïsme toonden aan, dat de optische activiteit in het zichtbare spectrum verschillend is voor de beide vormen, P_R en P_{FR}. Bovendien bleken de veranderingen in de optische activiteit parallel te verlopen met de photochemische omzetting van het pigment. Dit duidt erop, dat de conformatie van het pigment daarbij verandert.

Verdere gegevens over het mechanisme van de photochemische omzetting werden verkregen door meting van de veranderingen in het absorptiespectrum van het pigment bij de temperatuur van vloeibare stikstof. Bij de photochemische omzetting van P_R in P_{FR} wordt een intermediair gevormd, dat een maximum absorptie heeft bij 693 nm en alleen bij lage temperatuur stabiel is. Het intermediair gaat in een aantal stappen in het donker over in P_{FR} als de temperatuur tot boven -70°C verhoogd wordt. Dit reactieverloop kan verklaard worden door aan te nemen, dat door absorptie van licht bij lage temperatuur een isomeer van de chromophoor wordt gevormd en dat de overtollige energie van deze overgangstoestand een conformatieverandering in het eiwit bewerkt; deze conformatieverandering is echter alleen mogelijk bij hogere temperatuur, waarbij het eiwitgedeelte beweeglijker is. De terugreactie, $P_{FR} \rightarrow P_R$, bij lage temperatuur vertoont een dergelijk beeld, maar verloopt niet via hetzelfde intermediar. Bij de

omzetting van P_{FR} verdwijnt de absorptieband van deze pigmentvorm onmiddellijk, terwijl een nieuwe absorptieband in het rode gebied van het spectrum wordt gevormd. Wanneer het pigment daarna in het donker wordt opgewarmd, wordt de rode absorptieband scherper en neemt toe in intensiteit. Het is daarom aannemelijk dat tijdens de terugreactie eveneens een conformatieverandering in het eiwitgedeelte plaats vindt. Onze metingen bij lage temperatuur bevestigen de resultaten van anderen, die de omzettingen van het pigment bestudeerden met behulp van flash fotolyse. Aangezien aangetoond werd, dat bij denaturatie van phytochroom de P_{FR} vorm het snelst verbleekt en P_R spontaan wordt teruggevormd uit P_{FR} in een langzame donkerreactie, is de conclusie gerechtvaardigd dat de P_{FR} vorm van het pigment minder stabiel is.

Om een nauwkeuriger verklaring te kunnen geven van het reactiemechanisme was het belangrijk meer te weten te komen over de structuur van de chromophoor. Onze bijdrage daartoe en die van anderen is beschreven in *Hoofdstuk 4*. Omdat het afsplitsen van de phytochroom-chromophoor van het eiwit zeer moeilijk bleek te zijn, werden modelexperimenten uitgevoerd met phycocyanobiline, de chromophoor van een vergelijkbaar eiwitpigment uit blauwgroene algen. Voor phycocyanobiline werd een methode gevonden, waarmee deze chromophoor selectief van z'n eiwitgedeelte kon worden afgesplitst. Deze reactie verloopt in een oplossing van trifluorazijnzuur, waarin waterstofbromidegas wordt geleid onder uitsluiting van zuurstof. Het afgesplitste phycocyanobilin werd op semi-microschaal gezuiverd door gradientelutie op een silicagelkolom. De phytochroom chromophoor kon niet op dezelfde wijze afgesplitst worden, wat erop duidt dat deze chromophoor met andere covalente bindingen gebonden is aan z'n eiwit. De phytochroom-chromophoor kon wel ontkoppeld worden door koken in methanol, waarbij echter een zeer lage opbrengst wordt verkregen. De zeer kleine hoeveelheden van deze chromophoor konden op microschaal worden gezuiverd met dezelfde methode, die voor phycocyanobiline werd gebruikt. Een vergelijking van de phytochroom-chromophoor met phycocyanobiline en mesobiliverdine met behulp van dunnelaagchromatografie en absorptiespectra, bevestigde dat de phytochroom-chromophoor zeer veel lijkt op deze tetrapyrrolen, maar niet identiek is met één van deze twee.

Massaspectrometrie van phycocyanobiline in zure en veresterde vorm leverde het verrassende resultaat op, dat het molecuulgewicht van het zuur twee massaeenheden hoger ligt dan de op grond van het molecuulgewicht van de ester berekende waarde. Ook werden verschillen in het fragmentatiepatroon van beide componenten gevonden. Deze resultaten suggereren, dat de structuur van de diestervorm van phycocyanobiline, zoals die door anderen met behulp van NMR spectrometrie bepaald werd, niet identiek is met die van de zure vorm. NMR analyse laat zien dat phycocyanobiline een tetrapyrrol is, dat op één substituent na, ethylideen in plaats van ethyl, gelijk is aan mesobiliverdine-IX α. Met de aanwezigheid van twee 'extra' H-atomen kunnen echter een aantal bijzondere eigenschappen van het zuur verklaard worden. Met behulp van molecuulmodellen kon een ruimtelijke structuur van phycocyanobiline vastgesteld worden, die laat zien, dat een dergelijk tetrapyrrol in verschillende conformaties

kan voorkomen. Analyse van de phytochroom-chromophoor in de massaspectrometer toonde aan, dat deze stof in tegenstelling tot phycocyanobiline niet verdampt, maar afgebroken wordt door pyrolyse, wat overeenstemt met het gedrag van mesobiliverdine.

Toen het onderzoek dit stadium had bereikt, werd door RÜDIGER met behulp van een chemische afbraakreactie bewezen, dat de phytochroom-chromophoor een tetrapyrrolstructuur heeft met een substitutiepatroon, dat waarschijnlijk op één plaats verschilt van dat van phycocyanobiline.

Aan de hand van deze gegevens wordt in *Hoofdstuk 4* getracht de isomerisatie van de chromophoor nader te preciseren. Er wordt verondersteld, dat de lichtabsorptie door P_R een verandering van de ladingsverdeling in de chromophoor veroorzaakt, die zich uit in een verschuiving van het absorptiemaximum van 660 naar 693 nm. De eindring van de chromophoor met de ethylideen-substituent krijgt daarbij het karakter van een protondonor. Deze op zichzelf niet stabiele configuratie wordt gestabiliseerd door het naderbij komen van een proton-acceptor groep in de peptideketen, hetgeen mogelijk is door een conformatieverandering van het eiwit. Deze configuratie is de P_{FR} vorm. Karakteristiek voor deze reactie is een gedeeltelijke maar niet volledige uitwisseling van een proton tussen de chromophoor en het naburige peptideresidu.

In *Hoofdstuk 5* worden de resultaten van de experimenten tenslotte samengevat en wordt nagegaan in hoeverre de opheldering van de structuur en het reactiemechanisme van phytochroom kunnen bijdragen tot een verklaring van de functie van het pigment in planten.

SUMMARY

Experiments have been performed with the plant pigment phytochrome with the aim of elucidating its structure and the mechanism of its photoreversible transformation. The photoreversibility of phytochrome is the key to its function as a regulator of morphogenetic reactions in plants. Physiological experiments on the effect of low-intensity monochromatic light in plants, which led to the discovery of the pigment, and the results of recent studies of phytochrome-controlled reactions in excised plant parts are reviewed in *Chapter 1*. The review also includes work on the behaviour of the pigment in vitro.

A primary requirement for the experiments was a simple and fast method for extracting and concentrating sufficient amounts of the pigment. The development of a suitable method, involving batch adsorption on calcium phosphate gel, is described in *Chapter 2*. Techniques well known in protein chemistry, such as column chromatography and gel filtration, were employed as subsequent purification steps. It was found that the pigment loses its photoreversible properties very easily, although its stability increases upon purification. The molecular weight of phytochrome was estimated to be 55,500 by analytical gelfiltration on Sephadex G-200. During purification, a denatured pigment fraction with a molecular weight above 200,000 was formed; this fraction was no longer photoreversible.

More detailed studies of the photoreversible transformations of phytochrome are described in *Chapter 3*. Upon denaturation, photoreversibility is lost immediately, indicating that the protein moiety plays an important role. Optical rotatory dispersion and circular dichroic spectra showed that the optical activity of the pigment in the visible range of the spectrum is different in the P_R and the P_{FR} forms.

Moreover, changes in the optical activity of the pigment fully parallel its phototransformation, suggesting that the conformation of the pigment changes during these reactions.

Further information about the phototransformation mechanism was obtained by recording spectral changes at the temperature of liquid nitrogen. In the photochemical $P_R \rightarrow P_{FR}$ transformation, an unstable intermediate with an absorption maximum at 693 nm is formed. This intermediate is transformed into P_{FR} in a series of dark reactions if the temperature is raised above $-70\,^{\circ}C$. A possible explanation is that the chromophore is photochemically isomerized at the low temperature, and that the energy surplus of the resulting transition state induces a conformational change in the protein which is possible only at higher temperature. The reverse reaction, $P_{FR} \rightarrow P_R$, follows a similar pattern but does not involve the same intermediate. The P_{FR} absorption band disappears immediately upon transition at low temperature, while a broad absorption band appears in the red region of the spectrum. Upon subsequent warming in the dark, the red absorption band is sharpened and intensified, which suggests that a conformational change is involved in this reaction as well. The results of

the low-temperature measurements agree well with those of flash-photolysis studied by other workers. The findings that P_{FR} is bleached more quickly than P_R upon denaturation and that P_{FR} reverts spontaneously to P_R in a slow reaction in the dark indicate that the P_R form is more stable than the P_{FR} form.

Elucidation of the structure of the phytochrome chromophore would enable a more comprehensive explanation of the transformation mechanism. Our contribution in this respect is described in *Chapter 4* in connection with those of others. Since cleavage of the phytochrome chromophore from its protein moiety proved very difficult, model experiments were performed with phycocyanobilin, the chromophore of a similar pigment in blue-green algae. Phycocyanobilin could be split off very effectively by treatment of the algal pigment with hydrogen bromide in trifluoroacetic acid solution in the absence of oxygen. The phycocyanobilin separated in this way was purified on semi-micro scale by gradient elution on a silica gel column. The chromophore of phytochrome could not be separated by this method, which implies that it is coupled to its protein by other covalent bonds than is the case with phycocyanobilin. The only way in which we could cleave the phytochrome chromophore from its protein part was by boiling the pigment in methanol, but the yield was very low. Small amounts of phytochrome chromophore were purified by applying the method used for phycocyanobilin, on a micro scale. A comparison of the thin-layer chromatogram and the absorption spectrum of the phytochrome chromophore with those of phycocyanobilin and mesobiliverdin confirmed that it is structurally very similar to these tetrapyrroles but not identical to either one of them.

Mass spectrometry of phycocyanobilin, both in acid and esterified form, yielded the striking result that the molecular weight of the acid form was two mass units higher than the value based on the molecular weight of the ester. The two forms also differed in their fragmentation patterns. These results imply that the structure of the product formed by esterification of phycocyanobilin, which other workers had determined by NMR spectrometry, is not identical to that of the diacid. NMR analysis did show that phycocyanobilin is a tetrapyrrole, which differs from mesobiliverdin-IXα in that one ring substituent is ethylidene instead of ethyl. With the aid of molecular models and the knowledge of the presence of two 'extra' hydrogen atoms in the diacid form, a specific spatial configuration could be assigned to phycocyanobilin diacid which illustrated that such a tetrapyrrole does not necessarily exist in the conventional linear conformation. Analysis of the phytochrome chromophore by mass spectrometry showed that, in contrast with phycocyanobilin, it does not evaporate but is fragmented by pyrolysis. In this respect it resembles mesobiliverdin.

Meanwhile, RÜDIGER, with the aid of a chemical degradation method, had confirmed that the phytochrome chromophore is a tetrapyrrole, the substitution pattern differing from that of phycocyanobilin probably in just one place.

On the basis of these structural data an attempt was made towards a more specific characterisation of the isomerisation of the chromophore. It is proposed that absorption of light by P_R causes a redistribution of charge in the chromophore, which is expressed as a shift in the absorption maximum from 660 to

693 nm and which results in the outer pyrrole ring with the ethylidene substituent acquiring a proton-donating capacity. This configuration is stabilized by the approach of a proton-accepting group in the peptide chain of the protein, once the conformation of the latter has been changed. The reaction is characterized by a partial, rather than a full, transfer of a proton from the chromophore to an amino acid residue in the peptide chain.

The results of the experiments are discussed in a broader context in *Chapter 5*. An assessment is made of the extent to which the elucidation of the structure and the transformation mechanism of phytochrome can contribute to an understandingof the regulatory function of the pigment in plants.

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