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# BINDING PROPERTIES OF THE PLANT PHOTORECEPTOR PHYTOCHROME TO MEMBRANES

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Phytochrome (P), a chromoprotein of 120,000 MW, occurs at low concentrations in all higher plants. The chromophore is an open tetrapyrrole. The pigment exists in two light-absorbing forms: Pr, which absorbs at 660 nm, and Pfr, which absorbs at 730 nm. These forms are interconvertible by light. Pr, the physiologically inactive form, exists in dark-grown plants; Pfr, the active form, appears after irradiation with red light. P-mediated responses, of which about 80 are known, range from short-time effects (sec) such as bioelectric potentials, to long-time effects (hr) such as increases in enzymatic activity. Measurements of phototransformation in vivo with polarized light suggest that P is localized in the plasma membrane. Particulate cell fractions contain about 70% of total extractable P if Pfr is present and only 4% if Pr is present. Evidence indicates that the fraction containing Pfr may be the plasma membrane. One can isolate a partially solubilized membrane system, which can be reversibly reconstituted by adding Mg. The reformed vesicles bind Pfr in vitro. Pfr binding increases with decreasing pH and decreases with increasing monovalent cation concentration. Pfr is released from the membrane by far red light (Pr is formed) and by Triton X-100. We suggest that Pfr binding to a membrane induces conformational changes; the functional properties of this membrane are altered, which might lead to the observed phytochrome-mediated responses.

## INTRODUCTION

Light plays a very important role in the growth and development of all green plants: radiant energy is absorbed by the photosynthetic pigments and converted to chemical potential energy; the same ubiquitous radiant energy is used, at irradiances orders of magnitudes lower than those involved in photosynthesis, to convey selective information from the environment. These signals are used to regulate growth and development. One of these "photochromic sensors" (1) has been called phytochrome. Since its discovery in the 1950's (2, 3) many and varied physiological effects of phytochrome have been discovered. It participates in the control of stem elongation. Induction and release from dormancy are mediated through phytochrome by light. It also controls movement – for example, of the chloroplast of Mougeotia (4). Bioelectric potentials are generated (5) and enzymatic

 

activities can be increased or decreased (6). A rough estimate indicates more than 100 phytochrome-mediated responses.

Phytochrome is a chromoprotein of about 120,000 MW (7). The prosthetic group is an open-chain tetrapyrrole (8, 9, 10). The pigment can exist in darkness in either of two forms: as Pr, absorbing red light with a maximum at about 660 nm, and as Pfr, absorbing far red light with a maximum at about 730 nm. Both forms are interconvertible by light:

$$\mathrm{Pr} \underset{\text{far red}}{\overset{\text{red}}{\rightleftharpoons}} \mathrm{Pfr}$$

Since the absorption spectra of Pr and Pfr overlap throughout the visible range, continuous irradiation establishes a photostationary state depending on the quality and quantity of incoming light quanta. In dark-grown plants only Pr, which is believed to be physiologically inactive, is present (11). Irradiation with red light generates Pfr, which is the physiologically active form. The photochemical formation of Pfr involves the isomerization of the chromophore (12) and a number of dark reactions which lead at least to small conformational differences between Pr and Pfr (13, 14, 15, 16).

Investigations of physiological effects and measurements of the photochemical state of phytochrome in vivo have lead to several hypotheses for its mechanism of action. Two major explanations have been advanced for the "multiple action" (17) of phytochrome:

(1) Phytochrome operates on the level of differential gene expression, controlling synthesis or repression of specific enzymes (6, 18, 19, 20, 21).

(2) Phytochrome controls the conformational state of membranes and regulates their permeability, as a consequence of which physiological responses are mediated (22, 23, 24, 25, 26, 27). These hypotheses are not necessarily mutually exclusive.

The main findings which led to the membrane concept can be classified roughly into two groups. One concerns short-time effects such as the generation of phytochrome-mediated bioelectric potentials (5) and red-light–induced adhesion of isolated mung bean and barley root tips to a negatively charged glass surface (28, 29). Both effects can be observed within seconds. The second group concerns experiments on the localization of phytochrome. Haupt et al. (24) used microbeam irradiation of the plasma membrane and other regions of Mougeotia cells and observed the subsequent chloroplast movement. They concluded that the responsible phytochrome must be located and oriented in or very near to the plasma membrane. In vivo measurements of phototransformation with polarized light in cells of corn coleoptiles lead to the same conclusion (30).

The first evidence for particulate phytochrome after cell fractionation came from Gordon (31) and later from Rubinstein et al. (32). Marmé et al. (33) reported a correlation between pelletable phytochrome and the binding of naphthylphthalamic acid (NPA), which has been suggested to be a specific marker for plant plasma membranes in corn and zucchini (34). More recent studies demonstrated that phytochrome is able to bind specifically to membranes in vitro and in vivo (26, 35, 36, 37). Roux and Yguerabide (38) have demonstrated photoreversible conductance changes induced by phytochrome in a model membrane. These authors suggest as one possible explanation of their effects that Pfr is able to bind to the oxidized cholesterol membrane and Pr is not.

## METHODS

### Materials

Seeds of zucchini squash (Cucurbita pepo, L.cv. Black Beauty) and seeds of corn (Zea mays L., WF9x38 from Bear) were germinated in darkness for six days at 25°C. The hypocotyl hooks (1 cm in length) from the squash seedlings were harvested. The coleoptiles from corn were harvested and the primary leaves removed and discarded. All manipulations were carried out in green safe light. The freshly harvested tissue was immediately placed on ice.

### Buffers

The extraction buffer contained: 25 mM N-morpholino-3-propanesulfonic acid (MOPS), 10 mM disodium salt of ethylenediamine tetraacetic acid (EDTA), 14 mM 2-mercaptoethanol (except for samples used for protein assay), at pH 7.3 unless stated otherwise. Methods except those listed below are described in detail in previous papers (26, 36, 37).

### Protein

Protein is determined by the method of Lowry et al. (39).

### NADPH-cytochrome-c-reductase

NADPH-cytochrome-c-reductase assays are performed at 25°C by measuring the reduction of oxidized cytochrome c at 550 nm in a Perkin Elmer Model 356 (40). A 1.16 ml reaction mixture consists of 1 ml of 0.05 M phosphate buffer at pH 7.5, 0.05 ml of 0.05 M sodium cyanide, 0.05 ml of 0.45 mM cytochrome c, and 0.05 ml of enzyme. The reaction is started with the addition of 0.01 ml of 5 mM NADPH.

### Lumiflavin

Lumiflavin is obtained from each gradient fraction by photodecomposition in alkaline solution (41). The sample (0.5 ml) is mixed with 0.5 ml 1 M NaOH and exposed for 30 min to a fluorescent lamp. Following irradiation, 0.5 ml of glacial acetic acid is added. Then 4 ml of chloroform are added and the tubes are shaken to extract the lumiflavin. The fluorescence excitation spectrum is measured from 300 to 500 nm. Emission is detected at 540 nm. Relative lumiflavin contents were determined by measuring fluorescence intensity at 540 nm when the excitation wavelength was at 460 nm.

## RESULTS

The induction of binding of Pfr to particulate cell components can be achieved both in vivo and in vitro. When no red light is given to dark-grown seedlings of zucchini or

**TABLE I. Effect of Red Light In Vivo and In Vitro on the Pelletability of Particle-Bound Phytochrome**

| Red Light Treatment | Percentage of Pelletable Phytochrome (Pfr) | |
|---|---|---|
| | Zucchini | Corn |
| None | 5* | 5* |
| In Vivo | 70 | 20 |
| In Vitro | | |
| 20 KS | 70 | 5 |

*As Pr.

The amount of pelletable phytochrome is calculated per total extractable pigment. For the dark control (first row) and the red light treatment in vivo (second row) the pelletable phytochrome is determined after one centrifugation of the homogenate at 20,000 × g for 30 min. To assay for in vitro induction of Pfr binding by red light, dark-grown tissue is homogenized. The homogenate is centrifuged at 20,000 × g for 30 min. The resultant supernatant (20 KS) is irradiated with red light and centrifuged again at 20,000 × g for 30 min. The amount of pelletable phytochrome is indicated in the last row. For all three treatments 20 mM $Mg^{++}$ was present in the extraction buffer.

corn only about 5% of the total extractable phytochrome can be obtained in the first 20,000 × g pellet (Table I). The pigment is in the Pr form. Brief irradiation of the seedlings with red light in vivo causes an increase in pelletable phytochrome by a factor of 12 for zucchini and a factor of 4 for corn (Table I). Thus phototransformation in vivo to the physiologically active form, Pfr, leads to considerable sedimentable phytochrome.

Binding of phytochrome to particulate material can also be induced by irradiation in vitro. After extraction of dark-grown plants and centrifugation of the homogenate at 20,000 × g for 30 min, the remaining supernatant (20 KS) is irradiated with red light and then centrifuged again at 20,000 × g for 30 min. As a result of this irradiation 70% of the total phytochrome from zucchini, but only 5% from corn, is found in the resulting pellet (Table I). Corresponding centrifugations of nonirradiated supernatants yield 1–2% pelletable phytochrome for both zucchini and corn.

### Effect of $Mg^{++}$ on the Pelletability of Phytochrome

The divalent cations $Mg^{++}$ and $Ca^{++}$ play an important role in the pelletability of phytochrome extractable from zucchini seedlings after irradiation in vivo or in vitro (26, 36). These findings have been confirmed for corn coleoptiles after irradiation in vivo (35).

Dark-grown zucchini hypocotyl hooks and corn coleoptiles which received 3 min of red light are extracted in the absence of $Mg^{++}$ (10 mM EDTA is present). After a first centrifugation at 500 × g for 10 min, different $Mg^{++}$ concentrations are added to the remaining supernatant (0.5 KS). A second centrifugation at 20,000 × g for 30 min separates the pelletable phytochrome from the soluble phytochrome (Fig. 1; upper curve for zucchini and upper curve for corn). If this centrifugation is preceded by one at 20,000 ×

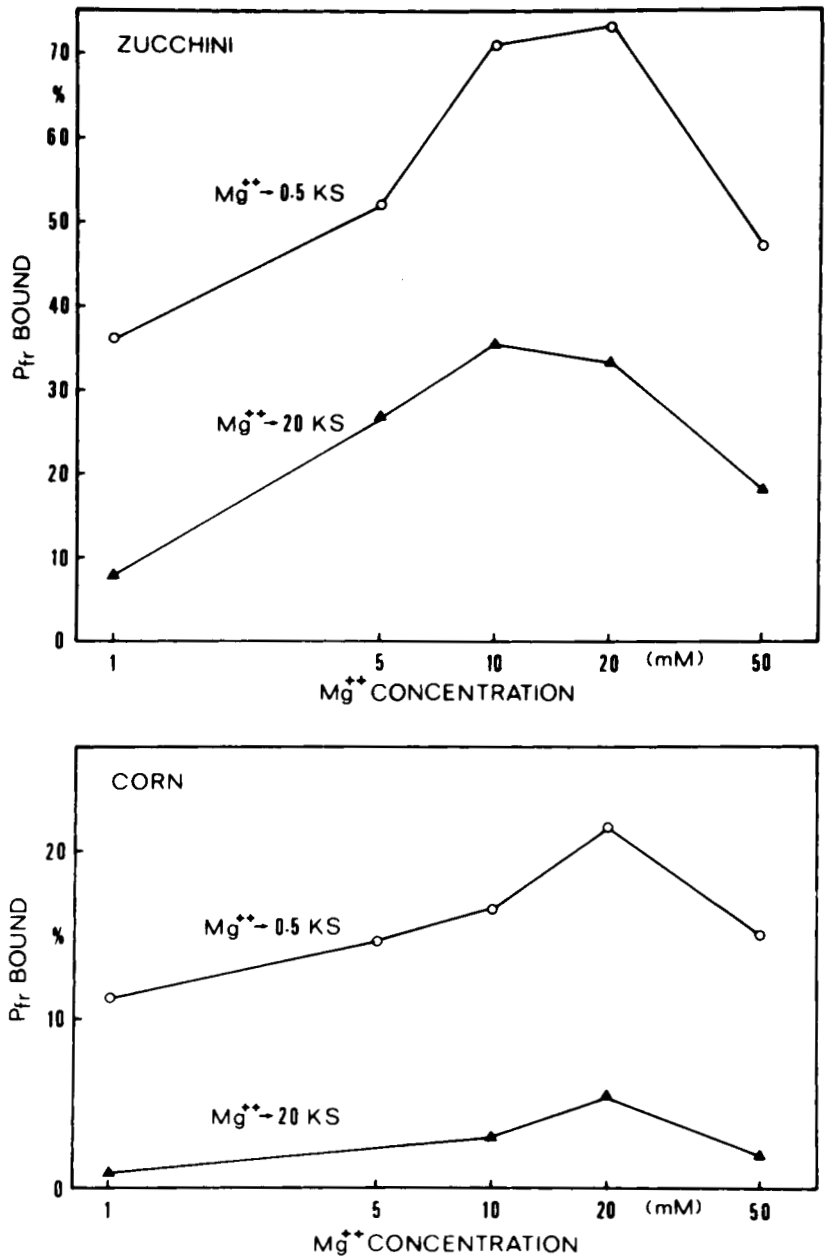


Fig. 1. Effect of $Mg^{++}$ on the pelletability of phytochrome from zucchini (upper curve) and corn (lower curves). $Mg^{++}$ is added before ($Mg^{++} \rightarrow 0.5$ KS) and after ($Mg^{++} \rightarrow 20$ KS) centrifugation at 20,000 X g for 30 min. In both cases bound Pfr is pelleted at 20,000 X g for 30 min after the addition of $Mg^{++}$.

g for 30 min in the absence of $Mg^{++}$ and the different $Mg^{++}$ concentrations are added to the resultant supernatant (20 KS) and centrifuged another time at the same acceleration and for the same time, the lower curves for zucchini and corn (Fig. 1) are obtained. The shapes of the curves with and without precentrifugation do not differ significantly. They are displaced by the amount of phytochrome which is pelleted in the absence of $Mg^{++}$: 30% for zucchini and about 10% for corn. This means that we are dealing with a $Mg^{++}$-independent fraction and a $Mg^{++}$-dependent fraction with respect to the pelletability of phytochrome.

### On the Nature of the $Mg^{++}$-Independent Fraction

We have studied the centrifugal separation of the membrane vesicles and cell organelles by rate-zonal and equilibrium density-gradient techniques. Zucchini hypocotyl hooks were irradiated in vivo with 3 min red light and extracted in standard buffer solution containing 18% sucrose. After removal of the larger cell debris at 500 × g for 10 min the resulting supernatant was centrifuged for 15 min at 12,000 × g. This differential centrifugation step removes about 90% of the mitochondria and about 20% of the total pelletable phytochrome. Mitochondria are found to trap phytochrome-containing particles, leading to false interpretations of gradient results. The 12,000 × g supernatant was diluted 1:1 with extraction buffer at pH 7.0 without sucrose to enable phytochrome-containing particles to sediment in 30 min at 20,000 × g to the bottom of the tube. The pellet was carefully washed and resuspended and layered on top of a linear sucrose gradient (20–50% sucrose w/w). After 16 hr at 23,000 rpm in the Beckman SW 25.2 rotor, isopycnic equilibrium was reached. Fig. 2 shows the distribution of phytochrome on a gradient prepared from zucchini seedlings. There is one main phytochrome peak. Its density corresponds to 34% sucrose. The remaining mitochondria, as measured by their cytochrome-c-oxidase activity, are clearly displaced towards higher densities (38.5% sucrose). The activity of NADPH-cytochrome-c-reductase, which is considered to be a marker enzyme for endoplasmic reticulum (42, 43), is distributed as a broad peak centered at 28.5%.Lumiflavin, extracted from membrane fractions, coincides with the phytochrome peak. The weak shoulder towards higher densities might be due to the mitochondria. Rate-zonal gradients show that mitochondria migrate faster than the bound Pfr. NADPH-cytochrome-c-reductase moves even more slowly into the gradient. Differential precentrifugation together with rate-zonal and equilibrium density centrifugation provides a suitable tool for isolation and concentration of phytochrome-containing particles.

Specifically bound NPA, which has been suggested to be a specific marker for plasma membrane (34), coincides within the error of the experimental measurement with the phytochrome peak under rate-zonal conditions and equilibrium conditions. Figure 3 shows an equilibrium density-gradient for zucchini. From these data it seems probable that Pfr binds in vivo to the plasma membrane. One obtains qualitatively the same results for corn coleoptiles.

### Binding Properties of Phytochrome to the $Mg^{++}$-Independent Fraction

Phytochrome-mediated physiological responses are mostly characterized by their reversibility under far red light. A short pulse of far red light given after the red light

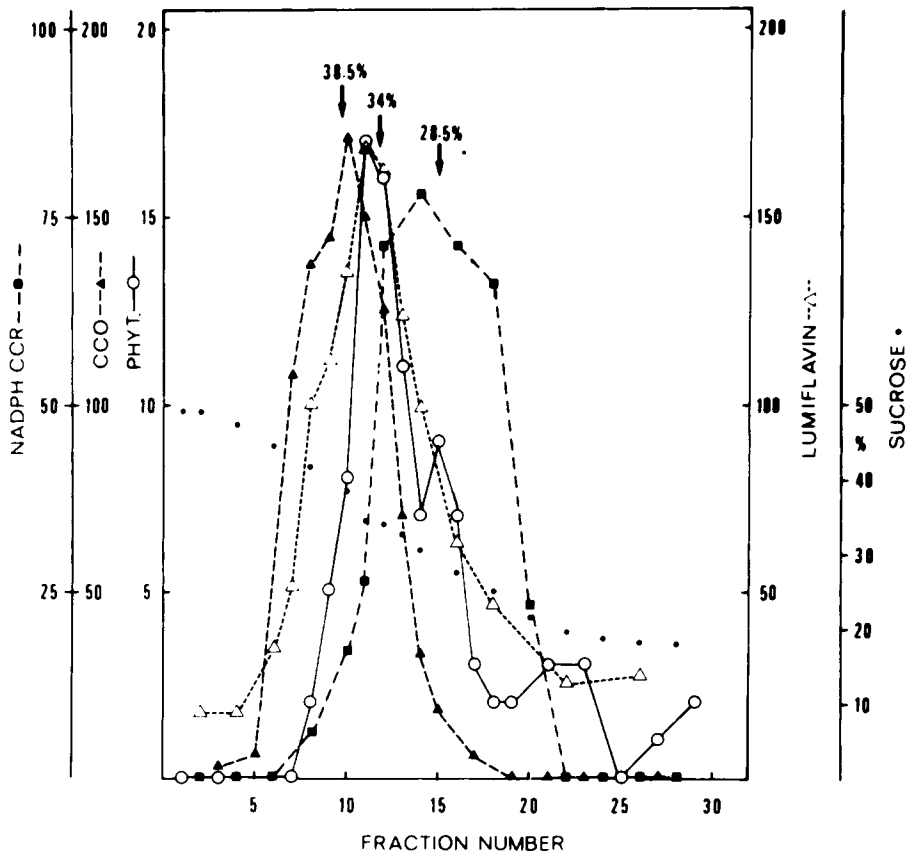


Fig. 2. Continuous sucrose equilibrium density-gradient from zucchini. Closed triangles: ctyochrome-c-oxidase; open triangles: Pfr; open circles: lumiflavin; closed squares: NADPH-cytochrome-c-reductase. The gradient was centrifuged for 16 hr at 23,000 rpm in a SW 25/2 Beckman rotor.

cancels the physiological effect. In a recent paper we demonstrated that far red light given after red light in vivo releases most of the bound phytochrome from the membrane (41). This is in good agreement with what one would expect from physiological experiments.

For physiological reasons Pfr is thought to bind to the inside of the plasma membrane. To determine whether Pfr is bound to the inside of the isolated plasma membrane vesicles, which are assumed to be "inside in," we have taken advantage of the pH-dependence of Pfr binding. It has been demonstrated that Pfr binding increases with decreasing pH (26).

Extraction of membrane-bound Pfr from red-light–treated zucchini seedlings is performed at different pH values. After removal of the larger cell fragments by low-speed centrifugation, the pH is readjusted to several different values. Subsequent centrifugation at 20,000 X g for 30 min yields the bound phytochrome in the pellet. Figure 4 shows that the amount of pelletable Pfr depends strongly on the pH of the extraction buffer and is more or less independent of the readjusted pH. The binding increases from 10% at pH 7.6 to 35% at pH 6.6. Readjusting the pH after extraction up to pH 8.0 releases

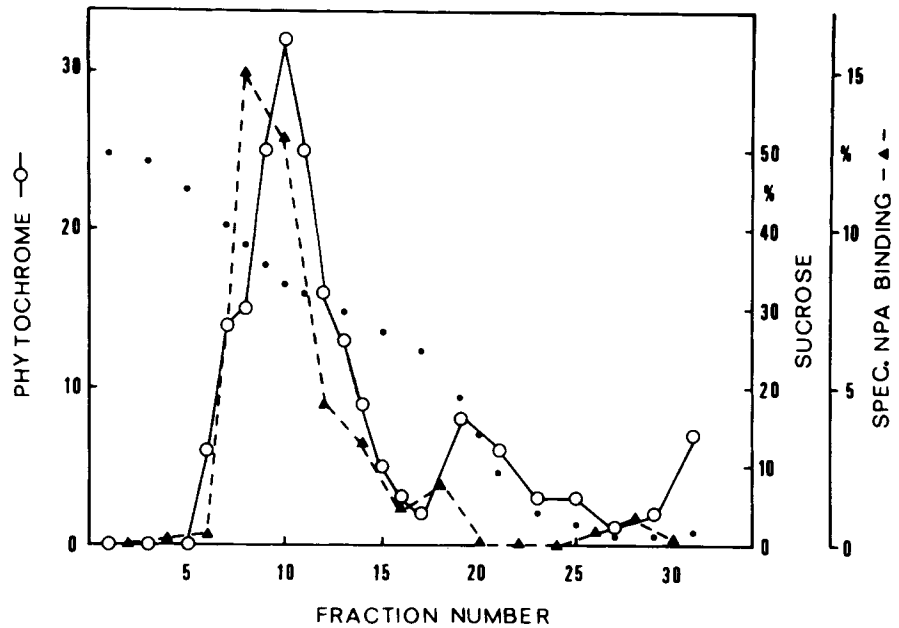


Fig. 3. Continuous sucrose equilibrium density-gradient from zucchini. Open circles: Pfr; closed triangles: specific NPA binding. The gradient was centrifuged for 16 hr at 23,000 rpm in a SW 25/2 Beckman rotor.

little Pfr to the soluble fraction. Neither far red light nor 0.1 M NaCl are able to decrease the amount of pelletable Pfr unless the membrane vesicles are opened by gentle sonication or hard resuspension. This indicates that the Pfr is bound to the inside of the plasma membrane vesicles. The bound pigment is released from the membrane by ionic strength, high pH, and far red light, but can leave the membrane vesicles only after they have been opened by sonication.

### Phytochrome-Binding Properties of the $Mg^{++}$ -Dependent Fraction

As previously mentioned, the amount of pelletable phytochrome depends strongly on the $Mg^{++}$ concentration in the homogenate (26). This is true for induction of binding with red light both in vivo (36) and in vitro (26). The results after irradiation in vivo are shown in Fig. 1. When cell-free extracts of dark-grown material are irradiated with red light, similar curves are obtained (Fig. 5).

To demonstrate that the increase in pelletability with increasing $Mg^{++}$ concentrations is not simply an aggregation of phytochrome itself, we have shown that precentrifugation of the homogenate from dark-grown material in the presence of $Mg^{++}$ removes structures which are able to bind Pfr (26). The homogenate was centrifuged before red irradiation at 50,000 X g for periods of 5 to 120 min. The supernatant from this precentrifugation, which still contains most of the originally present phytochrome, was then irradiated with red light, and a second centrifugation at 50,000 X g for 30 min was

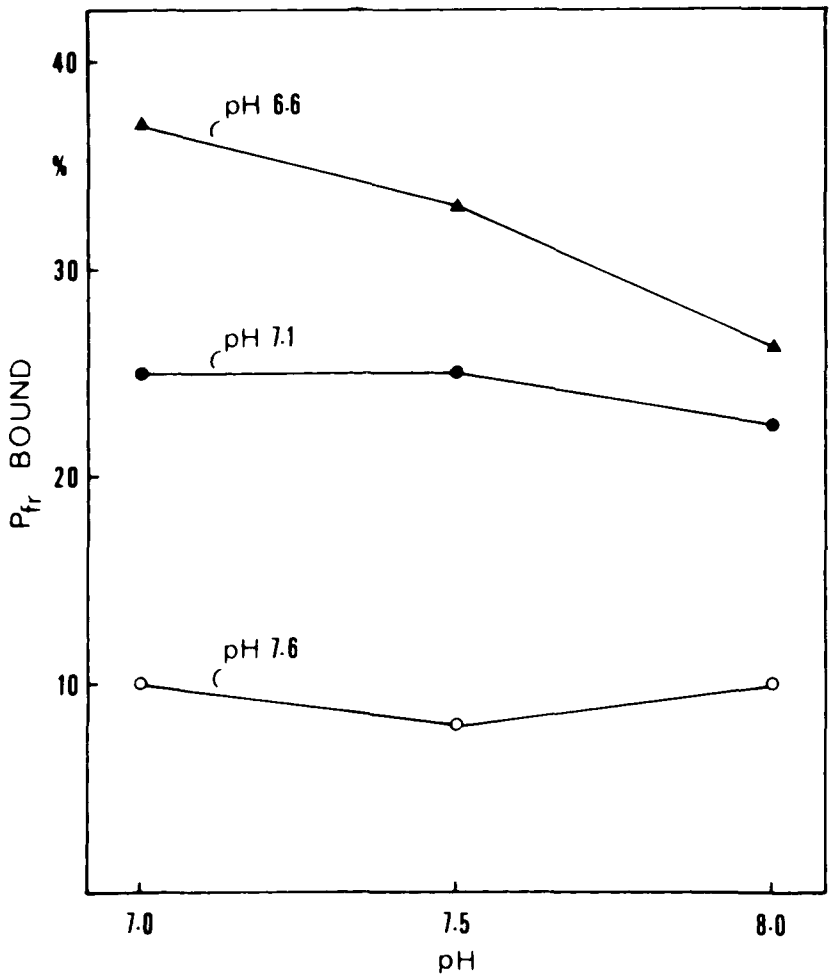


Fig. 4. Effect of pH on the Pfr binding to the $Mg^{++}$-independent fraction. The red-light-treated zucchini hypocotyl hooks are extracted at pH 6.6, 7.1, and 7.6. The pH of the homogenate is readjusted to 7.0, 7.5, and 8.0 before the assay for pelletable phytochrome is performed.

performed to assay for Pfr binding. Figure 6 shows that after 60 min of precentrifugation at 50,000 × g all Pfr-binding structures are removed from the homogenate.

In Fig. 5 the amount of sedimentable Pfr per mg protein after induction of binding in vitro is plotted against $Mg^{++}$ concentration. While total pelletable protein increases from 1 to 10 mM $Mg^{++}$ and then remains constant up to 50 mM $Mg^{++}$, the bound Pfr decreases sharply at higher $Mg^{++}$ concentrations. In a previous paper we have shown by electron microscopic investigations that $Mg^{++}$, in contrast to sodium and potassium, causes the formation of vesicles and their aggregation (36). Negatively stained preparations of precentrifuged homogenates show in the absence of $Mg^{++}$ only very small particles about 100 Å in diameter. At $Mg^{++}$ concentrations equal to the EDTA concen-

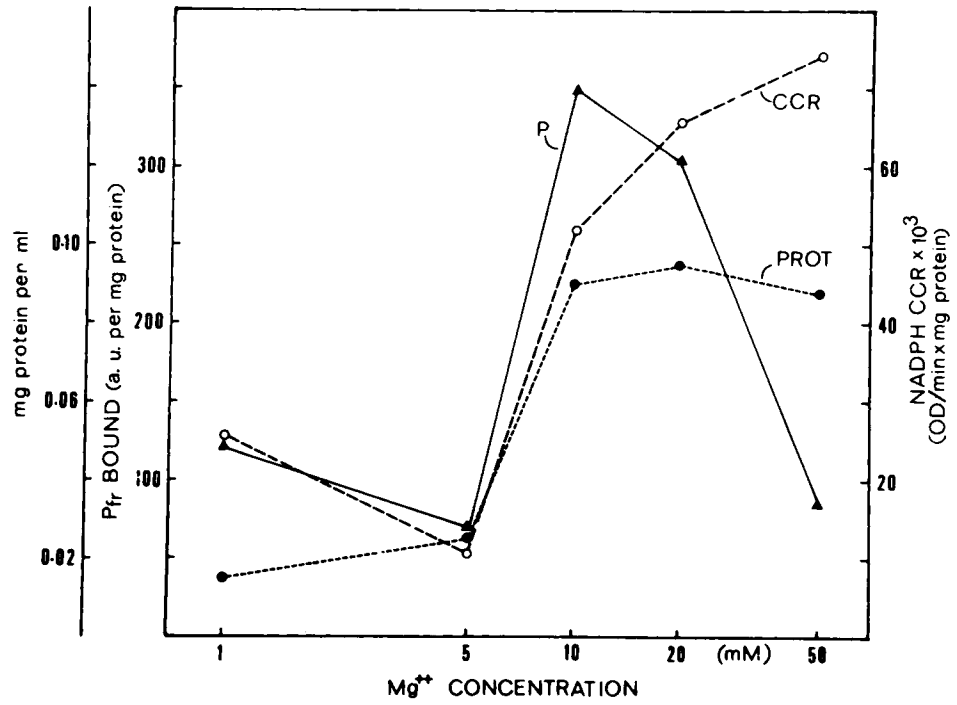


Fig. 5. $Mg^{++}$ effect on pelletability of protein (dark circles), phytochrome per mg protein (dark triangles), and NADPH-cytochrome-c-reductase per mg protein (open circles).

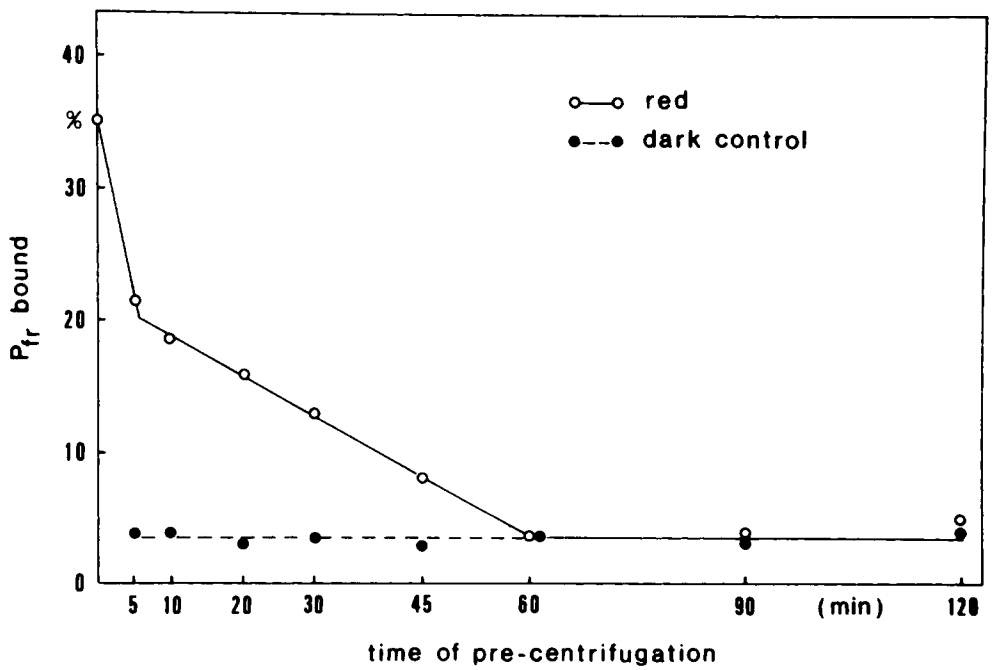


Fig. 6. Effect of precentrifugation of the homogenate before red irradiation on subsequently pelletable phytochrome after red treatment and a second centrifugation of 30 min at 50,000 X g. The acceleration of the precentrifugation was 50,000 X g. [This figure is taken from Marmé et al. (26).]

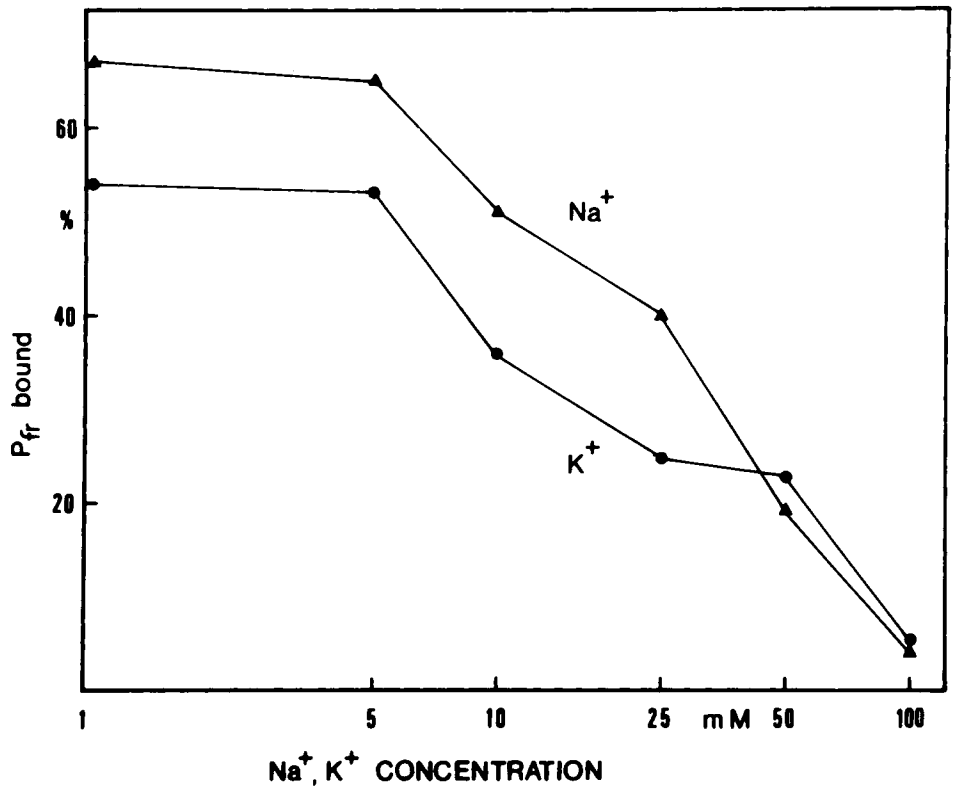


Fig. 7. Effect of $Na^+$ and $K^+$ on the binding of Pfr to the $Mg^{++}$-dependent vesicles.

tration in the homogenization medium, distinct vesicles with diameters between 400 and 600 Å can be observed. Higher $Mg^{++}$ concentrations cause aggregation of these vesicles and therefore facilitate their sedimentation. The clumped vesicles do not disappear at higher $Mg^{++}$ concentrations. These findings are in agreement with the $Mg^{++}$-dependent increase in sedimentable protein (Fig. 5). The influence of $Mg^{++}$ on vesicle formation and aggregation is reversible. The increase of Pfr in the sediment with increasing $Mg^{++}$ concentrations can therefore most probably be explained by the formation of Pfr-binding vesicles which clump together at higher $Mg^{++}$ concentrations and become more easily sedimentable.

It will be shown below that the decrease of sedimentable Pfr at higher $Mg^{++}$ concentrations is due to an ionic strength effect on the binding.

To investigate the effect of ionic strength on Pfr binding, $Na^+$ of $K^+$ is added at various concentrations to a red irradiated supernatant prior to the addition of a $Mg^{++}$ concentration which causes maximal pelletability of Pfr. Figure 7 shows the decrease of bound Pfr with increasing monovalent cation concentration. Almost no Pfr is pelletable at 100 mM $Na^+$ or $K^+$. The amount of sedimentable protein does not change significantly.

In a previous paper it was shown that a decrease in pH leads to an increase in Pfr

TABLE II. Effect of $Mg^{++}$ on the Specific Binding of Naphthylphthalamic Acid (NPA)

| Fraction | $Mg^{++}$ (mM) | % Specifically bound NPA |
|---|---|---|
| Homogenate | None | 10 |
| 20 KS | None | 2 |
| 20 KP | None | 11 |
| 20 KS | 10 | 1 |
| 20 KP | 10 | 9 |

The $Mg^{++}$ is added before the centrifugation at 20,000 × g for 30 min. The pellets 20 KP with and without $Mg^{++}$ are resuspended and assayed for specific NPA binding. Specific NPA binding is the difference in NPA binding between a sample containing only $^3$H-NPA (about $10^{-8}$ M) and a sample containing $^3$H-NPA ($10^{-8}$ M) and unlabeled NPA ($10^{-5}$ M). For further information on the NPA binding assay see Hertel et al. (34).

binding (26). In contrast to the pH effect during extraction, the pH effect on Pfr binding to the $Mg^{++}$-dependent fraction is completely reversible. This seems to indicate that the Pfr binds to the outside of the $Mg^{++}$-dependent vesicles.

Table II indicates that these vesicles do not bind NPA, as the $Mg^{++}$-independent, Pfr-binding plasma-membrane vesicles do. Figure 5 shows that the NADPH-cytochrome-c-reductase activity in the pellet increases with increasing $Mg^{++}$ concentration. It is suggested that this enzyme is a marker of the endoplasmic reticulum (42, 43). From these results one might conclude that the $Mg^{++}$-dependent fraction, which is able to bind Pfr, is the endoplasmic reticulum.

### Pfr Binding to a Constituent of the "Solubilized" $Mg^{++}$-Dependent Fraction

In a preceding paper (36) we showed by sucrose density-gradient centrifugation that after removal of the Pfr-binding plasma-membrane vesicles in the presence of $Mg^{++}$, the Pfr, bound to the $Mg^{++}$-dependent fraction, sediments to about 40% sucrose (w/w). In the absence of $Mg^{++}$, when no Pfr-binding vesicles are formed, one obtains a Pfr peak at about 15% sucrose (w/w). If Pfr binds via a specific receptor to the vesicles, formed in the presence of $Mg^{++}$, one should be able to bind Pfr to the "soluble" receptor in the absence of $Mg^{++}$. The $Mg^{++}$-dependent fraction was freed from cell organelles and membrane vesicles by centrifuging the dark-extracted homogenate for 1 hr at 50,000 × g. The resulting supernatant was separated into two parts. One sample was layered under dim green light (ligand Pfr is not formed) on top of a linear sucrose gradient from 4% to 16% sucrose (w/w). The second sample was irradiated with red light before layering on a gradient of the same type. Figure 8 shows the Pr profile (upper curve) and the Pfr profile (lower curve) for the corresponding gradients. The soluble Pr from the dark-treated sample migrates to the 7% sucrose position, whereas the Pfr profile from the red treated sample shows two peaks – one at 7% representing the soluble pigment (now in the form Pfr), the other at 14% corresponding to the 15% peak observed with steeper gradients (36). The Pfr-receptor complex can be dissociated by adding 100 mM $Na^+$ or by adjusting

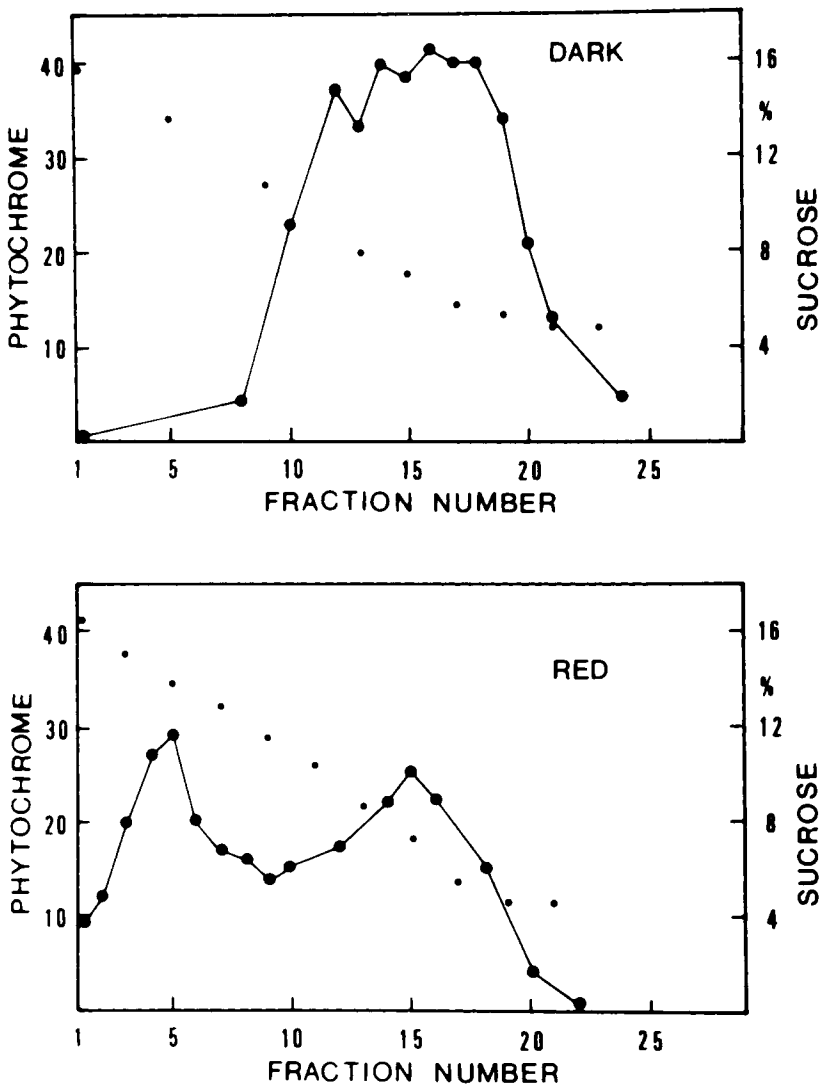


Fig. 8. Binding of Pfr to a "soluble" constituent of the $Mg^{++}$-dependent vesicles. Upper curve represents the Pr profile from a sucrose gradient (4–16% sucrose, w/w) which was run for 16 hr at 23,000 rpm in a Beckman SW 25/2 rotor. Lower curve represents the Pfr profile (after red treatment of the sample) from a sucrose gradient (4–16% sucrose, w/w) which was run for 16 hr at 23,000 rpm in the same rotor.

the pH of the gradient to 8. The same effect is obtained by irradiating the Pfr-receptor complex with far red light. Triton X-100 at 1% likewise splits the complex. The molecular nature of the receptor molecule is not yet known and needs further investigation.

### A Simple Method of Purifying Phytochrome from Zucchini and Corn

The various effects described in this paper suggest a simple procedure for purification

of phytochrome from zucchini (Fig. 9) and corn (Fig. 10). For zucchini the $Mg^{++}$ -dependent Pfr-binding fraction is used as a carrier system (Fig. 9). The dark-grown material is extracted in the presence of a low $Mg^{++}$ concentration (0.1 mM). The homogenate is centrifuged at 500 X g for 10 min in order to remove most of the larger cell debris. The resulting supernatant 0.5 KS is centrifuged at 17,000 X g for 30 min to remove most of the cell organelles. The $Mg^{++}$ -dependent Pfr-binding fraction is still in its "solubilized" state. The pH of the supernatant (17 KS) is adjusted to 6.5 and the $Mg^{++}$ concentration to 10 mM to form the vesicles. Binding of Pfr is induced at 0°C in red light. Under these conditions about 80% of the total extractable phytochrome is pelleted after 30 min at 50,000 X g (26). The resulting pellet is carefully washed in extraction buffer (0.1 mM $Mg^{++}$) at pH 7.5. At this pH most of the bound Pfr becomes soluble (26). Far red light for 5 min transforms Pfr to Pr, which is no longer able to bind. Addition of 10 mM $Mg^{++}$ and centrifugation at 100,000 X g for 1 hr sediments most of the protein into the pellet and leaves most of the phytochrome in the supernatant (100 KS). The purification factor for zucchini phytochrome with respect to total protein is about 400.

To purify phytochrome from corn coleoptiles, in contrast to zucchini, the plasma-membrane vesicles must be used as carriers. Whereas in zucchini hypocotyl hook extracts about 80% of the total phytochrome binds to the $Mg^{++}$ -dependent fraction under optimal conditions, only 5% to 10% of the total pigment binds to the corresponding fraction from corn coleoptiles (Fig. 1). Therefore, we propose to use the plasma-membrane vesicles as a carrier system, which is about 6 to 10 times as efficient. Figure 10 shows in detail the protocol. Corn coleoptiles are harvested in darkness and are irradiated on ice with red light prior to extraction. The homogenate is centrifuged at 500 X g for 10 min to remove large debris. The remaining supernatant (0.5 KS) is then centrifuged at 20,000 X g for 30 min. The pellet (20 KP) is vigorously resuspended in $Mg^{++}$ -free buffer at pH 8. Under these conditions Pfr is released from the plasma membrane and diffuses out of the open vesicles. Far red light transforms Pfr to Pr, which is no longer able to bind. $Mg^{++}$ up to 10 mM is added, and the particulate material is pelleted at 100,000 X g for 1 hr.

## DISCUSSION

It has been demonstrated that phytochrome in its physiologically active form, Pfr, is able to bind preferentially to a particulate fraction which can be extracted from zucchini hypocotyl hooks and from corn coleoptiles (Table I; 26, 35, 37). Investigations of the pelletability of the particles as a function of $Mg^{++}$ concentration in the extraction buffer have led to two kinds of Pfr binding structures. One is pelletable at 20,000 X g for 30 min in the absence of $Mg^{++}$; the other is pelletable at 20,000 X g only in the presence of $Mg^{++}$. The $Mg^{++}$ -independent fraction, to which Pfr can be bound only in vivo, can be separated on sucrose density gradients from mitochondria and endoplasmic reticulum (Fig. 2). Hertel et al. (34) concluded that NPA, which inhibits the transport of the plant hormone auxin within a few minutes, binds specifically to the plasma-membrane. Rate-zonal gradients and equilibrium density gradients (Fig. 3) show that Pfr activity and specific NPA binding activity migrate together and band at the same density.

For physiological reasons Pfr is thought to bind to the inside of the plasma-membrane.

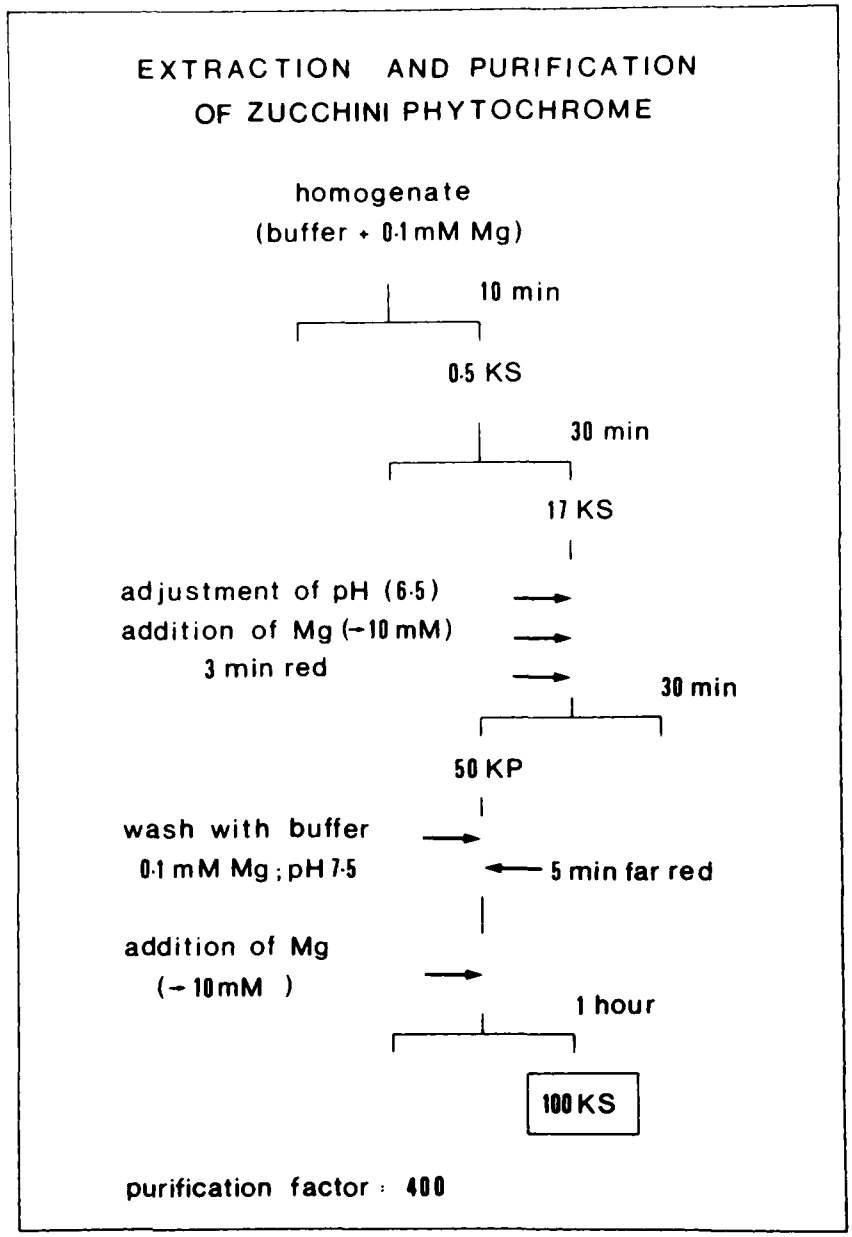


Fig. 9. Phytochrome purification protocol for zucchini. 0.5 Ks, 17 KS, 100 KS: supernatants after centrifugation at 500, 17,000 or 100,000 × g. 50 KP: pellet after centrifugation at 50,000 × g.

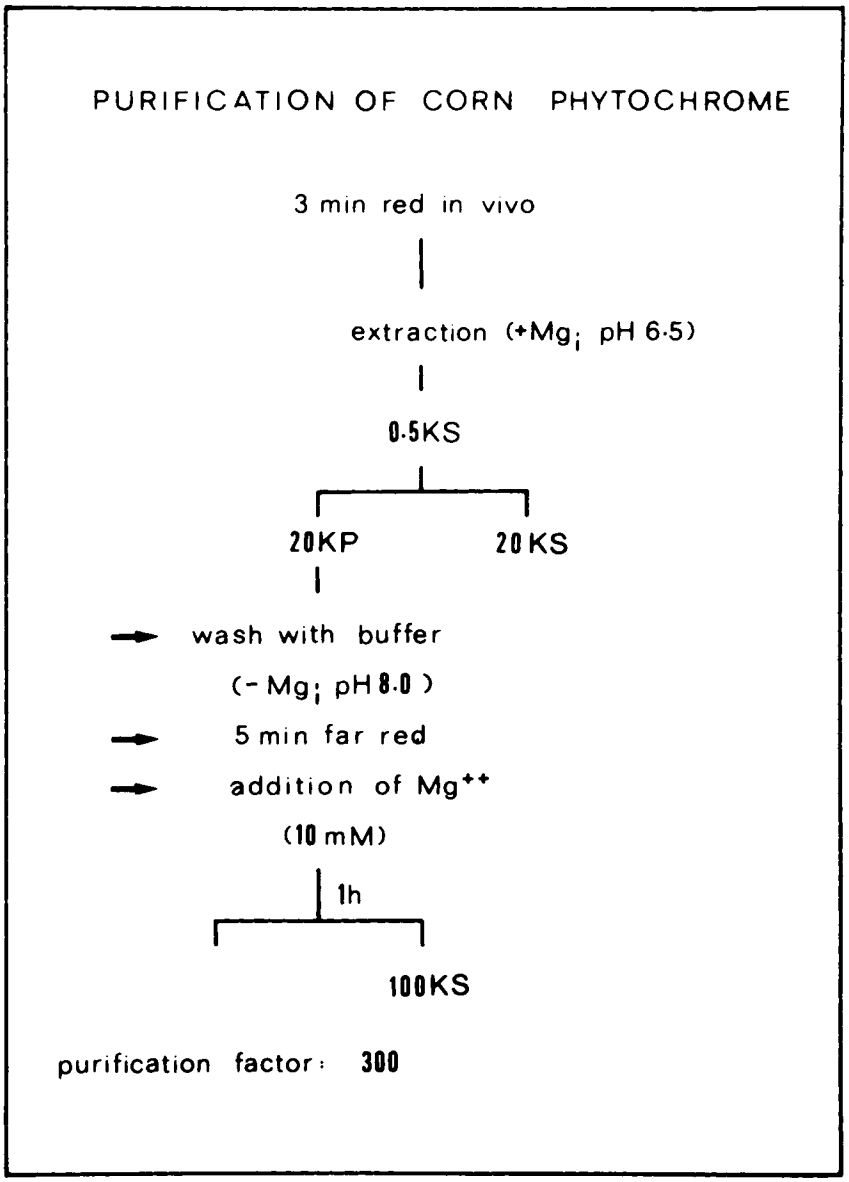


Fig. 10. Phytochrome purification protocol for corn. 20 KS, 100 KS: supernatants after centrifugation at 20,000 or 100,000 X g. 20 KP: pellet after centrifugation at 20,000 X g.

We have demonstrated that Pfr is bound to the inside of the plasma-membrane vesicles. Ionic strength, high pH, or far red light cannot prevent Pfr from being pelleted unless the membrane vesicles are opened up by vigorous resuspension or gentle sonication; the Pfr, after being released from the membrane, can diffuse out of the open vesicles. It seems very unlikely that the plasma-membrane vesicles are no longer "inside in." It has been demonstrated for adipocyte membranes (44) and for human red cell membrane vesicles (45),

using extreme sonication conditions, that "inside in" vesicles can be turned "inside out."

These plasma-membrane vesicles contain a flavoprotein (Fig. 2) whose absorption spectrum matches nicely the action spectrum of phototropism (unpublished). The fact that the phototropic pigment and the phytochrome are attached to or incorporated into the same membrane could account for the interference between both pigments, as observed in physiological experiments (46).

The effect of $Mg^{++}$ on the pelletability (Fig. 5) can be explained by assuming three mechanisms. First, at low $Mg^{++}$ concentrations the "soluble" particles form vesicles. Second, at higher $Mg^{++}$ concentrations these vesicles clump together and become easily pelletable. Third, at even higher $Mg^{++}$ concentrations $Mg^{++}$ acts by its ionic strength and prevents Pfr binding. The origin of the $Mg^{++}$ -dependent fraction is probably ER. It does not bind NPA, and it contains NADPH-cytochrome-c-reductase but no cytochrome-c-oxidase. Pfr can be bound in vitro to the outside of the vesicles. Ionic strength as well as high pH are able to release bound Pfr from the membrane.

In the absence of $Mg^{++}$ the vesicles are in a solubilized state. Among the constituents of the vesicles there is one which is able to bind Pfr (Fig. 8). The band can be dissociated by ionic strength, high pH, 1% Triton X-100, and far red light, which transforms Pfr to Pr.

Both Pfr binding systems are used to purify phytochrome from other proteins. For zucchini hypocotyl hooks the $Mg^{++}$ -dependent system is used as a carrier (Fig. 9). For corn the plasma-membrane vesicles are used as carriers because the $Mg^{++}$ -dependent fraction is vanishingly small in corn coleoptiles (Fig. 10).

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