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The Isolation and Partial Characterization of a Membrane Fraction Containing Phytochrome[1,2]

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ABSTRACT

If 4-day-old dark-grown zucchini squash seedlings (*Cucurbita pepo* L. cv. Black Beauty) are exposed briefly to red light, subsequent cell fractionation yields about 40% of the total extractable phytochrome in the far red-absorbing form bound to a particulate fraction. The amount of far red-absorbing phytochrome in the pellet is strongly dependent on the Mg concentration in the extraction medium. The apparent density of the Pfr-containing particles following sedimentation on sucrose gradients corresponds to 15% (w/w) sucrose with 0.1 mM Mg and 40% sucrose with 10 mM Mg. This particulate fraction could be readily separated from mitochondria and other particulate material by taking advantage of these apparent density changes with changes in Mg concentration. Electron microscopy of negatively stained preparations shows that with 1 mM Mg only minute particles are present. These were too small to reveal structural detail with this technique. With 3 mM Mg, separate membranous vesicles between 400 and 600 Ångstroms in diameter appear. At higher Mg concentrations, the vesicles aggregate, causing obvious turbity. The effect of Mg on vesicle formation and aggregation is completely reversible. Above 10 mM Mg, vesicle aggregation persists, but the percentage of bound Pfr decreases.

A number of studies during the past few years have suggested that an early consequence of the phototransformation of phytochrome from the red light-absorbing form to the far red light-absorbing form might be some change in the functional properties of plant membranes (7). For example, red light causes isolated root tips of barley (19) or mung bean (20) to adhere to a negatively charged glass surface. Subsequent far red irradiation causes their release. These effects occur within less than a minute of the start of irradiation. There are clear phytochrome-mediated alterations in the bioelectric potentials of oat coleoptiles (15) and the onset of these changes occurs within 15 sec of the onset of irradiation. Red light given during the middle of a 16-hr night inhibits the flowering of the Japanese morning glory (14). The effect can only be reversed by far red light if the far red is given within 60 sec after the beginning of red treatment (4). The rapidity of this escape from photoreversibility is consistent with membrane action for Pfr.

Other evidence comes from studies of nyctinastic leaflet movement in *Albizzia julibrissin* under the control of phytochrome (18). Turgor changes in the motor cells of the pulvinules can be directly related to massive fluxes of K into or out of these cells. Experiments on the localization of phytochrome in the filamentous green alga *Mougeotia* suggest that a major part of the pigment must be located at or very near the plasmalemma (6). Microbeams of red or far red light are only effective in regulating chloroplast rotation if they irradiate the region of the plasmalemma. Irradiation of corn coleoptile cells with polarized red and subsequent far red light reveals significant differences in the amount of phytochrome phototransformed, depending upon whether the plane of polarization is parallel or perpendicular to the longitudinal axis of the cells (11).

Evidence for particulate phytochrome after cell fractionation was published by Rubinstein *et al.* (17). Marmé *et al.* (12) later reported a correlation between pelletable phytochrome and the binding of naphthylphthalamic acid. Naphthylphthalamic acid binding has been suggested to be a specific property of plasmalemma membrane (8).

The present study was designed to isolate and characterize a particulate fraction which has the property of binding phytochrome in its Pfr form. Studies of the binding properties of phytochrome to this particulate fraction have been published elsewhere (10).

MATERIALS AND METHODS

Plant Material. Seeds of zucchini squash (*Cucurbita pepo* L. cv. Black Beauty) were placed on cellulose packing material (Kimpak 6223, Kimberly-Clark) moistened with distilled water in plastic boxes. The boxes were covered, and the seedlings were allowed to grow in darkness at 25 C for 4 days.

Extraction Medium. The extraction buffer normally contained 25 mM N-morpholino-3-propanesulfonic acid, 3 mM EDTA (disodium salt), 250 mM sucrose, 14 mM 2-mercapto-

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[2] Dedicated to Solon A. Gordon.

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ethanol, and 10 mM $MgCl_2$. The pH was adjusted to 7.35. In some cases, the Mg concentration was varied as indicated under "Results."

Resuspension Medium. The resuspension medium differed from the extraction medium only in pH and sometimes Mg concentration. The pH was 7.00, and the Mg concentration was 0.1 or 10 mM.

Isolation Procedure for the Pfr-binding Particulate Fraction. After any preillumination treatment, the seedlings were immediately chilled on ice as rapidly as possible. All subsequent manipulations were carried out between 2 to 4 C under dim green illumination. Hypocotyl hooks, about 1 cm in length, were harvested from appropriate light-treated plants or dark controls, and placed in Syracuse dishes. Four ml of extraction medium per g of tissue were added, and the tissue was chopped vigorously with a razor blade. The resulting coarse brei was ground gently in a chilled mortar for a few minutes. The homogenate was then filtered through four layers of cheesecloth plus one layer of Miracloth. If the above procedure was carefully followed, the pH of the filtrate was precisely 7.0. The standard protocol for subsequent manipulations is shown in Figure 1. The filtrate was centrifuged for 10 min at 500*g* to remove large fragments. The pellet which contained negligible amounts of phytochrome was discarded, and the supernatant (designated 0.5 KS) was centrifuged again at 17,000*g* for 30 min. The pellet (designated 17 KP) was resuspended and washed with resuspension medium, 0.1 mM Mg, pH 7.0. After a second centrifugation at 17,000*g* for 30 min, Mg was added to the supernatant (designated 17 KS') up to 10 mM. The 17 KS' was then centrifuged at 100,000*g* for 60 min. This final pellet (100 KP) was resuspended with medium containing either 0.1 or 10 mM Mg.

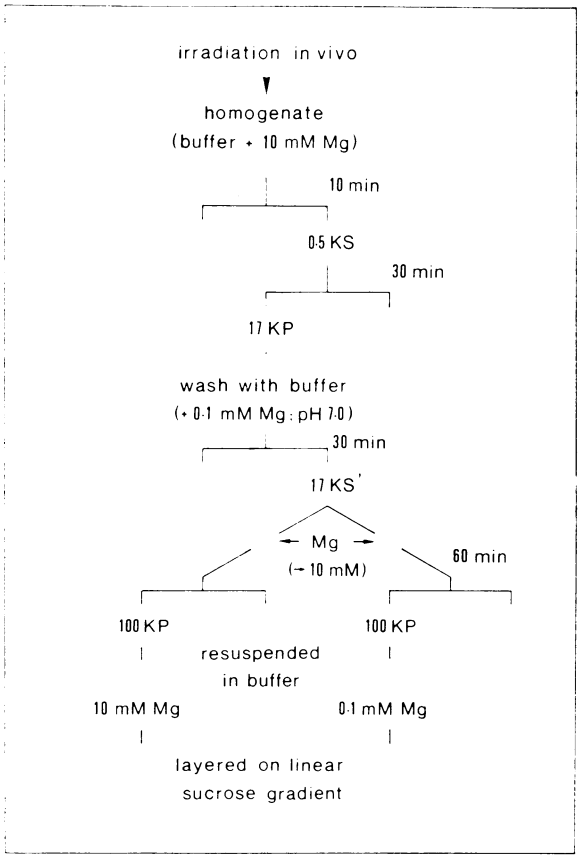

FIG. 1. Standard procedure for the partial purification of phytochrome-containing particles. Abbreviations: 0.5 KS supernatant after 10 min at 500 g; 17 KP pellet after 30 min at 17000 g; 17 KS' supernatant after a second centrifugation at 17000 g for 30 min; 100 KP pellets after 60 min at 100,000*g*.

Sucrose Density Gradients. Linear sucrose density gradients were prepared using the basic resuspension medium with sucrose from 10 to 45% (w/w), and either 0.1 mM Mg (for the 100 KP resuspended with 0.1 mM Mg) or 10 mM Mg (for the 100 KP resuspended with 10 mM Mg). Samples were then layered on top of the gradients. The gradient plus layered sample containing 10 mM Mg was centrifuged for 3 hr at 100,000*g* in a swinging bucket rotor (SW 27, Beckman). The gradients plus sample containing 0.1 mM Mg were centrifuged for 6 hr at 100,000*g*. After these centrifugation conditions, isopycnic equilibrium was reached in both cases. The sucrose density was measured with a refractometer.

Electron Microscopy. Samples at the appropriate Mg concentration were mounted on carbon-coated copper grids and negatively stained with a 2% solution of uranyl acetate. Comparisons were made with osmium-fixed artificial lecithin liposomes to determine criteria for preservation of small membrane vesicles (13). If the grid was introduced into the vacuum system while it was still moist, separate vesicles could be visualized without the coagulation usually seen in unfixed negatively stained membrane preparations. Pictures were taken at 20,500 diameters in a Phillips 300 electron microscope operating at 80 KV and calibrated with a cross-ruled grating. Representative fields were chosen from at least three separate preparations at each Mg concentration to ensure random sampling.

Cytochrome *c* Oxidase Assay. For the estimation of cytochrome *c* oxidase activity, the sample (normally 75 μl) was diluted with 0.1 M potassium phosphate buffer for use in the assay as described by Hackett (5). Activity is expressed as the first order rate constant for the disappearance of reduced cytochrome *c*, measured as decrease in absorbance at 550 nm.

Phytochrome Assay. Phytochrome was measured in a Ratiospect R-2 dual wavelength difference spectrophotometer. The measuring wavelengths were 660 and 730 nm. Since the sample volume was frequently low (300 μl) and pigment concentration likewise small, $CaCO_3$ powder was used to increase the optical pathlength (3). The optimal amount of powder to give a maximum signal for a 300-μl sample in an aluminum cuvette with a 1 cm^2 cross sectional area was 200 mg. The optimal amount of $CaCO_3$ has to be determined for every new lot. The $CaCO_3$ was added to a chilled cuvette and distributed evenly by gentle tapping. The 300-μl sample was added carefully, and the cuvette was allowed to remain on ice for 5 min to allow the liquid to penetrate throughout the $CaCO_3$. The cuvette was tapped again to complete penetration of the liquid and eliminate any light leaks caused by air bubbles. The specimen was ready for measurement when the mixture became a paste with a shiny surface. This procedure gave a maximal deviation for identical samples which was never larger than 10% of the mean value. Therefore, for most experiments, only two measurements were needed for any one phytochrome determination. Any intrinsic scattering differences between samples (*e.g.* between a 100 KP, resuspended, and a 100 KS) were eliminated by the high scattering component produced by the $CaCO_3$ if the resuspension volume was not smaller than one-third that of the supernatant. The percentage of bound phytochrome was calculated in relation to the sum of the phytochrome in the pellet plus that in the supernatant. All values were corrected for the volume of the resuspended pellet.

RESULTS

Isolation of Phytochrome-containing Particles. Following centrifugation of the 0.5 KS at 17,000*g* for 30 min (Fig. 1), an amount of phytochrome in the pellet is obtained which depends on the treatment of the seedlings before homogenization (2). Three minutes of irradiation of the seedlings with a broad band red source sufficient to bring about maximal phototransformation of Pr to Pfr increases the percentage of bound phytochrome by a factor of over 10 in standard extraction medium (10 mM Mg, Fig. 2). Figure 2 also shows that the amount of pelletable phytochrome varied substantially depending upon the Mg concentration in the extraction medium, with 10 mM being optimal. The role of Mg in this system has been considered in more detail elsewhere (10). The small amount of sedimentable phytochrome found in the dark controls could simply be contamination of the pellets from the supernatant, since the pellets are large.

Separation of the Phytochrome-containing Particles from Mitochrondria. The 17 KP, in the experiment shown in Table I, contained 38% of the total phytochrome and 95% of the cytochrome *c* oxidase activity. This pellet was carefully resuspended in resuspension buffer containing a 100-fold lower Mg concentration (0.1 mM; 0.1 ml of buffer per g fresh weight of the tissue). After a second centrifugation at 17,000*g* for 30 min, only 5.5% of the initially extracted phytochrome was found in the resulting pellet 17 KP' and 35% remained in the supernatant (17 KS'). Clearly, most of the phytochrome which was initially pelletable at 17,000*g* with 10 mM Mg was no longer pelletable at the lower Mg concentration. Not shown in Table I are the results from resuspending the 17 KP with resuspension medium containing 10 mM Mg. With the higher Mg concentration, all of the phytochrome remains pelletable. The mitochondria, as measured by their cytochrome *c* oxidase activity, behave differently. After the first 17,000*g* centrifugation, nearly all of the activity is in the 17 KP. After resuspension in the low Mg medium and the second centrifugation, about 90% of the mitochondria are still pelletable at 17,000*g*, (Table I). Reasonable separation from phytochrome is achieved.

When 10 mM Mg is added to the 17 KS' about 65% of the phytochrome in the 17 KS' is pelletable at 100,000*g* for 1 hr, suggesting that the effect of Mg on phytochrome pelletability is reversible. Indeed, the small amount of cytochrome *c* oxidase activity found in the 100 KP (Table I) can readily be removed by 1 hr of centrifugation at 100,000*g* prior to adding the Mg (not shown). The cytochrome *c* oxidase activity is all found in the pellet, but only a small amount of the phytochrome is sedimented. Thus separation of phytochrome-containing particles from mitochondrial cytochrome *c* oxidase activity is readily achieved by taking advantage of the change in sedimentation properties of these particles at different Mg concentrations. The sedimentation properties of the mitochondria are not affected under these conditions.

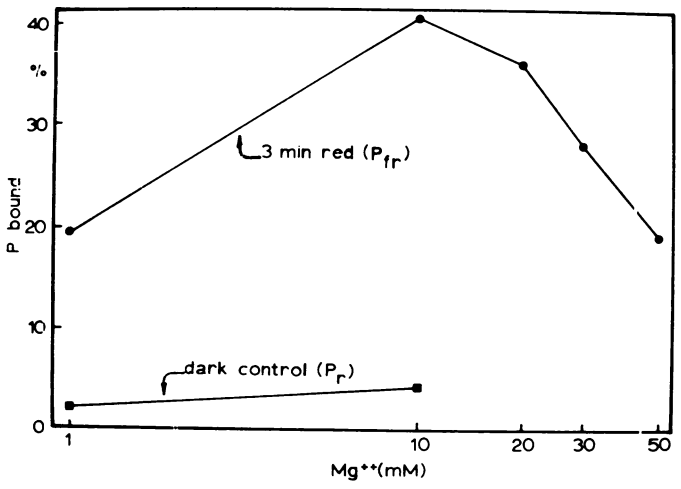

FIG. 2. Percentage of phytochrome bound as function of the magnesium concentration in the extraction medium. The upper curve represents the Pfr bound if the seedlings are preirradiated with 3 min red light just before extraction. The lower curve represents the Pr bound, when no light treatment was given.

Table I. *Influence of Magnesium Concentration on Sedimentation Properties of Phytochrome-containing Particles versus Cytochrome c Oxidase*

The 17 KP was resuspended in buffer containing only 0.1 mM Mg^{2+} and centrifuged a second time at 17,000*g* for 30 min; 17 KS' and 17 KP' were obtained. After addition of Mg^{2+} (up to 10 mM) to the 17 KS', another centrifugation for 1 hr at 100,000*g* yielded the 100 KS and the 100 KP. The table presents values obtained from one characteristic experiment.

Fraction	Mg^{2+}	Phytochrome	Cyt *c* Oxidase
	mM	*% 17 KP + 17 KS*	
17 KS	10	62	5
17 KP		38	95
17 KS'	0.1	35	7
17 KP'		5.5	62.5
100 KS	10	9.5	0
100 KP		19	4

Sucrose Density Gradients. Phytochrome-containing particles were isolated and partially separated from mitochondria following the procedure shown in Figure 1. The final pellets (100 KP) were resuspended in the usual buffer (pH 7.0) at two different Mg concentrations, either 0.1 mM or 10 mM. The samples were then layered on top of sucrose gradients, with the gradient Mg concentration matching that of the sample being layered. The low Mg gradient reached isopycnic equilibrium after 6 hr at 100,000*g* (Fig. 3). A clear separation of phytochrome from cytochrome *c* oxidase is apparent. The principal phytochrome peak corresponds to a sucrose concentration of about 15%. It is not yet clear whether the trace of phytochrome activity at higher density (about 38%) is related to different structures or whether it has been trapped by the remaining mitochondria. Before the gradient was fractionated, it was possible to observe four minor bands between the phytochrome band and the cytochrome *c* oxidase band. These minor bands did not contain significant phytochrome activity. When the phytochrome band was collected, magnesium added up to 10 mM, and centrifuged at 100,000*g* for 60 min, virtually all of the phytochrome was found in the pellet, again demonstrating the reversibility of the effect of Mg.

The gradient with 10 mM Mg reached isopycnic equilibrium after 3 hr at 100,000*g*. Analysis of this gradient showed both cytochrome *c* oxidase and phytochrome in a band at about 40% sucrose. Only with the low magnesium could separation be achieved.

In order to prevent trapping of phytochrome by the residual mitochondria, the 100 KP was first resuspended in the 0.1 mM Mg buffer. Phytochrome activity could then be separated from residual mitochondrial activity and from the four other bands by layering the resuspended pellet on a 25% sucrose cushion. All other bands were of higher density and

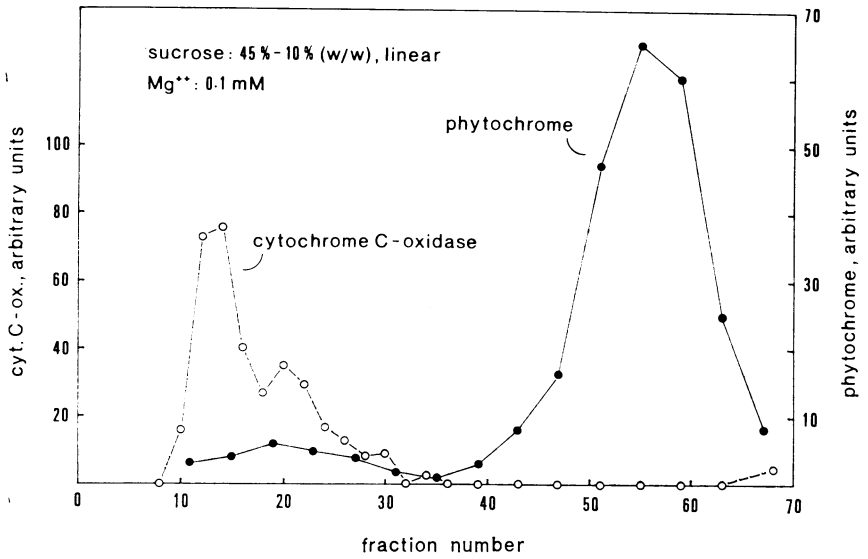

FIG. 3. Sucrose density gradient profile for partially purified phytochrome-binding particles and remaining mitcchondria at low Mg concentration. The main phytochrome peak is at about 15% sucrose, the small peak lies at about 38%. Maximal cytochrome *c* oxidase activity is found at about 40%.

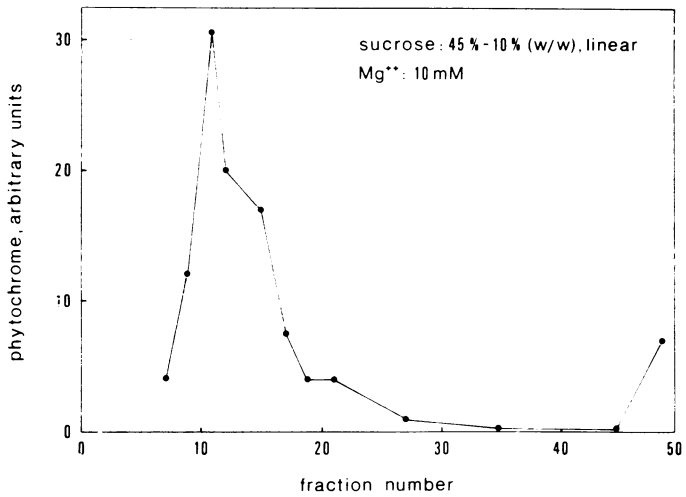

FIG. 4. Sucrose density gradient profile for purified phytochrome-containing particles in 10 mM Mg. The phytochrome peak is at 40% sucrose. No cytochrome *c* oxidase activity is detectable.

went through the sucrose cushion during 30 min at 50,000*g*. Phytochrome activity remained above the cushion. The material was carefully collected from the top of the cushion, made up to 10 mM Mg, and layered on a sucrose gradient with the same magnesium concentration. After 3 hr at 100,000*g*, the phytochrome again showed a strong peak of activity at roughly 40% sucrose (Fig. 4). No cytochrome *c* oxidase was detectable, however, nor were any other bands visible.

Effect of Magnesium on Turbidity. Phytochrome-containing particles were isolated and partially purified as shown in Figure 1. They were then further purified as described above by layering them in low Mg (0.1 mM) on a 25% sucrose cushion for another centrifugation, 30 min at 50,000*g*. They were then collected from the interface, and the influence of Mg on their turbidity was investigated. With 0.1, 1, or 3 mM Mg, very little turbidity was seen. At 5 mM, turbidity developed and at 10 mM, the solution became immediately turbid. However, if the turbid material was collected by centrifugation, resuspension in a buffer with 0.1 mM Mg essentially eliminated the turbidity.

These turbidity properties do not depend upon the presence of bound phytochrome. Similar particles can be isolated from dark-grown seedlings which received no light treatment prior to isolation and purification. Their phytochrome content was vanishingly small. Their turbidity behavior in response to different Mg concentrations was just the same.

Electron Microscopy. Particles with bound phytochrome were isolated and purified according to Figure 1 and the sucrose cushion procedure described in the preceeding section. The material was collected from the top of the sucrose cushion and Mg added to make final concentrations of 0.1, 1, 3, 5, or 10 mM. The particles were then negatively stained with uranyl acetate for electron microscopy. Figures 5 through 8 show representative micrographs. At 0.1 mM magnesium (Fig. 5), only very small particles, about 100 Å in diameter, are visible, and no detailed structure can be resolved. Similar particles were observed with 1 mM Mg. However, at 3 mM Mg, distinct vesicles with an average diameter of between 400 and 600 Å can be seen (Fig. 6 and inset). These vesicles are very similar in appearance to artificial lecithin liposomes of approximately the same size (13). At 5 mM Mg, the vesicles begin to form aggregates (Fig. 7), and at 10 mM (Fig. 8), the aggregation process is very pronounced. Though large membranous masses are formed, higher magnification reveals that vesicular structures can still be found.

Preliminary analyses done in collaboration with Dr. Jean Beck, Harvard University, show that the purified preparations contain substantial phospholipid and several steroids plus a number of polypeptides.

DISCUSSION

When the Mg concentration in the extraction medium is 10 mM, it is possible to obtain about 40% of pelletable phytochrome from etiolated zucchini squash seedlings which have been preirradiated before extraction with red light. Neither higher nor lower concentrations are as effective. From dark-

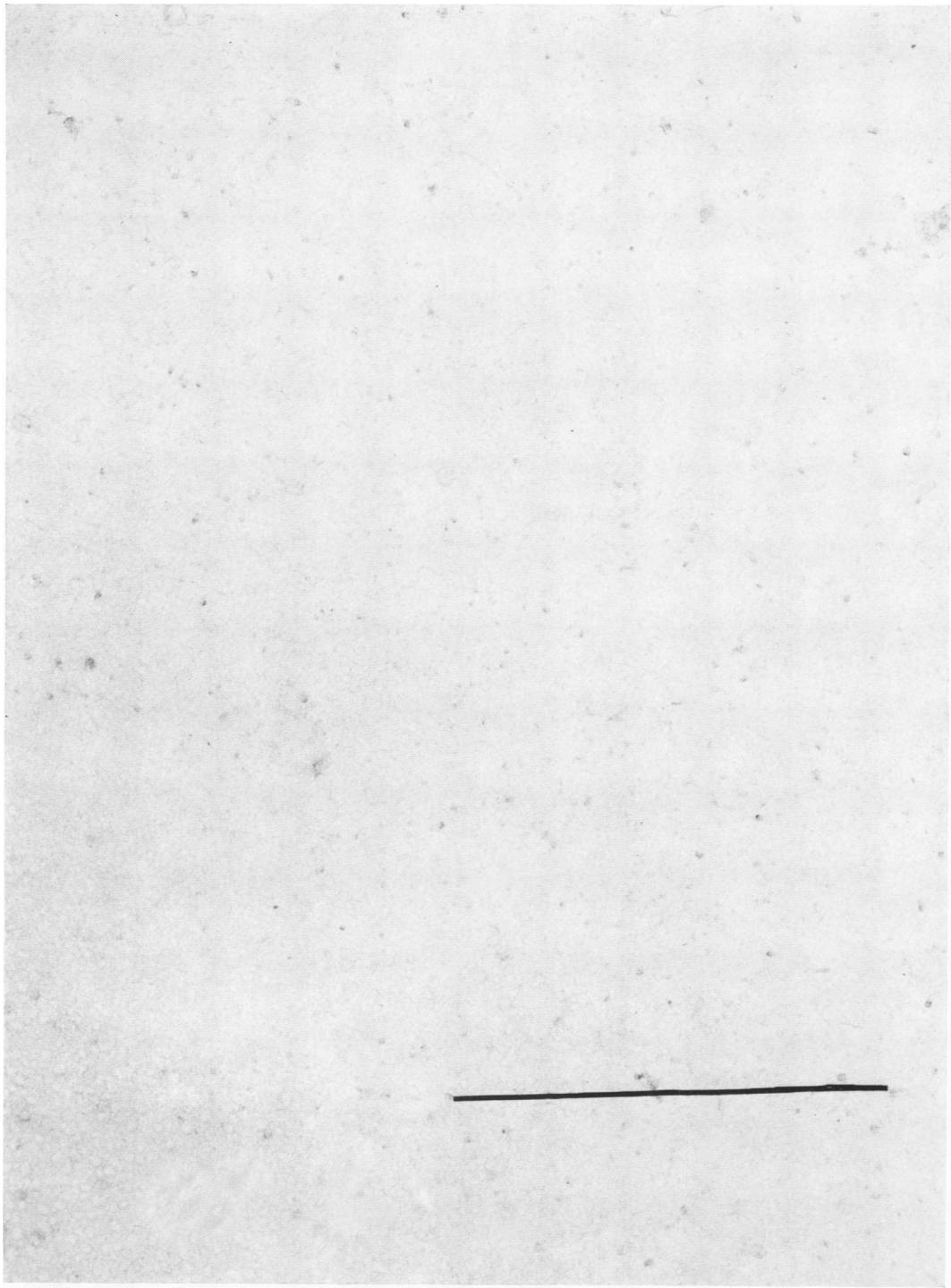

FIG. 5. Purified squash extract at 0.1 mM Mg, negatively stained with uranyl acetate. Small particles are present which are generally in the range of moderate sized globular proteins (about 100 Å). Bar represents 1 μm.

grown seedlings which received no light pretreatment, only about 4% of the phytochrome is pelletable, almost independent of Mg concentration. It is probable that for Mg concentrations between 1 and 10 mM, the results with preirradiated seedlings reflect the influence of Mg^+ upon vesicle formation and subsequent aggregation. Little can yet be said of the influence of magnesium on the binding of Pfr to these structures within that limited concentration range. Evidence presented elsewhere (10) suggests that at higher concentrations Mg may prevent binding without affecting the pelletability of the aggregated vesicles.

The differing sedimentation properties of the phytochrome-binding structures in high and low concentrations of Mg provide an elegant tool for purifying them. They can be separated from other organelles and organelle fragments by centrifugation in the presence of low Mg, and separated from soluble proteins and other substances by centrifugation at high Mg concentration.

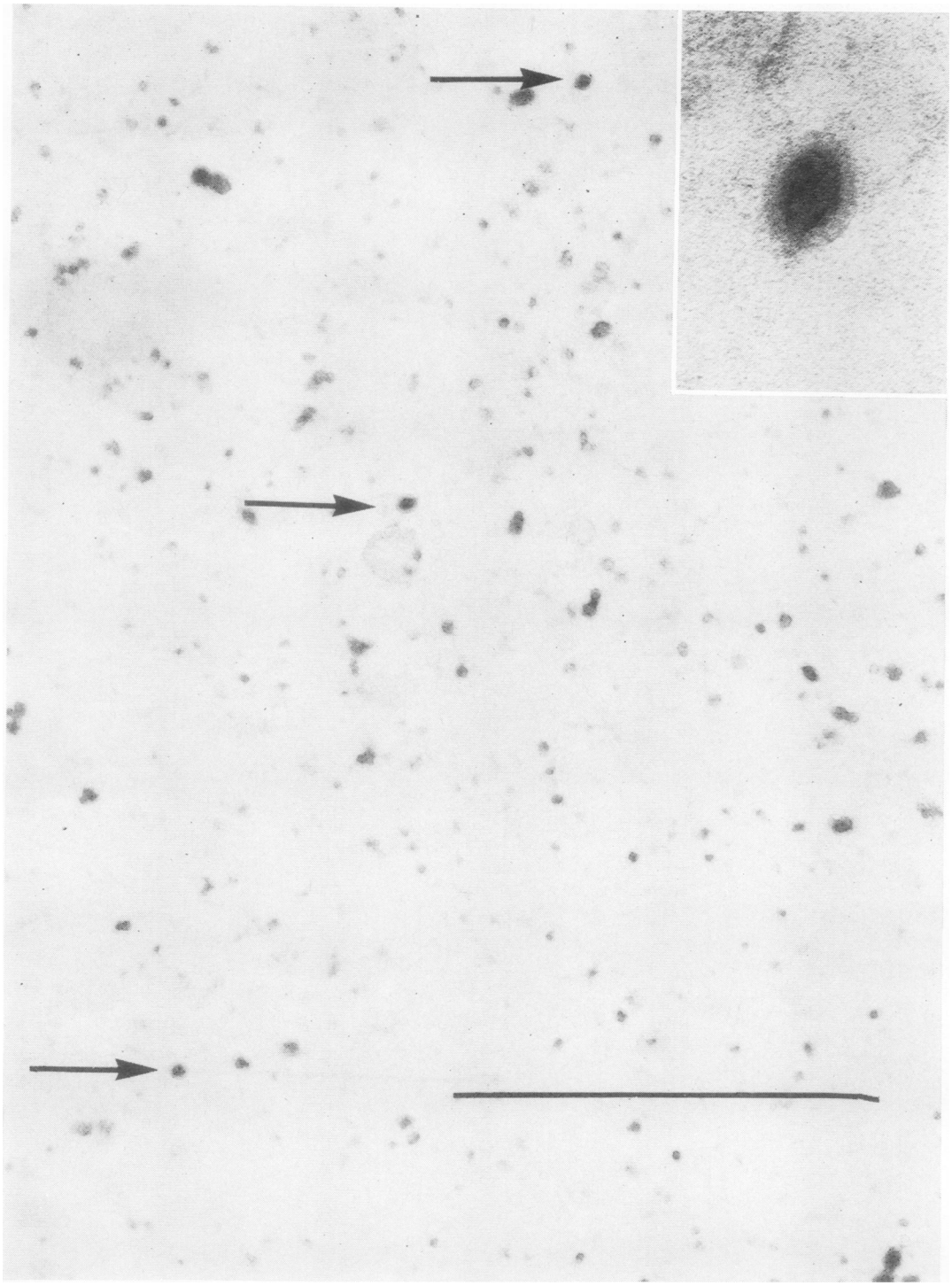

FIG. 6. Same purified squash extract as Figure 5, except at 3 mM Mg. Small vesicles (arrows) appear. Bar represents 1 μm. Inset: Higher magnification of vesicle at center of plate. Vesicle diameter about 500 Å.

It is clear that at different Mg concentrations, the Pfr-binding structures show different apparent densities on sucrose gradients. Several authors (*e.g.* 9) have suggested that higher Mg concentrations might yield cross-linking with other structures, *e.g.* mitochondria. However, apparent density changes in in the absence of mitochondria are seen in the purified phytochrome-binding preparations. The electron microscopy clearly shows changes in the size of the particles with increasing magnesium. The larger particles have a substantially higher density at isopycnic equilibrium. Partial solubilization of the membranes at low magnesium concentrations (1) might lead to lower apparent density.

Razin (16) has pointed out in a recent review on reconstitution of biological membranes that there is no direct evidence for a role for divalent cations in vesicularization. He summarizes, however, substantial data giving indirect evidence

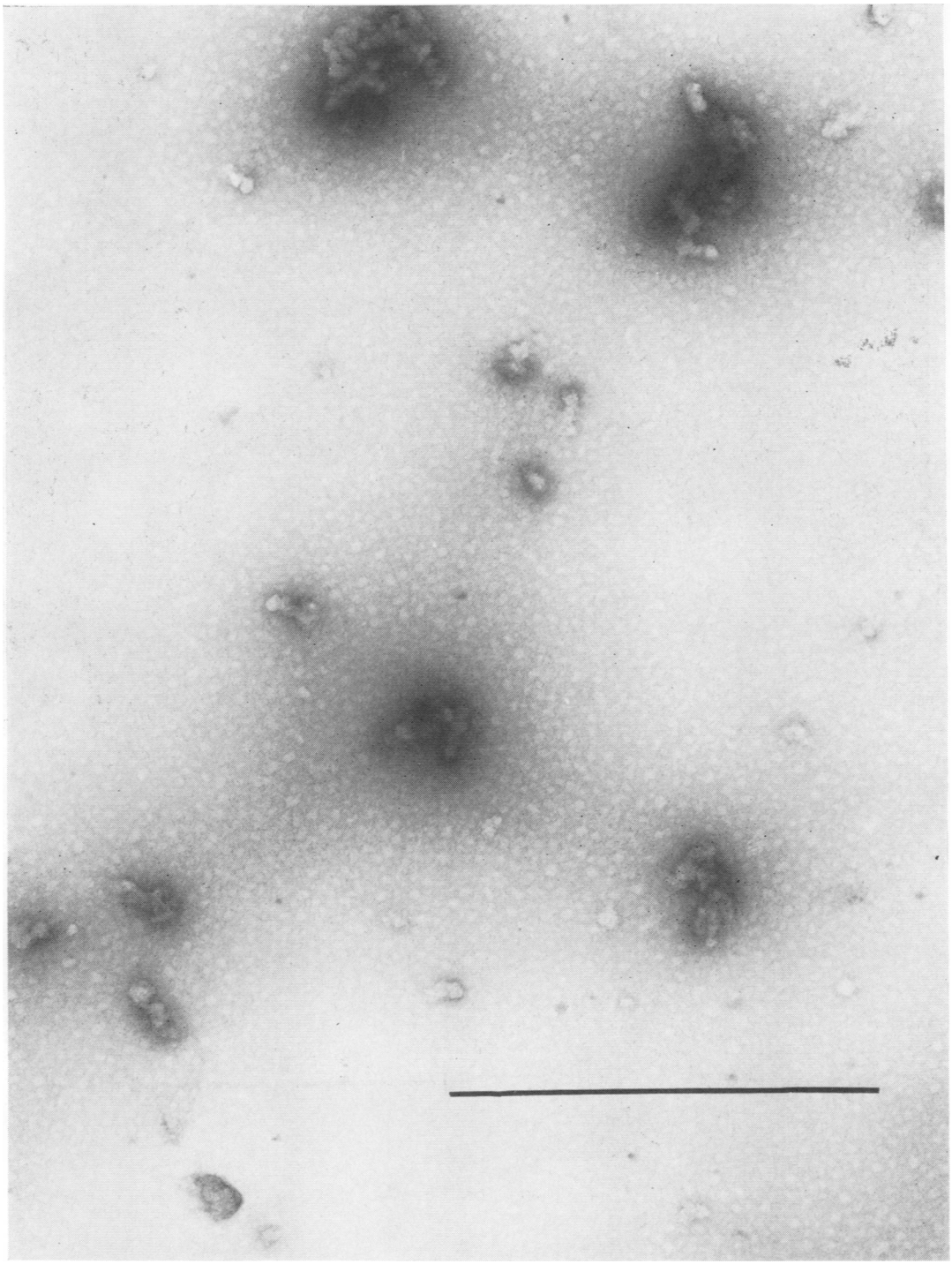

FIG. 7. Same extract as Figure 5, except at 5 mM Mg. The vesicles begin to clump. The vesicle do not coalesce, but rather agglutinate. Bar represents 1 μm.

that vesicle formation is associated with electrostatic interactions involving divalent cations and the polar groups of the phospholipids. The system described in this paper could be very useful in investigating these problems.

Although it has not yet been demonstrated that the Pfr molecules bind to the vesicles which can be seen in the electron microscope, the parallelism between vesicle formation and phytochrome pelletability is striking. The origin of these membranes is unknown and requires investigation.

Addendum. In 1961, Solon A. Gordon published a brief paper (*In:* B. C. Christensen and B. Buchmann, eds., Progress in Photobiology, Elsevier, Amsterdam. pp. 441–443) reporting that under appropriate conditions, a large percentage of the phytochrome in corn coleoptiles could be collected by centrifugation in a fraction enriched for mitochondria. Since that time, his paper has been very seldom cited and generally ignored. In dedicating this paper and the accompanying paper to him, we take pleasure in pointing out that the first experi-

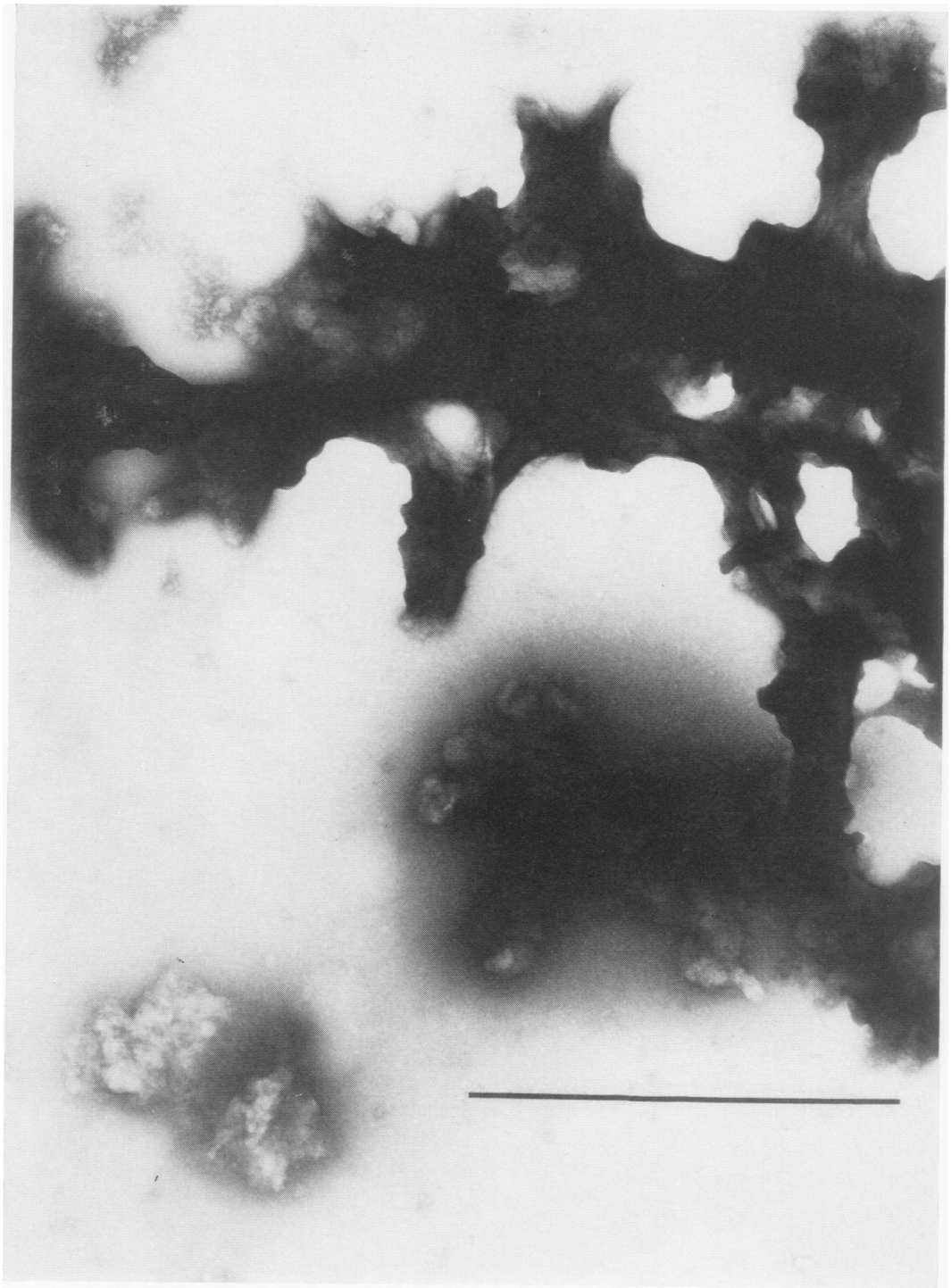

FIG. 8. Same extract as Fig. 5, except at 10 mM Mg. Clumping is now so pronounced that stain accumulation makes visualization of detail difficult. Vesicles are still not coalescing. Bar represents 1 μm.

ments demonstrating particulate and readily sedimentable phytochrome were those of Gordon, some 13 years before the present work.

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