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Association of Phytochrome with Rough-surfaced Endoplasmic Reticulum Fractions from Soybean Hypocotyls[1]

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ABSTRACT

Distribution of phytochrome (as Pfr) among membranes from soybean hypocotyls (*Glycine max* L. cv. Wayne) was determined by the combined techniques of cell fractionation, difference spectrometry, and electron microscopic morphometry. More than 90% of the phytochrome was found in the soluble fraction. With homogenates prepared in the presence or absence of Mg^{2+}, the portion associated with membrane was only 6.5% and 1%, respectively. In the presence of Mg^{2+}, the content of particulate phytochrome correlated with the amount of endoplasmic reticulum with attached ribosomes in the fractions but not with mitochondria or other membranes (including endoplasmic reticulum membranes from which the ribosomes may have been lost during cell fractionation). In the absence of Mg^{2+}, phytochrome was associated with a "heavy" plasma membrane fraction. The phytochrome content was sufficiently low to be accounted for by a contamination of less than 10% by rough-surfaced fragments of endoplasmic reticulum. The findings show association of phytochrome with a particulate fraction enriched in rough-surfaced fragments of endoplasmic reticulum but do not rule out cosedimentation of some unknown or unspecific phytochrome aggregate with this fraction.

Since the discovery of phytochrome and the elaboration of its ubiquitous role in the regulation of growth and development of flowering plants, its location within the cell and immediate mode of action have remained unknown. Information concerning its location has been mainly inferential, relating rapid phytochrome-mediated responses to physiological events at the plasma membrane (10–12) or the nuclear envelope (7). A part of the extracted phytochrome is associated with a particulate fraction (25, 26, 29); yet, a membrane or membrane fraction which binds phytochrome has not been identified.

We examined different fractions obtained from soybean hypocotyls by differential and sucrose gradient centrifugation and observed that phytochrome content in the presence of Mg^{2+} correlated with the presence of endoplasmic reticulum with attached ribosomes. In the absence of Mg^{2+}, phytochrome was found in the so-called "heavy" plasma membrane fraction, possibly as a result of contamination of this fraction by fragments of endoplasmic reticulum.

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MATERIALS AND METHODS

Plant Material. Soybean seeds (*Glycine max* L. cv. Wayne) were soaked in water for 6 hr and grown in moist vermiculite (9). After 4 days in darkness at 29 C, the cotyledons were removed, and the hypocotyl apices (approximately 1.5 cm) were harvested.

Membrane Isolation. Isolations of membrane fractions were either in fluorescent room light (Table I) or in dim green light after a 5-min exposure of the tissue to red light (Tables II to IV). By either procedure, the phytochrome was expected to be predominantly in the far red-absorbing form at the time of isolation. Fifty to 100 g of tissue were finely chopped with a razor blade and suspended in cold (0–4 C) homogenizing medium (1 ml/g tissue).

For isolations in the absence of Mg^{2+}, the homogenization and gradient media were freshly prepared 0.1 M K_2HPO_4 and 20 mM Na_2EDTA in Millipore-filtered coconut milk, pH 7.4, containing 0.5 M sucrose (13). For isolations in the presence of Mg^{2+}, the buffer used for homogenizing and gradient media was 50 mM tris-HCl, pH 7.4, 20 mM Na_2EDTA, and 30 mM $MgCl_2$ dissolved in coconut milk. However in the presence of Mg^{2+}, at pH 7.4, some components of the coconut milk were insoluble. The resulting precipitate was removed by sequential filtration through Whatman No. 1 filter paper, Millipore prefilter, a 0.45-μm Millipore filter, and a 0.22-μm Millipore filter. Sucrose was added to give a final concentration of 0.5 M in the homogenizing medium. Gradient solutions were prepared by appropriately diluting a 2 M solution of sucrose in the gradient medium with additional sucrose-free gradient medium. No phytochrome was detected in coconut milk.

Homogenization was for 2 min at approximately 5,000 rpm with a Polytron 20 ST (Kinematica, Lucerne, Switzerland). After homogenization, the brei was filtered through Miracloth, and the filtrate was centrifuged at 7,000*g* (Sorvall HB-4 rotor) for 15 min. The resulting supernatant was applied to a discontinuous gradient of sucrose in buffered coconut milk (Fig. 1) and centrifuged at 90,000*g* (Spinco SW-27.1 rotor) for 1 hr. Fractions were removed with a Pasteur pipette, pelletted at 90,000*g* for 45 min, and suspended in 0.25 M sucrose and 50 mM tris-HCl, pH 7. Pellets of the C, D, and P fractions from the gradient (Fig. 1) usually consisted of two distinct layers. The top (CT, DT, and PT) and bottom (CB, DB, and PB) layers were suspended separately. All homogenization and centrifugation steps were at 0 to 4 C.

Electron Microscope Morphometry. A representative section of each pellet was fixed in 2% glutaraldehyde in 0.1 M cacodylate

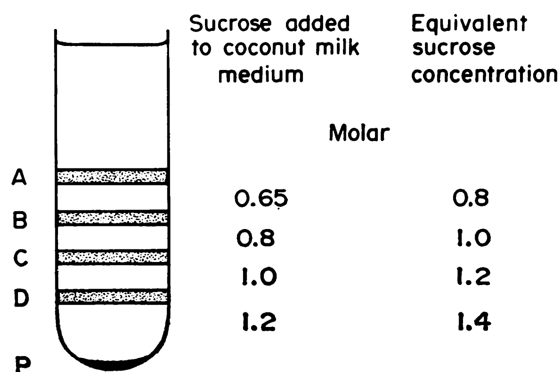


FIG. 1. Diagram of the discontinuous sucrose and coconut milk gradient used to separate cell components of soybean hypocotyl homogenates.

buffer, pH 7.2, for 1 to 2 hr and prccessed for lectron microscopy (27).

Electron micrographs of pelleted fractions (×35,000) were examined under a transparent overlay bearing lines 1 cm apart (13, 15). Intersections of the lines with different membrane types were counted. Mitochondria (Fig. 4) and dictyosomes (9) were identified by their morphology. Plasma membranes (Fig. 3) were identified by the staining procedure of Roland *et al.* (28). Rough endoplasmic reticulum was identified by the presence of membrane-associated ribosomes (Fig. 2). Membranes not categorized by these criteria were grouped together as "unidentified" and may include tonoplast, endoplasmic reticulum-lacking ribosomes, and fragments of plastids, dictyosomes, or mitochondria. Three micrographs from different parts of the pellets were examined for each fraction. The percentage of each membrane type was compared with the phytochrome content of the pellet ($\Delta(\Delta A)$/mg protein). Since phytochrome content was estimated on a protein basis, membrane compositions were also estimated in a manner approximating a protein basis. The major source of discrepancy was the protein matrix of the mitochondrial cristae. To compensate for mitochondrial matrix proteins (31), an intersection of an overlay line with both membranes of a mitochondrial crista was scored as 5 rather than 2.

Phytochrome Measurement. Phytochrome was assayed with a Ratiospect lent to us by Dr. Carl Norris, Plant Industry Station, United States Department of Agriculture, Beltsville, Md. In the experiments shown in Table I, phytochrome measurements were made on concentrated suspensions of membranes in a cuvette. However, the $\Delta(\Delta A)$ value was affected by the physical nature of the sample (32), and further phytochrome assays used 1-ml samples mixed with 1.5 g of dry $CaCO_3$ (1).

Other Techniques. Free Mg^{2+} was measured by EDTA titration with Eriochrome black T as indicator (35). Protein was measured by the Lowry method (16).

RESULTS

Distribution of Phytochrome among Fractions Prepared in Absence of Mg^{2+}. In initial experiments, phytochrome was found predominantly at the 1.2 to 1.4 M sucrose interface (D fraction) of the sucrose gradients (Table I). The spectral response of photoreversibility (Fig. 5) demonstrated that one form of the pigment in this fraction can only be reversed by red light and the other form by far red light. By these criteria, the pigment corresponded to phytochrome.

Electron microscope examination of the phytochrome-rich fraction showed abundant plasma membranes and mitochondria. Upon further fractionation, phytochrome was found associated with the plasma membrane-rich part (Table II). However, we could not conclude that phytochrome was associated with plasma membrane *per se*, since the fractions were only about 50% plasma membrane (9). High purity was not possible because of the nearly equal amounts of plasma membrane and tonoplast in this tissue and the tendency for these two membrane types to copurify along with substantial amounts of thinner membranes resembling endoplasmic reticulum (Table II). Also, the C top fraction from the 1 to 1.2 M sucrose interface was rich in plasma membrane but contained substantially less phytochrome than the plasma membrane-rich D top and P top fractions. This suggested either a functional heterogeneity of plasma membrane with respect to phytochrome location (36), or that particulate phytochrome cosedimented independently or in association with some component (*i.e.* tonoplast or endoplasmic reticulum) other than plasma membrane.

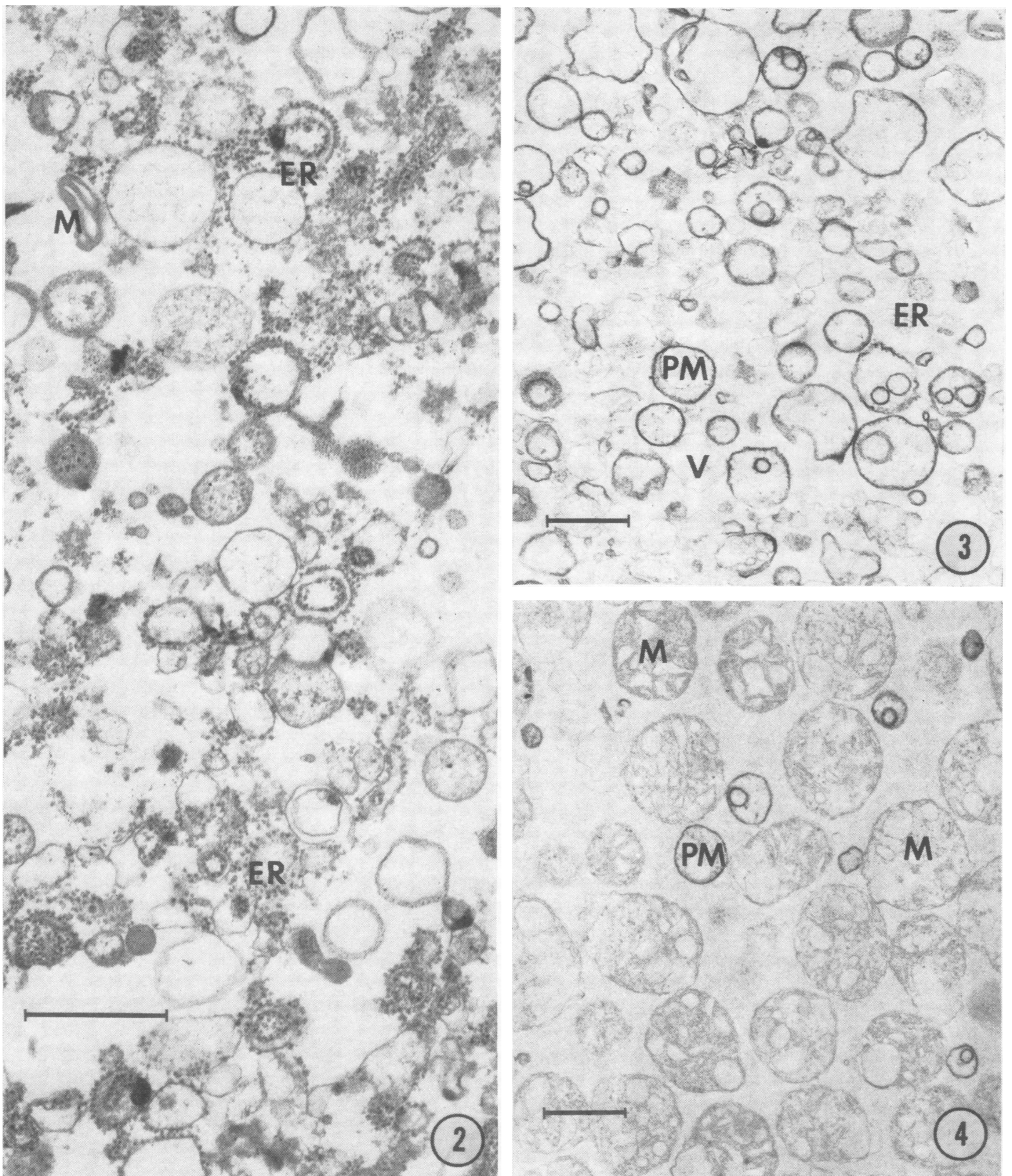
Distribution of Phytochrome among Fractions Prepared in Presence of Mg^{2+}. In the presence of Mg^{2+}, 6.5% of the total phytochrome was recovered as the sum of the gradient fractions in a microsomal pellet, compared to only 1% in the absence of Mg^{2+} (Table III). When the microsomal fraction was fractionated by density gradient centrifugation in the presence of Mg^{2+}, a large amount of material passed through the bottom layer of the gradient (equivalent to 1.4 M sucrose in density). Phytochrome content increased with increasing fraction density (Fig. 6) and was most concentrated in the gradient pellet (P fraction) (Table IV). The specific activity of the phytochrome ($\Delta(\Delta A)$/mg protein) in the P fraction was about twice that of the total homogenate.

Morphometric analyses of the fractions prepared in the presence of Mg^{2+} showed that the fraction consistently highest in phytochrome (P fraction, Fig. 2) contained up to 85% rough endoplasmic reticulum. The identity of the remainder of the fraction was variable, consisting of 5 to 30% plasma membrane, 0 to 15% mitochondria and 10 to 30% unidentified membrane. Other fractions containing significant amounts of phytochrome were rich in plasma membrane (up to 55%, Fig. 3) but always contained rough endoplasmic reticulum. Fractions rich in mitochondria but lacking rough endoplasmic reticulum (Fig. 4) were low in phytochrome (Table IV).

The correlation between endoplasmic reticulum and phytochrome in cell fractions was confirmed by plotting phytochrome concentration as a function of percentage of rough endoplasmic reticulum for all fractions. The correlation coefficient of 0.9491 (Fig. 7) is significant at the 1% confidence level. Similar plots for mitochondria (Fig. 8) showed no correlation ($R = -0.1327$). For plasma membrane (Fig. 9), there was a positive correlation ($R = 0.2171$) significant at the 5% confidence level, but the plasma membrane fractions were contaminated by endoplasmic reticulum. For fractions prepared in the absence of Mg^{2+}, we did not evaluate endoplasmic reticulum contamination because most of the ribosomes were detached from the membranes. The only membranes clearly identified as endoplasmic reticulum in these preparations were double vesicles concentrated in the D fraction with ribosomes attached to the inner, protected surface of the vesicle.

DISCUSSION

Cell Fractionation. Our procedure for plant cell fractionation in the presence of Mg^{2+} and coconut milk yields fractions rich in endoplasmic reticulum. The buoyant density of rough endoplasmic reticulum of soybean hypocotyl on sucrose gradients (about 1.19 g/cm³) is similar to that of rough endoplasmic reticulum from rat liver (5), but greater than that from castor bean endosperm (14). The fraction isolated by Lord *et al.* (14) from castor bean endosperm has a density of 1.16 g/cm³, whereas our rough endoplasmic reticulum fraction passes through 1.4 M sucrose (density at 20 C of about 1.18 g/cm³) but is retained by 1.6 M sucrose (density at 20 C of about 1.20 g/cm³). These differences in density between rough endoplasmic reticulum from soy-



FIGS. 2–4. Electron micrographs of cell fractions isolated in the presence of Mg^{2+} and recovered from the sucrose-coconut milk gradient of Fig. 1. Scale bar = 0.5 μm. Fig. 2: Gradient pellet fraction rich in endoplasmic reticulum stained with alkaline lead citrate (27); Fig. 3: fraction C top rich in plasma membranes stained by the procedure of Roland *et al.* (28); Fig. 4. fraction C bottom rich in mitochondria stained by the procedure of Roland *et al.* (28).

Table I. *Distribution of Phytochrome and Protein among Sucrose Gradient Fractions of Soybean Hypocotyls Prepared in Absence of Mg^{2+}*

Fraction Designation[1]	Gradient Interface	Protein				Phytochrome			
		Expt. I	Expt. II	Expt. III	Avg.	Expt. I	Expt. II	Expt. III	Avg.
	M sucrose	*mg*				Δ(Δ*A*) × *100*			
A	Homogenate/0.8	5.6	4.1	4.2	4.5	−2	4	0	0
B	0.8/1.0	7.7	6.0	4.7	6.1	0	−12	0	−4
C	1.0/1.2	4.5	4.9	4.1	4.5	4	−10	0	−2
D	1.2/1.4	5.0	8.7	3.2	5.6	22	14	12	16
P	1.4 pellet	2.2	1.8	1.2	1.7	−1	−2	1	−1
7,000*g* pellet		22.5				5			

[1] For an estimate of the average composition of fractions A–D, see Table 2 of Hardin *et al.* (9).

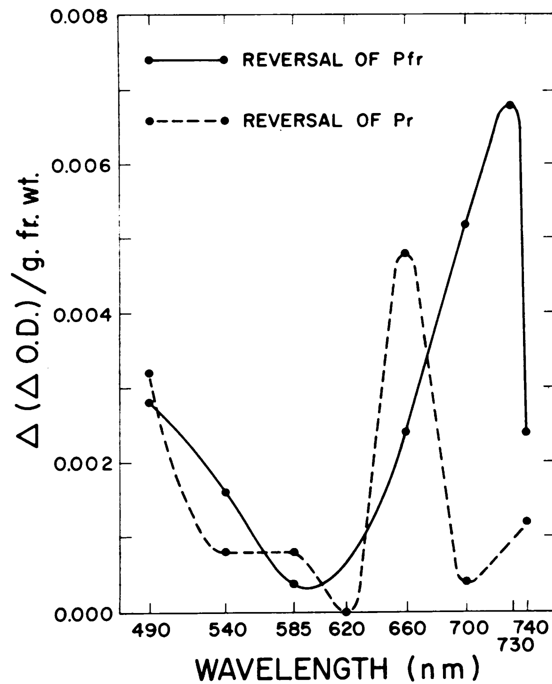


FIG. 5. Wavelength sensitivity of phytochrome photoreversibility of fraction D of the sucrose-coconut milk gradient of Fig. 1 from a homogenate of soybean hypocotyls prepared in the absence of Mg^{2+}. The suspension was irradiated *in vitro* for 60 sec with monochromatic light obtained with second order interference filters coupled with colored glass filters to screen out the unwanted peak. Irradiances at all wavelengths averaged 3400 ergs/cm²/sec.

bean hypocotyl and from castor bean ensosperm may reflect differences in the degree of retention of ribosomes by the membranes.

Coconut milk in the fractionation media stabilizes dictyosomes by preventing separation of the cisternae (19). The components of coconut milk which stabilize are unknown. The morphological preservation of plasma membrane and endoplasmic reticulum are also enhanced by coconut milk. The presence of free Mg^{2+} is essential to maintain attachment of ribosomes to endoplasmic reticulum during isolation. Endoplasmic reticulum with attached ribosomes is not a major component of any fraction prepared without Mg^{2+}, in the presence or absence of coconut milk. The free Mg^{2+} concentration of the coconut milk medium is about 9.5 mM, indicating that little Mg^{2+} is removed in the precipitate from the coconut milk. This Mg^{2+} concentration is based on the results of Quail *et al.* (26) which demonstrate a marked increase of particle-bound phytochrome in the presence of high Mg^{2+}. Our results also indicate an increase in bound phytochrome from 1 to 6.5% of the total upon inclusion of 10 mM free Mg^{2+} (30 mM $MgCl_2$ and 20 mM EDTA). However, 4 mM free Mg^{2+} is sufficient to maintain the integrity of the rough endoplasmic reticulum. Concentrations of free Mg^{2+} in the medium greater than 10 mM may cause membrane vesicles to aggregate (36).

Localization of Phytochrome. In the presence of Mg^{2+} at pH 7.4, membrane-associated phytochrome correlates with endoplasmic reticulum with attached ribosomes. When tissue is fractionated in the absence of Mg^{2+} there is no absolute correlation of phytochrome content with any specific type of membrane, although fragments of endoplasmic reticulum with attached ribosomes do appear almost exclusively in the phytochrome-rich

Table II. *Distribution of Cell Components and Phytochrome Content among Gradient Fractions of Soybean Hypocotyls Prepared in Absence of Mg^{2+}*

Fraction Designation	Plasma Membrane	Dictyosomes	Mitochondria	Other Membranes	Phytochrome
	profiles/100 profiles				Δ(Δ*A*)/*mg protein*
A	8.2	9.5	0	82.3	0
B	26.7	10.9	3.7	58.7	0
C top	39.1	0	0	60.9	0
C bottom	2.8	0	91.1	6.1	0
D top	48.5	0	0	51.5[1]	0.0584
D bottom	6.1	0	76.5	17.4	0
P top	49.0	0	1.3	49.9	0.0431
P bottom	18.5	0	32.2	49.3	0
Total homogenate					0.0558
90,000*g* supernatant					0.0762

[1] A mixture of thick membranes resembling tonoplast and thinner membranes of the endoplasmic reticulum type.

Table III. *Comparison of Distribution of Phytochrome among Cell Fractions Prepared in Presence or Absence of Mg^{2+}*

Fraction Designation	Fractionation in Absence of Mg^{2+}				Fractionation in Presence of Mg +			
	Protein		Phytochrome		Protein		Phytochrome	
	mg	Δ(Δ*A*)	*% total homogenate*	Δ(Δ*A*)/*mg protein*	*mg*	Δ(Δ*A*)	*% total homogenate*	Δ(Δ*A*)/*mg protein*
Total homogenate	398.64	22.24	100	0.0558	317.5	20.16	100	0.0635
90,000 *g* supernatant	335.3	25.55	115	0.0762	264.6	15.54	77.1	0.0587
7–90,000 *g* pellet (microsomes)	25.9	0.225	1	0.0085	32.31	1.313	6.5	0.0406

fraction. Fractions rich in phytochrome are also rich in plasma membrane, but not all fractions rich in plasma membrane are rich in phytochrome. In the presence of Mg^{2+}, low levels of phytochrome associated with plasma membrane may be masked by phytochrome associated with rough endoplasmic reticulum but, at best, less than 1% of the total phytochrome is ascribable to plasma membrane fragments. The unidentified membranes in many of the phytochrome-poor fractions prepared in either the presence or absence of Mg^{2+} may be rough endoplasmic reticulum stripped of its ribosomes. Ribosomes are easily detached from endoplasmic reticulum membranes especially in the absence of Mg^{2+} (30). The presence of membrane-associated ribosomes provides the only correlation with phytochrome content of the fractions (Fig. 7).

Rubinstein *et al.* (29) concluded that membrane-associated phytochrome, because of its sensitivity to detergents and lability, was distinct from soluble phytochrome. They isolated particulate fractions in a buffer containing EDTA and without Mg^{2+} which

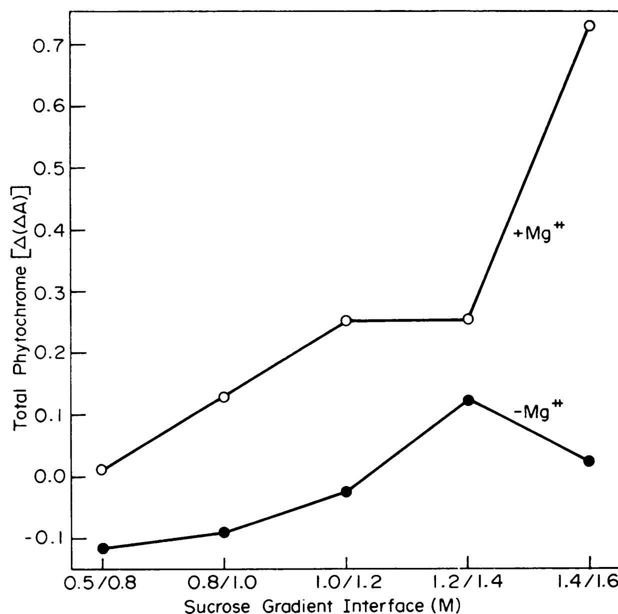


FIG. 6. Distribution of total sedimentable phytochrome on the sucrose-coconut milk gradient of Fig. 1. Homogenates prepared in the presence (+) and absence (−) of Mg^{2+} are compared.

Table IV. *Distribution of Phytochrome among Selected Cell Fractions Prepared in Presence of* Mg^{2+}

Fraction Designation	Major Cell Components	Phytochrome
		$\Delta(\Delta A)$/*mg protein*
500*g* pellet	Nuclei + starch	0
7,000*g* pellet	Proplastids + mitochondria	0.0138
Gradient fractions		
A	88% unidentified[1]	0
B	52% dictyosomes	0.0143
C top	55% light plasma membrane	0.0126
D top	47% heavy plasma membrane	0.0224
D bottom	80% mitochondria	0.0014
P	78% endoplasmic reticulum	0.137

[1] This fraction consists predominantly of membrane fragments with thin (about 80 Å) membranes. It contains little or no plasma membrane as identified by the staining procedure of Roland *et al.* (28). It, along with the B fraction, may contain endoplasmic membranes lacking ribosomes as well as plastid and outer mitochondrial membrane fragments.

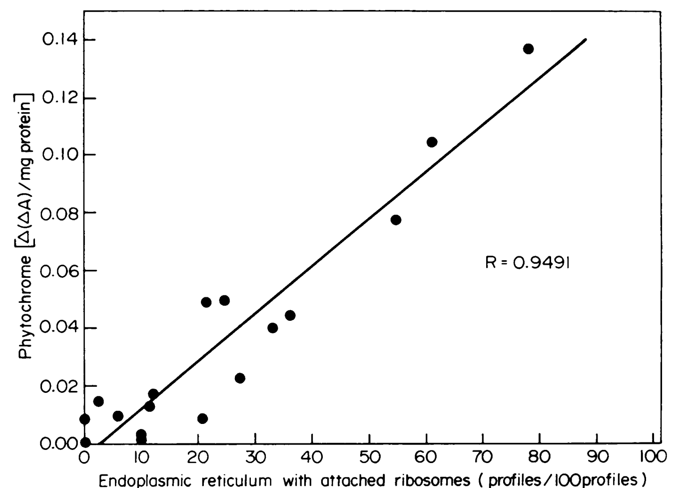


FIG. 7. Correlation between phytochrome concentration ($\Delta(\Delta A)$/mg protein) and content of endoplasmic reticulum with attached ribosomes (morphometry) for fractions prepared in the presence of Mg^{2+}.

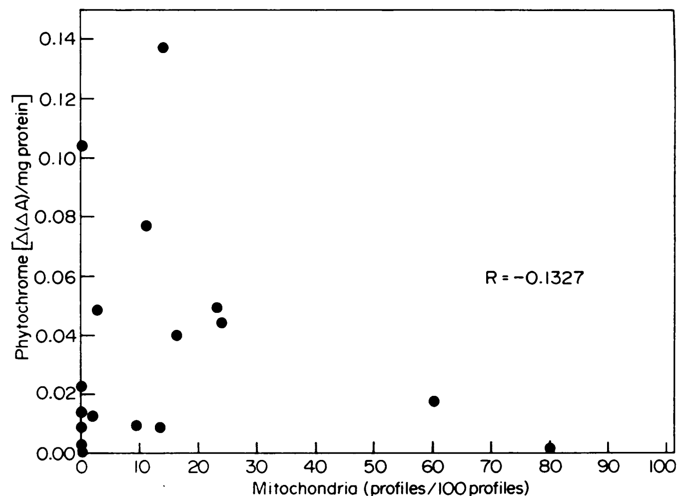


FIG. 8. Correlation between phytochrome concentration ($\Delta(\Delta A)$/mg protein) and mitochondrial content (morphometry) of fractions prepared in the presence of Mg^{2+}.

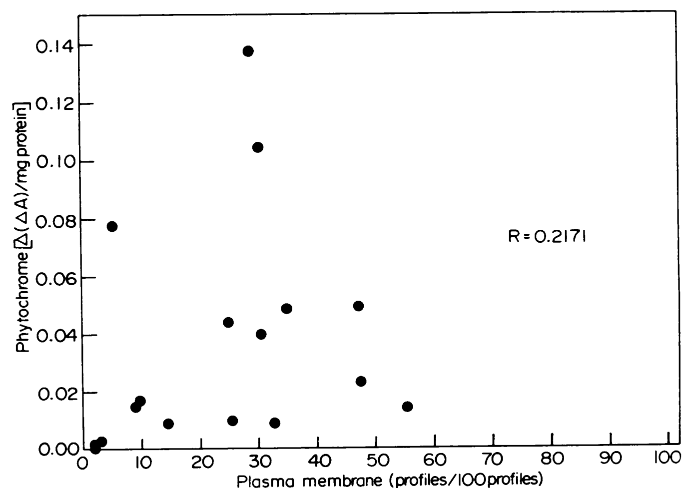


FIG. 9. Correlation between phytochrome concentration ($\Delta(\Delta A)$/mg protein) and content of plasma membrane (morphometry) as identified by the procedure of Roland *et al.* (28) for fractions prepared in the presence of Mg^{2+}.

was comparable to our Mg^{2+}-free buffers. They showed that phytochrome could not be extracted from the membranes isolated under these conditions. This contrasts with the membrane-associated phytochrome described by Marmé *et al.* (18) which was adsorbed from solution in the presence of Mg^{2+} in a manner influenced by irradiation with red light. The phytochrome could subsequently be removed by washing with buffer containing supraoptimal concentrations of Mg^{2+} (>10 mM) or at high pH (>6.5). Thus, a small fraction of the total phytochrome may exist as an integral part of some membranes, but the possibility that phytochrome is reversibly adsorbed from solution or lost under certain conditions of homogenization or light treatment cannot be decided from the present study. MacKenzie *et al.* (17) report that, when localized as Pr by indirect peroxidase-antiperoxidase antibody labeling methods, phytochrome is generally distributed throughout the cytoplasm (see also 2, 3, 24). However, after saturation with red light, Pfr becomes associated with discrete regions of the cytoplasm no larger than 1 μm. Their findings do not prove, but would be consistent with, a reversible association of Pfr with rough endoplasmic reticulum.

We determined only the correlation between phytochrome content and membrane composition of each of the fractions. On this basis, the correlation is between phytochrome and endoplasmic reticulum with attached ribosomes rather than between phytochrome and total endoplasmic reticulum, plasma membrane, mitochondria, or other types of membranes. An association between phytochrome and microbodies or nuclei was not eliminated; nor do we exclude the possibility that the phytochrome may be localized in some sedimentable, nonmembranous cell component such as microfilaments, microtubules, or lipoprotein micelles not revealed in the electron micrographs or with some nonmembrane cell component associated with membranes such as the ribosomes of the endoplasmic reticulum or subsurface cytoplasmic constituents associated with "heavy" fragments of plasma membranes (21).

The lack of correlation of phytochrome content with plasma membranes was unexpected in view of indirect experimentation which predicted such an association, *e.g.* phytochrome appears to control the electrical potential between the cell and its environment (12, 33), and appears to be located at the cell periphery in *Mougeotia* (10, 11). Such findings are also consistent with phytochrome associated with rough endoplasmic reticulum. Connections of endoplasmic reticulum with plasma membrane (4, 20) are evident at plasmodesmata (20; C. E. Bracker, unpublished). Additionally, higher plants and the alga *Mougeotia* contain peripheral cisternae of endoplasmic reticulum in near proximity and parallel to the cell surface. In *Mougeotia* the large central chloroplast especially restricts endoplasmic reticulum and other cytoplasmic components to a narrow band just under the plasma membrane (6; C. A. Lembi, unpublished).

An interaction between phytochrome and ribosomes is indicated by the stimulation of the protein synthetic activity of maize ribosomes (34) and the induction of polyribosome formation in bean leaves (23) by red light. Our data do not support an association of phytochrome with free ribosomes since no phytochrome was found in the fraction equilibrating at 0.8 M sucrose (A fraction) which contains ribosomes from the supernatant of the gradient.

Our data eliminate the possibility that phytochrome is localized in mitochondria (*e.g.* 8). An effect of red light on respiration in mung bean roots (37) and effects of red and far red light on rates of phosphorylation in *Avena* (8) may be explicable in terms of connections *in vivo* between endoplasmic reticulum and mitochondria (21).

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