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Reversible Redistribution of Phytochrome Within the Cell upon Conversion to Its Physiologically Active Form

(immunocytochemistry/peroxidase visualization/photomicrographs/oat shoot/rice shoot)

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ABSTRACT **The intracellular localization of phytochrome was seen in dark-grown oat (*Avena sativa* L., cv. Garry) and rice (*Oryza sativa* L., cv. unknown) shoots after various light treatments using an indirect peroxidase-antiperoxidase antibody labeling method. Phytochrome is generally distributed throughout the cytoplasm in cells of tissue that had not been exposed to light prior to fixation. Within, at most, 8 min after the onset of saturating red irradiation, phytochrome, now present in the far-red-absorbing form, becomes associated with discrete regions of the cell. These regions do not appear to be nuclei, plastids, or mitochondria. After phototransformation back to the red-absorbing form originally present, phytochrome slowly resumes its general distribution. It is possible that this discrete localization of the far-red-absorbing form of phytochrome represents a physiologically significant binding with a receptor site in the cell.**

For many years investigators have searched for an association of phytochrome, a morphogenically active plant chromoprotein, with a cellular organelle or membrane component. The rationale for this search was the belief that knowing the location of phytochrome within the cell would perhaps help in understanding the mechanism through which it exerts its influence on plant morphogenesis (1).

By standard aqueous extraction and cell fractionation techniques, phytochrome has been reported associated in its physiologically active (far-red-absorbing) form, Pfr, with rough endoplasmic reticulum (2, 3) and plasma membrane (3). Localization of phytochrome within the cell has also been inferred from physiological studies. Haupt and coworkers have demonstrated by microbeam irradiation that phytochrome is probably associated with the plasmalemma of the green alga *Mougeotia* (4). Wellburn and Wellburn (5), on the other hand, demonstrated an association with isolated, washed bean etioplasts by showing that development of the etioplasts is reversibly influenced by red and far-red light, while Manabe and Furuya (6) observed a phytochrome-mediated response in isolated pea mitochondria, implying an association of the chromoprotein with this organelle. Thus, there is at present no consensus regarding any possible unique subcellular localization of phytochrome.

Recent developments in immunocytochemical methodology have made it practical to localize phytochrome *in situ* (7–9). It has previously been reported that phytochrome as its red-absorbing form (Pr) is in most cases generally distributed throughout the cytoplasm and is often associated with amyloplasts and mitochondria (9, 10). The present paper investigates the fate of phytochrome distribution after photoconversion of the chromoprotein to Pfr.

MATERIALS AND METHODS

Oat (*Avena sativa* L., cv. Garry) and rice (*Oryza sativa* L., cv. unknown, Carolina Biological Supply) grains were germinated and grown in complete darkness at 25° (11). Shoots from 4-day-old oat and 5-day-old rice were treated with light if appropriate, excised, and then fixed overnight in darkness at 0° in 4% formaldehyde in 0.1 M sodium phosphate, pH 7.6. Except for the specified light treatments, plants were exposed only to green safelights (11) prior to fixation. In a few experiments, fixation was performed in a variety of other buffers as indicated below. Red light treatments were 8 min of exposure at 24° to three closely spaced 40 W Sylvania Gro-Lux lamps at a distance of 20 cm. Far-red treatments were 2 min of exposure at 24° to incandescent light filtered through Plexiglas (FRF-700) at a distance of 15 cm. Both treatments were saturating and produced photostationary states equivalent to those produced by 665 nm and 725 nm interference filters, respectively (ref. 12 and unpublished observations).

Fixed tissue was then embedded either in paraffin (8) or in polyethylene glycol (9). For localization, hydrated sections were treated sequentially with rabbit antiphytochrome serum, sheep antirabbit serum, and a solution of rabbit antiperoxidase–peroxidase complex. The peroxidase was then localized with 3,3′-diaminobenzidine and H_2O_2. Most visible contrast in the bright field photographs therefore represents oxidized diaminobenzidine reaction product associated ultimately with antigenically active phytochrome. Detailed presentations and justifications of the methodology used here may be found elsewhere (8, 9).

Controls for every experiment, with nonimmune rabbit serum as a substitute for antiphytochrome serum, showed no visible staining and, thus, are not presented. The controls thus demonstrated the absence of both nonspecific adsorption of immunoglobulins to the tissue as well as the absence of any endogenous peroxidase activity that might have survived the fixation and embedding procedures (8).

RESULTS

The tips of oat coleoptiles were chosen for most of this study because of their relatively high phytochrome content when fixed before any light exposure (Fig. 1a; ref. 7). In contrast, this apparent high activity decreases dramatically when

Abbreviations: Pr, red-absorbing form of phytochrome; Pfr, far-red-absorbing form of phytochrome.

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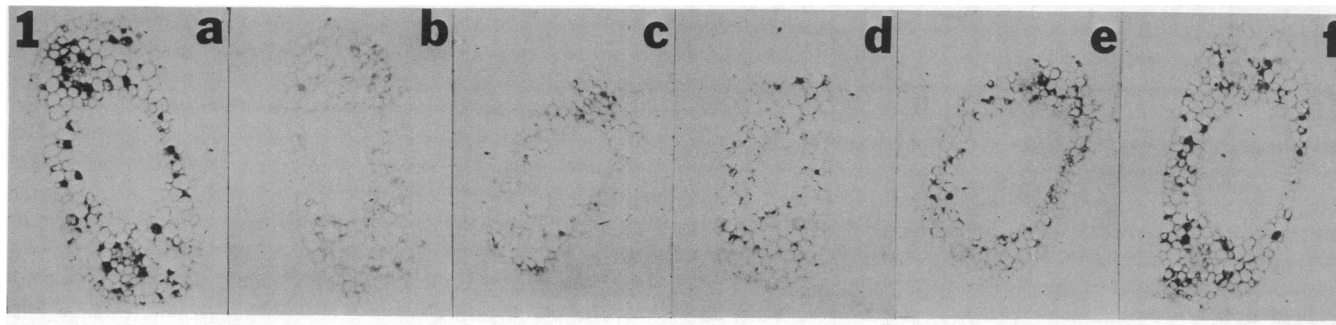

FIG. 1. Bright field micrographs of 3-μm cross-sections from near the tip of etiolated oat coleoptiles. Phytochrome was localized in tissue fixed: (a) prior to light exposure, (b) immediately after exposure to red light, (c) immediately after exposure to red followed by far-red light, (d) same as (c) but after 30 min in darkness at 24°, (e) same as (c) but 60 min in darkness, (f) same as (c) but 120 min in darkness. Magnification, ×38.5.

seedlings irradiated with saturating red light immediately prior to fixation are viewed at low magnification (Fig. 1b). Prompt photoconversion of the Pfr produced by the previous red light treatment back to Pr does not significantly change the level of activity when the tissue is fixed immediately at 0° after the far-red light treatment (Fig. 1c). Only gradually, during a subsequent period in darkness at 24°, does the activity return to the general level of the dark controls (Fig. 1d, e, and f).

If the parenchyma cells of oat coleoptile sections such as those presented in Fig. 1 are examined at higher magnification, it is clear that the phytochrome-associated activity is generally distributed throughout the cytoplasm in plants not exposed to light prior to fixation (Fig. 2a). This observation agrees with other immunocytochemical observations made by both light and electron microscopy (9, 10). However, in contrast to what is seen at low magnification, activity is now visible in the red-treated tissue as densely stained, discrete areas (Fig. 2b, arrows) which were apparently not resolved at low magnification and thus possibly accounted for the apparent low level of activity presented in Fig. 1b. Upon exposure to far-red light these areas of activity enlarge and appear to increase in number (Fig. 2c). (This apparent increase in number may simply represent an increase in size of some areas previously too small to resolve to ones that are resolvable.) After 30 min in darkness at 24° after the far-red irradiation, the areas enlarge further and their number begins to decrease (Fig. 2d). Within 2 hr, the phytochrome slowly redistributes throughout the cytoplasm, concomitant with the disappearance of these densely stained areas produced by the original red light treatment (Fig. 2e and f). Without the far-red irradiation, this redistribution is not seen. In addition, an initial exposure to far-red light alone has no effect on the distribution of phytochrome.

To determine whether the observed reversible redistribution of phytochrome after interconversion between Pr and Pfr might be a function of fixation conditions, we repeated the experiment with tissue fixed in the same buffer but at pH 6.5 and 7.0, or in 0.01 M sodium phosphate, pH 7.6 (at the same osmolarity), or in 0.1 M *sym*-collidine (2,4,6-trimethylpyridine) buffer, pH 7.4, in the absence of sodium. All conditions tested yielded results identical to those presented.

The reversible redistribution of phytochrome observed above is also seen in parenchyma cells of the nodal region of etiolated rice (Figs. 3 and 4). Again, this region was chosen because of its relatively high phytochrome content (8). The size of the discrete areas in rice (Fig. 4b) resulting from red light treatment appears to be slightly smaller and their number per cell greater than in the case of oats. It is also possible that the kinetics of redistribution after a subsequent far-red light treatment may be different since intermediate relaxation times were not tested. It is clear, however, that relaxation of the phytochrome back to the original general distribution (Fig. 4a) does take place (Fig. 4d) although the level of activity may not be as high (compare Fig. 3b to e).

DISCUSSION

Phytochrome responses have classically been defined as those that are induced by red light and reversed with far-red light (1). The subcellular redistribution of phytochrome in etiolated oat and rice shoots is also a classical phytochrome-mediated response. After red light treatment, phytochrome distribution in the cell changes from a general pattern (Figs. 2a and 4a) to one of discrete localization (Figs. 2b and 4b). This apparent redistribution to a discrete localization is reversed by far-red light followed by a subsequent period in darkness (Figs. 2c–f and 4c and d). While it is possible that the reappearance of generally distributed phytochrome represents newly synthesized material and that the loss of activity from the discrete areas represents degradation of the chromoprotein, the only measurements of the rate of turnover or resynthesis after red light treatment (13) indicate that it is much too slow to account for the observations presented here. In addition, all available spectrophotometric evidence suggests that this latter possibility is extremely unlikely (1, 14, 15). Finally, the apparent observed decrease in immunochemical activity (Fig. 1a → b and 3b → c) is to be expected if phytochrome becomes sequestered even without a loss of antigenic activity.

An alternate but less tenable hypothesis to explain the apparent decrease in antigenic activity after red light exposure would suggest that phytochrome, even as Pr, is associated with membranes. Upon conversion to Pfr, the chromoprotein would move deep within the lipophilic phase of the membranes such that virtually all antigenic activity would be lost. However, this hypothesis is not consistent with either the large size (about 240,000 daltons) or the characteristic hydrophilic nature of phytochrome (1, 11, 15). Nor does this hypothesis explain the apparent return of phytochrome-associated activity initially via the observed increase in size of the original discretely localized areas (Fig. 2b to d). Rather, this hypothesis would suggest simply a direct and uniform reappearance throughout the cytosol. Finally, while previous electron

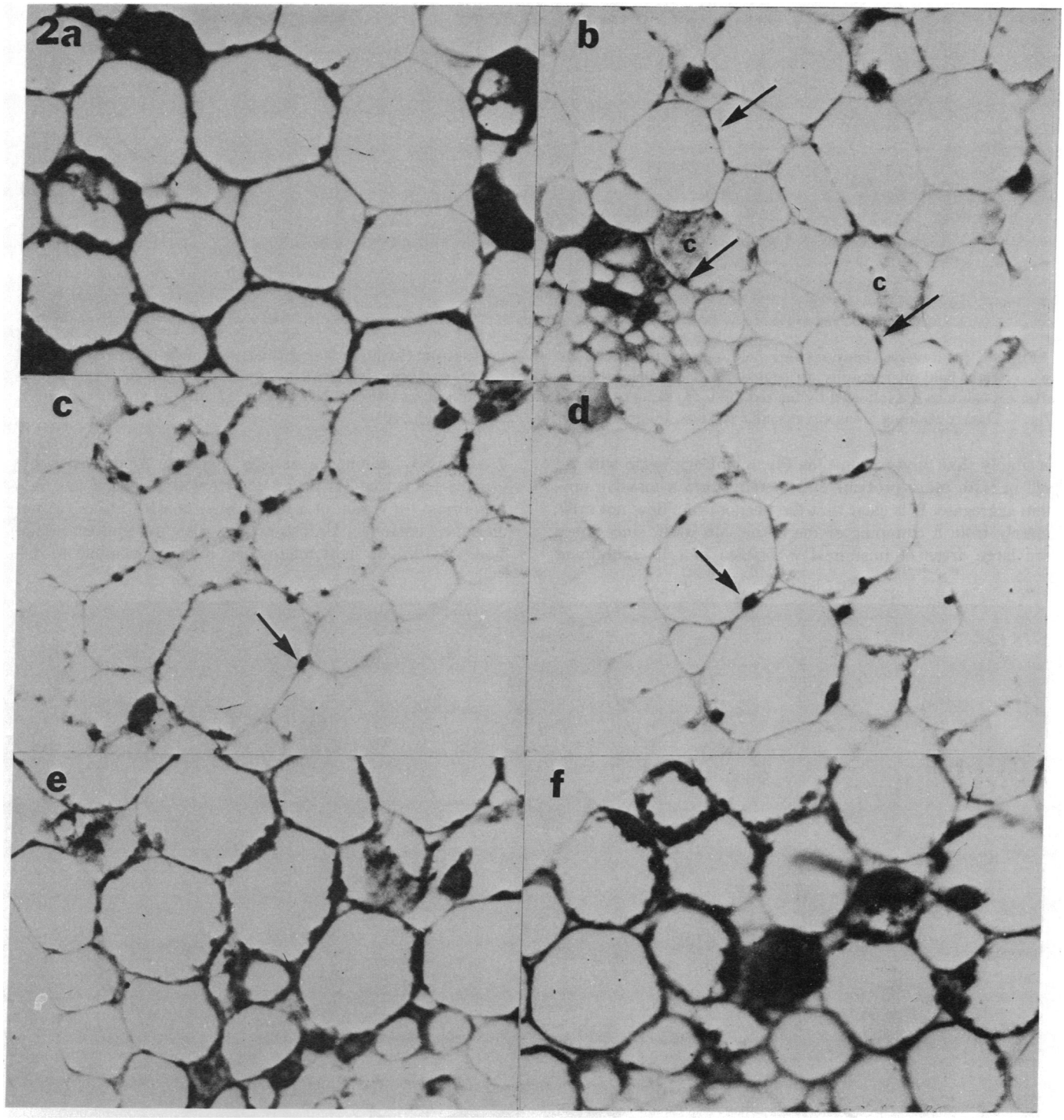

FIG. 2. Bright field micrographs (a–f) of comparable parenchyma cells from oat coleoptile, 3-μm cross-sections, treated as in the corresponding segments of Fig. 1. *Arrows* indicate discrete areas of dense phytochrome-associated stain. Unstained cytoplasm, *c*. Magnifications, ×493.

micrographs of Pr localization indicate that some of the phytochrome may possibly be associated with membranes, the micrographs, coupled with extensive biochemical data, suggest that Pr is a soluble protein (1, 3, 9–11, 16). Hence, the apparent loss in activity is more likely a result of redistribution rather than a loss of antigenic activity as the hypothesis above would suggest.

The apparent movement of phytochrome to and from these discrete areas is not influenced by any of the fixation conditions tested and, thus, does not appear to be an artifact arising from fixation conditions. This discrete localization of phytochrome also clearly does not result from the form in which phytochrome is fixed since the result is qualitatively the same whether phytochrome is fixed as Pr or Pfr (compare Fig. 1b to c and Fig. 2b to c).

There are at least two fundamental explanations for this discrete localization after transformation of phytochrome to its physiologically active Pfr form by red light. One possibility

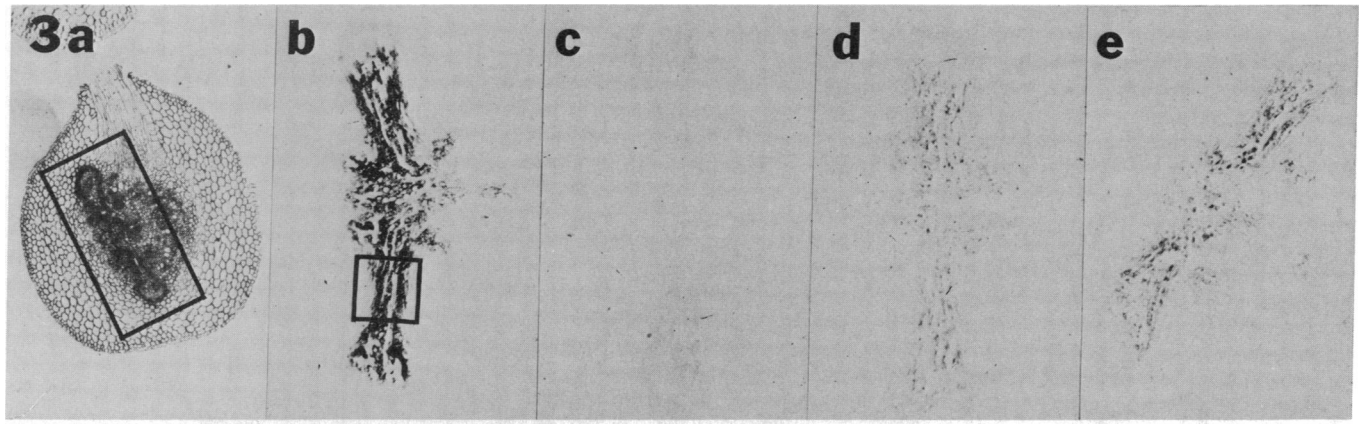

FIG. 3. (a) Reversed contrast dark field micrograph of an 8-μm cross-section from rice node. *Box* outlines area presented in (b) through (e). Bright field micrographs of rice node fixed: (b) prior to light exposure, (c) immediately after exposure to red light, (d) immediately after exposure to red followed by far-red light, (e) same as (d) but after 120 min in darkness at 24°. *Box* in (b) outlines area presented in Fig. 4. Darkly staining areas are vascular strands. Magnifications: (a), ×27; (b)–(e), ×61.5.

is simply that phytochrome, as Pfr, might aggregate with itself or with other proteins and thereby form a massive protein aggregate. It is clear that the phenomenon does not arise merely from a clumping of the cytoplasm itself, since there are large areas of unstained cytoplasm in cells containing these densely staining areas (e.g., Fig. 2b). Another possible explanation is that Pfr may associate with a specific site on a membrane (or region of a membrane) or with an as yet unidentified organelle. Unfortunately, with the limited resolution provided by light microscopy it is not possible to dis-

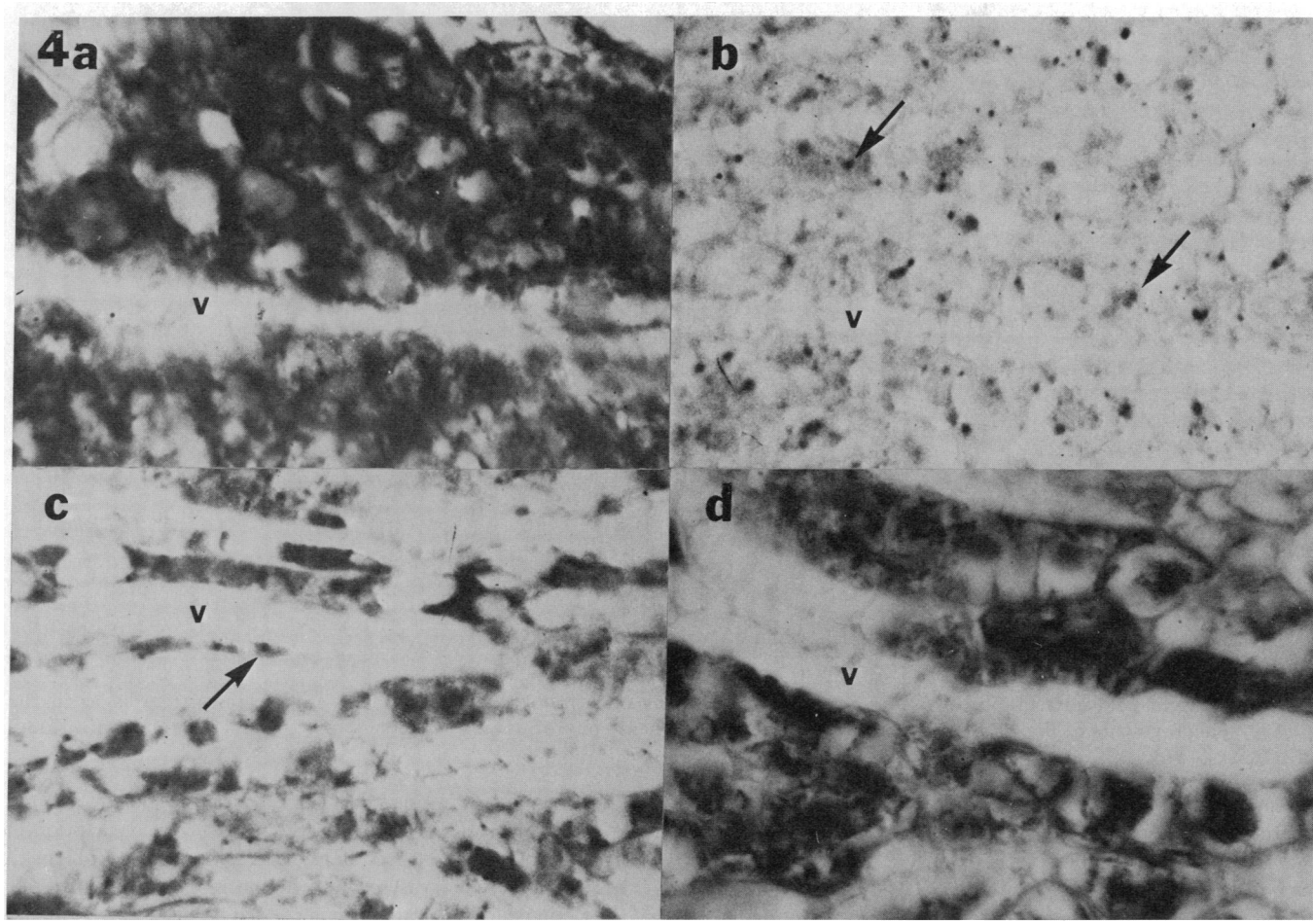

FIG. 4. Bright field micrographs of representative cells from rice node in 8-μm sections comparable to those presented in Fig. 3. Tissue fixed: (a) prior to light exposure, (b) immediately after red light, (c) immediately after red followed by far-red light, (d) as in (c) but after 120 min in darkness at 24°. *Arrows* identify discrete areas of dense phytochrome-associated stain. Vascular element, *v*. Magnifications, ×962.5.

tinguish between the above two possibilities. Recent investigations involving cell fractionation and spectrophotometric assay *in vitro* for phytochrome have suggested a specific association of Pfr, but not Pr, with the plasma membrane (3) and the rough endoplasmic reticulum (2, 3), but it is not yet possible to correlate our present observations with this work *in vitro*.

Since it is virtually impossible to fully represent in black-and-white the information that is present in color in the original slides, densely stained cells often appear virtually black in micrographs (e.g., Fig. 2a). In addition, organelles are not apparent in black-and-white micrographs of densely stained cells, although they may be readily identified in either color micrographs or phase-contrast views (7, 10). Direct observation of a large number of stained slides from several independent experiments does indicate that the heavily stained phytochrome-containing regions resulting from red light treatment are not mitochondria, plastids, or nuclei and that these regions are not present prior to irradiation.

Two recent studies (16, 17) report the binding of Pfr to an extractable membrane fraction after red light treatment *in vivo* and the subsequent dark release of this bound phytochrome to the soluble fraction after far-red irradiation immediately after initial red irradiation. In both cases, the half-time for release *in vivo* was approximately 1 hr, in rough agreement with the return of phytochrome to the distribution pattern seen in the untreated controls in the present study. It would be premature, however, to suggest that the two types of observations are the consequence of a single phenomenon. First, while the present study was with oat coleoptiles and rice nodes, the other studies were with squash hooks (16) and corn coleoptiles (17), and phytochrome dark reactions are well known to vary widely from one plant to another (1, 14). Second, there is no evidence that the extraction procedures used yielded quantitative recovery of all bound phytochrome in the corn and squash studies, nor is there evidence from the present study that the phytochrome observed as localized is membrane-bound, or even membrane bounded. Electron microscopy should help to resolve the situation.

At least three possible interpretations of the significance of this apparent redistribution of phytochrome may be suggested. The first is that it results from an aggregation of excess physiologically inactive phytochrome. The second is that Pfr is associating with a specific site that makes phytochrome accessible to the phytochrome destruction mechanism. [Destruction is the loss of photoreversibly detectable phytochrome *in vivo* which occurs when the pigment is present as Pfr (1). Destruction apparently results from a proteolytic degradation of Pfr (18, 19).] The third interpretation is again that Pfr is associating with specific binding sites but that this binding represents the initial event in the chain of reactions that ultimately lead to morphogenic activity. Although the latter interpretation is the most attractive, it is nevertheless premature to suggest that one or more of the preceding interpretations is correct.

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