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Red Light-enhanced Phytochrome Pelletability

RE-EXAMINATION AND FURTHER CHARACTERIZATION[1]

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ABSTRACT

Red light-enhanced pelletability of phytochrome was observed in extracts of all 11 plants tested: *Avena sativa* L., *Secale cereale* L., *Zea mays* L., *Cucurbita pepo* L., *Sinapis alba* L., *Pisum sativum* L., *Helianthus anuus* L., *Raphanus sativus* L., *Glycine max* (L.) Merr., *Phaseolus vulgaris* L., and *Lupinus albus* L. This enhanced pelletability was observed in all 11 plants following *in situ* irradiation (*in vivo* binding) but only in *Sinapis* and *Cucurbita* after irradiation of crude extracts (*in vitro* binding). *In vivo* binding was not strongly dependent upon pH and, with few exceptions, was not markedly sensitive to high salt concentration, whereas *in vitro* binding was completely reversed by both high pH and high salt concentration. However, both binding phenomena were observed only with a divalent cation in the extract buffer. *In vivo* binding was further characterized using *Avena* which showed an increase in pelletability from less than 10% in dark control extracts to more than 60% in extracts of red light-irradiated shoots. The half-life for binding was 40 seconds at 0.5 C and was strongly temperature-dependent, binding being complete within 5 to 10 sec at 22 C. If pelletable phytochrome in the far red-absorbing form was photoconverted back to the red-absorbing form *in situ*, phytochrome was released from the pelletable condition with a half-life of 25 minutes at 25 C and 100 minutes at both 13 C and 3 C. No cooperativity in red light-enhanced pelletability with respect to phytochrome–far red-absorbing form was observed.

The existence of a biologically active receptor for Pfr, the physiologically active form of phytochrome, has been postulated (Hendricks as cited in Ref. 35) both to explain the so-called phytochrome paradoxes (8) and the potential involvement of phytochrome in mediating the high irradiance responses (2, 7, 13, 31). Recent evidence indicating an enhanced association of phytochrome with particulate material (29), following red light irradiation of either intact tissue or crude extracts of *Cucurbita pepo* L., has led to the suggestions that such a receptor for Pfr has been found and that this receptor is an integral part of a membrane (14, 33). However, Quail (26, 27) has demonstrated that this enhanced association represents, at least in large part, an interaction between phytochrome and 31S ribonucleoprotein particles which displays the characteristics of a nonspecific, electrostatic phenomenon. As discussed in detail by Quail (25), it is premature to assume that an association between phytochrome and one or more membrane-bound receptors has been demonstrated.

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Enhanced associations of phytochrome with pelletable material have also been reported using maize coleoptiles (29), pea shoots (5), and oat shoots (6). Although the characteristics of these associations have not been examined as carefully as for *Cucurbita*, preliminary evidence has indicated that, at least in the case of maize and oats, the associations are of a different type from that described for *Cucurbita* (6, 15). In particular, the association between phytochrome and pelletable material from maize and oats is not markedly reversed by either high pH or high ionic strength nor has such an association been observed following irradiation of crude extracts at 0 C, in direct contrast to the observations utilizing *Cucurbita*.

The present report presents further evidence that two distinct types of phytochrome binding do occur. Only binding induced exclusively following irradiation of intact tissue (*in vivo* binding) is found to be a general phenomenon. This *in vivo* binding is characterized further here since it has not previously been well studied free of the complications of the *in vitro* binding already described for *Cucurbita*, and since it may yet reflect a biologically significant association of Pfr with a receptor. Oat shoots were chosen as a suitable system for this latter purpose because: (*a*) no *in vitro* binding has been observed in oat extracts (6); (*b*) the ratio of red light-induced to dark control binding is higher for oats than for any of 10 other plants tested; (*c*) immunocytochemical evidence obtained with oats has already led to the suggestion that Pfr has a unique distribution within the cell which might reflect binding with a receptor (12); and (*d*) oats have already been used for many biochemical and immunochemical studies of phytochrome (3).

MATERIALS AND METHODS

Plant Material. Etiolated tissue was obtained from plants grown at 25 C on moist, absorbent cellulose packing material in total darkness for 3 to 7 days (Table I). Eleven plants were used: Garry oats (*Avena sativa* L.), Nomaro winter rye (*Secale cereale* L.), maize (*Zea mays* L., WF9 × 38 from Bear Hybrid Corn Co., Decatur, Ill.), *Cucurbita pepo* L., cv. Black Beauty, mustard (*Sinapis alba* L., obtained from H. Mohr, Freiburg, BRD), pea (*Pisum sativum* L., purchased from Hambrecht, Freiburg, BRD), sunflower (*Helianthus anuus* L., purchased from Hambrecht), Carnita radish (*Raphanus sativus* L.), soybean (*Glycine max* (L.) Merr., Asgrow Z735264), bean (*Phaseolus vulgaris* L., cv. Dark Red Kidney), and lupine (*Lupinus albus* L.).

Standard Actinic Irradiations. Intact plant segments or crude extracts in an ice bath were irradiated as needed for 3 min with red light and 5 min with far red light. Red (660 nm) and far red (729 nm) light were obtained by filtering the output of a 500-w tungsten lamp through both a heat-absorbing filter and a 15 nm half-bandwidth interference filter. For both wavelengths, 15- to 30-sec irradiations were sufficient to establish photostationary equilibrium. Irradiated tissue was kept at 0 to 3 C until ex-

tracted. Exceptions to these standard conditions are noted below with each experiment where appropriate.

Standard Pelletability Assay. In the normal assay, which is similar to that used earlier (6), 1.5 g tissue was homogenized for 10 to 15 sec in a Bühler Homogenisator with 6 ml extraction buffer. Extraction buffer was prepared by mixing 25 mM *N*-morpholino-3-propane-sulfonic acid with 25 mM tris, each 10 mM in $MgCl_2$ and 14 mM in 2-mercaptoethanol, to obtain the desired pH (Table I). For other $MgCl_2$ concentrations or when $MnCl_2$ or $CaCl_2$ was used, the cations were added to $MgCl_2$-free extraction buffer from 1 M stock solutions. The crude homogenates were squeezed through nylon cloth and then centrifuged for 30 min (Figs. 1 and 2; Tables I and II) or 15 min (Figs. 3–8; Tables III–V) at 20,000*g*. Pellets were resuspended in 1 to 2 ml of extraction buffer with a Teflon and glass tissue homogenizer. Normally, all handling of the extracts was at 0 to 3 C. All work was performed under dim green light until the pellets and supernatants were separated. When Pfr was assayed, work continued under green light until measurements were complete. Deviations from the above conditions are noted below where appropriate.

Phytochrome Assay. Phytochrome measurements were made in 3-mm path length cuvettes using samples prepared with $CaCO_3$ as a scattering medium to eliminate effects of inherent scattering differences in the samples. Photoreversibility was measured between 662 and 739 nm (Schott IL interference filters) using a custom built dual wavelength spectrophotometer. The spectrophotometer is an automated version of that described elsewhere (10) with the following essential modifications: the light-chopping frequency is 75 Hertz and the photomultiplier high voltage is obtained from an Ortec model 456 supply equipped with feedback regulation to maintain the mean photomultiplier current at 2 μamp. Actinic red (662 nm) and far red (729 nm) light are obtained with Schott IL interference filters. One unit/ml of phytochrome is that concentration which gives a $\Delta(\Delta A)$ of 0.001 using the above conditions. The proportion of phytochrome in the pellet is the number of units in the pellet divided by the number in the pellet and supernatant combined. Pfr is measured as described elsewhere (4) assuming a photoequilibrium in red light of 75% Pfr to 25% Pr (20).

Each experiment was performed a minimum of two times.

RESULTS

SURVEY OF PHYTOCHROME PELLETABILITY

The pH of the extraction buffer and resulting supernatant, as well as the total amount of phytochrome extracted, are tabulated for each plant (Table I). All 11 plants studied show a clear enhancement of phytochrome binding by red light irradiation of tissue prior to extraction (*in vivo* binding), whereas only two (mustard and *Cucurbita*) show such enhancement following irradiation of crude extracts (*in vitro* binding) (Figs. 1 and 2; Tables II and III). Except for radish and *Cucurbita*, added $MgCl_2$ is required to observe this enhanced binding (Figs. 1–3; data for maize are reported elsewhere [15] and were confirmed here). Generally, the *in vivo* binding is not markedly reversed by high $MgCl_2$ concentration (Figs. 1 and 2), whereas the *in vitro* binding is completely reversed (Fig. 2a) as shown earlier for *Cucurbita* (15; confirmed here, data not shown). Since binding *in vitro* had already been documented for *Cucurbita*, an attempt was made to observe only *in vivo* binding in this case by converting all phyto-

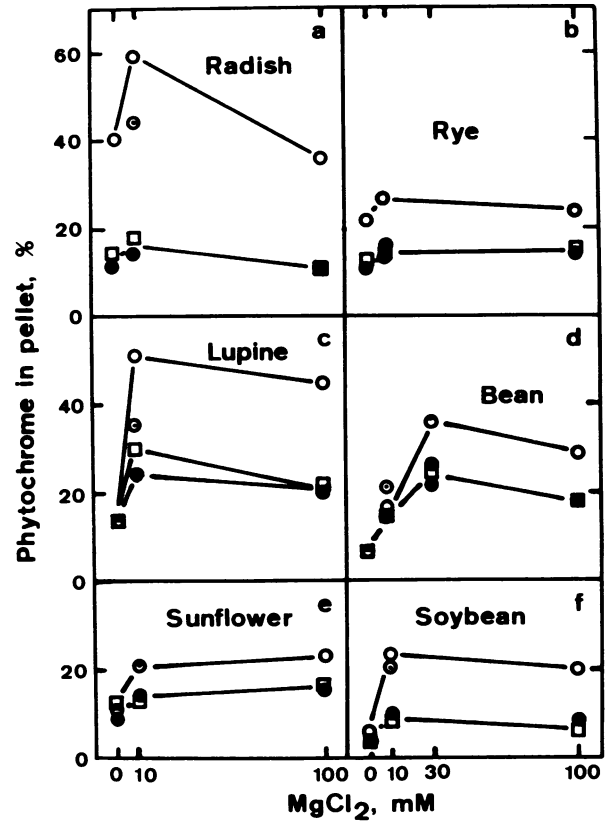

FIG. 1. Percentage of phytochrome pelletable in extracts of unirradiated (●), red-irradiated (○), or red-far red irradiated (⊙) radish (a), rye (b), lupine (c), bean (d), sunflower (e), or soybean (f) as a function of $MgCl_2$ concentration in the extraction buffer. Pelletability was also tested in red-irradiated extracts of unirradiated tissue (□). Extracts prepared 5 min after last irradiation with 30 min incubation between red and far red irradiations. Significance of red light-induced binding in sunflower verified by repeating the assay six times at 10 mM $MgCl_2$. Each point is the average of at least two independent observations.

Table I. Summary of Extraction Protocols

Plant	Tissue extracted	Age	Extraction buffer	Supernatant	Phytochrome in crude extract
		days	pH		units/g fresh wt
Radish	entire shoot	4	7.8	7.4	35
Rye	entire shoot	6	7.6	7.0	35
Lupine	hook only	6	7.8	7.3	35
Bean	hook only	5	7.6	7.2	25
Sunflower	hook only	5	7.3	7.3	30
Soybean	hook only	6	7.6	7.1	30
Mustard	entire shoot	3	8.0	7.3	20
Cucurbita	hook only	7	7.5	7.2	55
Pea	shoot apex with hook	6	7.6	7.2	60
Maize	coleoptile	7	7.3	6.8	20
Oat	entire shoot	5	7.5	7.2	100

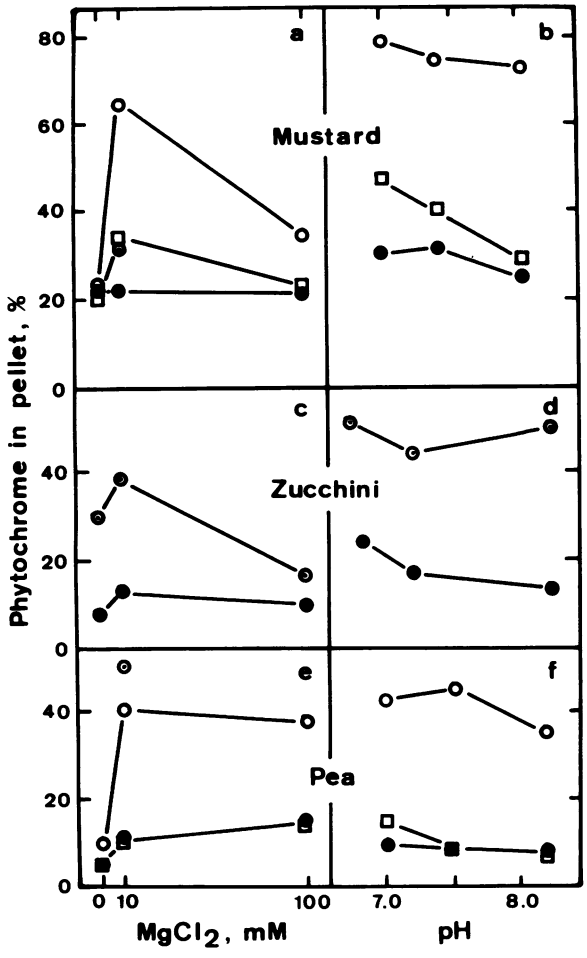

FIG. 2. Percentage of phytochrome pelletable in extracts of unirradiated (●), red-irradiated (○), or red-far red irradiated (⊙) mustard (a, b), *Cucurbita* (c, d), or pea (e, f) as a function of both $MgCl_2$ concentration in the extract buffer (a, c, e) and pH of the final supernatant (b, d, f), the latter in the presence of 10 mM $MgCl_2$. Pelletability in red-irradiated extracts of unirradiated tissue (□) was also measured with mustard and pea. Extracts prepared 5 min after last irradiation with 30 min incubation between red and far red irradiations. Each point is the average of at least two independent observations.

Table II. Percentage Phytochrome Pelletable in the Presence of 0.1 M NaCl

Red irradiations were given to intact tissue prior to extraction (oat, radish, Cucurbita-in vivo) or to crude extracts (Cucurbita-in vitro). Extracts prepared 5 min after red irradiation. Values are averages of at least two independent observations.

Plant	Addition and Irradiation: no addition dark	no addition red	NaCl added dark	NaCl added red	Inhibition of binding by NaCl
			%		
Oat	10	59	7	41	31[a]
Radish	23	74	12	32	61
Cucurbita	18		11		
in vivo		28		22	-10
in vitro		69		14	94

[a]Percentage inhibition of binding by NaCl is calculated after subtracting the dark control binding values.

chrome in red-irradiated tissue back to Pr by far red irradiation prior to extraction (Fig. 2c). Thus, unless the Pr formed by a red, far red cycle is different from that originally present, the possibility of *in vitro* binding may be excluded. Subsequent far red irradiation of other red-irradiated tissues was found to have little or no effect on the red-enhanced binding observed with 10 mM $MgCl_2$ in radish, sunflower, soybean, and pea whereas it resulted in virtually complete reversal in rye, lupine, bean (at 30 mM $MgCl_2$), and mustard.

The effect of pH on red light-induced *in vivo* binding is small (Fig. 2, b, d, and f; Fig. 4) whereas *in vitro* binding in mustard is completely eliminated at high pH as has been shown for *Cucurbita* earlier (16; confirmed here, data not shown).

In vivo binding in oat, radish, and *Cucurbita* was only partially inhibited by 100 mM NaCl whereas *in vitro* binding observed with *Cucurbita* was almost completely inhibited (Table II).

Separation of maize and radish shoots into different organs indicates that *in vivo* binding occurs at comparable levels in different organs of the same plant (Table III). The apparently reduced binding observed in maize leaves is not significantly different because of the very low phytochrome levels in this tissue (less than 10 units/g fresh weight).

FURTHER CHARACTERIZATION OF *IN VIVO* BINDING WITH OAT SHOOTS

Effect of Extraction Conditions on Observed Binding. Although Mg^{2+} has already been shown to be required for enhanced pelletability to be observed (Figs. 1 and 2; Ref. 6), this requirement is not specific. Both Ca^{2+} and Mn^{2+} can substitute (Fig. 3), although the latter also increases the dark control binding. (Other divalent cations tested [Co^{2+}, Cu^{2+}, Zn^{2+}] at least partially denatured the phytochrome and thus led to uninterpretable observations.) The divalent cation must be present at the moment of extraction; later addition to a crude extract of red-irradiated tissue is not sufficient (Table IV). Moreover, if 20 mM EDTA is present in the extraction buffer as well as 10 mM $MgCl_2$, no enhancement in pelletability is observed (Table IV). Addition of EDTA after extraction also reverses the $MgCl_2$-

Table III. Percentage Phytochrome Pelletable in Extracts of Different Organs from the Same Plant

Red irradiations given to intact organs prior to extraction. Extracts prepared 5 min after red irradiation. Values are averages of at least two independent observations.

	Maize: Coleoptile	Maize: Leaves	Maize: Mesocotyl	Radish: Cotyledons	Radish: Hypocotyl
			%		
Dark control	30	24	25	21	26
Red irradiated	81	51	72	84	82

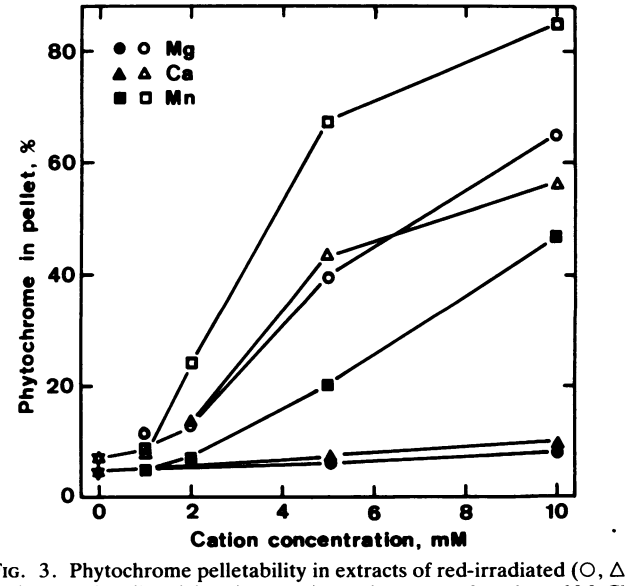

FIG. 3. Phytochrome pelletability in extracts of red-irradiated (○, △, □, ☆) or nonirradiated (●, ▲, ■, ✱) oat shoots as a function of $MgCl_2$ (○, ●), $CaCl_2$ (△, ▲), or $MnCl_2$ (□, ■) concentration in the extraction buffer. In no case was the pH of the crude extract changed by more than 0.1 pH unit from the controls without added cation (☆, ✱).

preserved pelletability although not as much as if it were present during extraction (Table IV). Finally, if pellets with high levels of phytochrome (prepared with 10 mM $MgCl_2$ in the extraction buffer using red-irradiated tissue) are resuspended in the absence of $MgCl_2$ and with 2.5 mM EDTA added, 94% of the phytochrome is released to the supernatant as determined by a second centrifugation at 20,000*g* for 15 min. By comparison, if replicate pellets are resuspended with 10 mM $MgCl_2$ present, only 8% of the phytochrome is found in the subsequent supernatant.

A more detailed examination of the dependence of observed phytochrome binding upon the pH of the extract confirms that *in vivo* binding is only partially reversed by values up to 8.5 (Fig. 4).

Kinetics of Phytochrome Binding. As little as 5 sec red actinic light given to shoots prior to extraction, which was found by direct assay to yield a photostationary equilibrium between Pr and Pfr, induces an approximately 8-fold increase in phytochrome pelletability (Table V) as observed with longer irradiation times (Table IV; Ref. 6). With the irradiations given at 0 C, this red-enhanced binding of phytochrome is almost fully reversed by an immediate irradiation with far red light (Table V). If a dark period is interposed between red and far red irradiations, no reversibility is seen when the tissue is extracted soon after the far red irradiation (Table V). Far red irradiation alone does not induce a significant increase in binding (Table V).

The kinetics of the binding process occurring during this interposed dark interval was examined by carefully controlling the duration of the dark interval (Fig. 5). The tissue was placed in the glass extraction vessel with extraction buffer and maintained at a constant temperature using a circulating, refrigerated water bath. The tissue was irradiated from both sides simultaneously with red light using two 500-w slide projectors and 656 and 658 nm interference filters with 12 and 24 nm half-bandwidths, respectively. Since the tissue was irradiated while in place on the homogenizer and since binding does not occur *in vitro* under these conditions (6), the binding reaction could be stopped within a few sec, when desired, by turning on the homogenizer. The half-life for binding at 0.5 C was about 40 sec and exhibited a strong temperature dependence (Fig. 5). At 22 C, the reaction was found to be more than 90% complete if extraction immediately followed the 5-sec red irradiation. Thus, the half-life at this temperature must be less than 4 to 5 sec. A semilogarithmic plot of the proportion of binding not yet completed as a function of time indicates that the rate-limiting step in the process exhibits first order kinetics (Fig. 5b).

Table IV. Effects on Phytochrome Pelletability of $MgCl_2$ and EDTA Added to the Extraction Buffer or the Crude Extract

Extracts of red-irradiated oat shoots were prepared with or without 10 mM $MgCl_2$ and 20 mM EDTA in the extraction buffer. Prior to centrifugation, $MgCl_2$ or EDTA from a neutral pH stock solution was added to some extracts to a concentration of 10 mM and 20 mM, respectively. Extracts prepared about 30 min after red irradiation. Values obtained in replicate experiments indicate variability.

Addition to extract buffer	Addition to crude extract	Pelletable phytochrome	
		%	
none	none	9.3	8.2
none	$MgCl_2$	10.1	12.6
$MgCl_2$	none	62	60
$MgCl_2$	EDTA	25	25
$MgCl_2$ + EDTA	none	9.3	10.9

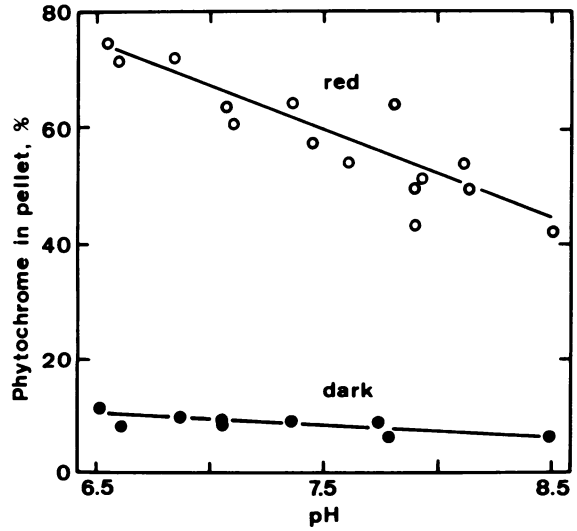

FIG. 4. Phytochrome pelletability as a function of pH in the supernatant fraction from red-irradiated (○) and nonirradiated (●) oat shoots. The pH was changed by varying the two components of the extraction buffer; no change was made in the extract itself.

Table V. Photoreversibility of a Red-induced Increase in Phytochrome Pelletability

Values obtained in replicate extracts of oat shoots indicate variability. All irradiations and treatments at 0 C. Extracts prepared about 30 min after single irradiations and about 5 min after irradiation with a red, far-red light sequence.

Treatment	Pelletable Phytochrome		
	%		
Dark control	9.0	8.1	6.4
5 sec red	64	67	57
5 sec red, 60 sec far-red (no intervening dark period)	10.6	9.7	8.8
60 sec far-red	9.0	7.5	8.8
5 sec red, 30 min dark, 60 sec far-red	63	61	62

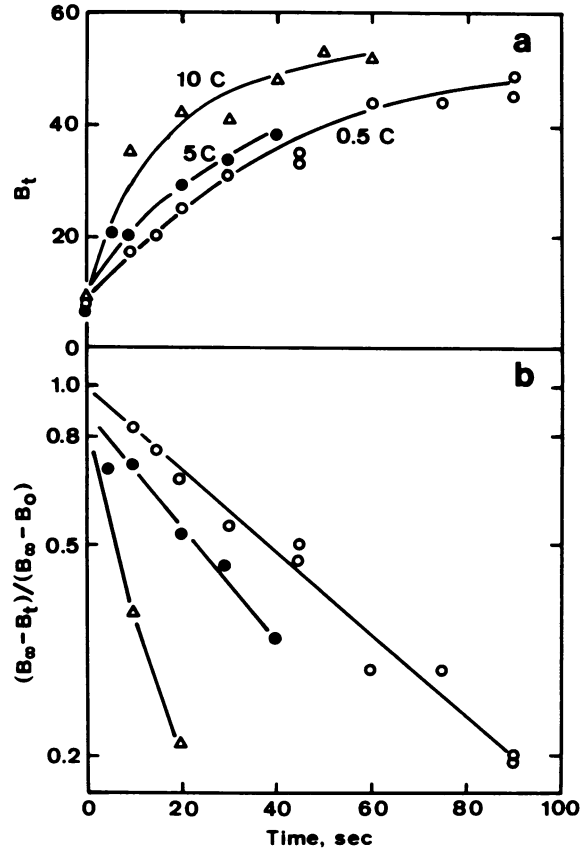

FIG. 5. a: Percentage of phytochrome bound in oat shoots at time *t* (B_t) as a function of time between the end of a 5-sec red irradiation and the beginning of homogenization at 0.5 C (○), 5 C (●), and 10 C (△); b: semilogarithmic plot of the amount of binding yet to take place as a function of time using the data presented in (a). B_0: percentage bound at zero time; B_∞: percentage bound at 30 min at 3 C.

Kinetics of Phytochrome Release. Although phytochrome is not released from the bound condition as Pr when extraction immediately follows a far red irradiation (Table V), it is released slowly when a dark period follows the far red irradiation and precedes tissue homogenization (Fig. 6). When the proportion of phytochrome remaining bound is semilogarithmically plotted as a function of time (Fig. 6b), the release displays first order kinetics and a relatively strong temperature dependence between 13 C (half-life, 100 min) and 25 C (half-life, 25 min). In contrast, the rate at 3 C is indistinguishable from that at 13 C. If the phytochrome is left as Pfr and the tissue incubated at 3 C prior to extraction, no release of bound phytochrome is seen. In addition, if crude extracts containing phytochrome in the bound condition are irradiated with far red light, the percentage of phytochrome bound (now as Pr) is still 57% when the extracts are centrifuged after a 2-hr incubation at 3 C. This result clearly contrasts with that obtained following a 1.5-hr incubation of Pr-bound phytochrome *in vivo* which results in a decrease in binding to 34% (Fig. 6) and by extrapolation to 28% after 2 hr.

Dependence of Binding upon Pfr Concentration. To determine the relationship between the amount of Pfr produced by a brief red actinic irradiation and the amount of phytochrome found in the pellet, whole oat shoots were irradiated at 0 C for up to 10 sec (although most irradiations were 3 sec or less) while floating in a thin layer on ice-cold extraction buffer. For all but the longest irradiation period, irradiation times were sufficiently short that binding did not occur during the irradiations (Table V; Fig. 5). The tissue was then extracted after a 15- to 30-min incubation on ice and the proportion of phytochrome in the pellet was determined as a function of the amount of Pfr experimentally measured in the crude extract (Fig. 7). Pfr determinations were made as rapidly as possible after extraction (normally within 2 hr).

The relative amounts of Pr and Pfr in both the supernatant and pellet fractions from the above experiment were also measured immediately after centrifugation so that both Pr and Pfr in the pellet could be independently expressed as a function of the total amount of Pfr produced by the brief red irradiations (Fig. 8). At low Pfr levels, it is apparent that the amounts of both Pfr *and* Pr in the pellet increase.

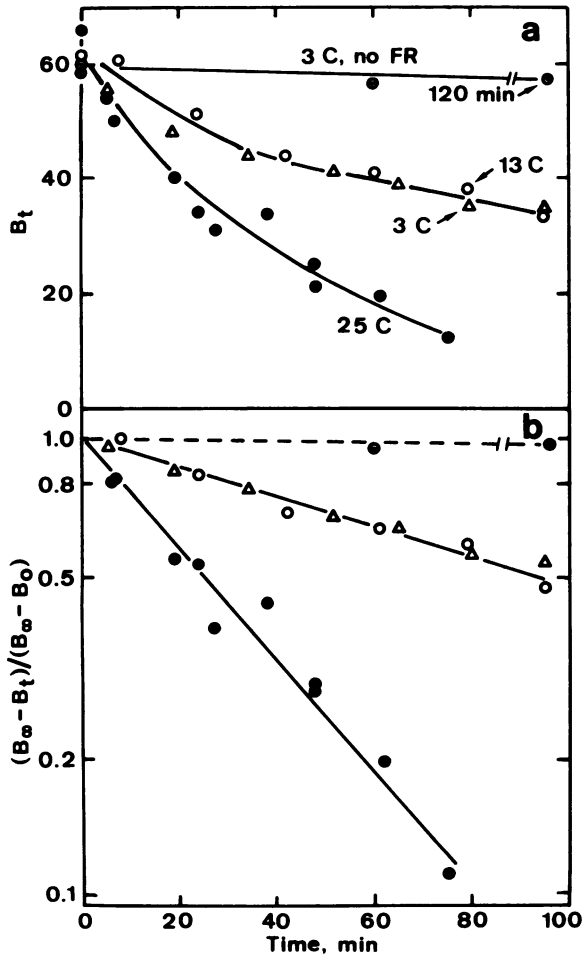

FIG. 6. a: Percentage of phytochrome bound at time t (B_t) as a function of time in darkness at 25 C (●), 13 C (○), and 3 C (△) between a 5-min irradiation of intact oat plants with "standard red light" (18) followed immediately by a 10-min irradiation with "standard far red light" (18) and extraction of the oat shoots. As a control, some plants were incubated in darkness at 3 C for 1 or 2 hr following a red irradiation and prior to extraction (⊙). Plants were harvested only at the time of extraction; b: semilogarithmic plot of the amount of phytochrome remaining bound as a function of time using the data presented in (a). B_0: percentage bound at zero time; B_∞: percentage bound in a dark control extract.

DISCUSSION

As first indicated by comparing results with oat (6), maize (15), and *Cucurbita* (15), there are two distinct types of phytochrome binding (Figs. 1 and 2). One type occurs *in vitro* and is completely reversed by high salt concentration and high pH (Fig. 2, a and b; Table II; Ref. 16). Since Quail (26, 27) has demonstrated that this binding is, at least in part, with degraded ribonucleoprotein material and exhibits the characteristics of an electrostatic, possibly artifactual, interaction, and since this binding is not observed in most plants studied, it would appear that this *in vitro* binding is likely to be of little biological interest. The other type of binding occurs only when Pfr is present within the cell (Figs. 1 and 2; Ref. 6), is not as strongly inhibited by high ionic strength and high pH (Figs. 1-4; Table II; Ref 6), and appears to be a widespread phenomenon, being observed even in *Cucurbita* using an appropriate protocol (Fig. 2, c and d). Thus, this second type of binding is not the same as that characterized by Quail (27) and may yet serve as a possible candidate for a Pfr-receptor system.

Since this second type of binding is observed only as a result of having Pfr present *in situ* for a defined period of time (Fig. 5, Table V) it is referred to here as *in vivo* binding. However, the observed increase in pelletability does not ncessarily represent a Pfr-receptor interaction. It may instead reflect a Pfr-induced change in the system which either stabilizes already "bound" phytochrome or permits binding to occur at the instant of extraction (but not later [Fig. 5, Table IV]). Use of the term *in vivo* "binding" should not be interpreted to exclude these other possibilities.

As pointed out in the introductory section, *in vivo* binding has not previously been well described, and for several reasons, oats have been chosen here for that purpose. *In vivo* binding in oats is a rapid association between phytochrome, only as Pfr and so far only *in situ* (Tables IV and V; Ref. 5), and one or more pelletable cellular constituents by a pseudo-first order, temperature-dependent process (Fig. 5). If far red light is given before binding has taken place, it is almost wholly reversible (Table V). Although the speed of binding is sufficiently slow that it may not wholly be explained on the basis of the rate of Pfr formation from phototransformation intermediates (21), it is sufficiently rapid that it would precede the fastest (within 10 sec) response yet described to red light (19). Hence, *in vivo* binding has the potential for being the first step leading to phytochrome-mediated responses.

Apart from the requirement for a divalent cation (Figs. 1-3),

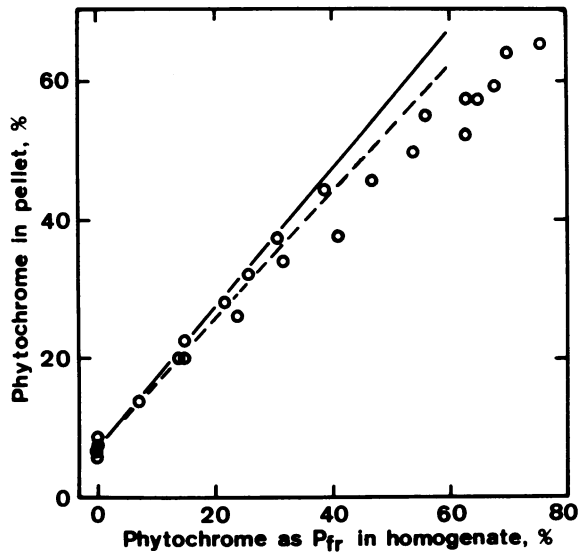

FIG. 7. Proportion of phytochrome in the pellet as a function of the proportion of phytochrome as Pfr measured in the crude oat shoot extracts before centrifugation. (—): Expected outcome if each molecule of Pfr measured results in 1 molecule of phytochrome being added to those already present in the dark control pellet. (- - -): expected outcome if the level of nonspecific binding (as represented by the dark controls, about 7%) decreases proportionately to the increase in red-induced binding, while simultaneously each molecule of Pfr measured results in 1 molecule of phytochrome being added to the pellet fraction. One sec red yields about 25% Pfr; 2 sec, 45%; 3 sec, 55%; 5 sec, 70%.

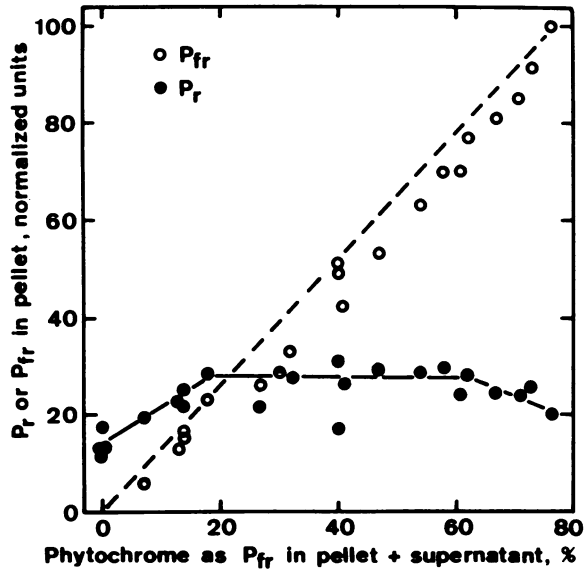

FIG. 8. Amounts of Pr (●) and Pfr (○) measured in the pellet as a function of the relative proportion of phytochrome as Pfr in the pellet and the supernatant combined. Pr and Pfr units were normalized to account for the different levels of total phytochrome in each oat shoot extract.

the composition of the extraction buffer had little effect on the level of *in vivo* binding (Figs. 2 and 4; Table II). Preliminary experiments showed that EDTA, sucrose, and mercaptoethanol, as used in most earlier studies (16, 26, 37), had no effect on the observed level of binding. (Mercaptoethanol is used here only to counteract the oxidative capacity of the crude extracts.) Cacodylate and phosphate buffers work as well as that used here, although the monovalent cations present in these buffers did not substitute for the divalent cation requirement. The requirement for a divalent cation is only to preserve the bound state after extraction. The divalent cation does not co-precipitate free or previously bound phytochrome with other cellular components since addition after extraction does not appreciably increase pelletability (Table IV) as it does in the case of *in vitro* binding (16). The requirement for a divalent cation to maintain the particulate state of a protein is known for other systems and is thus not inconsistent with a biologically significant association (30).

By contrast to binding, the release of phytochrome from the bound condition, which occurs at an appreciable rate only as Pr, is a slow process (Fig. 6). Release is also temperature-dependent and, as demonstrated by incubating Pr-bound phytochrome *in vitro* before centrifuging (see "Results"), occurs at an appreciable rate also only *in situ* (Fig. 6). The release of phytochrome from the bound condition has also been found to be relatively slow in maize both by Quail *et al.* (29) and Yu (37) who reported half-lives of 50 min at 25 C and 45 min at 30 C, respectively. The discontinuity in the temperature dependence of this process (Fig. 6, compare 3 C *versus* 13 C, and 13 C *versus* 25 C) might be interpreted to mean that a membrane is involved as has been done previously by Schäfer and Schmidt (34) for a discontinuity in another reaction involving phytochrome. However, since it is well established that soluble proteins may also exhibit discontinuities in Arrhenius plots (36), such an observation is neither evidence for nor against membrane involvement.

The data reported here are in direct disagreement with the previous report that binding is cooperative with respect to Pfr using maize, *Cucurbita*, and oats (32). We find, by contrast, what appears to be a simple titration of binding sites, at least up to 30 or 40% of the phytochrome as Pfr (Fig. 7). In addition, Boisard (personal communication) and Quail (23) also report an absence of cooperativity using *Cucurbita*. Thus, it appears that cooperativity may not be a fundamental property of the binding phenomenon.

Although we have not observed cooperativity in binding, we have seen that at low Pfr levels there is an apparent, enhanced binding of Pr (Fig. 8). It is possible that once Pfr has bound, it induces a binding of Pr, but many other interpretations are possible. In any event, the observation remains that at low Pfr levels, for each Pfr molecule produced by red light, one additional molecule of phytochrome (although some are Pr) is found in the pellet (Fig. 7). Because binding follows the irradiation period (Fig. 5), the trivial explanation that bound Pr was photochemically derived from previously bound Pfr may be discarded.

The possibility that some of the results presented above might reflect changes in absolute phytochrome levels occurring in either the supernatant or pellet fractions rather than true changes in per cent pelletability was excluded by examining the data obtained with oat extracts. Except in the case of a sequential red-far red light treatment (Fig. 6), no significant changes in phytochrome levels were observed as a result of varying treatments or extraction conditions (*e.g.*, pH or cation concentration). Thus, data were presented as percentages to facilitate direct comparisons of replicate experiments performed on different dates. (The approximately 30% loss of phytochrome following sequential red-far red irradiation will be reported elsewhere.)

A comparison to earlier reports of our description of *in vivo* binding is complicated by several factors. Since Furuya and Manabe (5) use different conditions in their experiments, it is not yet possible to correlate our observations with theirs. Since almost all other binding studies have utilized *Cucurbita* and maize, both of which exhibit *in vitro* binding under some conditions (16, 24) in addition to *in vivo* binding (Fig. 2; Ref. 15), one must exercise caution when comparing the present data with

earlier data. Three distinct possibilities arise. First, if irradiations are given only *in vitro*, *in vivo* binding is eliminated (9, 16), but one is then left with the probability of studying an artifactual, electrostatic interaction with 31S ribonucleoprotein particles (26, 27) as discussed above and elsewhere (25). Second, if, following red irradiation *in vivo*, extractions are at high pH, high ionic strength, or after a subsequent far red irradiation, one can be reasonably confident of studying only the *in vivo* system as was done earlier in a few experiments (11, 29, 37). In this case, however, it would be simpler to use an alternate plant tissue, as here, which does not exhibit *in vitro* binding under normal conditions. Third, if one uses a red irradiation *in vivo*, and then extracts without taking special steps to eliminate *in vitro* binding, one is left with the probability that the observed results are the sum of both systems. In this last case, any interpretation arising from the data becomes questionable because one can no longer be confident of what is being observed. Unfortunately, a large number of studies utilize this third approach (1, 17, 26-28, 32) and it may be necessary to repeat many, if not all, of these experiments to separate the contributions of the two phenomena. The third possibility is further complicated by the observation that some investigators have not observed *in vitro* binding in maize (15) while others have (24, 32).

Yet another series of problems in interpreting earlier experiments arises from methodological considerations. First, the use of low speed (500-1000*g*) precentrifugations of crude extracts in the presence of Mg^{2+} (26, 32), with this first pellet being discarded, is a common step in most binding protocols utilizing *in vivo* irradiations. Since a 500*g*, 5-min pellet from crude oat (6) and maize (Pratt, unpublished) extracts contains almost 25% of the red light-induced bound phytochrome whereas a 500*g*, 10-min pellet from oat extracts contains 45% (Pratt, unpublished), a large proportion of the bound phytochrome may have been discarded in these earlier experiments thus leading to possibly erroneous conclusions. Second, the common use of long actinic irradiation times (23, 28) means that binding can occur during the irradiation itself (Fig. 5). Thus, *e.g.* immediate reversibility by far red light should not be expected (33). In addition, complex, and potentially spurious, relationships may arise among light dose, steady state Pfr levels and binding (28). Third, the common use of time-consuming hand extraction methods (28, 32) prevents close control of the time of extraction. We have so far found that machine extraction yields results indistinguishable from those obtained by hand (15; Pratt, unpublished). Thus, problems in interpretation arising from variable or delayed extraction times may be readily eliminated when important (Figs. 5-8).

At high Pfr levels, not all Pfr becomes bound *in vivo* (Fig. 7). Among several possible explanations are: (*a*) cells contain more Pfr than binding sites; (*b*) the affinity of binding sites for Pfr is low; (*c*) more than one type of Pfr exists; and (*d*) binding occurs preferentially in only one region of the plant. It is not yet possible to test the first three hypotheses, but evidence regarding the fourth indicates that at least at the organ level, it is false (Table III).

It has already been reported (12) that Pfr reversibly associates with discrete subcellular regions within oat coleoptile cells using an entirely different, immunocytochemical approach. Mackenzie (22) has more recently observed by this technique that this association occurs within 1 to 2 min at 3 C, comparing well with the 40-sec half-life for binding measured at 0.5 C by the pelletability assay (Fig. 5). In addition, the immunocytochemically observed redistribution of phytochrome following a red-far red light cycle requires 1 to 2 hr at 25 C, again comparing well with the half-life of 25 min observed here at the same temperature (Fig. 6). Thus, it is possible that we are observing the same phenomenon by two entirely different approaches.

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