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In Vivo Properties of Membrane-bound Phytochrome[1,2]

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ABSTRACT

After a 3-minute irradiation with red light, which saturates the phototransformation from the red light-absorbing form of phytochrome to the far red light absorbing form of phytochrome, about 40% of the phytochrome extractable from hooks of etiolated squash seedlings (*Cucurbita pepo* L. cv. Black Beauty) can be pelleted as Pfr at 17,000*g* after 30 minutes. Dark controls yield only 2 to 4% pelletable phytochrome in the form Pr. If a dark period intervenes between red irradiation and extraction, the bound Pfr gradually loses its photoreversibility. The time course for this destruction parallels the time course for phytochrome destruction *in vivo* following saturating red irradiation. The soluble fraction of phytochrome remains constant. These results suggest that in squash seedlings phytochrome destruction is related exclusively to the fraction which becomes membrane-bound. The induction of phytochrome binding by red light is not completely reversible by far red. In plants given saturating red followed immediately by saturating far red light, 12% of the phytochrome is found in the bound fraction as Pr if the phytochrome extraction is immediate. If a dark period intervenes between red-far red treatment and extraction, the bound phytochrome is released within 2 hours. A model of the binding properties of phytochrome, based on molecular interaction at the membrane is proposed, and possible consequences for the mechanism of action of phytochrome are discussed.

In the preceding paper, Marmé *et al.* (14) presented evidence for the binding of Pfr *in vivo* to a membrane fraction extractable from etiolated squash seedlings following brief irradiation with red light. The membrane fraction could be isolated and purified under suitable conditions to yield a preparation free of mitochondria and other membranous structures. Studies demonstrating and partially characterizing *in vitro* binding of phytochrome to this membrane fraction have appeared elsewhere (13).

Boisard *et al.* (3) recently demonstrated by *in vivo* spectrophotometry that the kinetics for Pfr disappearance in etiolated squash seedling hooks are clearly not those of a single first order reaction. Rather there is an initial rapid phase followed by a slow phase. This kinetic behavior led the authors to propose two separate populations of phytochrome molecules, a suggestion made previously from other kinds of experimental evidence (10). In the present paper, the relative role of bound and soluble phytochrome with respect to phytochrome destruction *in vivo* is investigated. The reversibility of Pfr binding by subsequent far red irradiation prior to extraction is also examined. A model for the binding properties of phytochrome based on molecular interactions at the membrane level, as suggested by Changeux and co-workers (5–7) is proposed.

MATERIALS AND METHODS

Plant Material. Seeds of zucchini squash (*Cucurbita pepo* L. cv. Black Beauty) were obtained from Fontaine (Bourg-Archard-27-France). They were germinated and grown in darkness for 4 days as described previously (14).

Preparation of Samples. For each point shown under "Results," 4 g of squash seedling hooks were extracted with 16 ml of extraction medium. The details of sample preparation are described in the previous paper (14). The extraction protocol and the composition of the extraction medium are shown in Figure 1. The initial pH of the medium was 7.35, yielding a pH of 7.0 in the homogenate.

Phytochrome Assay. A method employing $CaCO_3$ and difference spectrophotometry was used to measure phytochrome content in the 17,000*g* pellet (17 KP) and the 17,000*g* supernatant (17 KS). Appropriate corrections for volume were made to determine total phytochrome in each fraction. This procedure is also described in detail in the preceding paper (14). The percentage of bound phytochrome was calculated in relation to the sum of the phytochrome in the pellet plus that in the supernatant. Each experiment was repeated at least two times. Separate experiments yielded the same result. The maximal deviation for identical samples was never larger than 10% of the mean value (calculated from four independent measurements).

RESULTS

Pfr Destruction after 3 Min Red Light. As was shown previously (14), etiolated squash seedling preirradiated with 3 min red light yield 40% of their subsequently extractable Pfr in pelletable form, as compared to 4% Pr for dark controls (homogenate pH 7.0, 10 mM Mg). These results are obtained

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either when the extraction is started immediately after irradiation or when the plants are stored on ice before extraction. To allow Pfr destruction to proceed in the present experiments, the intact plants were kept at 25 C in the dark for various periods of time following 3 min red treatment, before extraction. The extraction procedure then followed the protocol shown in Figure 1. The amount of bound Pfr was measured in the 17 KP and the amount of soluble in the 17 KS. The time course for both is shown in Figure 2, as is the over-all decrease in total phytochrome. The bound Pfr decreased rapidly in darkness while soluble phytochrome remained unchanged. After 60 min, bound Pfr was barely detectable. The time course for disappearance of total phytochrome parallels closely the disappearance of the bound fraction (Fig. 2).

Pfr Destruction during Continuous Red Irradiation. The time course for over-all loss of photoreversibility during continuous red irradiation and the distribution of this loss between

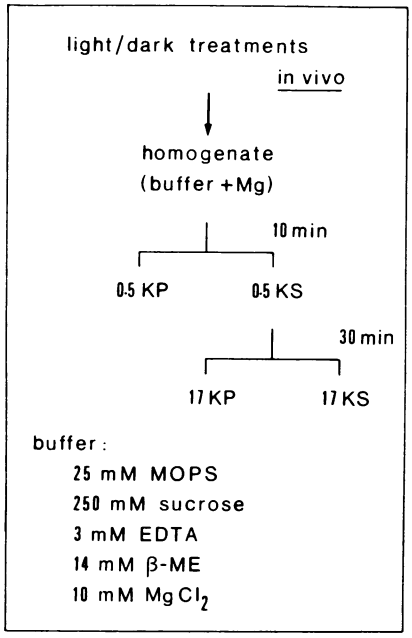

FIG. 1. Extraction procedure for membrane fraction containing phytochrome, and composition of extraction medium. Abbreviations: 0.5 KP: 500*g* pellet; 0.5 KS: 500*g* supernatatant; 17 KP: 17,000*g* pellet; 17 KS: 17,000*g* supernatant.

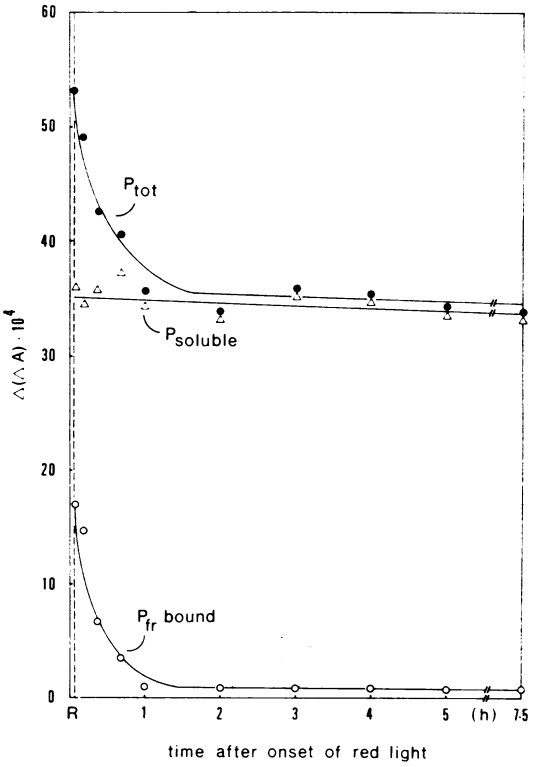

FIG. 2. Kinetics for phytochrome destruction in bound and soluble fractions, following incubation of intact seedlings in the dark after irradiation for 3 min with red light. ○: bound Pfr; △: soluble phytochrome; ●: total phytochrome.

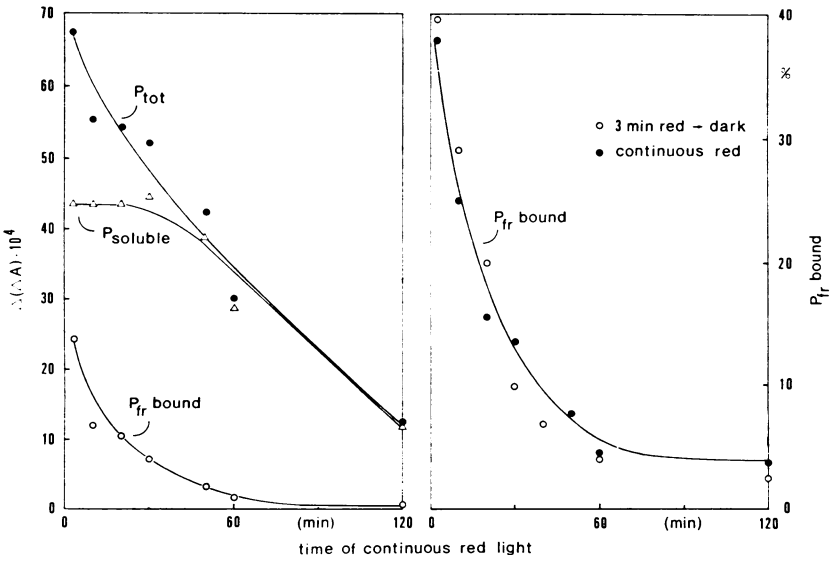

FIG. 3. (left) Kinetics for phytochrome destruction in bound and soluble fractions extracted from seedlings held for various times under continuous red light. ○: bound Pfr; △: soluble phytochrome; ●: total phytochrome. (right) Comparison of Pfr bound at various times after 3 min red irradiation with that bound at various times during continuous red irradiation. The percentages are of the total phytochrome extractable at time t.

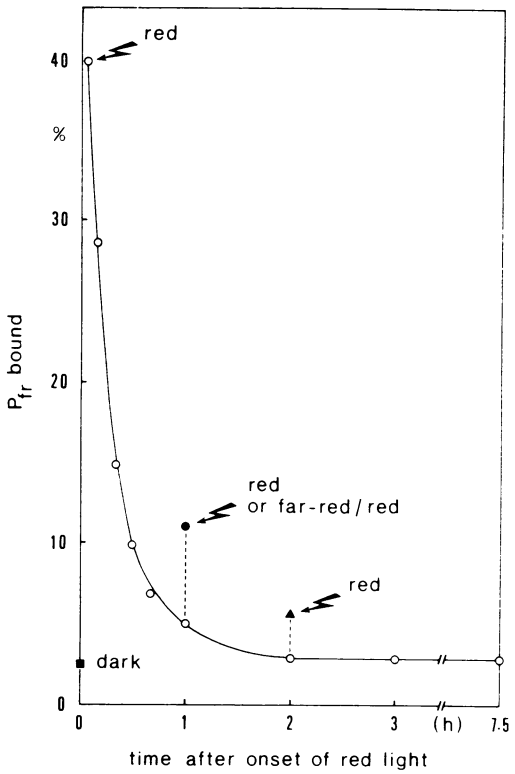

FIG. 4. Relative binding of phytochrome following several red irradiations. ■: dark control (Pf bound); ○: 3 min red plus dark; ●: 3 min red, 1 hr dark, 3 min red (or 3 min far red plus 3 min red for second light exposure); ▲: same as ●, plus 1 hr dark plus 3 min red. Twenty-four hr after the first red light, a second irradiation with 3 min red yields 35% Pfr bound of the total phytochrome present at this time (data not shown).

bound and soluble phytochrome is shown in Figure 3 (left). As in darkness following saturating red irradiation (Fig. 2), bound Pfr decreases to a very small amount during 60 min at 25 C. The soluble fraction remains constant for the first 30 min as before, but then decerases rapidly with a half life of about 30 min. Figure 3 (right) shows the disappearance of bound Pfr either in darkness following saturating red irradiation or under continuous red light. There is no detectable difference.

Pfr Binding after Repeated Red Irradiation. Figure 4 (open circles) again shows the kinetics for Pfr destruction following a saturating 3 min irradiation for the bound fraction of phytochrome. When the seedlings receive an additional 3 min of red light after 1 hr of darkness, the relative bound phytochrome increases from 4 to 12% (closed circle). Far red treatment just before this second red yields the same result. A third red irradiation 2 hr after the first yields an even smaller increase in bound Pfr (solid triangle). By contrast, if a second red treatment is given 24 hr after the first, up to 35% binding is obtained. At this time, total phytochrome is about one half the initial dark level, and has been partially newly synthesized (2).

Partial Reversal of Red-induced Binding by Far Red Light. A 3-min saturating far red exposure immediately following an initial 3 min of red light reduces the amount of bound phytochrome from 40%, as Pfr to roughly 12%, now bound as Pr (Fig. 5). However, the reversal of the red effect is not complete. During a subsequent dark period at 25 C, the remaining bound Pr decreases gradually, reaching the level of the dark control after 2 hr. There is no net loss of phytochrome. Clearly some Pr remains bound to the membrane fraction, and is released slowly *in vivo*. Note that 3 min of far red light alone produces only a slight increase in bound phytochrome (Fig. 5, open triangle). After the relaxation process is complete, a second red treatment (2 hr after the initial red-far red) yields almost normal binding, 30% in the form Pfr (Fig. 5, closed square).

DISCUSSION

The initial rapid phytochrome destruction observed by Boisard *et al.* (3) in the hooks of etiolated squash seedlings following red treatment can be related directly to the disappearance of the fraction of Pfr which is membrane bound. Total phytochrome declines within 1 hr to a stable 60% of the initial amount, and the membrane-bound phytochrome decreases from 40% (of total phytochrome at t = 0) to about 4% (of total phytochrome at t = 1 hr). The level of soluble phytochrome meanwhile remains unchanged during this period (though much of it is Pfr). After 1 hr of darkness the destruction process seems to be completed. One cannot obtain, at this time, more than a little binding by a second red irradiation, suggesting that most of the binding sites are either blocked or altered. After 24 hr of darkness a substantial increase of binding up to 35% can be observed.

The red light-induced binding of phytochrome cannot be fully reversed by far red light (Fig. 5). About 12% of the phytochrome (now as Pr) remains bound, and is only slowly released from the membrane during 2 hr in darkness. After this time, almost normal binding can be achieved by 3 min of red light (Fig. 5).

These observations might be explained on the basis of a series of interactions between macromolecules organized into a multimolecular complex. Such concepts have been developed in detail by Changeux and his co-workers (5, 6, 7). They compared chemically excitable membranes with regulatory enzymes. The biological activity of such membranes is

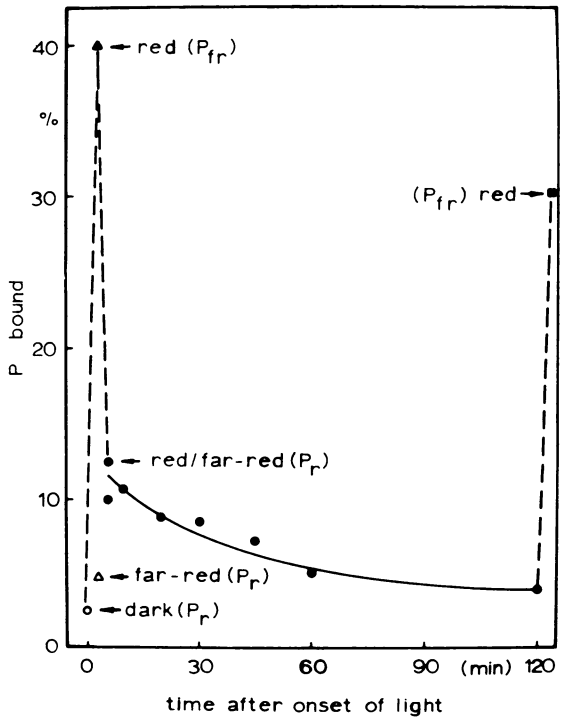

FIG. 5. Reversibility of red light-induced phytochrome binding by far red light *in vivo*. ○: dark control, Pr bound; ▲: 3 min red, Pfr bound; ●: 3 min red plus 3 min far red plus darkness, Pr bound; △: 3 min far red alone, Pr bound; ■: 3 min red plus 3 min far red plus 2 hr dark plus 3 min red, Pfr bound.

controlled by specific regulatory ligands. These ligands act not by formation of covalent bonds, but rather by reversible interaction with receptors on the surfaces of the membranes (6).

In the present case, the ligand is phytochrome (Pfr), and the receptor could be an organized region of membrane surface. Changeux and co-workers (6) propose that the affinity and stability of the association between ligand and membrane surface resemble the affinity and stability of subunit aggregation in a polymeric protein. In order to attempt interpretation of the binding properties of phytochrome to membranes in squash seedlings, the following assumptions have been made (6). (*a*) More than one conformational state is reversibly accessible to the receptor; (*b*) the receptor possesses a site for stereospecific recognition of the ligand; (*c*) the affinity of the receptor site for the ligand is altered when a transition occurs from one conformational state to another.

These assumptions are implicit in the model shown in Figure 6. In darkness, the membranes possess receptors principally in the conformational state "triangle." In this state, the membrane cannot bind Pr. The experiments described above show that binding of Pr in the absence of any light treatment is negligible (Fig. 4). Red light causes transformation of Pr to Pfr and induces a small but significant conformational change in the phytochrome protein moiety (1, 11, 12, 19) detectable by several different techniques. The ligand, Pfr, exhibits a high affinity for the receptor sites, and binding is increased to 40% (Fig. 4). Such binding could cause a transition of the conformational state of the receptor from "triangle" to "half circle."

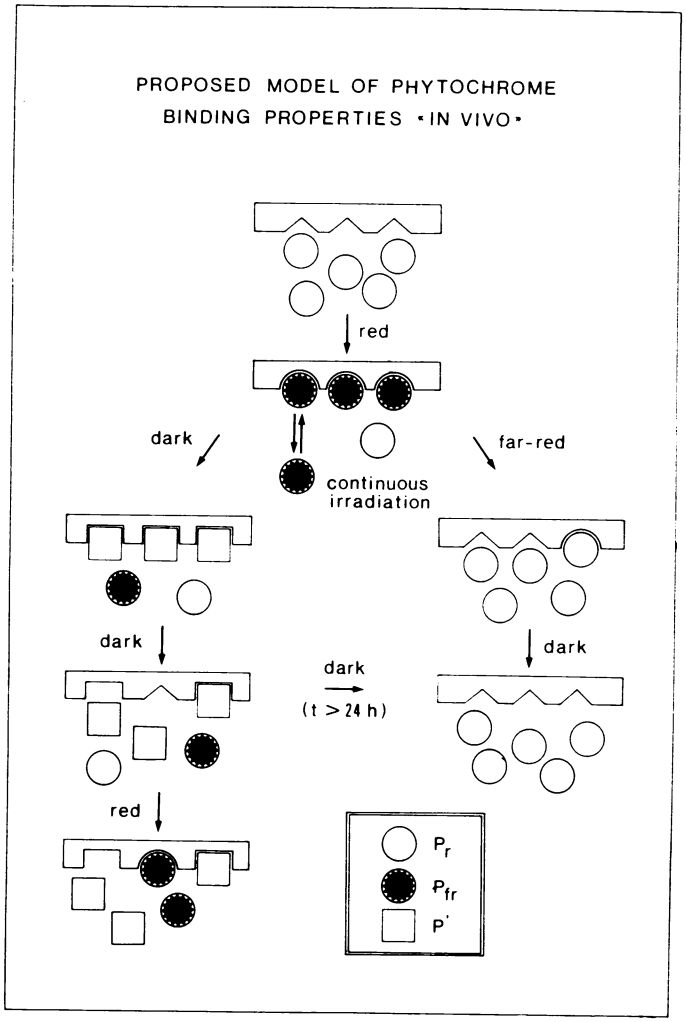

FIG. 6. Diagrammatic representation of a model for phytochrome binding to membranes *in vivo*. The three conformational states proposed for the phytochrome receptors are represented as "triangles," "half circles," and "squares." P′ is no longer photoreversible.

Subsequent irradiation with saturating far red light transforms virtually all of the bound phytochrome back to Pr, but a significant amount of Pr (25% of that bound, 12% of the total) remains on the membranes (Fig. 5) and is released slowly. After about 2 hr of darkness (Fig. 5) the system is again dark-adapted, and the triangle conformation recovered. Almost normal binding capacity is restored (Fig. 5).

To account for the loss of the photoreversible Pfr which occurs at the membrane level (Fig. 2), one might formally assume a third conformational change of the receptor site from half circle to square. This change could be related to the loss of photoreversibility of phytochrome. The relaxation of P′ (pigment without photoreversibility) from the membrane must be very slow. After rapid loss of photoreversibility, the sites do not become available to the remaining soluble Pfr, and a second pulse of red light 1 hr after the first causes only a slight increase in binding (Fig. 4). The state transition from square to triangle must be the limiting step. After 24 hr, the binding capacity is essentially recovered.

Molecular interactions at the membrane level suggest the possibility of functional changes in these membranes which could lead to observed photomorphogenic responses. Assuming allosteric mechanisms, models for cooperativity (6, 9) and for ion transport (8) have been proposed. On the basis of such models, phytochrome mediated effects such as ion movements in *Albizzia* leaflets (18), rapid electrical potential changes in oat coleoptiles (15), and suppression of lipoxygenase activity (16, 17) might be explained.

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LITERATURE CITED

1. ANDERSON, G. R., E. L. JENNER, AND F. E. MUMFORD. 1970. Temperature and pH studies on phytochrome *in vitro*. Biochemistry 8: 1182-1187.
2. BOISARD, J., B. MARCHAL, AND P. ROLLIN. 1974. Relation entre destruction et synthèse du phytochrome dans l'hypocotyle de courge. Physiologie Végétale. In press.
3. BOISARD, J., D. MARMÉ, AND E. SCHÄFER. 1971. The demonstration *in vivo* of more than one form of Pfr. Planta 99: 302-310.
4. CHANGEUX, J. P. 1966. Responses of acetylcholinesterase from *Torpedo marmorata* to salts and curarizing drugs. J. Mol. Pharmacol. 2: 369.
5. CHANGEUX, J. P., T. PODLESKI, AND L. WOFSY. 1967. Affinity labeling of the acetylcholine-receptor. Proc. Nat. Acad. Sci. U.S.A. 58: 2063-2070.
6. CHANGEUX, J. P. AND J. THIÉRY. 1968. On the excitability and cooperativity of biological membranes. *In:* J. Jarnefelt, ed., Regulatory Functions of Biological Membranes. BBA Library, Vol. 11: Elsevier, Amsterdam. pp. 116-138.
7. CHANGEUX, J. P., J. THIÉRY, Y. TUNG, AND C. KITTEL. 1967. On the cooperativity of biological membranes. Proc. Nat. Acad. Sci. U.S.A. 57: 335-341.
8. HILL, T. L. 1969. A proposed common allosteric mechanism for active transport, muscle contraction, and ribosomal translocation. Proc. Nat. Acad. Sci. U.S.A. 64: 267-274.
9. HILL, T. L., AND Y. CHEN. 1970. Cooperative effects in models of steady-state transport across membranes. II. Oscillating phase transitions. Proc. Nat. Acad. Sci U.S.A. 66: 189-196.
10. HILLMAN, W. S. 1967. The physiology of phytochrome. Annu. Rev. Plant Physiol. 18: 301-324.
11. HOPKINS, D. W. AND W. L. BUTLER. 1970. Immunochemical and spectroscopic evidence for protein conformational changes in phytochrome transformation. Plant Physiol. 45: 567-570.
12. KROES, H. H. 1968. Reversible changes in the circular dichroism of phytochrome during photoisomerization of the pigment. Biochem. Biophys. Res. Commun. 31: 877-883.
13. MARMÉ, D., J. BOISARD, AND W. R. BRIGGS. 1973. *In vitro* binding properties of phytochrome to a membrane fraction. Proc. Nat. Acad. Sci. U.S.A. 70: 3861-3865.

14. MARMÉ, D., J. M. MACKENZIE, JR., J. BOISARD, AND W. R. BRIGGS. 1974. The isolation and partial characterization of membrane vesicles containing phytochrome. Plant Physiol. 54: 000-000.
15. NEWMAN, I. A. AND W. R. BRIGGS. 1972. Phytochrome-mediated electric potential changes in oat seedlings. Plant Physiol. 50: 687-693.
16. OELZE-KAROW, H. AND H. MOHR. 1973. Quantitative correlation between spectrophotometric phytochrome assay and physiological response. Photochem. Photobiol. 18: 319-330.
17. OELZE-KAROW, H., P. SCHOPFER, AND H. MOHR. 1970. Phytochrome-mediated repression of enzyme synthesis (lipoxygenase): a threshold phenomenon. Proc. Nat. Acad. Sci. U.S.A. 65: 51-57.
18. SATTER, R. L., P. MARINOFF, AND A. W. GALSTON. 1970. Phytochrome controlled nyctinasty in *Albizzia julibrissin*. II. Potassium flux as a basis for leaflet movement. Amer. J. Bot. 57: 912-926.
19. TOBIN, E. M. AND W. R. BRIGGS. 1973. Studies on the protein conformation of phytochrome. Photochem. Photobiol. 18: 487.