Short Communication

Rapid Destruction of the P_{FR} Form of Phytochrome by a Substance in Extracts of Pisum Tissue[1]

Masaki Furuya[2] and William S. Hillman

Biology Department, Brookhaven National Laboratory, Upton, New York

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In summary, crude aqueous extracts of etiolated *Pisum* tissue contain a substance that almost instantaneously destroys phytochrome in the P_{FR} but not in the P_R form. This P_{FR}-killer is separable from the phytochrome by gel-filtration, is soluble in *n*-butanol but not in petroleum ether, and is itself probably inactivated by reaction with P_{FR}. *Avena* extracts yield no P_{FR}-killer. The physiological significance of these results is doubtful, but they illuminate several technical points concerning in vitro phytochrome assays. In particular, they cast doubt on studies of comparative phytochrome content by extraction unless the P_R state is maintained from homogenization through purification and assay.

In the tissue of seedlings grown in darkness, conversion of the chromoprotein phytochrome from the red-absorbing (P_R) form to the far-red-absorbing (P_{FR}) form by light is followed by a gradual destruction of P_{FR}. This gradual destruction, which has many of the characteristics of an enzymic process, has not been observed in cell-free systems (2, 4, 5). However, a rapid loss of P_{FR} in cell-free systems can be brought about in several ways. For example, addition of *p*-chloromercuribenzoate (pCMB) to a P_{FR} solution causes almost instantaneous loss of reversibility (and thus in effect of detectability) while P_R is stable in the same conditions until converted to P_{FR} by light (1). The experiments here demonstrate a similar rapid destruction by a substance or substances occurring in *Pisum* extracts together with phytochrome itself. This activity will be called P_{FR}-killer activity to distinguish it from the gradual destruction in intact tissue. Though the physiological relevance of such information is admittedly dubious, it may eventually bear on the in vivo transformations and is certainly of immediate technical significance.

The starting material for this work was derived either from 7-day-old seedlings of *Pisum sativum*, cultivar Alaska, or 5-day-old seedlings of *Avena sativa*, cultivar Clintland, grown in total darkness at 25 to 27°. At harvest the terminal 3 cm of the *Pisum* epicotyl, including plumule tissue, or the terminal 2 cm of the *Avena* tissue, including coleoptile, primary leaf, and some mesocotyl, was cut in the darkroom, wrapped in aluminum foil and kept at 2 to 4° in darkness for at least an hour. All steps up to and including gel-filtration were then conducted in the cold at 2 to 4° and under dim green safelights (2).

One hundred g portions of tissue were homogenized in a Waring Blendor with 50 ml of a medium consisting of 0.05 M Tris, 0.1 M 2-mercaptoethanol and 2 mM EDTA. After filtration through 2 thicknesses of cheesecloth, the homogenate was centrifuged for 15 minutes at 13,000 × *g* and the supernatant then filtered through a half-inch of diatomaceous silica (Celite filter aid, Johns Manville Company) on a Büchner funnel with the aid of a vacuum. The Celite filtrate was loaded on a column 5 cm in diameter and 30 cm in depth of Sephadex G-50 medium grade (Pharmacia, Uppsala, Sweden) already equilibrated with 0.05 M Tris-citrate buffer containing 0.05 M 2-mercaptoethanol and 2 mM EDTA, and the column was then eluted with the same buffer. Phytochrome was assayed as previously described (2) with a dual wavelength difference spectrophotometer (Ratiospect Model R2, Agricultural Specialty Company, Beltsville, Maryland). For each measurement, 1 ml of extract was pipetted into a metal cuvette with an inside radius of 3 mm, giving a pathlength of roughly 3 cm. The cuvettes were kept in a dark box at or close to 0° with crushed ice until and during the measurements. All data presented are means of at least duplicate samples, which in most cases did not differ.

In a preliminary screening experiment, crude (Celite filtration only) and partially purified (Sephadex G-50) extracts of both *Pisum* and *Avena* were held in either darkness or continuous white light (about 100 ft-c) at either ice-bath or room temperature (24°). At the end of 2 hours the phytochrome contents were compared with those at

[1] Research carried out at Brookhaven National Laboratory under the auspices of the United States Atomic Energy Commission.

[2] Present address: Biological Institute, Faculty of Science, Nagoya University, Nagoya, Japan.

the start. In the dark the crude *Pisum* extract lost no phytochrome at 0° and about 15 % at 24, but in the light it had lost about 30 and 75 %, respectively. The *Pisum* G-50 filtrate, on the other hand, lost essentially no activity. No significant loss was observed in the *Avena* extract under any of these conditions. These results suggested the presence in crude extracts of *Pisum,* but not of *Avena,* of a substance possessing P_{FR}-killer activity and separable from the phytochrome by gel-filtration on Sephadex.

Next, experiments were undertaken towards a separation and partial purification of the P_{RF}-killer in pea extracts. During the course of gel-filtration of 100 ml portions of Celite filtrate on Sephadex G-50, all phytochrome was recovered in tubes 20 to 35 (each tube collected 7 ml) and the killer activity presumably retained on the column. Elution was continued with double distilled water, and fractions 81 to 230 so obtained were combined and concentrated to 100 ml at 40° under reduced pressure. This aqueous concentrate, pH 7.8, was then extracted by shaking successively with portions of petroleum ether, ethylacetate and *n*-butanol. Each of these organic extracts and the aqueous residue of the original concentrate were evaporated to dryness under reduced pressure and each residue taken up into 5 ml of water. P_{FR}-killer activity was then assayed by mixing 0.5 ml of the material to be tested with 2.5 ml of a Sephadex G-50 phytochrome preparation and exposing the mixture to red light for 5 minutes and then incubating in darkness at 20° for 1 hour before the phytochrome assay. Typical results are presented in table I, which indicates that P_{FR}-killer activity is apparently highly soluble in *n*-butanol, much less so in ethylacetate, and essentially insoluble in petroleum ether. Blank experiments following the same techniques but omitting the initial aqueous extract showed that the activity was not present as a contaminant in the solvents used.

The procedure described was repeated quantitatively in order to obtain an estimate of the amount of tissue required for a given P_{FR}-killer activity in the assay system. The results are shown in figure 1 as a relation between P_{FR}-killer activity

Table I. *Recovery of P_{FR}-killer Activity from Aqueous Concentrates by Extraction with Various Solvents*

0.5 ml added*	$10^3 \times \Delta(\Delta OD)$/ml**
Water control	38
Petroleum ether fraction	35
Ethyl acetate fraction	28
n-Butanol fraction	10
Aqueous residue	38

* To 2.5 ml Sephadex G-50 filtrate, high phytochrome fraction.

** After 5 minutes red light, 1 hour dark at 20°. Initial (time 0) value for H_2O control 39.

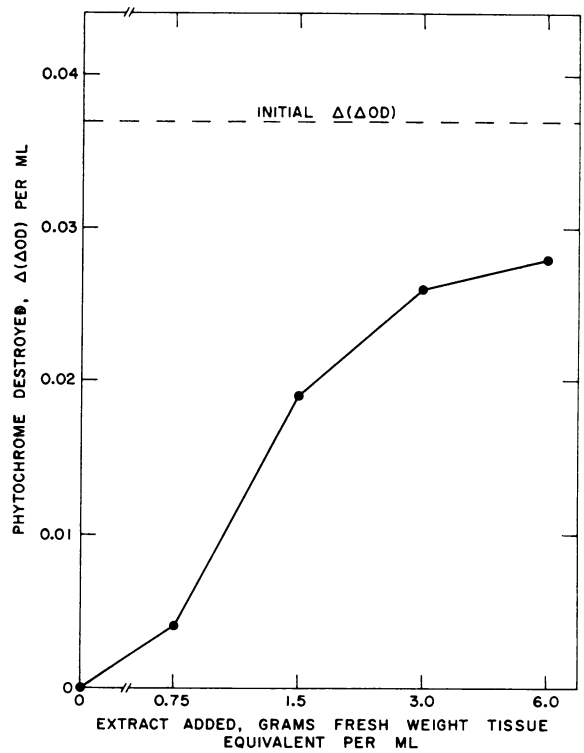

FIG. 1. Relationship between P_{FR}-killer activity and concentration of *n*-butanol-extractable material from etiolated *Pisum* tissue. Experimental conditions as in table I.

and the concentration of material extractable with *n*-butanol represented as fresh weight equivalents of *Pisum* tissue. It is clear that the effect increases with the concentration, though the data are insufficient to establish accurately the type of relationship involved.

In the preceding assays, the phytochrome readings were taken only some time after exposure to light for the convenience of establishing a standard procedure. It is clear, however, that the P_{FR}-killer effect observed here, like that of pCMB previously reported, is extremely rapid. It almost certainly accounts for the anomalous readings, often obtained on crude plant extracts, in which the apparent $\Delta(\Delta OD)$ drops with succeeding reversals and in which the drop is particularly noticeable after the pigment has been held in the P_{FR} form, even during a brief 1 or 2 minute illumination. Such anomalous readings, at least, can easily be reproduced by combining Sephadex-G-50 filtrates in which readings are essentially steady with various amounts of P_{FR}-killer extracts prepared as described previously. The situation is summarized in table II, which shows both that the destruction of phytochrome is rapid and that it is apparently P_{FR} that is destroyed. Thus, in the first section of the table, the $\Delta(\Delta OD)$ in the presence of killer is much lower when calculated using the second far-red value, which now reflects the destruction brought about by

Table II. *Effect of P_{FR}-killer on Successive Readings in 2-Wavelength Difference Spectrophotometer*

Reaction mixture*	$10^3 \times \Delta$ OD reading**				$10^3 \times \Delta(\Delta OD)$	
	Initial	After first F(F_1)	After R	After second F(F_2)	F_1-R	F_2-R
P_R only	60	60	28	57	32	29
P_R + ¼ unit killer	53	53	22	40	31	18
P_R + 1 unit killer	80	78	43	60	30	12
P_{FR} only	43	72	41	69	31	28
P_{FR} + ¼ unit killer	32	48	31	43	17	17
P_{FR} + 1 unit killer	48	53	43	51	5	3

* Sephadex G-50 filtrate, high phytochrome fraction, plus addenda. P_R means material held in darkness before mixing, P_{FR}, material saturated with red light before mixing. Killer units arbitrary, but all mixtures with constant initial phytochrome content. Measurements and illuminations at 0°; initial readings made within 1 minute of mixing.

** Δ OD = OD 660 − OD 730 + instrument response. The last term is subtracted out on calculating Δ (ΔOD). Red (R) and far-red (F) illuminations 1 minute each.

the first red illumination. In the second portion of the table, where the pigment is initially in the P_{FR} form before the addition of the killer solution, the first and second Δ (ΔOD) calculations show no such difference since the major part of the destruction has already taken place. These and similar experiments suggest that the P_{FR}-killer is itself inactivated during its action and does not continue indefinitely to destroy P_{FR}.

The technical significance of these observations for future studies is in providing at least a partial explanation for the greater stability of phytochrome in semipurified as compared to crude extracts. They also suggest the desirability of maintaining the phytochrome to be extracted in the P_R state until considerable purification is accomplished. Without this precaution, apparent differences in the phytochrome content of various plants as estimated by extraction (e.g. 3) may be due to differing P_{FR}-killer content.

In more physiological terms, the present results are difficult to evaluate. That there is probably no meaningful relationship between the P_{FR}-killer described here and the gradual P_{FR} destruction in intact tissue is suggested by at least 3 facts. First, the action of P_{FR}-killer is almost instantaneous. Second, it takes place in the presence of levels of mercaptoethanol and EDTA which are known to inhibit the in vivo destruction (*2*). Finally, while P_{FR}-killer activity occurs in *Pisum*, where destruction is probably the fate of only a portion of the P_{FR}, it is not evident in *Avena*, where destruction is the only process detectable (*2*, 5). That *Avena* phytochrome is not simply resistant to P_{FR}-killer is shown by the observation that it responds to active extracts from *Pisum* approximately as the *Pisum* phytochrome does. The chemistry of the P_{FR}-killer studied here is unknown, except for the indication that it is a small, relatively polar molecule but it is evident from other work that many substances might exert killer activity, since activity is largely a reflection of the instability of P_{FR} (1, 5). Hence it would be rash at this time to postulate any physiological function with respect to phytochrome for naturally-occurring substances of this kind, particularly since it seems unlikely that they are in direct contact with the molecule itself until the cells are disrupted. If such substances do play any role in controlling phytochrome level, the specificity and physiologically significant aspects would reside not in the substances themselves, but in the nature and lability of the system which separates them from the phytochrome.

Acknowledgment

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