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ABSORPTION SPECTRUM CHANGES DURING DARK DECAY OF PHYTOCHROME - 730 IN PLANTS

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INTRODUCTION

In a previous communication (1) we have presented evidence to show that, whereas the red absorbing form of phytochrome (P_{660}) is stable in excised plant parts for relatively long periods, the far red absorbing form (P_{730}) is rapidly inactivated in the dark, possibly by an enzymatic reaction. Strictly, our observations had only shown that the reversible interconvertibility by light of the two forms of the pigment is rapidly lost if the phytochrome is in the far red absorbing form but not if it is in the red absorbing form. We can visualize two ways to account for such an inactivation. Firstly, P_{730} may be broken down to a substance showing no absorption at 730 mμ (bleaching). Secondly, it might become transformed into a compound that, although absorbing in the far red, is no longer capable of transformation into P_{660} by far red radiation. The second alternative deserves special consideration since it has been shown by several investigators that there is no obvious relation between phytochrome concentration in plants and the physiological effects of light (2). It would not be unlikely that the bulk of the phytochrome in etiolated plants is a photochemically active but physiologically inactive form, that has first to be transformed by light into the active compound. In the latter case, such a transformation might or might not be accompanied by measurable changes in the positions of the absorption bands. The example of the transformation of protochlorophyll into chlorophyll may suffice to illustrate the possiblity of such a reaction. In this connection, the problem of the so-called back reaction of P_{730} to P_{660} in the dark warrants discussion. Whereas at the conclusion of the dark decay of P_{730} a certain fraction of the reversible absorption changes, originally found in the material, can still be observed, we have pronounced the opinion that this cannot be taken as proof of a back reaction (1).

On the other hand, the Beltsville group repeatedly has stressed the importance of this reaction in plants. (e.g. 3). Recently, HILLMAN (4) investigated a large number of plants and concluded that although in some of them little or no formation of P_{680} during a dark period following red irradiation could be observed, there appeared to be considerable regeneration of absorption in the red in some of them.

In order to obtain additional information regarding the problems mentioned above, we have measured difference spectra for the dark reactions following red irradiation of plant parts as well as the changes in the amounts of both red and far red absorbing forms of phytochrome.

MATERIALS, METHODS

Pea plants (*Pisum sativum* var. Krombek) were grown in complete darkness at 20 °C and 90 % R.H. for 6-7 days. The upper parts (plumules) were collected in the dark and either used immediately or stored in the dark at 2 °C for periods up to five days. The plumules were cut to a size of 2-3 mm with a razor blade and pressed rather firmly into a sample box of 10 mm path length in complete darkness, before transferring to the spectrophotometer.

Relative phytochrome concentrations and the decay of P_{730} were measured in a dual wavelength spectrometer, as reported previously (1). All measurements refer to the changes in optical density at 730 mμ with reference to that at 800 mμ. Intensities of the actinic light incident upon the front of the sample box in this instrument were 1.8×10^4 erg/cm².sec. at 650 mμ and 0.6×10^4, erg/cm². sec. at 737 mμ. The photomultiplier was an E.M.I. type 9558 B operated at about 800 V, depending upon the opacity of the sample.

The pea material proved so rich in phytochrome that absorption and difference spectra could be measured in a Cary model 14 spectrophotometer equipped with a 0-0.2 density slidewire. To this end, the scattered transmission accessory normally supplied with the instrument was somewhat modified to provide for sample illumination with a 500 W slide projector *via* interference filters. Difference spectra were obtained by subtracting the spectra of two successive runs between which the necessary treatment was given to the sample. In this instrument, actinic intensities incident at front of the sample cell were 3.2×10^4 erg/cm².sec at 650 mμ and 1.2×10^4 erg/cm².sec at 737 mμ. Duration of the actinic irradiation usually was 60 sec. The standard photomultiplier supplied with the instrument was replaced by a far red sensitive R.C.A. type 7102.

RESULTS AND DISCUSSION

Fig. 1 shows some spectra measured during dark decay of P_{730}. In the present discussion we will disregard any absorption changes below 600 mμ. In the first place it is evident that the absorption due to P_{730} rapidly declines in the dark. Although there may be a small change in the form of the 730 mμ band during decay, there is no clear indication of the formation of a form of the pigment with a different spectrum. On the other hand, disappearance of absorption at 730 mμ is not accompanied by formation of an equivalent amount of absorption at

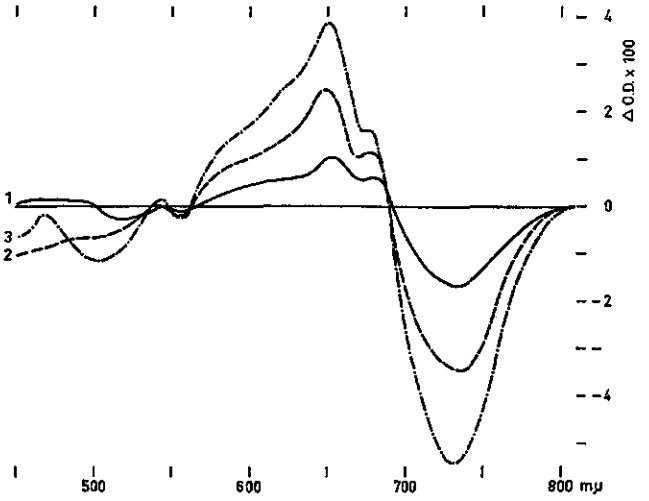

FIG. 1. Difference spectra during dark decay of phytochrome. Before measurement, both absorption cells received a saturating dose of 650 mμ. Immediately afterwards, the vessel in the reference beam was irradiated with 737 mμ to transform the phytochrome into the red absorbing form. Curve 1: spectrum change after 30 min. dark, curve 2 after 60 min and 3 after 120 min.

660 mμ. Although there is considerable increase in red absorption, the spectra demonstrate that this is to a large extent due to pigments with absorption maxima at other wavelengths, notably at 650 mμ. The difference spectra obtained upon renewed red irradiation of such samples reveal that a considerable part of the red absorption must be due to protochlorophyll formed during the dark period, fig. 2. This is not at all unexpected since red irradiation has been known

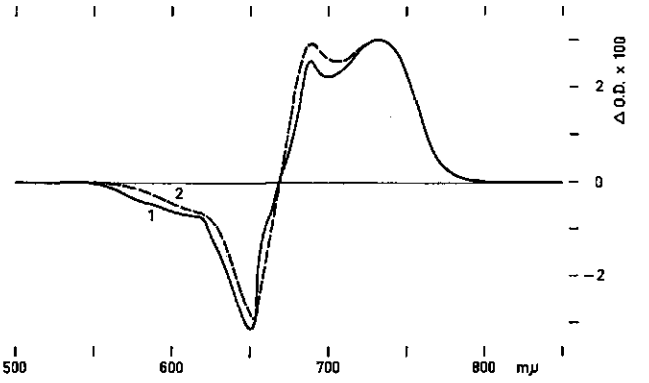

FIG. 2. Curve 1: difference spectrum upon red irradiation of a sample, 250 minutes after the first red dose. In the period between the two irradiations, the sample was kept in the dark at 25°C.
Curve 2: Sum of difference spectra for phytochrome transformation in pea plus protochlorophyl to chlorophyll-680 transformation in maize leaf. See text.

for a long time to stimulate resynthesis of protochorophyll. This is a reaction, also under the control of the red-far red system (5). We can now attempt to analyse the difference spectra of fig. 2 in terms of a combination of transformation of protochlorophyll to chlorophyll-680 plus that of P_{660} to P_{730}. Curve 2 in fig. 2 shows one of the possible linear combinations of the two difference spectra of the above mentioned transformations. The protochlorophyll-chlorophyll-680 difference spectrum used in this computation was measured with leaves of etiolated maize plants, that contain relatively little phytochrome. Although there is some degree of similarity between the 'synthetic' spectrum and the observed one, a perfect fit could not be obtained with any combination tried. In view of the accumulation of errors in such an analysis, a well as of the uncertainty involved in the use of a protochlorophyll-chlorophyll difference spectrum of a different plant, not much more can be said than that apart from phytochrome and protochlorophyll, other pigments may also be involved. Especially around 680 mμ, the absorption increase in darkness appears too high. If the pea material is stored at 2 °C for several days after harvesting, the amount of phytochrome only decreases slightly, but the capacity for protochlorophyll resynthesis after red irradiation is diminished considerably. Decay difference spectra for this material have a proportionally smaller contribution form protochlorophyll to the absorption increase in the red during darkness, fig. 3.

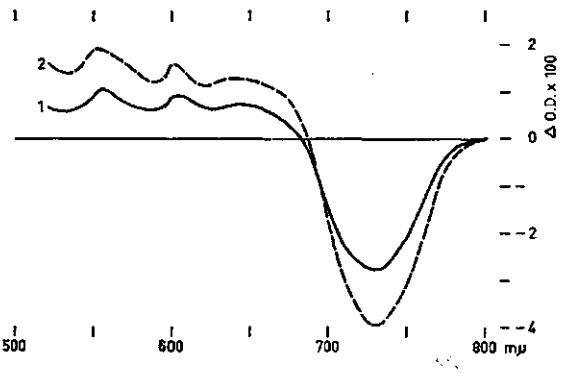

FIG. 3. Difference spectra for phytochrome decay in pea plumules, aged at 2 °C for five days. Curve 1: after 60 min. dark, curve 2: after 120 min. dark.

Also in this case, a satisfactory analysis of the spectra in terms of protochlorophyll-phytochrome is difficult. It is fairly evident that the absorption around 680 mμ again is too high. Difference spectra for red irradiation, both of fresh and of aged material, show that the absorption increase around 680 mμ, mentioned above, is not reversed by light.

It is clear that formation of pigments, other than P_{660}, during the dark decay of P_{730} seriously interferes with the estimation of newly formed P_{660}, if any. To overcome this difficulty, we have estimated 'newly formed' P_r by measuring the absorption increase at 730 mμ following a second red dose given at various times after the first. In this way, both total phytochrome and newly formed phy-

tochrome can be estimated in the same experiment. In a separate series of measurements, we have determined the dark decay of P_{730} directly from the optical density changes at 730 mμ. It was then possible to compare residual P_{730} and P_{730} formed from 'new' P_r with the total amount of spectroscopically detectable, reversible, pigment. Results are given in fig. 4. Decay of P_{730} starts immediately. The kinetics are not first order. At the same time, there is rapid formation of new phytochrome. From the two measurements, the total phytochrome at any moment can be computed. This is compared in curve 3 with phytochrome found by actual estimation. The agreement appears to be sufficient. Although the curve for total phytochrome for this material differs considerably from the one,

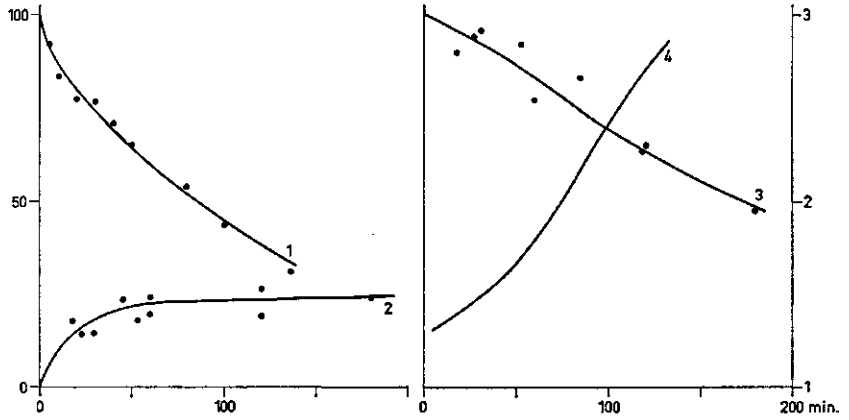

FIG. 4. Curve 1: decay of absorption at 730 mμ in the dark after red irradiation. Readings in per cent of values before treatment. Scale on the left. Curve 2: new phytochrome formed in the dark, measured as absorption increase at 730 mμ after red irradiation. Readings in per cent of phytochrome present before treatment. Curve 3: sum of newly formed plus residual phytochrome, computed from 1 and 2. The points represent experimental values for total phytochrome (spectroscopic). Curve 4: ratio of phytochrome disappeared to newly formed. Scale on the right.

found previously for maize mesocotyls, we believe that they are of fundamentally the same type, and that their unusual kinetics can be explained as resulting from a combination of rapid formation of new phytochrome after red irradiation plus steady decay of P_{730}. There are, however, two remarks to be made.

1. We have originally attempted to explain the observation that part of the total phytochrome appears resistant to dark decay, as due to the impossibility to convert P_{660} completely to P_{730} photochemically. This idea has now to be modified in such a way as to include formation of new P during the dark period following red irradiation. The latter reaction must be much more important in pea than in maize.
2. Where does the new phytochrome come from? If it is formed from P_{730} in a dark reaction, it appears reasonable to expect that there should be a more or less constant ratio between the amount of P_{730} disappearing and the amount

of P_r forming, irrespective of the progress of the reaction. Curve 4 of fig. 4 gives the ratio

$$\frac{P_{730} \text{ disappeared}}{P_{730} \text{ from new } P_r}$$

computed from curves 1 and 2. It is evident that this ratio is far from constant even if we allow for the relatively large errors inherent in this type of calculation. It appears, therefore that the two processes are unrelated. We would like to suggest that the new phytochrome formed results from resynthesis induced by red irradiation. As this resynthesis does not occur if the red illumination is followed immediately by far red, phytochrome resynthesis, like protochlorophyll resynthesis, is under the control of the red-far red mechanism. If this interpretation is correct, it obviously will have consequences for our understanding of the effects of repeated red and far red irradiations on the physiological responses of plants.

Summary

Difference spectra for the absorption changes following dark decay of phytochrome-730 in pea plumules show that the disappearance of photochemical activity of this pigment is due to a bleaching process and not to loss of reversibility of the photochemical reaction. No indications were found that the P_{730} originally formed by red irradiation, is further transformed into another far red absorbing compound in appreciable amounts. Accompanying this bleaching of P_{730}, there are absorption changes in the red, due to formation of protochlorophyll, a red absorbing form of phytochrome and possibly other pigments. The newly formed phytochrome was measured by observing the absorption increase at 730 mμ following a second red dose. A comparison of the amount of new phytochrome with the amount of P_{730} disappearing during the same period showed that there was no constant ratio between the two. It is, therefore, unlikely that new phytochrome was formed from P_{730} in a dark reaction. The results suggest that phytochrome resynthesis is under the control of the red-far red system, a finding of considerable significance for the interpretation of certain physiological plant reactions.

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