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PHOTOREACTIONS IN PHYTOCHROME-CONTAINING EXTRACTS FROM ETIOLATED PEA SEEDLINGS

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INTRODUCTION

In leaves of etiolated pea, phototransformation of P_{fr} by irradiation with far red light gives rise to an absorption band at about 650 nm (1). We have previously presented evidence tot show that this band is due to a special form of phytochrome (2). It appeared desirable to look for a confirmation of this conclusion by studying the photoreactions of this form of phytochrome in vitro with pigments, extracted from the leaves. There can be no interfering protochlorophyll resynthesis in such extracts, which reaction forms a complication in the interpretation of photoreactions of living material.

METHODS

Pea seedlings, var. 'Nunhems Krombek' were grown in the dark at 20°C for seven days. The plants were harvested and the leaves separated from the stems in complete darkness. During a period of two hours, about 1000 plants could be treated in this way, yielding 7-9 grams of leaves and about 500 g of stems. This amount of leaf material is just sufficient to obtain an extract that can be measured in the Cary 14 spectrophotometer.

The leaves were ground in a cooled mortar with some sand and a total volume of about 50 ml buffer. This buffer consisted of 0.2 M sodium pyrophosphate pH 8.1, containing 0.05 mol/l mercaptoethanol. The liquid was clarified by centrifugation at 4600 rpm and 2°C for 90 min. To the supernatant was then added one half volume of ammonium sulphate, saturated at room temperature and adjusted to pH 7.8. The precipitate was collected and dissolved in 6 ml of a buffer made up of one volume 0.4 M potassium phosphate pH 7.8, 0.05 M in mercaptoethanol, plus three volumes glycerol. After carefull resuspension, this solution was centrifuged for 30 min at 18.000 g to remove a small amount of undissolved matter. A similar proce-

dure was followed for the pea stems. All operations were performed in the presence of a weak green safelight.

Dithionite ($Na_2S_2O_4$) was used as a freshly prepared 0.1 M solution in a mixture of 1 volume potassium phosphate 0.4 M, pH 7.8 plus 3 volumes glycerol. To 3 ml pigment solution in an absorption cell, 0.3 ml of the dithionite solution were added, an equal volume of glycerol-buffer without dithionite being added to the reference cell.

Details of equipment and method of actinic irradiation were reported previously (3).

Results

Fig. 1 gives difference spectra for red irradiation of pea leaf extracts. A number of these extracts, prepared by the same procedure, so far have yielded difference spectra that clearly fall apart into two types. The first, fig. 1 a, has a red minimum at about 650 nm, and negative absorption changes in the blue-violet. The second type, fig. 1 b, has a red minimum at 665-670 nm and positive absorption changes in the blue-violet region. This second type is similar to the difference spectra that are obtained from type I extracts upon a repeated red irradiation, following far red, such as shown in fig. 2 c. During storage at 2°C in the dark, type I solutions undergo a gradual change in the direction of type II. Examining fig. 2 in more detail, shows that the first red irradiation has irreversibly changed the pigment(s), because the red peak, originally at 650 nm reappears upon far red irradiation at 665 nm, whereas the second red irradiation apparently attacks a pigment with its red peak at 670 nm. Obviously, the form of phytochrome in these extracts is unstable. We may suppose that in preparations such as those of fig. 1 b, a similar irreversible reaction has taken place in darkness also, during the preparation of the extract. We return to this point later on. It is undoubtedly significant that the magnitude of the red absorption change resulting from the second red irradiation was always larger than that of the first, compare fig. 2 a and c. This demonstrates that the irreversible part of the photo-

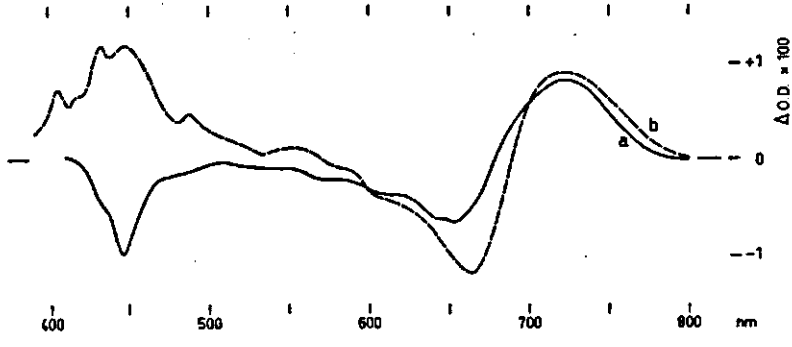

Fig. 1. Difference spectra upon red irradiation of pea leaf extracts.
a. Type I, b. type II.

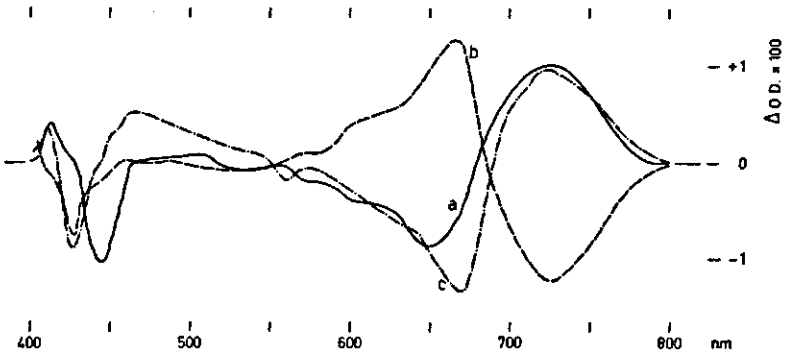

FIG. 2. Difference spectra of type I extract.
a. Upon first red irradiation, b. upon subsequent far red irradiation, c. upon second red irradiation.

transformations is not simply a destruction of part of the pigment, but rather – a phototransformation of the original pigment into a new one with a higher molar absorption coefficient, at a longer wavelength. There does not seem to be a considerable difference between the spectra of the far red absorbing forms of both pigments.

Undoubtedly one of the most interesting aspects of these photoreactions is formed by the absorption changes in the blue and violet regions, induced by red and far red irradiations. Are they due to absorption bands of phytochrome? This is not likely, because violet actinic light (440 nm) has but little effectiveness in inducing pigment transformations. The small effects can be explained by assuming that, like maize and oat phytochrome, the photoreversible pigments present in pea extracts have weak absorption bands in the violet. This point should be clarified by a study of the action spectra for phototransformations in pea extracts. In view of the limited availability of these pigments and their unstable nature, this will be an extremely laborious task, however.

Differences such as those between extracts corresponding to fig. 1 a and b may possibly be due to loss of a reducing substance during preparation of the extracts. After the difference spectra were recorded, the extracts of both types were irradiated with far red to reconvert the pigments to the red absorbing forms. A small amount of buffered dithionite was then added. The difference spectra upon red irradiation were very similar for both types of extracts treated in this way. An example is given in fig. 3 a. This treatment apparently restores the capacity of the solution to react to red irradiation with disappearance of an absorption band at 447 nm. For comparison, fig. 3 b gives a similar spectrum made with a pea stem extract. Evidently, the concentration of the violet pigment system is much smaller in this material. On the whole, stem extracts give difference spectra that suggest that the pigments in them are a mixture of 'normal' phytochrome with va-

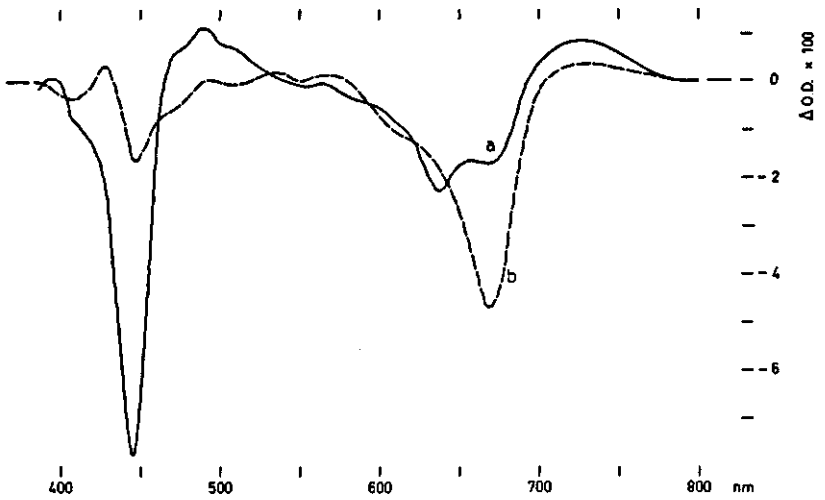

FIG. 3. Difference spectra of extracts in the presence of 0.01 M dithionite.
a. Pea leaf extract, b. pea stem extract.

rying amounts of the phytochrome type found in leaves. It is, therefore, possible that both organs contain mixtures of phytochromes, and that there is a concentration gradient in the direction leaf to cotyledons for both, the 'leaf type' pigment being preponderant in the leaves, and the 'normal' type being more typical for the stem.

During this photoreaction in the presence of dithionite, very little far red absorption is formed. Subsequent irradiation with far red does not restore red absorption, and the reaction evidently has become irreversible. This is not, however, due to an action of dithionite upon P_{fr}, as can be readily demonstrated by adding the dithionite *after* the P_{fr} has been formed. This treatment is not followed by bleaching of the far red absorption band. The action of the dithionite is, therefore, upon some intermediate of the photoreaction, rather than upon either the red or the far red absorbing forms of the pigment.

It appears likely that at least some of the violet absorption bands in the difference spectra belong to cytochromes. Difference spectra reduced-oxidized were obtained by adding dithionite in the dark to one of the absorption cells in the spectrophotometer, see fig. 4. Spectra of this type are similar to those, resulting from oxido-reduction of naturally occurring mixtures of cytochromes (8).

We can trace back some of the peaks in this red-ox spectrum to absorption bands in the photochemical difference spectra, viz. those at 445-449 nm, 425-430 nm, 405 nm and 555 nm. It appears that, depending upon (partly as yet unknown) conditions, phototransformations of the pigments in these extracts can be accompanied by either reduction of oxidized, or oxidation of reduced cytochromes, present in them. This may also explain why there are variations in the form of the short wave parts of the difference spectra

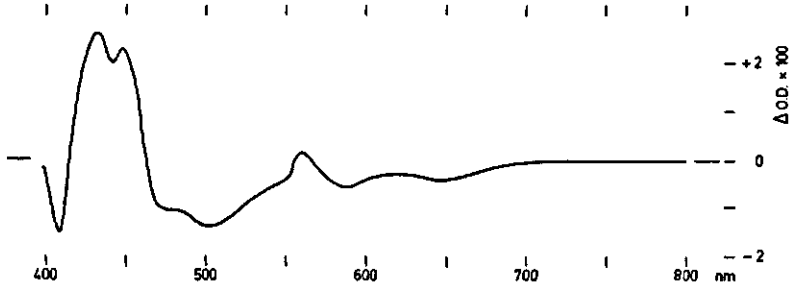

FIG. 4. Reduced minus oxidized difference spectrum of a pea leaf extract.

from one sample to the next. For, notwithstanding the presence of mercaptoethanol in the buffer solutions, we have very little control of the oxidation state of various components in the extract. Figures like number two seem to suggest that during illumination, cytochrome oxidations and reductions may occur at the same time, the phytochrome transformations thereby mediating coupled oxidoreductions.

It is, however, surprising that, as fig. 3 shows, there should be cytochrome oxidation (disappearance of the 446 nm absorption band) during irradiation in the presence of an excess of dithionite, or rather, that the cytochrome does not become completely reduced again upon darkening. We will have to be cautious in interpreting difference spectrum bands, therefore. We have also attempted to measure changes in oxidation-reduction potentials during illumination of purified maize phytochrome solutions, but we have been unable to find any effects larger than $\pm$ 1 mv.

If this cytochrome oxidoreduction is a reaction, occurring normally during illumination of plant organs, it might be of the utmost importance for the understanding of phytochrome action. We have, accordingly, looked for absorption changes in the violet region following red and far red irradiation of pea leaves, but without success. This may, in part, be due to technical difficulties, as the leaves contain large amounts of yellow pigments, which make it difficult to measure through sufficiently thick layers. The only absorption change, probably attributable to a cytochrome, that can be regularly observed in difference spectra of pea leaves is a band at 550-560 nm, usually close to 555 nm. It can be observed in various spectra in the present article, e.g. fig. 2, 4 and 6, as well as in those reproduced in earlier publications, the first in ref. 3, fig. 1, where it appears as a negative band at the wavelength indicated. This band is readily observed in difference spectra anaerobic-aerobic of pea leaves, where it is found at about 557 nm with an indication of a shoulder at 565 nm. This may be a cytochrome of types c or f.

Of special importance are the photoreactions of these pigment solutions at low temperature, where chemical reactions must be almost absent. Fig. 5 compares the difference spectra for red irradiation at —196°C of pea leaf

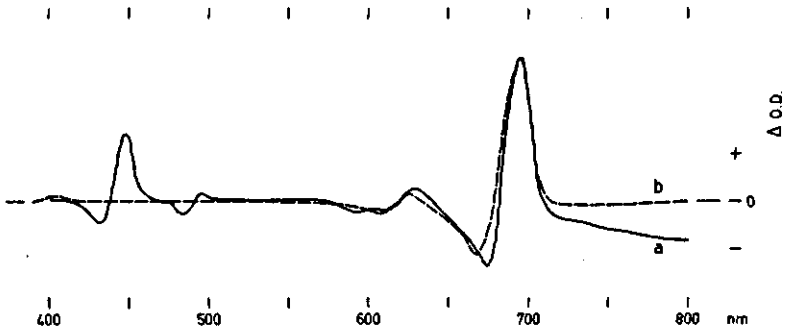

FIG. 5. Difference spectra upon red irradiation at liquid nitrogen temperature.
a. Pea leaf extract, b. solution of maize phytochrome.

extract and a purified extract of maize phytochrome. The pea material was of type II (fig. 1 b). Warming to —70°C of a solution, irradiated with red at —196°C followed by cooling to the original temperature, resulted in absorption changes that are shown in fig. 6 a. For comparison, a similar spectrum for purified maize phytochrome is given in the same figure. Apart from the changes in red and far red, the pea material also shows remarkably large absorption changes in blue and violet upon warming. The peaks in this part of the spectrum can be compared with those in the room temperature spectra, and we must conclude that the same compounds are involved in both types of reactions.

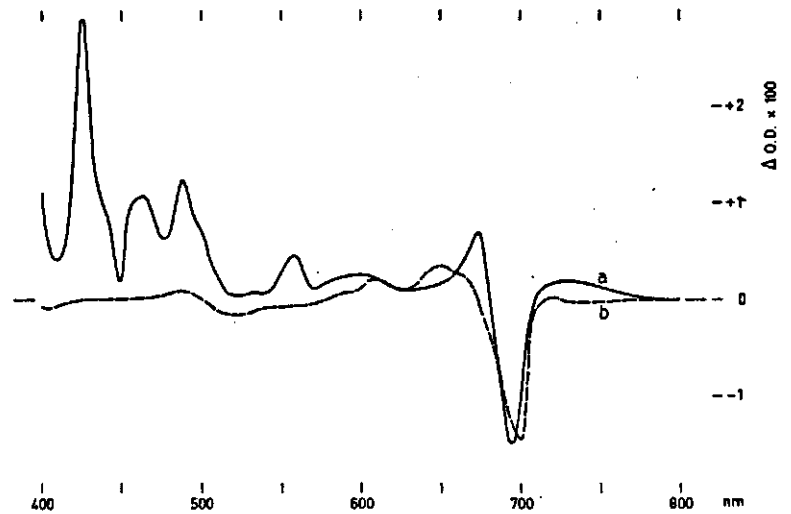

FIG. 6. Difference spectra for a warming and cooling cycle (-196°, -70°, -196°) following red irradiation at -196°.
a. Pea leaf extract, b. purified maize phytochrome solution.

In the wavelength region above 600 nm, the spectra of fig. 6 are also rather different. In the pea extracts, this part of the spectrum forms a rather accurate reversion of the spectral changes, induced previously by the red irradiation (fig. 5 a). In maize phytochrome, this correspondence is much less marked, cf. also (4).

In partly purified extracts of pea stems, as well as in pea leaf extracts, we observe the negative peak in the red upon irradiation at low temperature at 673–674 nm. This forms a contrast with similar spectra for purified phytochrome from maize and oats where this peak is at 667 nm, fig. 5 b. Only as far as the region above 600 nm is concerned, we have therefore the somewhat confusing situation, that whereas at room temperature, the difference spectra of pea stem extracts resemble those of phytochrome from grasses, at low temperature they are indistinguishable from those of pea leaf extracts. We must also conclude that the pigment found in pea stems is not exactly identical with the 'normal' phytochrome from grasses.

One of the peaks in the temperature cycling spectrum is at 449 nm and forms a near-perfect reversal of the peak at this wavelengh in the difference spectrum for red irradiation at low temperature (fig. 5 a). The low temperature experiments point to a very close association in this material of phytochrome with a pigment, absorbing in the violet.

Discussion

The significance of these findings is still far from clear. At first sight, they seem to support the conclusion, reached earlier (1,2) that the phytochrome in pea leaves differs from the form found in grasses. The difference spectra show peaks, similar to those, observed in intact leaves in the position of the maxima at about 650 nm and in the low ratio $\triangle$ O.D.$_{730}$/ $\triangle$ O.D.$_{650}$. Moreover, difference spectra at low temperature (fig. 5 a) show that the negative peak in the red (674 nm) in the pea extracts is different from a corresponding peak in maize phytochrome (667 nm). There can be no doubt about the non-identity of these two forms of phytochrome.

We have not observed a constant quantitative relationship between the absorption changes in blue and violet on the one hand and those in red and far red on the other hand. We believe, therefore, that the short wavelength peaks do not belong to the photoreversible pigments in the extracts. Undoubtedly, further purification will lead to pigment solutions that only show weak absorption bands in the violet. It cannot be denied, however, that red irradiation of the extracts induces absorption changes in the violet region. The simplest way to explain them, would be to assume that they are mediated by a different red absorbing pigment, also present in the crude extracts. As some of the blue and violet bands are most likely due to cytochromes, light absorption by this pigment would lead to reactions by which these cytochromes would enter into oxidations and reductions in which, presumably, colourless components of the solutions would also be involved.

Direct absorption spectra of the extracts show the presence of an absorption band at 635 nm, presumably due to some form of protochlorophyll. Several authors (5) (6) (7) have shown that aqueous buffers can extract photochemically active protochlorophyll complexes from etiolated plant material. The conditions of our extraction procedure (high ionic strength buffers)

does not appear to favour the isolation of active protochlorophyll holochrome.

Obviously, one should study more highly purified extracts. Unfortunately, the difficulty of obtaining sufficient starting material, together with the instability of the material, has made this approach unattractive so far. Spectra, such as fig. 3 a can be most easily understood by assuming that, in the presence of dithionite, red light in addition to transforming a phytochrome with absorption maximum 673 nm, also bleaches a pigment with absorption maxima at 635 and 445 nm. The latter fits the data in the literature for protochlorophyll-protein complexes in solution rather well. The correspondence of the 445 nm peak with a similar one in the red-ox difference spectra then might be a coincidence. We may, therefore, try to identify the red absorbing pigment, discussed above, with a form of protochlorophyll.

There are, however, also observations that seem to contradict the explanation given above.

a. Crude solutions of maize phytochrome, that also contain protochlorophyll, give no other blue or violet peaks in their difference spectra than a weak band around 415 nm, belonging to phytochrome.

b. The activity of far red light in inducing absorption changes in the violet as well as in the long wavelength region (fig. 2 b) appears to exclude protochlorophyll or any other red-absorbing pigment as the photoreceptor.

c. Starting with virgin extracts of type I, a series of alternate red and far red irradiations results in an increase in the magnitude of the red peak and a simultaneous displacement to longer wavelengths. This cannot be understood on the basis of two pigments, one of which becomes bleached during irradiation.

d. No formation of chlorophyll a was observed during red irradiation of the extracts.

e. The low temperature spectra demonstrate a close association between the phytochrome and at least part of the blue absorbing pigments in the extracts. They demonstrate, moreover, that the pea phytochrome, even the form with its red maximum at 664 nm (room temperature), is not identical with the form of phytochrome, found in grasses.

The experimental data, presented in this paper, appear insufficient to decide whether or not all light-induced absorption changes are mediated by the same pigment system. There seem to be some indications, however, that at least part of the blue and violet absorption changes are connected with the red-far red photoreversible pigment system in the extracts.

Summary

Crude extracts of leaves of etiolated pea seedlings contain a pigment, similar in its photochemical behaviour to phytochrome, but distinct from it in several respects. One of the most remarkable features of these extracts is the absorption changes in the blue and violet regions upon actinic irradiation with red or far red light.

Some arguments are presented favouring an identification of these blue and violet absorption changes with oxidations and reductions of cytochromes, present in the extracts. Difference spectra for reactions at low temperature indicate a close connection between the phytochrome-like pigment, here called 'leaf phytochrome', and one or more components of the blue-violet pigment system.

The possibility of protochlorophyll or some other red-absorbing pigment participating in these reactions is discussed.

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