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MEDEDELINGEN LANDBOUWHOGESCHOOL
WAGENINGEN • NEDERLAND • 66-15 (1966)

THERMAL REACTIONS FOLLOWING ILLUMINATION OF PHYTOCHROME

C. J. P. SPRUIT

Laboratory of Plant Physiological Research,
Agricultural University, Wageningen, The Netherlands,
254th Communication

(Received 28–XI–1966)

INTRODUCTION

We have previously shown (SPRUIT, 1965 b, 1966 a) that phytochrome, the pigment acting as photoreceptor for photomorphogenetic reactions in plants, can be converted by illumination of isolated plant parts at low temperature into pigment forms that are not observed during the phototransformations at room temperature. Apart from absorption bands at 670 and 744 mμ that can be attributed to the known forms of phytochrome (see e.g. SIEGELMAN et al. 1965) usually indicated with P_r and P_{fr}, the low temperature spectra show bands at about 650 mμ and at 698 mμ, apart from minor bands at a number of other wavelengths. The question arises as to the nature of these pigments, P_{650} and P_{698} and their possible function in the transformations of phytochrome at room temperature. In this preliminary communication we will describe observations of the changes in the absorption spectra following controlled warming of the photoproducts formed from either P_r or P_{fr} at low temperature. Such observations with isolated pea plumules have been supplemented by similar experiments with solutions of maize phytochrome.

MATERIALS, METHODS

Difference spectra were measured with the equipment, described earlier (SPRUIT, 1965 a). The absorption vessels were placed in a cryostat with flat windows at the bottom, mounted in the cell compartment of the spectrophotometer. Samples were cooled with either liquid nitrogen (-196°), dry ice-ethanol (–79°) or coarse lumps of dry ice (about –70°). As position and intensities of the absorption bands are dependent upon temperature, warming of a sample was always followed by cooling to the original temperature. In most cases, the following cycle was adopted : 1. Cooling to the desired temperature for 45–60 min. 2. First absorption spectrum run. 3. Actinic irradiation to effect the desi-

red pigment transformation. 4. Second absorption spectrum run. 5. Rapid warming to –20 or –15° followed by 15 min. at this temperature. 6. Cooling to the original temperature for 30 min. 7. Third absorption spectrum run. Difference spectra were computed as the difference between the spectra before and after a particular treatment.

Solutions of maize phytochrome were prepared by a simplified procedure, to be described elsewhere. The concentration was adjusted to give optical density changes of 0.1 to 0.2 at 725 mμ upon illumination at room temperature (10 mm path cells). The solutions were made up with a buffer containing 75 % glycerol (v/v). Such samples could be cooled to –196° in polyethylene absorption vessels without crystallization. In addition we observed that the presence of glycerol greatly increased the thermal stability of the phytochrome solutions, which could be kept at room temperature for considerable periods without much loss in activity.

Samples of pea plumules were prepared as described earlier (SPRUIT, 1965 a).

RESULTS

Warming of a sample containing P_{698}, obtained by irradiation of P_r at –70° is followed by the absorption changes shown in fig. 1. Whereas no P_{735} was formed by the actinic irradiation, this pigment develops during the subsequent warming step, both in phytochrome solution and in pea. The remainder of the difference spectra are apparently quite different for the two sources of phytochrome, however, the pea material only showing the expected disappearance of P_{698}. We will return to this discrepancy in due course.

Similar experiments were done with P_{735}, prepared by the photoconversion of

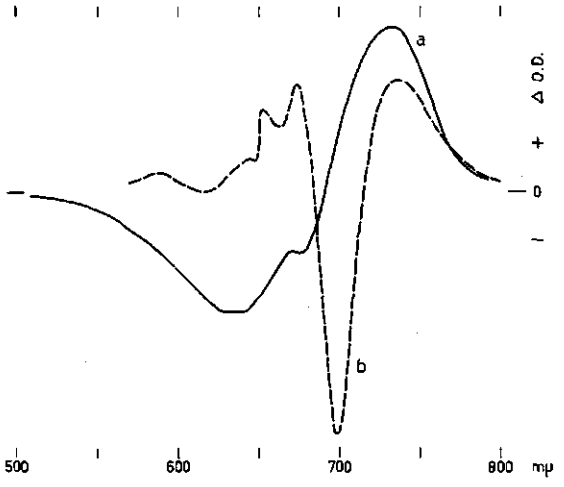

FIG. 1. Difference spectra for warming from –70° to –20° after photoconversion of P_r at –70 . a. For a solution of maize phytochrome, b. for isolated plumules of etiolated pea seedlings (not on the same scale).

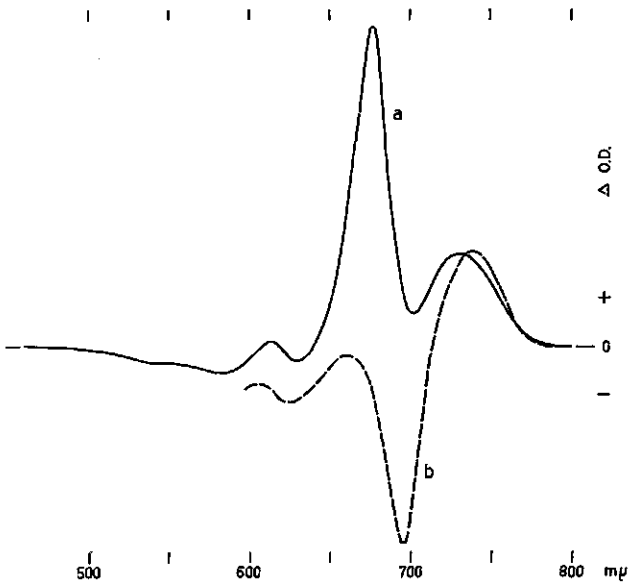

FIG. 2. Difference spectra for warming from –70° to –20° after photoconversion of P_{fr} at –70°. a. For a solution of phytochrome, b. for isolated pea plumules (not on the same scale).

P_r at room temperature. In this case, we do not obtain a 100% conversion, however, and the resultant pigment is a mixture containing some 20% unchanged P_r. This has to be kept in mind when interpreting the spectra. After cooling the photoproduct, P_{735} can be bleached by irradiation with far red. This is accompanied by a larger or smaller absorption increase in the red region around 650 mμ, fig. 3. Warming of this irradiation product gives rise to the absorption changes shown in fig. 2. Again, the absorption increases at 735 mμ i.e. part of the P_{735} bleached during the previous irradiation, is formed back. Although also in this case there is a difference between the spectra for phytochrome solutions and for pea, it is obvious that regeneration of P_{735} in this case is accompanied by a disappearance of P_{698} and formation of P_{670}.

Additional experiments showed that in both types of reactions the absorption changes in the dark occur in two steps. If the irradiation is done at –196°, the absorption changes during warming, shown in fig. 1 and 2, are preceded in the temperature interval –196 to –70° by reactions that are roughly a partial reversal of the absorption changes induced by the illumination. Also in this case, there is a difference between phytochrome solutions and pea in that these dark reactions in the latter occur only at temperatures, considerably higher than required for solutions. This may be attributed to a greater reactivity of the components in solution as compared with those in intact plant parts. This explains the discrepancy between fig. 1 a and b, where the negative absorption change at 698 mμ observed in pea, has already taken place to a large extent in the phytochrome

solutions at –70° during and immediately following the actinic irradiation. It therefore escapes measurement unless we do the illumination at a much lower temperature. This points to the occurrence of more than one temperature dependent step in the total process. For comparison, we have given, in fig. 3 and 4, the difference spectra for actinic irradiation both of P_r and P_{fr} at several temperatures and for the two sources of phytochrome.

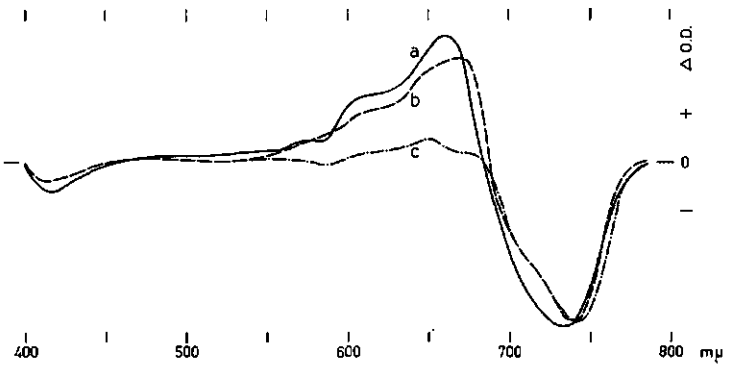

FIG. 3. Difference spectra for photoconversion of P_{735}.
a. In a solution of phytochrome at –70°, b. in a solution of phytochrome at –196°, c. in isolated pea plumules at –196° (not on the same scale).

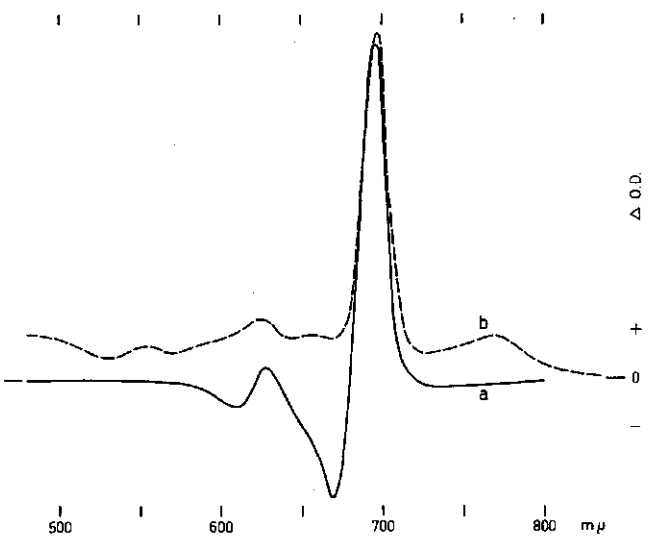

FIG. 4. Difference spectra for photoconversion of P_r.
a. in a solution of phytochrome at –196°, b. in isolated pea plumules at –196° (not on the same scale).

Discussion

It is evident from fig. 3 and 4, that the two photoreactions represented by

$$P_r \xrightarrow{h\nu_1} P_{fr} \xrightarrow{h\nu_2} P_r \qquad (1),$$

are replaced at low temperature by the separate reactions:

$$P_{670} \leftrightarrows P_{698} \qquad (2),$$

$$P_{735} \rightleftarrows P_{650} \qquad (3).$$

These two systems do not appear to have common intermediates at low temperature, as the sequence of actinic irradiation: far red followed by red does not form P_{698} from P_{735}, for instance (the formation af a trace of P_{698} under these conditions can be explained from the presence of a little P_r in the original sample, see above). On the other hand, the photoproducts of reactions 2 and 3 both form P_{735} on warming, which is accompanied by absorption increases at 670 mμ *(P_r)* in addition to changes at other wavelengths, notably around 650 mμ. Although we cannot, as yet, offer a complete explanation of the experiments reported here, we will attempt to give a qualitative interpretation on the basis of the following assumptions.

a. The difference spectra for actinic irradiation can be regarded as due to the mutual transformation of a limited number of components.
b. The phytochrome pigment system is made up of two components, viz. P_{670}–P_{698} and P_{650}–P_{735}.
c. Absorption changes resulting from warming of irradiation products are linked to chemical reactions between components. At the lowest temperatures the reactions seem to be mainly within each system separately, whereas above –70° the reactions are predominantly between the two systems.

Although it is recognized that other explanations are possible and that, in particular, some of the intermediates may have the nature of electronically excited, metastable molecules, we will now attempt to develop the above assumptions to see where they may lead us. The following scheme summarizes the picture presented above:

$$P_{670} \underset{h\nu_3}{\overset{h\nu_1}{\rightleftarrows}} P_{698} \quad \overset{\text{temp.}}{\longleftrightarrow} \quad P_{650} \underset{h\nu_4}{\overset{h\nu_2}{\leftrightarrows}} P_{735} \qquad (4).$$

Starting from P_{670}, irradiation gives P_{698}, this reaction being partially reversible in the dark at relatively low temperatures (between –196° and –70°) and by irradiation with wavelengths between 690 mμ and 740 mμ. Upon warming, the remaining P_{698} then reacts according to:

$$P_{698} + P_{650} \rightarrow P_{670} + P_{735} \qquad (5).$$

This reaction is illustrated by fig. 1. We can now interpret the negative part of fig. 1 a in the region 680 mμ downwards as a combination of the disappearance of P_{650} (broad band) and formation of P_{670} (narrow band). The simultaneous

disappearance of P_{698} required by the equation is only obvious in fig. 1 b (pea). As we have already mentioned, in the phytochrome solutions it precedes the other spectral changes.

Starting from P_{735}, irradiation forms P_{650}. Upon warming, this reacts according to equation (5) to form P_{670} and P_{735} with simultaneous consumption of P_{698}. This is illustrated by fig. 2. In this case, the disappearance of P_{650} is not very obvious, but it may be obscured by the other changes occurring at about the same wavelength. However, there is a serious obstacle to this interpretation in that our scheme does not account for the presence of P_{698} in this reaction. For we have assumed that P_{698} was a photoproduct of P_{670} and neither should the latter be present, nor has the appropriate illumination for its conversion been given. So, in order to explain the unquestionable consumption of P_{698} (fig. 2 b), we will have to introduce the further assumption that (some) P_{698} was present from the start, i.e. that it was already present in 'P_r' at room temperature, and that the reactions of equations (1) and (4) result in a regeneration of the pigment to about its original concentration. If P_{698} is a stable chemical compound there should be no objection to this idea. Summarizing, we must conclude that the view presented above, leads us to the assumption that the pigments designated with 'P_r' and 'P_{fr}' are mixtures, each containing at least three components. It is then likely that the four pigments in equation (4) form a chemical equilibrium in which, at room temperature, the concentration of P_{735} is small. Photoconversion of the mixture to 'P_{fr}' is followed by a (slow) reestablishment of the original equilibrium. This much debated point has been clarified recently by Mumford (1966) who demonstrated the 'reversal' reaction for phytochrome solutions, and we can fully confirm his findings. The only difficulty in this picture is that the reversal reaction has a very slow rate determining step, whereas the dark reactions of equation (4) all should be rapid.

Before we can accept the picture, presented above, several points require clarification. Perhaps the most serious objection to the scheme is, that equation (4) requires that the substance ultimately consumed in the reaction:

$$P_r \rightarrow P_{fr},$$

be P_{650}. Although the peak wavelength of this form at room temperature is not kown, room temperature difference spectra for the reaction point to the disappearance of a pigment with λ_{max} at about 650 mμ, rather broad-peaked, broader than our pigment P_{670}. A further observation, not accounted for by the scheme is, that at the lowest temperatures, red irradiation of P_r in pea, resulting in formation of P_{698}, is hardly followed by negative absorption changes at other wavelengths. Where then is this P_{698} formed from? In addition, the action spectra for this reaction point to P_{670} as the energy acceptor for P_{698} formation (SPRUIT, 1966 b). A similar situation exists for bleaching of P_{735} in pea where, at the lowest temperatures, hardly any P_{650} is formed, fig. 3 c. We may, therefore, have to consider 'colourless' components taking part in the reactions of equation (4). This, however, would considerably complicate the scheme.

Finally, the differences between spectra for light induced and dark transformations in pea as compared with pigment solutions remain unexplained. Remarkable also is the observation that the ratio $\Delta P_{670}/\Delta P_{698}$ for red irradiation of P_r is temperature dependent in pea, and much more so in phytochrome solutions. This is difficult to harmonize with the idea of a simple interconversion of the two pigments. Although these points will require additional experimentation, we feel that the picture, presented above may form a basis for further work. In the mean time, the recent observations by Linschitz et al. (1966) on the flash photolysis of phytochrome seem to supplement our work. In particular, they have demonstrated that P_{698} acts as an intermediate of reaction (1) also at room temperature. A more detailed discussion will be published elsewhere.

Summary

Warming of the products formed by photoconversion at low temperature of both forms of phytochrome is followed by absorption spectrum changes. These point to the occurrence of reactive intermediates that form, above a certain temperature, the same end products that are also obtained from the photoreaction at room temperature. An attempt has been made to interpret the difference spectra on the basis of reactions in which four coloured components participate. The scheme implies that all four components are, to some extent, present in the pigment mixture at room temperature, forming a chemical equilibrium.

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