Control of Phenylalanine Ammonia-lyase and Ascorbate Oxidase in the Mustard Seedling by Light and Hoagland's Nutrient Solution

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Hoagland's nutrient solution (HS) * was used as a tool to investigate whether or not the phytochrome-mediated appearance of phenylalanine ammonia-lyase (PAL) * and ascorbate oxidase (AO) * in the mustard seedling cotyledons is simply a modulation of the appearance of these enzymes in the dark (basal level). HS was applied to the mustard (*Sinapis alba* L.) seedling at sowing instead of distilled water. It was observed that HS causes a stimulation of enzyme disappearance (interpreted as degradation) in light and dark. This effect becomes obvious at approximately 48 h after sowing. On the other hand, however, HS exerts *specific* effects on the appearance of the enzymes in continuous far-red light (which is considered to operate exclusively *via* phytochrome). In the case of PAL there is a strong stimulation of the rate of enzyme appearance; however, the onset of activity increase remains precisely at 29 h after sowing irrespective of treatment. In the case of AO the onset of activity increase is shifted by approximately 6 h; however, the rate of enzyme appearance remains the same (up to 54 h after sowing). The data suggest that HS specifically affects phytochrome-mediated enzyme syntheses whereas syntheses of the same enzymes in the dark are not affected. It is concluded that the appearance of PAL and AO in the dark and phytochrome-mediated appearance of these enzymes are independent phenomena. Some consequences of these conclusions for the interpretation of density labelling data obtained with "inducible" enzymes are discussed.

Photoregulation of enzyme levels in plants by the photochromic sensor pigment, phytochrome [1], is a well established phenomenon [2]. However, the details of the mechanism involved are still under intense debate [3, 4]. A major issue at present is to what extent the phytochrome-mediated increases in observed enzyme activity are due to differences in the number of enzyme molecules ("synthesis") or due to an activation of preexisting molecules ("activation"). While the results of previous density or radioactive enzyme labelling [5, 6], and of the usual inhibitor experiments [7, 8] seemed to justify the concept of "synthesis" [9], the concept of "activation" has recently been favoured by H. Smith and associates [10, 11] in an interpretation of their recent density labelling data, including labelling of PAL * in mustard seedling cotyledons.

In the present paper, the validity of a basic assumption implicit in the "theory" of density labelling of inducible enzymes [11] will be investigated, namely, that the light-mediated induction of the enzyme is simply a *quantitative* modulation, *i. e.* an increase over the low basal level, rather than the appearance of enzyme activity which was totally absent in the dark. The alternative assumption that the appearance of an enzyme in the dark-grown tissue and the light-mediated appearance of the enzyme are unrelated phenomena (*e. g.*, occuring in different cells or tissues) was not seriously considered in the recent controversy [11] in spite of the fact that data in favour of this alternative are in print [12].

Since any model of the mechanism of phytochrome-mediated enzyme induction depends essentially on our knowledge of the relationship between the basal enzyme level and the light-mediated enzyme level, we have tried to approach this problem from a physilogical point of view, using a second external factor in addition to light. We have noticed in preliminary experiments that the application of a combination of inorganic ions known as Hoagland's solution [13] to the mustard seedling instead of distilled water leads to drastic changes of the PAL level in the light. In the present paper we investigate the extent to which application of HS * to the

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* *Abbreviations:* Phenylalanine ammonia-lyase, PAL (EC 4.3.1.5); Ascorbate oxidase, AO (EC 1.10.3.3); Hoagland's solution, HS; physiologically active phytochrome, P_{fr}; total phytochrome, P_{total}.

mustard seedling modifies the appearance of different enzymes in dark and light. The following enzymes were measured in the mustard cotyledons: PAL, an enzyme which shows rapid turnover [12], and AO *, an enzyme which seemed to be nearly stable during the period of investigation [14]. The basal level of PAL was very low whereas in the case of AO the basal level was relatively high.

Material and Methods

Standard techniques for photomorphogenic research with mustard seedlings (*Sinapis alba* L.) were used [15]. The mustard seeds were purchased in 1969 from Asgrow Company (Hamburg, Germany). The seedlings were grown at 25 ± 0.2 °C. The standard far-red source which maintains a low P_{fr}/P_{total} ratio * in the seedling [16] was used at an irradiance of $3.5\ W \cdot m^{-2}$. The standard methods of extraction and assay of PAL and AO described elsewhere [14, 17] were used. The mean values presented are those of 6 independent experiments. The vertical bars represent standard errors. The use of the biological unit (cotyledon) as a system of reference was justified previously [2, 18, 19]. Hoagland's nutrient solution (originally described by Hoagland and Arnon in 1939 [13]) was used instead of distilled water (*cf.* Standard Technique above). Fe-Sequestrene (sodium ferric diethylenetriamine pentaacetate) was used in the solution instead of $FeSO_4$ and tartaric acid. The photometric measurements of total phytochrome in the cotyledons were performed by B. Steinitz and E. Schäfer with a dual wavelength photometer ("Ratiospect") as described previously [20].

Results

1. Kinetics of PAL and AO levels in the mustard cotyledons in the dark. Fig. 1 shows that the application of HS * diminishes considerably the enzyme levels which can be extracted from the dark grown cotyledons after 48 h after sowing. Any positive effects of HS on the enzyme levels cannot be detected.

2. Kinetics of PAL levels in the mustard cotyledons under continuous far-red light (which is considered to operate *via* phytochrome [2, 16, 17, 21]). Fig. 2 shows that in the presence of phytochrome (operationally, continuous far-red light) the application of HS affects the kinetics of PAL levels in a different manner compared to the dark control (*cf.* Fig. 1). The main feature is that irrespective of the onset of light, PAL activity increases much faster in the presence of HS, and the peak of activity appears considerably earlier. However, although the *onset* of the phytochrome-mediated increase of activity (at approximately 29 h after sowing) is not changed by the treatment with HS, the decrease of activity (interpreted as enzyme degradation) occurs earlier (at approximately 48 h after sowing) in the presence of the nutrients. A closer inspection of the PAL kinetics in Fig. 2 a, b shows that the "bandwidth" of the up and down kinetics is considerably smaller in the presence of the nutrients. The kinetics in Fig. 2 c (onset of light at time of sowing) is difficult to conceive since both peaks (HS- and H_2O-curves) seem to be shifted to the right by approximately 6 h and the peak of activity is considerably lower in the

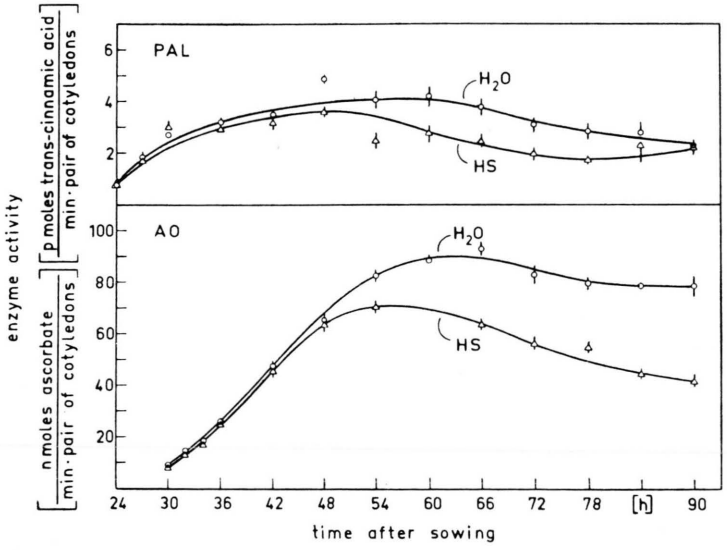

Fig. 1. Kinetics of phenylalanine ammonia-lyase (PAL) and ascorbate oxidase (AO) levels in the mustard seedling cotyledons in the dark supplied with distilled water (H_2O, –○–) or with Hoagland's nutrient solution (HS, –△–).

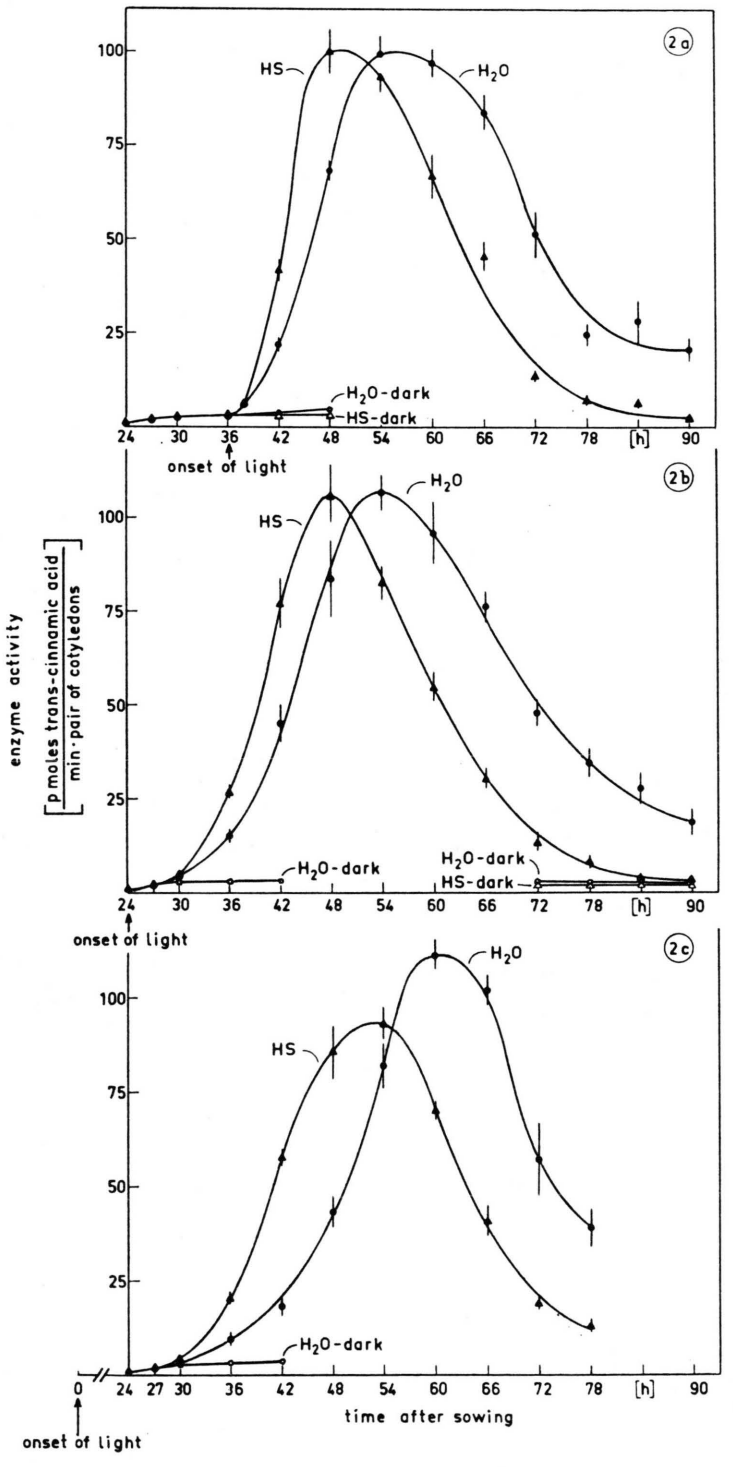

Fig. 2. Kinetics of PAL levels in the cotyledons of the mustard seedling under continuous far-red light. The seedlings were supplied with distilled water (H_2O, –●–) or with Hoagland's nutrient solution (HS, –▲–). Fig. 2 a, onset of light at 36 h after sowing; Fig. 2 b, onset of light at 24 h after sowing; Fig. 2 c, onset of light at time zero (sowing).

presence of the nutrients. The peak shifting effect of a far-red light pretreatment (between time zero and 24 h after sowing) on the kinetics of PAL levels was unexpected since in other cases, *e. g.* anthocyanin synthesis [2, 22], no effect of this kind was found. Irrespective of the difficulty in under-

standing these data in Fig. 2 c at present the main results of Figs 1 and 2 can be described as follows: In the presence of phytochrome HS stimulates the rate of appearance of PAL and it leads to an earlier onset and to some stimulation of disappearance of PAL. In the dark HS only stimulates the disappearance of PAL. HS does not shift the point in time at which the increase of enzyme activity in either light or dark becomes detectable. We further notice that the *increase* of the PAL level in the dark becomes detectable somewhat earlier than the far-red light mediated increase of the PAL level.

3. Kinetics of AO levels in the mustard cotyledons under continuous far-red light. Fig. 3 shows that under continuous far-red light the application of HS affects the kinetics of AO levels differently as compared to PAL. It is obvious that the AO levels tend to rise somewhat earlier in the presence of HS; however, the rate of increase up to 54 h after sowing does not seem to be significantly higher in the presence of the nutrients. The major change caused by the application of HS is that the AO levels tend to decrease around 60 h after sowing while in the water controls no decrease of the far-red "induced" AO level can be detected [14].

4. Kinetics of phytochrome levels in the mustard cotyledons. Effects of the application of HS upon the appearance of total phytochrome (P_{total}) in the mustard cotyledons were checked. Fig. 4 shows that there is a slight effect of HS on the level of P_{total} in the dark while under continuous far-red light no significant effect can be detected. It is improbable, therefore, that the effect of the nutrients on the enzyme levels in the presence of phytochrome can be attributed to an effect of HS on the phytochrome system. The data in Fig. 5 suggest that at 36 h after sowing the half-life of P_{total} is somewhat shortened in the presence of HS. This permits an understanding of the data in Fig. 4, namely, that there is no significant difference in P_{total} between HS treated

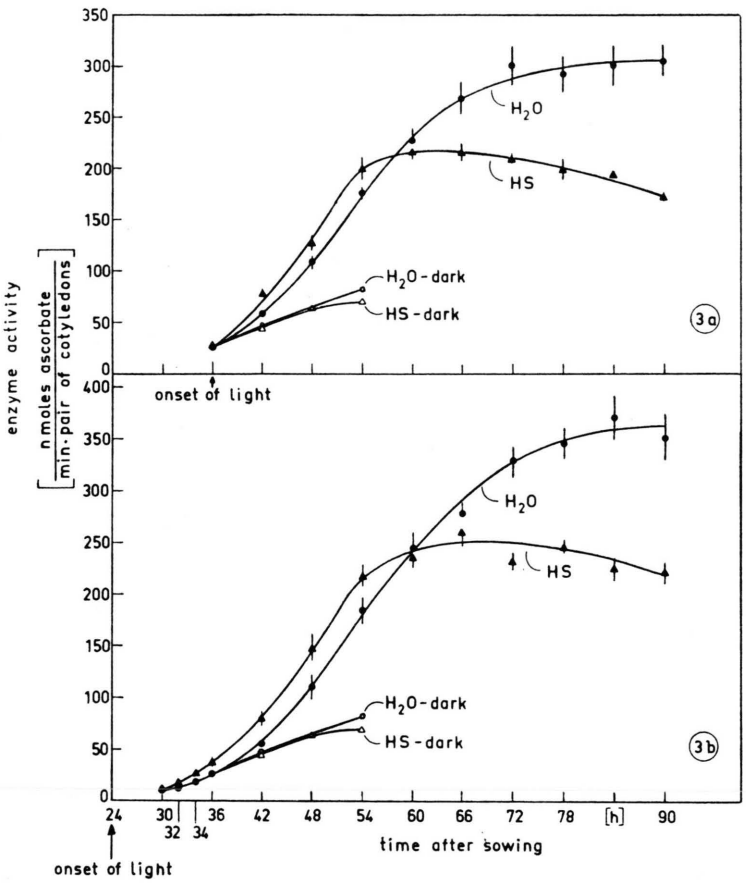

Fig. 3. Kinetics of AO levels in the cotyledons of the mustard seedling under continuous far-red light. The seedlings were supplied with distilled water (H_2O, –●–) or with Hoagland's nutrient solution (HS, –▲–). Fig. 3 a, onset of light at 36 h after sowing; Fig. 3 b, onset of light at 24 h after sowing.

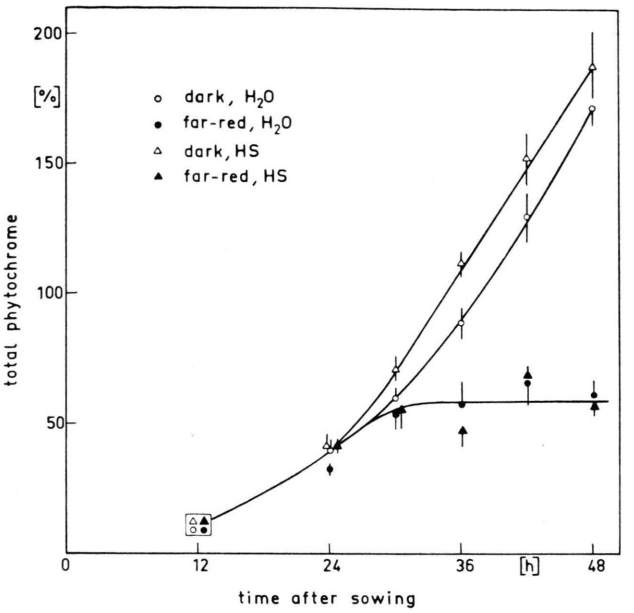

Fig. 4. Appearance of phytochrome (P_{total}) in the cotyledons of the mustard seedling in the dark and under continuous far-red light (onset of light at time zero). The seedlings were supplied with distilled water (H_2O) or with Hoagland's nutrient solution (HS). To ensure the fairest comparison between HS — and H_2O — data the mean value between P_{total} (H_2O) and P_{total} (HS) at 36 h after sowing was chosen as the reference point (100%) (data from B. Steinitz and E. Schäfer).

seedlings and H_2O controls in far-red light in spite of the fact that the rate of appearance of P_{total} in the dark is somewhat increased by HS.

5. Effects of single ions. It was tried to attribute the effect of HS on the kinetics of PAL to defined ion species, *e.g.* Mn^{2+}, K^+, NO_3^-, SO_4^{2-}. Unfortunately these experiments have not yielded useful results. Single ions or ion pairs had either no significant effect or inhibited at higher concentrations the appearance of PAL in the light as well as in the dark. The report by Engelsma [23] who observed a strong stimulation of PAL activity in the hypocotyl of gherkin seedlings under the influence of Mn^{2+} in the dark could not be confirmed with the mustard cotyledons (Table I as an example).

Table I. The influence of $MnSO_4$ at different concentrations on the level of PAL in the cotyledons of the mustard seedling in the dark and under far-red light. Experimental program: Sowing — 36 h dark — 12 h dark or continuous far-red light. The seedlings were transferred to the $MnSO_4$ containing medium at 36 h after sowing.

Medium	Concentration [mM]	pH	PAL levels at 48 h [%] Far-red	Dark
H_2O	—	5.2	100 ± 8	100 ± 12
$MnSO_4$	0.003	5.9	105 ± 9	103 ± 8
$MnSO_4$	3 *	5.6	92 ± 11	77 ± 6
$MnSO_4$	30 *	4.9	66 ± 18	62 ± 8

* These concentrations of $MnSO_4$ caused the highest increase of PAL in Engelsma's [23] investigations with gherkin hypocotyls.

6. Mixing experiments [3, 24]. In the previous and present work with mustard seedlings mixing experi-

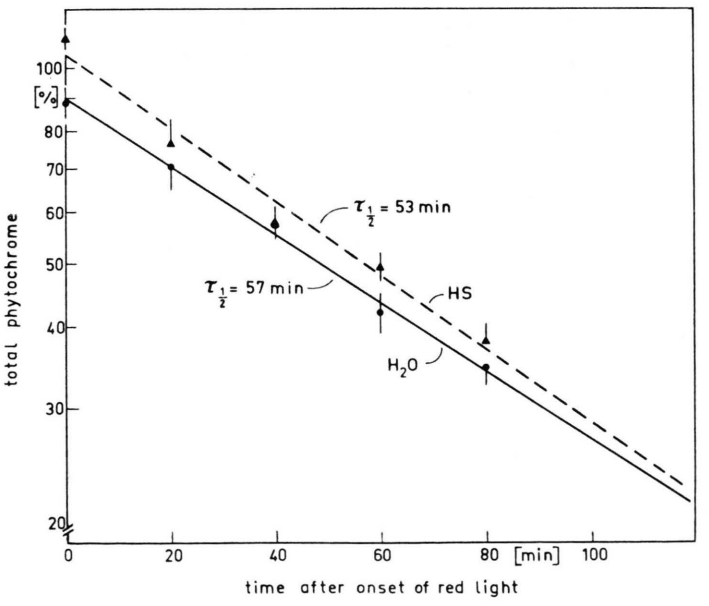

Fig. 5. Time course of change of total phytochrome (P_{total}) in the mustard cotyledons under continuous standard red light (*cf.* [15]). Onset of light: 36 h after sowing. ●, seedlings supplied with distilled water (H_2O); ▲, seedlings supplied with Hoagland's nutrient solution (HS) (data from B. Steinitz and E. Schäfer).

ments were routinely carried out to check for the presence of inhibitors or activators whose concentration is light- or HS-dependent. In these, extracts from dark-grown and light-grown plants grown with and without HS are mixed. If the activities are strictly additive it is concluded that there is at least no differential inhibition or activation of the enzyme due to the light- or HS-mediated presence of "factors" in the crude or partially purified extract. All mixing experiments performed previously (*cf.* Table 2.1 in [3]) and in connection with the present work yielded additive results.

Discussion

Hoagland's nutrient solution (HS) was used as a tool to investigate whether or not appearance of PAL and AO in the presence of phytochrome (operationally, continuous far-red light) is simply a modulation of the appearance of these enzymes in the dark (basal level). It was observed that the application of HS instead of water leads to an earlier onset and also to some stimulation of enzyme disappearance (interpreted as enzyme degradation) in light and dark. With respect to this effect the "dark" enzyme and the "light" enzyme behave very similarily. On the other hand, however, HS exerts *specific* effects on the appearance of the enzymes in the light. In the case of PAL there is a strong stimulation of the rate of enzyme appearance; in the case of AO the onset of activity increase is shifted by approximately 6 h. These effects have no counterparts in the dark. The data suggest that HS *specifically* affects enzyme syntheses in the light whereas syntheses of the same enzymes in the dark are not affected. The data are consistent with the hypothesis that the appearance of PAL and AO in the dark and the appearance of these enzymes in the presence of phytochrome are independent phenomena and that the effect of HS on the appearance of PAL and AO is specific for the particular enzyme and cannot be understood in terms of a non-specific stimulation of protein synthesis, intermediary metabolism, phytochrome synthesis or phytochrome action. It is realized, of course, that other (more complicated) interpretations of the data are not ruled out. However, irrespective of interpretation the data indicate that the *a priori* assumption that appearance of enzyme activity in the presence of phytochrome is simply a modulation of the basal enzyme level may not be made uncritically. Rather, the investigator must *prove* explicitly that this assumption is justified in his particular approach. The conclusion that dark and light-mediated syntheses are probably not related to each other was previously reached by Craker and Wetherbee [25] with respect to anthocyanin synthesis in red cabbage seedlings. These investigators found that treatment of the dark grown seedlings with ethylene had no effect on the amount of anthocyanin synthesized. However, ethylene had a profound effect on light-mediated anthocyanin synthesis in this system. It was concluded that the ethylene effect is mediated only through the light-initiated anthocyanin synthesis pathway. Data reported by Cheng and Marsh [26] are possibly also relevant for the present discussion. The investigators found that the activity of PAL and the extent of lignification was higher in gibberellin-treated dwarf pea plants grown under white or red light than in untreated dwarf plants. Gibberellic acid had no detectable effect on the activity of this enzyme and on lignification when the plants were grown in darkness. It was only in light that gibberellin had an effect: Under these conditions both lignification and PAL activity were promoted by the hormone.

Attridge and Smith [27] reported differences in properties of PAL extracted from the buds of 7-day-old dark-grown pea seedlings and the buds of 7-day-old dark-grown pea seedlings treated with 6 h white light. The extractable PAL activity in dark-grown pea seedlings was low, approximately 15% of that extracted from seedlings treated with 6 h white light. The authors have suggested that the differences in properties of PAL (differential inhibition by quercetin and differential loss of activity in dialysis) might be associated with a light-mediated activation of the enzyme. However, the data are equally consistent with the concept, that the light-induced enzyme activity results from the *de novo* synthesis of a PAL with slightly different properties possibly occuring in different cells or tissues. As far as the mustard seedling is concerned no indications of isoenzymes of PAL and AO in the organs of the mustard seedling were found [14, 28]. In disc electrophoresis on polyacrylamide gel PAL and AO move as a single homogeneous band under all circumstances of dark and far-red light treatment. These results do not exclude, of course, that the dark enzyme and the corresponding light-dependent

enzyme from mustard slightly differ in properties in the same way as those tested by Attridge and Smith [27]. The consequences of these conclusions for the "theory" of density labelling of "inducible" enzymes will now be considered briefly. The point is whether density labelling of an enzyme in the dark can be used as a system of reference for the density labelling in the light. In a recent paper, Attridge *et al.* (1974)[11] point out: "Where there is a significant basal level of enzyme activity, density labelling can be employed to test whether synthesis is occuring in the unstimulated material. An unequivocal demonstration of an increase in the rate of enzyme synthesis in response to the stimulus can only be obtained from a comparison of the two treatments" (in the present case, dark and far-red light). If the appearance of an enzyme activity in the dark and in the light are unrelated phenomena, this assumption is not justified. Interpretations of density labelling data based on this assumption may be misleading. It is possible, *e. g.*, that only certain cells or tissues respond to the light stimulus while some other cells produce the enzyme in the dark. In this case the possibility of synthesis of "bulk" and "basal" enzymes from essentially separate amino acid pools may not be overlooked. Blondel *et al.* (1973)[29] and Rollin (personal communication) have suggested that active PAL in light-grown radish cotyledons results from an inactive protein present in large amounts in the dark-grown cotyledons and even in the seed. It would require complicated (and improbable) assumptions to reconcile the present data (in particular Fig. 2) with this concept. We recall that the onset of far-red light mediated PAL increase (at approximately 29 h after sowing) is always the same, irrespective of HS and irrespective of the onset of light. On the other hand, phytochrome is present in considerable amounts much earlier (*cf.* Fig. 4), and there are numerous data, *e. g.* [30], which indicate that P_{fr} is fully active at 24 h and even earlier. It is more plausible to pursue the concept [2] that the increase of PAL activity in the mustard cotyledons reflects *de novo* synthesis of PAL and that the appearance of competence (at 29 h) reflects a step of primary differentiation, *i. e.* the gene for PAL becomes available for transcription and/or the messenger for PAL becomes available for translation. In the case of AO the few available data [14] also suggest that the increase of extractable enzyme activity is due to *de novo* synthesis of enzyme molecules.

We admit, of course, that the crucial experiments *pro* or *contra de novo* synthesis must be done by some sort of labelling. However, the data of the present paper should be considered in order to avoid a mis-interpretation of the data obtained in density labelling experiments.

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