Control by Phytochrome of the Level of Nicotinamide Nucleotides in the Cotyledons of the Mustard Seedling *

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(Z. Naturforsch. **29 c**, 392–398 [1974]; received April 4, 1974)

Phytochrome, Nicotinamide Nucleotides, Mustard Seedlings

Long-term and short-term effects of phytochrome on the levels ("tissue contents") of NAD, $NADH_2$, NADP and $NADPH_2$ were measured in the cotyledons of the mustard (*Sinapis alba* L.) seedling. It was found that long-term far-red light (which is considered to operate exclusively *via* active phytochrome, P_{fr}) strongly increases the levels of NADP and $NADPH_2$, whereas this light treatment suppresses the levels of NAD and $NADH_2$ below the levels present in the cotyledons of the dark grown seedling. The high levels of NADP and $NADPH_2$ as well as the low levels of $NADP_2$ (and probably also NAD) are actively being maintaned by the far-red light. In experiments with light pulses it was found that a red light pulse causes a rapid but transient rise in the level of $NADPH_2$ under all circumstances (dark-grown seedlings and seedlings pretreated with long-term far-red light). The operational criteria for the involvement of phytochrome (P_{fr}) are fulfilled. In the case of NADP a significant rise of the level following a red light pulse could only be observed if the seedlings were pretreated with long-term far-red light. Dark-grown seedlings do not significantly respond. In the case of NAD and $NADP_2$ no significant changes could be induced by light pulses either in dark-grown seedlings or in seedlings pretreated with long-term far-red light. It is concluded that $NADPH_2$ does neither originate from $NADP_2$ nor from NADP. It is further concluded that it is unlikely that nicotinamide nucleotides are links in any causal chain originating from P_{fr} and leading to phenomena of photomorphogenesis. We favour the concept that the phytochrome-mediated changes caused by light pulses occur in the plastids.

It is well established that many biochemical changes in developing plants are controlled by phytochrome [1]. However, our knowledge about the causal sequence between active phytochrome (P_{fr}) ** and the biochemical photoresponses which are actually being measured is still quite limited. Recent reports [2–4] suggest that in some cases rapid changes in the level of nicotinamide nucleotides might be intermediary events in the causal chain between phytochrome (P_{fr}) and the final photoresponses. Tezuka and Yamamoto [3] reported that in light-grown seedlings of Pharbitis nil a brief irradiation with red light in the middle of the night period will raise the level of NADP. This effect was attributed to a phytochrome control of NAD kinase. The $NADPH_2$ level was not appreciably affected by the red light treatment. On the other hand, Fujii and Kondo [2] observed a red light mediated increase of the $NADPH_2$ level in etiolated oat tissue. Manabe and Furuya [4] reported that a rapid phytochrome-mediated stimulation of $NADPH_2$ formation from NADP will occur in a particle fraction isolated from etiolated bean hypocotyl. The aim of the present investigation was to examine long-term and short-term effect of phytochrome (P_{fr}) on the levels on NAD, $NADP_2$, NADP, and $NADPH_2$ in the cotyledons of the mustard *(Sinapis alba* L.) seedling. Phytochrome-mediated photomorphogenesis of the mustard seedling has been thoroughly investigated also at the molecular level [1, 5]. However, so far no information is available about the extent to which photochrome-metiated changes in the level of nicotinamide nucleotides occur in the mustard seeedling and whether such changes can be causally related to the phenomenon of photomorphogenesis.

Material und Methods

Standard techniques for photomorphogenic research with mustard seedlings were used [6]. The seeds of *Sinapis alba* L. were purchased in 1969 from Asgrow Company (Hamburg, Germany). The

* Herrn Prof. H. Borris, Greifswald, mit guten Wünschen zum 65. Geburtstag.

** *Abbreviation:* P_{fr}, far-red absorbing form of the phytochrome system.

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seedlings were grown at 25.0 ± 0.2 °C in the dark and experiments were started 36 h after sowing which is taken as time zero in the figures.

The standard far-red source [6] which maintains a low P_{fr}/P_{total} ratio in the seedlings [7, 8] was used at an irradiance of 3.5 $W \cdot m^{-2}$. The standard red source [6] which maintains a P_{fr}/P_{total} ratio of about 0.8 [7] was used at an irradiance of 675 $mW \cdot m^{-2}$. Under continuous far-red light (onset 36 h after sowing) a steady state of the photochrome system can be maintained in the mustard cotyledons and hypocotylar hook over at least 11 h [8]. Continuous far-red light is considered to operate exclusively *via* phytochrome (P_{fr}) [1, 9]. The time requirement for the shift from red light to far-red light and *vice versa* was approximately 1 min.

The use of the biological unit "pair of cotyledons" as a system of reference for the biochemical data was justified previously (*cf.* [1]). The major arguments in favour of the biological unit as a system of reference are the following: 1. During the experimental period (between 36 and 60 h after sowing) there is no significant increase of the DNA contents in the cotyledons [10]. For this reason the biological unit "cotyledon" can be used as a system of reference instead of "cell" or "unit DDA". There are enzymes, *e. g.* isocitrate lyase [11], in the mustard cotyledons whose temporal development, *i. e.* increase and decline of extractable activity, is not influenced by phytochrome even if continuous far-red light is applied throughout the experimental period. The occurence of enzymes whose rise and fall of extractable activity is phytochrome-independent shows that the phenomena of photomorphogenesis (photoresponses) are specific and that phytochrome control of development of an organ does not affect every aspect of metabolism and not even protein synthesis as a whole.

The data to be reported represent the nicotinamide nucleotide contents of whole cotyledons ("tissue contents") without considering compartmentation and in situ binding of the nucleotides to protein. These disadvantages cannot be overcome at present since more sophisticated indirect measurements of the redox state of the NAD- and NADP-couples in rat liver cytoplasm and mitchondria [12, 13] cannot readily be applied to plant tissue. Moreover the significance of these results and deductions is still under debate [14].

Extraction and determination of nicotinamide nucleotides: 40 seedlings were harvested under dim green safelight and 20 seedlings each were selected for extracting the oxidized or reduced nucleotides respectively. The organs (pairs of cotyledons, hypocotyls and roots) were isolated and frozen to liquid nitrogen temperatures in a precooled 9 ml teflon cell. The time needed for isolating the organs was about 15 min. The frozen organs were reduced to a power together with a 9 mm tungsten carbide ball (pre-colled) for 20 sec in a Micro-Dismembrator (Braun Melsungen A.G., Melsungen, West Germany). Extraction was accomplished by adding 3.5 ml 50% alcoholic 0.1 N HCl (oxidized nucleotides) or 3.5 ml 50% alcoholic 0.1 N KOH (reduced nucleotides) and immediately reshaking for 10 sec. The then liquified axtracts were quantitatively transferred to centrifuge tubes and kept at 60 °C for 5 min. They were subsequently cooled to 0 °C. After centrifugation for 10 min at 39 100 × *g* and 0 °C, 2.5 ml of the supernatant was removed and 0.5 ml buffer (0.5 M triethanolamine, 0.4 M KH_2PO_4, 0.1 M $K_2HPO_4 \cdot 3\,H_2O$) added. The buffered extract of the oxidized nucleotides was adjusted to pH 7.2 – 7.4 with 6 N KOH; the extract of the reduced nucleotides was adjusted to pH 7.4 – 7.6 with 1 N HCl. After centrifugation for 10 min at 39 100 × *g* and 0 °C the clear supernatant was decanted and used in the assay.

Nucleotide determination

NAD(H_2) and NADP(H_2) wase assayed after a modified method of Slater and Lawyer [15]. The oxidized nucleotides allow the oxidation of alcohol with alcohol dehydrogenase (NAD) or the oxidation of glucose-6-phosphate with glucose-6-phosphate dehydrogenase (NADP). The nucleotides which are reduced during these reactions are reoxidized through phenazine-methosulfate while the hydrogen is accepted by 2,6-dichlorophenolindophenole. The reduction of 2,6-dichlorophenolindophenole was proportional to the concentration of NAD + $NADH_2$ or NADP + $NADPH_2$. The assays were carried out in semimicro cuvettes at 25 °C and the reactions followed with an Eppendorf photometer (Hamburg, West Germany) at 546 nm. The concentrations of the nucleotides were calculated from standards run at the same time. The reaction mixtures for NAD(H_2) contained in a test volume of 1.06 ml: 0.17 mmol phosphate buffer pH 7.4; 0.857 mmol ethanol; 0.185 μmol 2,6-dichloropheiolindophenole; 0.8 μmol phenazine-methosulfate; 60 U alcohol dehydrogenase. For NAD 20 μl – 200 μl extract and for $NADH_2$ 50 μl-200 μl extract were added and the difference in volume was adjusted by adding the appropriate amount of neutralized 50% alcoholic 0.1 N HCl or neutralized 50% alcoholic 0.1 N KOH. The reaction mixtures for NADP(H_2) contained in a test volume of 1.02 ml: 0.07 mmol tris buffer pH 8.0; 0.185 μmol 2,6-dichlorophenolindophenole; 0.8 μmol phenazine-methosulfate; 2 μmol

glucose-6-phosphate; 1.4 U glucose-6-phosphate-dehydrogenase. The test mixture for NADP contained 2×10^{-2} mmol neutralized 50% alcohol 0.1 N KOH. 50 μl – 200 μl extract were used for both NADP and $NADPH_2$ and the difference in volume was adjusted by adding the appropriate amount of neutralized 50% alcoholic 0.1 N HCl or neutralized 50% alcoholic 0.1 N KOH.

The following results were obtained in methodological experiments:

1. The kinetics of the test reaction are linear over 20 min. The velocity of the reaction is proportional to the nicotinamide nucleotide concentration up to 0.15 nmol/reaction mixture.

2. The recovery of standards added during the extraction was between 94% and 102% and proportional to the amount added.

3. Mixing experiments show that light treatment does not influence the assay of the nicotinamide nucleotides through formation of inhibitors or activators. Standards added to the reaction mixture are additive.

4. The nicotinamide nucleotide concentration is proportional to the number of seedlings or organs.

The average values given in the Figures are based on 8 – 12 independent experiment. Standard errors, as indicated by the bars, are in the range of 2 to 7 per cent.

Results

1. Long-term exposure of the seedlings to standard far-red light (onset of light at 36 h after sowing). Continuous standard far-red light which is considered to operate exclusively *via* phytochrome (P_{fr}) [1, 9, 16] leads to the following effects on the level of nicotinamide nucleotides in the cotyledons: The levls of NADP and $NADPH_2$ are strongly increased as compared to those in the dark-grown seedling (Figs 1, 2); the increase of the levels of NAD and $NADH_2$ is retarded as compared to those in the dark-grown seedling (Figs 3, 4). If the far-red light is turned off at 12 h after the onset of light there is a rapid drop in the levels of NADP and $NADPH_2$ (Figs 1, 2). In the case of $NADH_2$ the level is increased after the light was turned off (Fig. 4) while in the case of NAD no significant change could be found (Fig. 3).

2. Short-term exposure of the seedlings to standard red and far-red light (5 min light pulses). Figs 2 and 5 show that a red light pulse causes a rapid but transient rise in the level of $NADPH_2$. This effect can always be obtained irrespective of the time of treatment. However, if the seedling was pretreated with long-term far-red light the peak rise is higher. Since the operational criteria for the involvement of phytochrome are clearly fulfilled (reversion of the inductive effect of a red light pulse by immediately following with a far-red light pulse)

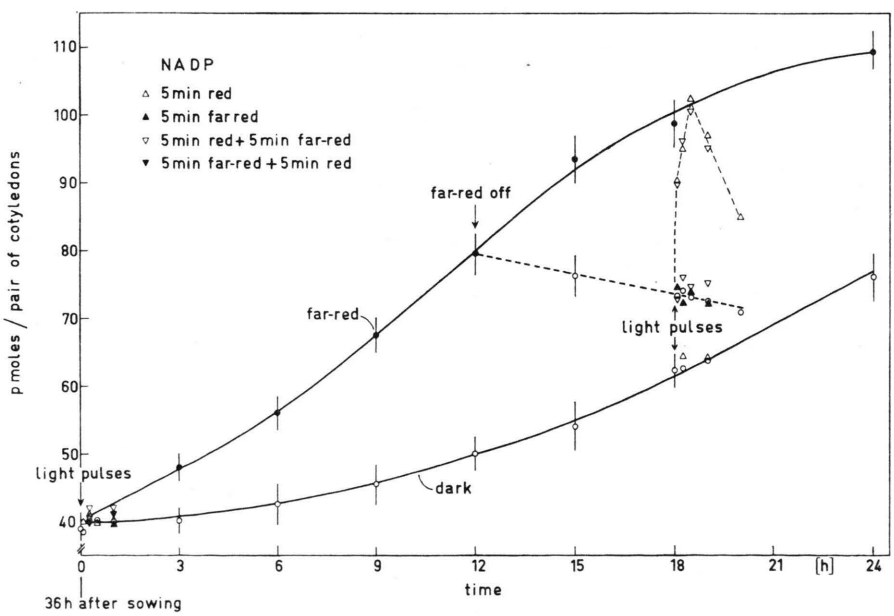


Fig. 1. Kinetics of NADP contents in the mustard cotyledons in the dark and under continuous far-red light (onset of light at 36 h after sowing = time zero). Light pulses (5 min each) with red and/or far-red light were given at time zero and at 18 h (following the sequence: 12 h far-red + 6 h dark).

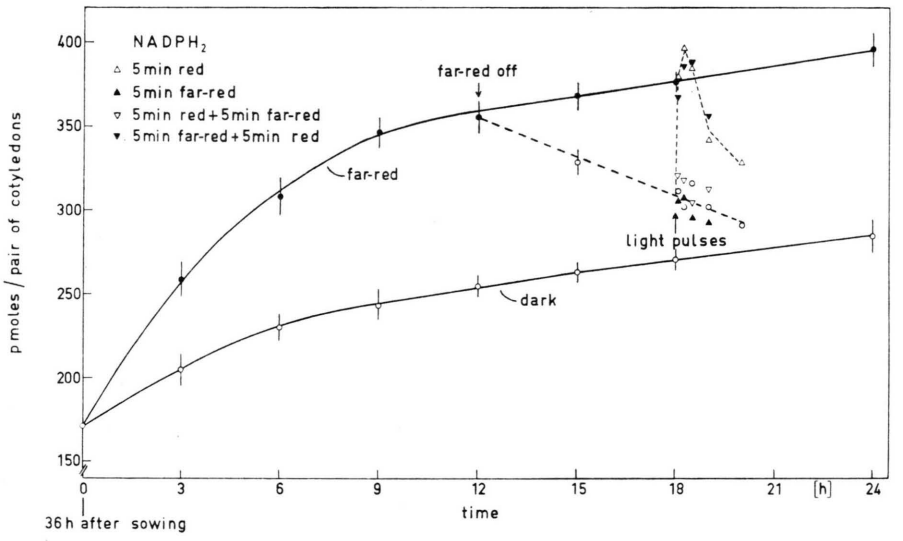
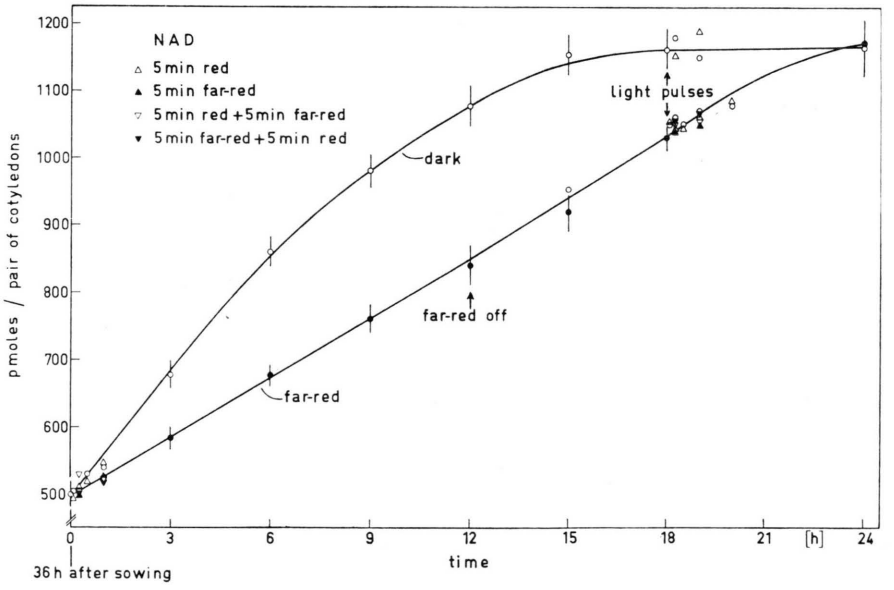


Fig. 2. Kinetics of $NADPH_2$ contents in the mustard cotyledons in the dark and under continuous far-red light (onset of light at 36 h after sowing = time zero). Light pulses (5 min each) wtih red and/or far-red light were given at 18 h following the sequence, 12 h far-red — 6 h dark.

Fig. 3. Kinetics of NAD contents in the mustard cotyledons in the dark and under continuous far-red light (onset of light at 36 h after sowing = time zero). Light pulses (5 min each) with red and/or far-red light were given at time zero and at 18 h.



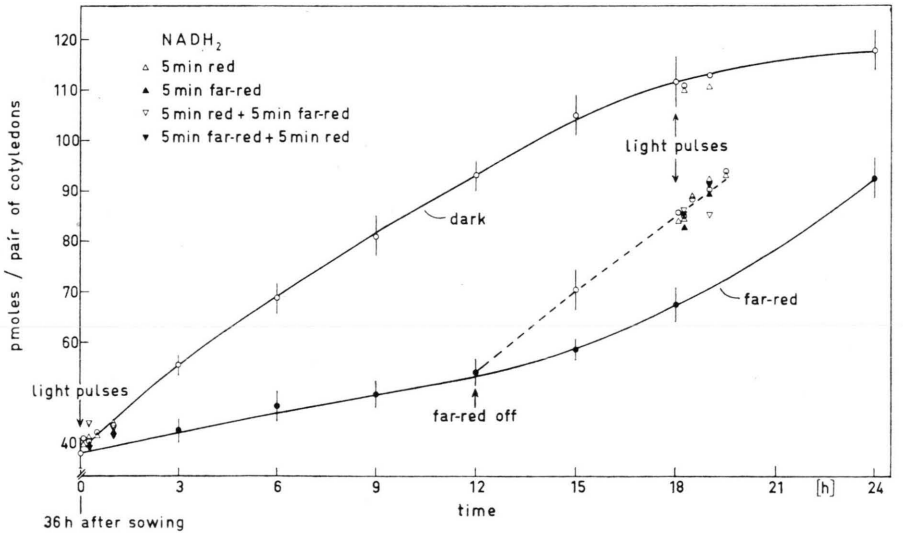


Fig. 4. Kinetics of $NADH_2$ contents in the mustard cotyledons in the dark and under continuous far-red light (onset of light at 36 h after sowing = time zero). Light pulses (5 min each) with red and/or far-red light were given at time zero and at 18 h and following the sequence 12 h far-red — 6 h dark.

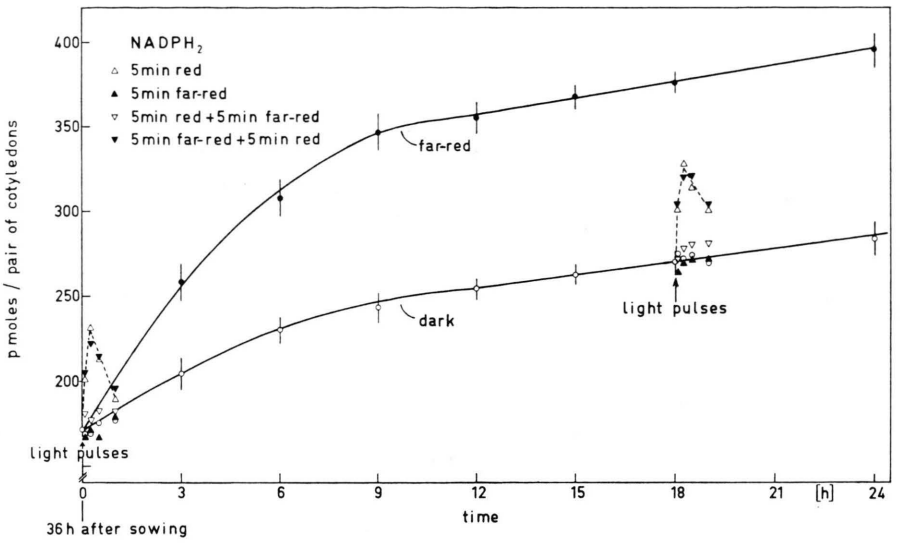


Fig. 5. Kinetics of $NADPH_2$ contents in the mustard cotyledons (*cf.* Fig. 2). Light pulses (5 min each) with red and/or far-red light were given at time zero and at 18 h in the dark.

it can be concluded that the appearance of P_{fr} rapidly leads to a transient increase of the $NADPH_2$ level in the mustard cotyledons. At this point it must be remembered (*cf.* Methods) that for technical reasons there is a time lag of approximately 15 min between the end of the light treatment and the freezing of the isolated cotyledons to liquid nitrogen temperature. This time lag which cannot be shortened further precludes more precise statements about the actual rapidity of the P_{fr}-mediated response.

In the case of NADP (Fig. 1) a significant rise of the level following a red light pulse could only be observed if the seedlings were pretreated with long-term far-red light. Dark-grown seedlings do not respond significantly at either 36 h after sowing or at 54 h after sowing. In the case of NAD and $NADH_2$ (Figs 3 and 4) no significant changes could be induced by light pulses either in dark-grown seedlings or in seedlings pretreated with long-term far-red light.

3. Nicotinamide nucleotide levels in hypocotyl and radicle (taproot). The changes caused by long-term far-red light in the cotyledons can be observed in the other major organs of the mustard seedling only as a tendency. As an example Figs 6 and 7 show the amounts of $NADPH_2$ and $NADH_2$ in hypocotyl and radicle of the dark grown and far-red light grown mustard seedling. We notice that in the radicle (which has no potential to develop chloroplasts) hardly any increase of the $NADPH_2$ level is caused by the far-red light treatment.

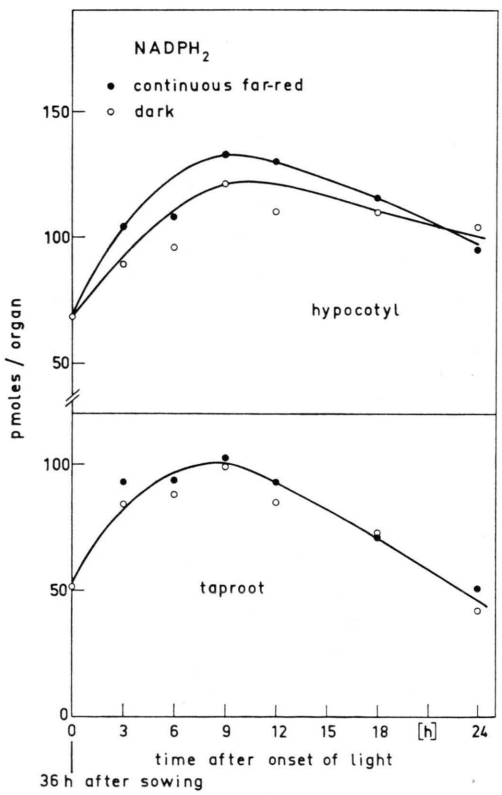


Fig. 6. Kinetics of the $NADPH_2$ contents in the mustard hypocotyl and radicle in the dark and under continuous far-red light (onset of light at 36 h after sowing = time zero).

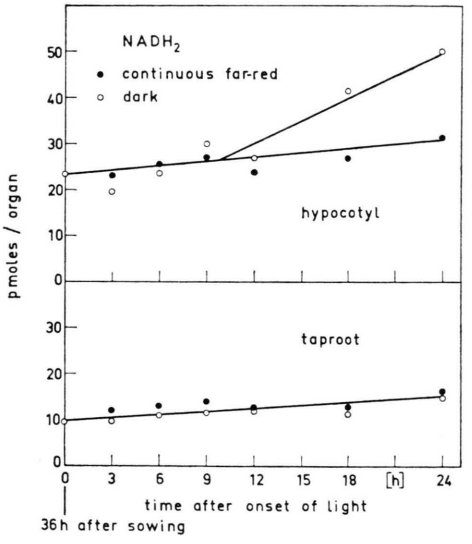


Fig. 7. Kinetics of the $NADH_2$ contents in the mustard hypocotyl and radicle in the dark and under continuous far-red light onset of light at 36 h after sowing = time zero).

Discussion

The present work shows that long-term far-red light which is considered to operate exclusively *via* phytochrome (P_{fr}) [1, 9, 16] controls the levels of NAD, $NADH_2$, NADP and $NADPH_2$ in the mustard seedling cotyledons. While the "mechanism" of this control is still obscure the data suggest that the increase of NADP and $NADPH_2$ mediated by far-red light is related to the far-red mediated growth of the plastids [17, 1]. We assume that the NADP and $NADPH_2$ "induced" by far-red light is predominantly located in the growing plastids. The suppressive effect of far-red light on the level of NAD and $NADH_2$ is possibly related to the fact observed previously [18, 19] that long-term far-red light transiently depresses the rate of respiration of the mustard seedling cotyledons.

Figs 1 and 2 document that the high level of NADP and $NADPH_2$ is maintained by the far-red light. If the far-red light is turned off, the level decreases and nearly reaches the dark level within 8 h. In th case of $NADH_2$ (Fig. 4) it is also obvious that the *low* level in far-red light is maintained by the light since the level tends to increase as soon as the light is turned off. It is concluded that the actual level of the nicotinamide nucleotides in the mustard cotyledons is determined throughout the experimental period by the rates of synthesis and destruction. The data are consistent with the view that far-red light increases the rate of synthesis in the case of NADP and $NADPH_2$ whereas the light increases the rate of destruction in the case of $NADH_2$ (and possibly of NAD). It is well known [12–14] that the interpretation of the measured ratios of reduced and oxidized forms of the nicotinamide nucleotides remains a matter of debate even in the case of liver tissue in particular since the redox ratios of free nicotinamide nucleotides associated with various dehydrogenase reactions in the cytoplasm and in the mitochondria are different. For technical reasons in the present work only the "tissue contents" of the nicotinamide nucleotides could be determined. Therefore the notorious difficulties – the distinction between free and bound nucleotides and the uneven distribution within the cell (cytoplasm, mitochondria, plastids) – could not be overcome. Thus any attempt to interpret the measured ratios of the NAD- and NADP-couples at the present stage of our work would probably lead to an overinterpretation of the data. One may only notice that the ratios for the NADP-couple (of the order of $4-5$) and for the NAD-couple (of the order of $1/10-1/20$) differ greatly. This confirms the general observation, *e. g.* with liver tissue using the substrate couple method [12, 13], that the NADP-couple is much more reduced *in situ* than the NAD-couple.

Concerning the effects of light pulses we confirm previous reports that red light pulses will raise the *in situ* levels of NADP [3] and $NADPH_2$ [2]. However, we question the interpretation given previously [3, 4] that the effect of a red light pulse is to be attributed to a phytochrome control of NAD kinase since no corresponding decreases of the NAD and/or $NADH_2$ levels could be detected. At least in the case of $NADH_2$ the decrease of the level vould easily be detected if the rapid rise of the $NADPH_2$ level was due to a rapid phosphorylation of $NADH_2$. While the interpretation of the previous investigators favouring a phytochrome activation of NAD kinases is probably not correct at least in case of the mustard seedling, it is difficult to suggest an alternative mechanism for the rapid rise of the NADP and $NADPH_2$ levels upon the formation of P_{fr}. At present no substantiated suggestion for the underlying mechanism can be made.

The results of the present paper sem to exclude NAD and $NADH_2$ as links in any causal chain be-

tween P_{fr} and the final biochemical photoresponses such as end product accumulation, enzyme "induction" and enzyme "repression" (*cf.* [1]). The fact that the NAD-couple does not significantly respond to light pulses makes it unlikely that it is a direct link (close to P_{fr}) in some causal chain between P_{fr} and the final photoresponses.

In the case of NADP/$NADPH_2$ the fact is that both forms of the couple respond strongly and rapidly upon the formation of P_{fr} in the system by a red light pulse. The response is positive in both cases. This excluedes the possibility that $NADPH_2$ originates from NADP as suggested by *in vitro* data recently reported by Manabe and Furuya [4]. We recall (Fig. 1) that the NADP level does not significantly respond if the red light pulse is given to a dark-grown seedling which has not received any light treatment before. It is only the $NADPH_2$ level which responds to P_{fr} under all circumstances in principally the same manner. Thus one may consider only $NADPH_2$ as a candidate for a link in the causal chain originating from P_{fr}. However, we must recall that the $NADPH_2$ level changes with endogenous rhythms [20–22] and with variation in many environmental conditions (*cf.* [3]). In addition by its very nature as a basic metabolite with rapid turnover $NADPH_2$ would not be a likely candidate for a very specific [1] and very precise [23, 24] metabolic transformation of the P_{fr} signal eventually leading to photomorphogenetic phenomena. Therefore an alternative interpretation of the P_{fr} effect upon the $NADPH_2$ level must be considered.

We suggest that the changes of the NADP-couple following red light pulse treatments reflect a control by phytochrome of some parts of the photosynthetic apparatus. Most of the constituents of the photosynthetic apparatus except chlorophyll are already present in the plastids of dark-grown or far-red light grown mustard seedlings (*cf.* [1]). There are other data in print which possibly require a similar interpretation, *e.g.*, the red far-red antagonistic effects observed by Michel and Thibaut [25] in kinetic studies on *in vivo* ATP synthesis in detached *Zea mays* leaves. The data suggest that photosynthetic phosphorylation is under the control of phytochrome.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 46). The competent technical assistance of Miss R. Loyal is gratefully acknowledged. We thank Mrs. Gloria Acton for checking of the manuscript. We are obliged to our colleagues at the Institute for critically reading the manuscript.

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