Enzymic Capacities for Chlorophyll Biosynthesis. Activation and *de novo* Synthesis of Enzymes

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(Z. Naturforsch. **31 c**, 55–63 [1976]; received September 1, 1975)

δ-Aminolaevulinic Acid Synthese, δ-Aminolaevulinic Acid Dehydratase, Porphobilinogenase, Protochlorophyllide, Cotyledons of *Helianthus*

A previously published working model for the regulation of chlorophyll formation has been tested studying early steps of chlorophyll and porphyrin biosynthesis in developing cotyledons of *Helianthus annuus*. The activities of δ-aminolevulinate synthetase (ALAS), δ-aminolevulinate dehydratase (ALAD), and the porphobilinogenase complex (PBGase) at any given time have been found to be strongly associated with endogenous developmental processes. Highest activities in darkness have been observed at times when maximum chlorophyll formation would have occurred had the plants been exposed to light. Only in the case of ALAS was the maximum activity in light much greater than that observed in the dark.

Density labeling experiments and other data suggest that enzyme synthesis is mediated both by development and by illumination. Moreover, ALAS activity appears to be subject to inhibition, presumably by products of the porphyrin biosynthesis, as indicated by halflife experiments. Rapid enzyme degradation in the absence of light seems to be less probable. Slight ALAS activity in darkness is present as long as the plastids are not fully developed.

In contrast to findings with cell cultures of tobacco, in *Helianthus* cotyledons ALAS certainly plays the main role in the regulation of chlorophyll biosynthesis. Nevertheless, increasing activities of the succeeding enzymes, located in the plastids, ensure that increased concentrations of δ-aminolevulinate (ALA) are drawn into the chlorophyll biosynthetic pathway. The experiments corroborate the suggestion that chlorophyll biosynthesis is controlled by different but interdependent mechanisms. The dominant regulatory mechanism is dependent on the stage of development.

Introduction

Recently a tentative working model for the regulation of chlorophyll biosynthesis has been published [1, 2]. It is based on new results and integrates other known observations concerning the influence of light and inhibitors of nucleic acid and protein biosynthesis on chlorophyll formation. The following new informations have been made available: In contrast to general postulations, the first enzyme of porphyrin and chlorophyll biosynthesis, δ-aminolevulinate synthetase (ALAS), which is thought to be the most essential enzyme in the regulation of chlorophyll biosynthesis, is active without illumination in certain organs of higher plants [3]. This is also true of cell cultures of tobacco containing leucoplasts and amyloplasts [4]. In leaves possessing etioplasts, however, ALAS activity can only be demonstrated during illumination of the leaves [3, 5, 6]: only a few hours of illumination are needed. In tobacco cells, however, increasing ALAS activities have been shown only after illumination for some days [4].

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The activities of later enzymes of chlorophyll biosynthesis, δ-aminolevulinate dehydratase and the porphobilinogenase complex (ALAD and PBGase), have also been found to be light-dependent in tobacco cells [7, 8] but are little affected or unaffected by light in corn leaves [3]. These conflicting observations led to the assumption that activities of chlorophyll-synthesizing enzymes in higher plants are affected not only by illumination but also by developmental processes in the cell and the plastids (for detailed discussion see 2). This view is supported by the fact that enzymes of chlorophyll biosynthesis are located in the plastids but synthesized in the cytoplasm [2, 9, 10]. Chlorophyll biosynthesis should therefore be dependent on both cell and plastid development. Consequently light-mediated enzyme inductions may be regarded as modifications of a developmental process regulated by endogenous factors *via* transcription and translation as demonstrated by experiments including inhibitors of nucleic acid and protein synthesis (for literature see 2).

This model for the regulation of chlorophyll biosynthesis and the hypotheses involved in it are tested in the experiments of the present paper. The paper is concerned with enzyme induction during a deve-

lopmental process which can be assumed to include transformations from early plastid development to etioplasts and chloroplasts. Further experiments with density labelling of enzymes are hoped to give new information about the nature of light-induced enzyme activity. Particularly we asked the following questions: Firstly, are increasing enzyme activities caused by enzyme activation or enzyme synthesis? Secondly, are decreasing enzyme activities caused by enzyme degradation or inhibition?

Experimental

Values presented are the means of four to eight independent experiments.

Germination

The experiment were performed with cotyledons of *Helianthus annuus*. Achenes were soaked for 20 hours then placed on moist leaf-mould and lightly covered with sand. To get a constant climate, the dishes with the achenes were covered by clear or dark plastic boxes, respectively. The temperature was adjusted to 25 °C, light intensity to 3500 lx.

Determination of ALAS activity

Cotyledons were cut from the seedlings in the dark or in the light, according to the experimental program, and transferred to petri-dishes (10 cm in diameter) lined with filter paper and filled with 20 ml of levulinic acid 2×10^{-2} mol/l) solution adjusted to pH 5.2 with NaOH. After equilibrating for 15 min, the experiment was started (under 3500 lx) and was finished by transferring the cotyledons (x grams) to a test tube containing 0.2 M sodium acetate buffer pH 4.6 (3 to 5 x ml) and acetylacetone (0.1 ml/ml). The mixture was heated to 98 °C for 10 min (see ref. 11), cooled and centrifuged. The supernatant was finally mixed with Ehrlich's reagent (1:1) and the extinction of the coloured ALA pyrrole read at 555 nm after 10 min [11].

Determination of ALAD and PBGase activity

Cotyledons were ground in a cooled mortar with sand and a solution containing 2.5 ml of tris (1 M) and 0.2 ml of mercaptoethanol per 100 ml of distilled water: caution must be exercised because freezing destroys ALAD. The slurry was filtered under pressure and centrifuged. Since the enzyme activities are normally high and only small quantities (10 μl) of filtrate are necessary for enzyme assays, purification by gelt centrifugation could sometimes be omitted.

The assay mixture for ALAD (see ref. 8) contained: 0.5 ml of Tris-HCl buffer (pH 8.2, 0.2 M), 0.05 ml of $MgCl_2$ (0.1 M), 0.05 ml of mercaptoethanol (0.1 M), 0.1 ml of ALA (2 mg/ml), H_2O and enzyme to a final volume of 1 ml.

For PBGase (see ref. 8) the reaction mixture contained: 0.5 ml of Tris-HCl buffer (pH 8.2, 0.2 M), 0.1 ml of EDTA (0.025 M, adjusted to pH 8.2), 0.05 ml of PBG (1 mg/ml, solved by a small amount of tris), H_2O and enzyme to a final volume of 1 ml.

Incubation at 32 °C was terminated by addition of 0.1 ml of TCA (3 M) and 0.1 ml of $HgCl_2$ (0.1 M). After centrifugation the supernatants were diluted (1:1) with Ehrlich's reagent for PBG assays and 5 N HCl for Urogen assays, respectively. PBG was estimated after reading the extinction at 555 nm 10 min after mixing [12]. Urogen was estimated 2 – 3 hours later after autoxidation to uroporphyrin by reading at 406 nm [13]. PBG values were corrected for losses due to conversion to urogen.

Labelling with deuterium

The achenes were soaked for 20 hours in D_2O and then placed in petri-dishes lined with filter paper moistened with D_2O. The same procedure was performed with H_2O controls. The temperature was adjusted to 25 °C. The percentage of germinating achenes in D_2O was less than in H_2O.

Deuterium label of the enzymes was determined by CsCl density gradient centrifugation. Enzyme extracts were prepared as described under "determination of ALAD and PBGase activity".

Before the 72-hour centrifugation at 10^5 g in a Spinco SW 39 rotor, the gradients consisted of two layers of CsCl (0.5 g/2.2 ml and 1.65 g/2.2 ml) in buffer (0.05 M Tris-HCl, pH 8.2; 0.05 M mercaptoethanol). The upper layer was mixed with aliquots of enzyme extract. To shorten centrifugation time, it proved advantageous to mix the gradient before centrifugation and thus the two concentrations were used to compose a linear gradient which was centrifuged over night. Then the centrifuge tubes were punctured and single drops collected in test tubes. Every second drop was used to determine the refractive index which is linearely correlated to density by the formula: $\varrho_{(25°)} = 10.8601\, n_{D(25°)} - 13.4974$ [14].

Care must be taken not to overload the gradients: overloading causes an apparent shift to higher densities despite extrapolation of the refractive index.

Determination of growth parameters

Protochlorophyllide, chlorophylls and carotenoids were extracted from the cotyledons by 90 percent

acteone. The spectra were recorded on a Spectronic 606 spectrophotometer and the extinction read at 626 nm (protochlorophyllide), 345 and 663 nm (chlorophylls) and 436 nm (carotenoids). For protochlorophyllide an extinction coefficient of 39.9 l/g·cm was used [15] and for carotenoids a specific extinction coefficient $E^{1\%}_{1\,cm}$ of 2000 was assumed (see ref. 16). Chlorophyll determination was done by means of nomogram [17].

Soluble protein was determined by a modified Lowry [18] method. Soluble protein was precipitated in 0.3 M TCA, centrifuged and then the pellet solubilized by 0.2 ml of 1% Na-dodecylsulfate. 1 ml of a reagent (4 g of Na_2CO_3 in 50 ml of 0.1 N NaOH + 5 mg of $CuSO_4 \cdot 5\,H_2O$ and 10 mg of K-Na tartrate in 1 ml of 0.1 N NaOH) was added. After 10 min the sample was mixed with 0.1 ml of Folin-Ciocalteus reagent adzusted to 1 N acid. 30 min thereafter the extinction was read at 578 nm and compared with a standard (100 μg $\approx$ ext. 0.7).

Results

Development and parameters of development

Enzyme activities are commonly given per unit weight of protein. Using developing organs such as cotyledons, however, there are difficulties in interpreting such values. During development neither the protein level nor any other possible reference remains constant. Therefore it seems best to refer enzyme activities to the biological unit of one cotyledon and to characterize development and growth by several parameters, for example by fresh weight, the quantity of protochlorophyllide and chlorophyll, and by soluble protein content.

Changes in fresh weight and pigment content of the cotyledons have been followed for 7 to 8 days (Fig. 1). Zero time values were obtained with dry cotyledons withdrawn from the achenes; first day values were obtained with soaked cotyledons. First light effects can be seen on about the 3rd day.

Cotyledons under illumination expand more rapidly than those in darkness due to morphogenetic and later to photosynthetic effects (Fig. 1 a). After a growth period of 7 days fresh weights for light-grown cotyledons are twice the values of dark-grown cotyledons. Traces of protochlorophyllide have been detected at the 3rd day of development in the dark, traces of carotenoids at the 2nd day (Fig. 1 b). The following days, up to the 5th day, are characterized by high accumulation rates of these pigments; subsequently accumulation rates decrease. Chlorophyll

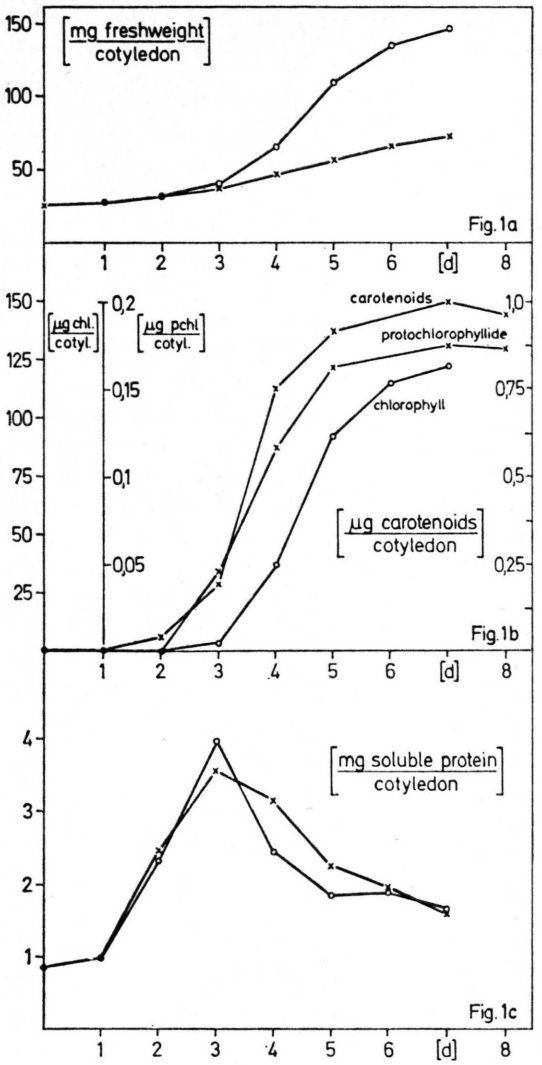

Fig. 1. Fresh weight, protochlorophyllide, carotenoids and chlorophyll content, and soluble protein in cotyledons of *Helianthus* under the influence of development and light. ○, Experiments with cotyledons grown under continous illumination; ×, experiments with cotyledons grown in darkness.

accumulation rates under illumination follow a similar time course. Protein content of the cotyledons has almost been unaffected by light treatment. Soluble protein content (Fig. 1 c) reaches a maximum at the 3rd day indicating a general activation of metabolic processes. The peak may be less sharp with dark-grown cotyledons. Total protein continuously decreases with the most rapid decrease occurring in the first days of development (not shown).

Development and the activity of δ-aminolevulinate synthetase (ALAS)

ALAS has always been thought to play an important role in the regulation of chlorophyll biosynthesis. Rapid changes in the activity of this enzyme have been ascribed not only to allosteric inhibition caused by products synthesized in excess of requirements for chlorophyll and haem biosynthesis, but also to a short half life of the enzyme and light-mediated induction of enzyme synthesis [20–22]. Changes in ALAS activity can also be affected by repression by haem [23].

As far as experiments with cotyledons of *Helianthus* are concerned, inhibition by products seems to be the most probable control mechanism for rapid changes. Nevertheless the experiments exhibit features which may be ascribed to enzyme degradation and light-mediated enzyme synthesis.

ALAS activity in cotyledons of *Helianthus* varies with the age of the seedlings (Fig. 2). Protochloro-

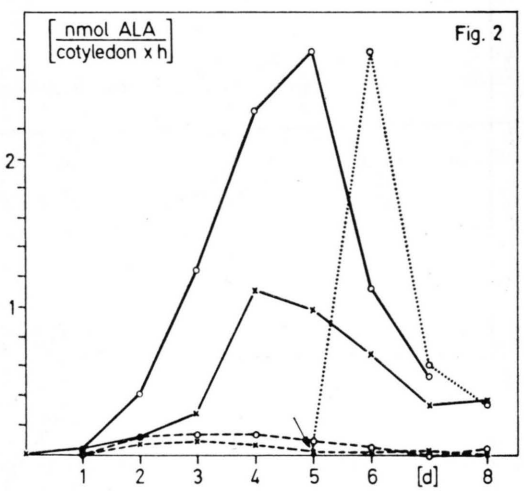

Fig. 2. Accumulation of ALA in cotyledons of *Helianthus* during a 4 hours incubation in 2×10^{-2} M levulinic acid, pH 5.2. ○, Experiments with light grown cotyledons; ×, experiments with dark grown cotyledons; ——, incubation under illumination; — — —, incubation in darkness; ····, cotyledons grown in darkness, illuminated after 5 days and incubated under illumination.

phyllide regeneration also depends on the age of leaves (l. c. 24, for literature see ref. 2). ALAS activity reaches a maximum at the 4th to the 5th day in dark-grown cotyledons as well as in light-grown ones. The difference is that light grown cotyledons show higher activities.

Dark- and light-grown cotyledons incubated in levulinic acid in darkness show slight activities with a maximum about the 3rd day, but then the activities gradually disappear. Incubation under illumination, however, results in much higher activities with the maximum between the 4th and the 5th day. When the activity detectable during dark incubation fades, ALAS activity under illumination reaches highest values. As shown in Fig. 2, preillumination enhances subsequent ALA formation. This is also true when 5 day old etiolated cotyledons are illuminated for 24 hours. This effect can surely be ascribed to the action of phytochrome and should also be present if the cotyledons are illuminated for a shorter period before a dark interval (*cf.* 22). Under prolonged illumination, however, always a decrease of activity is observed. Endogenous processes dominate over light induction. The great differences between ALA accumulation rates in darkness and light indicate rapid break down of enzyme activities without irradiation.

Cotyledons illuminated during incubation in levulinic acid and then transferred to darkness show rapid decrease in ALAS activity, comparable to the decrease in corn leaves [3]. Presupposed a linear increase of ALA accumulation during illumination, ALA accumulation rate in darkness is reduced with a halflife of less than a quarter of an hour in etiolated cotyledons (Fig. 3 a). But there is only little congruence between the experimental values (means of 6 experiments) and the theoretical halflife curve and the variation is even higher with green cotyledons (Fig. 3 b). The experimental values indicate that ALAS activity is blocked nearly immediately.

Other experiments (Fig. 3 c) show that decreasing accumulation of ALA may in part be the result of enzyme degradation. Six day old *Helianthus* seedlings grown under illumination have been transferred to darkness and at the beginning of the dark period, and after different intervals of time, cotyledons have been cut and incubated for 4 hours under illumination. The amount of accumulated ALA decreases only slightly and moderately significant dependent on the duration of the dark period. Assuming a halflife of a quarter of an hour for enzyme degradation in cotyledons after 4 hours of darkness, ALA accumulation should start at best with traces of the activity under continous illumination. Since induction and synthesis of enzymes cannot be a rapid process in higher plants accumulation of ALA

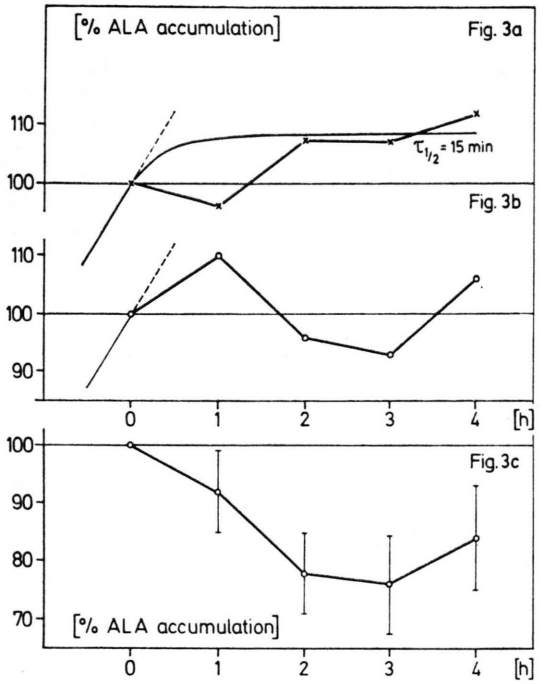

Fig. 3. The influence of different light programs on the accumulation of ALA during incubation in levulinic acid. Codyledons aged 5 days. 3 a. Dark-grown, 3 b. light-grown cotyledons were incubated for 4 hours under illumination and thereupon transferred to darkness. 100% represent the quantity of ALA accumulated after 4 hours of illumination. A theoretical halflife curve for 15 min is given in 3 a. 3 c. Light-grown cotyledons were transferred to darkness for different periods and thereupon incubated under illumination for 4 hours. Vertical lines represent the standard deviation.

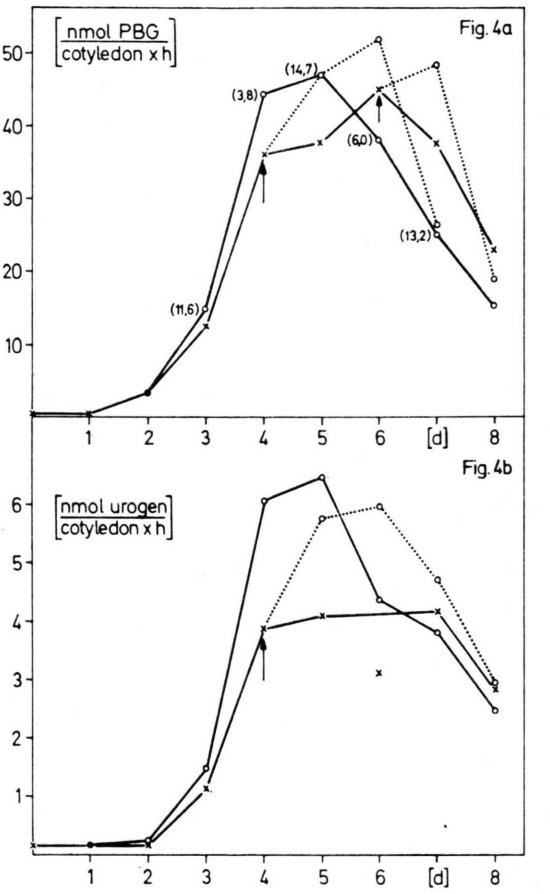

Fig. 4. Activity of ALAD (4 a) and PBGA (4 b) under the influence of development and light. ○, Experiments with light-grown cotyledons; ×, experiments with dark-grown cotyledons. ····, cotyledons illuminated after a dark period. Numbers in brackets represent the percent reliability between values of light and dark grown cotyledons.

after a dark period should be appreciable lower than occurring without a dark period. Accumulation should be lower than indicated by the experimental values. Repression may also be excluded hence it is also a slow process. Thus the inhibition of ALA accumulation in darkness seems more likely to be the result of an inhibition by products synthesized from ALA than of enzyme degradation. Without illumination ALAS may be present in a latent state. (Inhibition may be delayed by the block given to ALAD.)

δ-Aminolevulinate dehydratase (ALAD) and porphobilinogenase (PBGase) during develpoment and illumination

The activities of ALAD and PBGase in etiolated cotyledons increase during development and growth, attaining maximum activity between the 4th to the 6th day (Fig. 4) and then the activities decrease. Similar results are obtained with light-grown cotyledons. Activities of illuminated cotyledons, however, increase and decrease more rapidly than in dark-grown tissue. At the 4th to the 5th day, when maximum activity is attained activities of dark-grown tissue are surpassed by about 25% for ALAD and 50% for PBGase, respectively. The decrease in activity of light-grown cotyledons, after the maximum is attained is so rapid that lower activities are found than is the case in dark-grown cotyledons. The percent reliability for the differences in ALAD activities of light- and dark-grown cotyledons have been computed (see Fig. 4) [25]. Although enzyme activities decrease under the influence of development a light-mediated increase can be initiated during this period in etiolated cotyledons. The increase is in the same

order of magnitude of illuminated cotyledons in the first days. In general the time course of ALAD and PBGase activities resemble those of latent ALAS activity; the light-mediated effect, however, is much smaller. Increasing activities are associated with periods of rapid chlorophyll accumulation and decreasing activities with low rates of accumulation.

Density labelling and de novo synthesis of enzymes

When enzyme activities increase under the influence of development and illumination the question is raised whether preformed enzymes are activated or whether enzymes are synthesized *de novo*. This question may be answered by density labelling experiments. Enzymes synthesized *de novo* by plants growing in D_2O will incorporate unexchangeable deuterium enclosed in the tertiary structure: preformed enzymes cannot because of the inaccessibility of the preformed tertiary structure.

As ALAD is rather stable, most of the density labelling experiments have been done with this enzyme. ALAS would have been more interesting since it plays a more major role in the regulation of chlorophyll biosynthesis, but the *in vitro* lability of ALAS and the lack of knowledge about the nature of this enzyme (for references see 2) do not permit such experiments.

The density of ALAD synthesized by plants grown in H_2O has been found to be 1.324 (g/cm^3) corresponding to a refractive index of 1.348 in CsCl, but in plants grown in D_2O the densities are about 1.348 (g/cm^3) corresponding to a refractive index in 1.3670. A typical experiment is represented in Fig. 5. Experiments with succinyl CoA synthetase and PBGase have also shown density differences. Profiles of PBGase in CsCl strongly deviate from a symmetrical distribution: They have a shoulder on the side of higher densities which may be the result of dissociation of the PBGase complex (profile c in Fig. 5).

Assuming a protein composed of amino acids with an average molecular weight of 140 and 10 hydrogen atoms and presupposing no change in volume by deuterium incorporation, the results suggest that about 3 hydrogens per amino acid have been substituted by unexchangeable deuterium. Since the plants have been grown in 99.8 percent D_2O for 13 days, the other 7 hydrogen positions should be accessible to H_2O during experimental procedures.

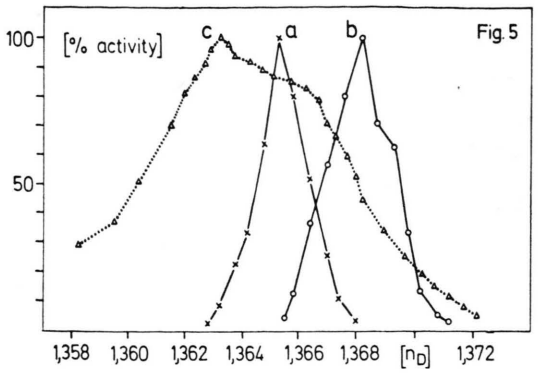

Fig. 5. Typical density gradient experiment. a. ALAD synthesized from plants grown in H_2O; b. ALAD synthesized from plants grown in D_2O; c. PBGA synthesized in H_2O.

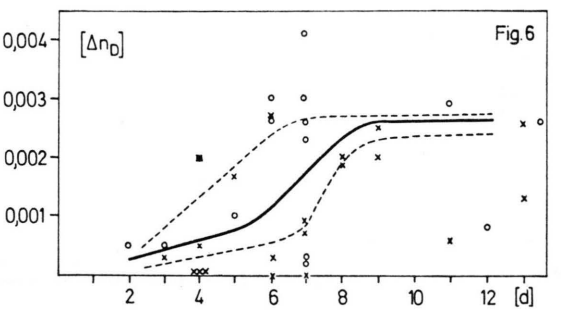

Fig. 6. Density labeling of ALAD in cotyledons grown in light (○) and darkness (×). By the heavy line most of the values obtained with light-grown cotyledons are separated from most of the values obtained with dark-grown cotyledons. Δn_D, refractive index of CsCl corresponding to the density of D_2O enzyme — refractive index of CsCl corresponding to density of H_2O enzyme.

As shown in Fig. 6, density labelling begins about the 3rd day after soaking the achenes although cultivation in D_2O delays growth and development. Labelling increases during the following days. Though seedlings have been soaked and grown with 99.8 percent D_2O, the amino acids used for enzyme synthesis during the first days have not yet been wholly labelled.

The results demonstrate that increasing enzyme activities observed in earlier experiments are probably correlated with enzyme synthesis and that ALAD is certainly synthesized *de novo*. Preformed enzyme stored in the cotyledons but activated during germination would exhibit the same density as the enzyme synthesized in H_2O. The small activities in early development seem to be due to preformed enzyme.

More interesting, however, is the finding that light accelerates the incorporation of label into the

enzyme. That the experiments are significant was checked by the chi-square test [26]. This means that light enhances in fact developmental processes connected with enzyme synthesis. Consequently a light-mediated activation of the enzyme formed during development seems less probable. This finding is supported by the following considerations: Light-mediated increase in activity is small compared with the increase during development and compared with light stimulation of ALA accumulation; in corn leaves [3] ALAD activity has not been influenced by light and even negative effects of light on enzyme activity have been demonstrated. It may be concluded that ALAD activity is regulated by effects on transcription and translation rather than on the enzyme protein itself. The same may be true for PBGase but not exclusively for ALAS.

Common features of the results

The 3rd to the 5th day of development seems to be the most important period in the life cycle of *Helianthus* cotyledons. Protochlorophyllide and chlorophyll but also carotenoids show maximum accumulation rates during this period and maximum accumulation rates are correlated with highest enzyme activities of the chlorophyll biosynthetic pathway. The synthesis of these enzymes is synchronous. Smaller accumulation rates, possibly caused by limited incorporation capacities of mature chloroplasts, coincide with decreasing enzyme activities. Enzyme induction by light has been shown possible at all stages of development, but prolonged illumination always reduces enzyme activity. The duration of the period of increasing activities under illumination is age-dependent. The 3rd day is characterized by maximum soluble protein indicating a general mobilization of metabolic processes. Some enzymes involved in other metabolic pathways show highest activities at this point [27] as well as ALAS tested in darkness. The experiments, thus, indicate a part of the schedule of enzyme activation in the course of development. Different biosynthetic pathways are not set going synchronously.

Discussion

Visible changes during development are associated with changes in the level of enzymes. Consequently also changes in the enzyme activities of the chlorophyll biosynthetic chain have been measurable during germination and development. Light mediated and developmental processes have been shown, both separately and together, to control chlorophyll formation. Evidence for enzyme activation, synthesis and degradation has been found.

The influence of de novo synthesis and activation on activities of ALAD and PBGase

Although cotyledons of *Helianthus* are storage organs they do not store enzymes or proenzymes for chlorophyll and protochloride biosynthesis which takes place very early after germination. Some enzymes of this pathway are synthesized *de novo* during development as demonstrated by density labeling experiments with ALAD. Maximum enzyme activity, however, does not coincide with maximum soluble protein accumulation correspondent with maximum activation of other metabolic pathways. Moreover the finding that light accelerates incorporation of deuterium label into ALAD supports the argument for a light-induced enzyme synthesis. The possibility that light activates the enzyme, newly synthesized in the course of development, loses credibility. Irrespective of this conclusion it seems unlikely that the structures of all the enzymes enhanced by light would all permit changes resulting in additional activation. In the case of ALAD, depending on conditions, light may increase activity, may be ineffective or even diminish activity indicating that endogenous and light mediated processes affecting enzyme synthesis are interwoven and interdependent and that such processes effect enzyme synthesis rather than activation.

Problems concerning de novo synthesis, degradation and activation of ALAS

Density labeling experiments with ALAS cannot be done due to the wellknown impossibility to conservate ALAS activity in homogenates (for literature see l. c. 2). However, experiments with protein and nucleic acid inhibitors [9, 10, 17, 20, 28, 29] show that protein synthesis is also involved in the light-mediated increase of ALAS activity though there is no doubt that ALAS is also subjected to other control mechanisms. Preilluminated cotyledons have greater ability to synthesize ALA indicating higher quantities of latent enzyme after preillumination. When the activities of ALAD and PBGase increase there is concurrently a greater ability to synthesize ALA.

In contrast to the activities of ALAD and PBGase, however, ALAS activity has also been shown to be subjected to rapid changes caused by light. The mechanisms discussed are: inhibition by products like heme, protochlorophyllide or other intermediates of the chlorophyll and porphyrin pathway [21–23] and a labile enzyme with a short halflife dependent on light-mediated synthesis [20] (for detailed discussion of the regulation problem see l. c. 2). Enzyme degradation during dark periods almost certainly takes place in *Helianthus* cotyledons as has been shown by experiments with light-grown cotyledons transferred to darkness, but the decrease in latent ALAS activity is small. Assuming the rate of enzyme degradation matches the half life of ALAS activity there should always be a high difference in ALA accumulation between cotyledons under continous illumination and cotyledons illuminated after a dark period. Such a difference, however, could not be demonstrated in experiments. In contrast to this finding, however, the rapid decrease after prolonged illumination and growth may be caused by enzyme degradation in the course of development. The results suggest that the ratio of synthesis and degradation rates of ALAS are strongly dependent on endogenous processes. Release from an inhibition of ALAS by consumption of inhibitory products during illumination seems more plausible. Consequently the decreasing activity of ALAS in the dark in *Helianthus* cotyledons after the 3rd day may be connected with increasing accumulation of protochlorophyllide. This, however, does not exclude the possibility that dark activity also depends on endogenous control mechanisms; for example on turn over rates of ALAS isoenzymes located in cytoplasm and plastids. Maximum activity of ALAS in the dark coincides with maximum accumulation of soluble protein.

The concert of ALAS, ALAD and PBGase in the regulation of chlorophyll biosynthesis

Of the enzymes of chlorophyll biosynthesis ALAS certainly appears to play the most important role in regulating the biosynthetic pathway. More minor roles may be played by enzymes such as ALAD and PBGase and finally by succinyl CoA synthetase assayed in previous experiments [3, 4]. During the development of *Helianthus* cotyledons, ALAD and PBGase are also formed at higher rates and thus increasing amounts of ALA can be drawn into the chlorophyll pathway. Under illumination this effect is supported by further increasing activities. Although the various enzyme activities fall once maximum chlorophyll or protochlorophyllide accumulation has occurred, etiolated cotyledons still preserve the ability to synthesize enzymes on subsequent illumination. However, as already demonstrated with corn leaves [3], PBGase and ALAD seem to be formed in abundance and their activities are mainly controlled by factors other than light. It is plausible to assume that increasing enzyme activities are correlated with the development of plastids, especially because the enzymes are located in these organelles. There are common features about the regulation of all the enzymes studied, but ALAS in cells with matured plastids will only work under illumination.

Dependent on species and stage of development, one or other of the discussed control mechanisms becomes dominant.

The work was supported by the Deutsche Forschungsgemeinschaft. Thanks are due to Dr. R. J. Porra (Canberra) for reading and discussing the manuscript and Mrs. Hanna Mansi-Wothke for skillful technical assistance.

[1] Hj. A. W. Schneider, Enzyme **16**, 108–114 [1973].
[2] Hj. A. W. Schneider, Ber. Deutsch. Bot. Ges. **88** [1975].
[3] Hj. A. W. Schneider, Z. Naturforsch. **28 c**, 45–58 [1973].
[4] Hj. A. W. Schneider, Z. Pflanzenphysiol. **69**, 68–76 [1973].
[5] E. Harel and S. Klein, Biochem. Biophys. Res. Commun. **49**, 364–370 [1972].
[6] S. I. Beale and P. A. Castelfranco, Plant Physiol. **53**, 291–296 [1974].
[7] Hj. A. W. Schneider, Z. Pflanzenphysiol. **62**, 133–145 [1970].
[8] Hj. A. W. Schneider, Phytochemistry **10**, 319–321 [1971].
[9] Hj. A. W. Schneider and W. W. Beisenherz, Biochem. Biophys. Res. Commun. **60**, 468–473 [1974].
[10] W. W. Beisenherz and Hj. A. W. Schneider, Ber. Deutsch. Bot. Ges. **87**, 161–166 [1974].
[11] D. Mauzerall and S. Granick, J. Biol. Chem. **219**, 435–446 [1956].
[12] D. Shemin, Methods in Enzymology, **vol. V**, pp. 883–884 (S. P. Colowick and N. O. Kaplan, eds.), Academic Press 1962.
[13] L. Bogorad, Methods in Enzymology, **vol. V**, pp. 885–895 (S. P. Colowick and N. O. Kaplan, eds.), Academic Press 1962.
[14] J. Vinograd, Methods in Enzymology, **vol. VI**, pp. 854–870 (S. P. Colowick and N. O. Kaplan, eds.), Academic Press 1963.
[15] C. S. French, Encyclopedia of Plant Physiology, **vol. V/1**, pp. 279–280 (W. Ruhland, ed.), Springer 1960.

[16] T. W. Goodwin, Modern Methods of Plant Analysis, **vol. III**, pp. 295–297 (K. Peach and M. V. Tracey), Springer 1955.
[17] J. T. O. Kirk, Planta **78**, 200–207 [1968].
[18] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem. **193**, 265–275 [1951].
[19] M. Jabben, M. Masoner, H. Kasemir, and H. Mohr, Photochem. Photobiol. **20**, 233–239 [1974].
[20] K. Nadler and S. Granick, Plant Physiol. **46**, 240–246 [1970].
[21] J. Duggan and M. Gassman, Plant Physiol. **53**, 206–215 [1974].
[22] H. Mohr, H. Drumm, and H. Kasemir, Ber. Deutsch. Bot. Ges. **87**, 49–69 [1974].
[23] J. Lascelles, Porphyrins and Related Compounds, pp. 61–74 (T. W. Goodwin, ed.), Academic Press 1968.
[24] C. Sundqvist, B. Odengård, and G. Persson, Plant Science Letters **4**, 89–96 [1975].
[25] A. Mudra, Statistische Versuche für Landwirtschaftliche Versuche, p. 67 f, Parey 1958.
[26] L. Sachs, Statistische Auswertungsmethoden, 3rd edition, p. 269 f, Springer, Berlin-Heidelberg 1972.
[27] B. Gerhardt, Planta **110**, 15–28 [1973].
[28] M. Gassman and L. Bogorad, Plant Physiol. **42**, 774–780 [1967].
[29] M. Gassman and L. Bogorad, Plant Physiol. **42**, 781–784 [1967].