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# The inhibitory effect of 4,5-dioxovalerate on 5-aminolevulinate dehydratase and its implication in the regulation of light-dependent chlorophyll formation in pigment mutant C-2A′ of *Scenedesmus obliquus*

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**Besides the known role of 4,5-dioxovalerate as an intermediate in the $C_5$-pathway [1], a novel regulatory function in light-dependent chlorophyll biosynthesis is described. Considerable amounts of protochlorophyllide accumulate in dark grown cultures of the yellow mutant C-2A′ of *Scenedesmus* [2]. This accumulation is almost completely blocked in darkness by the addition of 4,5-dioxovalerate in vivo. Likewise, light-dependent chlorophyll biosynthesis is strongly inhibited by the addition of this compound during greening. The considerable increase of protochlorophyllide formation in darkness upon the addition of 5-aminolevulinate (Kotzabasis, K. and Senger, H. (1989) Z. Naturforsch., in press) is also drastically reduced by external 4,5-dioxovalerate. It is shown by in vitro experiments that concentrations of dioxovalerate, above the physiologically relevant level, inhibit 5-aminolevulinate dehydratase. The $K_i$-value was determined to be $60 \pm 5$ μM. From these results it is concluded that besides the predominant control of $^{glu}$t-RNA-ligase by protochlorophyllide [4] a second regulatory mechanism is involved in chlorophyll biosynthesis. Under excessive concentrations of 5-aminolevulinate and 4,5-dioxovalerate further porphobilinogen and subsequently chlorophyll biosynthesis is inhibited.**

## Introduction

The formation of chlorophylls is probably the largest biosynthetic process on earth and is estimated at more than $10^{10}$ metric tons per year [5]. This biosynthesis is efficiently regulated to avoid any overproduction of intermediates. Nevertheless, the mechanism of the regulation is still a matter of debate (see Ref. 6).

For organisms in which chlorophyll biosynthesis proceeds only to the stage of protochlorophyllide in darkness, it has been suggested that protochlorophyllide exerts a negative feedback on one of the early steps of the pathway [7].

The formation of chlorophyll precursors in dark-grown cells of a pigment mutant of the green alga *Scenedesmus obliquus* is efficiently controlled by negative feedback of protochlorophyllide on the $^{glu}$t-RNA-ligase [4]. The ubiquity of this mechanism for the control of chlorophyll biosynthesis in darkness has still to be shown.

This regulatory mechanism, however, is removed in the light, when protochlorophyllide is immediately reduced to chlorophyllide. Nevertheless, the biosynthesis of chlorophyll and its precursors is equally well regulated, as can be deduced from the fact that free chlorophyll, not bound to an apoprotein, has never been found.

To our knowledge so far only vague theories exist which explain the regulation of chlorophyll biosynthesis in the light. In the current paper we report evidence for the inhibition of 5-aminolevulinate dehydratase and hence chlorophyll biosynthesis by 4,5-dioxovalerate in whole cells of pigment mutant C-2A′ of *S. obliquus* as well as in cell free preparations. 4,5-Dioxovalerate, known as an essential anabolic intermediate of the

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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$C_5$-pathway to 5-aminolevulinate [1,8], thus obtains an additional regulatory function.

## Materials and Methods

*Chemicals.* 4,5-Dioxovalerate was prepared as described in Ref. 9. 5-Aminolevulinate was purchased from Fluka, Buchs, Switzerland. Aminooxyacetate was obtained from Sigma, München, F.R.G. Yeast extract was provided by Merck, Darmstadt, F.R.G. All other chemicals were p.a. grade.

*Cultivation of cells.* All experiments on the accumulation of protochlorophyllide and the inhibition of its biosynthesis by the action of 4,5-dioxovalerate on 5-aminolevulinate dehydratase (porphobilinogen synthase) (EC 4.2.1.24) were performed with the X-ray induced mutant C-2A′ of the unicellular green alga *S. obliquus* [10]. Cells were grown heterotrophically in darkness in an inorganic medium supplemented with 0.5% glucose and 0.25% yeast extract as described earlier [11].

Cells were harvested 20 h after inocculation in the dark. Protochlorophyllide accumulation is maximum at this stage and decreases afterwards [2,12]. In some experiments the growth period was prolonged for 6 h and in another series of experiments dark growth was followed by an illumination period, using white fluorescent light of an intensity of 20 $W \cdot m^{-2}$.

*Determination of protochlorophyllide.* Cells were harvested by centrifugation for 5 min at $1400 \times g$. In vivo-measurements of protochlorophyllide were carried out at 77 K with a Shimadzu RF-540 spectrofluorometer. Excitation was fixed to 440 nm, excitation and emission bandwidth being 10 nm. Furthermore, fluorescence emission spectra were recorded in acetone at room temperature. Conditions were as described above. Protochlorophyllide was isolated from the cells and purified as described earlier [13]. Assays were performed at room temperature in methanol with an Uvikon 820 spectrophotometer (Kontron, München, F.R.G.) using the absorption coefficient of Brouers and Michel-Wolwertz [14].

*Inhibition experiments in whole cells.* For these experiments cells were grown for 20 h in darkness in the presence or absence of 1 mM 4,5-dioxovalerate or 1 mM 5-aminolevulinate. To overcome the effect of traces of 5-aminolevulinate in the heterotrophic medium (yeast extract) on the controls containing no added intermediate, cells were spun down after 20 h of growth, resuspended in Hepes-buffer (20 mM, pH 7.0) and grown for another 6 h as described above. In another series of experiments, cells were grown for 20 h in darkness and then illuminated for 2 h with white light (fluence rate, 20 $W \cdot m^{-2}$) to transform the accumulated protochlorophyllide to chlorophyll; the cultures were then again transferred to darkness for another 6 h with the above-mentioned additions. For specific substrate combinations and culturing conditions see Table I.

To test directly the influence of 4,5-dioxovalerate on chlorophyll biosynthesis during greening, cells were again grown heterotrophically in darkness for 15 or 20 h, and then greened in the presence or absence of 4 mM 4,5-dioxovalerate (see Figs. 2 and 3).

*Inhibition experiments in cell-free preparations.* To test 4,5-dioxovalerate inhibition directly on 5-aminolevulinate dehydratase, the enzyme fraction that does not bind to the affinity column system described by Wang et al. [15] run-off fraction) was used. This preparation contains, besides other proteins, glutamate-1-semialdehyde aminotransferase, 4,5-dioxovalerate transaminase, 5-aminolevulinate dehydratase and porphobilinogenase. This preparation, 600 $\mu$g protein in Tricine/NaOH-buffer (pH 7.9) containing glycerol (0.3 M), dithiothreitol (1 mM) and $MgCl_2$ (25 mM), was incubated for 30 min at 27 °C with 4,5-dioxovalerate concentrations ranging from 0.25 mM to 2 mM in each test series performed with 5-aminolevulinate concentrations 50 $\mu$M, 100 $\mu$M or 200 $\mu$M in the different experiments: the final volume was 1.3 ml. Incubations were stopped by the addition of 100 $\mu$l of 70% $HClO_4$. Porphobilinogen was determined by the method of Mauzerall and Granick [16] and uroporphyrin was determined spectrophotometrically as follows: 10 $\mu$l aliquots were mixed with 900 $\mu$l of 0.5 M HCl and determined at 405 nm ($\epsilon_{405} = 541\ mM^{-1} \cdot cm^{-1}$) [17].

## Results and Discussion

*In vivo influence of 4,5-dioxovalerate on protochlorophyllide formation in darkness*

Dark-grown cultures of pigment mutant C-2A′ of *S. obliquus* accumulate up to 20 nmol protochlorophyllide per ml packed cell volume [2]. This protochlorophyllide strictly regulates the ligation of $^{glu}$t-RNA with glutamate catalyzed by a t-RNA ligase [4]. When this mutant is grown in darkness in the presence of 1 mM 4,5-dioxovalerate, an intermediate of the $C_5$-pathway [4], protochlorophyllide formation is slightly increased. However, the addition of 1 mM 5-aminolevulinate, the next intermediate in the biosynthetic pathway, causes a 16-fold increase in protochlorophyllide. This increase is diminished to one fourth in the presence of both, 1 mM 4,5-dioxovalerate and 5-aminolevulinate. The amount of protochlorophyllide formed was determined by the in vivo fluorescence of whole cells at 77 K and is presented in Table I. These results suggest, that 4,5-dioxovalerate can act not only as a presursor of 5-aminolevulinate [1,8], but also as an inhibitor of chlorophyll biosynthesis when excess 5-aminolevulinate and 4,5-dioxovalerate are present. This suggests that 4,5-dioxovalerate inhibits an enzyme of the chlorophyll biosynthetic sequence subsequent to the formation of 5-aminolevulinate.

TABLE I

*Relative amounts of protochlorophyllide formed in the presence of various metabolites in whole cells of mutant C-2A′ measured as its in vivo fluorescence at 77 K*

Controls with no metabolite present were considered to be 100%, while data obtained with metabolites are given as percent of the control. Cells were grown in the presence of intermediates of the $C_5$-pathway. For measurement, samples were adjusted to identical packed cell volume. A, 20 h dark grown (pH 7.0), intermediates present during growth. B, 20 h dark grown, harvested and resuspended in Hepes-buffer (20 mM, pH 7.0) to provide 5-aminolevulinate-free medium, then grown dark for another 6 h with additions. C, 20 h dark grown, illuminated for 2 h (20 $W \cdot m^{-2}$, white light) to reduce accumulated protochlorophyllide, subsequently grown in darkness for 6 h with additions. D, 20 h dark grown in the presence of 4,5-dioxovalerate + glutamate to assure the supply of the aminodonor of 4,5-dioxovalerate transaminase. E, 20 h dark grown with additions to determine the influence of 4,5-dioxovalerate on 5-aminolevulinate consumption. Abbreviations: ALA, 5-aminolevulinate; DOVA, 4,5-dioxovalerate; Glu, glutamate.

| Culturing conditions | A | B | C | D | E |
|---|---|---|---|---|---|
| Control | 100 | 100 | 100 | 100 | 100 |
| Additions: | | | | | |
| +1 mM DOVA | 96 | 109 | 119 | – | 75 |
| +1 mM ALA | 169 | 195 | 346 | – | 1666 |
| +1 mM DOVA+1 mM Glu | – | – | – | 107 | – |
| +1 mM DOVA+1 mM ALA | – | – | – | – | 416 |

To reconfirm the reported measurements with whole cells of mutant C-2A′, the extracted protochlorophyllide was determined fluorometrically. Cultures were again grown in the presence or absence of 5-aminolevulinate (1 mM) or 4,5-dioxovalerate (1 mM) and together (all 1 mM) for 20 h in darkness. Cultures were then harvested, extracted and the accumulated protochlorophyllide separated. All extracts were obtained from identical packed cell volumes. Fluorescence emission spectra of the extracts in acetone are shown in Fig. 1. Again, it is evident that the addition of 5-aminolevulinate produces a 4 times higher yield of protochlorophyllide than occurs in the presence of both compounds. The control experiments with and without 4,5-dioxovalerate show the presence of only small amounts of protochlorophyllide. This demonstrates that 4,5-dioxovalerate plays a regulatory role in the $C_5$-pathway in the presence of high 5-aminolevulinate concentrations. Little is known about the 4,5-dioxovalerate concentration in plants. Also the $K_i$-values might change drastically under in vivo conditions. Taking this into consideration it might well be that the proposed regulatory mechanism works in plants to avoid overproduction of chlorophylls after the photosynthetic apparatus is completed.

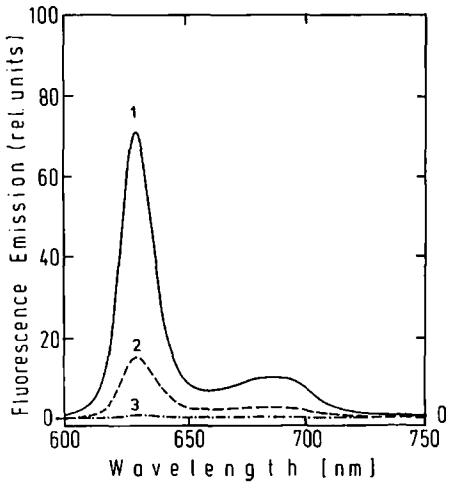


Fig. 1. Fluorescence emission spectra at room temperature of protochlorophyllide in acetone of methanol extracts from cultures of mutant C-2A′ grown in the presence of 1: 1 mM 5-aminolevulinate, 2: 4,5-dioxovalerate and 5-aminolevulinate (1 mM each), 3: 4,5-dioxovalerate and without any addition. Excitation wavelength was 440 nm.

*In vivo influence of 4,5-dioxovalerate on chlorophyll formation*

To verify that 4,5-dioxovalerate is not only inhibiting protochlorophyllide formation in darkness, the influence of this compound on light-dependent greening of mutant C-2A′ was tested. Cultures were grown for 20 h in darkness and upon the onset of light one of the cultures was incubated with 4 mM 4,5-dioxovalerate. As

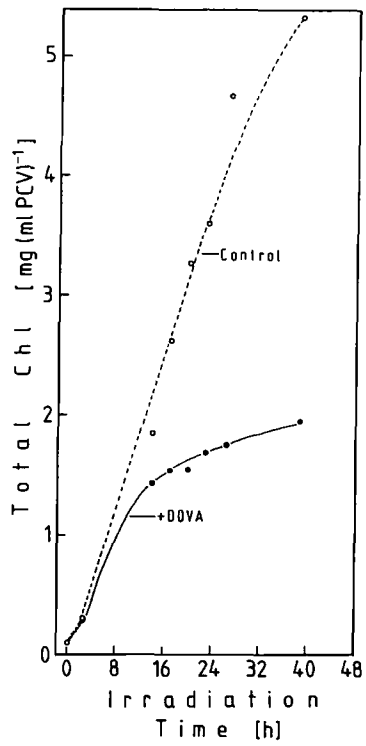


Fig. 2. Influence of 4,5-dioxovalerate on chlorophyll biosynthesis during greening of mutant C-2A′ of *Scenedesmus*. Cultures were grown in darkness for 20 h and then illuminated with white light (20 $W \cdot m^{-2}$) in the presence (●———●) and absence (○- - - - - -○) of 4 mM 4,5-dioxovalerate (final concentration). For experimental details, see Materials and Methods.

shown in Fig. 2 during the first stages of greening no significant difference in chlorophyll biosynthesis can be observed. After 8 h, however, the difference in chlorophyll content amounts already to about 30% and increases further with time. After a 36 h incubation, chlorophyll synthesis in the 4,5-dioxovalerate-grown culture reaches 35% of the control (i.e., there is 2,7-fold more chlorophyll in the control). That the inhibitory effect during the early stage of greening is quite small can be explained by the high demand for porphyrin precursors by the chlorophyll synthesizing system: during this initial period chlorophyll formation rate is maximum, but the rate decreases after about 8 h of greening [18], when the photosynthetic apparatus is largely completed. At this stage chlorophyll biosynthesis is slowed down. This regulation might well be mediated by the proposed mechanism. Still, the in vivo concentration of 4,5-dioxovalerate has to be assumed to be low and to be compensated by a low $K_i$-value in vivo.

This interpretation becomes more convincing with the results obtained after a 20 h dark-grown culture was illuminated for 2.5 h without metabolites to initiate chlorophyll biosynthesis. Subsequently, the culture was divided and incubated with and without 4 mM 4,5-dioxovalerate in darkness for 2 h. In both, the presence and absence of 4,5-dioxovalerate the chlorophyll, content decreased slightly during the dark incubation (Fig. 3). Upon illumination chlorophyll formation in both cultures was nearly parallel for 8 h, but then a decrease in the rate of chlorophyll formation occurred in the 4,5-dioxovalerate-treated culture. This can be explained by a decrease in the chlorophyll formation rate as described above. The competitive inhibition of 5-aminolevulinate dehydratase by 4,5-dioxovalerate causes even a decline of the total chlorophyll content. When, however, 4 mM 5-aminolevulinate was added to the culture after 30 h of incubation with 4,5-dioxovalerate, an immediate increase in chlorophyll biosynthesis was observed, that was equal to that of the control at the 8th hour of treatment. After the added 5-aminolevulinate was metabolized, chlorophyll biosynthesis again decreased. This clearly demonstrates that under conditions when both, 4,5-dioxovalerate and 5-aminolevulinate, are metabolized rapidly to chlorophyll, no competition between these intermediates for the substrate binding site of 5-aminolevulinate dehydratase takes place. When, however, chlorophyll formation slows down, intermediates accumulate and 4,5-dioxovalerate can compete with 5-aminolevulinate for 5-aminolevulinate-dehy-

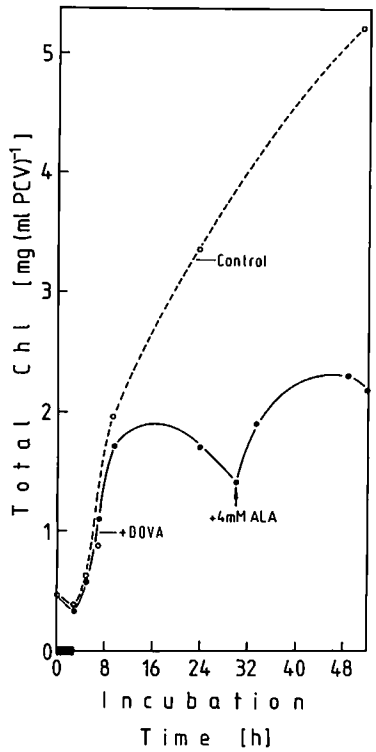


Fig. 3. Influence of 4,5-dioxovalerate on chlorophyll biosynthesis during greening of mutant C-2A′ of *Scenedesmus*. Cultures were grown in darkness for 15 h, then illuminated for 2.5 h with white light (20 W·m$^{-2}$) and then kept dark for another 3 h in the presence (●———●) and absence (○- - - - -○) of 4 mM 4,5-dioxovalerate (final concentration): After 3 h of darkness cultures were again illuminated (20 W·m$^{-2}$) and the 4,5-dioxovalerate treated culture supplemented with 5-aminolevulinate to a final concentration of 4 mM.

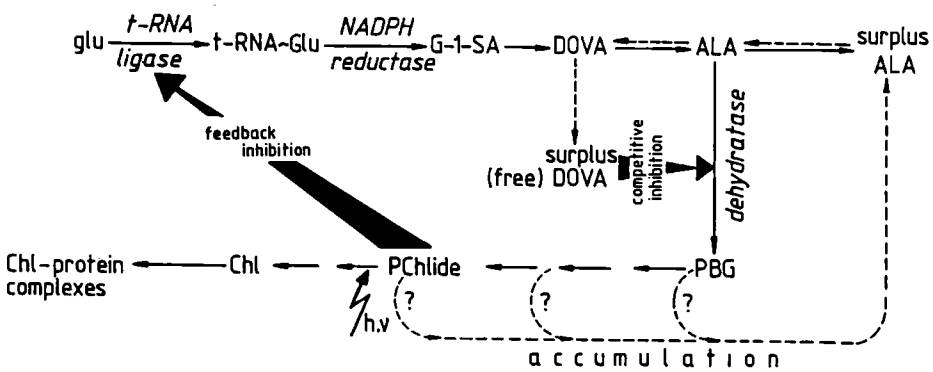


Fig. 4. Regulation scheme of chlorophyll biosynthesis under light and dark conditions. The dashed lines do not represent a reversal of the reaction sequence, but a possible accumulation of intermediates, finally causing a surplus of then non-enzyme-bound (free) 4,5-dioxovalerate. PBG, porphobilinogen.

dratase thus regulating the formation of porphobilinogen. By this mechanism an accumulation of chlorophyll precursors is prevented and a regulation of tetrapyrrole biosynthesis via the $C_5$-pathway under light conditions is achieved. From our recent results on regulation of chlorophyll biosynthesis by protochlorophyllide [4] and the current data in this paper a regulation scheme for chlorophyll biosynthesis under all naturally occurring conditions (light and darkness) is proposed (Fig. 4). Accumulated protochlorophyllide in darkness acts directly on $^{glu}$t-RNA-ligase [4] as a feedback inhibitor and so blocks further 5-aminolevulinate formation and thus chlorophyll biosynthesis. Under saturating light conditions chlorophyll precursor formation and accumulation can become excessive, leading to a final surplus of $C_5$-intermediates. Such a surplus of 4,5-dioxovalerate can competitively inhibit 5-aminolevulinate dehydratase activity and block further synthesis of porphobilinogen and thus chlorophylls.

*Inhibition of 5-aminolevulinate dehydratase in cell-free preparations*

The competitive inhibition of 5-aminolevulinate dehydratase by 4,5-dioxovalerate can be confirmed in vitro using an enzyme preparation containing all the enzyme activities needed to convert glutamate-1-semialdehyde to uroporphyrinogen III. The oxidation of uroporphyrinogen II to uroporphyrin III is accomplished by exposure to UV-light (366 nm) in the presence of oxygen. The enzyme preparation was incubated in the presence of 5-aminolevulinate concentrations of 50, 100 and 250 $\mu$M, while 4,5-dioxovalerate concentrations were varied between 250 $\mu$M and 2 mM in each case. The results showed an increasing inhibitory effect on 5-aminolevulinate dehydratase and also on the slope of the different curves in dependence of 5-aminolevulinate concentration. From inhibition curves for the formation of porphobilinogen and uroporphyrin shown in Fig. 5a and b a $K_i$-value for 4,5-dioxovalerate on 5-aminolevulinate dehydratase of $60 \pm 5$ $\mu$M was derived. This value is much lower than that reported by Shioi et al. [19] who found in *Chlorella* a $K_i$-value for the inhibition of 5-aminolevulinate dehydratase by 4,5-dioxovalerate of 1.4 mM. Superficially this $K_i$-value seems to be in contradiction to the $K_m$-value of 4,5-dioxovalerate transaminase for this substrate, which we reported to be 600 $\mu$M with the isolated enzyme [3,20]. However, in vivo free 4,5-dioxovalerate can only be detected in the presence of competitive inhibitors of 5-aminolevulinate dehydratase like levulinate [9]. This means that under physiological conditions 4,5-dioxovalerate is bound to the enzyme and does not occur in relevant concentrations as free substrate in the stroma. When excessive porphyrin precursor formation occurs with consequent 5-aminolevulinate and 4,5-dioxovalerate accumulation, the latter compound acts as a

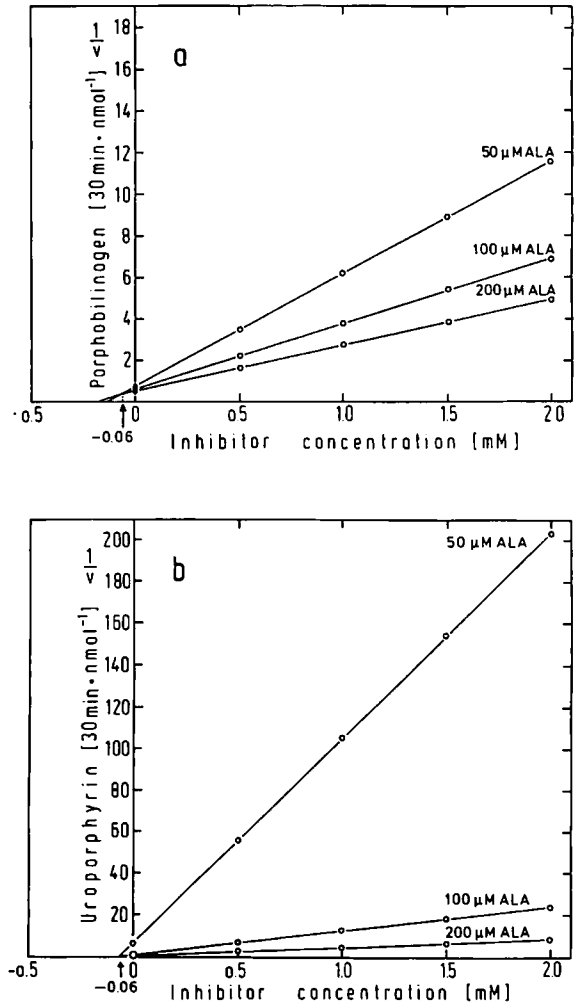


Fig. 5. Determination of the $K_i$-value of 4,5-dioxovalerate on 5-aminolevulinate dehydratase. In Fig. 5a and b the inhibition of porphobilinogen and of uroporphyrinogen III formation, respectively, was determined. The values in both figure panels are taken from identical experiments using aliquots of the incubation mixture. For details see the Materials and Methods section.

competitive inhibitor on 5-aminolevulinate dehydratase, preventing further formation of tetrapyrroles in the plastid. Perusal of Fig. 4 shows that the regulation of chlorophyll biosynthesis can be achieved by two different regulating steps. Under light conditions excessive formation of tetrapyrrole precursors for chlorophyll biosynthesis could principally take place. However, it is a fact that chlorophyll synthesis takes place only to the extent that is required for the chlorophyll-protein complexes. This can be explained by the competitive inhibition of 5-aminolevulinate dehydratase by excessive 4,5-dioxovalerate, an earlier intermediate in the $C_5$-pathway. The concentration of 4,5-dioxovalerate necessary for its competitive action must derive from the accumulation caused by backing up of the final product. In darkness, when no chlorophylls are needed, accumulated protochlorophyllide [4] completely blocks the function of the $C_5$-pathway by its direct feedback

inhibition on $^{glu}$t-RNA-ligase. By these two proposed mechanisms chlorophyll biosynthesis can be controled under all naturally occurring conditions.

## Conclusions

Regarding the proposed regulatory effect of 4,5-dioxovalerate on chlorophyll biosynthesis the objection could be made that the applied concentrations of 4,5-dioxovalerate and 5-aminolevulinate were, compared to physiological conditions, considerably high and that the observed inhibitory effect of 4,5-dioxovalerate were not relevant under physiological conditions within the cell. Because of the high turnover rate during chlorophyll formation accumulation of 5-aminolevulinate and 4,5-dioxovalerate hardly occurs. Their formation rates, however, can be estimated from chlorophyll biosynthesis (see Fig. 2). When the rate of chlorophyll formation is maximum, about 0.22 $\mu$mol of chlorophyll *a* synthesized per ml packed cell volume per h. This is equivalent to 1.8 $\mu$mol of 5-aminolevulinate and 4,5-dioxovalerate, respectively. Based on the chloroplast or thylakoid volume this rate is still higher. When chlorophyll biosynthesis ceases because it has filled all available sites of the apoproteins of the pigment-protein complexes, one can assume rather high temporary concentrations of 5-aminolevulinate and 4,5-dioxovalerate. Such high concentrations in combination with a lower $K_i$-value in vivo could well accommodate the inhibitory effect of 4,5-dioxovalerate on 5-aminolevulinate dehydratase. Hence the shown inhibitory effect of 4,5-dioxovalerate on chlorophyll formation in vitro might well be considered to occur in vivo to prevent excessive and wasteful formation of intermediates.

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