Labeling and Isolation of Hemes from *Scenedesmus*

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Scenedesmus obliquus, Protoheme, Heme *a,* ALA Biosynthesis, Protoheme Turnover

Protoheme and small quantities of heme *a* were isolated and purified from the wild type and the developmental mutant, C-2A′, of *Scenedesmus obliquus*. In fast growing synchronized WT cells and greening cells of the mutant protoheme could be labeled with [1-^{14}C]glutamate and [2-^{14}C]glycine. The labeling of heme *a* was ambiguous and of minor quantity. During rapid chlorophyll biosynthesis the turnover of protoheme was determined by a pulse-chase experiment to have a half-life of 2 h. At the current stage of investigations the question, whether [2-^{14}C]glycine is incorporated into protoheme *via* the Shemin pathway or *via* photorespiration or refixation of $^{14}CO_2$ during general metabolism remains open.

Introduction

The major porphyrin pigment groups in plants are the magnesium porphyrins (chlorophylls) and the iron porphyrins (hemes). Two hemes are well known, heme *a,* the prosthetic group of cytochrome *c* oxidase, and protoheme, the prosthetic group of the enzymes peroxidase and catalase and of the cytochromes which are involved in both, the mitochondrial and the photosynthetic electron transport chain.

5-Aminolevulinic acid (ALA) is the common precursor of both the magnesium and the iron porphyrins. ALA can be synthesized either from glutamate *via* the C_5-pathway [1] or from glycine and succinyl CoA *via* the Shemin pathway [2]. It was shown for various organisms that chlorophylls and hemes can be labeled by feeding radioactive precursors of porphyrin biosynthesis [3, 4].

In *Scenedesmus* in which both pathways to ALA have been demonstrated [5–8] we tried to label the hemes with both radioactive precursors, [1-^{14}C]-glutamate and [1-^{14}C]glycine or [2-^{14}C]glycine. These labelings and the purification of the labeled hemes are supposed to lay the foundation for later experiments to identify the pathways by which hemes are synthesized in *Scenedesmus obliquus*.

Abbreviations: ALA, 5-aminolevulinic acid; WT, wild type; PCV, packed cell volume; HPLC, high performance liquid chromatography.

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Experimental

Organisms and growth conditions

Cells of the wild type (WT) and the developmental mutant, C-2A′ [9], of the green alga *Scenedesmus obliquus* were employed for the experiments described in this communication. Synchronized cultures of the WT [10] were grown under a 14 h light (20 $W \cdot m^{-2}$) to 10 h dark regime in liquid inorganic medium and percolated with air enriched with 3% CO_2 [11]. At the beginning of each life cycle (onset of illumination) cultures were diluted to a density of 3.7×10^6 cells $\times$ ml^{-1} with a photoelectrically controlled dilution device [12]. Cells of C-2A′ were cultured under heterotrophic conditions [13] in the dark for 60 h. At this stage the end of the logarithmic growth phase is reached with a density of 10–11 µl packed cell volume (PCV) per ml.

Conditions of incubations with labeled precursors

Heterotrophically grown cells (60 h) of the mutant were harvested by centrifugation (1400 $\times$ *g*; 5 min), the pelleted cells resuspended in 250 ml of the standard inorganic growth medium and transferred to regular culture tubes (∅ 3.7 cm; length 42 cm). Greening was initiated by illumination with white light (20 $W \cdot m^{-2}$); the cell suspension was continuously aerated with 3% CO_2 in air.

Both, synchronized wild type and mutant cells were supplemented with 1 mM glutamate and 1 mM glycine and incubated in parallel experiments for 6 h with either [1-^{14}C]glutamate, [1-^{14}C]glycine or [2-^{14}C]glycine. The uptake of the radioactive precursors during the incubation was assayed by de-

termining the amount of radioactivity left in 100 µl of medium separated from the cells. Radioactivity was measured by liquid scintillation counting using 5 ml Aqualuma (Baker, Groß-Gerau).

Pigment extraction

Following to incorporation of labeled substrate the cells were harvested by centrifugation. For pigment extraction the algal pellet was suspended in 80% aqueous acetone containing 0.01 M NH_3. Cells (approx. 75 µl PCV · ml^{-1}) were disrupted in this medium in a Vibrogen cell mill (Bühler, Tübingen) for 15 min as described before [14]. The resulting crude cell-free homogenate was centrifuged and the resulting pellet was then reextracted 10 times with cold 80% aqueous acetone to remove all the chlorophyll.

Non-covalently bound hemes were extracted afterwards by a modified method described first by Stillman and Gassman [15]: The pellet was suspended in cold 90% aqueous acetone containing 5% conc. HCl (v/v). The mixture was stirred for 10 min on ice and for an additional 10 min at room temperature. After centrifugation (1400 × *g*; 5 min) the supernatant was collected and the pellet was reextracted in the same way. Both supernatants were combined as the crude heme extract.

Purification of hemes

The crude heme extract in acidified acetone was mixed with diethyl ether and cold water was added until the phases separated. The heme-containing ether phase was collected and extraction of the acidified acetone–water phase was repeated two times. The combined ether phases were stored for at least 1 h at −17 °C to freeze out the excess water.

Subsequently, the ether was separated from the ice and evaporated under reduced pressure. The residue was dissolved in a mixture of pyridine and acetone (1:20 (v/v)). For anion exchange chromatography a Fractogel TSK 650 column (diameter: 0.5 cm; length: 0.5 cm) in the acetate form, as described for DEAE-cellulose by Omata and Murata [16], was used. The solvent system described for the elution of a DEAE-sepharose column by Weinstein and Beale [17] was employed in a modified form.

The heme containing solution was applied to the column and washed with acetone to remove the non-binding β-carotene which is partly extracted together with the hemes. Then 80% aqueous acetone (v/v) was used to elute oxidation products of chlorophyll which were sometimes present in the extract. Afterwards hemes were eluted with 90% acetone (v/v) containing 2% (v/v) glacial acetic acid.

For the preparation of standards of the iron porphyrins large amounts of unlabeled culture material of mixotrophically grown *Euglena gracilis* or heterotrophically grown *Scenedesmus* mutant C-2A′ were used. The protoheme and heme *a* of these extracts were separated by elution of the Fractogel column with a step gradient of glacial acetic acid (0.25%, 0.5%, 2.0% (v/v)) in 90% acetone (v/v). The column material was normally discarded after chromatography of radioactively labeled samples.

The heme-containing fraction was reextracted with diethyl ether and the diethyl ether extract then treated as described above prior to evaporation. The residue was dissolved in 20 µl 0.1 M NH_3, 60 µl ethanol and 20 µl 2.0 M acetic acid. It was further purified by HPLC on a reversed phase Nucleosil 100-10 C 18 column (∅ 0.4 mm; length 25 cm) with a particle size of 10 µm. The HPLC equipment consisted of 2 pumps (Kontron LC 410), a programmer (Kontron Model 200), an absorbance detector (Kontron Uvikon 720 LC), a 20 µl loop and a precolumn (Merck, Darmstadt, Germany). The solvent system was 98% ethanol:2 M acetic acid = 70:30 (v/v). The flow rate was 0.8 ml · min^{-1} and the absorbance of the eluted fractions was measured at 402 nm.

For the preparation of heme *a* it was necessary to collect the heme *a* containing fraction of the first HPLC separation, reextract it with ether and prepare it for a second chromatography by HPLC as described above. The samples were rechromatographed and the heme *a* containing fractions retained. The heme containing fractions of 5 HPLC-runs were pooled and used for quantification.

Quantification of the hemes

The purified hemes were identified by their absorption maxima as reduced pyridine-hemochromogens. Spectra of protoheme were recorded as

described by Furhop and Smith [18]. The sample was dissolved in 0.5 ml pyridine and 2.1 ml water. Just before the recording, 0.25 ml NaOH (1 M) were added and the solution was divided into two aliquots. One was oxidized with ferricyanide and one was reduced by adding a few crystals of dithionite which was stored in small portions under vacuum at −17 °C. Then the reduced minus oxidized difference spectrum was recorded. Heme *a* was measured in 90% aqueous pyridine after reduction with a few crystals of dithionite against a reference without porphyrin.

The concentration of the hemes in the eluted fractions from HPLC were determined in this solvent using the absorption coefficients for air oxidized hemes: 144 mM^{-1} at 398 nm for protoheme and 123 mM^{-1} at 406 nm for heme *a* [17].

Low concentrations of heme *a* were determined by measuring the peak area of the HPLC elution profile. The correlation between peak area and concentration was determined by a standard curve measured with different concentrations of a heme *a* standard that had been previously isolated from mixotrophically grown *Euglena gracilis* and purified with the system described above.

Chemicals

Radioactive compounds were purchased from Amersham (Braunschweig, Germany), Fractogel from Merck (Darmstadt, Germany) and all other reagents and solvents of purified grade from Merck or Aldrich (Steinheim, Germany).

Results

Uptake of radioactive substrates

In order to measure the uptake ability, cells of the synchronized WT and of the developmental mutant C-2A′ during greening were incubated with ^{14}C-labeled substrates. The uptake of substrates during the incubation period was determined by measuring the remaining radioactivity in the culture medium after different periods of incubation. The time course of the uptake of substrates in greening mutant cells and synchronized WT cells is shown in Fig. 1. In both cases the labeled glycines were taken up faster and more effectively than glutamate. After a 6 h incubation period more than 90% of the glycines were taken up in both cultures, whereas the uptake of [1-^{14}C]glutamate reached only about 60%.

Purification of the hemes

Protoheme and heme *a* were extracted with acidified acetone (see Materials and Methods) and were further purified by a combination of gel- and ion-exchange-chromatography. In contrast to the systems described by Weinstein and Beale [17] and Schneegurt and Beale [19] we used Fractogel TSK-650 instead of DEAE-Sepharose. When preparing

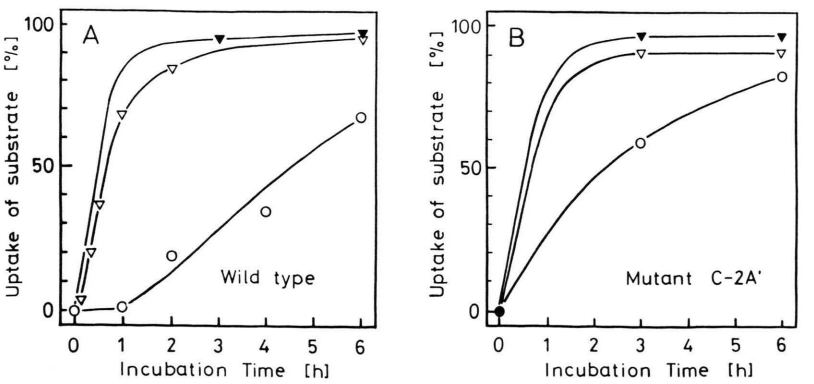

Fig. 1. A: Uptake of radioactive substrates by synchronized cells of the wild type of *Scenedesmus obliquus* during the first 6 h of irradiation. The uptake was determined by measuring the activity of the culture medium after different times of incubation. The culture medium contained 1 mM unlabeled glutamate and glycine. Symbols: ○ [1-^{14}C]glutamate; ▽ [2-^{14}C]glycine; ▼ [1-^{14}C]glycine.
B: Uptake of radioactive substrates by the developmental-mutant C-2A′ of *Scenedesmus obliquus* during the early phase of greening in white light (20 $W \cdot m^{-2}$). See also Fig. 1A. Symbols: ○ [1-^{14}C]glutamate; ▽ [2-^{14}C]glycine; ▼ [1-^{14}C]glycine.

standards of heme *a* and protoheme from large amounts of unlabeled culture material both tetrapyrroles were separated by elution of the Fractogel-column with a stepwise gradient of acetic acid (0.25%; 0.5%; 2.0%) in 90% acetone. The elution of the different hemes was monitored by the change of the absorption maximum in the eluted fractions (heme *a:* 406 nm; protoheme: 398 nm).

Weinstein and Beale [17] described the separation of protoheme and heme *a* by elution from DEAE-Sepharose with 0.5% acetic acid in 80% acetone. Protoheme was eluted before heme *a* in that system. In our system heme *a* was eluted before protoheme using 0.25% and 0.5% acetic acid in 90% acetone. Protoheme was retained on the column after the elution of heme *a*. In order to elute it in a small volume and a sharp band, 2% (v/v) acetic acid in 90% acetone (v/v) was used. The elution profile shown in Fig. 2 was obtained with heme extracts from *Euglena gracilis,* because from this organism greater amounts of hemes could be isolated. However, identical profiles were also obtained with *Scenedesmus*.

For identification the heme *a* containing fraction was extracted with ether. After evaporation of the ether the residue was dissolved in 90% (v/v) aqueous pyridine and a pyridine-hemochromogene spectrum [20] was recorded. The heme-*a*-fraction we obtained, following the separation from protoheme on the Fractogel-column, shows two major absorption bands at 426 and 584 nm, a small peak at 524 nm and a shoulder at 559 nm indicating a small amount of protoheme present in this fraction. For the preparation of a pure heme *a* standard this fraction was further purified by HPLC.

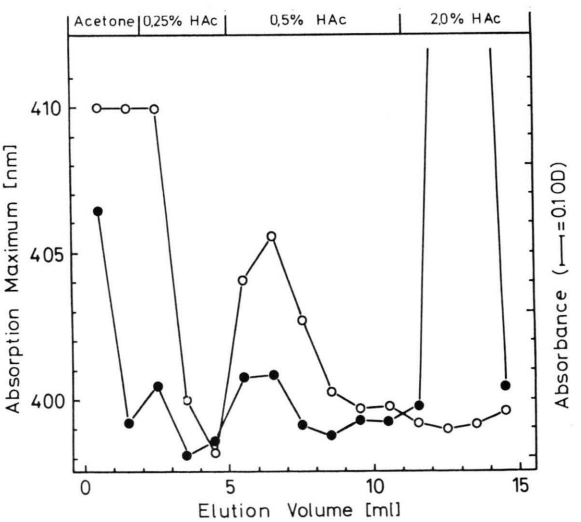

Fig. 2. Elution profile of a chromatographic separation of a heme-extract prepared from *Euglena gracilis* on a Fractogel-TSK-650-column (for details see Materials and Methods). The column was eluted with a stepwise gradient of acetic acid in acetone (concentrations are shown at the top of the figure). Flow rate was 1 ml·min^{-1}. Symbols: —○——○— absorption maximum; —●——●— absorbance.

In labeling-experiments we used small amounts of culture material (100 or 250 ml). To avoid a loss in labeled products, the heme extracts we prepared in these experiments were not separated into protoheme and heme *a* by ion-exchange chromatography on Fractogel. Rather, lipids, carotenes and degradation products of chlorophylls, which were also labeled, were separated from the hemes by elution of the Fractogel-column with pure, respectively 80% aqueous acetone. The hemes retained on the column during this procedure were eluted with 2% acetic acid in 90% acetone and subsequently separated by HPLC.

With the HPLC-system described in Materials and Methods, protoheme and heme *a* could be separated. Because of the high amount of protoheme relative to heme *a* in the samples the hemes were not fully separated from each other by a single run on the reversed-phase column. Therefore, the heme *a* containing fractions were collected from the first HPLC elution and separated from remaining protoheme in a second HPLC run. The elution diagrams of two subsequent runs of a protoheme and heme *a* containing sample are shown in Fig. 3.

The absorption spectra of the hemes purified by HPLC as reduced pyridine hemochromogens, are shown in Fig. 4. The absorption maxima agree with published data for heme *a* [20] and protoheme [18].

No radioactive remnants were recognized on the column material under our experimental conditions.

Incorporation of labeled precursors into the hemes

In all our experiments we were able to recover radioactivity in the protoheme containing fractions of the HPLC eluate. Table I shows the specific activities of the ^{14}C-labeled substrates, the

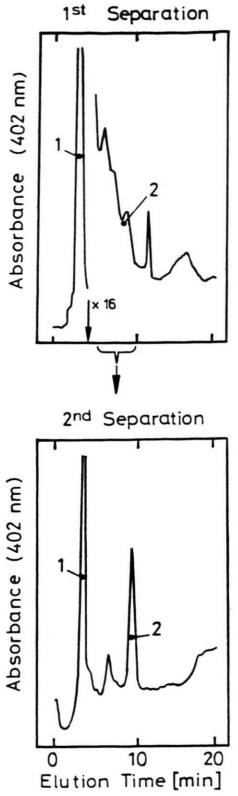

Fig. 3. Elution profiles of protoheme (indicated by "1") and heme *a* (indicated by "2") after separation by HPLC with a reversed phase column (Nucleosil 100-10 C 18 (Macherey-Nagel, Düren, Germany); particle size 10 μm). The mobile phase was 98% ethanol and 2 M acetic acid 70:30 (v/v). Flow rate was 0.8 ml·min^{-1}. The hemes were extracted from *Scenedesmus obliquus* and purified first by ion-exchange-chromatography on Fractogel.

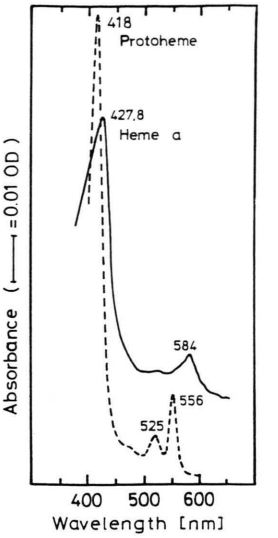

Fig. 4. Absorption spectra of protoheme and heme *a* extracted from *Scenedesmus obliquus* after separation by HPLC (see Fig. 3). Spectra were recorded with an UVIKON dual beam spectrophotometer (Kontron, Eching, Germany). Light path was 1 cm.

amount of uptake of the substrates and the incorporation of radioactivity into protoheme, summarized from different experiments. The degree of incorporation is expressed as specific radioactivity of the labeled protoheme and as percentage of protoheme synthesized from exogenous substrates of the total amount of protoheme. The method for calculating the amount of product synthesized from exogenous substrate described by Weinstein and Beale [17] is based on the assumptions that for

Table I. Specific activity and uptake of the substrate applied to algal cultures in *in vivo* labeling experiments (see Materials and Methods section for methods employed) and the incorporation of these substrates into protoheme (for isolation and purification see Materials and Methods). The amount of protoheme synthesized from exogenous substrate was calculated as described by Weinstein and Beale [17].

	Specific activity of substrate [dpm·nmol^{-1}]	Uptake of substrate [%]	Specific activity of protoheme [dpm·nmol^{-1}]	Protoheme synthesized from exogenous substrate [%]
Mutante C-2A′				
[1-^{14}C]glutamate	269 ± 4	81.5 ± 7.6	268 ± 67	14.5 ± 3.6
[2-^{14}C]glycine	226 ± 49	89.6 ± 4.2	37 ± 11	2.3 ± 0.3
[1-^{14}C]glycine	293 ± 134	95.6 ± 1.3	9 ± 6	0.3 ± 0.2
Wild type				
[1-^{14}C]glutamate	352 ± 100	64.5 ± 5.8	245 ± 62	13.0 ± 1.7
[2-^{14}C]glycine	299 ± 85	96.2 ± 1.7	72 ± 9	3.0 ± 0.5
[1-^{14}C]glycine	270 ± 51	96.7 ± 1.7	28 ± 16	1.4 ± 1.0

the synthesis of 1 mol of product, 8 mol of substrate are needed and that the intracellular pool of substrate is negligible relative to the amount of exogenous substrate added to the cell suspensions. Thus the amount of product synthesized from the exogenous substrate is calculated according to the following formula:

$$\frac{\text{Radioactivity}_{\text{Product recovered}}\,[\text{dpm}]}{\text{Spec. activity}_{\text{Substrate}}\,[\text{dpm nmol}^{-1}] \times 8} = \text{product}_{\text{exog. Substrate}}\,[\text{nmol}].$$

This value, when expressed as percentage of the total amount of isolated product, allows the comparison of the isolated amounts of protoheme, even if the specific activity of substrates or the amount of culture material applied, differs. It is also normalized for possibly existing differences in intracellular pool sizes of protoheme.

The values in Table I show that a high amount of radioactivity was incorporated into protoheme when cells were incubated with [1-^{14}C]glutamate and much less when incubated with [2-^{14}C]glycine. The amount of radioactivity incorporated into the porphyrin in the experiments with [1-^{14}C]glycine was negligible.

When the incorporation of different substrates into heme *a* were compared ambiguous results were noted. In some experiments no significant incorporation of radioactivity into heme *a* was detected. In two experiments, one with wild type cells and the other one with mutant cells, the heme *a* containing fraction eluted by HPLC was radioactive. Activity in these fractions was increased by a factor of 10 relative to the fractions eluting before and after the heme *a*. The specific activities for heme *a* calculated from these measurements were 1200 dpm · nmol^{-1} and 1040 dpm · nmol^{-1} in the mutant cells and 570 dpm · nmol^{-1} and 800 dpm · nmol^{-1} in the wild type cells when incubated with [1-^{14}C]glutamate or [2-^{14}C]glycine, respectively. No incorporation of ^{14}C from [1-^{14}C]-glycine was found in the experiment with the mutant cells, but in the wild type cells the specific activity of the isolated heme *a* was calculated to be 400 dpm · nmol^{-1}. The amount of heme *a* isolated in these experiments was about 0.3 nmol.

Turnover of protoheme

Because of the long incubation time we used in these experiments a possible turnover of the protoheme had to be taken into account. Therefore, pulse chase experiments were carried out with the fast greening mutant cells. The mutant cells were chosen since Castelfranco and Jones [21] reported that a turnover of protoheme in greening barley leaves is favoured under conditions of a high rate of chlorophyll synthesis. [1-^{14}C]glutamate was used as substrate since it had been shown to be the most effective precursor for the labeling of protoheme. The time course for the development of the specific activity of protoheme in a pulse-chase-experiment is shown in Fig. 5. Cells were incubated for 6 h with the labeled substrate (pulse) which was then washed out and replaced by unlabeled substrate (chase). Protoheme was isolated from samples taken at various times after the radioactive substrate was washed out and the amount of radioactivity incorporated was determined. During the first hour after the chase the specific radioactivity of protoheme rose by about 20%. However, in the following 2 h the specific activity of protoheme dropped to 27% of the peak value obtained 1 h after the chase of radioactivity. After 18 h the specific activity of protoheme was only 16% of the peak value.

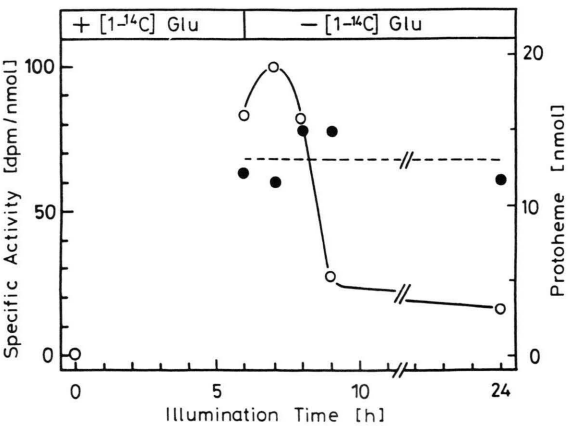

Fig. 5. Pulse-chase-experiment with [1-^{14}C]glutamate applied as radioactive substrate to greening cells of the pigment-mutant C-2A′ of *Scenedesmus obliquus* (for incubation conditions see Materials and Methods). The incubation period with and without the radioactive substrate is indicated at the top of the figure. The specific activity of the protoheme isolated at different times after the chase (○) was calculated as percentage of the highest specific activity which was reached 1 h after the chase. The total amount of protoheme isolated (●) is also shown.

Discussion

The presence of the different biosynthetic pathways leading to ALA, the C_5-pathway [1] and the Shemin-pathway [22], has been investigated in many plants. The synthesis of chlorophylls *via* the C_5-pathway seems to be common in plants, while the mechanisms of heme synthesis were found to vary in different organisms.

For the unicellular green alga *Scenedesmus obliquus* it was also shown that chlorophylls are synthesized *via* the C_5-pathway [23], but the biosynthesis of hemes has not been investigated in this organism.

In this investigation a modified method for the isolation of hemes from *Scenedesmus* was employed. The results show that protoheme can be labeled by different radioactive precursors of ALA-biosynthesis. Future studies will be required to evaluate the participation of both pathways to ALA in the synthesis of the hemes in *Scenedesmus*.

The system described here for the separation of protoheme and heme *a* by anion-exchange-chromatography with Fractogel TSK 650 is very useful for the preparation of a crude heme *a* preparation starting from large amounts of plant material. Since the ratio of protoheme to heme *a* is high in plants, the removal of the majority of protoheme by anion-exchange chromatography facilitates the subsequent preparation of pure heme *a* by HPLC. Only one run on HPLC is sufficient to separate the heme *a* in these extracts from the remaining traces of protoheme.

Weinstein and Beale [17] report an inverse elution pattern of protoheme and heme *a* from the DEAE-Sepharose column. We could not confirm their results with either the Fractogel-column, nor with separations on the DEAE-matrix with respect to the expected polarity characteristics of the substances to be separated. Since no details are given by Weinstein and Beale [17], the discrepancy remains unexplained.

Our labeling-experiments with ^{14}C-substrates show that [1-^{14}C]glutamate and [2-^{14}C]glycine are incorporated into protoheme in wild type and mutant cells of C-2A′ of *Scenedesmus*. [1-^{14}C]glutamate was more effective in labeling protoheme (3.5 times in WT cells; 7 times in C-2A′); however, a significant incorporation of label from [2-^{14}C]glycine was always detected.

Similar labeling patterns were obtained by Weinstein and Beale [24] with *Cyanidium caldarium* when the culture material was incubated for 7 h with radioactive substrates. However, the authors explain the labeling of protoheme with [2-^{14}C]glycine by a distribution of activity in the general metabolism rather than through the Shemin-pathway. This discrepancy will be subject of further investigations.

Our results concerning labeling of heme *a* with labeled glycine and glutamate varied. In some experiments the specific activity was evenly distributed between both hemes while in others the specific activity of heme *a* was even higher than that noted for the protoheme fraction. Since the amount of heme *a* isolated in our experiments was always very low (about 2% of the amount of protoheme), we do not consider the labeling of these small amounts of heme *a* meaningful enough to draw any conclusion from it.

Heme *a* was shown to be synthesized from protoheme in *Staphylococcus* [25]. If this would also be true for plant material, the specific activities of protoheme and heme *a* should be similar if both porphyrins would turn over with the same rate. However, our results would be consistent, if heme *a* were lacking in contrast to protoheme any turnover. The existence of a turnover of protoheme was indeed deduced by Castelfranco and Jones [21] from the incorporation of ^{14}C-labeled precursors into protoheme in greening barley leaves. They also found a labeling of protoheme in the absence of a net synthesis. These results are, however, not fully conclusive, since the authors did not do pulse-chase experiments.

In greening cells of C-2A′ we showed by a pulse-chase experiment that a considerable part of the cellular protoheme has a turnover. This explains why protoheme can be labeled with radioactive precursors of porphyrin-biosynthesis even though the cytochrome level of the cells does not change during greening [13]. Our results also show that approximately 20% of the cellular protoheme has no turnover. Whether this portion of the protoheme pool fulfills a special function in the cell or what kind of mechanism prevents it from being degraded cannot be deduced from our data.

It is assumed from these results that the major part of the protoheme in *Scenedesmus* is synthesized *via* the C_5-pathway and a smaller portion is

synthesized *via* the Shemin-pathway. However, labeling of protoheme by [2-^{14}C]glycine *via* reactions of photorespiration, *via* the general metabolism of glycine or refixation of $^{14}CO_2$ in photosynthesis cannot be totally excluded. Since the role of the Shemin-pathway in plant-porphyrin biosynthesis is still a matter of controversy. A detailed study on the participation of the two biosynthetic pathways to ALA in the synthesis of protoheme in *Scenedesmus obliquus* will be a subject of future investigations.

Acknowledgements

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