Biosynthesis of Protoheme and Heme *a* Precursors Solely from Glutamate in the Unicellular Red Alga *Cyanidium caldarium*[1]

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ABSTRACT

Two biosynthetic routes to the heme, chlorophyll, and phycobilin precursor, δ-aminolevulinic acid (ALA) are known: conversion of the intact five-carbon skeleton of glutamate, and ALA synthase-catalyzed condensation of glycine plus succinyl-coenzyme A. The existence and physiological roles of the two pathways in *Cyanidium caldarium* were assessed *in vivo* by determining the relative abilities of [2-^{14}C]glycine and [1-^{14}C]glutamate to label protoheme and heme *a*. Glutamate was incorporated to a much greater extent than glycine into both protoheme and heme *a*, even in cells that were unable to form chlorophyll and phycobilins. The small incorporation of glycine could be accounted for by transfer of label to intracellular glutamate pools, as determined from amino acid analysis. It thus appears that *C. caldarium* makes all tetrapyrroles, including mitochondrial hemes, solely from glutamate, and there is no contribution by ALA synthase in this organism.

Two biosynthetic pathways to the heme and Chl precursor, ALA[2], are known. In animals, fungi and bacteria, ALA is formed by condensation of succinyl-CoA and glycine, catalyzed by the enzyme ALA synthase (succinyl-CoA:glycine *C*-succinyltransferase [decarboxylating], EC 2.3.1.37). In greening plants and algae, most or all ALA is synthesized via a five-carbon pathway that utilizes the intact carbon skeleton of glutamate or a closely related compound (2). This pathway supplies ALA for both Chl and hemes in greening plants (6). Although the five-carbon pathway has been found to exist in all O_2-evolving photosynthetic organisms examined (2), it is uncertain whether it is the only route for ALA formation in these organisms. It has been suggested that ALA synthase is used for ALA formation in the dark and/or for mitochondrial hemes, which are only a minor component of the total hemes in photosynthetic tissue (14).

One organism that utilizes both ALA biosynthetic pathways in the manner suggested above is the photosynthetic phytoflagellate *Euglena gracilis*. Cell-free extracts from *E. gracilis* catalyze the formation of ALA via the five-carbon pathway (17) and via ALA synthase (4). It was recently demonstrated that the five-carbon pathway is used for chloroplast tetrapyrroles and ALA synthase for ALA that is destined for mitochondrial and perhaps other nonplastid tetrapyrroles (20). However, details of morphology and biochemistry place *E. gracilis* phylogenetically closer to some animal-like protists than to green plants and algae (7, 8, 13). Therefore, these results cannot be justifiably extrapolated to plants and algae.

Cyanidium caldarium is a unicellular red alga which uses Chl *a* and phycacyanobilin as its photosynthetic pigments. The alga grows rapidly in liquid culture and efficiently takes up amino acids from the medium. *In vivo* labeling experiments have shown that *C. caldarium* uses the five-carbon pathway to make ALA in the light (11). A mutant strain of *C. caldarium* that is unable to make Chl or phycocyanobilin in the dark or light was also found to synthesize ALA via the five-carbon route (18). The approach used in these experiments was to block ALA utilization and cause its accumulation with levulinic acid, a competitive inhibitor of ALA dehydrase, and then measure incorporation of radioactivity into ALA from specifically labeled exogenous potential precursors. These procedures do not allow unambiguous determination of the physiological role of the accumulated ALA. Also, accumulation of the ALA into one pool may mask the existence of separate pools, each having a different intracellular biosynthetic origin, especially if the pool sizes are vastly different. Finally, in the experiments with the nonpigmented mutant, the cells were transferred to minimal medium without glucose for 24 h prior to incubation (18). In *E. gracilis*, ALA synthase activity reaches its lowest levels in cells just before stationary phase, and ALA synthase is subject to rapid turnover, with a half-life of 55 min (9). Thus, starvation of *C. caldarium* for 24 h before incubation might have decreased ALA synthase activity to undetectable levels. For these reasons, it was important to reinvestigate the route of ALA synthesis in *C. caldarium* with methods that are sensitive and specific enough to detect small amounts of ALA synthase that may be specific for certain functions.

Specifically labeled potential ALA precursors were administered to rapidly growing heterotrophic cultures of wild-type and nonpigmented *C. caldarium*. Relative incorporation of radioactivity into highly purified protoheme and heme *a* was used to monitor each ALA biosynthetic pathway. Heme *a* is the prosthetic group of Cyt oxidase, and is generally believed to exist only within the mitochondria in eucaryotic cells. Incorporation of radioactivity into heme *a* was used as a specific indicator of mitochondrial heme synthesis. Portions of this work were previously reported in abstract form (19).

MATERIALS AND METHODS

Cells. Wild-type cells of *C. caldarium* and a nonpigmented mutant (GGB-Y), incapable of forming Chl or phycobilins, were a generous gift from Dr. R. F. Troxler (Boston University School of Medicine). The cells were grown axenically in the dark at 42°C on a glucose-based heterotrophic medium as described previously (3). Each culture consisted of 60 ml of cell suspension in a 125-ml flask. Wild-type cells form reduced amounts of phycobilins and Chl when grown in the dark. Maximum cell density at stationary phase in this medium was 2×10^8 cells/ml.

Incubation Conditions. Cells were incubated under the conditions described above for 4 or 7 h at initial cell densities selected so that the final cell density would be close to 1×10^8 cells/ml.

[2] Abbreviation: ALA, δ-aminolevulinic acid.

Each culture was supplemented with both unlabeled L-glutamic acid and glycine together at 0.5 mM each for the 4-h incubations or 1 mM each for the 7-h incubations. For each incubation, the algae were administered L-[1-^{14}C]glutamic acid, [1-^{14}C]glycine, or [2-^{14}C]glycine in two separate flasks for each isotope. Uptake was monitored hourly by sampling 30-μl portions of the centrifuged cell medium as described previously (20). After incubation, the cultures were cooled to 0°C and stored on ice overnight.

Pigment Extraction. The two flasks of cells for each isotope administration were combined and the cells harvested by centrifugation. The combined cell pellets were washed with 20 ml of cold deionized H$_2$O. The cells were suspended in 3 ml of dimethyl sulfoxide followed by 12 ml of cold 99% (v/v) aqueous acetone containing 12.5 mM NH$_4$OH, and allowed to stand for 5 min at room temperature before centrifugation. The cell pellets were extracted in this manner a total of seven times. The first three extracts were saved for future analysis. Noncovalently bound hemes were next extracted by suspending the cells in 2 ml of dimethyl sulfoxide and adding 0.5 ml of concentrated HCl and 10 ml of cold acetone with mixing. The mixture was allowed to stand for 10 min at room temperature in the dark before centrifugation. The extraction was repeated three times with half volumes and the combined extracts were filtered through a plug of glass wool.

Purification of Hemes. Fifteen ml of peroxide-free diethyl ether were added to the acid-acetone extract and, after mixing, 75 ml of cold water and 2 ml of saturated NaCl were added to achieve phase separation. The top ether phase, containing the hemes, was removed, and the bottom phase was re-extracted with 10 ml of ether. The combined ether extracts were washed twice with two 10-ml portions of water. A volume of 95% (v/v) aqueous ethanol equal to one-third of the ether volume was added to the ether. The hemes in ether-ethanol were applied to a DEAE-Sepharose (acetate form) column (20) which had been equilibrated previously with diethyl ether:95% ethanol (3:1, v/v). The column was washed with 10 ml of the same solvent mixture, then with 5 ml of ether:95% ethanol (1:1, v/v), and finally with 1 ml of 95% ethanol. The hemes were eluted with a minimal volume of ethanol:acetic acid:water (81:9:10, v/v/v). The solvent was evaporated in a stream of N$_2$ until 0.5 ml remained.

Protoheme and heme *a* were further purified and separated from each other by reverse phase HPLC as previously described (20).

HPLC eluate fractions corresponding to the heme peaks were collected and the absorbance spectrum recorded. Concentrations were determined from the absorption coefficients of the air-oxidized hemes in HPLC solvent: 144 mM^{-1} at 398 nm for protoheme, and 123 mM^{-1} at 406 nm for heme *a*. These absorption coefficients were determined from standards as described previously (20). The solvent was evaporated and radioactivity was determined by liquid scintillation counting (20).

Amino Acid Analysis. Free amino acids were recovered from the first three basic dimethyl sulfoxide-acetone cell extracts. The combined extracts were diluted with 75 ml of water, acidified to pH 3 with HCl, and extracted with five 15-ml portions of ether. The remaining aqueous phase was reduced in volume on a vacuum rotary evaporator and then applied to a 1-cm-diameter × 8.8-cm-long column of Dowex 50X8 400 mesh (hydrogen form). The column was washed with 5 ml of 25% (v/v) aqueous ethanol and then 5 ml of water. Amino acids were eluted with 1 M NH$_4$OH. The radioactive fractions were dried by rotary evaporation and redissolved in 1 ml of 0.1 N HCl.

A 10-μl sample was applied to a Waters HPLC amino acid analyzer. Separation was achieved on a 0.4-cm-diameter × 15-cm-long sulfonated polystyrene column (Waters model 80002) with an increasing pH gradient of 0.2 M Na citrate, and detection was by post-column reaction with *o*-phthalaldehyde and contin-

uous monitoring of absorption at 338 nm and fluorescence at 455 nm (5). Fractions were collected and counted in a Triton X-100 based scintillation mixture (10).

Chemicals. Radioactive isotopes were purchased from New England Nuclear, and all other reagents and solvents from Fisher or Sigma.

RESULTS

Growth of Cells and Uptake of Substrates. Cultures were inoculated with either wild-type or nonpigmented cells and grown in the dark for at least 3 d prior to incubation.

Details of substrate supply, uptake, and final cell number are shown in Table I. At least 92% of the substrate was taken up during the 4-h incubations with 0.5 mM substrates. In other experiments, at least 43% of the substrate was taken up during the 7-h incubations with 1 mM substrates. Differences in the apparent uptake between [1-^{14}C]glycine and [2-^{14}C]glycine are most likely due to excretion of the metabolized portion of the molecule. This effect is greater in the longer incubations.

Recovery and Identification of Hemes. Results of a typical separation of *C. caldarium* protoheme and heme *a* by reverse phase HPLC are shown in Figure 1. The first major peak which eluted at 11.1 ml was collected and its Soret absorbance spectrum recorded (Fig. 2a). The spectrum was identical to that of commercially available protoheme with an absorption maximum at 398 nm in HPLC solvent. The second major peak in Figure 1 eluted at 26.5 ml and had an absorption spectrum identical to that of heme *a* purified from beef heart (Fig. 2b). The absorption maximum in HPLC solvent was 406 nm. Peak elution volumes of standard protoheme and heme *a* were 11.3 and 26.5 ml, respectively. Dithionite-reduced pyridine-hemochrome spectra of HPLC-purified commercial protoheme and beef-heart heme *a* are shown in Figure 2c. Identical spectra were obtained from hemes purified from *C. caldarium* cells (Fig. 2d).

The amounts of hemes recovered were determined from the absorption at the Soret maxima and were approximately equal for wild-type and nonpigmented cells: 10.2 ± 2.3 and 0.60 ± 0.119 nmol/10^{10} cells for protoheme and heme *a*, respectively.

Incorporation of Radioactivity into Hemes during 4-Hour Incubations. Coelution of the major peaks of radioactivity with the two heme peaks is shown in Figure 1. Eighty-six per cent of the radioactivity that was recovered from the HPLC eluate was associated with the two heme peaks.

Radioactivity incorporation and recovery of hemes from 4-h incubations with wild-type cells is shown in the first three lines of Table II. The amounts of radioactivity incorporated and the specific radioactivities of the substrates were used to calculate the amounts of product synthesized from each exogenous substrate (20). These values, shown in the first three lines of Table III, are normalized for differences in substrate specific radioactivity. Also shown in Table III is the specific radioactivity of the recovered hemes. The ratio of incorporation of [1-^{14}C]glutamate to [2-^{14}C]glycine was 25 for protoheme and 5 for heme *a*. For heme *a*, labeling by [1-^{14}C]glycine, an indicator of nonspecific or indirect incorporation, was 76% of the value for labeling by [2-^{14}C]glycine. In the incubation with [1-^{14}C]glutamate, the specific radioactivity of protoheme and heme *a* were within 13% of each other.

Incorporation of Radioactivity into Hemes during 7-Hour Incubations. In separate experiments, incubation times were extended to 7 h with both wild-type and nonpigmented cells. In these experiments, the concentrations of unlabeled substrates were increased to 1 mM to ensure the presence of sufficient substrate for uptake to occur throughout the entire incubation period. Both cell types preferentially utilized [1-^{14}C]glutamate as a precursor for both protoheme and heme *a* (Table III). In each case, incorporation of [1-^{14}C]glutamate was at least 3-fold higher

Table I. *Incubation Conditions*

Cells were grown in liquid culture and incubated in the dark with the radioactive substrates shown. The percentage of substrate taken up and the cell number at the end of the incubations are tabulated.

| Cell Type and Incubation Time (h)	^{14}C-Substrate	Radioactivity Supplied	Specific Radioactivity of Substrate	Substrate Taken up	Cell No.
		μCi	$cpm/nmol$	%	$\times 10^{10}$
Wild-type (4)	1-Glutamate	44.7	1640	98.1	1.34
	1-Glycine	43.6	1600	98.6	1.34
	2-Glycine	42.1	1550	92.1	1.36
Wild-type (7)	1-Glutamate	50.8	938	96.6	1.61
	1-Glycine	49.9	922	66.1	1.55
	2-Gycine	44.1	815	43.2	1.60
Nonpigmented (7)	1-Glutamate	36.3	668	85.3	1.36
	1-Glycine	33.8	621	67.3	1.36
	2-Glycine	32.5	598	75.1	1.33
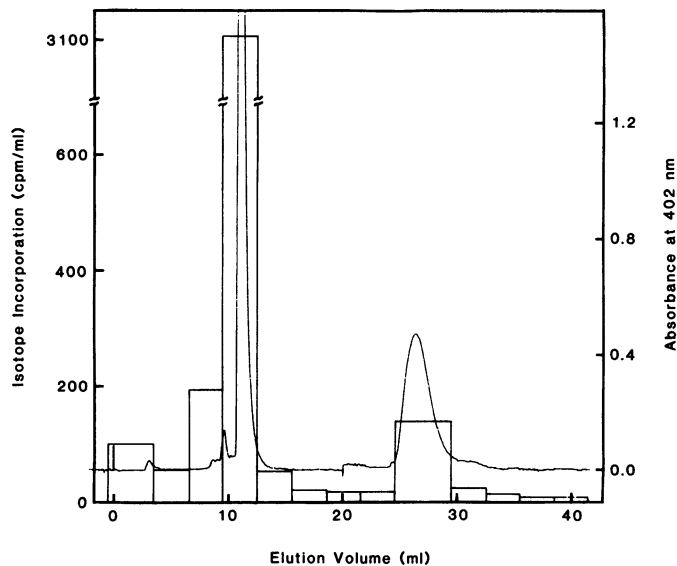
FIG. 1. Separation of protoheme and heme *a* by reverse phase HPLC. Elution volumes of protoheme and heme *a* were 11.1 ml and 26.5 ml, respectively. At 20 ml elution volume, the absorbance sensitivity was increased 10-fold. Radioactivity incorporation from [1-^{14}C]glutamate is shown by the vertical bars. This sample was from the 4-h incubation of wild-type cells.

than the incorporation of the next best precursor, [2-^{14}C]glycine. The ratio of incorporation of [2-^{14}C]glycine to [1-^{14}C]glycine was never higher than 19.3 and in three of four cases was less than 11.0. In the experiment with wild-type cells, the specific radioactivity of protoheme was equal to that of heme *a* whether the label was from [1-^{14}C]glutamate or [2-^{14}C]glycine.

Comparison of Label Incorporation Patterns in the Different Incubations. The results of all three experiments are summarized in Figure 3. The percentages of the products synthesized from each exogenous substrate are normalized for differences in specific radioactivity of the substrate and differences in the amount of product recovered. Clearly, [1-^{14}C]glutamate was the best precursor for both protoheme and heme *a* in all cases. The most pronounced difference between the effectiveness of [1-^{14}C]glutamate and [2-^{14}C]glycine was in the 4-h incubation with wild-type cells. Protoheme in this instance was effectively labeled only by [1-^{14}C]glutamate. In the 7-h incubations, the relative effectiveness of incorporation of [1-^{14}C]glutamate over [2-^{14}C]glycine de-

creased compared to the 4-h incubations.

Labeling of the Free Amino Acid Pool. Free amino acids recovered from the cell extracts after the 7-h incubations of wild-type and nonpigmented cells were separated by HPLC. Individual fractions were collected and radioactivity was determined in each. When [1-^{14}C]glutamate was the substrate, one major peak of radioactivity appeared, coinciding with proline, and a small amount of radioactivity coeluted with glutamate (data not shown). The radioactivity under the glycine peak was not above the baseline level. In contrast, when [2-^{14}C]glycine was the substrate, there were many radioactive fractions, some of which did not correspond to protein amino acid peaks. However, significant amounts of radioactivity coeluted with aspartate, threonine, serine, glycine, and glutamate.

DISCUSSION

Green plants and algae synthesize ALA that is destined for Chl and chloroplast hemes via a five-carbon pathway (2, 6). The existence and possible physiological role of the enzyme ALA synthase in plants and algae have remained uncertain. It was recently demonstrated that the photosynthetic phytoflagellate, *E. gracilis*, uses the 'plant' five-carbon pathway to synthesize ALA for chloroplast products, while simultaneously using ALA synthase to manufacture ALA destined for nonplastid hemes, including mitochondrial heme *a* (20). Simultaneous operation of the two pathways was demonstrated by administration of labeled potential ALA precursors to growing cultures and determining the relative degree of label incorporation into the tetrapyrrole end products specific to chloroplasts and mitochondria. *In vivo* activity of ALA synthase was indicated by the preferential incorporation into ALA of radioactivity from C_2 of glycine, compared to C_1 of glycine or C_1 of glutamate. The C_2 of glycine becomes C_5 of ALA, which is subsequently incorporated into eight positions in the porphyrin macrocycle. Activity of the five-carbon pathway is indicated by preferential incorporation of C_1 of glutamate, which also becomes C_5 of ALA and is subsequently incorporated into the porphyrin macrocycle. Compared to the more commonly used technique of radioactive precursor administration and blocking ALA utilization with levulinic acid (1, 2), the above procedure is more sensitive in its ability to detect the contribution of minor amounts of ALA that are destined for a specific product, even when much greater amounts of ALA are simultaneously being produced via a different pathway. For example, it was possible to detect heme *a* biosynthesis via ALA synthase under conditions where the ratio of Chl to heme *a* is 6400 and the Chl is synthesized via the five-carbon pathway (20).
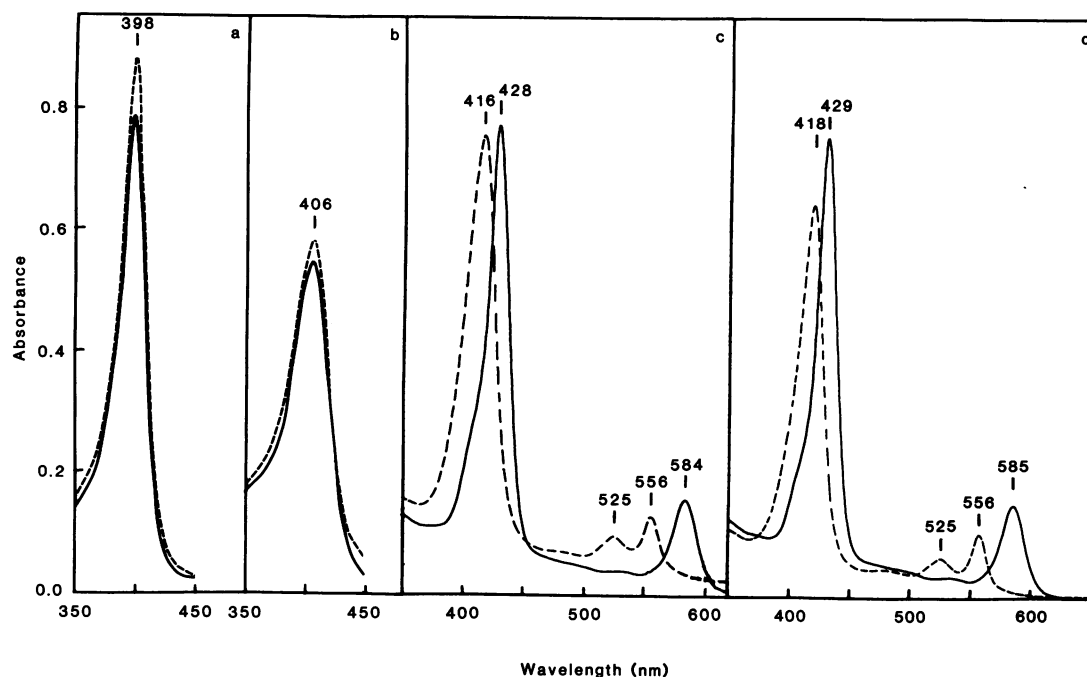
Wavelength (nm)

Fig. 2. a, Soret band absorption spectra in HPLC solvent of standard protoheme (---) and protoheme extracted from *C. caldarium* cells
(———). b, Soret band absorption spectra in HPLC solvent of standard heme *a* extracted from beef heart (---) and heme *a* extracted from *C. caldarium* cells (———). c, Absorption spectra in 90% aqueous pyridine of dithionite-reduced HPLC-purified beef heart heme *a* (———) and standard protoheme (---). d, Absorption spectra in 90% aqueous pyridine of dithionite-reduced HPLC-purified *C. caldarium* heme *a* (———) and protoheme (---). Absorbance scale is expanded 20-fold in (b), and 2- to 10-fold in (d). Peak absorption wavelengths in nanometers are indicated in the figure.

Table II. *Incorporation of ^{14}C into Hemes*

Hemes were extracted from the algae and purified. The total amount of radioactivity incorporated into each heme is tabulated.

Cell Type and Incubation Time (h)	^{14}C-Substrate	Protoheme		Heme *a*	
		Product recovered	Radioactivity	Product recovered	Radioactivity
		nmol	*cpm*	*nmol*	*cpm*
Wild-type (4)	1-Glutamate	18.3	9320	1.19	695
	1-Glycine	15.9	150	1.16	102
	2-Glycine	15.5	350	1.04	130
Wild-type (7)	1-Glutamate	12.8	5120	1.33	547
	1-Glycine	14.1	121	1.14	18
	2-Glycine	10.1	1140	1.17	135
Nonpigmented (7)	1-Glutamate	15.0	6020	1.31	775
	1-Glycine	12.7	96	1.18	20
	2-Glycine	15.4	1780	1.47	130

If this experiment had been performed by blocking ALA utilization with levulinic acid and measuring label incorporation into the accumulated ALA pool, it could have been concluded that ALA synthase was not present in *E. gracilis*.

In light of recent findings in *E. gracilis* (20) and the continuing uncertainty about the possible existence of two pathways for ALA synthesis in plant tissues, we have used the techniques of labeling purified end products *in vivo* to test the possibility that *C. caldarium* uses ALA synthase to make mitochondrial hemes. Both wild-type and nonpigmented cells were grown in the dark to minimize the contribution of chloroplast development to total ALA synthesis. The concentration of unlabeled substrate mixture was chosen to expose the cells to equivalent concentrations of both glutamate and glycine to minimize effects due to different endogenous pool sizes and rates of uptake. The addition of

unlabeled substrate was also necessary to ensure the availability of exogenous substrate throughout the entire incubation time. For the 7-h incubations, the concentrations had to be increased from 0.5 to 1.0 mM. The particular incubation times were selected to minimize label scrambling and still allow time for sufficient cell growth to permit observation of label incorporation into products present in very small amounts. The 7-h incubation of wild-type cells was a separate experiment and not a continuation of the 4-h incubation. Valid comparisons between experiments are possible only with data that have been corrected for differences in substrate specific radioactivity and recovery of the hemes. These data are presented as percentage of the recovered products from exogenous substrate in Figure 3. Comparison of specific radioactivity is valid only with reference to the two heme products from the same flasks of cells.

Table III. *Effectiveness of Labeled Substrates as Heme Precursors*

Values are calculated from the data of Tables I and II, assuming that 8 mol substrate are required for 1 mol heme, and that the endogenous substrates contribute negligibly to the specific radioactivities of the hemes.

| Cell Type and Incubation Time (h)	^{14}C-Substrate	Protoheme		Heme a	
		Specific radioactivity	Synthesis from exogenous substrate	Specific radioactivity	Synthesis from exogenous substrate
		cpm/nmol	*pmol*	*cpm/nmol*	*pmol*
Wild-type (4)	1-Glutamate	509	709	584	52.9
	1-Glycine	9.43	11.7	87.9	7.96
	2-Glycine	22.6	28.3	125	10.5
Wild-type (7)	1-Glutamate	400	682	411	72.9
	1-Glycine	8.58	16.0	15.8	2.44
	2-Glycine	113	174	115	20.7
Nonpigmented (7)	1-Glutamate	401	1130	592	145
	1-Glycine	7.56	19.3	16.9	4.03
	2-Glycine	115	372	88.0	27.2
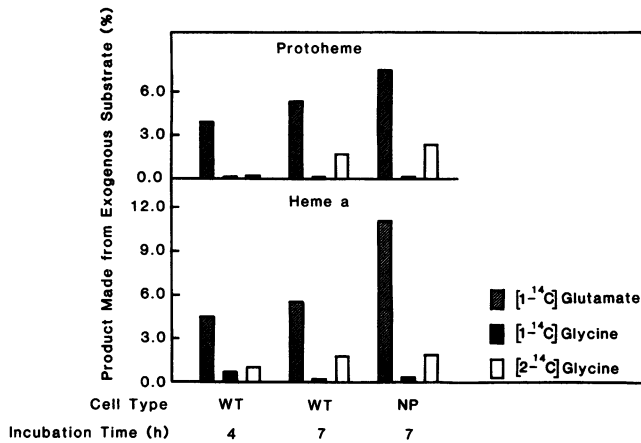
FIG. 3. Percentages of hemes made from exogenous substrates. Each value was calculated from the quantity of product synthesized from exogenous substrate and the total product recovered. Abbreviations: WT, wild-type; NP, nonpigmented.

In the 4-h incubation of wild-type cells, [1-^{14}C]glutamate was the best precursor of protoheme by a factor of 25. Thus, the five-carbon pathway provided ALA for at least 96% of the protoheme which would be found in both chloroplasts and mitochondria. [1-^{14}C]Glutamate was also the best precursor of heme a, the prosthetic group of mitochondrial Cyt oxidase. Thus, the five-carbon pathway was responsible for at least 83% of the heme a which was presumably mitochondrial in origin. The small contribution of [2-^{14}C]glycine to heme a was probably not due to ALA synthase because the contribution of [1-^{14}C]glycine is almost as great. One criterion for the detection of ALA synthase activity *in vivo* is that the ratio of incorporation of the C_2, compared to the C_1, of glycine must be high. In *E. gracilis*, which has ALA synthase and uses it to synthesize heme a, this ratio was twenty to one or greater (20).

The specific radioactivities of protoheme and heme a in the 4-h incubation with [1-^{14}C]glutamate were within 13% of each other, suggesting that both end products were derived from the same ALA pool. These results also indicate that very little additional radioactivity was incorporated into the farnesyl group of heme a.

The results of the 7-h incubations with both wild-type and nonpigmented cells were similar to the 4-h incubation. The best

precursor for both protoheme and heme a was [1-^{14}C]glutamate, indicating that most of the ALA destined for these products was derived via the five-carbon pathway. However, in the 7-h incubations the labeling of both hemes by [2-^{14}C]glycine was significant and the ratio of incorporation of the C_2 compared to the C_1 of glycine was high enough to suggest the possible involvement of ALA synthase. It is difficult to rationalize the involvement of ALA synthase in the 7-h incubations but not in the 4-h incubation, especially since the cultures were at the same cell density at the end of the experiments. A more likely explanation is that the longer incubation period allowed more time for metabolism of the exogenous precursors and subsequent indirect incorporation of [2-^{14}C]glycine into glutamate and then into hemes via the five-carbon pathway. The most likely fate of the label from [1-^{14}C] glutamate or [1-^{14}C]glycine would be removal from the system as CO_2. However, the C_2 of glycine would be expected to stay in the system longer.

This possibility was investigated by determining radioactivity remaining in the free amino acid pools after the incubations. When [2-^{14}C]glycine was the substrate, several amino acids were apparently labeled, including aspartate, serine, threonine, glutamate, and glycine. There were also several peaks of radioactivity which did not coincide with amino acid peaks. Therefore, it was impossible to confidently ascribe the radioactivity which did elute with amino acids to those amino acids rather than to contaminants which happened to coelute. When [1-^{14}C]glutamate was the substrate, only glutamate and proline appeared to be radioactive. Thus, the pattern of labeling of the free amino acid pools was consistent with the hypothesis that labeling of hemes from [2-^{14}C]glycine, but not from [1-^{14}C]glutamate, was via an indirect process.

In the light, wild-type cells of *C. caldarium* synthesize Chl, phycocyanobilin, and hemes from ALA. In these cells, ALA utilization can be partially blocked by the ALA dehydratase inhibitor levulinic acid (11). When specifically labeled radioactive precursors were administered to the cells under these conditions, the distribution of radioactivity in ALA indicated that it was made via five-carbon pathway rather than via the ALA synthase reaction (11). Because the total amount of photosynthetic pigments are vastly greater than mitochondrial hemes, it is possible that a potential contribution from ALA synthase to mitochondrial heme precursor ALA might have been overlooked. Therefore, similar experiments were repeated with a mutant strain which is unable to make phycocyanobilin or Chl

(18). The results were similar to those with wild-type cells: In the light or in the dark, the nonpigmented cells, which accumulated small amounts of ALA in the presence of levulinic acid, synthesized that ALA via the five-carbon pathway (18). However, these cells had been resuspended in minimal medium minus glucose for 24 h prior to the incubation with radioactive precursors. ALA synthase is subject to rapid turnover *in vivo* (half-life of 55 min) in *E. gracilis* (9), and it is possible that the same might be true in *C. caldarium* when growth was limited by removing its energy source. We therefore re-examined the possible role of ALA synthase in providing ALA for mitochondrial hemes in growing cells by methods which were successful in showing the role of ALA synthase in providing ALA for mitochondrial heme *a* in *E. gracilis*. The results show that ALA synthase does not perform a similar function in *C. caldarium*, and there is no apparent contribution by ALA synthase to the formation of tetrapyrroles in this organism.

A recent report by Meller and Gassman (14) suggests that ALA synthase may be active in dark-grown barley leaves. The experimental approach in this study was to label accumulated ALA with radioactive precursors. Although the evidence suggested that the five-carbon pathway was not very active in the dark, a strong case for the participation of ALA synthase could not be made, due to uncertain purity of the recovered ALA.

Klein and Senger (12) have reported *in vivo* ALA synthase activity in the light-grown mutant strain of *Scenedesmus obliquus*. Although the labeling pattern in the accumulated ALA was indicative of ALA synthase, the originally suggested physiological role for this ALA in Chl biosynthesis has since been rejected (15, 16), and no other role has been determined. Thus, separate roles for two ALA synthetic routes have yet to be demonstrated in higher plants and algae other than *E. gracilis*.

The results obtained with *C. caldarium* are incompatible with the dual-pathway hypothesis for ALA formation that has been shown to occur in *E. gracilis* (20) and suggested for higher plants (14). The existence and role of ALA synthase in other plants and algae remain hypothetical. In order to test the dual-pathway hypothesis in a higher plant, it will be necessary to find a tissue that is undergoing heme *a* synthesis at a sufficiently high rate to permit significant label incorporation. We have attempted to label barley leaf heme *a in vivo* with [U-^{14}C]glutamate, which can label ALA formed by either pathway. Although protoheme became labeled, no radioactivity could be detected in heme *a* (J. D. Weinstein and S. I. Beale, unpublished observations).

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