Biosynthesis of Protoheme and Heme \(a\) from Glutamate in Maize

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ABSTRACT

The heme and chlorophyll precursor \(\delta\)-aminolevulinic acid (ALA) can be formed by two biosynthetic routes: from the intact carbon skeleton of glutamate via a five-carbon pathway (4, 16), and by condensation of succinyl-CoA and glycine catalyzed by ALA synthase (succinyl-CoA:glycine C-succinyltransferase [decarboxylating] EC 2.3.1.37) (11, 14). Animals and some bacteria, including photosynthetic bacteria, form ALA exclusively via ALA synthase (11, 14, 19). On the other hand, plants and algae form ALA and plastid hemes from ALA made via the five-carbon route (1, 6, 18, 21, 25, 26). The phytoplankter Euglena gracilis has both pathways: plastid tetrapyrroles are made from glutamate, while mitochondrial hemes, including heme \(a\), are made from glycine (25). In contrast, the unicellular red alga Cyanidium caldarium forms all cellular tetrapyrroles, including mitochondrial heme \(a\), from glutamate (26). It is not possible to extrapolate from these disparate results to the situation occurring in higher plant mitochondria.

Recent refinements of the techniques developed for determining the biosynthetic routes of hemes in algae have made it possible to experimentally determine the pathway in etiolated plant tissues. We now report that in etiolated maize epicotyl sections, all noncovalently bound hemes, including mitochondrial heme \(a\), are made from ALA that is synthesized from glutamate.

MATERIALS AND METHODS

Plant Material. Seeds of Zea mays cv 31001 (Blue Seal 12995) were soaked for 1 h in a 0.05% (w/v) aqueous solution of the anti-mold agent Botran, then thoroughly rinsed with deionized H\(_2\)O and soaked overnight in the dark at 25°C. The rehydrated seeds were sown in moistened vermiculite and grown for 8 d in the dark at 25°C.

Incubation. All preincubation manipulations were carried out under dim green safelight illumination. Epicotyls excised from above the first node were cut into 1 cm sections. For each incubation, approximately 5 g of material was used. The tissue sections were placed on the bottom of a 400-ml beaker to which 2.5 ml of incubation solution was added. Incubation solutions contained 150 \(\mu\)Ci of either \(\mathrm{L}^{-1}\)\(^{14}\)Cglutamic acid or \(\mathrm{L}^{-1}\)\(^{14}\)Cglycine. Each incubation contained both amino acids at final concentrations of 5 mM, added from 100 mM unlabeled stock solutions. Final incubation solution volume was adjusted to 2.5 ml with tissue culture medium (15) supplemented with naphthylalanine (2.0 g/L), kinetin (0.1 mg/L), and TWEEN-80 (0.01%, v/v). The beakers were loosely covered and returned to the dark. After 12 h, some incubations were terminated and others were allowed to incubate for another 12 h after addition of 1 ml of water to compensate for evaporation. All harvested samples were washed for 10 min in 100 ml of ice-cold water. Washed samples from the 24 h incubations were stored at –80°C before extraction, while the 12 h samples were extracted immediately. Uptake of the amino acids was measured by comparing the radioactivity of a 10 \(\mu\)l portion of the incubation solution to that of a 100 \(\mu\)l aliquot of the postharvest wash solution. Prior to counting, each sample was acidified with a drop of concentrated acetic acid and evaporated to dryness, to remove CO\(_2\) and other volatile metabolites.

Pigment Extraction. All postincubation manipulations were carried out at temperatures between 0 and 4°C. Washed tissue sections were homogenized in 250-ml centrifuge bottles with a Polytron Homogenizer (Brinkmann) in 50 ml of 99% (v/v) aqueous acetone. An additional 50 ml of acetone were added and samples were centrifuged at 4000g for 10 min. The supernatants were set aside at ~20°C in the dark for later lipid analysis. Tissue pellets were thoroughly extracted with eight 30-ml portions of 80% (v/v) aqueous acetone. Noncovalently bound hemes were next extracted by resus-

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2 Abbreviations: ALA, \(\delta\)-aminolevulinic acid; Botran, 2,6-dichloro-4-nitroaniline; gabcuslinc, 3-amino-2,3-dihydrobenzoic acid; Tween-80, polyoxethylene sorbitan monoooleate.
pending the pellets in 10 ml of cold 36% (w/v) aqueous HCl:acetone:water (5:80:15, v/v/v), and centrifuging for 2 min at 1600g (24). The pellets were reextracted with a 5-ml portion of the acid-acetone solution and the two extracts were combined.

**Purification of Hemes.** Hemes were purified according to previously reported methods with slight modifications (25, 26, 28). To each acid-acetone extract, an equal volume of dichloromethane:butanol (2:1, v/v) was added, and after mixing, 30 ml of water were added. Phases were separated by centrifugation for 2 min at 1600g. The bottom heme-containing phase was put aside while the upper aqueous phase was reextracted with 15 ml of the dichloromethane:butanol solution. The organic phases were combined and washed twice with 40-ml portions of water, bringing the pH of the last water wash to above 4.5. An equal volume of 95% (v/v) aqueous ethanol was added to the heme-containing organic phase. This solution was applied to a 0.5 cm diameter × 8.0 cm long column of DEAE-Sepharose (acetate form) that was previously equilibrated with ethanol (20). The column was washed with 5 ml of dichloromethane:ethanol (1:1, v/v) and then with 5 ml of 95% (v/v) aqueous ethanol. The hemes retained at the top of the column were eluted with a minimal volume of HPLC solvent, which was 95% (v/v) aqueous ethanol:acetic acid:water (70:17:13, v/v/v).

Protopheme and heme α were further purified and separated from each other by reverse-phase HPLC as previously described (25, 26, 28). The HPLC apparatus was thoroughly washed between samples to minimize carryover contamination.

HPLC eluate fractions containing protopheme and heme α were next purified individually by another cycle of purification as described above, beginning with extraction into dichloromethane:butanol (2:1, v/v), but modified by using half-volumes and including five water washes instead of two. The organic phases were again applied to DEAE-Sepharose columns and subjected to reverse-phase HPLC. Small amounts of biliverdin were added to the samples containing only heme α to aid in visually monitoring the elution of dicarboxylic-acid tetrapyroles from the DEAE-Sepharose columns. Two-milliliter fractions of the HPLC eluate were collected for quantitative spectrophotometry and determination of radioactivity. Heme concentrations were determined by recording the absorbance spectra and using the absorption coefficients previously reported (25) for air-oxidized hemes in HPLC solvent: 144 mm⁻¹ at 398 nm for protopheme, and 123 mm⁻¹ at 406 nm for heme α. The solvent was evaporated and radioactivity determined by liquid scintillation counting. Sufficient counts were accumulated to assure a counting error of less than 3%.

**Extraction of Total and Nonsaponifiable Lipids.** Each initial acetone extract of the tissue section homogenates (100 ml) was filtered through glass fiber filter paper. The filtrate was combined with 100 ml of diethyl ether, mixed, and then combined with 100 ml of water. After phase separation, the lower aqueous phase was reextracted with 50 ml of ether. The combined ether phases were washed with three 100-ml portions of water. The ether was evaporated in vacuo at 30°C and the residue (containing 2 ml of water) was dissolved in 15 ml of absolute ethanol. This fraction was designated 'total lipids' (9) and 0.15 ml was reserved for scintillation counting. Saponification of the remainder was carried out according to standard methods (9), by addition of 1.5 ml of 60% (w/v) aqueous KOH and heating the mixture to refluxing for 10 min. Upon cooling to room temperature, 20 ml of diethyl ether were added, mixed, and then combined with 45 ml of water. After phase separation, the lower aqueous phase was reextracted with another 20 ml portion of ether. The combined ether extracts were washed with four 40-ml portions of water until the pH of the aqueous phase was below 7. The ether was evaporated in vacuo at 30°C and the residue was dissolved in absolute ethanol to final volume of 5 ml. This fraction was designated 'nonsaponifiable lipids,' and consists primarily of isoprenoid compounds (9). A 1-ml portion was used for liquid scintillation counting.

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

**RESULTS**

**Uptake of Substrates and Recovery of Hemes.** At least 70% of the substrate was removed from the bathing solution during the 12 h incubation period. After 24 h, almost all (99%) of the substrate had disappeared from the incubation solution.

The amounts of hemes recovered were 1.033 ± 0.234 nmol/g fresh weight for protopheme and 0.037 ± 0.009 nmol/g fresh weight for heme α. The protopheme/heme α ratio was 26.4 ± 2.9. The elution profile of a typical separation of maize protopheme and heme α by reverse-phase HPLC is shown in Figure 1. The major peaks are protopheme and heme α, eluting at 8.1 and 20.0 min, respectively. Dithionite-reduced pyridine hemochrome spectra of the protopheme and heme α peaks are shown in Figure 2. The major absorption bands for protopheme occurred at 419 and 557 nm, and for heme α at 429 and 588 nm, which correspond to the values previously reported for authentic protopheme (26) and heme α (7). The Soret maxima in HPLC solvent for protopheme and heme α were 398 nm and 406 nm, respectively, in agreement with values previously determined (25, 26, 28).

**Incorporation of Radioactivity into Hemes.** Recovery of hemes and incorporation of radioactivity are shown in Table I. In the case of protopheme, the values tabulated were determined solely from a single 2 min fraction of the HPLC eluate collected

![Figure 1](https://www.plantphysiol.org)  
**FIG. 1.** Reverse-phase HPLC elution profile of hemes extracted from etiolated maize. Peak elution times were 8.1 min for protopheme and 20.0 min for heme α. At the break in the trace, the absorbance sensitivity was increased by a factor of 20.
between 8 and 10 min, which contained most of the protoheme, although some radioactivity was measured in other fractions (Figs. 3, 4). Similarly, two 2 min HPLC eluate fractions eluting between 18 and 22 min were used to calculate the radioactivity incorporation and recovery of heme a. In the heme a samples, the absorbance peak eluting at about 7 min is due to biliverdin which was added to the samples as a visual marker before elution from the second DEAE-Sepharose column. The biphasic peak appearing at 3 min in the heme a elution profiles is due to refractive index differences between the HPLC solvent and the injected solution, and is not seen in the lower-sensitivity elution profiles for protoheme.

Incorporation of radioactivity into protoheme from L-1-[14C]glutamate was far greater than that from 2-[14C]glycine. In the 12 h incubations, the specific activity of protoheme isolated from tissue incubated with labeled glutamate was 29.7-fold greater than that from the tissue incubated with labeled glycine (Table I). The specific activity of the heme a from the incubation with labeled glutamate was 4.1-fold greater than that from the sample incubated with labeled glycine.

Heme recovery and radioactivity incorporation from the 24 h incubations generally followed the pattern observed for the 12 h incubations (Table I). However, in the longer incubations, the specific activity ratios of the glutamate versus glycine-labeled samples were not as great (18.9-fold for protoheme and 2.3-fold for heme a), indicating that label randomization occurred between 12 and 24 h, and suggesting that a similar degree of randomization probably occurred during the first 12 h. Also, in the elution profile of the heme a sample from the 24 h incubation, it is apparent that a considerable fraction of the radioactivity eluted near to, but not coincidently with heme a, indicating that a contaminant was present (Fig. 4). This nonabsorbing radioactive component of the heme a isolate was much smaller in the sample obtained from the 12 h incubation with labeled glycine, and was not apparent in the profiles obtained from the tissue incubator for 12 or 24 h with labeled glutamate. Since this contaminant was not completely resolved from heme a, it is possible that it might be responsible for some of the radioactivity included in the calculation of the specific activity of heme a in the incubations with glycine.

Incorporation of Radioactivity into Lipids. Ether-extractable materials were collected from the first acetone extracts of the tissue samples after incubation. Radioactivity incorporation into these fractions is indicated in the column designated total lipids in Table II. Compared with L-1-[14C]glutamate, 2-[14C]glycine was 25 times more effective at labeling the total lipids fraction in the 12 h incubations, and 32 times more effective in the 24 h incubations. Radioactivity was measured in the isoprene-containing nonsaponifiable lipids fractions separated from the total lipids fractions of the 12 h incubations. 2-[14C]Glycine was 11.3 times more effective than L-1-[14C]glutamate in labeling this fraction (Table II).

DISCUSSION

From these results it appears that etiolated maize epicotyl sections, cellular hemes, including all protoheme and most or all mitochondrial heme a, are formed from ALA that is made from glutamate via the five-carbon pathway, and there is little or no contribution to heme precursors by ALA synthase-catalyzed condensation of glycine and succinyl-CoA. Indirect evidence suggests that the relatively small transfer of label from exogenous glycine to heme a can be explained by label randomization and incorporation into the non-tetrapyrrole farnesyl moiety of this heme.

ALA was first shown to be formed from succinyl-CoA and glycine in a reaction catalyzed by ALA synthase in bacteria (including photosynthetic bacteria) and animal mitochondria (11, 14). Much later, it was found that plants and algae, including prokaryotic cyanophytes, were capable of forming ALA via another route from the intact carbon skeleton of glutamate (1, 2, 4, 16). In vivo studies have shown that ALA is formed predominantly, and perhaps exclusively, via the five-carbon path in plants and most algae investigated. Cell-free plastid systems from greening plant tissues have corroborated the central role of the five-carbon route in ALA production (12, 13, 29). By the use of 13C-NMR spectroscopy of 13C-enriched Chl a, the exclusive operation of the five-carbon pathway (and the absence of the ALA synthase pathway) in the biosynthesis of the tetrapyrrole moiety of Chl has been substantiated (18, 21).

After the discovery that in plants, ALA is made predominantly from the intact carbon skeleton of glutamate, rather than via

Table I. Quantity and Specific Radioactivity of Recovered Hemes

<table>
<thead>
<tr>
<th>Compound Administered</th>
<th>Incubation Time</th>
<th>Protoheme</th>
<th>Heme a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Amount recovered</td>
<td>Specific radioactivity</td>
</tr>
<tr>
<td>L-1-[14C]Glutamate</td>
<td>12 h</td>
<td>3.78 nmol</td>
<td>1032 cpm/nmol</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>5.28</td>
<td>1264</td>
</tr>
<tr>
<td>2-[14C]Glycine</td>
<td>12 h</td>
<td>5.28 nmol</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>5.14</td>
<td>67</td>
</tr>
</tbody>
</table>

Fig. 2. Dithionite-reduced pyridine hemochrome absorption spectra of protoheme (- -) and heme a (---) isolated from etiolated maize and purified by reverse-phase HPLC.
ALA synthase-catalyzed condensation of succinyl-CoA and glycine, the question naturally arose whether all plant ALA is made from glutamate, or whether the precursor to one class of tetrapyrroles (e.g. Chl) might be made from glutamate, while that destined to form hemes is made from glycine. Alternatively, precursor ALA for all plastid tetrapyrroles (Chl and hemes) might be made from glutamate, while that for the less abundant mitochondrial hemes is made from glycine. Castelfranco and Jones (6) found that in greening barley tissue, glutamate, rather than glycine, is the predominant precursor of protoheme as well as Chl. The methods employed by these workers could not exclude the possibility that some small subfraction of cellular hemes (e.g. mitochondrial hemes) is made from glycine.

The microbial toxin gabaculine is a powerful inhibitor of ALA formation via the five-carbon pathway in vitro (27). Flint (10) reported that gabaculine inhibited Chl formation in (unspecified) leaves by more than 99.9%, but did not alter the respiratory activity or mitochondrial Cyt oxidase level. This result was interpreted to indicate that the ALA which serves as precursor to mitochondrial Cyt hemes is synthesized by a different route, one that is insensitive to gabaculine. However, it is possible that the very low cellular levels of mitochondrial Cyt hemes could be supplied even by a system that is severely inhibited. Alternatively, the leaves might have already attained their normal complement of mitochondrial Cyt at the time of gabaculine application.

To establish the biosynthetic route of mitochondrial Cyt...

**Fig. 3.** Reverse-phase HPLC elution profiles (smooth curves) and radioactivity in 2-min column eluate fractions (histograms) of purified protoheme and heme a from 12-h incubations of etiolated maize epicotyl sections with L-1-[14C]-glutamate and 2-[14C]glycine. The peak eluting at 7 min in the heme a samples is due to biliverdin which was added as a visual marker to aid in previous purification steps.
hemes, methods were devised to isolate and quantitate very small amounts of heme $a$, the prosthetic group of Cyt oxidase, as a marker of mitochondrial hemes. Incorporation of label from 2-$[^{14}C]$glycine is consistent with the operation of ALA synthase. $C_2$ of glycine becomes $C_1$ of ALA, which is subsequently incorporated into eight positions of the porphyrin macrocycle (22). Incorporation from $C_1$ of glutamate, which also enters the porphyrin macrocycle as $C_3$ of ALA, was used as a specific indicator of the operation of the five-carbon pathway, because, under nonphotosynthetic conditions, there is no known route whereby the $C_1$ atom of glutamate could be incorporated into ALA via the operation of ALA synthase. The first organism to be examined was the phytoflagellate Euglena gracilis, chosen because it contains relatively large amounts of heme $a$ for a photosynthetic organism, and also because from in vitro studies this organism was known to possess both routes of ALA synthesis (3, 23). The
Table II. Radioactivity of Total and Nonsaponifiable Lipids

<table>
<thead>
<tr>
<th>Compound Administered</th>
<th>Incubation Time</th>
<th>Radioactivity Incorporation in Lipids</th>
<th>Total Nonsaponifiable</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>h</td>
<td>cpm $\times 10^4$</td>
<td></td>
</tr>
<tr>
<td>L-$[14C]$Glutamate</td>
<td>12</td>
<td>0.17</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>2-$[14C]$Glycine</td>
<td>12</td>
<td>4.32</td>
<td>1.36</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>8.20</td>
<td></td>
</tr>
</tbody>
</table>

results obtained with *E. gracilis* indicated that while plastid tetrapyrroles are synthesized exclusively from glutamate, mitochondrial hemes, as indicated by heme $a$, are made exclusively from glycine (25). When a similar experiment was performed with *Cyanidium caldarium*, a unicellular rhodophyte, different results were obtained. In this organism, all cellular tetrapyrroles, including mitochondrial hemes, are made from glutamate, and there is no evidence for a contribution from glycine through ALA formed via ALA synthase (26).

The disparate results from the two algal systems made it impossible to extrapolate to the situation in higher plants, and a plant system was sought having the necessary features for successfully carrying out a similar experiment. These features include rapid uptake of both glycine and glutamate, sufficient cellular content of heme $a$, and sufficient rates of heme $a$ formation to incorporate detectable amounts of exogenous precursors during an incubation period that is long enough to permit synthesis, but not so long as to permit a high degree of label randomization through general cellular metabolism. After several unsuccessful trials employing suspension-cultured tobacco cells, cucumber roots, and barley leaves (MA Schneegurt, SI Beale, unpublished data), etiolated maize epicotyls were found to have the requisite properties.

During 12 h of incubation with exogenous radiolabeled precursors, L-$[14C]$Glutamate contributed label to total cellular protoporphyrin almost 30-fold more effectively than 2-$[14C]$Glycine, indicating that 97% of the radiolabeled extractable protoporphyrin was formed from ALA derived via the five-carbon pathway. In this same time period, the predominant precursor of mitochondrial heme $a$ was also glutamate. Comparison of the relative incorporation of radioactivity from L-$[14C]$Glutamate and 2-$[14C]$Glycine indicates that over 80% of the radiolabeled extractable heme $a$ was produced via the five-carbon pathway. Radioactivity incorporation in the 24-h incubations generally followed the pattern observed for the 12 h incubations. However, in the longer incubations, the specific activity ratios of glutamate- versus glycine-labeled samples were not as great, indicating that label randomization occurred between 12 and 24 h, and suggesting that a similar degree of randomization probably occurred during the first 12 h. Also, in the radioactivity elution profile of the heme $a$ sample from the 24 h incubation, a considerable amount of radioactivity did not coincide with the heme $a$ elution peak, indicating that a contaminant could have been responsible for some of the radioactivity in the fractions used to calculate the specific activity of the heme $a$ formed from glycine. This nonabsorbing radioactive component of the heme $a$ isolate was much lower in the profile obtained from the 12 h incubation with labeled glycine, and was not obvious in the profiles from either of the incubations with labeled glutamate.

An important consideration in the interpretation of the data from experiments of this type is that it is possible for label from 2-$[14C]$Glycine to find its way into ALA and tetrapyrroles indirectly, via glycine catabolism and reutilization of the carbon for glutamate biosynthesis. On the other hand, there is no known mechanism whereby, under nonphotosynthetic conditions, 1-$[14C]$Glutamate could contribute label to ALA that is made via ALA synthase (26). Thus, a small incorporation of C$_1$ of glycine into hemes, especially at longer incubation times, cannot be accounted for in ALA biosynthesis via ALA synthase, whereas incorporation of C$_1$ of glutamate in the absence of photosynthetic carbon fixation is strong evidence for the participation of the five-carbon pathway. When these considerations are not taken into account, other conclusions can be reached (17).

In the incubations with labeled glycine, the relatively small amount of radioactivity appearing in heme $a$ could have been due to incorporation into the farnesyl moiety. Protoheme does not contain the hydroxylfarnesyl group which is attached to position 2 of the tetrapyrrole macrocycle of heme $a$ (7). It was previously shown that, like other polyisoprenoids, the farnesyl moiety of heme $a$ in chick liver cells is biosynthesized from mevalonic acid (28), the most likely precursors of which are acetate and acetoacetate (5). Because glycine can enter the Krebs cycle via catabolism to acetyl-CoA, it is reasonable to propose that glycine can be used to synthesize isoprenoids. Moreover, in the case of Chl, glycine was reported to efficiently label the polyisoprenyl phytol moiety while not contributing label to the tetrapyrrole macrocycle (18, 21). To test whether exogenous 2-$[14C]$Glycine can label polyisoprenoids under our incubation conditions, nonsaponifiable lipids were isolated from the radiolabeled maize epicotyls. Label from glycine appeared in this fraction to a much greater extent than label from glutamate. The extensive incorporation of glycine into the nonsaponifiable lipids fraction, which comprises primarily polyisoprenoids (9), supports, but does not prove, the proposal that the small amount of radioactivity incorporated into heme $a$ from labeled glycine is due to the preferential labeling of its farnesyl moiety, and not incorporation into the heme $a$ tetrapyrrole macrocycle.

The quantities of heme $a$ isolated and the specific radioactivity values in these experiments were far too small to permit direct confirmation of this hypothesis by chemical degradation (8).

The results presented here demonstrate that in etiolated maize epicotyls, both protoheme and heme $a$ are biosynthesized from ALA formed via the five-carbon pathway. Even when no corrections are made for indirect incorporation of label from glycine into heme $a$ via the farnesyl moiety, or possible lipid contamination of the final heme $a$ fractions, over 80% of the heme $a$ synthesized during 12 h was derived from ALA formed by the five-carbon route. There appears to be little or no contribution from ALA formed via ALA synthase, and no evidence has been found for the operation of this enzyme in maize.

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