This study examined the possibility that generation of heme within mitochondria may provide a local concentration sufficient to inhibit the activity of δ-aminolevulinic acid (ALA) synthase, the enzyme that catalyzes the rate-limiting step in hepatic heme biosynthesis. This was accomplished by simultaneously running ALA synthase and heme synthase activities in intact mitochondria isolated from rat liver. Radiochemical assays were used to measure the enzyme activities. ALA synthase activity did not decrease as the rate of heme formation was increased by varying the concentration of substrates for heme synthase. Even at a rate of heme generation estimated to be at least 75 times the rate occurring in vivo, ALA synthase activity was unchanged. We conclude that end product inhibition of ALA synthase activity by heme is not an important physiological mechanism for regulation of hepatic heme biosynthesis.

The first step in the biosynthesis of heme is the condensation of glycine with succinyl-CoA to form δ-aminolevulinic acid. A number of studies indicate that the formation of ALA, catalyzed by the mitochondrial enzyme ALA synthase, is the rate-limiting step in the hepatic production of heme (1-4). Three mechanisms have been proposed by which heme may regulate hepatic ALA synthase (Fig. 1): 1) repression of the synthesis of enzyme; 2) prevention of the transfer of newly synthesized enzyme from cytosol to mitochondria; 3) end product inhibition of ALA synthase activity. The studies which demonstrated inhibition of ALA synthase activity by heme utilized solubilized and partially purified enzyme (5, 6). A relatively high concentration of heme must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

The present study examined this possibility. ALA synthase activity was measured in intact mitochondria isolated from rat liver while heme was simultaneously generated at different rates via heme synthase activity.

**METHODS**

Preparation of Mitochondria from Rat Liver (10-12)—Male Sprague-Dawley rats, weighing 175 to 275 g, fed, and given water ad libitum, were killed by decapitation. Livers were perfused through the portal vein with ice cold saline (0.9% NaCl solution) to remove blood, excised, and weighed. All subsequent manipulations were performed on ice. The liver was minced and rinsed twice with ice cold sucrose/Tris/EGTA solution (0.25 M sucrose, 0.05 M Tris-Cl, 0.001 M EGTA, pH 7.4). A 10% (w:v) homogenate in sucrose/Tris/EGTA was prepared using a glass Potter-Elvehjem homogenizer and a motor-driven Teflon pestle run at 500 rpm. The homogenate was centrifuged for 10 min at 700 x g, and the pellet was discarded. The supernatant was centrifuged at 6100 x g for 10 min, and the pellet from this spin was retained. The pellet was resuspended sequentially in sucrose/Tris/EGTA and sucrose/Tris, each time centrifuging at 6100 x g. The final suspension was in 0.25 M sucrose, 0.05 M Tris-Cl, pH 7.4. Electron microscopy of the fraction showed it to be enriched with structurally preserved mitochondria.

Assay of Enzymatic Activities—The radiochemical assay of Ebert et al. (13) was used to measure ALA synthase activity in the mitochondria. This was measured in the presence of 50 μM succinate containing 1 to 2 μCi of [2,3-3H]succinate (New England Nuclear, Boston, Mass.), 100 mM glycine, 0.2 mM pyridoxal phosphate, and 100 mM Tris-Cl, pH 7.4. Samples were run in duplicate. An enzyme blank, to which was added 0.5 ml of 25% trichloroacetic acid before the start of the incubation, was run for each set of conditions. Flasks containing 0.01 μCi of [4-14C]ALA (New England Nuclear, New Haven, Connecticut) were incubated for 10 min at 37°C with 1 to 2 μCi of 57Fe (New England Nuclear, Boston, Mass.) in 0.05 ml through a cap on the flask. The reaction was terminated by the addition of 0.5 ml of 25% trichloroacetic acid to the flask. The method of Ebert et al. (13) was used to isolate ALA from the trichloroacetic acid supernatant on Dowex 50 columns with the following modifications: 1) use of 4:1, rather than 2:1, methanol, 0.1 M acetate buffer, pH 3.9, in a wash; 2) elution of ALA with 4 ml of 0.1 M NaOH rather than 1 M NaOH. The NaOH eluate was counted in a Beckman LS-255 liquid scintillation counter with a counting efficiency of 69 to 77%, and the formation of ALA was calculated after correcting for the loss of the [4-14C]ALA standard.

In addition, ALA in the NaOH eluate was converted to the pyrrole and isolated on Dowex 1 columns by the procedure of Marver et al. (14). A second [4-14C]ALA standard was used to correct for losses in this procedure.

Heme synthase activity was measured using a radiochemical assay previously described from this laboratory (15). Flasks containing the mitochondria along with 1 to 8 mM porphyrin and 100 mM Tris-Cl, pH 7.4, in a total volume of 3.95 ml were kept on ice in the dark and flushed with N2 for 2 min to remove air. This step is critical to ensure that heme synthase activity will proceed under the conditions employed, as well as to prevent lipid peroxidation of mitochondrial membrane which may occur in the presence of ferrous salts or ascorbate, or both (16, 17). They were then placed in a shaking water bath at 37°C, and the reaction was started by injecting 25 to 1600 nmol of ferrous sulfate containing 1 to 2 μCi of 57Fe (New England Nuclear, Boston, Mass.) in 0.05 ml through a cap on the flask. The

The abbreviations used are: ALA, δ-aminolevulinic acid; EGTA, ethylene glycol bis(ethyleneimino)ether)N,N'-tetraacetic acid.
iron solution was prepared as previously described (15). Samples were run in triplicate, together with an enzyme blank which contained mitochondria which had been boiled for 10 min. All results were corrected for nonenzymatic formation of heme (less than 4% of enzymatic formation).

Two methods were used to prepare porphyrin. Protoporphyrin and deuteroporphyrin in 0.3 M Tris-Cl, pH 7.4, were prepared by dissolving 8 mg of free porphyrin (Porphyrin Products, Logan, Utah) in 5 ml of 0.6 M Tris-Cl, adjusting the pH to 7.4 with HCl, filtering, and adding distilled water. The final porphyrin concentration was approximately 1 mM. Protoporphyrin in micelles of lecithin was prepared by dissolving 10 mg of free protoporphyrin in 10 ml of 3 N HCl, adjusting the pH to 3.2 with crystalline sodium acetate, and extracting with ethyl ether. Lecithin (50 mg) was dissolved in the ether extract, and the ether was blown off under an N₂ stream with gentle heating. The residue was dissolved in 9 ml of 0.15 M Tris-Cl, the pH was adjusted to 7.4, distilled water was added to a final volume of 10 ml, and the solution was sonicated until clear.

**Dual Assay**—In each experiment, two sets of parallel flasks were run. All flasks contained the complete system for ALA synthase and heme synthase described above, with the exception that the set of synthesis of new enzyme

\[ \text{ALA SYNTHASE} \]

protoporphyrin III (90)

\[ \text{protoporphyrinogen I} \]

8-oxomethylquinonic acid (ALA)

\[ \text{porphobilinogen} \]

\[ \text{coproporphyrinogen I} \]

\[ \text{uroporphyrinogen I} \]

\[ \text{coproporphyrinogen I} \]

**FIG. 1.** Schematic representation of the heme biosynthetic pathway, showing three mechanisms by which heme may regulate hepatic ALA synthase: 1) repression of synthesis of enzyme; 2) prevention of transfer of newly synthesized enzyme from cytosol to mitochondria; 3) feedback inhibition of activity.

**RESULTS**

Preliminary experiments were done to determine optimal conditions for the individual enzymatic assays. The formation of ALA in the mitochondria was linear with time through 25 min, and with protein up to 3 mg. These conditions were employed for the dual assay.

Heme synthase activity in the mitochondria was maximal with a protoporphyrin concentration of 4 to 8 \( \mu \text{M} \) (Fig. 2). The activity was similar whether protoporphyrin was dissolved in Tris buffer or presented in micelles of lecithin, and the former method was adopted for the dual assay. Heme synthase activity was varied by changing the concentration of ferrous ion (Fig. 2), maintaining porphyrin at 4 \( \mu \text{M} \).

The mean value of ALA synthase activity for all experiments was 384 ± 8 pmol of ALA formed/mg of protein/h (±S.E. for 82 measurements), using the method of Ebert et al. (13). Reproducibility was excellent, duplicates agreeing within 8%. The recovery of \([4^-{\text{14C}}]\)ALA which had been incubated with the flasks ranged from 75 to 87%.

When ALA in the NaOH eluate from the Dowex 50 column was converted to the pyrrole and subsequently isolated on a Dowex 1 column (14), only 55% of the radioactivity appeared to be ALA, however. Thus, under the conditions of our assay, the method of Ebert et al. (13) appeared to overestimate the production of ALA. Using the addition of the procedure of Marver et al. (14), formation of ALA was 212 ± 14 pmol/mg of protein/h.

Irrespective of which measurement was used for ALA synthase activity, the effects of the individual heme synthase substrates, as well as heme generation, were the same (Table...
which had hepatic ALA synthase activity previously induced by allylisopropylacetamide, ALA synthase activity in the mitochondria decreased whereas that in the extramitochondrial fraction increased considerably. This could not be demonstrated in rats which had enzymatic activity induced by treatment with 1,4-dihydro-3,5-dicarbachoxycolidine, however (19), raising questions about its importance as a general mechanism by which heme regulates hepatic ALA synthase.

Inhibition of ALA synthase activity by heme has been demonstrated using solubilized, partially purified enzyme (5, 6). In addition to heme, various other metalloporphyrins, porphyrins and bilirubin also inhibited activity (5). Heme showed an apparent $K_i$ for ALA synthase of $2 \times 10^{-8}$ M, which is 200 times the concentration required to produce repression of synthesis of the enzyme in tissue culture, raising doubts about end product inhibition as an important physiological mechanism for regulation of ALA synthase. Nevertheless, since both ALA synthase and heme synthase, the enzyme which catalyzes the chelation of ferrous ion to protoporphyrin to form heme, appear to be located on the inner mitochondrial membrane (20-23), generation of heme within the mitochondria might provide a local concentration sufficient to inhibit ALA synthase.

The present study investigated whether this may occur. The unique feature of the study was that heme synthase and ALA synthase activities were run in the same mitochondrial sample. Our results show this was accomplished satisfactorily. However, formation of ALA was apparently overestimated by the radiochemical assay of Ebert et al. (13), in which the product is isolated on a single column. This may reflect the conditions of the assay, although a recent study by Bishop et al. (24), as well as our own studies, indicate that labeled succinate is metabolized to other products that may elute chromatographically on Dowex 50 with ALA.

Irrespective of whether ALA formation was measured by the method of Ebert et al. (13), or after the additional step of conversion to the pyrrole (14), generation of heme in the mitochondria did not affect the activity of ALA synthase. Further study is required to determine if the induced enzyme behaves in a similar fashion. We have found in one experiment that enzyme induction by single or repeated administration of allylisopropylacetamide to the rat is also not affected.

Regarding the level of heme synthase activity achieved in the mitochondria used in our study, studies of hepatic heme turnover in the rat indicate a requirement for heme synthesis of 2.5 to 3.0 nmol/g of liver/h (25). Assuming that rat liver mitochondria contain about one-fifth of the total cell protein (26), this requires generation of 0.1 nmol of heme/mg of mitochondrial protein/h. Thus, levels of heme generation in our mitochondrial preparations were at least 75 times the basal rate occurring in vivo, making it unlikely that end product inhibition is an important physiological mechanism for regulation of hepatic ALA synthase activity, and hence for control of hepatic heme biosynthesis.

REFERENCES

7. J. Wolfson and J. R. Bloomer, manuscript in preparation.

I and Fig. 3). Neither the presence of iron nor protoporphyrin alone, nor heme generation when the substrates were combined, altered ALA synthase activity.

DISCUSSION

Of the three ways by which heme may regulate hepatic ALA synthase (Fig. 1), repression of the synthesis of enzyme has been established as an important physiological mechanism. Using an antibody prepared against enzyme isolated from chick embryo liver mitochondria, Whiting and Granick (9) showed that increases in ALA synthase activity in chick embryos produced by the drugs 2allylisopropylacetamide and 1,4-dihydro-3,5-dicarbachoxycolidine were accompanied by proportional changes in the amount of enzyme protein. Hemin blocked both the increase in enzyme activity and amount of new enzyme protein without changing general protein synthesis. Previous studies from the same laboratory had demonstrated that 50% repression of induced ALA synthase activity was obtained at a hemin concentration of $10^{-7}$ M in culture of chick embryo liver cells (8).

Studies by Kurashima et al. (18) have suggested that heme may also affect hepatic ALA synthase by interfering with the transfer of newly synthesized enzyme from cytoplasm to the mitochondria so that ALA synthase accumulates in the extra-mitochondrial fraction. When hemin was injected into rats

\[
\begin{array}{|c|c|}
\hline
\text{Substrate} & \text{% of control ALA synthase activity}\tabularnewline
4 \mu M \text{ protoporphyrin} & 95 \pm 9 \\
25 \mu M \text{ Fe}^{2+} & 109 \pm 5 \\
50 \mu M \text{ Fe}^{2+} & 106 \pm 5 \\
100 \mu M \text{ Fe}^{2+} & 102 \pm 4 \\
400 \mu M \text{ Fe}^{2+} & 103 \pm 10 \\
\hline
\end{array}
\]

*a Control ALA synthase activity, measured in the absence of both protoporphyrin and Fe$^{2+}$, was 374 ± 14 pmol of ALA formed/mg of protein/h (mean ± S.E. of 15 measurements).

FIG. 3. Effect of endogenous heme generation (heme synthase activity) on ALA synthase activity in intact mitochondria isolated from rat liver. Data from seven experiments are shown (six with protoporphyrin as substrate, and one with deuteroporphyrin). See Table I for control levels of ALA synthase activity.

Effect of heme synthase substrates on ALA synthase activity in mitochondria from rat liver

<table>
<thead>
<tr>
<th>Substrate</th>
<th>% of control ALA synthase activity</th>
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<tbody>
<tr>
<td>4 μM protoporphyrin</td>
<td>95 ± 9</td>
</tr>
<tr>
<td>25 μM Fe$^{2+}$</td>
<td>109 ± 5</td>
</tr>
<tr>
<td>50 μM Fe$^{2+}$</td>
<td>106 ± 5</td>
</tr>
<tr>
<td>100 μM Fe$^{2+}$</td>
<td>102 ± 4</td>
</tr>
<tr>
<td>400 μM Fe$^{2+}$</td>
<td>103 ± 10</td>
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Effect of Heme Generation on ALA Synthase in Mitochondria

Effect of endogenous heme generation on delta-aminolevulinic acid synthase activity in rat liver mitochondria.
S J Wolfson, A Bartczak and J R Bloomer


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