6-Aminolevulinic Acid Synthetase

III. SYNERGISTIC EFFECT OF CHELATED IRON ON INDUCTION

(Received for publication, October 30, 1969)

JEFFREY A. STEIN, DONALD P. TSCHUDY, P. LYNN CORCORAN, AND ANNIE COLLINS

From the Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

SUMMARY

Orally administered ferric citrate exerts a pronounced synergistic effect on the induction of hepatic δ-aminolevulinic acid synthetase produced by two compounds. The effect appears to be specific for iron citrate, as opposed to other metals in complex with citrate, and specific also in affecting the level of δ-aminolevulinic acid synthetase as compared with another induced enzyme, tyrosine transaminase. Inorganic iron salts did not produce augmentation of the induction of hepatic δ-aminolevulinic acid synthetase. The data suggest that iron augments the rate of synthesis of the enzyme during induction rather than activating or stabilizing the enzyme.

δ-Aminolevulinic acid synthetase is a mitochondrial enzyme which is rate-controlling in heme biosynthesis (1, 2). The enzyme level is greatly increased in mammalian liver after administration of a number of compounds (3–5). Previous studies by Brown in chicken erythrocytes (6, 7) indicated that iron is necessary for the activity of this enzyme, but later studies in a microorganism (8) could not show such a requirement.

There is no present evidence that hepatic ALAS synthetase requires iron for its activity. However, the findings presented below show that administration of a large dose of chelated iron, particularly ferric citrate, produces a marked synergistic effect on the induction of hepatic ALAS synthetase. It is thought that this results from augmented synthesis of the enzyme.

EXPERIMENTAL PROCEDURE

Materials

Allylisopropylacetamide was provided by Hoffmann-La Roche and sulfonmethane (Sulfonal) was obtained from K and K Laboratories. Cycloheximide and iron-deficient diet were purchased from Nutritional Biochemicals. Ferric citrate was purchased from Fisher. Diethylthiocarbamate was purchased from Eastman Chemicals. Zinc citrate, copper citrate, and magnesium citrate were prepared by adding zinc sulfate, copper sulfate, and magnesium chloride in half-molar amounts to a 0.3 M citric acid solution. Succinyl coenzyme A was prepared by the method of Simon and Shemin (9).

All experiments were performed with Sprague-Dawley female rats of 100 to 120 g weight.

Methods

Enzyme Assays—ALA synthetase activity was measured in liver homogenates by the method of Marver et al. (10), ALA dehydrase (EC 4.2.1.24) by the method of Gibson, Neuberger, and Scott (11), and tyrosine transaminase (EC 2.6.1.5) by the method of Diamondstone (12). ALA synthetase was also measured in isolated rat liver mitochondria as described previously (2). ALA was determined by the method of Marver et al. (13).

Administration of Compounds—Allylisopropylacetamide was dissolved in 0.9% NaCl solution at a concentration of 15 mg per ml and administered subcutaneously in a dosage of 400 mg per kg. Hydrocortisone was injected subcutaneously in a dosage of 100 mg per kg. Sulfonmethane dissolved in 2.5% ethanol was administered subcutaneously in a dosage of 250 mg per kg. Cycloheximide was injected intraperitoneally at a dose of 30 mg per kg every hour. Ferric citrate was dissolved in water at a concentration of 70 mg per ml and administered by stomach tube at a dose of 700 mg per kg. In attempts to compare the effect of other metal citrate complexes with that of ferric citrate on the induction of ALA synthetase, it was found that amounts of these metals which were equimolar with the amount of iron used were too toxic (mortality at least 80%). Therefore, the amount of citrate was kept approximately constant in all experiments (550 to 600 mg per kg) and the amount of the other metals was decreased to one-half the molar amount of iron used. These metal chelates were prepared by adding half-molar amounts of the metal salts to citric acid. The metal chelates were administered by stomach tube.

Lead acetate was administered as an aqueous solution in a dose of 600 mg per kg as a single daily subcutaneous injection for 5 days.

Other Procedures—Mitochondria were isolated by the method of Schneider (14) and were washed with 0.25 M sucrose to remove EDTA (Versene) when iron compounds were added for incubation.

RESULTS

The lower curve in Fig. 1 shows the change of hepatic ALA synthetase level after a single subcutaneous injection of allyliso-
TABLE I

Effect of in vitro addition of various compounds on ALA synthetase activity in liver homogenates and mitochondria

A, six Sprague-Dawley female rats weighing 110 to 120 g were fasted for 40 hours before injection of allylisopropylacetamide, 400 mg per kg, and fasting continued until death 5 hours later. Liver homogenates pooled from these six animals were divided and the same homogenate was used for all studies. Ferric chloride, ferrous sulfate, citric acid, and ferric citrate were dissolved in water and the pH was adjusted to 6.7 to 6.9 with HCl or NaOH. The final concentration in the standard incubation mixture is indicated and the final pH of the incubation mixture was unchanged from that of the buffered homogenate. B, six Sprague-Dawley female rats weighing 110 to 120 g were treated as described above and killed 3 hours after injection of allylisopropylacetamide. Mitochondria were prepared as previously described (2). Ferric chloride, ferric citrate, and citric acid were added so as to give a final concentration in the mitochondrial incubation mixture as indicated.

<table>
<thead>
<tr>
<th>Addition</th>
<th>ALA synthetase (nmol/g liver/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>150</td>
</tr>
<tr>
<td>Iron-citrate</td>
<td>375</td>
</tr>
<tr>
<td>Copper-citrate</td>
<td>198</td>
</tr>
<tr>
<td>Zinc-citrate</td>
<td>136</td>
</tr>
<tr>
<td>Magnesium citrate</td>
<td>190</td>
</tr>
</tbody>
</table>

Table II

Effect of various metal-citrate complexes on induction of ALA synthetase by allylisopropylacetamide

Sprague-Dawley female rats weighing 100 to 120 g were fasted for 40 hours prior to the injection of allylisopropylacetamide, 400 mg per kg subcutaneously. Fasting was continued until death 5 hours later. Each group consisted of five animals and ALA synthetase levels were determined on pooled homogenates. At the time of injection of allylisopropylacetamide the following compounds were administered orally with citric acid, 600 mg per kg: magnesium chloride, 150 mg per kg; copper sulfate, 200 mg per kg; zinc sulfate, 800 mg per kg.
Sprague-Dawley female rats weighing 100 to 120 g were fasted for 40 hours prior to the administration of allylisopropylacetamide. Fasting was continued until death 5 hours later. Each group consisted of four animals and ALA synthetase activity was determined in pooled liver homogenates. At the time of injection of allylisopropylacetamide ferric chloride (600 mg per kg) with citric acid (600 mg per kg) was administered orally, as were ferric chloride (600 mg per kg) with malic acid (1 g per kg), ferric chloride with succinic acid (1.5 g per kg), ferric chloride with fumaric acid (1.2 g per kg), and ferric chloride with α-ketoglutaric acid (2 g per kg).

<table>
<thead>
<tr>
<th>Compound administered</th>
<th>ALA synthetase (μmoles ALA/g liver/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>204</td>
</tr>
<tr>
<td>Iron + citrate</td>
<td>720</td>
</tr>
<tr>
<td>Iron + malate</td>
<td>310</td>
</tr>
<tr>
<td>Iron + succinate</td>
<td>247</td>
</tr>
<tr>
<td>Iron + fumarate</td>
<td>275</td>
</tr>
<tr>
<td>Iron + α-ketoglutarate</td>
<td>410</td>
</tr>
</tbody>
</table>

Fig. 3. The effect of cycloheximide on ALA synthetase 17 hours after allylisopropylacetamide (400 mg per kg subcutaneously) or allylisopropylacetamide and ferric citrate (700 mg per kg orally) administered simultaneously. Conditions were similar to those described in Fig. 3. •, ALA synthetase levels in pooled homogenate from four rats. In animals not given cycloheximide ALA synthetase following allylisopropylacetamide and ferric citrate alone rose during the 2-hour period shown above.

Fig. 4. The effect of cycloheximide on ALA synthetase 5 hours after allylisopropylacetamide (400 mg per kg subcutaneously) and ferric citrate (700 mg per kg orally) administered simultaneously. Conditions were similar to those described in Fig. 3. •, ALA synthetase levels in pooled homogenate from four rats. In animals not given cycloheximide ALA synthetase following allylisopropylacetamide and ferric citrate alone rose during the 2-hour period shown above.

The effect of administration of various compounds alone or in combination on the induction of hepatic ALA synthetase is presented in Fig. 2. Ferric citrate alone produced little effect on the level of hepatic ALA synthetase. Sodium citrate, citric acid, or ferric chloride given individually along with allylisopropylacetamide do not enhance the magnitude of ALA synthetase induction above that produced by allylisopropylacetamide alone. Likewise, ferrous sulfate (not shown in Fig. 2) does not augment hepatic ALA synthetase induction produced by allylisopropylacetamide.

It is seen in Table I that inorganic iron, citrate, and ferric citrate produced no activation or inhibition of ALA synthetase at the concentrations studied. This was true for the enzyme when measured in both homogenates and mitochondria. The mitochondria did not contain EDTA (Versene) during incubation.

Copper, zinc, and magnesium citrates administered by stomach tube alone with a subcutaneous injection of allylisopropylacetamide...
FIG. 5. The effect of glucose on the induction of ALA synthetase. With the exception of glucose-treated animals, Sprague-Dawley female rats weighing 100 to 120 g were fasted for 40 hours prior to drug administration, and fasting was continued until death. Each point depicts hepatic ALA synthetase activity of pooled liver homogenates from four rats. Allylisopropylacetamide was administered subcutaneously in a dose of 400 mg per kg. *Left panel: •, ALA synthetase after allylisopropylacetamide alone; ○, ALA synthetase after allylisopropylacetamide and glucose (2 g by intubation 2 hours before and 2 g simultaneously with allylisopropylacetamide). *Right panel: •, ALA synthetase after allylisopropylacetamide and ferric citrate (700 mg per kg orally); ○, ALA synthetase after allylisopropylacetamide and ferric citrate with glucose treatment as described above. Allylisopropylacetamide and ferric citrate were administered at the same time.

FIG. 6. The effect of ferric citrate on the induction of hepatic tyrosine transaminase produced by hydrocortisone and allylisopropylacetamide. Sprague-Dawley female rats weighing 100 to 120 g were allowed food and water until the time of injection of the various compounds. Each point represents the mean activity of duplicate determinations of tyrosine transaminase in liver homogenates pooled from three animals. *Left panel: •, tyrosine transaminase activity in liver homogenates following hydrocortisone (100 mg per kg subcutaneously); ○, tyrosine transaminase activity after simultaneous administration of hydrocortisone and ferric citrate (700 mg per kg orally). *Right panel: tyrosine transaminase activity after allylisopropylacetamide (400 mg per kg subcutaneously); ○, tyrosine transaminase activity after simultaneous administration of allylisopropylacetamide and ferric citrate (700 mg per kg orally).

FIG. 7. The effect of ferric citrate on the induction of hepatic ALA synthetase caused by allylisopropylacetamide.

As seen in Table III ferric citrate augmented the allylisopropylacetamide-mediated induction of hepatic ALA synthetase to a much greater extent than any of the other iron (ferric) complexes of the tricarboxylic acid cycle intermediates studied. These complexes were more toxic than equimolar amounts of ferric citrate in that a mortality of at least 80% was seen in the animals receiving amounts of these metals which were equimolar with the amounts of iron used.

The half-life of hepatic ALA synthetase has previously been measured in intact rats with a compound (puromycin) which inhibits protein synthesis at the translational level (4). Cycloheximide is also known to inhibit protein synthesis at the translational level and has been used to inhibit protein synthesis in rat liver (15). By a method with the use of cycloheximide in similar fashion to that previously described with puromycin (4), the half-life of hepatic ALA synthetase was measured 17 hours (Fig. 3) and 5 hours (Fig. 4) after induction by the combined administration of ferric citrate and allylisopropylacetamide. It was found that the half-life (57 and 62 min, respectively) was not longer than that seen during induction by allylisopropylacetamide alone (Fig. 4) (75 min).

Glucose has previously been shown to block the induction of hepatic ALA synthetase caused by allylisopropylacetamide
(2, 4). Oral glucose administration (2 g) 2 hours before and at the time of allylisopropylacetamide injection prevents induction of hepatic ALA synthetase and is equally effective in preventing the pronounced induction produced by simultaneous administration of allylisopropylacetamide and ferric citrate (Fig. 5).

As seen in Fig. 6 the induction of hepatic ALA synthetase produced by sulfonamethane (Sulfonal) is markedly augmented by ferric citrate. The induction of hepatic tyrosine transaminase produced by hydrocortisone or by allylisopropylacetamide was not augmented by ferric citrate when administered simultaneously with either of these compounds (Fig. 7).

Animals raised on an iron-deficient diet for 6 weeks (average hematocrit 25 volumes %) showed no impairment of induction of ALA synthetase by allylisopropylacetamide as compared with littermates raised on a normal laboratory diet (average hematocrit 45 volumes %). Conversely, animals raised on normal laboratory diets supplemented with ferrous sulfate (10 mg per animal per day) and ferric citrate (15 mg per animal per day) for 4 weeks still showed the synergistic effect of the large single doses of ferric citrate when administered with allylisopropylacetamide.

ALA dehydrase activity in livers of animals given allylisopropylacetamide and ferric citrate 6, 12, and 18 hours prior to death was 40, 70, and 60% of the activity seen in animals killed at similar times after administration of allylisopropylacetamide alone. At concentrations of $10^{-3}$ M and $5 \times 10^{-3}$ M ferric citrate added in vitro to liver homogenates produced 20 and 100% inhibition of ALA dehydrase.

Hepatic ALA dehydrase activity in animals given lead acetate subcutaneously for 5 days was 60% of that in control animals. There was no increase of hepatic ALA synthetase activity in these lead-treated animals.

DISCUSSION

The present findings indicate that large doses of orally administered ferric citrate produced a synergistic effect on the induction of hepatic ALA synthetase produced by two compounds. Thus, ferric citrate produces little or no induction by itself, but greatly enhances the induction produced by other compounds. The observed data can be discussed in terms of possible mechanisms related to (a) chemical artifact, (b) specificity of synergistic effect, (c) possible activation of ALA synthetase, and (d) effects of ferric citrate on the synthesis or degradation of the enzyme during induction.

The possibility of iron producing a chemical artifact in ALA determination is ruled out by several pieces of evidence. First is the fact that all measurements of the enzyme are calculated on the basis of the difference between a "live" and "killed" incubation sample. The latter contains the same amount of iron as the former. Second, ferric citrate and other iron salts added to homogenates did not alter the measured amounts of ALA. Another type of artifact could result from the fact that iron chelates enter mitochondria readily (16) and might delver high enough concentrations of intramitochondrial citrate to augment succinyl-CoA generation, which in turn might increase ALA formation. This possibility is ruled out by the fact that the addition of ferric citrate (or sodium citrate) in isolated mitochondria did not augment ALA formation from glycine and succinyl-CoA (Table I). Furthermore, the direct measurement of catalytic activity of ALA synthetase in isolated liver mitochondria with glycine and succinyl-CoA as substrates was greater in animals given ferric citrate and allylisopropylacetamide than in those given allylisopropylacetamide alone. This again corroborates the fact which has previously been shown that succinyl-CoA generation from endogenous substrates in rat liver homogenate is close to optimal for ALA production, even when ALA synthetase levels are markedly increased (10). It is thus clear that the effect of ferric citrate is to augment catalytic activity of ALA synthetase during induction.

The synergistic effect is not related to alterations in the metabolism of allylisopropylacetamide, since it was also seen with sulfonamethane (Sulfonal) (Fig. 6). This finding, along with the fact that induction of tyrosine transaminase by hydrocortisone or allylisopropylacetamide was unaffected by ferric citrate (Fig. 7), suggests that the effect may be specific for ALA synthetase. The fact that other metal citrate complexes did not augment hepatic ALA synthetase induction (Table II) is evidence of the specificity of iron in producing the synergistic effect.

Although Brown's previous studies demonstrating an iron requirement for ALA synthetase in erythrocytes (6, 7) would suggest that ferric citrate might augment by activation ALA synthetase activity observed during induction, no such effect could be produced by addition of various iron compounds to liver homogenates or mitochondria from animals treated with allylisopropylacetamide (Table I).

These considerations suggest that the effect of ferric citrate is not to augment the rate of synthesis or to decrease the rate of degradation of ALA synthetase during induction. The rate of degradation of the enzyme was measured when protein synthesis was blocked by means of cycloheximide (15) and was not found to be prolonged in animals treated with allylisopropylacetamide and ferric citrate as compared with those given allylisopropylacetamide (Figs. 3 and 4). The half-life of hepatic ALA synthetase has been previously measured at different levels of enzyme induced by allylisopropylacetamide and is essentially constant (17). Since no consistent prolongation of the half-life of hepatic ALA synthetase could be shown to result from the combined administration of ferric citrate and allylisopropylacetamide, it is thought that the synergistic effect of ferric citrate probably results from augmented synthesis of the enzyme.

The present data exclude the possibility that the synergistic effect of ferric citrate results from rapid correction of an iron deficiency, since induction was not impaired in iron-deficient rats. Furthermore, rats given supplemental oral iron (ferrous sulfate, ferric citrate) chronically still showed the synergistic effect on ALA synthetase when given a single large dose of ferric citrate with allylisopropylacetamide.

It is conceivable that iron might augment hepatic ALA synthetase induction by inhibiting one or more of the steps of heme biosynthesis. Although ferric citrate administered with allylisopropylacetamide in these experiments inhibited ALA dehydrase by about 60%, a similar degree of inhibition produced by lead acetate caused no induction of hepatic ALA synthetase. These studies, however, do not rule out the possibility that a partial inhibition of heme synthesis which is not sufficient to induce hepatic ALA synthetase might nevertheless exert a synergistic effect during induction produced by another compound. Other possible mechanisms causing the synergistic effect might result from reactions between iron and components.
of the closed negative feedback loop (3) controlling ALA synthetase production.

The effectiveness of ferric citrate in producing the synergistic effect compared with the lack of such a response to lethal and sublethal doses of ferric chloride suggests that the chelated form of iron reaches a certain intracellular locus in higher concentration. This may result from the absence of ionic charge in this compound which may facilitate passage across membranes. Numerous studies have shown rapid passage of iron chelates across gastrointestinal membranes (18-21) and ready entrance into liver (18, 22, 23) and mitochondria in vitro (16).

The findings reported above may be relevant to the disease porphyria cutanea tarda. In this disorder there is an increased excretion of porphyrins, but no increase of porphyrin precursors (ALA and porphobilinogen). Excessive iron is often demonstrated in the liver and the patients experience clinical and biochemical remissions after repeated phlebotomies, perhaps as a result of iron removal (24). Several investigators have reported increased levels of hepatic ALA synthetase in this disease (25-27), but no enzymatic mechanism by which iron overload may be related to the overproduction of porphyrins had ever been demonstrated. Further studies will be required to determine whether iron overload has effects on the enzymes beyond ALA dehydrase in the heme biosynthetic pathway.

REFERENCES
δ-Aminolevulinic Acid Synthetase: III. SYNERGISTIC EFFECT OF CHELATED IRON ON INDUCTION
Jeffrey A. Stein, Donald P. Tschudy, P. Lynn Corcoran and Annie Collins


Access the most updated version of this article at http://www.jbc.org/content/245/9/2213

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/245/9/2213.full.html#ref-list-1