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Studies on the Role of Heme in the Regulation of δ-Aminolevulinic Acid Synthetase during Fetal Hepatic Development

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SUMMARY

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The role of heme in the regulation of δ-aminolevulinic acid (ALA) synthetase was studied in fetal rat liver. The activity of ALA synthetase, the first and rate-limiting enzyme in the heme-biosynthetic pathway, is 10 times higher in fetal rat liver mitochondria than in adults. Hemin injection depresses mitochondrial ALA synthetase activity in the adult but not in the fetus. Cycloheximide, a selective inhibitor of protein synthesis in cytoplasmic ribosomes, but not in mitochondria, causes a rapid decrease in fetal mitochondrial ALA synthetase activity. When hemin is given prior to cycloheximide, a slower rate of turnover of mitochondrial ALA synthetase activity occurs than after cycloheximide alone. Treatment with 3-amino-1,2,4-triazole, an inhibitor of δ-aminolevulinic acid dehydratase, the second enzyme in heme biosynthesis, causes a decrease in fetal mitochondrial ALA synthetase activity but no decrease in the extramitochondrial enzyme levels. These studies indicate that heme may facilitate the development of mitochondrial ALA synthetase in fetal rat liver. It is suggested that ALA synthetase may not become rate-limiting in hepatic heme biosynthesis until it becomes susceptible to repression by heme, a phenomenon which does not develop until near the time of birth.

INTRODUCTION

Knowledge of the mechanisms which regulate fetal hepatic development is essential to understanding the effects of drugs and other environmental stimuli on cellular growth and function. Many enzyme systems which are easily measurable in the adult are low or undetectable in fetal liver cells (1–4). This functional immaturity has impeded studies of the biochemical events which characterize perinatal development. Previous studies (5) from our laboratory, however, have shown that the activity of hepatic δ-aminolevulinic acid synthetase, the first and rate-limiting enzyme in the heme-biosynthetic pathway in adults (6), is significantly elevated in fetal mammalian liver and declines to adult levels near the time of birth. During the period of elevated activity, ALA[1] synthetase is refractory both to induction by chemicals (7, 8) and to repression by the end product, heme (7). These facts suggest that regulation of ALA synthetase during the fetal period may be quite different from that of the adult.

In the current studies the effects of hemin in the regulation of ALA synthetase in fetal liver were investigated. Factors affecting the

[1] The abbreviation used is: ALA, δ-aminolevulinic acid.

subcellular distribution of ALA synthetase activity in fetal liver were studied using drugs, enzyme inhibitors, and other agents which specifically alter the hematopoietic process in mammalian cells. The possible role of ALA synthetase as a rate-limiting enzyme in heme biosynthesis in fetal liver was investigated, and the pharmacological implications of alterations of heme synthesis during fetal hepatic development are discussed.

MATERIALS AND METHODS

Materials. Succinyl-CoA synthetase (succinic thiokinase) (EC 6.2.1.4), pyridoxal 5′-phosphate, adenosine 5′-triphosphate, guanosine 5′-triphosphate, coenzyme A (free acid), cycloheximide, and 3-amino-1,2,4-triazole (aminotriazole) were obtained from Sigma Chemical Company. Glycine, δ-aminolevulinic acid, glutathione, and hemin were purchased from Calbiochem. Other chemicals were of reagent grade and were purchased from standard commercial sources.

Treatment of animals. Sprague-Dawley rats (CD strain) were date-bred by Charles River Laboratories and were utilized on the 18th day after breeding date unless otherwise indicated. Pregnant animals were housed in individual cages and were allowed food and water ad libitum. Crystalline hemin was dissolved in a small volume of 0.01 M NaOH and adjusted to pH 7.5 with 0.01 M HCl in 0.9% NaCl to a final concentration of 10 mg/ml. It was administered to pregnant rats by intravenous injection at a dose of 20 mg/kg. Other chemicals were administered intraperitoneally to the mother. Aminotriazole was dissolved in 0.9% NaCl and given in a dose of 3 g/kg; cycloheximide was dissolved in 50% ethanol and administered in a dose of 50 mg/kg. All drugs were given within 6 hr after preparation.

Preparation of tissues. Animals were killed by decapitation. Livers of adult and fetal rats were rapidly excised, washed, weighed, and homogenized in 9 volumes of 0.25 M sucrose containing 0.02 M Tris-HCl buffer (pH 7.6), 0.1 mM EDTA, and 0.1 mM pyridoxal phosphate (buffer A) in a Potter-Elvehjem homogenizer fitted with a Teflon pestle. The homogenate was centrifuged for 10 min at 600 × *g*, and the mitochondria were sedimented from the resulting supernatant solution by centrifugation at 9000 × *g* for 15 min. Mitochondria were resuspended and washed twice with the same solution and finally resuspended in 0.02 M Tris-HCl buffer (pH 7.6) containing 0.1 mM pyridoxal phosphate so that each milliliter of suspension contained 10–20 mg/ml of mitochondrial protein.

The microsomal fraction was prepared from the 9000 × *g* supernatant solution by centrifugation at 105,000 × *g* for 60 min. The microsomes were resuspended in 15 ml of 150 mM Tris-HCl buffer (pH 7.4) and recentrifuged at 105,000 × *g* for 60 min. The final pellet was gently resuspended in the same buffer, using a Potter-Elvehjem homogenizer and a Teflon pestle, so that the final suspension contained approximately 10 mg/ml of microsomal protein. Proteins were determined by the method of Lowry *et al.* (9).

Assay of enzyme activities. ALA synthetase activity in subcellular fractions of adult and fetal liver was assayed by a modification of the method of Scholnick *et al.* (10). Reaction mixtures contained 0.05 M Tris-HCl buffer (pH 7.5), 0.01 M $MgCl_2$, 0.1 M glycine, 0.01 M sodium succinate, 0.2 mM pyridoxal phosphate, 1 mM β-mercaptoethanol, 0.25 M NaCl, 0.1 mM GTP, 5 mM EDTA, 60 μM coenzyme A, 3 mM ATP, sufficient succinyl-CoA synthetase to generate 1 μmole of succinyl-CoA in 30 min, and 0.5 ml of subcellular preparation in a final volume of 2.5 ml. Mixtures were shaken in a metabolic incubator (American Optical Company) for 60 min at 37°. Reactions were terminated by addition of 0.5 ml of cold 10% trichloracetic acid solution. The ALA produced was converted to 2-methyl-3-acetyl-4-propionic acid pyrrole by reaction with sodium acetate and acetylacetone and was then determined colorimetrically by reaction with modified Ehrlich's reagent (11). In some experiments ALA was purified by column chromatography prior to conversion to the corresponding pyrrole (7). Identical results were obtained using either method. Aminoacetone has been previously shown (7) to represent less than 10% of the total aminoketone present. Hence aminoketone levels were taken to

represent the levels of ALA. All assays were carried out in duplicate, using zero-time samples as blanks. The molar extinction coefficient for the ALA pyrrole was 5.3×10^4.

ALA dehydratase activity was measured in 9000 × *g* supernatant fractions of adult and fetal livers by a modification of the method of Gibson *et al.* (12) as described by Baron and Tephly (13).

RESULTS

In previous studies (5) it was shown that fetal hepatic ALA synthetase activity is 6–8 times the adult level in rats. In order to delineate more clearly these differences in enzyme activity at different stages of development, an analysis of the subcellular distribution of ALA synthetase activity in adult and fetal rat liver was made (Table 1). In both adult and fetal rat liver ALA synthetase activity is localized primarily in the mitochondrial fraction. The most notable aspect of these measurements is the 10:1 difference observed between fetal and adult mitochondrial enzyme levels. In the adult, enzyme activity is distributed between the mitochondrial and postmitochondrial fractions in a ratio of approximately 2:1. High-speed centrifugation (105,000 × *g*) of the postmitochondrial 9000 × *g* supernatant fraction revealed that approximately 50% of the extramitochondrial ALA synthetase activity resides in the soluble fraction, whereas the remainder is found in the microsomes. In contrast, fetal mitochondrial ALA synthetase levels are 20 times those measured in the postmitochondrial supernatant fraction. The enzyme activities in the soluble and microsomal fractions are approximately equal to those observed in the adult.

The pattern of decline in fetal mitochondrial ALA synthetase activity to adult levels is shown in Fig. 1. Mitochondrial ALA synthetase activity was measured in rats ranging in age from 5 days antepartum to 13 days postpartum. Enzyme levels equivalent to those observed in adults are reached approximately 3 days after birth.

Studies with adult rat liver (14) have shown that, although ALA synthetase is localized primarily in the mitochondrial fraction, it is initially synthesized in the endoplasmic reticulum and accumulates transiently in the soluble fraction of the cell prior to incorporation by the mitochondria. In addition, it has been shown that heme plays an important role in regulating ALA synthetase, both by suppressing its synthesis (15) and by limiting its incorporation into the mitochondria (16). It was therefore of interest to determine whether heme plays a role in the regulation of ALA synthetase activity in fetal liver. Previous studies (7) have shown that hemin does not alter the over-all activity of fetal ALA synthetase, as measured in whole liver homogenates. As an initial approach to this problem, therefore, the effects of hemin on the subcellular distribution of ALA synthetase activity in fetal liver were determined. The effects of hemin treatment in adult liver were analyzed for

TABLE 1

Subcellular distribution of hepatic ALA synthetase activity in adult and fetal rats

Subcellular fractions were prepared as described under MATERIALS AND METHODS. Actual optical density values of samples minus blanks at 553 nm of adult and fetal mitochondrial enzyme activities were 0.020 and 0.090, respectively. Corresponding values of samples minus blanks for adult and fetal postmitochondrial supernatant activities were 0.010 and 0.005, respectively. Values in this table and subsequent figures represent the means ± standard errors of at least six experiments. Livers of four adults or four litters of fetuses were pooled for each experimental value or time point.

Rats	ALA synthetase activity			
	Mitochondrial fraction	9,000 × *g* supernatant	105,000 × *g* supernatant	Microsomal fraction
	nmoles ALA/mg protein/hr			
Adult	0.52 ± 0.09	0.23 ± 0.05	0.15 ± 0.09	0.12 ± 0.01
Fetal	5.10 ± 0.11	0.26 ± 0.09	0.16 ± 0.06	0.18 ± 0.02

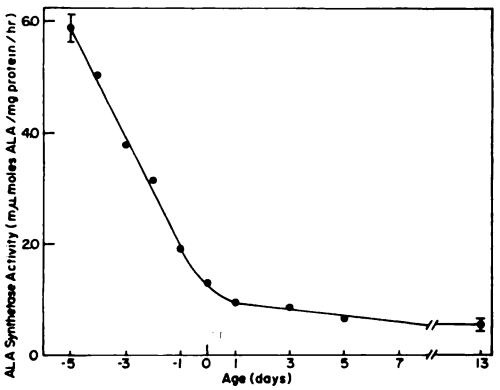

FIG. 1. *Changes in rat hepatic mitochondrial ALA synthetase activity during perinatal period*

Points represent hepatic mitochondrial ALA synthetase actvity in rats ranging in age from 5 days prior to 13 days after delivery. All points were determined concurrently.

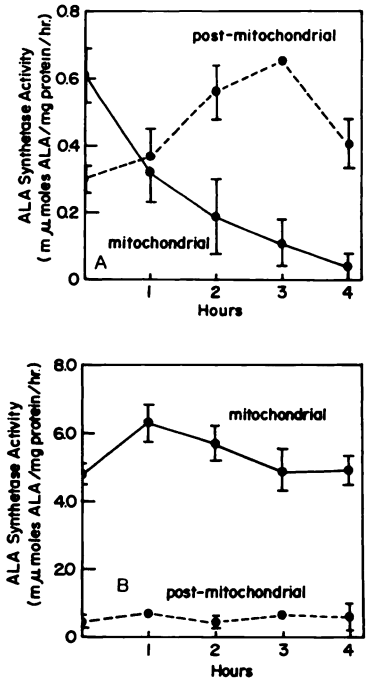

FIG. 2. *Effects of hemin on mitochondrial and postmitochondrial ALA synthetase activity in adult (A) and fetal (B) rat liver*

Hemin (20 mg/kg) was administered to pregnant rats by intravenous injection at zero time. Controls received an equal volume of 0.9% NaCl. Animals were killed at the times indicated.

comparison. The results of time-course studies following administration of hemin to adult rats are shown in Fig. 2A. A continual decrease in mitochondrial ALA synthetase activity occurred for at least 4 hr following hemin injection. A concomitant increase in enzyme levels in the postmitochondrial fraction was observed for 3 hr, but decreased to control levels during the subsequent hour. In contrast, hemin did not depress mitochondrial ALA synthetase activity in fetal liver (Fig. 2B). Enzyme levels 1 hr following hemin treatment were either similar to (five of eight experiments) or greater than (three of eight experiments) those of zero-time controls. The over-all result, therefore, was one of slightly increased mitochondrial ALA synthetase activity 1 hr after hemin injection. The significance of this increase ($p < 0.05$) is difficult to interpret because of the difficulties associated with hemin administration. At no time, however, did mitochondrial ALA synthetase levels decrease below those of zero-time controls. ALA synthetase levels in the postmitochondrial fraction of fetal liver were not significantly altered by hemin administration. A higher dose of hemin (50 mg/kg) did not produce a more pronounced alteration in ALA synthetase activity in any subcellular fraction of fetal rat liver. Hemin has been shown to cross the placenta readily and to reach a maximal concentration in the fetus within 1 hr after injection into the mother (17).

In order to investigate more directly the possible influence of hemin on fetal mitochondrial ALA synthetase *in vivo*, the effects of hemin on the rate of turnover of the mitochondrial enzyme were studied in fetal livers in which extramitochondrial protein synthesis was inhibited. The latter process was accomplished by treatment of fetal rats with cycloheximide, a selective inhibitor of cytoribosomal protein synthesis in mammalian cells (18). Following cycloheximide injection, ALA synthetase activity declined rapidly in both mitochondrial and postmitochondrial fractions of fetal liver. The decline in the relative activity of mitochondrial ALA synthetase after cycloheximide treatment is shown in Fig. 3. A linear rate of decay occurred for approximately 90 min following

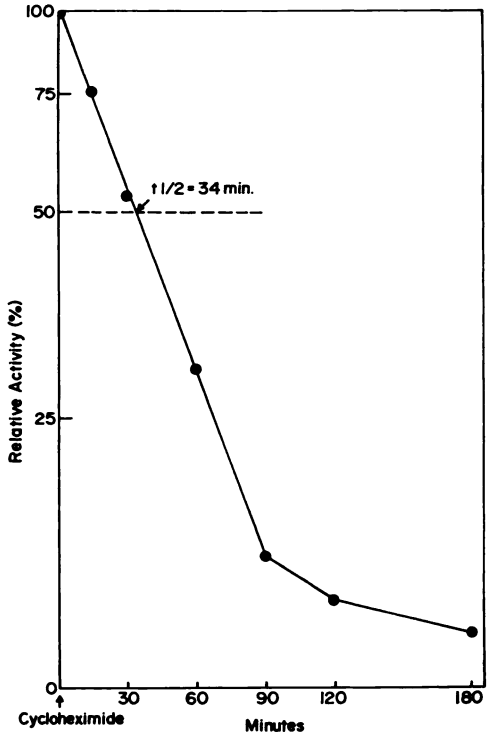

FIG. 3. *Decay of fetal mitochondrial ALA synthetase activity following cycloheximide treatment*

Pregnant rats were treated with cycloheximide (50 mg/kg) by intraperitoneal injection at zero time and were killed at 30- or 60-min intervals thereafter. Half-life ($t_{1/2}$) represents the time following cycloheximide treatment at which 50% of the initial ALA synthetase activity remained. The actual activity of the 100% level was 5.00 ± 0.13 nmoles/mg/hr.

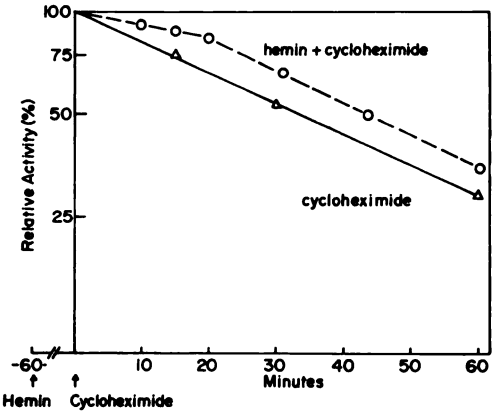

FIG. 4. *Effects of hemin on decay of fetal mitochondrial ALA synthetase activity following cycloheximide treatment*

Pregnant rats were treated with hemin (20 mg/kg) 1 hr prior to cycloheximide injection (50 mg/kg). Animals were killed at the times indicated. The decay curve for ALA synthetase activity following treatment with cycloheximide alone is given for comparison. The actual activity for the 100% level was 5.33 ± 0.12 nmoles/mg/hr.

cycloheximide injection, after which a slower rate of decline in activity was observed. During the initial period of decay, enzyme activity decreased with an approximate half-life of 34 min. This turnover rate is considerably greater than that reported for the adult enzyme (19). Cycloheximide (200 μM) was not effective in altering ALA synthetase activity when incubated with fetal mitochondria and the appropriate substrates for 1 hr *in vitro*.

The effects of hemin on fetal mitochondrial ALA synthetase activity prior to inhibition of protein synthesis *de novo* with cycloheximide are shown in Fig. 4. The decay of mitochondrial ALA synthetase activity following treatment with cycloheximide alone is included for comparison. Data in the two treatment groups were compared using a Wilcoxon signed rank test and were found to differ significantly at the $p < 0.05$ level. When hemin was given 1 hr prior to cycloheximide treatment, a much slower rate of decay of mitochondrial ALA synthetase activity was observed immediately following cycloheximide injection.

Studies with adult rats (6, 14, 15, 19) have shown that mitochondrial ALA synthetase levels are substantially elevated following treatment with chemicals which induce synthesis of the enzyme in the endoplasmic reticulum. Previous studies (7, 20), however, have shown that ALA synthetase in fetal mammalian liver is refractory to induction by agents which induce the enzyme in adult liver. Thus the possible stimulatory effect of hemin on mitochondrial ALA synthetase activity in fetal liver was further investigated by blocking heme synthesis, hence reducing over-all heme levels. This process was accomplished by administration of aminotriazole, an inhibitor of ALA dehydratase, the second enzyme in the heme-bio-

synthetic pathway (21). Although ALA dehydratase is not rate-limiting in heme biosynthesis, the activity of this enzyme can be reduced by aminotriazole sufficiently to limit heme production *in vivo* (13). Preliminary studies indicated that 40% inhibition of ALA dehydratase activity could be achieved in both adult and fetal rat liver within 1 hr following aminotriazole administration at a dose of 3 g/kg. This dosage has been shown not to have an inhibitory effect on protein synthesis in general in adult rats (13). In addition, no direct effects on ALA synthetase activity were observed in either adult or fetal liver when aminotriazole (5 mM) was incubated with mitochondria and the appropriate substrates *in vitro*. Inhibition of ALA dehydratase activity *in vivo* was sustained for at least 24 hr following treatment with aminotriazole.

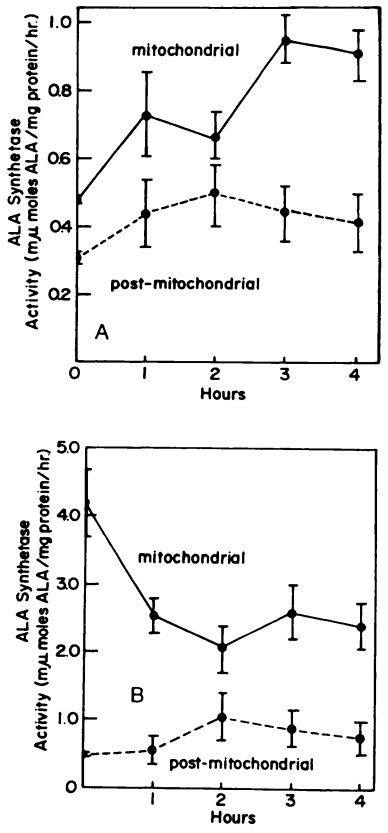

FIG. 5. *Effects of aminotriazole on mitochondrial and postmitochondrial ALA synthetase activity in adult (A) and fetal (B) rat liver*

Aminotriazole (3 g/kg) was administered to pregnant rats by intraperitoneal injection at zero time. Controls received an equal volume of 0.9% NaCl. Animals were killed at hourly intervals thereafter.

The effects of reduced heme synthesis by aminotriazole on the subcellular distribution of ALA synthetase in adult rat liver are shown in Fig. 5A. Mitochondrial ALA synthetase activity was doubled within 3 hr after treatment. In addition, a slight increase in the enzyme activity in the postmitochondrial fraction was observed. On the other hand, a significant decrease in fetal mitochondrial ALA synthetase activity was observed following aminotriazole treatment (Fig. 5B). A 50% decrease was observed 2 hr following drug injection, and this decrease was sustained during the 4-hr experimental period. ALA synthetase activity in the postmitochondrial fraction of fetal liver increased from 50% to 100% in six of eight experiments within 2 hr following aminotriaxole injection, but the over-all increase was not significant. At no time, however, did ALA synthetase activity drop below levels observed in zero-time controls.

Simultaneous administration of hemin and aminotriazole produced no significant alteration of ALA synthetase activity in the subcellular fractions in either adult or fetal rat liver. A reversal of the aminotriazole-induced decrease in mitochondrial ALA synthetase activity in fetal liver by hemin could be observed when hemin was given 1 hr after aminotriazole injection. In this experiment mitochondrial ALA synthetase activity in zero-time controls was 5.50 nmoles/mg of protein per hour. One hour following aminotriazole injection enzyme activity had dropped to 3.40 nmoles/mg/hr, or 62% of control levels. If hemin was given 1 hr after aminotriazole, ALA synthetase activity 1 hr later was 4.40 nmoles/mg/hr, or 80% of control values.

Responsiveness to the effects of hemin and aminotriazole on the subcellular localization of ALA synthetase activity during the perinatal period was investigated in rats ranging in age from 5 days before to 18 days after birth. The results of these experiments showed that the facilitative effects of hemin

on mitochondrial ALA synthetase levels are restricted to the fetal period prior to the decline of over-all ALA synthetase levels. Neither hemin nor aminotriazole produced alterations in the subcellular distribution of ALA synthetase activity in the livers of newborn rats or of fetal rats 1 day prior to delivery. Eighteen-day-old rats exhibited responses similar to those seen in adults.

DISCUSSION

Numerous reports regarding the regulation of hepatic ALA synthetase in adult mammalian liver have appeared in recent years (14–16, 22, 23). Convincing evidence has been provided that heme plays a major role in this process (15, 16, 24). Although it is well established that ALA synthetase is the rate-limiting enzyme in heme biosynthesis in adult liver, the current studies, in which fetal mitochondrial ALA synthetase levels are 10 times those seen in the adult, suggest that this enzyme may not play a rate-limiting role in heme biosynthesis throughout fetal development. Freshney and Paul (25) have recently shown in fetal mice that ALA synthetase, ALA dehydratase, and ferrochelatase, the final enzyme in the heme-biosynthetic pathway, are elevated maximally at 14, 15, and 17 days of gestation, respectively. The sequential elevation of these enzymes during gestation suggests that each may play a rate-limiting role in heme biosynthesis at a different time during hepatic development. The current studies suggest that ALA synthetase may not become rate-limiting in fetal liver until it becomes susceptible to repression by heme, a phenomenon which does not develop until near the time of birth (7).

A unique role for heme in fetal liver is indicated by the strikingly different effects of hemin and aminotriazole on the distribution of ALA synthetase in mitochondrial and postmitochondrial subcellular fractions during different stages of development. The rapid decrease in mitochondrial ALA synthetase levels in adult rats following hemin injection, and the corresponding increase in enzyme activity in the postmitochondrial fraction, support the hypothesis that hemin limits the mitochondrial incorporation of ALA synthetase in adult rat liver (16). Conversely, the observation that hemin did not inhibit and, in fact, may have enhanced ALA synthetase activity in fetal mitochondria suggests that hemin may play a quite different role during fetal hepatic development. The studies with cycloheximide, in which a slower turnover rate of ALA synthetase was observed after treatment with hemin (Fig. 4), suggest that hemin enhances the activity of the mitochondrial form of the enzyme rather than inhibiting it.

The mechanism through which hemin acts in this process, however, cannot be determined from the results of these studies. One possibility is that hemin directly stabilizes fetal mitochondrial ALA synthetase or slows its degradation. This concept is supported by studies currently in progress in this laboratory, which indicate that the activity of ALA synthetase partially purified from fetal mitochondria is increased, rather than inhibited, by hemin, as has been reported in the case of the adult enzyme (26, 27). Alternatively, hemin might stabilize the mitochondrial membrane to which ALA synthetase is attached, or may in some manner promote the conversion of ALA synthetase from the soluble to the mitochondrial form. The latter possibility is supported by the studies of Beattie and Stuchell (14), which demonstrated the stimulatory effects of induced heme synthesis on the mitochondrial incorporation of cytochromes and other proteins, including ALA synthetase, in adult rat liver. Such studies suggest that both the rate of mitochondrial protein synthesis and the incorporation of proteins synthesized outside the mitochondria are increased when heme levels are elevated by induction of ALA synthetase. Recent studies by Mathews *et al.* (28) suggest that hemin may stimulate a general increase in mammalian protein synthesis.

The use of aminotriazole in these studies was based on the observation that the primary mechanism of action of this chemical is inhibition of ALA dehydratase without concomitant inhibition of over-all protein synthesis (13). On the basis of this consideration, it is concluded that the 50% decrease in fetal mitochondrial ALA synthetase levels

observed following aminotriazole treatment (Fig. 5B) occurred as an indirect result of inhibition of heme synthesis. However, it is not currently known whether aminotriazole directly inhibits protein synthesis in fetal liver. This possibility could also account for the observed decline in mitochondrial ALA synthetase levels after aminotriazole treatment, although no concomitant decline in enzyme levels in the postmitochondrial fraction was observed. Another consideration is the possibility that aminotriazole might specifically inhibit other enzymes of the heme-biosynthetic pathway in fetal liver, aside from ALA dehydratase, to reduce heme synthesis effectively. Studies in progress in this laboratory, as well as those of other investigators (25), indicate that in 18-day fetal liver ALA dehydratase may not be present in great excess in comparison with other enzymes of the heme-biosynthetic pathway; hence inhibition of this enzyme may reduce over-all heme synthesis more effectively than in the adult. The rapid clearance of [^{14}C]hemin from fetal serum following injection into the mother (17) suggests that a rapid turnover of heme occurs in fetal liver and supports the contention that fetal mitochondrial ALA synthetase activity decreases when heme levels are reduced (Fig. 5B). Aminotriazole is also known to be an inhibitor of thyroid function (29). The possible influence of the goitrogenic effects of aminotriazole on fetal ALA synthetase have not been investigated.

The possible contribution of hemopoietic cells to the high levels of ALA synthetase seen in fetal liver has been considered previously (7) and does not seem very likely. Data on the ratio of hemopoietic to parenchymal cells in 18-day fetal rat liver are not currently available. Recent histological studies (4) have shown that, whereas hemopoietic activity in rats declines toward the end of gestation, a small number of developing blood cells remain in the liver during the first week of life. Thus the elevated hepatic ALA synthetase activity observed in the late gestational period could, in part, reflect the gradual decline in the function of the liver as an erythropoietic organ. On the other hand, changes in ALA synthetase activity in fetal liver do not seem to be closely correlated with changes in the cell population of the liver during late fetal development (25). In addition, hemopoietic cells appear to contain too few mitochondria, in comparison with fetal parenchymal cells (30), to account for the differences observed.

In conclusion, these studies suggest that heme may play a role in the regulation of ALA synthetase in developing fetal rat liver which is quite different from that proposed for the adult. The possible facilitative or stabilizing role of heme in the development of fetal mitochondrial ALA synthetase activity may, in part, explain the elevated enzyme levels measured in fetal mitochondria in comparison with those of the adult. The developmental changes which render ALA synthetase susceptible to repression by hemin near the time of birth are not yet known. However, exposure during gestation to drugs or other environmental chemicals, such as aminotriazole which interfere with heme synthesis might preclude the development of normal hepatic hemopoietic activity. On the other hand, the identification and utilization of agents which specifically alter hepatic heme synthesis may permit further clarification of the role played by heme in cellular growth and function.

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