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DEVELOPMENTAL ASPECTS OF HEPATIC HEME BIOSYNTHETIC CAPABILITY AND HEMATOTOXICITY

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Abstract—δ-Aminolevulinic acid (ALA) synthetase is considered to be rate-limiting in heme biosynthesis in normal adult mammalian liver. However, under certain pharmacological or pathological circumstances, other enyzmes of the heme biosynthetic pathway have been shown to be limiting in this process. In the current studies, the developmental patterns of ALA dehydratase, uroporphyrinogen I synthetase and heme synthetase were measured in rat liver, and the potential influence of these enzymes on heme biosynthetic capability under various conditions in adult, neonatal and fetal liver was assessed. In addition, a comparison of the activities of these enzymes with those of ALA synthetase was made as a means of assessing the relative influence of hematotoxic agents on hepatic heme biosynthetic capability at different stages of development.

Hepatic heme biosynthesis in normal adult mammals is regulated by δ-aminolevulinic acid (ALA) synthetase, the first and rate-limiting enzyme in this pathway [1–3]. ALA synthetase, a mitochondrial enzyme, catalyzes the synthesis of ALA from glycine and succinyl coenzyme A. The ALA (2 moles) is converted to porphobilinogen (PBG) by ALA dehydratase, a soluble fraction enzyme. PBG (4 moles) is then converted in the presence of uroporphyrinogen I synthetase (uro I synthetase) and other soluble fraction enzymes to protoporphyrin IX, into which iron is incorporated to form heme. The final step in this process is catalyzed by heme synthetase (ferrochelatase), a mitochondrial enzyme. The heme thus formed is utilized in the synthesis of various hemoproteins, including the mitochondrial and microsomal cytochromes, which are essential to cellular growth and function.

Recently, various investigators have reported that enzymes of the heme biosynthetic pathway other than ALA synthetase might limit heme biosynthetic capability under a variety of pharmacological or physiological conditions. ALA dehydratase, for example, is known to limit heme production in the presence of certain heavy metals [4] or organic compounds such as the herbicide, aminotriazole [5]. Uro I synthetase has been shown to be rate-limiting in heme biosynthesis in livers [6] and red blood cells [7] of humans suffering from acute intermittent porphyria and in the livers of mice treated with the porphyrogenic drug diethyl -1,4-dihydro-2,4,6-trimethylpyridine -3,5-dicarboxylate (DDC) [8]. Heme synthetase is inhibited by cobalt compounds [9], DDC, griseofulvin, EDTA and various other agents [10].

Previous studies from this laboratory have shown that the activity of ALA synthetase in perinatal mammals is highly elevated in comparison with that of the adult [11, 12] and does not become susceptible to regulation by the end-product heme until near the time of birth [13, 14].

These observations question the role of ALA synthetase as the rate-limiting enzyme in hepatic heme biosynthesis in perinatal liver, and suggest that other enzymes of the hepatic heme biosynthetic pathway may, under various pharmacological or physiological conditions, limit hepatic heme biosynthetic capability with particular susceptibility in this regard at different stages of development.

In the current studies these possibilities were examined by first measuring the ontogenic development of hepatic ALA dehydratase, uro I synthetase and heme synthetase activities in rats. A comparision of the activities and other known properties of these enzymes with those of ALA synthetase in adult, neonatal and fetal rat liver was then made in order to assess the relative rate-limiting influence of each enzyme in heme biosynthesis at each stage of development. Finally, the potential of each enzyme for limiting heme biosynthetic capability in the presence of specific hematotoxic agents at each stage of development was assessed.

MATERIALS AND METHODS

Materials. Succinyl coenzyme A synthetase (succinic thiokinase) (EC 6.2.1.4), pyridoxal 5′-phosphate (PLP), ATP, GTP, coenzyme A, cytochrome *c*, PBG, aminotriazole and ALA were obtained from Sigma Chemical Co., St. Louis, Mo. Glycine, succinic acid, glutathione, hemin, protoporphyrin IX and *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) were purchased from CalBiochem, San Diego, Calif. 4-Dimethylaminoantipyrine (aminopyrine) was purchased from Aldridge Chemical Co., Milwaukee, Wisc. $^{59}FeCl_3$ (529 mCi/m-mole) was obtained from New England Nuclear, Boston, Mass. Cobaltous chloride ($CoCl_2$) was purchased from K & K Laboratories, Inc., Plainview, N.Y. Other chemicals were of reagent grade and were obtained from standard commercial sources.

Preparation of animals and tissues. Sprague–Dawley rats (CD strain) were date-bred by the Charles River Laboratories, Boston, Mass. Pregnant animals were housed in individual cages and were allowed food and

water *ad lib*. All animals were killed by decapitation. Livers of adult and fetal rats were excised, washed, weighed and homogenized in 9 vol. of 0.25 M sucrose containing 0.05 M Tris–HCl buffer, pH 7.5, using a Potter–Elvehjem homogenizer fitted with a Teflon pestle. When ALA synthetase was assayed, the buffered sucrose also contained 0.1 mM EDTA and 0.1 mM pyridoxal phosphate. The preparation of hepatic mitochondria, microsomes and other subcellular fractions was performed as previously described [14]. A minimum of four adult animals or litters was utilized for each experimental point.

Assessment of hepatic heme biosynthetic capability. The effects of hematotoxic agents on heme biosynthetic capability were determined through assessment of mitochondrial and microsomal hemoprotein function after treatment of neonatal or 18-day pregnant rats with inhibitors of heme biosynthetic pathway enzymes. Aminotriazole and $CoCl_2$ were chosen as specific inhibitors of ALA dehydratase [5] and heme synthetase [9] respectively. No selective inhibitor of uro I synthetase is currently known. Cytochrome *c* oxidase and aminopryine demethylase activities were assessed as indicators of mitochondrial and microsomal (cytochrome P-450) hemoprotein function respectively. Animals were treated by subcutaneous injection with aqueous solutions of aminotriazole (3 g/kg) or $CoCl_2$ (50 mg/kg) either 24 hr or 1 week prior to sacrifice. Control animals received 0.9% sodium chloride.

Assay of enzyme activities. Mitochondrial ALA synthetase activity was measured by previously described methods [14]. Reaction mixtures contained about 4 mg of mitochondrial protein/ml.

ALA dehydratase activity was measured in the 9000 *g* supernatant fraction of adult and fetal liver homogenates by a modification of the method of Gibson *et al.* [15] as described by Baron and Tephly [5].

Uroporphyrinogen I synthetase was assayed by a modification of the method of Levin and Coleman [16] wherein the rate of disappearance of PBG was measured. The incubation mixture contained 1×10^{-4} M PBG, 0.2 M potasium phosphate buffer, pH 7.65, and 0.4 ml of a 9000 *g* supernatant solution prepared by centrifugation of a 10% liver homogenate. The total reaction volume was 0.8 ml. Reactions were terminated after a 1-hr incubation by the addition of 1 ml of 10% trichloroacetic acid (TCA) solution containing 0.1 M $HgCl_2$. After centrifugation, the PBG remaining was assayed by reaction with an equal volume of Ehrlich's reagent and quantitated spectrophotometrically at 553 nm. Zero time and zero degree incubations were run in all experiments in order to measure the PBG initially present. In all experiments, the 9000 *g* supernatant solutions were heated at 65° for 15 min in order to inactivate the uroporphyrinogen III cosynthetase present, prior to assay of uro I synthetase activity.

Heme synthetase activity was measured by a modification of the method described by Freshney and Paul [17]. Reaction mixtures containing 0.1 ml of hepatic mitochondrial suspension (20 mg/ml) and 0.4 ml of 0.1 M reduced glutathione prepared in 150 mM Tris buffer, pH 7.5, were incubated with shaking at 37° in a nitrogen atmosphere. After 20 min, 0.4 ml of a solution containing 0.05 mM $FeCl_3$, 0.25 μCi/ml $^{59}FeCl_3$ and 0.5 mM protophorphyrin IX was added, and the mixture was incubated for an additional hr under the same conditions. Reactions were then terminated by the addition of 0.1 ml of 1 M HCl. Zero time and zero degree incubations were run simultaneously. After centrifugation, supernatants were removed and extracted with 4, followed by 3, vol. butanone at 4°. Pooled butanone extracts were then extracted with 3 ml of 0.1 N HCl and centrifuged, and 0.5 ml of the butanone supernatants was transferred to individual counting vials. Radioactivity was determined after the addition of 10 ml PPO* POPOP–toluene counting solution in a Packard Tri-Carb liquid scintillation spectrometer.*

Cytochrome *c* oxidase activity in mitochondrial preparations was assessed polarographically by measuring oxygen consumption with a Clark electrode, essentially as described by Schnaitman *et al.* [18], with the exception that assays were performed at 30°.

Aminopryine demethylase activity in microsomal preparations was measured by a modification of the procedure of Orrenius [19] as described by Lucier *et al.* [20]. Reaction mixtures contained approximately 3 mg of microsomal protein/ml.

Protein determinations were made by the method of Lowry *et al.* [21] using bovine serum albumin (Fraction V) as a standard.

RESULTS

The developmental patterns of hepatic ALA synthetase activity in a variety of mammalian species have been previously described [11, 14]. In the rat the level of activity at 5 days prior to delivery is approximately ten times that of the adult enzyme. This activity declines to adult levels near the time of birth.

The ontogenic development of ALA dehydratase activity is illustrated in Fig. 1. An initial decline in enzyme levels occurs just prior to birth and reaches a nadir 2 days after delivery. Activity then increases to levels observed in prenatal liver, reaching a maximum by 8–10 days after birth and subsequently declines to adult levels during the second postnatal week.

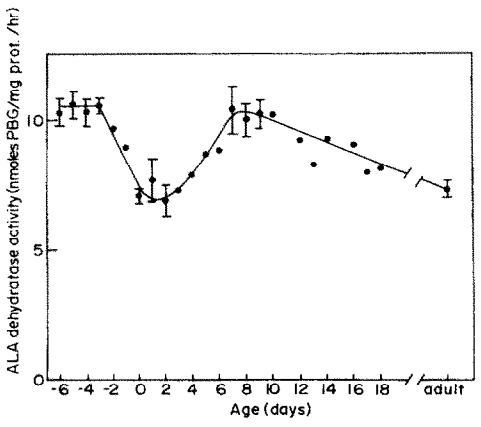

Fig. 1. Ontogenic development of hepatic ALA dehydratase activity in the rat. Values express enzyme activity as nmoles PBG produced/mg of protein/hr.

* PPO = 2,5-diphenyloxazole; and POPOP = 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene.

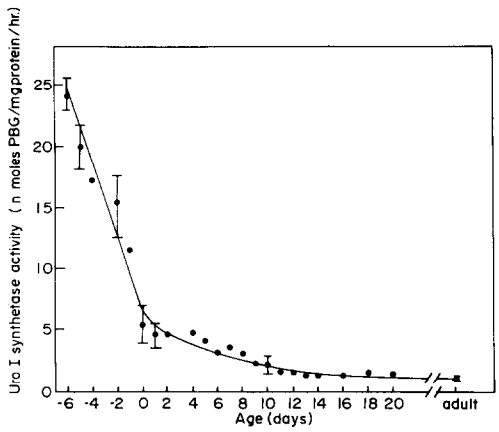

Fig. 2. Ontogenic development of hepatic uroporphyrinogen I synthetase activity in the rat. Values express enzyme activity as nmoles PBG utilized/mg of protein/hr.

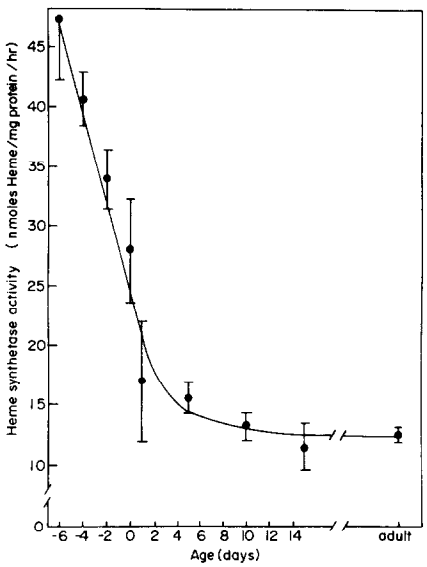

Fig. 3. Ontogenic development of hepatic heme synthetase in the rat. Values express enzyme activity as nmoles heme produced/mg of protein/hr.

The pattern of development of uro I synthetase, shown in Fig. 2, is somewhat similar to that of ALA synthetase inasmuch as a rapid decline in activity occurs just prior to birth. In contrast to the development of ALA synthetase activity, however, adult levels of uro I synthetase are not attained until 10–12 days postpartum.

Heme synthetase activity is also highly elevated in fetal liver in comparison with that of the adult enzyme (Fig. 3). Activity declines to adult levels near the time of birth.

Consideration of the developmental patterns of heme biosynthetic pathway enzymes presented here indicates that, although ALA synthetase levels are elevated in fetal liver, the activity of this enzyme remains the lowest relative to those of other enzymes investigated at any stage of development. This fact, taken together with the exceptional lability of ALA synthetase ($T_{1/2} = 34$ and 72 min for fetal and adult enzymes, respectively [14]), and the relatively low affinity of this enzyme for the substrate glycine ($K_m \cong 10^{-2}$ M) in both adult [22] and fetal [23] liver, suggest that ALA synthetase is likely to be the most limiting of the enzymes considered here in terms of heme biosynthetic capability in perinatal liver. Under this assumption it becomes possible to assess the potential role of enzymes other than ALA synthetase in limiting hepatic heme biosynthetic capability at each stage of development. In order to make such an assessment, the activities of ALA dehydratase, uro I synthetase and heme synthetase in adult, neonatal and 18-day fetal lever were compared with those of ALA synthetase at equivalent stages of development. Comparisons were made by first calculating total enzyme activities (total activity in the mitochondrial or soluble fraction/g of liver/hr), which were then converted to an ALA molar basis (nmoles of ALA utilized/g of liver/h) by multiplying ALA dehydratase activity by 2 (2 ALA → 1 PBG), uro I synthetase activity by 2 (2 ALA → 1 PBG → $\frac{1}{4}$ URO, as assayed), and heme synthetase activity by 8 (8 ALA → heme). Total activities are given in Table 1. Values in parentheses represent ALA molar activities. Enzyme acti-

Table 1. Activities of hepatic heme biosynthetic pathway enzymes in adult, neonatal and fetal rat liver

	ALA synthetase (nmoles ALA/g liver/hr)	ALA dehydratase (nmoles PBG/g liver/hr)	Uro I synthetase (nmoles PBG/g liver/hr)	Heme synthetase (nmoles heme/g liver/hr)
Adult	21	602*	88	529
		(1204)†	(176)	(4232)
Neonatal	20	404	321	643
		(808)	(642)	(5144)
Fetal	96	454	759	657
		(908)	(1518)	(5256)

* Total enzyme activities were calculated by multiplying specific activities (nmoles/mg of protein/hr) for each enzyme by total protein levels of mitochondrial or soluble fraction pools/g of adult, neonatal or fetal liver. Values for protein concentrations were as cited by Knox [24] or as calculated using hepatocellular preparations.

† ALA molar activities (values in parentheses) were calculated by multiplying total enzyme activities by the number of moles of ALA utilized in the specific enzymatic reaction measured as described in the text.

Table 2. Ratios of activities of ALA dehydratase, uro I synthetase and heme synthetase to those of ALA synthetase in adult, neonatal and fetal rat liver*

	ALA dehydratase / ALA synthetase	Uro I synthetase / ALA synthetase	Heme synthetase / ALA synthetase
Adult	57.33	8.38	201.53
Neonatal	40.40	32.10	257.20
Fetal	9.46	15.81	54.75

* Ratios were calculated by dividing ALA molar activities (Table 1) by ALA synthetase activity for each age group.

vity ratios were then calculated by dividing ALA molar activities of ALA dehydratase, uro I synthetase and heme synthetase by those of ALA synthetase for each age group (Table 2). These calculations permit the comparison of enzymes on the common basis of activity required to produce or utilize molar equivalents of ALA during the synthesis of heme; thus, they serve to predict the extent to which heme synthesis might be inhibited by specific enzyme inhibitors at each stage of development.

As a test of this hypothesis, neonatal or pregnant rats were treated with either aminotriazole or $CoCl_2$, as described in Materials and Methods. The effects of these agents on mitochondrial and microsomal hemoprotein function, as reflected in cytochrome *c* oxidase and aminopyrine demethylase activities, respectively, in adult, neonatal and fetal rats, are seen in Table 3. Fetal animals are clearly the most sensitive to inhibition of hepatic hemoprotein function by both inhibitors. Aminotriazole treatment caused cytochrome *c* oxidase and aminopyrine demethylase activities to be reduced to 84 and 70 per cent of control levels, respectively, 24 hr after injection; administration of $CoCl_2$ to pregnant rats diminished fetal cytochrome *c* oxidase and aminopryine demethylase levels to 61 and 29 per cent of control levels respectively. This reduction in hemoprotein function in the fetus after treatment on day 18 of gestation persisted into the postnatal period with little apparent recovery of activity 1 week after treatment. As would be predicted from Table 2, the neonate, on the other hand, is surprisingly resistant to the effects of inhibitors of heme biosynthetic capability, expressing less than 10 per cent alteration in either cytochrome *c* oxidase or aminopryine demethylase activities 24 hr after treatment with aminotriazole and approximately 25 per cent after treatment with cobalt chloride. Recovery in the neonate occurred within 1 week after treatment with either hematotoxic agent. Adults experienced up to a 20 per cent reduction in hemoprotein function, but, like the newborn animals, also recovered rapidly within 1 week after treatment.

DISCUSSION

Numerous studies documenting the role of ALA synthetase as the rate-limiting enzyme in hepatic heme biosynthesis in normal adult mammals have appeared in recent years [1–3, 25, 26]. Previous studies from this laboratory [14, 27] regarding the properties of ALA synthetase in fetal liver have suggested that this enzyme may not become rate-limiting in hepatic heme biosynthesis until it becomes susceptible to end-product regulation during the postnatal period. Identification of the actual rate-limiting event(s) in fetal heme biosynthesis has thus far been rather inconclusive, and it has been interesting to speculate that another enzyme of the heme biosynthe-

Table 3. Effects of aminotriazole and cobalt chloride on hepatic heme biosynthetic capability in adult, neonatal, and fetal rats*

	Enzyme activity (% control)			
	Aminotriazole		Cobalt chloride	
	Cytochrome *c* oxidase	Aminopyrine demethylase	Cytochrome *c* oxidase	Aminopyrine demethylase
Adult	100 (100)†	79 (100)	93 (100)	84 (93)
Neonatal	100 (100)	92 (95)	74 (84)	78 (88)
Fetal	84 (86)	70 (78)	61 (64)	29 (52)

* Heme biosynthetic capability was assessed by measurement of cytochrome *c* oxidase and aminopyrine demethylase activities in hepatic mitochondrial and microsomal preparations, respectively, 24 hr after treatment, as described in the text. Control levels of cytochrome *c* oxidase were 1.31 ± 0.17, 1.04 ± 0.14 and 0.98 ± 0.06 μgatoms O_2 consumed/min/mg of mitochondrial protein ± standard deviation in adult, neonatal and fetal liver respectively. Corresponding control values for aminopyrine demethylase were 6.87 ± 0.4, 1.82 ± 0.09 and 0.33 ± 0.02 nmoles formaldehyde liberated/min/mg of microsomal protein respectively.

† Values in parentheses are enzyme activities measured 1 week after treatment. A minimum of four pregnant animals or litters was combined for each experimental value.

tic pathway may play such a role. The demonstration in the current studies that the activities of ALA dethyratase, uro I synthetase and heme synthetase are highly elevated relative to that of ALA synthetase, however, suggests that these enzymes are even less likely than is ALA synthetase to limit the rate of fetal hepatic hematopoiesis under normal physiological circumstances.

Consideration of other known properties of the heme biosynthetic pathway enzymes in a variety of species tends to substantiate this view. The K_m values of ALA dehydratase [4, 28], uro I synthetase [7, 16] and heme synthetase [29, 30] for their respective substrates are 10^2 to 10^4 times less than that of ALA synthetase for glycine, suggesting that the functional capabilities of these enzymes are probably not restricted by the availability of substrates *in vivo*. Although the exact endogenous levels of all heme biosynthetic pathway substrates are not currently known, it is generally accepted that the enzyme assays utilized herein employ substrate concentrations which closely approximate those found under normal physiological conditions. Thus, the enzyme activities measured in these studies, as well as the activity ratios calculated at different stages of development, are a likely representation of conditions which exist *in vivo*. On the other hand, the endogenous glycine concentration ($\sim$ 4 mM) in fetal liver is known to be less than the K_m of ALA synthetase for this substrate [27]; thus, the availability of this amino acid to ALA synthetase could conceivably influence heme biosynthetic capability in the fetus. In this vein, it is interesting that fluctuations in the glycine concentration *in vivo* have been shown to modulate the rate of heme biosynthesis in porphyric adult animals [9].

The potential regulatory properties of the heme biosynthetic pathway enzymes studied here might also be influenced by their turnover rates, which reflect their responsiveness to physiological demands for rapid changes in the rate of heme biosynthesis *in vivo*. In this regard, it is of interest that the turnover rates of ALA synthetase in both adult [31] and fetal [14] liver are among the highest observed for mammalian enzymes.

These considerations, while they do not identify ALA synthetase as the rate-limiting enzyme in fetal hepatic heme biosynthesis, support the conclusion that this enzyme, in comparison with the others studied herein, is most likely to limit heme biosynthesis in perinatal liver under normal circumstances. It is upon this conclusion that the hypothesis for the assessment of relative hematotoxicity at different stages of development is based. The validity of this approach appears to be justified in consideration of the data presented in Table 3. Table 2 predicts that agents which selectively inhibit either ALA dehydratase or heme synthetase will have the greatest probability of limiting heme biosynthesis in the fetus. As seen in Table 3, both aminotriazole and $CoCl_2$ exerted the most profound effects on the expression of hemoprotein function in the fetus. These inhibitory effects were expressed not only 24 hr after treatment but up to one week after administration of these chemicals, suggesting that late gestation may be a period of enhanced susceptibility to the effects of hematotoxic agents on the postnatal development of hemoprotein function. It is interesting that the neonate is relatively unaffected by either enzyme inhibitor; as predicted from Table 2, neither the adult nor the neonate would be expected to experience a substantial compromise of hemoprotein function after acute exposure to inhibitors of ALA dehydratase or heme synthetase.

Less easily demonstrated, due to a lack of a known specific inhibitor of uro I synthetase, is the prediction that agents which inhibit this enzyme may be of greatest concern to the adult, wherein the ratio of uro I synthetase to ALA synthetase activities is the lowest of all three age groups. It is interesting, however, that uro I synthetase becomes the rate-limiting enzyme in heme biosynthesis in adult humans suffering from acute intermittent porphyria, a genetic disease characterized by a primary diminution in the activity of this enzyme in the liver [6, 32]. Thus, porphyria patients may be particularly susceptible to a reduction of heme biosynthetic capability by agents which restrict uro I synthetase activity.

When all enzymes are considered together, the fetus appears to be most highly susceptible of all age groups to restriction of heme biosynthetic capability through inhibition of enzymes other than ALA synthetase. This point may be of particular importance when considering the effects of drugs and environmental toxicants on the development of drug-metabolizing capability or other metabolic functions wherein hemoproteins play a vital role. This point is also of importance in light of recent arguments regarding the proposed role of heme in the initiation of protein synthesis [33] and in the stimulation of mitochondrial biogenesis [34] in rapidly developing fetal cells.

Finally, it is interesting to note that selective prenatal inhibition of single heme biosynthetic pathway enzymes may result in inhibited or delayed postnatal development of both mitochondrial and microsomal hemoprotein function, and a compromise of metabolic processes which are essential to the normal growth and development of the organism. Thus, the identification of mechanisms by which gestational exposure to hematotoxic agents induces delayed or incomplete development of hepatic hemoprotein function may provide a focus for investigation of the mechanisms by which transplacental exposure to other types of toxic agents predisposes to specific organ dysfunction at different stages of development.

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