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DEVELOPMENTAL ASPECTS OF HEPATIC HEME BIOSYNTHETIC CAPABILITY AND HEMATOTOXICITY—II

STUDIES ON UROPORPHYRINOGEN DECARBOXYLASE

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Abstract—δ-Aminolevulinic acid (ALA) synthetase is considered the rate-limiting enzyme in heme biosynthesis in mature mammalian liver. However, under various physiologic or toxicologic conditions, other enzymes of the heme biosynthetic pathway may become rate-limiting in this process. In the present studies, the ontogenic development of uroporphyrinogen (uro) decarboxylase was measured in rat liver, and the properties and potential influence of this enzyme on heme biosynthetic capability in adult and fetal liver were assessed. In addition, a quantitative comparison of the activity of uro decarboxylase with that of ALA synthetase was made as a means of estimating the relative effects of specific inhibitors of uro decarboxylase on hepatic heme biosynthetic capability at each stage of development. The results indicate that fetal uro decarboxylase activity is over three times that of the adult and that enzyme activity declines to the adult levels concomitant with a decrease in the hematopoietic cell composition of the liver near the time of birth. Moreover, fetal uro decarboxylase may be substantially more susceptible to physiologic or toxicologic alteration than is the adult enzyme. The fetus may, therefore, be at greater risk with respect to compromise of heme biosynthetic capability by agents which alter uro decarboxylase activity.

The regulation of heme biosynthesis in perinatal mammalian liver and other tissues has been investigated in previous studies from these laboratories [1–5]. Among the more interesting findings from these studies is the observation that in the fetus the activity of δ-aminolevulinic acid (ALA) synthetase, which is the rate-limiting enzyme in heme biosynthesis in mature liver [6], is highly elevated, compared with the adult, and is refractory to both feed-back regulation by the end-product heme and to induction by exogenous chemicals. These observations have called into question the rate-limiting role of ALA synthetase in perinatal heme biosynthesis [5] and have stimulated further research which has suggested that other enzymes of this process may limit heme biosynthetic capability during perinatal development. This prospect is of particular interest in light of studies which have shown that both mitochondrial and microsomal hemoprotein function [7, 8], as well as drug-metabolizing capability [4, 9] during postnatal development, may be compromised by maternal exposure to chemicals which selectively inhibit specific heme biosynthetic pathway enzymes other than ALA synthetase in fetal liver.

Among the enzymes of the heme biosynthetic process which have not as yet been investigated in this regard is uroporphyrinogen (uro) decarboxylase (EC 4.1.1.37), which catalyzes the step-wise decarboxylation of uroporphyrinogen to coproporphyrinogen [10]. Evaluation of the role played by this enzyme in perinatal heme biosynthesis is of particular interest in light of recent studies from these laboratories [11] and others [10, 12, 13] which demonstrate that uro decarboxylase in adult tissues can be selectively and substantially inhibited by trace metals, organohalogens and other toxic environmental chemicals. Moreover, uro decarboxylase in various tissues has been reported to be inhibited by excess tissue iron [14–16], the concentration of which in perinatal mammalian liver exceeds that of the mature adult by more than 10-fold [17]. This circumstance could render the fetus more susceptible to the effects of porphyrinogenic chemicals which are known to alter the activity of this enzyme.

The present studies were undertaken to investigate the ontogenic development of uro decarboxylase in rat liver and to compare the fetal and adult enzymes with respect to susceptibility to alteration by various factors. The activity of uro decarboxylase relative to that of ALA synthetase in adult and fetal liver was also calculated in order to estimate quantitatively the extent to which uro decarboxylase could become rate-limiting in hepatic heme biosynthesis under conditions of selective inhibition of that enzyme at each stage of development.

MATERIALS AND METHODS

Materials. Succinyl coenzyme A synthetase (succinic thiokinase, EC 6.2.1.4), pyridoxal 5′-phosphate, ATP, GTP, coenzyme A and porphobilinogen (PBG) were obtained from the Sigma Chemical Co.,

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St. Louis, MO. Glycine, succinic acid, glutathione, mercuric chloride and *p*-dimethylaminobenzaldehyde were purchased from CalBiochem, San Diego, CA. 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, MA. 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 and washed in ice-cold 0.05 M Tris–HCl buffer, pH 7.5, and then blotted in filter paper placed on ice. Livers were then weighed, minced 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 held in ice. 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 and other subcellular fractions was performed as previously described [4]. Pooled livers from a minimum of four postnatal or adult rats or from the fetuses of four pregnant animals averaging eight to ten fetuses each were utilized for each experimental point.

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

Uro decarboxylase activity in mitochondria-free extracts of rat liver was assayed by a modification of our previously described method [18]. For these studies the substrate, uroporphyrinogen, was first derived enzymatically from exogenous PBG, using a partially purified preparation of uro I synthetase from adult rat spleen. The reaction mixture for this step contained 2 ml of 25,000 *g* postlysosomal rat spleen supernatant solution containing 4–8 mg protein/ml (pretreated at 65° for 15 min to inactivate spleen decarboxylase), 160 nmoles PBG and 50 μmoles potassium phosphate buffer, pH 7.65, in a total volume of 3 ml. The mixture was incubated in a shaking metabolic incubator for 40 min at 37° in the dark. Strict anerobic conditions were maintained during the incubation by the use of Thunberg tubes (Kontes Glass Co., Vineland, NJ), which were flushed with nitrogen prior to beginning the incubation procedure. Following this reaction, which resulted in complete conversion of PBG to uroporphyrinogen, the pH of the reaction mixture was lowered to 6.8 by addition of 0.125 ml of 0.025 M phosphoric acid through the side-arm of the Thunberg tube, and 0.5 ml of 25,000 *g* liver supernatant solution containing 16–20 mg protein/ml was then also added for assay of uro decarboxylase activity. Thunberg tubes were again flushed with nitrogen and tightly closed, and the reaction mixture was gently shaken for 60 min in a shaking metabolic incubator at 37° in the dark, as before. The reaction was terminated by addition of 8 ml of sodium acetate buffer, pH 4.8, to the assay mixture. Porphyrinogens were oxidized to porphyrins by exposure to room air and fluorescent (overhead) light for 30 min. No special light source was required. The coproporphyrin produced was quantitated after extraction from the aqueous phase with ethyl acetate, as previously described [18]. Recovery of coproporphyrin was greater than 90%.

It should be noted that, since pretreatment at 65° during the substrate preparation step destroys uro III cosynthetase, only uroporphyrinogen I is formed in the reaction mixture; hence, the subsequent reaction catalyzed by uro decarboxylase involves only the non-physiological substrate, uroporphyrinogen I. Previous studies from these [18] as well as other [19] laboratories have shown, however, that the uro decarboxylases from both hepatic and erythropoietic tissues are equally active when either the I or III isomer of uroporphyrinogen is used as substrate. It seems unlikely, therefore, that the destruction of cosynthetase in the substrate preparation step limits the assessment of uro decarboxylase activities in adult and perinatal liver as measured in these studies.

Protein determinations were made by the method of Lowry *et al.* [20] using bovine serum albumin as a standard.

Determination of hepatic hematopoietic cell composition. The fetal hepatic hematopoietic cell composition was determined as described by Doyle and Schimke [21].

Studies with mercuric chloride and glutathione. Studies to ascertain the effects of mercuric chloride and glutathione on uro decarboxylase activity were performed by adding appropriate concentrations of aqueous solutions (0.01 ml) of the test substance directly to the incubation mixture at the same time as the enzyme source was added. Control reaction mixtures received distilled water only. Solutions or water were added by injection into the reaction medium through the arm of the Thunberg tube in which the assay was performed, as described above. Final concentrations of substances tested are given in Table 2.

Statistical analysis. Analysis of significance of differences between groups was performed by means of Student's *t*-test. The level of significance was chosen as $P < 0.05$.

RESULTS

The developmental pattern of hepatic ALA synthetase activity in perinatal rats and other species has been described previously [1–3, 8]. In the rat, ALA synthetase activity at 4 days prior to delivery is approximately ten times that of the adult and decreases to adult levels near the time of birth. In the present studies, adult and fetal ALA synthetase activities were 0.53 ± 0.06 and 5.96 ± 0.61 nmoles ALA per mg protein per hr respectively.

The ontogenic development of uro decarboxylase is illustrated in Fig. 1. As in the case of other hepatic heme biosynthetic pathway enzymes which have been investigated in these laboratories, uro decarboxylase activity was elevated in fetal liver and exceeded that of the adult by approximately 3-fold up to 2 days prior to delivery. Enzyme activity declined rapidly at the time of birth but remained at approximately twice the adult level until at least 2 weeks following delivery.

The decline in hepatic uro decarboxylase activity

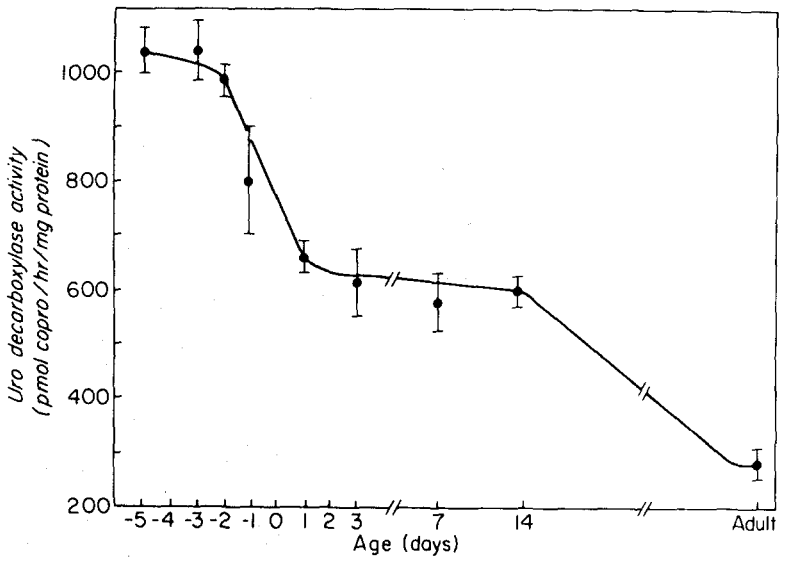

Fig. 1. Ontogenic development of hepatic uro decarboxylase activity in the rat. Enzyme activity in this and subsequent tables and figures is expressed by pmoles coproporphyrinogen formed per mg protein per hr. Values represent means ± S.E. of at least four replicate experiments. Pooled livers from at least four postnatal rats or from the fetuses of four pregnant animals were used for each point in each replicate experiment.

at the time of birth was closely correlated with the change in the function of the liver from an erythropoietic to a hepatocytic organ. As illustrated in Fig. 2, hepatic erythropoietic function, as assessed by the percent composition of hematopoietic cells in the liver, declined rapidly during the 4-day period immediately surrounding the time of birth. The decrease in decarboxylase activity closely paralleled the reduction in the hepatic composition of hematopoietic cells.

To compare fetal and adult uro decarboxylase with respect to regulatory properties and other characteristics, several experiments were performed on enzyme preparations from adult and fetal liver. As indicated in Table 1, increased fetal enzyme activity did not appear to be due to the presence of a specific activator of the enzyme in fetal liver. The total activity measured in a 1:1 mixture of enzyme preparations from adult and fetal rats was equivalent to that expected from addition of the activities measured in individual preparations. Moreover, the

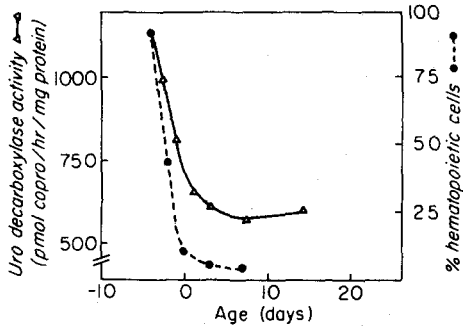

Fig. 2. Changes in uro decarboxylase activity and hematopoietic cell composition of perinatal rat liver.

activity of the enzyme from adult liver was not altered when incubated with a preparation of fetal liver in which uro decarbosylase was inactivated by prior heat treatment (65° for 15 min) (results not shown). This experiment indicates that endogenous iron present in the fetal liver did not directly inhibit uro decarboxylase in the adult liver preparation.

The heat stability characteristics of uro decarboxylase from adult and fetal livers were also investigated. As indicated in Fig. 3, when the fetal liver preparation was incubated at 43°, uro decarboxylase activity declined to approximately 50% of the zero-time level within 5 min; in contrast, adult enzyme activity declined to only 80% of the initial level during the same time interval. However, the activity of the enzyme from both sources decreased to approximately 20% of zero-time levels after heat treatment for 20 min. Equivalent concentrations of protein were used for adult and fetal enzyme heat stability determinations.

Previous studies from these laboratories [11] have demonstrated that uro decarboxylase from adult rat liver is highly susceptible to inhibition *in vitro* by mercury, cadmium and other toxic trace metals. Mercuric chloride was most potent in this respect, inhibiting uro decarboxylase to approximately 50% of the control level at 10^{-4} M and to 5% of control at 10^{-3} M. As demonstrated in Table 2, fetal uro decarboxylase was even more susceptible to inhibition by mercury (mercuric chloride) than was the adult enzyme. Uro decarboxylase activity was inhibited to 26% of the control level at 10^{-4} M and to 10% at 5×10^{-4} M. Essentially no activity could be detected at higher concentrations. Inhibition of either adult or fetal uro decarboxylase by mercuric chloride was prevented by concomitant addition of reduced glutathione (10^{-2} M) to the reaction mix-

Table 1. Effects of mixing equivalent amounts of fetal and adult liver preparations on uro decarboxylase activity*

Preparation	Uro decarboxylase activity [pmoles copro·hr^{-1}·(mg protein)$^{-1}$]
Fetal (4 days)	1050 ± 72
Adult	285 ± 37
Fetal:adult mixture (1:1)	1243 ± 63

* Enzyme activities were determined as described in Materials and Methods. In this and subsequent tables and figures, pooled livers from at least four adult rats or from fetuses of four pregnant rats were used for each enzyme determination.

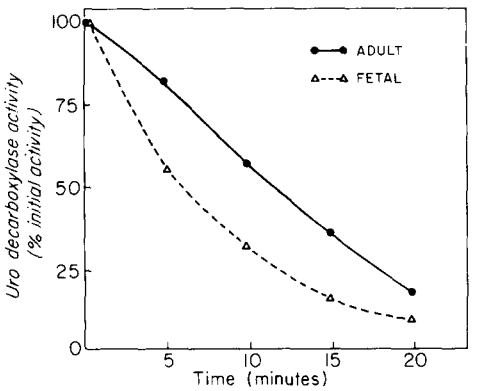

Fig. 3. Effects of heat (43°) on uro decarboxylase activity in adult and fetal rat liver.

ture, suggesting that inhibition by mercury is the result of metal binding to sulhydryl groups of the enzyme.

DISCUSSION

The present studies demonstrate that uro decarboxylase activity in fetal liver is elevated by several times above that seen in the adult and declines rapidly near the time of birth. This developmental pattern is similar to that of other heme biosynthetic pathway enzymes which have been investigated in these laboratories [8]. The concomitant decrease in uro decarboxylase activity with the reduction in the hematopoietic cell composition of the liver suggests that elevated decarboxylase activity reflects the function of the liver as an hematopoietic organ during the prenatal period. This view is further substantiated by the observation that uro decarboxylase activities were additive when fetal and adult enzyme preparations were combined, suggesting that the elevated fetal uro decarboxylase activity was not due to the presence of a specific activator of the enzyme in fetal liver.

Although fetal uro decarboxylase activity was approximately three times that of the adult enzyme, it is interesting that the 3:1 activity ratio is among the lowest observed when the activities of other heme biosynthetic pathway enzymes in fetal and adult livers are compared. In contrast, fetal ALA synthetase, uro I synthetase and heme synthetase activities exceed adult levels by 10-, 20- and 5-fold respectively [4, 8]. A similarly low ratio of fetal-to-adult enzyme activity is observed with respect to ALA dehydratase [8], which, as in the case of uro decarboxylase, has been postulated to be regulated *in vivo* by tissue iron levels [22–24]. Since both the non-heme iron and the total iron concentrations of fetal liver exceed that of the adult by greater than 10-fold [17], it is interesting to postulate that the

Table 2. Effects of reduced glutathione and mercuric chloride on adult and fetal (4 days) hepatic uro decarboxylase activity

	Uro decarboxylase activity [pmoles copro·hr^{-1}·(mg protein)$^{-1}$]			
	Adult		Fetal	
Reagent	Activity	% Control	Activity	% Control
Control	296 ± 17	100	936 ± 16	100
Glutathione (10^{-2} M)	305 ± 13	103	1021 ± 30	109
Mercuric chloride (10^{-6} M)	290 ± 16	98	930 ± 28	99
Mercuric chloride (10^{-5} M)	254 ± 21	86*	599 ± 22	64*
Mercuric chloride (10^{-4} M)	164 ± 17	55*	248 ± 18	26*
Mercuric chloride (5×10^{-4} M)	92 ± 12	31*	94 ± 11	10*
Mercuric chloride (10^{-3} M)	15 ± 4	5*	0	0*
Mercuric chloride (10^{-4} M) plus glutathione (10^{-2} M)	298 ± 19	100	922 ± 21	99

* Significantly ($P < 0.05$) different from control value.

Table 3. Activities and ratios of heme biosynthetic pathway enzymes in adult and fetal liver

	ALA synthetase [nmoles ALA·hr^{-1}·(g liver)$^{-1}$]	Uro decarboxylase [nmoles copro·hr^{-1}·(g liver)$^{-1}$]	Uro decarboxylase / ALA synthetase
Adult	21.3*	24.6* (196.8)†	9.24‡
Fetal	100.1	44.2 (353.6)	3.53

* Total enzyme activities were calculated by multiplying specific activities (nmoles per hr per mg protein) for each enzyme by total protein concentrations (mg/g) of mitochondria or soluble fraction pools of adult and fetal liver, as previously described [8].

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

‡ Ratios were calculated by dividing ALA molar activity by ALA synthetase activity for each age group.

relatively low ratio of fetal-to-adult uro decarboxylase activity, as compared with those of other heme biosynthetic pathway enzymes, reflects the large excess concentration of iron in the fetal organ. Such circumstances would be consistent with evidence from other studies that iron may inhibit uro decarboxylase both *in vivo* and *in vitro* in a variety of tissues [14, 15, 25, 26] and that hepatic iron overload exacerbates inherited or chemically induced porphyria cutanea tarda (PCT) [26–29], which is characterized by deficient uro decarboxylase activity [15, 30]. Since iron may also mediate the metabolic conversion of various environmental chemicals, particularly organohalogens, to porphyrinogenic intermediates which further inhibit uro decarboxylase [13, 31–33], the circumstance of elevated iron concentration in the prenatal liver could predispose the fetus to increased susceptibility to the effects of such agents during maternal exposure. Further studies of this issue are required to evaluate the effects of tissue iron levels on specific heme biosynthetic pathway enzyme activities both *in vitro* and *in vivo*, and to investigate the role of iron balance in the overall regulation of heme biosynthesis in the adult and fetal tissues.

Consideration of the immaturity of regulatory control of ALA synthetase in fetal liver [4, 5], and of the increased susceptibility of fetal uro decarboxylase to inhibition by mercury and, possibly, other porphyrinogenic chemicals, suggests that uro decarboxylase could become rate-limiting in fetal hepatic heme biosynthesis under conditions of exposure to such substances. To quantitatively assess this possibility, the activities of uro decarboxylase in both adult and fetal liver were compared with those of ALA synthetase at both stages of development to determine the extent of reduction in activity required for uro decarboxylase to become rate-limiting in this process. Comparison of enzyme activities was made, as previously performed with other heme biosynthetic pathway enzymes [8], by first calculating the activity of uro decarboxylase in adult or fetal liver in terms of total activity per gram of liver per hour and, then, expressing that activity on an ALA molar basis (nmoles of ALA utilized per g liver per hr) so that a direct comparison with ALA synthetase activity could be made. The latter calculation was performed by multiplying uro decarboxylase activity as assayed by 8 (8 ALA → 1 coproporphyrinogen). The activities for adult and fetal enzymes expressed in this manner are presented in Table 3. Values in parentheses represents ALA molar activities of uro decarboxyalse. Enzyme activity ratios can thus be calculated by dividing the uro decarboxylase activities, expressed on an ALA molar basis, by that of ALA synthetase for each age group. 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 inhibitors of uro decarboxylase in adult and fetal liver.

From these calculations, it can be estimated that the adult liver has at least nine times the uro decarboxylase activity required to sustain heme biosynthesis, based on the activity of ALA synthetase as the rate-limiting enzyme in this process. In contrast, fetal liver has only three times that capacity. This observation suggests that the fetus may be at substantially greater risk than the adult with respect to compromise of heme biosynthetic capability by agents which inhibit uro decarboxylase. Such compromise may be of special concern when compounded by an inherited deficiency in uro decarboxylase activity, such as occurs in PCT, a most common disorder of porphyrin metabolism in humans [34]. In light of the wide range of clinical and environmental agents which are known to precipitate this syndrome in both human and experimental animal populations, it is essential that factors affecting the activity and regulation of uro decarboxylase in fetal tissues be understood in order that the effects of such agents on perinatal heme biosynthesis and drug metabolism may be accurately predicted and evaluated.

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