THE INFLUENCE OF POSTNATAL DEVELOPMENT ON DRUG-INDUCED HEPATIC PORPHYRIA AND THE SYNTHESIS OF CYTOCHROME P-450

A BIOCHEMICAL AND MORPHOLOGICAL STUDY*

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An experimental form of hepatic porphyria which biochemically resembles the genetic disease of man, acute intermittent porphyria (1, 2), can be induced readily in rats by the administration of allylisopropylacetamide (AIA),[1] a chemical agent structurally related to Sedormid (3, 4). The increase in urinary output of δ-aminolevulinic acid (ALA) and porphobilinogen (PBG) and the hepatic accumulation of porphyrins that result from treatment with this agent are due to the induction of hepatic δ-aminolevulinate synthetase (ALAS) (5), the initial and rate-limiting enzyme in the biosynthesis of porphyrins and heme (6, 7).

The production of experimental porphyria in rats with AIA is accompanied by a concomitant hypertrophy of the liver (8) which can be accounted for almost entirely by an increase in the volume of hepatocytes (9). Ultrastructural studies of the liver in AIA-treated rats have revealed substantial proliferation of the smooth endoplasmic reticulum (SER), enlargement of various intracellular organelles, and accumulation of lipid droplets in the hepatocytes (9–11).

During the course of studies on drug-induced experimental porphyria in animals, we noted that neonatal rats were refractory to the porphyria-inducing properties of various drugs and chemicals including the potent ALAS-inducing agent, AIA (12, 13). The observation prompted the present study, which is

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[1] Abbreviations used in this paper: AIA, allylisopropylacetamide; ALA, δ-aminolevulinic acid; ALAS, δ-aminolevulinate synthetase; DDC, diethoxycarbonyl dihydrocollidine; PBG, porphobilinogen; SER, smooth endoplasmic reticulum.

concerned with the biochemical and morphological alterations in the livers of AIA-treated rats during various stages of postnatal development. The occurence of developmental changes in the inducibility of hepatic ALAS which we report here also provided us with a unique opportunity to examine the relationship between ALAS activity in liver cells and the drug-mediated induction of the hepatic hemoprotein, cytochrome P-450.

Materials and Methods

Animals.—Female Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass.) were obtained on the 16th day of pregnancy and housed singly in cages free of softwood bedding (14). The times of birth of the neonates were carefully noted for each pregnant female, and the neonates were allowed free access to maternal milk after birth. The age of each liter of neonates was determined with an accuracy of ± 12 hr.

On the day before experiments, the neonates were removed from their mothers and fasted for various periods up to 24 hr. Drugs and chemicals were administered intraperitoneally. The neonates were studied for induction of hepatic ALAS at 5-day intervals from birth until 20 days of age and at 10-day intervals thereafter until 40 days of age. Fetuses were examined for response to AIA *in utero* by administering the chemical to pregnant rats 5 days before the date of expected delivery. For the microsomal cytochrome P-450, the neonates were examined at ages 3, 6, 10, and 30 days. Female rats weighing 100–200 g were used as adults in all experiments.

Drugs and Chemicals.—Phenobarbital (100 mg/kg) and AIA (400 mg/kg) were administered as aqueous solutions of 0.85% NaCl. Diethoxycarbonyl dihydrocollidine (DDC)[2] (250 mg/kg) was administered as a solution in propylene glycol. ALA (30–120 moles/100 g) was given as a solution in 0.85% NaCl.

Enzyme Assay.—ALAS was assayed in liver homogenates according to the method of Marver et al. (15). Some reaction mixtures for this enzyme assay contained succinyl coenzyme A synthesized according to the method of Simon and Shemin (16). ALA and amino acetone, synthesized in the reaction mixtures, were separated and determined by solvent extraction as described by Granick (7). The unit of ALAS activity was expressed as millimicromoles ALA formed per hour per gram liver.

Determination of Microsomal Cytochrome.—Cytochrome P-450 was quantitated in dithionite-reduced preparations of liver microsomal fractions by its carbon monoxide difference spectrum as determined in a Cary 15 spectrophotometer (Cary Instruments, Monrovia, Calif.) according to the method of Omura and Sato (17). Incorporation of ALA-3,5-^{3}H and glycine-2-^{14}C (New England Nuclear Corp., Boston, Mass.) into microsomal cytochrome P-450 was carried out as follows: 50 μCi/100 g of ALA-3,5-^{3}H or 30 μCi/100 g of glycine-2-^{14}C was injected intraperitoneally and the rats sacrificed 3 hr later. Microsomal preparations from their livers were then converted to carbon monoxide–binding particles by digestion with 0.3% (w/v) crude pancreatic lipase (Steapsin, Nutritional Biochemicals Corporation, Cleveland, Ohio), as described by Omura and Sato (17). The resulting preparations, containing 80–95% of original cytochrome P-450 in the form of cytochrome P-420 (18) and virtually free of cytochrome b_5, were counted in a Beckman LS-250 Liquid Scintillation System (Beckman Instruments, Inc., Fullerton, Calif.) as in the method of Levin and Kuntzman (19).

Cytochrome b_5 was determined from the difference spectrum between nicotinamide adenine dinucleotide (reduced form)- (NADH)-reduced and air-saturated suspensions of microsomes (18). In some preparations of microsomal fractions as well as of CO-binding particles, NADH-

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[2] Gift from Professor S. Granick, The Rockefeller University.

cytochrome b₅ reductase, purified according to Takesue and Omura (20), was added to ensure complete reduction of cytochrome b_5. Protein was measured according to the method of Lowry et al. (21).

Porphyrin Determination.—Porphyrin content of the liver was determined spectro-fluorometrically as follows. Homogenates of livers were prepared in distilled water and small aliquots, containing approximately 10 mg of protein, were freeze-dried. Porphyrins from the lyophilates were then extracted with a solvent containing equal volumes of N-perchloric acid and absolute methanol (Spectral Grade, Matheson Coleman & Bell, Matheson Co., Inc., East Rutherford, N. J.). After filtration through Whatman No. 2 filter paper, fluorescence emission spectra of porphyrins contained in the solvent were traced in a Hitachi–Perkin-Elmer MPF-2A fluorescence spectrophotometer (Hitachi, Ltd., Tokyo, Japan; Perkin-Elmer Corp. Instrument Div., Norwalk, Conn.) (22).

Bilirubin Determination.—Serum bilirubin was measured by coupling with diazotized sulfanilic acid (23).

Electron Microscopic Studies.—Livers from 27 animals were examined in the electron microscope 16 hr after administration of 0.85% NaCl solution (9 animals), phenobarbital (9 animals), and AIA (9 animals). With the exception of 3 adult animals (1 control and 2 AIA-treated), the remaining animals were 5 or 7 days old.

The liver specimens were cubed into 1 mm blocks and fixed in 3% (w/v) glutaraldehyde in a modified Tyrode's solution (pH 7.2) for 3 hr (24), rinsed in 7.5% (w/v) sucrose solution buffered with 0.01 M phosphate buffer (pH 7.2), and then postfixed in Millonig's osmic acid fixative (25). After dehydration in graded ethanol solutions, tissue blocks were passed through propylene oxide and embedded in Maraglas (Polysciences, Inc., Rydal, Pa.). Mid-zonal areas of the hepatic lobule were selected for thin sectioning from 1-μ-thick sections after examination by phase-contrast microscopy. Thin sections stained with uranyl acetate and lead citrate were examined in a Philips EM 300 electron microscope (Philips Electronic Instruments, Mt. Vernon, N. Y.).

RESULTS

Induction of Hepatic ALAS.—There was a progressive increase in the activity of hepatic ALAS after a single administration of AIA to adult rats. The activity reached a peak level, approximately 12 times the control values, by 12–16 hr after the administration of the chemical (Fig. 1). Fig. 1 also shows the response of hepatic ALAS activity after a single injection of an equivalent dose of AIA to neonatal rats. No induction of ALAS of a magnitude comparable to that seen in adult rats was noted. The mean value of ALAS activity did approximately double ($P < 0.01$) by the 9th hr after AIA. No further increase in ALAS activity was noted during an observation period of up to 30 hr after injection of the inducing agent.

Fig. 2 illustrates the response to AIA in rats during various perinatal and developmental stages. An essentially complete lack of full responsiveness to the induction of hepatic ALAS characterized the fetuses and the newborn animals, despite the great potency of AIA for induction of this enzyme in adult rats. This refractoriness lasted until about 10 days of age. Some induction was seen by 15 days of age, and the inducibility of hepatic ALAS rapidly increased thereafter during weaning, approaching adult levels with further physical maturation.

Of interest were the observations on the basal hepatic ALAS activity in the immediate perinatal periods (Fig. 2). The ALAS activities in fetal rats 5 days before delivery were significantly higher than those noted during later periods in development. The mean activity of ALAS in the fetal liver was 55 mμmoles/ hr per g. The activity progressively declined after delivery to reach one third of this level by the 15th day. Similar observations on hepatic ALAS activities of fetal rats, guinea pigs, and rabbits have recently been made by Woods and Dixon (26).
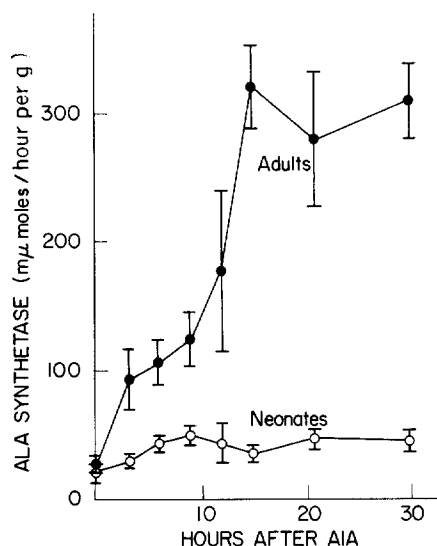

FIG. 1.   Time course of induction of ALAS in adult and neonatal rats by AIA. 30 adult female rats weighing 130–140 g and 40 7-day-old rats were starved for 24 hr before administration of AIA (400 mg/kg) intraperitoneally. Rats were killed in groups of 3–5 at indicated intervals and hepatic ALAS activity was determined in each liver. Each point represents the mean value of ALAS activity ± one standard deviation.

Fig. 3 shows the refractoriness of neonates to the induction of ALAS by phenobarbital, a drug which caused an approximately four-fold increase in hepatic ALAS activity in adult rats. No statistically significant increase in ALAS activity was noted in neonates treated with phenobarbital. The newborn rats were also refractory to the induction of ALAS by DDC, an extremely potent porphyria-inducing chemical known to induce readily this enzyme in adult animals (6) and other experimental preparations (7).

The following manipulations had no influence on the neonatal refractoriness to drug induction of hepatic ALAS: (a) variations of substrate concentrations in the enzyme assay, (b) variations of the interval between drug administration and the enzyme assay, (c) addition of succinyl coenzyme A to the ALAS reac-

tion mixture, (d) administration of glucocorticoids to the newborn animals, and
(e) withdrawal of the neonates from maternal milk or feeding on an artificial
formula[3] for 60–72 hr. Moreover, unlike adult animals, in whom fasting enhances
the degree of ALAS induction by drugs (27), the ALAS activities in fasted and
nonfasted neonates did not differ significantly.

The absence of possible inhibitors of ALAS in neonatal livers (28) was as-
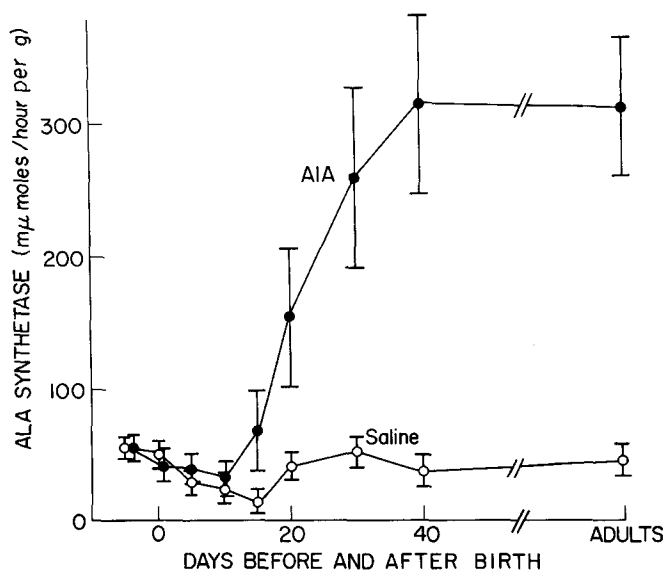certained by addition experiments in which varying amounts of homogenates

Fig. 2. Relation between perinatal development and induction of hepatic ALAS by AIA.
5–10 rats of indicated age before and after birth were starved for 24 hr and given AIA (400
mg/kg) intraperitoneally. Adult rats were mature virgin female rats weighing 150–200 g. 16
hr after AIA, the animals were killed and hepatic ALAS activity was determined in each liver.
Each point represents the mean value of ALAS activity ± one standard deviation.

of neonatal liver were added to ALAS reaction mixtures containing adult liver
homogenates with high ALAS activities. The latter activities were not inhibited
by the neonatal homogenates. The gestational hormones, estradiol and pro-
gesterone, to which the neonates may have been exposed, did not inhibit the
drug induction of ALAS in adult animals when they were pretreated with these
hormones,[4] both singly and in combination, for 7 days.

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[3] Artificial formula was prepared as follows: 60 g egg yolk, 60 g dry milk, 90 g corn oil, 6 g
casein, 6 g commercial vitamin mixture for rats, 6 g choline, and 372 g distilled water to make
600 g of formula.

[4] Estradiol (1 mg) or progesterone (10 mg) in 0.5 ml propylene glycol administered sub-
cutaneously.

Changes in Liver Weight after the Administration of AIA.—A substantial enlargement of liver weight is a well-known concomitant of the hepatic porphyria produced by AIA in adult rats (9, 27). As shown in Table I, a greater than 100% average increase in liver weight was noted in adult rats approximately 16 hr after a single administration of AIA. In contrast, no significant
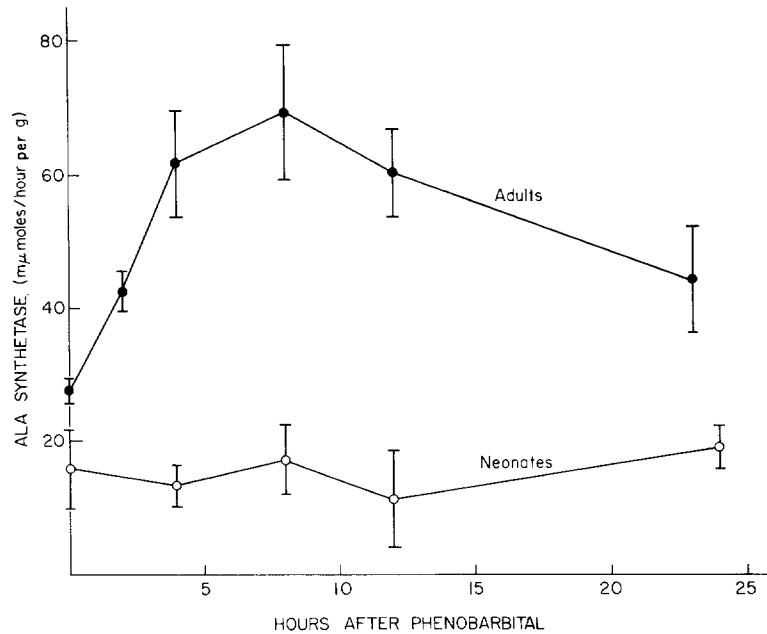

FIG. 3.   Time course of induction of ALAS in adult and neonatal rats by phenobarbital. 30 adult female rats weighing about 100 g and 20 5-day-old rats were starved for 16 hr before administration of AIA (400 mg/kg) intraperitoneally. Rats were killed in groups of 4–5 at indicated intervals and hepatic ALAS activity was determined in each liver. Each point represents the mean value of ALAS activity ± one standard deviation.

TABLE I

Mean Liver Weights in Rats of Various Ages Treated With AIA*

Age	Control	AIA	Change in liver weight
	g ± standard error	g ± standard error	%
5 days	0.21 ± 0.02 (11)	0.19 ± 0.01 (10)	Not significant
20 days	1.20 ± 0.11 (9)	1.50 ± 0.05 (9)	+25 ($P = 0.025$)
Adults‡	2.04 ± 0.23 (8)	4.11 ± 0.21 (8)	+103 ($P < 0.005$)

* All animals were fasted 24 hr before AIA administration. Liver weights were determined 16 hr after AIA. Figures in parentheses indicate number of animals.
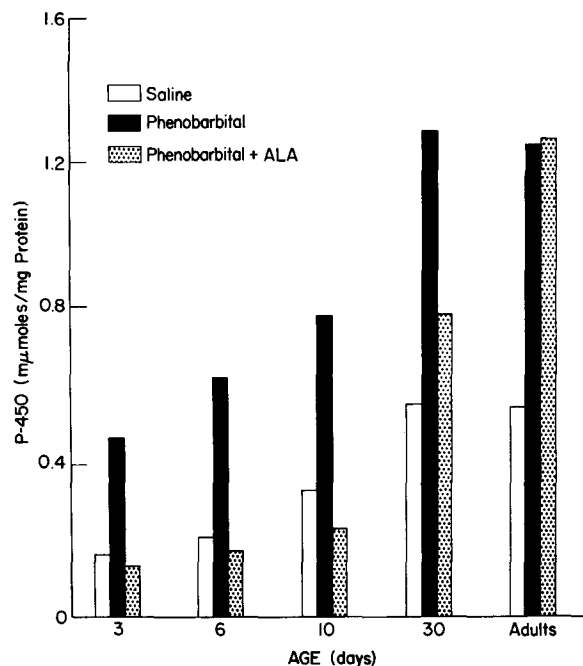
‡ 100–200 g in body weight.

Fig. 4. Induction of microsomal cytochrome P-450 by phenobarbital in developing rats. Animals of indicated age were starved for 16 hr and divided into 3 groups: the "saline" group received 0.85% NaCl intraperitoneally; the "phenobarbital" group received 100 mg/kg of phenobarbital in 0.85% NaCl intraperitoneally; the "phenobarbital + ALA" group received, in addition to phenobarbital, 120 μmoles of ALA in 0.85% NaCl intraperitoneally in three divided doses, the first dose immediately after, the second and third at 4 and 8 hr after, phenobarbital treatment. Each group of animals was killed 24 hr later, livers were pooled, and the microsomal fractions isolated. Cytochrome P-450 was determined in each pooled preparation of liver microsomes. The number of animals ranged from 5 adults (100–150 g) to 18 for the neonates.

TABLE II

Incorporation of Glycine-2-^{14}C and ALA-3,5-^{3}H into Carbon Monoxide–Binding Particles of Neonatal Rat Liver*

	ALA-3,5-^{3}H		Glycine-2-^{14}C	
	cpm/g liver		cpm/g liver	
Control	6088	(17)	6404	(15)
Phenobarbital-treated	19,869	(14)	11,838	(16)

* 5-day-old (ALA-3,5-^{3}H) and 7-day-old (glycine-2-^{14}C) rats were given the labeled precursors intraperitoneally 16 hr after treatment with saline (control) or phenobarbital. The rats were killed 3 hr later and livers were pooled and carbon monoxide–binding particles isolated from pooled livers as described. Figures in parentheses indicate the number of animals.

change in liver weight was detectable in AIA-treated neonatal rats of 5 days of age. By 20 days of age, however, at which time a significant AIA-mediated induction of hepatic ALAS was detectable (Fig. 2), a smaller (25%) but significant increase in liver weight was apparent in the developing animals.

Relationship between Induction of Hepatic ALAS and Microsomal Cytochrome P-450.—ALAS is the rate-limiting enzyme in the hepatic biosynthesis of porphyrins and heme (6, 7); it has therefore been suggested that the induction of
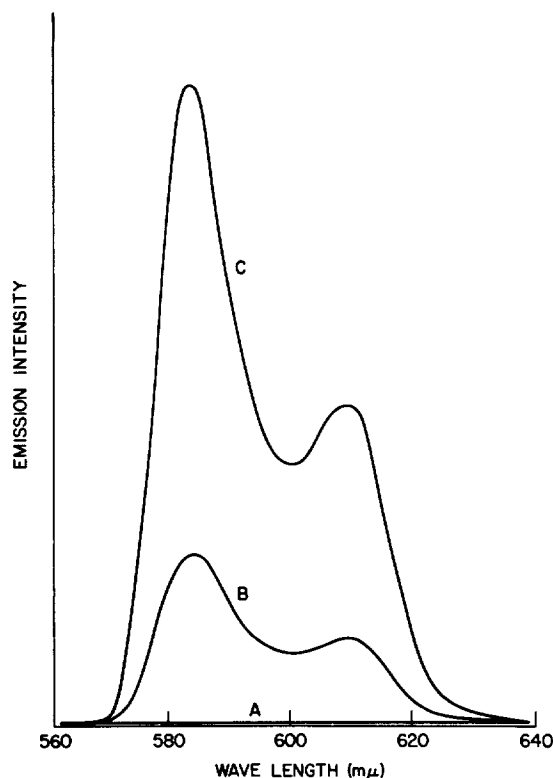

Fig. 5.   Fluorescence emission spectra of porphyrins isolated from livers of neonatal rat. treated with phenobarbital and phenobarbital plus ALA. Three groups of 17–18 rats of 5 days of age were treated with 0.85% NaCl, phenobarbital (100 mg/kg in 0.85% NaCl), and phenobarbital plus ALA (90 μmoles/100 g in 0.85% NaCl). ALA was administered in three divided doses as indicated in the legend for Fig. 4. Each group of animals was killed 24 hr later and the homogenates from the pooled livers were lyophilized and extracted with perchloric acid–methanol solvent. The fluorescence emission spectrum was traced for each solvent at identical sensitivity setting. *A* represents tracings for saline- and phenobarbital-treated groups, showing no porphyrin fluorescence; *B* represents a tracing for the phenobarbital plus ALA -treated group. The emission spectrum is similar to that obtained for the protoporphyrin IX standard, curve *C*.

this enzyme, which would then lead to an increase in availability of heme, might be a necessary event preceding the increase in the hepatic microsomal hemoprotein, cytochrome P-450, which is brought about by exposure of animals to drugs such as phenobarbital (29). This relationship between ALAS activity and cytochrome P-450 of the liver was examined in neonatal and developing rats (Fig. 4) by quantitating the hemoprotein level after treatment of the animals with phenobarbital. Untreated newborn rats had very little P-450 in the liver, amounting to about one third of the level present in adult hepatic microsomes when expressed in terms of microsomal protein. Despite the refractoriness of the neonates to induction of hepatic ALAS by phenobarbital,

TABLE III

*Serum Total Bilirubin in Neonatal Rats Treated with ALA**

	No. of animals	Total bilirubin
		(mg/100 ml)
Control	(17)	0.4
Phenobarbital	(18)	0.1
Phenobarbital plus:		
120 μmoles/100 g ALA	(18)	4.3
90 μmoles/100 g ALA	(18)	1.4
60 μmoles/100 g ALA	(18)	1.7
30 μmoles/100 g ALA	(18)	0.6
120 μmoles/100 g ALA	(17)	11.3

* 5–6-day-old rats weighing 11–15 g were used. Control animals received 0.1–0.15 ml of 0.85% NaCl. Phenobarbital was given intraperitoneally at 100 mg/kg. ALA was administered intraperitoneally in three divided doses at 4-hr intervals, beginning immediately after the phenobarbital treatment. All animals sacrificed by decapitation 24 hr later. Blood from animals in each group was pooled and the bilirubin concentration in the pooled sera was determined.

an increase in microsomal cytochrome P-450 of the liver mediated by this drug was demonstrable throughout the neonatal period (Fig. 4). A more than two-fold increase in this cytochrome was noted as early as 3 days after birth after treatment with phenobarbital, although the drug-stimulated level of P-450 at that age was still substantially less than the P-450 level in the microsomal fractions of untreated adult rat livers (Fig. 4). Phenobarbital-treated neonates also incorporated the heme precursors, ALA and glycine, into microsomal cytochrome P-450 at rates significantly increased over those of untreated litter mates (Table II).

Effect of ALA on Phenobarbital-Mediated Induction of Microsomal Cytochrome P-450.—To examine further the relationship between hepatic ALAS and the degree of phenobarbital-stimulated induction of cytochrome P-450, the rate of the ALAS step was arbitrarily increased in the liver by exogenous ad-

ministration of the product of this enzymic reaction, ALA. Treatment with
various doses of ALA up to 30 μmoles/100 g of body weight, administered in
3–4 divided doses during the 24 hr after exposure to phenobarbital, had no in-
fluence on the drug-mediated increase in cytochrome P-450 of neonatal rats.
When it was given in doses in excess of 60 μmoles/100 g, however, the ALA in
fact blocked the drug-induction of P-450 in the microsomal fractions of the
neonates (Fig. 4).

Hepatic uptake and conversion of the exogenous ALA to porphyrins and
heme was established by the following observations. First, after the administra-
tion of ALA, there was a progressive and large increase in the amount of
porphyrins found in liver cells (mostly in the form of protoporphyrin IX) as
shown by the representative fluorescence emission spectra of liver extracts
depicted in Fig. 5. Second, the conversion of this protoporphyrin IX to heme
was demonstrated by the rapid appearance of hyperbilibubinemia and jaundice
in the ALA-treated neonates (Table III). This hyperbilirubinemia, which was
an unexpected finding, implies the presence of a protoporphyrin IX-to-heme
conversion catalyzed by ferrochelatase[5] and then a heme-to-bilirubin conver-
sion catalyzed by the heme oxygenase (30, 31) in the livers of the neonatal ani-
mals. Protoporphyrin IX itself is not a substrate for heme oxygenase (31).

Electron Microscopic Observations.—The electron microscopic appearance of
a hepatic parenchymal cell from an adult rat treated with 0.85 % NaCl solution
is illustrated in Fig. 6. Fasting for the 40 hr experimental period resulted in
depletion of a major portion of the visible glycogen rosettes; no other ultra-
structural changes were noted.

Fig. 7 depicts a representative hepatocyte from the liver of an adult rat 16
hr after a single dose of AIA. The hepatocytes were hypertrophied because of
an increase in both the nucleus and cytoplasm; the nucleoli were markedly
enlarged with the increase disproportionately greater than that of the total cell.
The most striking morphologic change noted was a marked proliferation of
SER membranes so that masses composed almost exclusively of SER were
prominent within the cytoplasm. The cisternae of the hypertrophied SER had
a more regular diameter than those of controls and thus appeared tubular in
nature. Golgi-associated vesicles of porphyric livers contained dense lipid-like
material, and apparent lipoprotein granules were present within SER cisternae
as well as in the Golgi vesicles. There was also a patchy accumulation of large
lipid vacuoles within the cytoplasm, usually within or adjacent to areas of
hypertrophied SER. These hepatocellular alterations were entirely similar to

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[5] In a preliminary study, the presence of ferrochelatase activity (32) was demonstrated in
the liver homogenates of the newborn rats. In 5-day-old rats, the activity of this enzyme was
approximately 50% of that noted in the adult rats weighing 100 g, when expressed in terms of
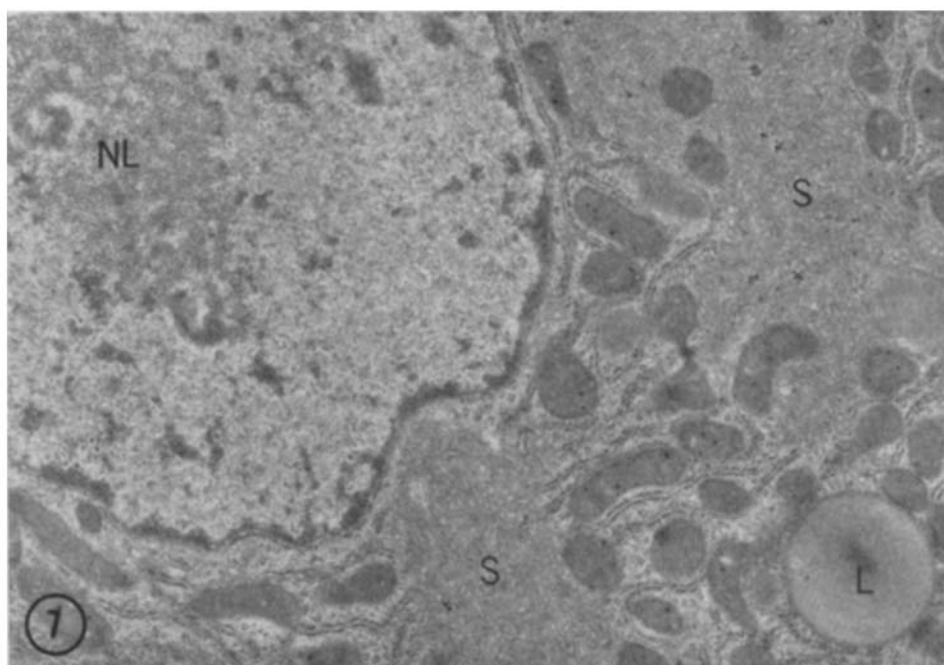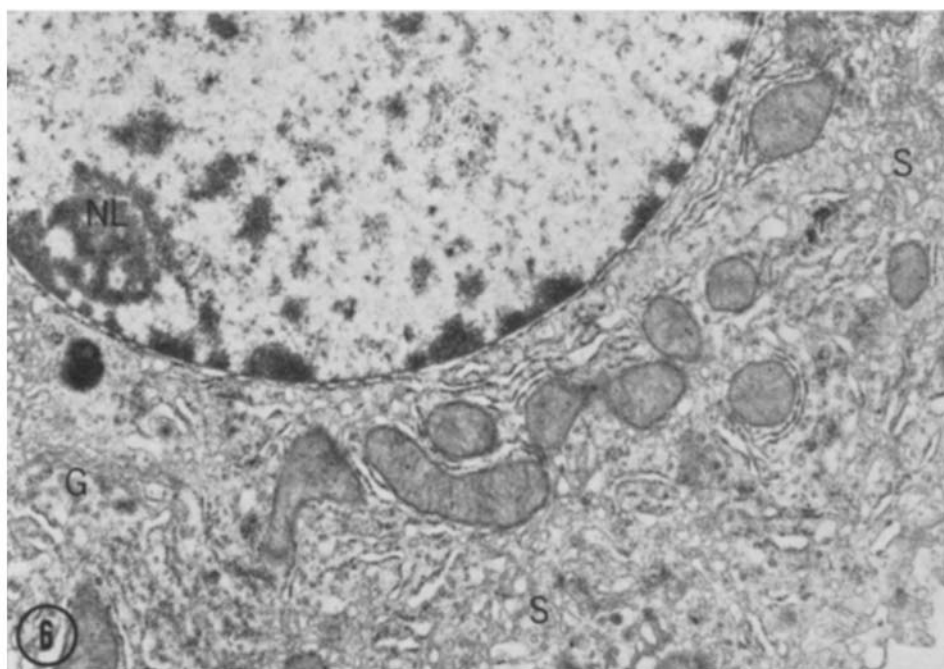wet weight of the liver.

FIG. 6. Adult liver, control. Hepatocyte from an adult rat treated with 0.85% NaCl solution. Note the relatively small nucleolus (*NL*) and the Golgi apparatus (*G*) containing small granules of medium density. Small islands of SER (*S*) are present. × 15,000.

FIG. 7. Adult liver, AIA effect. Hepatocyte from an adult rat treated with AIA 16 hr before sacrifice. Note the prominent enlargement of the nucleolus (*NL*) and the large cytoplasmic masses of hypertrophied SER (*S*). Lipid vacuole (*L*). × 15,000.
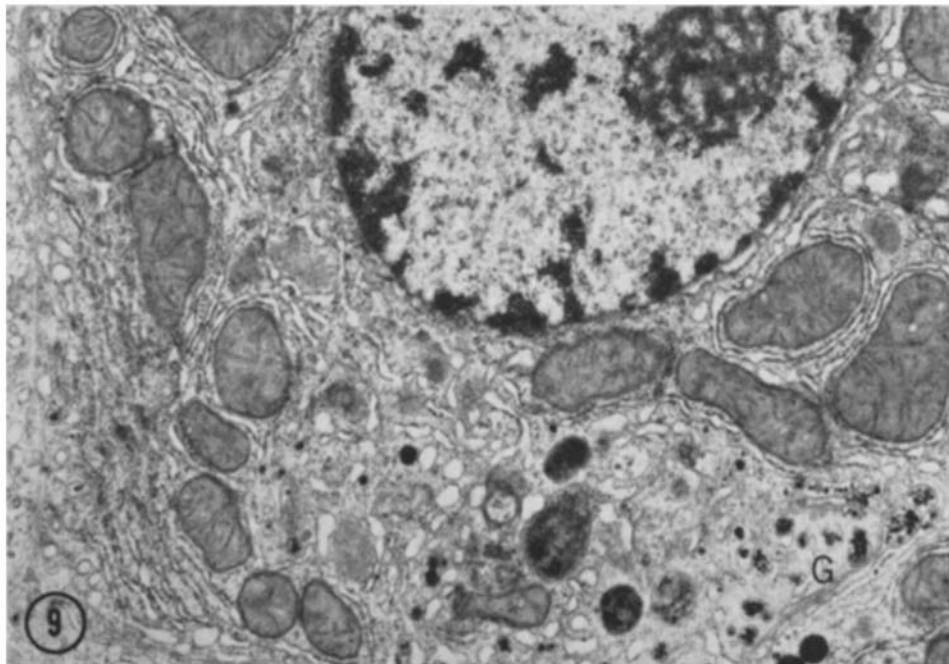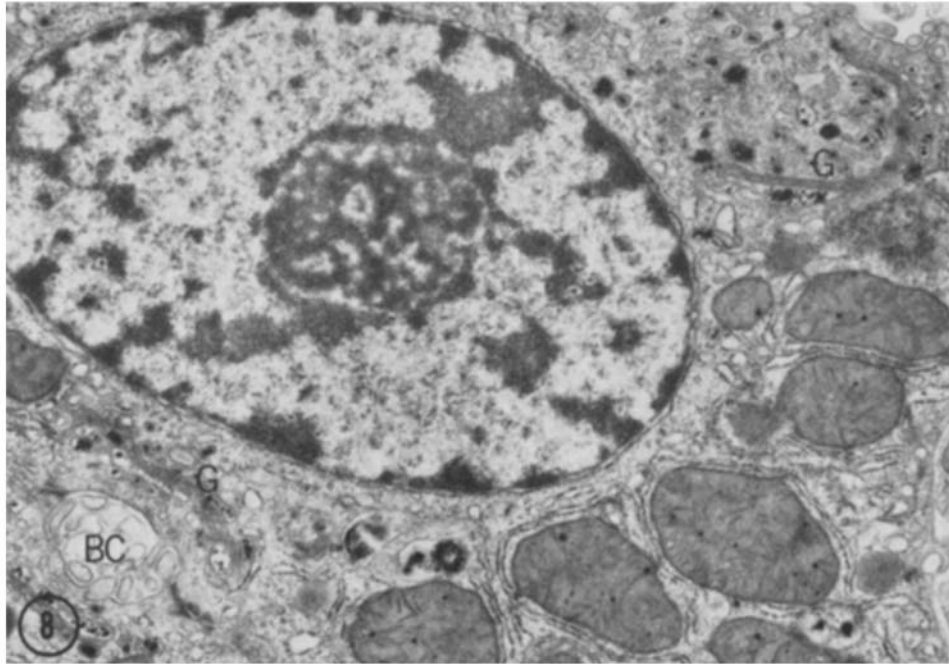
FIG. 8.   Neonatal liver, control. Parenchymal cells of neonatal animals were much smaller than those of adult animals and contained prominent nucleoli. Neonatal liver cells generally contained relatively small quantities of rough-surfaced endoplasmic reticulum and very scant SER. The Golgi apparatus (G) and associated vesicles very frequently contained numerous lipoprotein-like granules (40–60 mμ in diameter) of high density. A bile canaliculus (BC). × 15,000.

FIG. 9.   Neonatal liver, AIA effect. No morphologic changes attributable to AIA treatment were detected in neonatal hepatocytes. × 15,000.
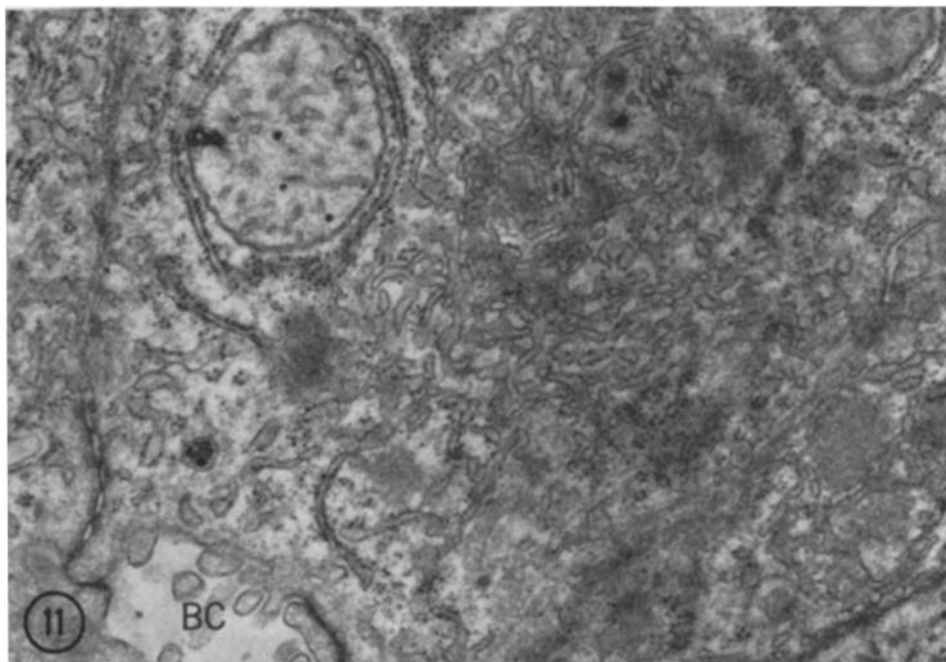
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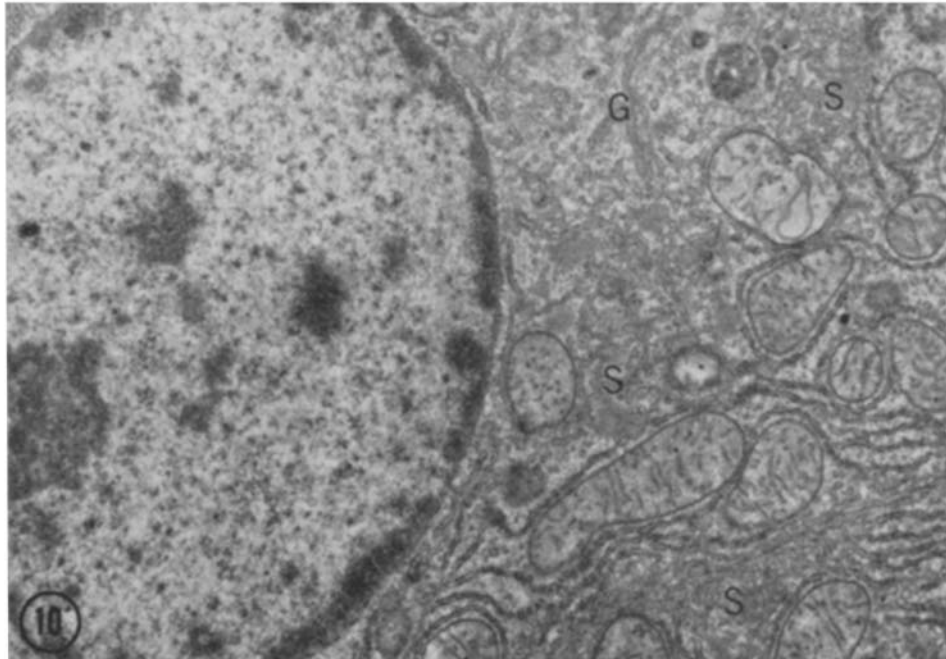
FIG. 10.   Neonatal liver, phenobarbital effect. Livers of neonatal rats treated with pheno-barbital occasionally showed greater development of SER (S) than was observed in any of the control animals. Tubular hypertrophy of the SER as seen in hepatocytes of adult rats treated with phenobarbital was not observed in the neonatal animals. The Golgi apparatus (G) and associated vesicles showed a relative depletion of lipoprotein-like granules. × 15,000.

FIG. 11.   Same as Fig. 10. This higher magnification of the SER shows connections between the smooth and the rough-surfaced endoplasmic reticulum. Many membrane-bound and un-bound polysomes are present. A bile canaliculus (BC). × 30,000.

those previously described in detail in rats treated with the porphyria-inducing agent, AIA (9).

The parenchymal cells of neonatal livers had an ultrastructural appearance different from that of adult livers, and the neonatal liver cells responded differently to AIA administration. The neonatal cells were smaller in size, with a higher nucleo-cytoplasmic ratio. There was less rough endoplasmic reticulum in the cytoplasm. Particularly striking was the paucity of SER (Fig. 8). Relative to the other cytoplasmic components, the Golgi apparatus appeared especially well developed and contained numerous lipoprotein-like granules of high density.

Administration of AIA to neonatal rats utilizing the same experimental conditions which induced pronounced morphological changes in adult rat livers produced no detectable structural alterations (Fig. 9). Livers from the 7 AIA-treated neonatal rats appeared identical in all respects with those of untreated litter mates.

Of the livers from 9 neonatal rats treated with phenobarbital, 3 demonstrated development of the SER in excess of that observed in any of the 8 untreated litter mates (Figs. 10 and 11). This increase in SER was patchy, involving some hepatocytes but not others in a given section; however, the livers of phenobarbital-treated neonates exhibited no tubular hypertrophy of the SER such as that observed in adult animals treated with phenobarbital (33). Golgi-associated vesicles appeared to contain fewer lipoprotein-like granules than were noted in untreated litter mates.

DISCUSSION

Drug induction of hepatic ALAS and the production of experimental hepatic porphyria in animals can be modified by a number of nutritional, endocrine, genetic, and other factors. Thus, starvation before the administration of porphyrinogenic drugs or chemicals enhances the induction of ALAS (27), while feeding or the administration of glucose diminishes or abolishes this drug effect (3, 27). Glucocorticoid hormones have a permissive effect on the drug induction of ALAS (34), i.e., adrenalectomy abolishes the drug effect, which can be restored by subsequent replacement of the glucocorticoid hormone, hydrocortisone. Administration of heme, the end product of the porphyrin-heme pathway, also abolishes the drug induction of ALAS (29, 35), an effect that has been attributed to the role of heme as a corepressor agent in the regulation of ALAS synthesis in liver cells (7, 36). Heme also inhibits the apparent incorporation of ALAS found in the soluble fraction of liver cells into the mitochondria after drug induction of this enzyme (37). Ferric citrate has a marked synergistic effect in the induction of ALAS (38). Finally, genetic factors have recently been invoked to explain differences in the degree of inducibility of ALAS in various strains of inbred mice (39). To this list must now be added the age and

the developmental stage of animals which, as shown in the present study, also have a profound effect on the drug induction of hepatic ALAS.

The perinatal period of the mammal is generally characterized by a lack in many tissues of the full enzymatic constitution of the adult (40, 41), and the process of parturition or the physical development after birth correlates specifically with the appearance or the increase in activity of a number of hepatic enzymes. Those hepatic enzymes which show increases in their specific activity during certain stages in the postnatal period include histidase (42), acetyl coenzyme A carboxylase (43), tyrosine aminotransferase (44, 45), tryptophan oxygenase (46), serine dehydratase (47), uridine diphosphate glucuronyl (UDP-glucuronyl) transferase (48, 49), certain drug-metabolizing enzymes (50), and others (51–61).

The induction of some of these enzymes which accompanies endocrine, nutritional, pharmacological, or other manipulations does not become apparent until after specific stages in perinatal maturation have been reached. For example, the induction of hepatic tyrosine aminotransferase (45) and tryptophan oxygenase (46) by administration of hydrocortisone or L-tryptophan in the rat is not possible until several days after birth or until about the 15th postnatal day, respectively. Various factors such as hormone secretion during puberty (42), dietary changes associated with weaning (43), and secretion of glucocorticoid hormones and glucagon during perinatal periods (62, 63) have been proposed as underlying these alterations in hepatic enzyme activity and their inducibility by various agents.

Our findings in this study suggest that the nutritional or endocrine alterations associated with physical maturation in the rat do not constitute the basis for the refractoriness of the neonates to the porphyrinogenic effects of various drugs and chemicals. Dietary and endocrine manipulations had no effect on the failure in the neonates of full induction of hepatic ALAS by drugs until the approximate time of weaning.

ALAS is localized in the mitochondria of hepatocytes (6, 64). The synthesis of this enzyme appears to take place in the endoplasmic reticulum, and the enzyme accumulates transiently in the soluble fraction of the liver cells (64). It is then incorporated into mitochondria. It is obvious that the induction of hepatic ALAS involves a complex series of events that include, in the final stages, protein synthesis in the endoplasmic reticulum and subsequent transfer of this enzymic protein into highly specialized subcellular organelles, the mitochondria. Functional maturation of the endoplasmic reticulum for expressing the genetic material for ALAS and of the mitochondria for incorporation of the synthesized ALAS would be necessary before chemicals such as AIA could manifest their full pharmocological potential, viz., excessive hepatic synthesis of porphyrins and their precursors. Thus, an "immaturity" in one or more of the steps in the sequence of events leading to an increase in the amount

of mitochondrial ALAS would account for our findings. In this respect, it is of interest that enzymes concerned with the mitochondrial electron transport system in the rat do not reach specific activities corresponding to those of adult animals until about 20–25 days after birth or until weaning (65, 66); however, an isolated defect of the neonatal liver mitochondria involving an inability to incorporate newly synthesized ALAS could not explain the low over-all activity of the enzyme in the livers of immature rats as noted in our experiments.

The induction of ALAS by AIA in the rat is accompanied by an array of morphological alterations that are demonstratable by electron microscopy. These include: (a) hypertrophy of hepatocytes, (b) volume enlargements of cytoplasm, nucleus, and nucleoli of hepatocytes, (c) accumulation of lipoprotein-like granules within the cisternae of the SER, (d) formation of lipid droplets in the cytoplasm, and (e) proliferation of the SER (9–11). The mitochondria, however, show surprisingly little morphological change depsite the fact that the newly synthesized ALAS localizes in these structures.

Some of the manipulations that have been shown to prevent the AIA-mediated induction of hepatic ALAS also prevent the morphological changes known to accompany the increase in the activity of this enzyme. Thus, in adrenalecto-mized rats or rats treated with metabolic inhibitors such as puromycin and actinomycin D, AIA fails to induce ALAS and does not produce the electron microscopic changes in the liver cells described above. Cultured rat hepatoma cells (67), which do not respond to AIA by induction of ALAS, also fail to develop these morphological alterations.[6] The present observations in neonatal rat livers are similar in that AIA produced neither an induction of ALAS nor ultrastructural changes in the hepatocytes.

The association of the induction of ALAS with the morphological changes found in hepatocytes suggest that these two seemingly disparate phenomena may be causally related in the production of experimental porphyria in the rat by AIA, although it is not possible at the present time to elucidate the basis of this relationship or the sequence in which the two phenomena take place. It is clear, however, that the AIA-mediated induction of ALAS is associated with alterations in biochemical pathways that are not directly involved in porphyrin-heme biosynthesis, and that such alterations, e.g. changes in the rates of synthesis of cholesterol and phospholipids (68), may find morphological expression in the ultrastructural changes observed in the liver cells.

Cytochrome P-450, the hepatic hemoprotein that acts as the terminal oxy-genase in the microsomal mixed-function oxidase system (19, 69), can be induced by a variety of steroids as well as drugs, including barbiturates, polycyclic aromatic hydrocarbons, etc. (70, 71). The induction of P-450 is associated with increased activity of the liver for oxidative and, to a certain extent, reductive metabolism of many drugs, although this relationship is not strictly stoichio-

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[6] Unpublished observations.

metric (72–74). Although heme is the prosthetic group for cytochrome P-450 and is thus obviously required for the synthesis of this hemoprotein, it can also block the drug-mediated induction of P-450 when it is administered exogenously (75).

We have shown in the present investigation that the phenobarbital-mediated induction of microsomal cytochrome P-450 does take place in neonatal rats despite the refractoriness of these animals to the effects of drugs which induce ALAS and would thus be expected to increase hepatic concentrations of porphyrins and heme. The level of P-450 in the hepatocytes of the newborn rats is low, with a corresponding scantiness of the SER demonstrable by electron microscopy (Fig. 8). On a single treatment with phenobarbital, however, the P-450 levels in neonatal livers more than doubled (Fig. 4) and islands of SER became discernible in some of the hepatocytes (Figs. 10 and 11). These findings are compatible with the recently described increases in hepatic rates of metabolism of aniline, ethylmorphine, and phenobarbital (76) in suckling rats exposed to phenobarbital. Our observations further indicate that the synthesis of P-450 in the neonates appears to be under a regulatory control different from that of ALAS and that the basal production of ALA in the neonatal liver is sufficient to meet the heme requirements imposed by drug-induced increases in P-450 synthesis. Increasing the hepatic concentration of ALA, porphyrins (Fig. 5), and presumably free heme by administration of exogenous ALA to neonatal rats did not enhance the phenobarbital induction of P-450. Indeed, when the amount of administered ALA exceeded 60 μmoles/100 g body weight, the induction of P-450 was actually blocked (Fig. 4) and the neonatal animals developed hyperbilirubinemia (Table III). Since bilirubin itself has no effect on the phenobarbital-mediated induction of P-450 (29), such an inhibitory effect of exogenous ALA must be due to its conversion to free heme in the liver and the action of this heme as a repressor of P-450 synthesis. It is not clear why this repressive effect of ALA was not observed in adult rats (Fig. 4). It is possible that there is a more rapid renal clearance of exogenous ALA in the adult animals.

Thus, the relationship between the activity of the porphyrin-heme biosynthetic pathway on the one hand and the rate of synthesis of cytochrome P-450 on the other in the mammalian liver appears to be a complex one. In contrast to the hemopoietic system in which the enhancement of porphyrin and heme formation by the induction of ALAS increases the rate of synthesis of the hemoprotein, hemoglobin (77, 78), no such direct relation seems to hold in the liver at least as far as the formation of the hemoprotein, P-450, is concerned. Increasing the heme concentration in the liver by ALA administration, as evidenced by the rapid appearance of hyperbilirubinemia (Table III), did not increase hepatic P-450 levels. Such manipulations in fact resulted in repressive effects on P-450 formation. This finding contrasts also with the case of another hepatic hemoprotein, tryptophan oxygenase, the activity of which increases in

the rat on treatment with AIA (79, 80) as well as with ALA (81). There is no ready explanation for the paradoxical phenomenon in which free heme can on the one hand act as a prosthetic group and on the other act as an apparent repressor in the synthesis of cytochrome P-450. The presence within hepatocytes of more than one heme pool with distinct metabolic functions could explain these findings. Further studies concerning the existence of such functionally distinct heme pools, however, are hampered by the lack of sensitive means for quantitating free heme levels in biological materials.

SUMMARY

The mitochondrial enzyme δ-aminolevulinate synthetase (ALAS) controls the rate-limiting step in the synthesis of porphyrins and heme. An experimental form of hepatic porphyria can be readily elicited in laboratory animals, such as the rat, by drugs and foreign chemicals which are known to enhance the *de novo* formation of this enzyme in the liver.

The present study shows that there is a striking refractoriness to the induction of ALAS during the perinatal period in the rat. Chemicals which have potent porphyria-inducing activity in adult animals have no significant inducing effect on hepatic ALAS in neonates. The ultrastructural changes which accompany the induction of ALAS by drugs and chemicals in adult liver also fail to take place in the livers of neonates. A progressive capacity for responding to the action of chemical inducers of hepatic ALAS does, however, develop in neonatal animals so that by approximately 5–6 wk of age experimental porphyria can be elicited as effectively in them as in adults.

The reasons for the refractoriness of hepatic ALAS to induction in the perinatal period are not known; but the findings of this study make it clear that ALAS belongs to that increasingly large group of liver enzymes in mammals whose appearance, increase of activity, or inducibility is developmentally determined.

The occurrence of developmental changes in the indicibility of ALAS in the liver of neonates also provided an opportunity to study the relationship of this enzyme activity to the drug-mediated induction of the hepatic hemoprotein cytochrome P-450. This inducible hemoprotein serves as the terminal oxygenase in the microsomal mixed-function oxidase system in the liver. The results of this study indicate that, in contrast to the refractoriness of ALAS to induction, significant drug-induced changes of hepatic P-450 content and of heme-precursor incorporation into this cytochrome do take place in neonates. The synthesis of P-450 thus appears to be under a regulatory control different from that of ALAS in neonates, and the relation between ALAS activity and P-450 formation is not therefore a direct one.

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