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METABOLISM OF DRUGS IN RAT LIVER DURING THE PERINATAL PERIOD*

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Abstract—Hepatic activities for the *N*-demethylation of aminopyrine and UDP-glucuronyl conjugation have been determined *in vitro* during the perinatal period. UDPglucuronyltransferase activities measured with *p*-nitrophenol and bilirubin as acceptors in ultrasonicated homogenates are relatively high shortly after birth and subsequently decline to the adult level at day 10 *post partum*. The *N*-demethylating activity remains low during the first 20 days after birth. In the 20 to 30-day period the enzyme activity of either sex suddenly rises. The hepatic activities in female rats reach their adult level during this period. As far as the males are concerned, the increase of their *N*-demethylating activity proceeds more gradually from the 30th day to a maximum at about 60 days, resulting in a marked sex difference in *N*-demethylation. A change in the time of weaning did not influence drug metabolism in the developing rats. It is suggested that the lower level of oxidative *N*-demethylation in the new-born rat is related to the presence of hemopoietic cells and to the rate of growth of the liver, rather than to a postnatal interference by maternal substances during the nursing period.

NEWBORNS of both animal and man commonly appear more sensitive to drugs than adults.[1,2] The longer duration of action and the greater toxicity of drugs in the fetus and the newborn are mainly attributed to an apparent absence or low activity of the drug metabolizing enzymes in the liver.[3,4]

About the cause of this lack of enzyme activity divergent opinions have been reported. Fouts and Hart[4] considered that the low enzyme concentrations might be due to a lack of stimulus to the enzyme-forming systems. However, it was demonstrated earlier that liver homogenates of newborn rabbits contain some material which inhibits the metabolism of amphetamine, hexobarbital, aminopyrine and acetanilid.[5] More recently Feuer and Liscio[6,7] suggested that the depression of the hydroxylation of 4-methylcoumarin in the liver of fetal and newborn rats is caused through an interference by maternal inhibitory substances.

Glucuronide synthesis, too, seems deficient in mammalian fetuses and newborns.[3,8,9] Remarkably, Dutton[10] found high levels of UDPglucuronyltransferase in rat liver homogenates just after birth of the animals. The contradictory reports induced us to re-study hepatic UDPglucuronyltransferase activities *in vitro* in the developing rat. In the present study the conjugation rates, using *p*-nitrophenol and bilirubin as acceptors, were measured with sonicated liver homogenates, to exclude any interference by differences in transport at the microsomal membranes.[11,12]

Furthermore, the oxidative *N*-demethylation of aminopyrine was followed in crude microsomal fractions from the liver of the rat during the postnatal development.

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Efforts were made to correlate the observed alterations of the metabolic activities with the developmental status, represented by the rate of liver growth, the histologic changes in the liver, and the sexual differentiation of the animals.

EXPERIMENTAL PROCEDURE

Animals

The animals employed in this study were Wistar rats, kept in an environment of constant temperature and humidity. The animals received water and a constant pellet diet *ad lib.*

The fetal age was estimated from the time of mating.

Liver preparations

The animals were decapitated under light ether anaesthesia and exsanguinated. The livers were rapidly removed, weighed and finely minced. Homogenates (20%) were prepared in 0·25 M sucrose, containing 5×10^{-2} M tris(hydroxymethyl)aminomethane-HCl (pH 7·4) and 10^{-3} M sodium ethylenediamine tetraacetate (EDTA-Na) using a Teflon-glass Potter–Elvehjem type of homogenizer. In the preparation of homogenates from fetal rats three to nine livers were pooled to obtain sufficient amounts for analysis.

Crude microsomal fractions were prepared from the homogenates by centrifugation at 9000 *g* for 20 min. The supernatant was employed in the enzyme assay of the *N*-demethylation of aminopyrine. The enzyme preparations used in the *in vitro* estimation of the glucuronyl conjugation were liver homogenates further disintegrated by ultrasonic vibrations.[12]

Enzyme assays

UDPglucuronyltransferase activity with *p*-nitrophenol as acceptor was determined as described previously.[12] The reaction mixture consisted of tris–HCl buffer (pH 7·4) (5×10^{-2} M), $MgCl_2$ ($3{\cdot}3 \times 10^{-3}$ M), saccharo-1,4-lactone (3×10^{-3} M) and saturating levels of *p*-nitrophenol ($1{\cdot}4 \times 10^{-3}$ M) and uridine-5′-diphosphoglucuronate (6×10^{-3} M). The reaction was started by adding the sonicated homogenate, equivalent to 60 mg of fresh tissue, to a final volume of 1·5 ml. The incubation was at 37° for 20 min and the reaction was stopped with 1 ml ethanol, followed by centrifugation. The glucuronide production was measured indirectly by determining the disappearance of free *p*-nitrophenol. Aliquots of the deproteinized supernatant were diluted with 0·1 N NaOH and assayed spectrophotometrically at 398 nm.

The rates of glucuronyl conjugation with bilirubin as acceptor were determined according to the method of Lathe and Walker[8] with slight modifications. The incubation mixture had the same composition as the medium used in the assay of *p*-nitrophenol glucuronidation, except for the acceptor and its concentration. Bilirubin was added to a concentration of 5×10^{-4} M. The incubation was at 37° for 30 min. The conjugated bilirubin was estimated by the direct diazo reaction.

The *N*-demethylation of aminopyrine was measured as described previously.[11,13] The assay mixture, containing tris–HCl (pH 8·0) (5×10^{-2} M), semicarbazide (5×10^{-3} M), $MgCl_2$ (8×10^{-4} M), $MnCl_2$ (8×10^{-6} M), sodium isocitrate (5×10^{-3} M), 10 μg isocitric dehydrogenase/ml (Sigma type 4, capable of reducing

$5{\cdot}7 \times 10^{-3}$ m-moles NADP/min/mg), NADP ($8{\cdot}8 \times 10^{-5}$ M), and a saturating level of aminopyrine ($16{\cdot}7 \times 10^{-3}$ M), was preincubated for 10 min at 37° to ensure reduction of all NADP. The reaction was started by the addition of the 9000 *g* supernatant fraction, equivalent to 80 mg fresh tissue. The total volume of the reaction mixture was 3 ml. After 10 min incubation at 37° the reaction was stopped by adding 0·5 ml 25% $ZnSO_4$ and 0·5 ml of a saturated $Ba(OH)_2$ solution. The precipitated protein was removed by centrifugation and the amount of formaldehyde was determined in the supernatant according to the method of Nash[14] as modified by Cochin and Axelrod.[15]

Standard solutions containing all reagents and heat-denatured homogenate were carried through the whole procedure, and served as blanks.

Protein assay

Protein content of the livers was determined following the method of Lowry *et al.*[16] Bovine serum albumin (Sigma) was used as a reference standard.

Histological preparations

Liver tissue, about 300 mg, was fixed for 2 hr in Carnoy's solution. After fixation the tissue was embedded in paraffin. Histological sections, 5 μm thick, were made, and stained with hematoxylin and eosin.

RESULTS

Liver growth

In Fig. 1a the liver weights are plotted logarithmically as a function of the age of the rat. Two main phases can be distinguished in the postnatal liver growth. From birth until about 30 days *post partum* considerable increase in liver wet weight occurs with a doubling time of about 7 days. This stage of relatively rapid growth is followed by a period of much slower growth. During the latter period the time of doubling amounts to nearly 140 days.

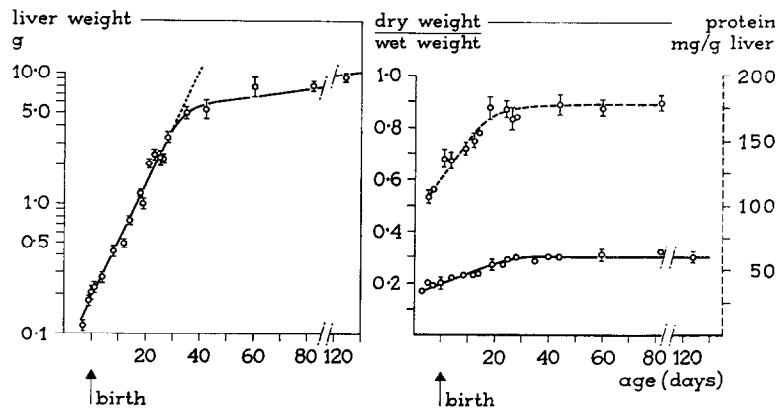

FIG. 1. (a) Liver weight of the postnatal rat. The liver wet weights are plotted logarithmically as a function of age. Each circle represents the mean (±S.E.M.) of three to twelve samples. Male rats were employed. (b) Change of the amount of liver dry weight in relation to liver wet weight and change of protein concentration (*broken line*) during the postnatal development of the rat liver. The protein concentrations are expressed as the amounts of protein per gram fresh liver. Each circle with bar represents the mean (±S.E.M.) of three to six animals. Rats of either sex were employed.

For the first period of growth, the neonatal growth, an increase of the liver dry weight has been observed, which is accompanied by a rise in liver protein concentration (Fig. 1b). Thereupon the composition of the liver remains constant as far as dry weight and amount of protein are concerned.

The rate of liver growth presented in Fig. 1a has been measured with male rats. It has to be remarked, however, that no sex difference for liver weight has become apparent until after 40 days of age. Weights of livers from adult female rats (60 days old) were found to be 70–75 per cent of those from adult males.

In the determinations of dry weight and protein concentrations rats of either sex were employed and no differences between male and female animals became apparent.

Glucuronyl conjugation

Hepatic UDPglucuronyltransferase activities were measured, with *p*-nitrophenol as acceptor, in whole homogenates, which were treated with ultrasound prior to incubation. The ultrasonic treatment of the liver preparation results in a considerable activation of the glucuronidating activity, as has been described previously.[12] The conjugating capacity during rat liver development is presented in Fig. 2. The glucuronyltransferase activities of 14 and 16 days old fetal livers calculated on the basis of liver dry weight are about 25 per cent of the adult level. Remarkably high activities (until about 300 per cent of the adult level) are found during the first week after birth. Sex of the animals was not identified until after 1 day of age. With respect to *p*-nitrophenol glucuronyltransferase activities no sex differences have been observed during the neonatal period. For adults, however, in this respect relatively small differences have been found (Fig. 2) at day 60 $0{\cdot}1 > P > 0{\cdot}05$; at day 90 $P < 0{\cdot}01$, Wilcoxon two-sided, two-sample test. The average rates of *p*-nitrophenol conjugation with sonicated liver homogenates from adult females were 80 per cent of those from male rats.

A relatively high UDPglucuronyltransferase activity, too, has been found in liver homogenates from newborns using bilirubin as acceptor. These enzyme activities,

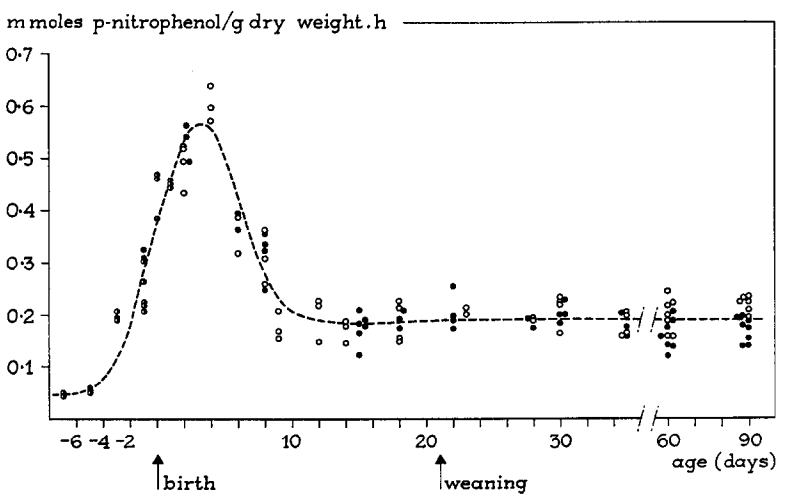

FIG. 2. *p*-Nitrophenol UDPglucuronyltransferase activity during the perinatal development of rat liver. The enzyme activity is given as m-moles *p*-nitrophenol conjugated per hour per gram dry weight: ○ male; ● female; ◑ sex not identified.

expressed as μmoles bilirubin glucuronide produced per gram dry tissue per hr, were 41·5 ± 3·9 and 17·2 ± 1·7 for male rats at day 4 and day 60 respectively.

N-*demethylation of aminopyrine*

The developmental pattern of the oxidative *N*-demethylation of aminopyrine is shown in Fig. 3. The enzymatic activities of male and female rats, measured with 9000 *g* supernatants, are low during the neonatal period. An increase of the activities can be observed between the 20th and 30th day after birth. During this period the young female animals already reach their adult level. However, as far as the males are

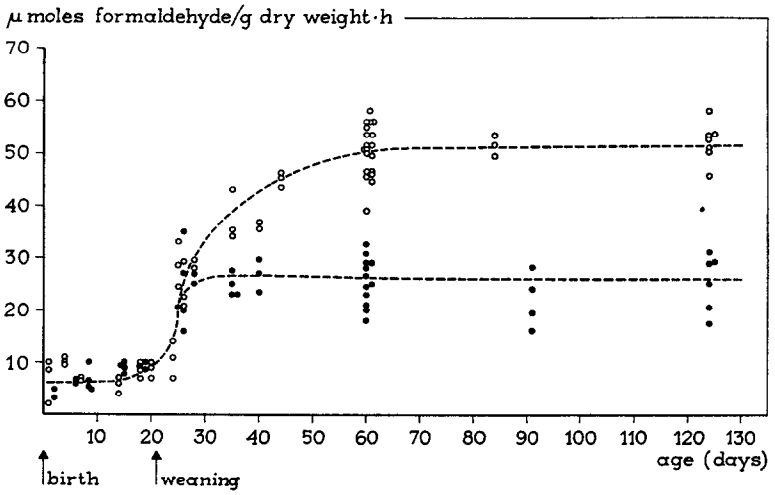

FIG. 3. Postnatal development of hepatic *N*-demethylation of aminopyrine in the rat. The enzymatic activities, measured with 9000 *g* supernatants, are given as μmoles formaldehyde produced per hour per gram dry liver: ○ male; ● female.

concerned, the relatively rapid rise of their *N*-demethylating capacity is followed by a more gradual increase to attain maximal levels of the enzyme system at about 2 months of age. It is noteworthy that, in contrast with the UDPglucuronyl conjugation, a sex difference in the rate of aminopyrine metabolism becomes manifest starting at about 30 days *post partum*.

It has been suggested by Feuer *et al.*, that the lower rate of conversion in the liver of the newborn is caused by an interference of maternal inhibitors. The intake of these substances by the newborn should be suddenly reduced by weaning. Consequently it was to be expected that a postponement of the time of weaning would result in a delay of the increase of enzyme activity. Two groups of animals of the same age were tested. Rats of the control group were normally weaned at 21 days after birth. The group of non-weaned animals were kept with the mother and mainly fed with mother's milk. Body weight, liver wet weight, liver dry weight and enzymatic activities of the weanling and suckling rats were determined and compared 10 days later. We were not able to detect any significant influence of a prolonged nursing period on the *N*-demethylation or UDPglucuronidation in developing rats (Table 1).

TABLE 1. METABOLISM OF DRUGS IN DEVELOPING RATS* AFTER NORMAL AND PROLONGED NURSING PERIODS

	Normally weaned at 21 days after birth	Non-weaned
Body weight (g)	54·5 ± 3·2 (8)†	56·5 ± 4·8 (7)
Liver wet weight (g)	3·07 ± 0·25 (8)	3·06 ± 0·34 (7)
Liver dry weight (g)	1·05 ± 0·09 (8)	0·99 ± 0·13 (7)
N-demethylation of aminopyrine‡	29·3 ± 3·3 (18)	29·4 ± 3·9 (17)
UDPglucuronidation of *p*-nitrophenol§	187·8 ± 17·7 (8)	179·6 ± 16·7 (11)

* Male 31-day-old rats were used in the assays.
† All values are means ±S.D.; numbers of animals are given in parentheses.
‡ Expressed as μmoles formaldehyde produced per hour per gram dry tissue.
§ Expressed as μmoles *p*-nitrophenol disappeared per hour per gram dry tissue.

Histological changes

The liver is one of the main sites of blood formation during the development of the mammalian fetus.[17] A great number of extravascular blood cells of varying types and stages of differentiation is located between the strands of parenchymal liver cells. Toward the end of gestation the hemopoietic activity of the liver declines. For the rat we observed that a considerable amount of foci of erythroblasts, however, remains in the liver during the first week after birth. About 20 per cent of all cells in the liver were found to be developing blood cells at the 3rd postnatal day. These cells are smaller than the hepatocytes, and can be easily recognized by their strong basophilia. Further it is noteworthy that at this time the hepatic cords are more widely separated by sinusoids than at later stages. The hemopoietic elements gradually disappear and are only sporadically found after 3 weeks *post partum.*

DISCUSSION

The observations of the increased and subsequently declining levels of *p*-nitrophenol UDPglucuronyltransferase shortly after birth of the rat (Fig. 2) are consistent with the data of Dutton.[10] In the present study, however, sonicated liver homogenates were applied. The ultrasonic treatment only increased the apparent enzyme activity but did not alter the developmental pattern as indicated by Dutton.[10] It can be concluded, therefore, that the varying rates of conjugation during the perinatal period of the rat must principally be ascribed to different levels of the catalytic proteins, rather than to differences in transport at the microsomal membranes.

During some preliminary studies the *in vitro* rates of bilirubin glucuronidation, too, appeared strongly increased at day 4 after birth. These observations raise the teleologic question, whether there is any relationship between the perinatal peak of glucuronidating activity and the neonatal jaundice caused by the extensive destruction of erythrocytes usually occurring immediately following birth. Bakken *et al.*[18] have supposed that the accumulation of unconjugated bilirubin acts as a trigger for the conjugating system. Accordingly, the temporary equipment of the neonatal rat with a high conjugating capacity might be considered as an adaptive response to the rapid rise of the bilirubin content.

The pattern of the oxidative *N*-demethylation in relation to age and sex greatly differs from that of the drug-conjugating enzyme UDPglucuronyltransferase. The finding of the relatively low *in vitro* activity of *N*-demethylation in the rat liver shortly after birth is in accordance with that reported by Soyka[19] and by Bresnick and Stevenson.[20] However, as is illustrated in Fig. 3, the present study reveals a sudden elevation of the enzyme activity for either sex in the 20 to 30-day period after birth. As far as the young male animals are concerned, this increase then proceeds more gradually to a maximum level at about 60 days, with as a result a marked sex difference in *N*-demethylation. It has been reported by Kato *et al.*[21,22] that the activity of drug oxidation in the liver is enhanced by male sex hormones. Most likely, therefore, the sexual differentiation underlies the latter phase in the postnatal development of *N*-demethylation of the male rat. This idea is supported by the fact, that this period corresponds to the time of testicular descent in the rat, which has been reported to take place between the 30th and 40th day.[23] As already mentioned, Feuer and Liscio[6,7] have suggested that the deficiency of drug oxidation in the liver of justborn rats is connected with an inhibition derived from the maternal environment. It was supposed that weaning of the animals results in an acceleration of the hepatic drug metabolism through a reduction of the maternal inhibitory effects. Our studies, however, have failed to reveal any influence on drug metabolism of the change of diet from mother's milk to solid food. No differences could be observed between weanling and pre-weanling rats of the same age (Table 1).

Presumably, the origin of the low enzyme activity is more complex. Although the liver has lost its major function in hemopoiesis after birth, developing blood cells still form an important part of the hepatic tissue during the first days postnatally. Hence it is understandable, that the relatively lower quantity of parenchymal cells—the main sites of drug oxidation—results in a lower capacity of the neonatal liver for *N*-demethylation.

In addition, evidence has been presented previously by Henderson and Kersten,[13] that a decreased level of drug oxidation during rat liver regeneration is associated with the rapid cellular proliferation of the liver. As appears from the present study the same holds true for the *N*-demethylation during the neonatal development of the rat liver. The period of poor *N*-demethylating activity from birth until 30 days *post partum* corresponds exactly with the period of rapid growth (Fig. 1). An increase of the enzymatic activity can be observed concurrent with the decline of the liver growth.

It might be concluded, therefore, that the level of hepatic drug oxidation in the developing rat is related—at least in part—to the rate of liver growth. The recent findings of Wilson,[24] which strongly indicate that a relatively high level of the growth hormone (STH) in blood has to be considered as one of the regulating conditions of hepatic drug oxidation, support this view.

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