Perinatal Developmental Changes in Hepatic UDP-Glucuronyltransferase

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Postnatal developmental changes in hepatic microsomal UDP-glucuronyltransferase were studied in the rat. The previously reported postnatal decline in the capacity of microsomal fractions to glucuronidate *p*-nitrophenol was found to be observable in unperturbed preparations only at non-saturating concentrations of the substrate UDP-glucuronic acid. At saturating concentrations of UDP-glucuronic acid, activity is identical in newborns and adults. Kinetic analysis revealed that the enzyme from liver of newborns has a much higher affinity for UDP-glucuronic acid than does the enzyme in adults, but the same activity at $V_{\max}$. On the other hand, the enzyme from adult liver microsomal fractions can be activated by the physiological allosteric effector UDP-*N*-acetylglucosamine, whereas the enzyme from newborns is largely unaffected by it. Thus it appears that the number of enzyme active sites is not changing; rather, the enzyme is maturing to a more highly regulable form. There were also differences between the enzymes in newborns and adults in their response to perturbation of the membrane-lipid environment by detergent and phospholipase A. Possible interpretations of these differences are discussed.

Foetal and neonatal liver was found to have a greatly decreased capacity for conjugating some compounds with glucuronic acid (Karunairatnam *et al.*, 1949); for more recent studies, see the review by Dutton (1966). The glucuronidating capacity of the liver is largely an expression of the activity *in vivo* of the microsomal enzyme UDP-glucuronyltransferase (EC 2.4.1.17). Lucier & McDaniel (1977) and Wishart (1978) investigated the perinatal development of rat UDP-glucuronyltransferase activity towards a variety of substrates and found two characteristic activity patterns. In the 'early' group of substrates, activities rose to values higher than those in the adult just before birth, returning after a few days to adult values. This group of substrates includes *p*-nitrophenol, *o*-aminophenol, *o*-aminobenzoate, 1-naphthol and 4-methylumbelliferone. In the 'late' group of substrates, activities did not develop until after birth, and never exceeded adult values. This group includes bilirubin, morphine and a number of steroids.

The initial rise in the early group of UDP-glucuronyltransferase activities appears to occur in response to glucocorticoids, and to be dependent on amino acid incorporation (Wishart *et al.*, 1977). However, the basis for the readjustment after the

initial surge in the 'early' group of enzyme activities is not understood. In the present paper, we have examined the development of UDP-glucuronyltransferase activity in the rat and guinea pig, using *p*-nitrophenol, an 'early'-group substrate. The results demonstrate that structural rearrangements in UDP-glucuronyltransferase may account for the readjustment in apparent activity which occurs after birth.

Materials and Methods

UDP-glucuronic acid (ammonium salt), *p*-nitrophenol, Trizma base and UDP-*N*-acetylglucosamine were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. CDP-choline was purchased from Calbiochem, San Diego, CA, U.S.A.

The microsomal cell fraction was isolated from the livers of Wistar rats in 0.25 M-sucrose as previously described (Zakim & Vessey, 1973). No significant increase in yield was obtained by reprocessing the mitochondrial fraction. For foetal and newborn rats, five to ten livers were pooled and processed as a single sample. All studies were done with freshly prepared microsomal fractions. Initial rates of *p*-nitrophenol conjugation were determined by using an assay procedure previously described

(Zakim & Vessey, 1973), with the exception of 20mM-CDP-choline being added to most assays to inhibit nucleotide pyrophosphatase activity. This did not affect *p*-nitrophenol conjugation at any developmental stage. Protein was determined by the biuret procedure (Gornall *et al.*, 1949).

Phospholipase A was isolated from *Naja naja kaouthia* venom by the procedure of Cremona & Kearney (1964). Activation by phospholipase A treatment or by UDP-*N*-acetylglucosamine is described elsewhere (Zakim & Vessey, 1973, 1974).

Results

UDP-glucuronyltransferase activity towards *p*-nitrophenol was determined for microsomal fractions isolated from livers of foetal rats (20 days gestation), newborns (less than 24 h *post partum*), 5- or 20-day-old rats, or adults. These activities are compared in Table 1. It should be noted that these rates are not absolute activities but rather rates of glucuronidation at fixed, non-saturating concentrations of UDP-glucuronic acid and *p*-nitrophenol. At 20 days of gestation, *p*-nitrophenol-conjugating activity is already present and, for this particular concentration of UDP-glucuronic acid, activities in both foetus and newborn are considerably greater than in the adult. The decrease from newborn to adult activities seems to occur between days 5 and 20 *post partum* and is nearly complete by day 20.

To delineate the biochemical basis for the apparent developmental changes shown in Table 1, we compared the kinetic properties of the enzyme in newborns and adults. The concentration of UDP-glucuronic acid varied, from 0.2 to 20mM, at a fixed concentration of *p*-nitrophenol (Figs. 1a and 1b). The results reveal that the rate of conjugation at saturating concentrations of UDP-glucuronic acid is the same for newborns and adults. The differences seen at lower concentrations arise from the fact that

the enzyme in the newborn (and foetus) has a higher affinity for UDP-glucuronic acid than does the form of the enzyme in the adults.

The activity of UDP-glucuronyltransferase in adults appears to be efficiently regulated *in vivo* by the allosteric activator UDP-*N*-acetylglucosamine (Zakim & Vessey, 1977; Vessey *et al.*, 1973). Therefore it was important to evaluate the response of the enzyme in foetuses and newborns to UDP-*N*-acetylglucosamine. The microsomal fractions from foetuses, newborns and adults were assayed at 2mM-UDP-glucuronic acid in the presence of Mn^{2+} , which is required for UDP-*N*-acetylglucosamine binding. Mn^{2+} had a small but equivalent effect on the enzyme from all developmental stages. Addition of UDP-*N*-acetylglucosamine to these assays enhanced the rate of *p*-nitrophenol conjugation over 3-fold with microsomal preparations from adults, in agreement with previous observations (Nakata *et al.*, 1976). However, only a small (approx. 20%) increase in activity resulted from the addition of UDP-*N*-acetylglucosamine to assays with microsomal fractions from foetuses and newborns.

It has been shown that UDP-*N*-acetylglucosamine activates UDP-glucuronyltransferase by enhancing its affinity for UDP-glucuronic acid (Vessey *et al.*, 1973). Thus failure of UDP-*N*-acetylglucosamine to stimulate conjugation with microsomal fractions for foetuses and newborns occurred either because the enzyme was already saturated with UDP-glucuronic acid, or because affinity for UDP-glucuronic acid was already as high as that generated by UDP-*N*-acetylglucosamine stimulation. To investigate these possibilities, we assayed the microsomal fraction from the liver of newborns in the presence and absence of UDP-*N*-acetylglucosamine at several concentrations of UDP-glucuronic acid, ranging from 0.2 to 20mM (Fig. 1a). The stimulation by UDP-*N*-acetylglucosamine was only approx. 20% at all concentrations of UDP-glucuronic acid,

Table 1. *Rate of glucuronidation of p-nitrophenol as a function of age*

Initial rates of activity were determined at 37°C by using 2mM-UDP-glucuronic acid, 0.2mM-*p*-nitrophenol and 100mM-Tris/HCl, pH 7.4. Activities are nmol of *p*-nitrophenol glucuronidated/min per mg of microsomal protein.

Age of rats	Sex	No. of observations	Activity
Foetuses (20 days of gestation)	Mixed	5	2.24–2.64
1 day old	Mixed	3	2.0–2.95
5 days old	F	1	2.62
	M	1	2.24
20 days old	F	1	1.6
	M	2	1.22–1.77
Adults	F	5	0.4–1.86
	M	1	0.8

Fig. 1. Double-reciprocal plot of UDP-glucuronyltransferase activity as a function of the concentration of UDP-glucuronic acid for the microsomal fraction from the liver of newborn (a) or adult (b) rats

The microsomal fractions were assayed at 0.4 mM-*p*-nitrophenol and 1 mM-MnCl₂. (a) Microsomal fraction from the liver of newborn rats assayed with the standard assay (●) or in an assay containing 5 mM-UDP-*N*-acetylglucosamine (○). (b) Microsomal fraction from the liver of adult rats assayed with the standard assay (■) or in an assay containing 5 mM-UDP-*N*-acetylglucosamine (□). The units of *v* are nmol of *p*-nitrophenol conjugated/min per mg of microsomal protein.

even at 0.2 mM, a concentration well below that required for saturation of the active site with UDP-glucuronic acid. Thus the enzyme in liver microsomal fraction of newborns does not respond to UDP-*N*-acetylglucosamine in a manner similar to the enzyme in adults.

The kinetic properties of UDP-glucuronyltransferase in adult-liver microsomal fraction also depend on interactions between the enzyme and its membrane-lipid environment (Zakim & Vessey, 1974, 1976). One way to examine this interaction is to perturb the microsomal lipid phase by treating the microsomal fraction with phospholipase A or detergent. In adult-rat liver microsomal fraction, limited digestion with phospholipase A leads to extensive activation of the enzyme (Hanninen & Puukka, 1971; Vessey & Zakim, 1972). The same is true for treatment with detergents (Winsnes, 1969; Zakim *et al.*, 1973a). This 'latency' is possible because the catalytic potential of the enzyme is constrained by the lipid environment in exchange for an enhanced substrate specificity and for sensitivity to the allosteric activator UDP-*N*-acetylglucosamine (Zakim & Vessey, 1974). The enzyme from liver microsomal fraction from newborns and fetuses resembles the detergent-activated forms of the enzyme from adults (Zakim & Vessey, 1975) in certain respects. It is not stimulated by UDP-*N*-acetylglucosamine, has a relatively high affinity for UDP-glucuronic acid, and is not inhibited by UDP-glucose,

Therefore, we were interested in comparing the enzyme in liver of newborns with the detergent-treated form.

Treatment of microsomal fraction from the liver of newborns with increasing concentrations of Triton X-100 produced the normal biphasic response of activation followed by a decline in activity. UDP-glucuronyltransferase activity, assayed at 1 mM-UDP-glucuronic acid, could be enhanced approx. 6–8-fold with Triton X-100. Under the same conditions, Triton X-100 activated microsomal fraction from adult rat approx. 10-fold.

Thus, as observed by Winsnes (1971), the enzyme from the newborn still shows considerable latency. Latency was also evident after phospholipase A treatment of microsomal fraction from liver of newborns. The time course of the phospholipase digestion, as reflected in changes in UDP-glucuronyltransferase activities, is shown in Fig. 2 for microsomal fractions from both newborns and adults. The latter showed greater latency than the former. However, in maximally activated microsomal fractions, the activity in those of newborns was still higher than in those of adults, and this difference was found to be unaffected by the concentration of UDP-glucuronic acid in the assay over the range 1–20 mM. After exhaustive digestion with phospholipase A, the differences between the microsomal fractions of newborns and adults are less apparent.

Fig. 2. Effect of treatment with phospholipase A on the UDP-glucuronyltransferase activity of microsomal fraction from newborn- and adult-rat liver

The microsomal fraction from the liver of newborn (O) and adult (●) rats was treated with 1 mg of phospholipase A protein/50 mg of microsomal protein. At the times indicated, a sample was taken and the digestion stopped by making it 5 mM in EDTA. The samples were then assayed for UDP-glucuronyltransferase activity with *p*-nitrophenol in the presence of 0.4 mM *p*-nitrophenol and 1 mM-UDP-glucuronic acid. The S.E.M. is indicated for analysis of the same sample of pooled microsomal fractions in quadruplicate. The units of *v* are nmol of *p*-nitrophenol conjugated/min per mg of protein.

Discussion

UDP-glucuronyltransferase activity in detergent-treated microsomal fractions with *p*-nitrophenol and certain other aglycones as acceptor substrates appears between days 16 and 20 of gestation, and rises to a very high value. Subsequently, between days 5 and 10 *post partum*, UDP-glucuronyltransferase activity appears to decline to adult values (Wishart, 1978). However, data in the present paper suggest that with *p*-nitrophenol as substrate this decline is not the result of a change in enzyme titre. No decline is seen when activities are assayed in untreated microsomal fractions at close to saturating concentrations of UDP-glucuronic acid. Rather, kinetic analysis has revealed that the enzyme undergoes a developmental change in its affinity for UDP-glucuronic acid. The enzyme of newborns has a higher affinity for UDP-glucuronic acid than does that of adults.

The *p*-nitrophenol-conjugating enzyme appears to undergo a major postnatal structural alteration. Consistent with this is the fact that the enzyme in the newborn is not significantly activated by UDP-*N*-acetylglucosamine, an allosteric effector of the mature enzyme (Vessey *et al.*, 1973). This observation may, in fact, be the key to understanding the nature of the enzyme's maturational changes. The synthesis of a UDP-*N*-acetylglucosamine-binding subunit may be under separate genetic control, which may not be expressed until after day 5 *post*

Fig. 3. Developmental profile for microsomal UDP-glucuronyltransferase activity determined in the presence or absence of UDP-*N*-acetylglucosamine

The microsomal fraction from livers of rats at the indicated stage of development was assayed for UDP-glucuronyltransferase activity in an assay containing 2 mM-UDP-glucuronic acid, 0.2 mM *p*-nitrophenol and 6 mM-MnCl₂ in either the presence (●) or the absence (O) of 5 mM-UDP-*N*-acetylglucosamine. Note that the activities (nmol of *p*-nitrophenol conjugated/min per mg of microsomal protein) are higher than in Table 1, where Mn²⁺ was not present in the assay.

partum. Alternatively, interactions between the enzyme and its lipid environment, which are known to be important for regulation of the enzyme by UDP-*N*-acetylglucosamine in the microsomal membrane of the mature rat, may not be fully operational before day 20 *post partum* owing to the known differences in membrane-lipid composition (Dallner *et al.*, 1965). It is noteworthy that, after complete disruption of the membrane-lipid phase by exhaustive phospholipase A digestion, the enzymic differences between the membranes of newborns and adults appears to be decreased. This might be taken to indicate that the enzyme proteins are identical but display different properties because they are in different lipid environments. However, perturbation of the structure of the microsomal membrane by phospholipases or detergents is known to produce a form of the enzyme that is no longer sensitive to UDP-*N*-acetylglucosamine (Zakim & Vessey, 1974; Zakim *et al.*, 1973b). Thus the phospholipase digestion might be expected to lead to a loss of those structural differences between the enzymes of adults and newborns that are associated with sensitivity to UDP-*N*-acetylglucosamine, and these seem to be the primary differences between the two enzymes.

An interesting aspect of the developmental alteration is the appearance of sensitivity to UDP-*N*-acetylglucosamine. If the *p*-nitrophenol-conjugating

activity at each stage of development is assayed under conditions resembling those *in vivo*, i.e. non-saturating concentrations of UDP-glucuronic acid, and in the presence of UDP-*N*-acetylglucosamine, then no developmental change in glucuronidating capacity is noted (Fig. 3). Post-natally, the catalytic potential is not changing; rather, the enzyme is being converted into a more highly regulated form.

In contrast with our studies, Winsnes (1971) reported a 6-fold increase in UDP-glucuronyltransferase activity towards *o*-aminophenol when 2 mM UDP-*N*-acetylglucosamine was added to neonatal-rat liver homogenates. However, he did not measure activity, but rather net synthesis over a 60 min time period. Whole homogenates contain a large number of active hydrolytic enzymes which can degrade substrate, product and enzyme. Therefore, initial rates of reaction must be determined. An alternative explanation for Winsnes's (1971) data is based on the fact that UDP-*N*-acetylglucosamine inhibits the breakdown of UDP-glucuronic acid catalysed by the potent nucleotide pyrophosphatase activity of rat liver (Zakim & Vessey, 1973). Thus the apparent stimulation noted by Winsnes (1971) may reflect the protection of UDP-glucuronic acid by UDP-*N*-acetylglucosamine over the 60 min assay period rather than a direct effect on the neonatal enzyme. This effect must therefore be re-examined.

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