The temporary postnatal decline in glucuronidation of certain phenols by rat liver

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(Received 28 March 1983/Accepted 9 May 1983)

1. A temporary but marked postnatal decline in UDP-glucuronosyltransferase activity occurs in homogenates and microsomes from rat liver. The profile of this trough and its time of occurrence (maximal over 13–16 days) are almost identical with the two substrates 2-aminophenol and 1-naphthol, whose rates of glucuronidation differ 10-fold. The trough is greatest with digitonin-activated preparations, least with fresh latent ('native') enzyme and intermediate when the native enzyme is treated with its specific activator UDP-*N*-acetylglucosamine (UDP-GlcNAc). Less detailed evidence supports similar conclusions with 4-nitrophenol as substrate. 2. The trough is not due to the presence of an inhibitor of the transferase in rat liver at 15 days of age. Over the whole perinatal period, including the time of the trough, the enzyme in homogenates can be activated by UDP-GlcNAc; the microsomal enzyme is activated to a rather lesser degree perinatally, and evidence suggests this may be due to artefacts introduced during tissue fractionation. 3. When the overall process of glucuronidation is studied in snips of intact liver offered high concentrations of the two different phenols, the trough is again evident over the same period as observed with broken cells, and of equal depth for both substrates. 4. The infant rat is therefore probably less able to glucuronidate hepatically these phenols over the suckling or early weaning period than are the adult, late foetus or newborn, and may be especially incompetent at 13–16 days of age.

Activities of UDP-glucuronosyltransferase (EC 2.4.1.17) exhibit at least two patterns of development in rat liver (see Dutton & Leakey, 1981). Activities to certain substrates, including simple phenols, surge late-foetally to reach a peak around birth at adult values or above, and then appear to fall during infancy, before climbing again to adult values (Wishart, 1978); activities to a second group of substrates, including phenols of a 'less-planar' molecular structure (Wishart & Campbell, 1979), surge neonatally, and do not exhibit the pronounced trough associated with activities towards the first group (Wishart, 1978).

The pattern of this trough has not previously been fully documented. Indeed, despite similar observations during hormonally-controlled enzyme developments (Greengard, 1971), its reality has been questioned on kinetic grounds (Goldstein *et al.*, 1980).

Abbreviations used: UDP-GlcA, UDP-glucuronic acid; UDP-GlcNAc, UDP-*N*-acetylglucosamine.

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We have therefore examined the development in rat liver of the transferase activities towards substrates of the first group, employing preparations of both broken cells and intact tissue. The results indicate that a temporary decline in glucuronidation may indeed occur in the liver of infant rats.

Materials and methods

Rats were Wistar strain and were time-mated. Only litters of more than three pups were used, and livers were pooled. After weaning (21–23 days), only male animals were employed.

UDP-GlcA (ammonium salt), digitonin and 4-nitrophenol were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.; 2-aminophenol (resublimed twice before use) was from BDH Chemicals, Poole, Dorset, U.K.; 1-[1-¹⁴C]-naphthol was from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were analytical reagent grade. Miracloth was from Calbiochem-Behring Corp., La Jolla, CA, U.S.A.

Unless otherwise stated, cell-free homogenates (10 or 20%, w/v) were prepared in a medium containing

0.25 mM-sucrose, 1 mM-EDTA, 5 mM-KH₂PO₄ (pH 7.4), using eight to ten strokes of a manually-operated glass/Teflon homogenizer and filtering the suspension through one layer of Miracloth. Unless otherwise stated, the microsomal fraction was prepared by spinning a 20% (w/v) homogenate at 10000g for 20 min, followed by spinning the supernatant at 100000g for 30 min; the resulting pellet was resuspended and washed in 0.15 M-KCl containing 5 mM-KH₂PO₄ (pH 7.4) and then spun at 100000g for 30 min. This washed microsomal fraction was resuspended in 20% (v/v) glycerol containing 0.1 M-KH₂PO₄ (pH 7.4) to give a protein concentration of 4–6 mg/ml in the suspension. Homogenates and microsomal fractions were used within 3–5 h of liver excision. For 2-aminophenol and 1-naphthol as substrates, reaction media and time of incubation were as described previously (Dutton *et al.*, 1981), with the following modifications. For 2-aminophenol, 25 µl of 20% (w/v) homogenate was incubated for 30 min, or 15 min with digitonin present. For 1-naphthol, 25 µl of 10% (w/v) homogenate was incubated for 20 and 10 min respectively. For 4-nitrophenol as substrate, conditions of tissue preparation, incubation and assay were as described by Zakim & Vessey (1973), except that microsomes were never stored as frozen suspensions.

Liver snips were prepared, and their glucuronidation assayed, as described by Pollard & Dutton (1982). Saturating substrate concentration for each age was found from a corresponding substrate-saturation curve. Conjugates were identified as β -glucuronides by hydrolysis with β -glucuronidase and by inhibition of that hydrolysis with 20 mM-mgucarolactone as indicated by Dutton *et al.* (1981); glucuronides constituted over 90% of the conjugated material.

Protein was determined by the Lowry method, with bovine serum albumin as standard.

Reaction rates were linear with respect to time and tissue concentration. For determination of the enzyme-development profiles, activity of each pool of liver samples was assayed in triplicate.

Results and discussion

UDP-glucuronosyltransferase activity in broken-cell preparations

Figs. 1(a) and 1(b) illustrate transferase activity towards 0.5 mM-2-aminophenol and 0.5 mM-1-naphthol respectively, as measured in liver homogenates containing 4 mM-UDP-GlcA and with the enzyme either latent, specifically activated with 4 mM-UDP-GlcNAc or activated by membrane perturbation with optimal concentrations of digitonin at each age.

In all cases the trough is evident, being most marked when the preparation has been optimally activated by perturbation, and least when it has been untreated. The profile of the curve is remarkably

similar for both substrates, with the trough of activity being deepest over 13–16 days. The two substrates were deliberately chosen, because the specific activity of the transferase was 10-fold greater towards 1-naphthol than towards 2-aminophenol at these assay concentrations; persistence of the similarity is therefore the more marked.

The results clearly confirm previous less-documented observations (Wishart, 1978). However, a slow decline, rather than a trough, had been reported when 4-nitrophenol and rat liver microsomes were employed (Goldstein *et al.*, 1980). We therefore examined the developmental profile of untreated, UDP-GlcNAc-activated and digitonin-activated transferase in microsomal preparations, using both 2-aminophenol (Fig. 1c) and 4-nitrophenol. In each case we found the trough deepest over 13–16 days. The reported absence of the trough (Goldstein *et al.*, 1980) could have been due to the few developmental stages examined.

Factors affecting UDP-glucuronosyltransferase activity over the period

The reproducibility of the pattern with substrates of widely different rates of reaction suggests it may be due to simple changes in enzyme activity rather than to the artefactual exigencies of the assay, which is known to exhibit kinetic irregularities (Zakim & Vessey, 1973). A previous study had suggested (Goldstein *et al.*, 1980) that on extrapolation to theoretically saturating concentrations of the donor substrate UDP-GlcA, with untreated microsomal preparations as enzyme source, no decline would occur. Unfortunately only two time points were taken, for newborn and adult, omitting the critical period of the trough.

In homogenates or microsomes from adult liver the transferase is specifically activated by UDP-GlcNAc (Winsnes, 1969; Vessey *et al.*, 1973), which may be necessary in the adult liver cell for efficient functioning of the enzyme by increasing its affinity for UDP-GlcA (Zakim & Vessey, 1976). Figs. 1(a), 1(b) and 1(c) show considerable activation of the transferase by UDP-GlcNAc throughout the perinatal period. However, Goldstein *et al.* (1980) found <20% activation by UDP-GlcNAc of microsomal transferase activity towards 4-nitrophenol at birth and concluded that the enzyme was 'largely unaffected' by the nucleotide at that time; moreover, they suggested that the enzyme of newborns (<2 h *post partum*) exhibited a higher affinity for UDP-GlcA than did that of adults, and thus might not need UDP-GlcNAc for effective functioning *in vivo*.

We investigated this apparent disagreement. Although UDP-GlcNAc activates the transferase perinatally for both substrates, it is less effective with 1-naphthol (Fig. 1b) than with 2-aminophenol (Fig.

Fig. 1. Development profile for UDP-glucuronosyltransferase activity towards 2-aminophenol (a) and 1-naphthol (b) in rat liver homogenates and (c) development profile for UDP-glucuronosyltransferase activity towards 2-aminophenol in the washed microsomal fraction from rat liver

(a) The enzyme assays were performed with 0.5 mM-2-aminophenol and 4 mM-UDP-GlcA in a standard assay (●), with the addition of 4 mM-UDP-GlcNAc (○) or maximally activating concentrations of digitonin (■). Points with error bars represent average values; otherwise points show the range of values in cases where three or more separate experiments were performed. (b) The enzyme assays were performed with 0.5 mM-1-naphthol and 4 mM-UDP-GlcA in a standard assay (●) with the addition of 4 mM-UDP-GlcNAc (○) or maximally activating concentrations of digitonin (■). (c) The enzyme assays were performed with 0.5 mM-2-aminophenol and 4 mM-UDP-GlcA in a standard assay (●) with the addition of 4 mM-UDP-GlcNAc or with optimal concentrations of digitonin (■).

Table 1. The effect of UDP-N-acetylglucosamine on developing UDP-glucuronosyltransferase activity towards 4-nitrophenol
Preparation of homogenates, washed microsomes and assay of enzyme activity were as described by Zakim & Vessey (1973). Homogenates and washed microsomes were prepared from at least nine pooled rat livers or four for 15-day-rat liver preparations. Values in parentheses show the fold stimulation of the values with UDP-GlcNAc compared with controls.

Age of rat liver	UDP-glucuronosyltransferase activity (nmol/min per mg of protein)					
	Homogenates			Microsomes		
	Control	+ 4 mM-UDP-GlcNAc	(Fold activation)	Control	+ 4 mM-UDP-GlcNAc	(Fold activation)
21-day-foetal	0.48, 0.47	1.40, 1.38	(2.9)	3.77	6.43	(1.7)
1 day	0.54, 0.64	2.58, 3.09	(4.8)	3.50, 7.27	5.55, 8.66	(1.3)
15 days	0.34, 0.34	0.96, 1.15	(3.1)	1.21, 1.12	2.88, 3.47	(2.8)
35 days	0.38, 0.38	1.92, 1.92	(5.1)	2.32, 2.38	7.98, 11.20	(4.1)

1a). We then studied its activation for the substrate 4-nitrophenol, used by Goldstein *et al.* (1980). Table 1 shows that, at 1 day of age and with this substrate, activation by UDP-GlcNAc of the enzyme in homogenates was as high as 4.8-fold. Difference in substrate does not therefore cause the disagreement. Another possible reason concerns

centrifugation; with liver microsomes as enzyme source, we found that indeed UDP-GlcNAc was not so effective an activator in early life as in the adult, with 2-aminophenol as substrate (Fig. 1c). We then employed 4-nitrophenol as substrate and the centrifugation procedure of Goldstein *et al.* (1980). With microsomes from adult (35-day) livers, activation, at

4-fold, was comparable with that with liver homogenates from rats of the same, or younger, age (Table 1); but with microsomes from late-foetal or 1-day-rat livers, activation was well below that level (Table 1).

The process of fractionation itself was responsible. In one experiment the activation seen with homogenates (4.8-fold) was markedly lessened when seen in the 10000g supernatant (1.6-fold) and further lessened (1.3-fold) when seen in the washed microsomes themselves. This loss was not due to aging, for homogenates simply stored at 0°C for 5h could still be activated 4–5-fold by UDP-GlcNAc. It is presumably due to the well known (see Dutton & Leakey, 1981) developmental differences in the components of the vesicle harvest comprising 'microsomes' when a centrifugation procedure designed for adult liver is employed. For example the perinatal population of vesicles harvested may be more susceptible to damage during centrifugation. This is a major argument for the use of homogenates in such exploratory developmental studies.

It was therefore evident that UDP-GlcNAc activates the transferase in preparations of 'native' perinatal and infant rat liver, even over the period of the trough. It was also evident that, until more is known of the local concentrations of UDP-GlcA and UDP-GlcNAc at the site of the transferase in the membrane throughout development, little more useful evidence concerning the physiological reality of the trough could be gained with broken-cell preparations. We did, however, ascertain from experiments mixing homogenates from 1-day or adult (35-day) rat liver with those from 15-day rat liver that no inhibitor of the homogenate enzyme was present at the period of its minimum activity.

Glucuronidation in liver snips

We therefore assayed the overall glucuronidation performed by snips of rat liver, sampled throughout the perinatal and infant stages. In these snips the local concentrations of sugar nucleotides and the degree of transferase latency might be considered approximating to those *in vivo* (see Pollard & Dutton, 1982) at each age chosen.

The developmental curves indicate that, at a concentration of 2-aminophenol, either fixed (0.25 mM) or found apparently to saturate the process of glucuronidation at each chosen age (0.25–1.0 mM), the pattern with this substrate (Fig. 2a) almost exactly reproduces that previously seen (Fig. 1a) for the transferase in broken-cell preparations, the peak, trough and subsequent surge corresponding in time. With 1-naphthol as substrate, at concentrations apparently saturating for each age (0.1–0.3 mM), the pattern (Fig. 2b) again resembles that previously seen (Fig. 1b) for the transferase itself in broken-cell preparations.

Fig. 2. Development profile for glucuronidation of 2-aminophenol (a) and 1-naphthol (b) by rat liver snips (a) Two concentrations of 2-aminophenol were examined: a fixed non-saturating concentration of 0.25 mM (○); and a concentration (0.25–1.0 mM) found to give maximum activity at each age (●). (b) A concentration of (0.1–0.3 mM) 1-naphthol was examined, found to give maximum activity at each age.

These marked similarities between the glucuronidation patterns in broken cells and in snips suggest that the temporary postnatal decline in glucuronidation seen in the snips could well be due to the coincident temporary decline of transferase activity observed with broken cells. They are therefore consistent with the physiological 'reality' of this temporary decline.

As liver is a major glucuronidation site in the infant rat (see Dutton, 1980), the results further suggest that the animal may be more susceptible to any toxic effects of such free phenols over 12–16 days of age than at other periods from late foetal to adult life. The importance of monitoring the activity of drug-metabolizing enzymes in each species, including man, throughout maturation, and not just until high activities have been attained, is therefore evident.

Further work will be needed to elucidate the reason(s) for this temporary deficiency in glucuronidation. It could, for example, be due to a fall in transferase concentration; to the changeover from a late-foetal to an adult form of the enzyme; to a change in the membrane in which the enzyme is positioned and which regulates its constraint and the access of substrates; or to lack of availability of UDP-GlcA or UDP-GlcNAc for the enzyme.

Further work should also pursue the other implications of the present findings: for example, the identity or otherwise of the enzyme form(s) developing activity so similarly towards 2-aminophenol and 1-naphthol; the possible factors, such as limitation of UDP-GlcA or UDP-GlcNAc or of transport, responsible for the similarity in maximal glucuronidation by snips of two substrates for which

the enzyme itself possesses such a marked difference in specific activity; and the suggestion that the transferase in liver snips behaves throughout perinatal and infant life in rat liver as if it were not wholly latent in the cell but activated there, as shown above to be possible *in vitro* by UDP-GlcNAc.

We thank Mr. D. Gardiner for his excellent technical assistance, the M.R.C. for a grant and the S.E.R.C. for a studentship to I. S.

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