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AGE AND SEX DEPENDENT VARIABILITY OF THE ACTIVATION CHARACTERISTICS OF UDP-GLUCURONYLTRANSFERASE *IN VITRO**

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Abstract—Addition of detergents, UDP-*N*-acetylglucosamine and diethylnitrosamine to liver homogenates *in vitro* have been reported to increase the activity of UDP-glucuronyltransferase severalfold. In the present report the activation characteristics of rat-liver glucuronyltransferase *in vitro* is shown to vary between the sexes and during postnatal development.

With *p*-nitrophenol and *o*-aminophenol as substrates a different degree of activation of glucuronyltransferase was obtained in liver homogenates from female and male rats, while no sex difference was found with bilirubin, 4-methylumbelliferone and phenolphthalein as substrates.

While detergents and UDP-*N*-acetylglucosamine have a strong activating effect on *o*-aminophenol glucuronyltransferase in homogenates from newborn rats, no activation by diethylnitrosamine is found at this age. During the first 14 days postnatally the activating effect of diethylnitrosamine gradually appears and increases concomitantly with a decrease in the activation revealed by addition of digitonin, while during weaning there is a rise in the effect of digitonin and a fall in that of diethylnitrosamine. Approaching adulthood the activating effect of digitonin again decreases and that of diethylnitrosamine increases. Consequently quite different developmental patterns of *o*-aminophenol glucuronyltransferase were revealed for "native" enzyme and digitonin supplemented enzyme with and without addition of diethylnitrosamine.

The observation that the activation characteristics of glucuronyltransferase (*o*-aminophenol and *p*-nitrophenol as substrates) *in vitro* vary with the age and the sex of the animal points towards an importance of the activation *in vitro* beyond its ability to increase the sensitivity of the enzyme assay, and also raises the question of what assay conditions should be used to determine the functional level of glucuronyltransferase *in vivo*.

THE CONJUGATION of several endogenous and exogenous compounds with glucuronic acid is catalysed by the microsomal enzyme glucuronyltransferase (UDP-glucuronate glucuronyltransferase, EC 2.4.1.17). This enzyme system is most active in liver.[1] Present knowledge strongly suggests the presence of multiple glucuronyltransferases.[2–7]

Several glucuronyltransferases have been activated *in vitro* by a variety of methods, UDP-*N*-acetylglucosamine,[8–10] snake venom,[2,3] detergents,[10–13] dialysis against EDTA at pH 9·0,[7,14,15] preincubation[10,11] and diethylnitrosamine.[16] Activation by preincubation, detergents and UDP-*N*-acetylglucosamine has been shown to be non-additive towards each other.[10] The activation of rat-liver *o*-aminophenol glucuronyltransferase by diethylnitrosamine was, however, increased when detergents or UDP-*N*-acetylglucosamine were added as well.[10] Presumably therefore the activation by diethylnitrosamine is mediated by another mechanism. At present the mechanism(s)

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underlying the activation *in vitro* remain(s) obscure, although the results with detergents presented so far are best explained by an unmasking of active sites.[10]

If the response *in vitro* of glucuronyltransferase towards various activating agents varies at different ages or between the sexes, this might indicate that differences in the "internal environment" for instance hormonal factors have a regulating effect on the enzyme or its membrane environment. In this connection it is interesting to note that detergents when added during enzyme assay, were able to eliminate the difference in "native" rat-liver glucose-6-phosphatase activity which resulted from cortisol treatment.[17]

A reinvestigation of the activities of glucuronyltransferase (*o*-aminophenol, 4-methylumbelliferone, *p*-nitrophenol, phenolphthalein and bilirubin as substrates) at different ages and in both sexes was therefore undertaken to find out whether the activating agents had the same effect or not in these situations.

Preliminary experiments with glucuronyltransferase in homogenates from newborn and adult female rat-liver (bilirubin and *p*-nitrophenol as substrates) gave little indication of such variation in degree of activation.[10] However, in the case of rat-liver *o*-aminophenol glucuronyltransferase obvious differences between the degree of activation in the newborn and adult rat-liver were found, and moreover quite different developmental patterns of the enzyme activity were revealed depending on the assay method used, as will be described.

In the present study differences in activation characteristics were also demonstrated between enzyme from females and males when *p*-nitrophenol and *o*-aminophenol were used as substrates.

MATERIALS AND METHODS

The animals used were NMRI/BOM mice and Wistar rats given a standard pelleted laboratory diet and tap water. Newborn rats of either sex were allowed to stay together with their mothers for the whole experimental period (40 days). When necessary, litters were pooled to yield sufficient material. Tissue homogenates (in 0·154 M KCl solution) were centrifuged at 2000 *g* for 15 min and the supernatant was used for enzyme assay.

Acceptor substrates were commercial samples of the purest grade. Donor substrate was UDP-glucuronate sodium salt from Boehringer.

Glucuronyltransferase assays were performed with the following substrates: *o*-aminophenol,[18,19] 4-methylumbelliferone,[20] *p*-nitrophenol,[2] phenolphthalein[21] and bilirubin.[12] For purpose of standardization, the methods described in Refs. 2, 12, 18–21 were modified in the following respects. The final concentration of acceptor substrates was 0·5 mM and that of UDP-glucuronate 2·0 mM. The buffer was tris-maleate (pH 7·4) in a final concentration of 75 mM. Incubation mixtures (final volume 0·5 ml) were kept in stoppered glass tubes and incubated aerobically for 30 min at 37° in a shaking water bath. Incubations usually were in duplicate.

Blanks containing all reagents except UDP-glucuronate were kept at 0°, and stopping reagents were added immediately after addition of enzyme. Bilirubin and *o*-aminophenyl glucuronides were estimated after diazotization as described in Refs. 12 and 18. In routine assays, *p*-nitrophenyl and phenolphthalein glucuronides were estimated by the reduction of the absorbance at 400 and 550 nm, respectively, while

TABLE 1. ACTIVITIES OF NON-ACTIVATED AND DETERGENT-ACTIVATED RAT-LIVER UDP-GLUCURONYLTRANSFERASES

Acceptor substrate:	Bilirubin		*p*-Nitrophenol		4-Methylumbelliferone		Phenolphthalein	
Additions to standard assay mixtures:	None	0·075% Tr X-100	None	0·050% Tr X-100	None	0·050% Tr X-100	None	0·050% Tr X-100
Newborn rats, less than 24 hr old	0·15 ± 0·07 (13)	0·36 ± 0·17 240% (13)	5·31 ± 1·30 (6)	27·1 ± 4·00 510% (5)	5·60 ± 0·46 (6)	46·9 ± 7·20 836% (6)	0·28 ± 0·16 (6)	1·87 ± 0·37 668% (6)
Adult female rat (250–300 g)	0·60 ± 0·11 (7)	1·78 ± 0·10 296% (5)	3·04 ± 0·69 (8)	10·9 ± 2·09 359% (8)	5·51 ± 1·04 (7)	93·0 ± 15·8 1690% (7)	2·06 ± 0·59 (6)	20·6 ± 5·30 1000% (6)
Adult male rat (370–430 g)	0·87 ± 0·23 (8)	2·48 ± 0·28 285% (8)	2·35 ± 0·50 (11)	18·7 ± 5·21 796% (11)	4·89 ± 0·88 (8)	82·1 ± 21·4 1680% (8)	1·22 ± 0·32 (9)	12·3 ± 2·47 1010% (9)

Glucuronyltransferase activities are given as μmoles/g wet weight/hr (mean ± S.D.), and for detergent-activated enzyme the activities are also given as per cent of the "native" enzyme activity. The number of pools of tissue or single animals analysed is given in parenthesis. The assay conditions were as described in Materials and Methods. Abbreviation: Tr X-100 = Triton X-100.

4-methylumbelliferyl glucuronide was estimated by the reduction of fluorescence at 460 nm.

For more detailed description of methods see Ref. 10.

RESULTS

With bilirubin as substrate approximately the same degree of activation was obtained in liver homogenates from newborn and adult rats of either sex (Table 1). With *p*-nitrophenol the activation by Triton X-100 was twice as effective in homogenates from males as from females, while the degree of activation was intermediate in homogenates from newborn rats (Table 1). The detergent activation of phenolphthalein and 4-methylumbelliferone glucuronyltransferases was less pronounced in homogenates from newborns than from adults, while similar results were obtained with homogenates from female and male rats (Table 1).

In the case of *o*-aminophenol glucuronyltransferase, however, detergents and UDP-*N*-acetylglucosamine were three times as effective in homogenates from newborns as from adult female rats, while the difference between liver homogenates from newborns and adult males was less pronounced (Table 2). In mouse-liver homogenates similar activation factors were obtained with tissue from newborns and adults of both sexes (Table 2). As usual with *o*-aminophenol as substrate Triton X-100 exhibited less activating effect than digitonin and UDP-*N*-acetylglucosamine.[10]

The results presented in Tables 2 and 3 are consistent with the hypothesis that detergents and UDP-*N*-acetylglucosamine activate glucuronyltransferase *in vitro* by a similar mechanism,[10] since the effect of these agents exhibited the same variations in the different age periods and sexes tested.

o-Aminophenol glucuronyltransferase in homogenates from adult female and male rat-liver was activated to the same degree by diethylnitrosamine (Table 3), while no activation of the mouse-liver enzyme occurred as described earlier.[16]

Neither alone nor in combination with digitonin or UDP-*N*-acetylglucosamine did diethylnitrosamine activate glucuronyltransferase in homogenates from newborn rat-liver (Table 3). Somewhat increased effect of diethylnitrosamine and/or Triton X-100 was, however, obtained when these agents were added together in the assay of the enzyme from newborn rats. This mutual potentiating effect of diethylnitrosamine and Triton X-100 was, however, not so striking in the liver homogenates from newborn rats as in the homogenates from adult rats and mice where about the same activity was obtained whether diethylnitrosamine was combined with Triton X-100, digitonin or UDP-*N*-acetylglucosamine. The potentiating effect of diethylnitrosamine and Triton X-100 revealed in liver homogenates from newborn rats and adult mice may be unrelated to the more genuine activating effect of diethylnitrosamine observed in liver homogenates from adult rat.

Figure 1 illustrates that quite different developmental patterns were obtained for rat-liver *o*-aminophenol glucuronyltransferase postnatally when slightly different assay methods were used. Although all four curves exhibit a rise in activity during the weaning period, the degree of this rise and the time when maximum activity was obtained differed. The dissimilarities were, however, more pronounced during the first 15 days postnatally. Little change was found in "native" enzyme activity during this period, while the activity of digitonin-supplemented enzyme exhibited a steady decline. After having reached maximum activities during weaning, a decline towards adult

TABLES 2 and 3. ACTIVATION OF *o*-AMINOPHENOL UDP-GLUCURONYLTRANSFERASE *in vitro*

TABLE 2.

	Additions to standard assay mixtures:			
	None	0·20% digitonin	0·05% Triton X-100	2 mM UDPNAG
Newborn rat, less than 24 hr old	0·32 ± 0·12 100% (15)	2·17 ± 0·67 678% (15)	0·89 ± 0·30 278% (6)	2·01 ± 0·72 628% (6)
Female rat, 110–140 g	0·51 ± 0·15 100% (8)	2·38 ± 0·32 467% (8)	—	—
Adult female rat, 250–300 g	0·50 ± 0·09 100% (10)	1·01 ± 0·19 202% (8)	0·46 92% (4)	0·97 194% (3)
Adult male rat, 370–430 g	0·59 ± 0·12 100% (8)	2·49 ± 0·44 422% (8)	—	2·03 ± 0·31 344% (8)
Newborn mouse, less than 24 hr old	0·56 100% (3)	2·20 393% (3)	—	1·57 280% (1)
Adult female mouse, 25–30 g	1·29 ± 0·28 100% (12)	3·56 ± 0·94 276% (12)	1·56 ± 0·41 121% (5)	3·59 278% (4)
Adult male mouse, 35–40 g	1·79 ± 0·20 100% (6)	5·57 ± 0·69 312% (6)	—	—

TABLE 3.

	Additions to standard assay mixtures:			
	15 mM DEN	15 mM DEN +0·20% digitonin	15 mM DEN +0·05% Triton X-100	15 mM DEN +2 mM UDPNAG
Newborn rat, less than 24 hr old	0·31 ± 0·12 97% (14)	2·02 ± 0·37 631% (15)	1·05 328% (3)	1·41 440% (3)
Female rat, 110–140 g	0·91 ± 0·22 179% (8)	5·97 ± 0·67 1170% (8)	5·35 ± 0·29 1050% (8)	4·80 ± 0·36 942% (8)
Adult female rat, 250–300 g	0·85 ± 0·14 170% (10)	3·04 ± 0·96 607% (10)	3·31 662% (3)	3·31 662% (3)
Adult male rat, 370–430 g	0·99 ± 0·31 168% (8)	7·35 ± 1·25 1245% (8)	—	—
Adult female mouse, 25–30 g	1·17 91% (3)	3·20 248% (3)	2·69 208% (3)	3·01 234% (3)

Glucuronyltransferase activities are given both as μmoles/g wet weight/hr (mean ± S.D.) and as relative activity in per cent ("native" enzyme = 100%). The number of pools of tissue or single animals analysed is given in parenthesis. The assay conditions were as described in Materials and Methods. The following abbreviations are used: UDPNAG = UDP-*N*-acetylglucosamine and DEN = diethylnitrosamine.

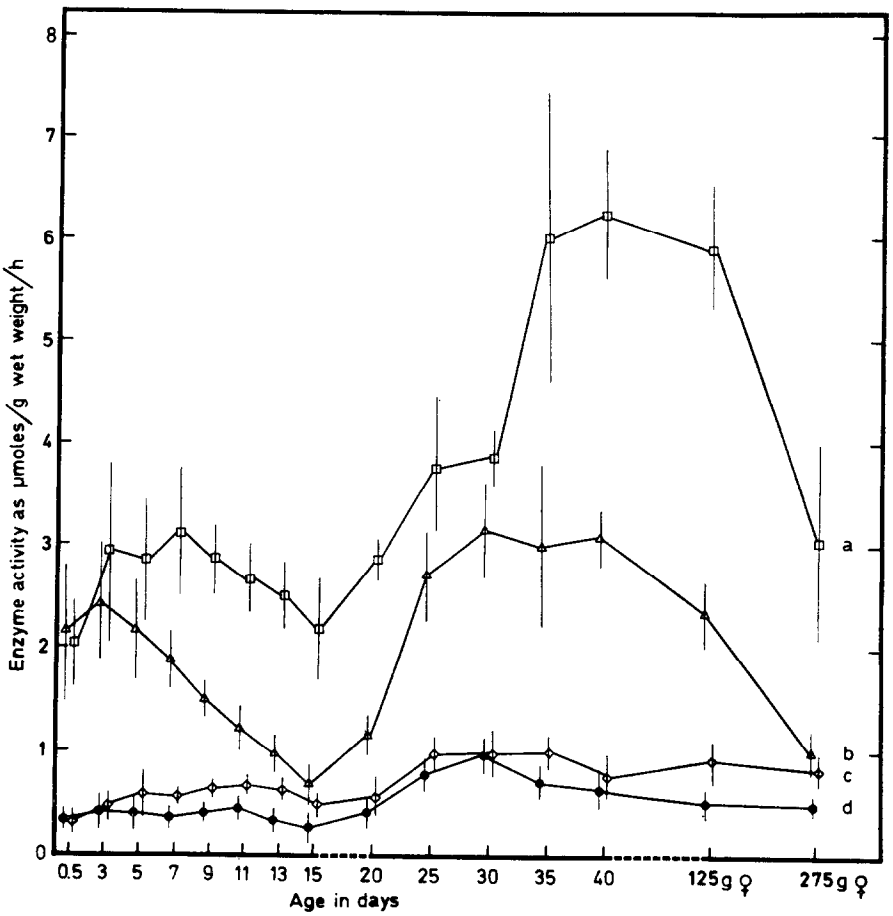

FIG. 1. The development of *o*-aminophenol glucuronyltransferase activity in rat-liver postnatally. The symbols represent the mean ±S.D. of 6–15 (mean 8·3) pools of tissue or single animals analysed. The enzyme activities for animals weighing 125 g (110–140) and 275 g (250–300) are given only for females. Females and males were not separated in the younger age groups. The curves represent enzyme supplemented with: 0·2% digitonin and 15 mM diethylnitrosamine (a), 0·2% digitonin (b), 15 mM diethylnitrosamine (c). Curve d represents "native" enzyme activity.

values was revealed for curve a, b and d. This decline was most pronounced for the enzyme from female rats, which are represented as adults in Fig. 1.

The different patterns presented in Fig. 1 are due to the varying effect of the two activating agents digitonin and diethylnitrosamine as illustrated in Fig. 2. The effect of digitonin is at maximum in newborns and in 40-day-old rats, while minima are revealed at 15 days of age and in adults. Diethylnitrosamine on the other hand does not activate glucuronyltransferase in homogenates from newborn rats and has almost no effect at 30 days, while maxima are seen at 15 days and in adulthood. To some extent there is a reciprocal relationship between these two activating agents.

Although a slight difference in the optimal concentration of digitonin was found between homogenates from newborn and adult rats (Fig. 3), this does not explain the different effect of digitonin in these age groups. In view of the effect of diethylnitros-

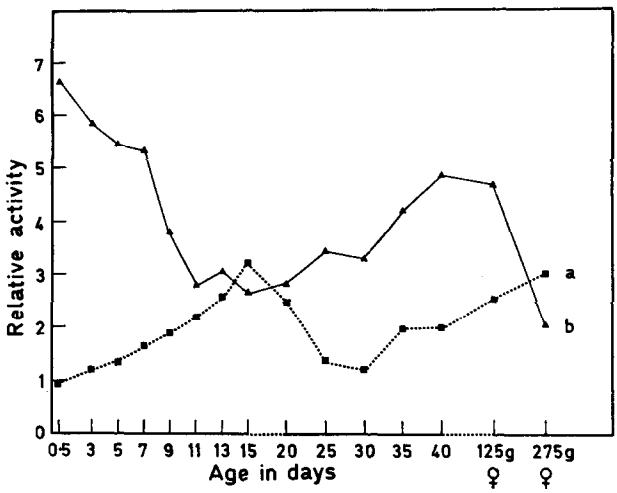

FIG. 2. The variation in the effect of digitonin and diethylnitrosamine on rat-liver *o*-aminophenol glucuronyltransferase activity at different age periods. Curve a represents the effect of diethylnitrosamine, i.e. the activity of enzyme supplemented with both digitonin and diethylnitrosamine relative to that of digitonin-supplemented enzyme. Curve b represents the effect of digitonin, i.e. the activity of digitonin supplemented enzyme relative to "native" enzyme activity.

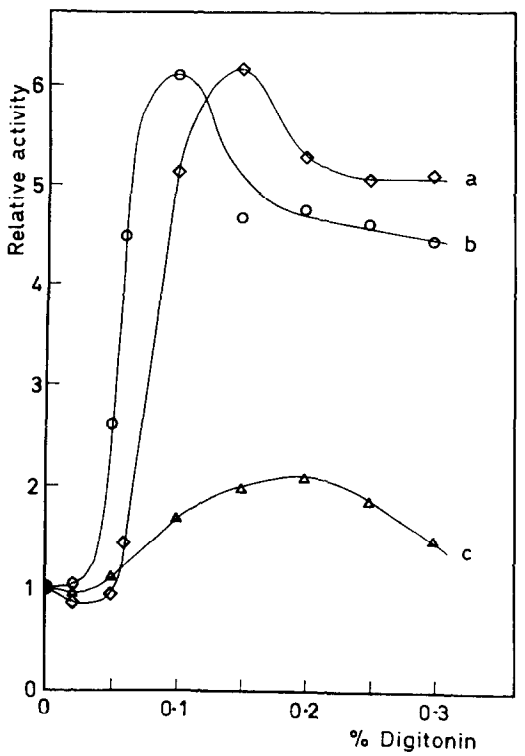

FIG. 3. The effect of varying concentrations of digitonin on rat liver *o*-aminophenol glucuronyltransferase *in vitro*. Curve a represents liver homogenates (from adult females) supplemented with 15 mM diethylnitrosamine. Curve b represents enzyme from newborns and curve c enzyme from adult females.

amine on *o*-aminophenol glucuronyltransferase in homogenates of Gunn rat-liver[16] (eliminating the difference between normal Wistar rats and Gunn rats) it is interesting to note the similarity between glucuronyltransferase from newborn (curve b) and adult rats (curve a) after addition of diethylnitrosamine to the latter, Fig. 3.

The different activating characteristics of glucuronyltransferase in homogenates from newborn and adult rats have been controlled with washed microsomes. The same results as for homogenates were obtained. In experiments where liver fractions (high-speed supernatant and boiled extract) and serum from one age group were combined with liver homogenate from the other age group and *vice versa*, no modification of the activation characteristics at the different age groups occurred.

Kinetic experiments with varying amounts of *o*-aminophenol showed K_m to be identical for glucuronyltransferase in homogenates (supplemented with both digitonin and diethylnitrosamine) from newborn and adult female rats. The results of kinetic experiments with varying concentrations of UDP-glucuronate were more complex as shown in Fig. 4. With UDP-*N*-acetylglucosamine added straight curves showing the same K_m (1·8 mM) were obtained with enzyme from both newborn (b) and adult female rats (d). Without UDP-*N*-acetylglucosamine added the curves were bended. If only that part of the bended curves where the UDP-glucuronate concentration was above 2 mM is taken into consideration, it is seen that the enzyme from adult rats (e) exhibited a K_m of 1·8 mM, while the enzyme from newborn rats (a and c) exhibited a K_m of 3·6 mM.

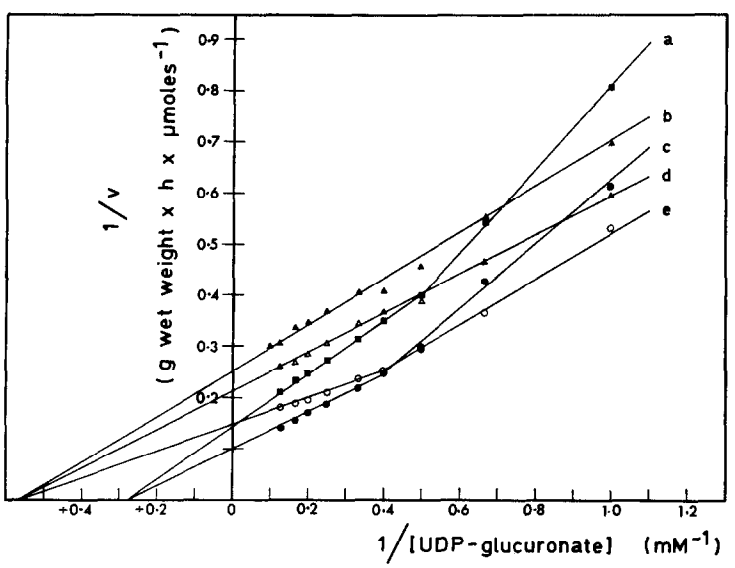

FIG. 4. The affinity of rat-liver *o*-aminophenol glucuronyltransferase for UDP-glucuronate. The enzyme activity was measured with concentrations of UDP-glucuronate in the assay solutions varying from 1 to 10 mM. Blanks were enzyme suspensions incubated without addition of UDP-glucuronate. The curves a, b and c represent liver homogenates from newborn rats supplemented with: 0·2% digitonin and 15 mM diethylnitrosamine (a), 2 mM UDP-*N*-acetylglucosamine (b) and 0·2% digitonin (c). The curves d and e represent homogenates (from adult female rats) supplemented with: 2 mM UDP-*N*-acetylglucosamine and 15 mM diethylnitrosamine (d) and 0·2% digitonin and 15 mM diethylnitrosamine (e).

The kinetic experiments with varying concentrations of UDP-glucuronate (Fig. 4) will be disturbed if hydrolysis of the nucleotide was taking place during the incubations. This may be the reason why the curves a, c and e deviated from the straight course below 2 mM concentration of substrate, since the curves were made straight throughout the whole concentration range when UDP-*N*-acetylglucosamine was added, Fig. 4b and d. UDP-glucuronic acid breakdown by pyrophosphatase present in rat-liver microsomes is thought to be inhibited by UDP-*N*-acetylglucosamine.[8]

However, in conclusion, neither the kinetic experiments nor the experiments with combination of different liver fractions and serum gave any clue to the mechanism underlying the observed differences in activation characteristics of glucuronyltransferase.

DISCUSSION

The development of the UDP-glucuronyltransferase system in the foetal and neonatal period has been the subject of several earlier studies. While the activity is low at birth and increases during the first days of life in most species and with most substrates (guinea pig,[20] rabbit[24] and mouse[25]), exceptions are found with some species (notably rat) and some substrates (*o*-aminophenol and *p*-nitrophenol).[21–23,26,27]

With one exception,[15] earlier studies on the developmental pattern of glucuronyltransferase have been performed on "native" non-activated enzyme. Halac and Sicignano,[15] however, reinvestigated the developmental patterns of activated bilirubin and *p*-nitrophenol glucuronyltransferases in the newborn rat. Their findings agreed well with previous studies performed with non-activated enzyme, and is in good agreement also with the present study showing that similar degrees of activation were obtained with homogenates from newborn and adult animals when *p*-nitrophenol and bilirubin were used as substrates.

When using *o*-aminophenol as substrate, however, quite different developmental patterns of glucuronyltransferase in the neonatal period of rats were revealed for "native" enzyme and enzyme activated by digitonin and diethylnitrosamine respectively. Accordingly great differences in the activity of glucuronyltransferase in liver homogenates from neonatal rats relative to that of adult animals were encountered with slightly different assay procedures.

It remains uncertain to what extent the different assay conditions reflect the functional level of glucuronyltransferase *in vivo*. However, it has been observed[15] that the activities obtained *in vitro* with bilirubin-glucuronyltransferase in rat-liver homogenates activated by dialysis against EDTA, corresponds best with the activity expected from calculations on the bilirubin-excretion capacity of the rat-liver.[28] Furthermore, it has been suggested by Nordlie *et al.*[17,29] that naturally occurring detergents may participate in the regulation of the activities of some microsomal enzymes *in vivo*, and the effects of such detergents may be drastically modified by dilution prior to assay of enzyme activities *in vitro*. Such considerations might as well apply to the detergent activation of glucuronyltransferase *in vitro*.

Although no explanation for the differences in the activation characteristics of glucuronyltransferase in homogenates from newborn compared with adult rats can be given at the present time, the results presented do stress the importance of being careful in judgment of the functional level of glucuronyltransferase *in vivo* in different situations if only one *in vitro* assay method is used. Until a better understanding of the

activation *in vitro* of glucuronyltransferase is reached, it would be advisable to do parallel studies of both "native" and activated enzyme.

The rise in activity of rat-liver *o*-aminophenol glucuronyl transferase during the weaning period, revealed irrespective of what assay method was employed, may be related to the change in diet taking place in this age period. Other enzymes have been shown to increase in activity during weaning,[30,31] and the possibility that glucuronyltransferase activity may be influenced by dietary factors deserves further study.

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