Biochemical Pharmacology, Vol. 35, No. 21, pp. 3777–3782, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

THE INDUCIBILITY AND ONTOGENY OF RAT LIVER UDP-GLUCURONYLTRANSFERASE TOWARD FUROSEMIDE*

AVINOAM RACHMEL† and GEORGE A. HAZELTON‡

Department of Pediatrics, and ‡ Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, U.S.A.

(*Received* 27 *January* 1986; *accepted* 12 *May* 1986)

Abstract—Furosemide (F) conjugation with glucuronic acid is the main pathway of F metabolism in humans and experimental animals. In order to study rat liver microsomal UDP-glucuronyltransferase (UDP-GT) activity towards F we developed an *in vitro* assay in which the conjugation product, furosemide 1-0-acyl glucuronide (FG) was separated and quantitatively determined by reverse phase high pressure liquid chromatography. The optimal conditions of the reaction were established and the apparent K_m for F and UDP-glucuronic acid (UDPGA) were 0.22 and 1.76 mM, respectively. Substrate inhibition of UDP-GT toward F occurred at F concentrations higher than 1.5 mM. Developmental changes in F glucuronidation were compared to the ontogeny of UDP GT activity toward two other acceptors, 1-naphthol and esterone that are known to have different patterns of maturation. F glucuronidation was 26% of adult activity at 18 days of gestation, reached 48% at birth and gradually increased to 250% of adult activity at 22 days of age. Glucuronidation of 1-naphthol and estrone attained 87% and 44% of adult activity at 22 days of gestation, 37% and 66% in six-day-old rats and 100% and 427% of adult activity in 22-day-old rats, respectively. The effect of 3-methylcholanthrene (3-MC), phenobarbital (PB) and pregnenolone-16α-carbonitrile (PCN) on F UDP-GT was studied and compared to their effect on 1-naphthol and estrone glucuronidation. PB, 3-MC and PCN increased F-UDP-GT activity to 208%, 282% and 342% of vehicle-treated animals, respectively, while F pretreatment did not affect the conjugation of F. In comparison, 1-naphthol glucuronidation was preferentially induced by 3-MC (4.4-fold of control) while estrone glucuronidation was induced by PB and PCN (4.9- and 2.5-fold of control, respectively). These studies suggest that several forms of UDP-GT activities, which differ in their ontogeny and inducibility patterns, are involved in the glucuronidation of F *in vitro*.

F§ is a potent diuretic agent which is widely used in all age groups for the treatment of congestive heart failure, hypertension and edema of various causes. F is actively secreted by the renal organic acid secretory mechanism in the proximal tubule. Variable amounts of the drug metabolize into a glucuronic acid conjugate which has been detected in the urine of infants, adults and laboratory animals [1–6]. It has been suggested that changes in the glucuronidation of F that occur during the neonatal period [2, 7], prolonged use of the drug [8] or compromised renal function [9] might affect the disposition of the drug. Saturation of the biotransformation of F has been offered as a cause for the dose dependent pharmacokinetics of F in the rat [10].

The functional heterogeneity of uridine-diphospho-glucuronyl-transferase, is well established and is reflected by substrate specific activities of purified proteins and the species' specific regulatory properties [11]. Thus, perinatal development and inducibility by xenobiotics or endogenous inducers has been used to characterize different enzyme forms of UDP-GT [12]. The time of appearance and the developmental pattern of GT activity during the perinatal period were used to characterize enzyme activities into three different 'clusters'—the late fetal cluster, the early neonatal cluster and the weaning cluster [13, 14]. In addition, at least three groups of GT activities can be distinguished according to their response following pretreatment with different inducers. Group 1 activities are mainly inducible by 3-methylcholanthrene type inducers and include substrates such as 1-naphthol, 4-nitrophenol and other planar phenols. These substrates are identical to substrates of the late fetal cluster. Group 2 activities are induced mainly by PB and include acceptors which are not planar phenols such as estrone, morphine and chloramphenicol. The activity toward these substrates appears early in the neonatal period, attaining adult levels some time after birth (the early neonatal cluster). Recently it has been suggested that PCN induces a third group of UDP-GT which include substrates like digitoxigenin-monodigitoxoside and bilirubin [15]. The enzymatic activities toward digitoxigenin-monodigitoxoside develop postnatally and reach only 10% of adult activity at the age of 20 days [16].

The present study was undertaken to characterize the UDP-GT activity toward F in rat liver. We estab-

* Portions of this work were presented at the Combined Meeting of the American Pediatric Society and the Society for Pediatric Research, Washington, DC, 6–9 May 1985.

† Address for correspondence: Dr A. Rachmel, Department of Pediatrics A, Beilinson Medical Center, Petah Tiqva 49100, Israel.

§ Abbreviations: F, furosemide; FG, furosemide 1-0-acyl glucuronide; UDP-GT, UDP-glucuronyltransferase; UDPGA, UDP-glucuronic acid; 3-MC, 3-methylcholanthrene; PB, phenobarbital; PCN, pregnenolone-16α-carbonitrile; CHAPS, 3 [(cholamidopropyl) dimethylammonio]-1-propranesulfonate.

lished the optimal assay conditions and examined the ontogeny and inducibility of hepatic UDP-GT activity toward F and compared these features with the glucuronidation of 1-napththol and estrone which are known substrates for specific UDP-GT activities.

MATERIALS AND METHODS

Trizma base, maleic acid, UDPGA sodium salt, sucrose, D-saccharic acid-1, 4-lactone, 3[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 1-naphthol, estrone, sodium cholate, Triton X-100, glutathione, β-glucuronidase type B3 and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, MO; EDTA was purchased from Aldrich Chemical Co., Milwaukee, WI; Pregnenolone-16α-carbonitrile was a gift from the Upjohn Co., Kalamazoo, MI; 3-Methylcholanthrene was obtained from Eastman Chemicals, Rochester, NY; Phenobarbital was purchased from Amend Drug & Chemical Co., Irvington, NJ; Desmethylnaproxene was a gift from Syntex Co., Palo Alto, CA; Furosemide, USP was generously supplied by Hoechst–Roussel Pharmaceuticals Inc., Sommerville, NJ; [2, 4, 6, 7-^{3}H]estrone (88.5 Ci/mmol) and [1-^{14}C]1-naphthol (4.3 mCi/mmol) were obtained from New England Nuclear, Boston, MA; 3a70 Scintillation fluid was from Research Products International Corp., Mount Prospect, IL. All other reagents and solvents were reagent or HPLC grade. Furosemide glucuronide (FG) was biosynthesized and purified as previously described [17]. Calibration of FG concentration in the stock solution was done by complete enzymatic hydrolysis of the conjugate (3000 units of β-glucuronidase, 0.1 M Tris–maleate buffer pH 5.0 at 37° for 4 hr). The concentration of F that was released was determined by HPLC according to Rapaka *et al.* [18] using desmethylnaproxene as an internal standard. FG solution (12.74 μM) was kept at pH 3–4 (3.5 M acetic acid) in light protected vials, at −20° and was stable during the experimental period. It was later diluted and processed by the same method as the microsomal incubation samples and was subjected to HPLC analysis (*vide infra*).

Animals. Pregnant Sprague–Dawley rats were obtained from Sasco (Omaha, NE). They were kept at a 12-hr light–dark cycle at 24–26° and constant humidity and were allowed food (laboratory Rodent Chow, Ralston-Purina, St. Louis, MO) and water *ad libitum* for at least 5 days before sacrifice.

Gestational age was determined according to the first day in which vaginal smears showed the presence of sperm cells, and was counted as day 0. Pregnant rats (N = 28) were individually kept in plastic cages that were lined with processed corncob bedding. Delivery time was determined to within 12 hr and the birth date was considered as postnatal day 0. The mean length of gestation determined for the animals delivered within the study was 21 ± 1.5 days (mean ± SD).

Pretreatment. Male rats, 200–280 g, were pretreated i.p. for 4 days with the following daily doses: 3-MC, 20 mg/kg in corn oil; PB 75 mg/kg in saline; PCN, 75 mg/kg suspended in 2% Tween 80 in saline; F 5 mg/kg in 0.05 N NaOH; and saline and corn oil as controls. All compounds were administered in a total volume of 5 ml/kg.

Preparation of liver microsomes. After pretreatment each rat was decapitated and the liver was excised, blotted, weighed and homogenized (25% w/v) in ice-cold 0.25 M sucrose containing 1.15% KCl with a Potter–Elvehjem glass mortar and Teflon pestle. All further manipulations were done at 4°. The postmitochondrial (9000 *g* for 20 min) supernatant was centrifuged at 105,000 *g* for 60 min. Microsomal pellets from 1 g of liver were suspended in 3 ml 0.25 M sucrose and were kept at −70° until used. In the study of UDP-GT activity during the perinatal period, livers from whole litters were pooled and processed as one sample with the following modifications. Livers were homogenized in ice cold 0.05 M Tris–HCl pH 7.4 containing 1.15% KCl; the microsomal pellets were further washed in 10 mM EDTA pH 7.4 in 1.15% KCl and were reisolated by ultracentrifugation as previously described. This method was previously found to yield minimally contaminated microsomal pellets from fetal rat liver [19]. UDP-GT activity was also determined in microsomes that were similarly prepared from five adult male rats. No significant difference in UDP-GT activity toward aglycones studied was found between adult liver microsomes prepared in either way.

The effect of detergents was studied by adding sodium cholate, Triton X-100 or CHAPS to fresh microsomal suspensions from adult male rats. After shaking for 20 min at 4°, the enzyme suspensions were added to the assay mixture.

Determination of UDP-GT. Reaction mixtures (0.5 ml) contained 0.1 M Tris–maleate buffer, pH 7.4, 10 mM $MgCl_2$, 1.5 mM furosemide dissolved in 0.05 N NaOH (20 μl), microsomes and 4 mM UDPGA. D-saccharic acid-1,4-lactone (1.2 mM) was added to inhibit any β-glucuronidase activity. Reaction was started after 5 min of preincubation at 37° by the addition of UDPGA and was terminated after 10 min with 0.5 ml of ice-cold 3.5 M acetic acid. Control incubation received water instead of UDPGA. The incubation and sample preparation were done under strict protection from light, in screw-top glass tubes. Unreacted furosemide was extracted twice with 7 ml of ethyl ether:*n*-hexane (65:35). After centrifugation of the aqueous phase at 3000 rpm for 10 min in 0.5 ml polypropylene tubes, 10–20 μl were subjected to HPLC as described below.

Determination of UDP-GT activity toward 1-naphthol and estrone were assayed radiometrically at 0.5 mM and 0.15 mM acceptor concentration, respectively, according to previously described methods [20, 21]. Protein concentration was measured according to Lowry [22] using bovine serum albumin as standard.

Determination of FG. FG was separated by reverse phase HPLC on C_{18} μBondapak column (Waters Associates, Milford, MA) and quantitated with a fluorescence detector (model FS970 Schoeffel Instruments, Westwood, NJ) set at 233 nm and 389 nm excitation and emission cut-off filter, respectively. The mobile phase of acetonitrile/0.08 M phosphoric acid (30:70) was delivered at 2 ml/min through a

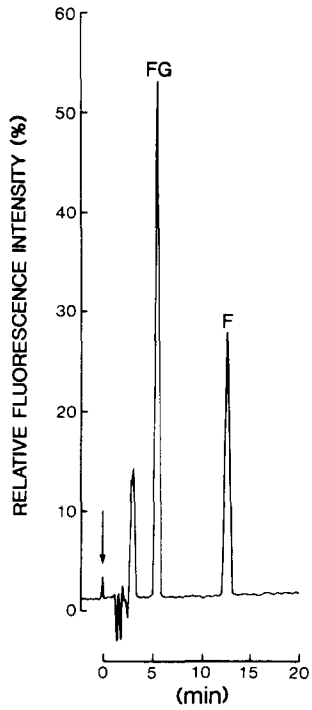

Fig. 1. HPLC separation of F and FG in microsomal incubation mixture, details of chromatographic conditions are given in the text.

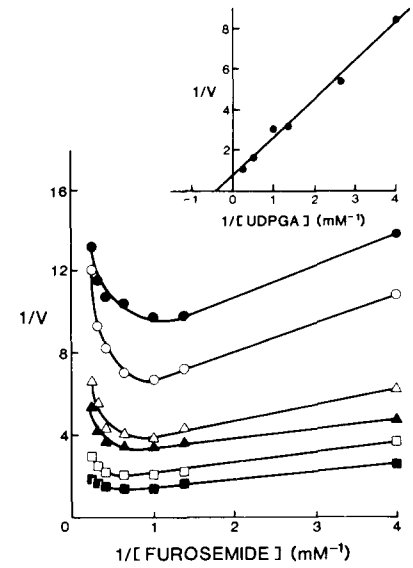

Fig. 2. Double reciprocal plot of initial rates of F-UDP-GT as function of the concentration of F. Different concentrations of UDPGA were held constant at either 0.25 mM (●), 0.375 mM (○), 0.75 mM (△), 1 mM (▲), 2 mM (□), and 4 mM (■). Initial velocity (V) is expressed as pmoles FG formed per mg protein per min. CHAPS activated rat liver microsomal protein was used at about 0.6 mg per incubation. A secondary plot (inset) of the intercepts on the $1/V$ axis vs reciprocal values of the concentrations of UDPGA was used to calculate the apparent K_m. Each point represents the mean of three experiments.

U6K injector and M45 solvent delivery system (Waters Associates, Milford, MA). Retention time of FG and F were 5.2 and 12 min, respectively (Fig. 1). The production of FG was linear in respect to protein concentration and incubation time, up to 5 mg/ml microsomal protein and for more than 20 min, respectively (data are not shown). Standard curves for FG determination were prepared by measuring FG peak height at a concentration range of 0.32–7.6 μM which were fitted to a straight line by the method of least-square linear regression analysis ($r \geq 0.99$). The coefficient of variation for the assay was 5.5% (N = 8).

Statistical analyses were performed using an analysis of variance followed by Duncan's new multiple range test.

RESULTS

Determination of K_m

Kinetic parameters of the F-UDP-GT activity were derived by bisubstrate kinetic analysis of the initial rates of enzyme activity as a function of varying concentrations of furosemide at several fixed concentrations of the UDPGA (Fig. 2) as described by Dixon [23]. K_m (mean ± SE of three enzyme preparations) was 0.223 ± 0.019 mM and 1.76 ± 0.55 mM for furosemide and UDPGA, respectively. Substrate inhibition curves were observed for furosemide as the variable substrate when present in concentrations higher than about 1.5 mM (Fig. 2). However, the linearity of the enzyme activity during the incubation period was not affected. In addition, the same pattern of inhibition was maintained when reduced glutathione (10 mM) was included in the incubation (data not shown).

Under the assay conditions chosen for subsequent studies (1.5 mM and 4 mM UDPGA), the normalized initial rate of the reaction as a percentage of V_{max} [21] was 87% and 69.4% for F and UDPGA, respectively.

Effect of detergents and Mg^{2+} *on F-UDP-GT*

Enzymatic activity toward F in fresh microsomal preparations rose by the treatment with increasing sodium cholate, Triton X-100 or CHAPS concentration (Fig. 3a). The optimal detergent concentration for the activation procedure was determined and maximal increase of 2.12-, 2.51- and 6.04-fold of native activity was found for sodium cholate (0.125%), Triton X-100 (0.5%) and CHAPS (0.25%), respectively. At higher concentrations of detergent, there was a gradual decrease in UDP-GT activity. While the activation caused by sodium cholate or Triton X-100 was abolished at higher concentrations of detergent, the activity in CHAPS activated preparations was still significantly higher than control non-activated microsomes, even at detergent concentrations that were four times greater than the optimum.

Addition of Mg^{2+} (4–20 mM) increased the enzymatic activity three-fold in CHAPS activated microsomal preparations (Fig. 3b). Therefore, all further experiments were carried in the presence of 10 mM $MgCl_2$ after activation of the microsomal protein in the presence of 6 mM CHAPS.

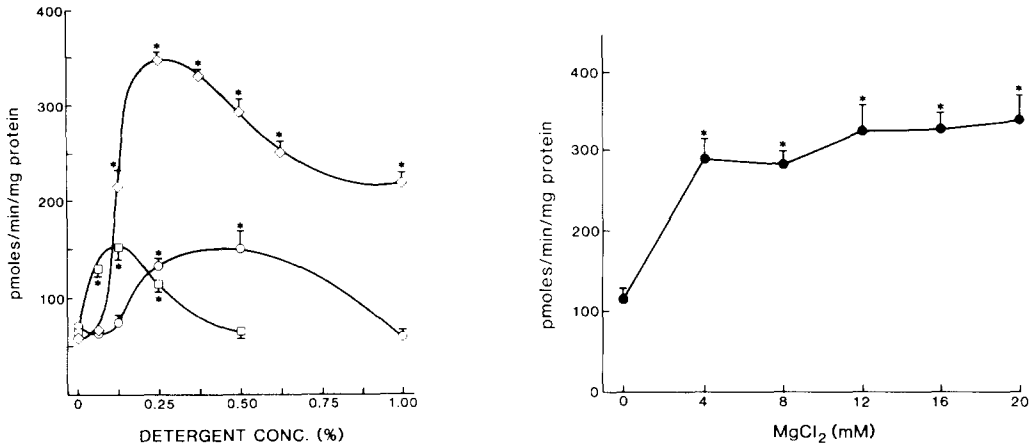

Fig. 3. (a) Effect of detergents on UDP-GT activity toward F. Fresh microsomal preparations were incubated for 20 min at 4° with different concentration of CHAPS (◇) (N = 4), sodium cholate (□) (N = 3) or Triton X-100 (○) (N = 3). 0.6–0.7 mg protein was added to the reaction mixture as described under methods and preincubated for 5 min at 37° before the reaction was started. * Significantly higher than native microsomes at $P < 0.01$. (b) Effect of $MgCl_2$ on UDP-GT activity toward F. Optimally activated (CHAPS, 6 mM) fresh microsomes were used; other details are in the text (N = 5; * $P < 0.01$).

The inducibility of UDP-GT activity

Pretreatment of young adult rats with 3-MC, PB or PCN changed UDP-GT activity toward F, estrone and 1-naphthol in detergent activated rat liver microsomes (Fig. 4). While 5 days treatment with F did not affect F-UDP-GT activity, PCN, 3-MC and PB pretreatment increased F-UDP-activity to 342%, 291% and 208% of control animals, respectively. UDP-GT activity toward estrone increased five-fold following the pretreatment with PB while 3-MC and PCN increased the enzyme activity 2- and 2.5-fold, respectively. 1-Naphthol glucuronidation increased 4.4-fold after treatment with 3-MC and 1.6-fold after PB treatment, whereas pretreatment with PCN had no effect.

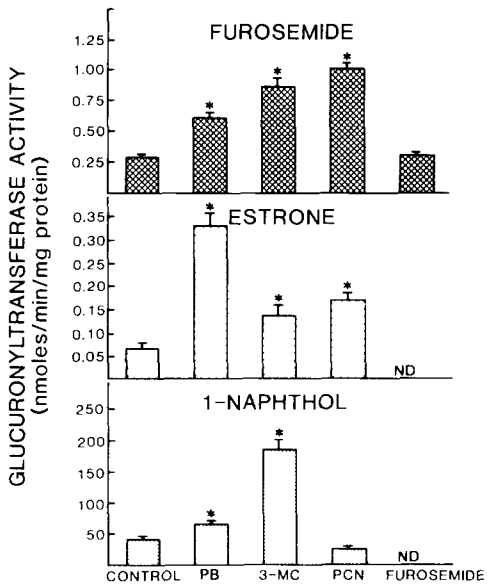

Fig. 4. Effect of enzyme inducers on glucuronidation of furosemide, 1-naphthol and estrone. Enzyme activities in rats pretreated with saline or corn oil were not different and were combined in the control group. Bars represent means ± SE of five or six male rats. * Values that are significantly higher than control activity ($P < 0.01$); ND—not determined.

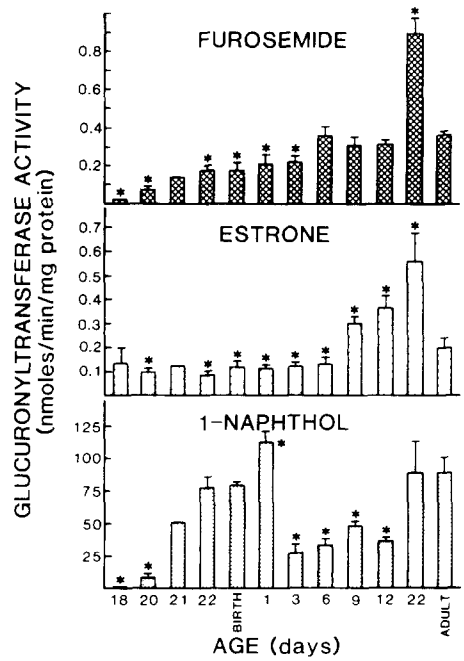

Fig. 5. Development of UDP-GT activity toward furosemide, 1-naphthol and estrone in fetal and neonatal rat liver. Bars represent mean ± SE of 3–6 determinations with the exception of 21-day-old fetus (2) and adult male rats (N = 11). Livers from whole litter were pooled for each determination (regardless of sex). * Significantly different from adult activity ($P < 0.05$).

Ontogeny

Rat liver microsomal UDP-GT activity toward furosemide, estrone and 1-naphthol in the pre- and postnatal period is illustrated in Fig. 5. Enzymatic activity toward estrone and 1-naphthol are shown for comparative purposes. Low activities of furosemide glucuronidation were detected at day 18 of pregnancy and gradually increased to 48% of adult levels at birth. Continuous increase in enzyme activity was observed with a 2.5-fold adult level at the age of 22 days. A similar pattern of development of enzyme activity toward estrone was found, though at a much slower rate, while 1-naphthol glucuronidation reached adult levels at birth, and then decreased for about two weeks to rise again to adult levels of activity around the weaning period.

DISCUSSION

Conjugation of F with glucuronic acid has been suggested to be the main metabolic pathway for F biotransformation [1, 5, 6]. Alternative metabolic routes such as formation of 4-chloro-5-sulfamoylanthranilic acid (CSA), which is a breakdown product of F, was found to be an artifact of the analytic process [1], while P-450-mediated formation of metabolites that covalently bind to macromolecules occurred only in mice treated with very high doses of the drug [24]. We have recently identified and characterized the glucuronic acid conjugate of F [17], and thus were able to study the glucuronidation of F by rat livers microsomal UDP-GT. We eliminated the formation of putative metabolite of F, CSA, by conducting the experiment under minimal exposure to light and with no use of hydrochloric acid, factors which were previously found to cause degradation of F [1, 25]. In addition, the short incubation period prevented the isomerization of the acyl type glucuronide that was formed to several isomers. This has been shown to occur at neutral pH at half-life of 5.3 hr [17]. Moreover, the reaction was terminated by prompt acidification with acetic acid, as it has been previously shown that the F-1-0-acyl glucuronide is much more stable at pH values lower than 6 [17]. The sensitivity of the assay enabled us to detect FG at a minimal concentration of 0.25 μM and enzymatic activities as low as 5 pmol/min/mg protein.

CHAPS, a non-denaturating zwitter-ion detergent with superior solubilizing properties [26] increased UDP-GT activity toward F more than Triton X-100 or sodium cholate. The relative broad range of detergent concentration at which maximal enzymatic activity was maintained (4–8 mM) was mainly useful in the study of the microsomal activity in the perinatal period where microsomal yield and protein concentration varies.

Substrate inhibition of UDP-GT is uncommon. Previous studies have shown that certain substrates of UDP-GT such as bile acids [27] or 7-hydroxy chlorpromazine [28] carry detergent activity, which at increasing concentrations decrease their own glucuronidation by UDP-GT activity of liver microsomes. The effect was even more prominent when detergent-treated microsomes were used [28]. Moreover, substrate inhibition of UDP-GT by the bile acid chenodeoxycholic acid was maintained even when a purified enzyme preparation of UDP-GT was used [29]. A dose-dependent inhibitory effect of F on microsomal UDP-GT activity toward several other substrates has been previously described but enzyme kinetic analysis did not disclose any common inhibitory pattern [8].

The relevance of the inhibitory effect of F on UDP-GT activity *in vivo* is not clear. Mitchell *et al.* [24] who studied the hepatotoxicity of F in mice found that it was apparent only at liver F concentrations higher than 1.8 mM (600 μg/g liver). They have suggested that P-450-mediated formation of reactive metabolite of F occurs at these concentrations. Since an alternative detoxification process was not examined, it remains speculative whether, at these liver concentrations, F might have caused inhibition of the conjugation with glucuronic acid and thus contributed to the extent of hepatic necrosis.

There is a well-established functional heterogeneity of UDP-GT activity in crude enzyme preparations and several classifications of the enzymatic activity have been suggested on the basis of their inducibility by three prototype xenobiotic inducers—3-MC, PB and PCN [11]—and on the basis of the ontogeny of the enzyme activity [16]. We have found that 3-MC and PCN pretreatment maximally increased F glucuronidation while PB had only minimal effect. We have also found that F glucuronidation was detected as early as day 18 of gestation and gradually increased and reached adult levels on day 6. This pattern was markedly different from the ontogeny of 1-naphthol glucuronidation, which like F, was markedly induced by 3-MC.

It seems, therefore, that different enzyme forms of UDP-GT which are independently regulated are involved in F glucuronidation. Additional studies are needed in order to clarify the role of FG formation in the non-renal elimination of the drug.

Acknowledgements—The authors would like to thank Dr Zoltan Gregus for helpful discussions, and Mrs Meliana Yong for her excellent technical assistance.

REFERENCES

1. D. E. Smith, E. T. Lin and L. Z. Benet, *Drug Metab. Dispos.* **8**, 337 (1980).
2. J. V. Aranda, C. Lambert, J. Perez, T. Turmen and D. S. Sitar, *J. Pediat.* **101**, 777 (1982).
3. G. Y. Yakatan, D. D. Maness, J. Scholler, W. M. J. Norick, Jr. and J. T. Doluisio, *J. pharmac. Sci.* **65**, 1456 (1976).
4. T. P. Green, L. P. Rybak, B. L. Mirkin, S. K. Juhn and T. Morizono, *J. Pharmac. exp. Ther.* **216**, 537 (1981).
5. J. Perez, D. S. Sitar and R. I. Ogilvie, *Drug Metab. Dispos.* **7**, 383 (1979).
6. B. Beerman, E. Dalen, B. Lindstrom and A. Rosen, *Eur. J. clin. Pharmac.* **9**, 57 (1975).
7. S. Tuck, P. Morselli, M. Broquaire and P. Vert, *J. Pediat.* **103**, 481 (1983).
8. J. A. Boutin, J. Thomassin, G. Siest, and A. Batt, *Pharmac. Res. Commun.* **16**, 227 (1984).
9. D. E. Smith and L. Z. Benet, *Eur. J. clin. Pharmac.* **24**, 787 (1983).

10. M. M. Hammarlund and L. K. Paalzon, *Biopharmac. Drug Dispos.* **3**, 345 (1982).
11. K. W. Bock, B. Burchell, G. J. Dutton, O. Hanninen, G. J. Mulder, I. D. Owens, G. Siest and T. R. Tephly, *Biochem. Pharmac.* **32**, 953 (1983).
12. G. Dutton, in *Glucuronidation of Drugs and other Compounds*. CRC Press, Boca Raton, FL (1980).
13. G. J. Wishart, *Biochem. J.* **174**, 485 (1978).
14. G. J. Wishart and M. T. Campbell, *Biochem. J.* **178**, 443 (1979).
15. J. B. Watkins and C. D. Klaassen, *Drug Metab. Dispos.* **10**, 590 (1982).
16. J. B. Watkins and C. D. Klaassen, *Drug Metab. Dispos.* **13**, 186 (1985).
17. A. Rachmel, G. A. Hazelton, A. L. Yergey and D. J. Liberato, *Drug Metab. Dispos.* **13**, 705 (1985).
18. R. S. Rapaka, J. Roth, C. T. Viswanathan, T. J. Goehl, V. K. Prasad and B. E. Cabana, *J. Chromatog.* **227**, 463 (1982).
19. T. Cresteil, J. P. Flinois, A. Pfister and J. P. Leroux, *Biochem. Pharmac.* **28**, 2057 (1979).
20. K. W. Bock, G. Brunner, H. Hoensch, E. Huber and D. Josting, *Eur. J. clin. Pharmac.* **14**, 367 (1978).
21. G. S. Rao, G. Haueter, M. L. Rao and H. Breuer, *Analyt. Biochem.* **74**, 35 (1976).
22. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
23. M. Dixon and E. C. Webb, *Enzymes*, 3rd edn. Academic Press, New York (1979).
24. J. R. Mitchell, W. L. Nelson, W. Z. Potter, H. A. Sasame and D. J. Jollow, *J. Pharmac. exp. Ther.* **199**, 41 (1976).
25. A. L. M. Kerremans, Y. Tan, C. A. M. Van Ginneken and F. W. J. Gribnau, *J. Chromatog.* **229**, 129 (1982).
26. L. M. Hjelmeland, *Proc. natn. Acad. Sci. U.S.A.* **77**, 6368 (1980).
27. H. Matern, S. Matern and W. Gerok, *Analyt. Biochem.* **133**, 417 (1983).
28. J. V. Dingell and N. Sossi, *Drug Metab. Dispos.* **7**, 61 (1979).
29. H. Matern, S. Matern and W. Gerok, *J. biol. Chem.* **257**, 7422 (1982).