The Submicrosomal Distribution of Hepatic Uridine Diphosphate Glucuronyltransferases in the Rabbit

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1. Rabbit liver microsomes were subfractionated into rough- and smooth-surfaced types, and glucuronyltransferase activity in each microsomal subfraction was determined with *p*-nitrophenol, *o*-aminophenol and phenolphthalein as substrates. The glucuronyltransferase activity measured with *p*-nitrophenol and *o*-aminophenol as substrates was localized predominantly in rough-surfaced microsomes. Glucuronyltransferase measured with phenolphthalein as substrate was equally present in rough- and smooth-surfaced microsomes. 2. Phenobarbital pretreatment of rabbits did not stimulate any of the glucuronyltransferase activities measured in either rough- or smooth-surfaced microsomes. 3. Preincubation of rabbit liver microsomes for 30–60 min. at 37° under oxygen did not cause any loss of glucuronyltransferase activity. Such preincubation caused either no change or increased enzyme activity in both submicrosomal fractions. The relative distribution of transferase activity in rough- and smooth-surfaced microsomes was not affected by preincubation.

In previous work, we investigated the submicrosomal distribution of a variety of hepatic NADPH-dependent drug-metabolizing enzymes and found many of these enzymes to be markedly concentrated in the smooth-surfaced microsomal subfraction (Fouts, 1961; Fouts, Rogers & Gram, 1966; Gram, Rogers & Fouts, 1967*a,b*; Gram & Fouts, 1967). A few of the enzyme activities studied were evenly distributed between smooth- and rough-surfaced microsomes, but none were preferentially concentrated in rough-surfaced microsomes.

UDP-glucuronyltransferase (EC 2.4.1.17) is a hepatic microsomal enzyme that catalyses the transfer of glucuronic acid from UDP-glucuronic acid to a variety of phenolic, alcoholic, carboxylic acid and amine acceptors (Levy & Storey, 1949; Dutton, 1961; Inscoc & Axelrod, 1960*a,b*; Isselbacher, 1956). Endogenous compounds such as bilirubin (Flint, Lathe, Ricketts & Silman, 1964), thyroxine and steroid hormones (Isselbacher, 1956) as well as foreign compounds (Williams, 1959) serve as substrates for this enzyme. Because of this broad substrate spectrum and other properties such as rate of post-natal development (Dutton, 1966*b*) and relative ease of solubilization (Isselbacher, Chrabas & Quinn, 1962), many investigators feel that

hepatic microsomes contain a family of UDP-glucuronyltransferases. It thus seemed possible that the transferases might have different submicrosomal distributions.

To our knowledge such a study of the submicrosomal localization of UDP-glucuronyltransferases in liver has not previously been made (see Dutton, 1966*a*, p. 217).

METHODS

Animals. Adult male Dutch rabbits weighing 1–2 kg. were used for all experiments and were allowed free access to laboratory chow and tap water. They were killed by a blow on the head, and livers were immediately excised and placed in beakers chilled in crushed ice. All subsequent tissue manipulations were performed at 0–4°.

Microsomal subfractionation. Smooth- and rough-surfaced microsomal subfractions were prepared from liver homogenates by the method of Dallner (1963) as described previously (Gram *et al.* 1967*a*; Gram & Fouts, 1967). Electron micrographs of the pellets and descriptions of their morphology and purity have been published (Dallner, 1963; Gram *et al.* 1967*a,b*). The subfractions were resuspended in 1.15% (w/v) KCl by gentle homogenization so that rough- or smooth-surfaced microsomes isolated from 1 g. wet wt. of liver were contained in 0.5 ml. of the final suspension. Because of the reported instability of the UDP-glucuronyltransferases (Isselbacher, 1956), incubations were always carried out within 1 hr. after resuspension of the microsomes.

Enzyme assays. UDP-glucuronyltransferase activity was studied with *p*-nitrophenol, *o*-aminophenol and phenolphthalein as substrates, essentially by the methods of

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Isselbacher *et al.* (1962), Dutton & Storey (1962) and Talalay, Fishman & Huggins (1946) respectively. Incubation mixtures consisted of the following components: 0.5 ml. of 0.5 M-tris-HCl buffer, pH 7.5 (containing 0.15 M-MgCl₂ when the substrate was *o*-aminophenol); 4 μ moles of UDP-glucuronic acid (ammonium salt, 98% pure, supplied by Sigma Chemical Co., St Louis, Mo., U.S.A.); 0.5 ml. of microsomal suspension; substrate, either *p*-nitrophenol (0.5 μ mole), *o*-aminophenol (0.42 μ mole, plus 2.3 μ moles of ascorbic acid as an antioxidant) or phenolphthalein (0.5 μ mole in 95% ethanol); water to a final volume of 3.0 ml. *p*-Nitrophenol (spectrophotometer grade) and phenolphthalein were obtained from Sigma Chemical Co. Technical grade *o*-aminophenol (Eastman Chemicals, Rochester, N.Y., U.S.A.) was purified by sublimation, as suggested by Dutton & Storey (1962). Incubations, in duplicate, were at 37° for 30 min. under N₂. *o*-Aminophenol glucuronide was assayed as described by Dutton & Storey (1962); after precipitation of the proteins from the incubation mixtures, samples were clarified by centrifugation at about 25000g for 20 min. before addition of the chromogenic reagents. Glucuronide formation from *p*-nitrophenol or from phenolphthalein was measured by substrate disappearance. Incubations were terminated by the addition of 95% ethanol, the mixtures were centrifuged, samples of the supernatant were made alkaline with 0.1 N-NaOH, and the extinction was determined at 400 m μ (for *p*-nitrophenol; Isselbacher *et al.* 1962) or 540 m μ (for phenolphthalein; Talalay *et al.* 1946). Glucuronide conjugation was estimated by ΔE between incubated and zero-time samples. That the disappearance of substrate was indeed the result of enzymic glucuronide formation was demonstrated by experiments with boiled microsomes and by omission of UDP-glucuronic acid from the reaction mixture. Under both these sets of conditions, no substrate disappearance occurred. Similarly, no synthesis of *o*-aminophenol glucuronide occurred in the absence of added UDP-glucuronic acid or when boiled microsomes were used as the tissue source.

Preliminary kinetic experiments demonstrated that substrate concentrations were at saturating values and that enzyme activities were proportional to the amount of enzyme protein used and to the incubation time employed.

Other methods. Nitrogen or protein content of the microsomal suspensions was determined by a micro-Kjeldahl procedure (Juchau, Cram, Plaa & Fouts, 1965) or by the technique of Lowry, Rosebrough, Farr & Randall (1951). Data were analysed statistically by Student's *t* test (Snedecor, 1956).

RESULTS

Submicrosomal distribution. Table 1 presents data on the submicrosomal distribution of UDP-glucuronyltransferase activities with *o*-aminophenol, *p*-nitrophenol and phenolphthalein as substrates. It is clear that the apparent distribution of enzyme activity varies with the substrate studied; thus with *o*-aminophenol or *p*-nitrophenol as substrate, activities were significantly concentrated in rough-surfaced microsomes, but the enzyme(s) catalysing glucuronide formation from phenolphthalein appeared to be evenly distributed between the two subfractions. These observations parallel a large body of information indicating that the behaviour and characteristics of UDP-glucuronyltransferases depend to a great extent on the substrate employed (Dutton, 1966a). Included in the present experiments for comparative purposes were assays of aniline hydroxylase, a microsomal NADPH-dependent drug-metabolizing enzyme that we have previously shown to be significantly concentrated in smooth-surfaced microsomes (Gram *et al.* 1967a; Gram & Fouts, 1967). The data in Table 1 seem to differentiate aniline hydroxylase and the UDP-glucuronyltransferases clearly on the basis of submicrosomal distribution. Fig. 1 shows that the relative differences in submicrosomal distribution of the UDP-glucuronyltransferases (Table 1) are independent of the UDP-glucuronic acid concentration. Thus with *p*-nitrophenol as substrate, the rough/smooth ratio of microsomal enzyme activities remained

Table 1. *Distribution of UDP-glucuronyltransferases in smooth- and rough-surfaced microsomes prepared from rabbit liver*

Microsomal subfractionation and incubation conditions are described in the Methods section. Values in parentheses are the numbers of animals used. Enzyme activities are expressed as μ mole of substrate metabolized (means $\pm$ S.D.)/mg. of microsomal N/30 min. No reference standard of *o*-aminophenol glucuronide was available; values in the Table for this pathway represent ΔE_{550} (incubated sample minus zero-time sample)/mg. of microsomal N/30 min. (means $\pm$ S.D.). Aniline hydroxylase activity was assayed as described by Gram *et al.* (1967a); assays for this enzyme activity require addition of an NADPH-generating system, as described by Gram *et al.* (1967a). Statistical comparisons (*P* values) were made on activity in rough-surfaced versus that in smooth-surfaced microsomes.

Substrate	Enzymic activities		<i>P</i>
	Smooth microsomes	Rough microsomes	
<i>p</i> -Nitrophenol	0.17 $\pm$ 0.03 (9)	0.35 $\pm$ 0.06 (9)	< 0.001
<i>o</i> -Aminophenol	0.104 $\pm$ 0.045 (8)	0.189 $\pm$ 0.058 (8)	< 0.005
Phenolphthalein	0.12 $\pm$ 0.03 (10)	0.12 $\pm$ 0.05 (10)	—
Aniline (hydroxylase)	0.23 $\pm$ 0.04 (5)	0.06 $\pm$ 0.03 (5)	< 0.001

relatively constant (about 2:1) at UDP-glucuronic acid concentrations varying from 2 to 12 μ moles/3 ml. (0.7–4 mM). Similar results were obtained with *o*-aminophenol as substrate. Fig. 1 also shows that with phenolphthalein as substrate the distribution of transferase activity in microsomal subfractions remains relatively constant at about unity across the range of UDP-glucuronic acid concentrations employed.

Effect of phenobarbital pretreatment. It has been demonstrated in a variety of animal species that the activities of many hepatic microsomal mixed-function oxidases can be markedly enhanced by the

administration to the animals of many foreign chemical compounds (Conney & Burns, 1962). Examples of such enzyme stimulators are barbiturates such as phenobarbital and aromatic hydrocarbons such as 3,4-benzopyrene and 3-methylcholanthrene. In addition, administration of 3,4-benzopyrene and 3-methylcholanthrene to rats has been shown (Inscoc & Axelrod, 1960b) to enhance significantly UDP-glucuronyltransferase activity with *o*-aminophenol as substrate. Therefore we studied the effect of pretreatment of rabbits with phenobarbital on UDP-glucuronyltransferase activities in microsomal subfractions. Rabbits were injected intraperitoneally with 15mg. of phenobarbital (sodium salt)/kg. once daily for 4 days and killed approximately 24 hr. after the last dose. Uninjected rabbits served as controls. In each experiment, two animals were used, one control and one phenobarbital-treated. Livers were homogenized, microsomes subfractionated and incubation and assay of the preparation from control rabbits and the preparation from phenobarbital-treated rabbits carried out side by side. The data are summarized in Table 2, and show that although aniline hydroxylase activities in both smooth- and rough-surfaced microsomes were significantly enhanced by phenobarbital administration, no significant increases were observed in UDP-glucuronyltransferase activities for any of the three substrates in either microsomal subfraction. Indeed, transferase activity toward *p*-nitrophenol was significantly decreased in rough-surfaced microsomes by the phenobarbital pretreatment. Additional experiments showed that injection of rabbits with phenobarbital in doses as high as 50mg./kg. daily for 4 days failed to stimulate UDP-glucuronyltransferases in microsomal subfractions even

Fig. 1. Relationship between UDP-glucuronic acid (UDPGA) concentration in the reaction mixture and UDP-glucuronyltransferase activity in hepatic microsomal subfractions. —, Rough-surfaced microsomes; ----, smooth-surfaced microsomes. ●, *p*-Nitrophenol; ▲, phenolphthalein. Experimental conditions were as described in the Methods section.

Table 2. Effect of pretreatment of rabbits with phenobarbital on the activities of several enzymes in hepatic microsomal subfractions

Treated animals received 15 mg. of phenobarbital/kg. intraperitoneally once daily for 4 days, and were killed 24 hr. after the last dose. See Table 1 for the definition of enzyme activities and the reference to the method used to assay for aniline hydroxylase activity. Phenobarbital pretreatment caused a significant decrease ($P < 0.001$) in glucuronidation of *p*-nitrophenol by rough-surfaced microsomes and significant increases ($P < 0.01$) in hydroxylation of aniline by both microsomal subfractions as compared with control activities in these fractions. No other changes in microsomal subfraction enzyme activity in the Table were significant ($P > 0.05$). Results are given as means $\pm$ S.D.; values in parentheses are the numbers of animals used.

Substrate	Enzyme activities			
	Control animals		Phenobarbital-pretreated animals	
	Rough microsomes	Smooth microsomes	Rough microsomes	Smooth microsomes
<i>p</i> -Nitrophenol	0.35 $\pm$ 0.06 (9)	0.17 $\pm$ 0.03 (9)	0.25 $\pm$ 0.07 (7)	0.14 $\pm$ 0.04 (7)
<i>o</i> -Aminophenol	0.189 $\pm$ 0.058 (8)	0.104 $\pm$ 0.045 (8)	0.227 $\pm$ 0.065 (5)	0.113 $\pm$ 0.031 (5)
Phenolphthalein	0.12 $\pm$ 0.03 (10)	0.12 $\pm$ 0.05 (10)	0.13 $\pm$ 0.04 (7)	0.13 $\pm$ 0.01 (7)
Aniline (hydroxylase)	0.06 $\pm$ 0.03 (5)	0.23 $\pm$ 0.04 (5)	0.14 $\pm$ 0.03 (5)	0.34 $\pm$ 0.06 (5)

though aniline hydroxylase activities were markedly enhanced under these circumstances.

Effect of preincubation on UDP-glucuronyltransferase activities in hepatic microsomal subfractions. Dutton (1966a) emphasized that the behaviour and characteristics of UDP-glucuronyltransferases depend largely on both the animal species and the substrate (acceptor) studied. It was thus thought that preincubation experiments might divulge some differences in enzyme stability either between substrates or between microsomal subfractions. Previous work in our laboratory has demonstrated differences in enzyme stability between microsomal subfractions for certain of the NADPH-dependent drug-metabolizing enzymes (Gram *et al.* 1967a).

Microsomal subfractions were preincubated with buffer and water (see the Methods section) at 37° under O₂ for either 30 or 60 min. Controls were stored in ice during this period. At the completion of preincubation, substrates and UDP-glucuronic acid were added, and the incubation was continued for an additional 30 min. under N₂. With none of the substrates used, *o*-aminophenol, phenolphthalein or *p*-nitrophenol, nor with either microsomal subfraction did we see any loss of enzyme activity due to preincubation; either control and preincubated samples had the same enzyme activity, or, in some cases, the preincubated preparations had slightly higher activity.

DISCUSSION

Previous investigations have distinguished the various UDP-glucuronyltransferases on the basis of the substrate (acceptor) employed. With *o*-aminophenol, *p*-nitrophenol, phenolphthalein, bilirubin and sterols, a family of transferases has been discovered having apparently different characteristics. Thus the various UDP-glucuronyltransferases have been differentiated on the basis of ease of solubilization (Isselbacher *et al.* 1962), rates of pre- and post-natal development (Dutton, 1966b), metal requirements (Tomlinson & Yaffe, 1960) and kinetic behaviour (Tomlinson & Yaffe, 1966). Our data indicate that the transferases may be further differentiated on the basis of submicrosomal distribution (Table 1). The distribution of the transferases differs diametrically from many of the microsomal NADPH-dependent drug-metabolizing enzymes, the latter being rather highly concentrated in the smooth-surfaced microsomes in both rats and rabbits (Gram *et al.* 1967a).

That some glucuronyltransferases may be in an entirely different kind of microsome from many of the NADPH-requiring drug-metabolizing enzymes suggests that normal transferase activity may be present in a liver having markedly depressed microsomal enzyme activity for many other drugs, and

vice versa. That some glucuronyltransferases may be equally distributed between smooth- and rough-surfaced microsomes (e.g. the transferase that conjugates phenolphthalein) suggests that significant differences in transferase activity may be seen in the same liver between transferase enzymes predominantly in rough-surfaced and those in smooth-surfaced microsomes, or transferases equally distributed between the two types of microsome. For example, if the transferase for the formation of sterol glucuronides were in smooth-surfaced microsomes, then a liver having little or no transferase for *p*-nitrophenol or *o*-aminophenol might have normal activity towards sterols and only slightly decreased activity toward phenolphthalein. Thus Drucker (1965) found that, in the Gunn rat, UDP-glucuronyltransferase activity towards *p*-nitrophenol was depressed, whereas that towards tetrahydrocortisone was normal in activity.

In support of Drucker's (1965) observations are those by Novikoff & Essner (1960), who found the parenchymal cells of Gunn rat liver to be relatively deficient in rough-surfaced endoplasmic reticulum, but to have relatively normal amounts of smooth-surfaced endoplasmic reticulum. In our opinion, a deficiency of rough-surfaced endoplasmic reticulum would be manifested as a deficiency of enzymes in rough-surfaced microsomes (e.g. *p*-nitrophenol glucuronyltransferase activity), and normal amounts of smooth-surfaced endoplasmic reticulum would suggest normal enzyme activities in smooth-surfaced microsomes (e.g., NADPH-requiring drug-metabolizing enzymes and any glucuronyltransferases localized there). The data of Inscoc & Axelrod (1960b) suggest that Gunn rats have normal enzyme activity in metabolizing acetanilide (an enzyme predominantly in smooth-surfaced microsomes), but are deficient in glucuronyltransferase measured with *o*-aminophenol as substrate (an enzyme in rough-surfaced microsomes).

Our experiments on the effect of preincubation of microsomes on glucuronyltransferase activity suggest that rabbit liver transferases in both rough- and smooth-surfaced microsomes are relatively stable enzymes. Rao & Taylor (1965), with progesterone as the acceptor (substrate), found that rat liver microsomes have a thermolabile glucuronyltransferase. Isselbacher (1956) also reported that rat liver microsomal glucuronyltransferases are unstable. Our present studies on the glucuronyltransferases are in agreement with our previous studies on NADPH-requiring microsomal drug-metabolizing enzymes (Gram *et al.* 1967a), in which we showed that the rabbit liver enzymes were more stable to preincubation than those from rat liver.

Our observation apparently indicating some activation of glucuronyltransferase activity in rough- and smooth-surfaced microsomes during

preincubation is in agreement with the work of Lueders & Kuff (1967). These authors reported a spontaneous activation of hepatic glucuronyltransferase (with *p*-nitrophenol as substrate) that occurs during storage of microsomes at 0–4°. Preincubation of microsomes may speed up this activation process, which occurs slowly (over several days) at 0–4° (Lueders & Kuff, 1967). What remains to be studied is whether such activation would complicate interpretation of induction of glucuronyltransferases by drugs such as phenobarbital. The discrepancy between our results, showing no effect of phenobarbital on glucuronyltransferases, and the report of Zeidenberg, Orrenius & Ernster (1967), who found that phenobarbital does stimulate *p*-nitrophenol conjugation, may be due to differences in degree of activation of the microsomes resulting from different preincubation conditions. In addition, there may be a species difference involved, since Zeidenberg *et al.* (1967) used rats and we used rabbits. It should be emphasized, however, that preincubation did not change the relative sub-microsomal distributions of the transferases that we studied. Before or after preincubation, *o*-aminophenol and *p*-nitrophenol glucuronyltransferases were predominantly in rough-surfaced microsomes and phenolphthalein glucuronyltransferase was equally distributed between rough- and smooth-surfaced microsomes.

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