Some Properties of the Uridine Diphosphate Glucuronyltransferase Activity Synthesizing Thio- β -D-glucuronides

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1. Some properties of the UDP-glucuronyltransferase synthesizing thio- β -D-glucuronides were investigated and compared with those of the enzyme synthesizing the *O*-glucuronides of analogous phenols. 2. Enzyme activity was generally similar for both classes of substrate in tissue distribution, intracellular location, optimum pH, perinatal development and induction by organ culture or by phenobarbital. 3. Certain differences were noted between the two types of activity in behaviour on storage and on activation, in kinetic behaviour and in distribution between Wistar and Gunn rats; the Gunn rats were not deficient in hepatic UDP-glucuronyltransferase activity towards *o*-aminothiophenol. 4. These differences are no greater than those exhibited in the synthesis of various *O*-glucuronides; therefore thiolic substrates could compete *in vivo* with phenolic compounds for access to the UDP-glucuronyltransferase complex as well as for UDP-glucuronic acid.

The microsomal enzyme UDP-glucuronyltransferase (EC 2.4.1.17) catalyses the glucuronidation of a wide variety of substrates. Its activity is markedly and often specifically altered by physical, chemical or enzymic treatments capable of changing microsomal membrane structure (Winsnes, 1969; Vessey & Zakim, 1971; see Dutton, 1971). As the enzyme has not been purified (Mowat & Arias, 1970; Howland *et al.*, 1971), the differing behaviour of UDP-glucuronyltransferase from any one species towards different substrates could be due to the existence of several related enzymes (Howland *et al.*, 1971) or to the physicochemical effect of the substrates on a single enzyme protein (Mulder, 1971). As many substrates are of metabolic, pharmacological or toxicological importance, the specificity of UDP-glucuronyltransferase has been widely studied.

Evidence for this specificity has come from work on the biosynthesis of *O*- or *N*-glucuronides.

UDP-glucuronyltransferase activity has been found to form *S*-glucuronides (Dutton & Illing, 1969, 1972; Illing & Dutton, 1970). We now report some properties of this enzyme towards substrates containing thiol or carbodithioic groups, and compare them with activities exhibited towards analogous phenolic substrates. A preliminary report of this work has appeared (Illing & Dutton, 1972).

Experimental

Materials

Acceptor and donor substrates. These were obtained and purified as described previously (Dutton & Illing, 1972).

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Other compounds. These were: diethylnitrosamine (Eastman Organic Chemical Co., Kodak Ltd., Kirkby, Liverpool, U.K.); digitonin, A.R. (BDH Chemicals Ltd., Poole, Dorset, U.K.); dithiothreitol [Sigma (London) Chemical Co., London S.W.6, U.K., or BDH]; phenobarbitone (Na⁺ salt) (BDH); Triton X-100 (Sigma); UDP-*N*-acetylglucosamine (Sigma). β -Glucuronidase and its specific inhibitor D-glucaro-(1 $\rightarrow$ 4)-lactone were prepared as described by Dutton & Illing (1972).

Animals. Mice were mixed laboratory stock of either sex; foetal ages were estimated by morphology and crown-rump length (Gruneberg, 1943). Rats, of either sex, were of the Wistar or Gunn strain; the latter were bred from stock kindly given by Dr. B. H. Billing. Chickens and chick embryos were Brown Leghorn; eggs were collected at day 0 and incubated at 39°C.

Methods

Tissue culture. Five segments (approx. 15 mg wet wt.) of 11-day chick-embryo liver were cultured on Millipore rafts (25 μ m thick, 0.45 μ m pore size) supported on a nylon-mesh platform in an organ-culture dish (Falcon Plastics) containing 1.2 ml of chemically defined medium (with or without 5.5 mM-sodium phenobarbital), as described by Burchell *et al.* (1972). Cultured tissue was removed at intervals, washed and sonicated; separate portions of the sonicate were used for enzyme and for protein assay.

Tissue preparation for enzyme assay. Homogenates were prepared as previously described (Dutton & Illing, 1972); they were 10, 30 and 30% (w/v) for liver, kidney and washed stomach respectively. For centrifugal fractionation, homogenates were prepared in 0.25 M-sucrose–1 mM-EDTA (pH 7.0–7.5); they

were centrifuged at 400*g*_{av.} for 5 min, the debris was discarded and the supernatant spun at 8000*g*_{av.} for 20 min; the resulting pellet was washed once and resuspended in the sucrose-EDTA medium (the 'nuclear-mitochondrial fraction'). The 'microsomal fraction' was spun down from the supernatant at 100000*g*_{av.} for 30 min, washed once and resuspended similarly. The remaining supernatant was the 'soluble fraction'. Sonicates were prepared with a Dawe Soniprobe 1130A apparatus of 6 mm ($\frac{1}{4}$ in) micro-tip. Ten liver segments were removed from culture rafts and washed. They were then placed in 2.8 ml of 43 mM-Tris-HCl buffer, pH 7.4, and sonicated at 0°C, with three bursts of 10 s each; 0.23 ml samples of sonicate were added, in place of the quoted buffer and tissue sample, to the incubation medium described below for *p*-nitrophenol or *p*-nitrothiophenol.

Enzyme incubation media. With *o*-aminothiophenol or diethyldithiocarbamic acid as substrate, the media (unless otherwise indicated) were as previously described (Dutton & Illing, 1972). The medium for *p*-nitrothiophenol and *p*-nitrophenol contained 0.20 mM-substrate, 2.9 mM-UDP-glucuronic acid, 32 mM-Tris-HCl buffer, pH 7.4, 2.6 mM-dithiothreitol and mouse liver microsomal suspension (containing 1.2–1.8 mg of protein) in a total volume of 0.30 ml. For thiophenol, the medium was 0.39 mM-thiophenol, 2.9 mM-UDP-[U-¹⁴C]glucuronic acid (93 μ Ci/mmol unless stated otherwise), 52 mM-Tris-HCl buffer, pH 7.4, 16 mM-MgCl₂ and 0.1 ml of 10% (w/v) liver homogenate (or corresponding microsomal preparation) in a total volume of 0.38 ml. Incubations were for 20 min at 37°C under N₂.

Results quoted are means of duplicates which did not differ by more than $\pm 5\%$. Controls were as used by Dutton & Illing (1972).

Isolation of thioglucuronides. The thio- β -D-glucuronides of diethyldithiocarbamic acid, *p*-nitrothiophenol and thiophenol were isolated and identified as described by Dutton & Illing (1972). Glucuronides of *o*-aminophenol and *o*-aminothiophenol were isolated by t.l.c. on silica gel G in ethylmethyl ketone-propanol-water (15:5:6, by vol.), in which they had *R_F* values of 0.30 and 0.32 respectively, against *R_F* values of 0.90 and 0.92 for the respective free aglycones, and 0–0.10 for UDP-[U-¹⁴C]glucuronic acid and its radioactive breakdown products.

Assays of glucuronide formation. With *o*-aminophenol, *p*-nitrothiophenol and *p*-nitrophenol, these were the colorimetric procedures described by Dutton & Illing (1972). Glucuronides of the other substrates were isolated as the radioactive conjugates and counted for radioactivity; the conditions and efficiencies are described by Dutton & Illing (1972), who demonstrate the quantitative nature of this procedure. Identification procedures clearly distinguished the thioglucuronides from their *O*-analogues.

Protein assay. This was by Oyama & Eagle's

(1956) modification of Lowry's method (Lowry *et al.*, 1951).

Results

Distribution of enzyme in tissues and species

Tissue distribution of the UDP-glucuronyltransferase activity forming *S*-glucuronides resembled that forming the *O*-conjugates (Stevenson & Dutton, 1962). Liver, kidney and stomach formed *S*-glucuronides of the substrates offered. Broken-cell preparations from mouse liver displayed activity towards *o*-aminophenol, diethyldithiocarbamic acid, *p*-nitrothiophenol and thiophenol; from Wistar rat liver, towards *o*-aminothiophenol and *p*-nitrothiophenol; and from chick liver, towards *p*-nitrothiophenol and thiophenol. Similar preparations from mouse kidney and stomach displayed activity towards *p*-nitrothiophenol and thiophenol; and from sheep kidney, towards thiophenol.

In kidney, the activity towards thiophenol, like that towards *o*-aminophenol (Stevenson & Dutton, 1962), occurs preferentially in the cortex. Sheep kidney cortex and medulla were homogenized separately or together and assayed for enzyme activity towards thiophenol; 0.46 nmol of glucuronic acid was conjugated/20 min per mg of protein with cortex + medulla, 0.17 nmol with medulla and 0.84 nmol with cortex.

The conjugate formed with *p*-nitrothiophenol in mouse kidney microsomal preparations was hydrolysed by β -glucuronidase. Hydrolysis was measured colorimetrically (Hétu & Gianetto, 1970). Of a total of 11 nmol of conjugate, 9.9 nmol (90%) was hydrolysed by β -glucuronidase after 3 h in the absence of glucaro-(1 $\rightarrow$ 4)-lactone; when the lactone (17 mM) was present only 1.1 nmol (10%) was hydrolysed. Similarly, the mouse stomach microsomal fraction formed a conjugate with *p*-nitrothiophenol (9.4 nmol), of which 9.1 nmol (97%) was hydrolysed by β -glucuronidase in 3 h; when 10 mM-glucaro-(1 $\rightarrow$ 4)-lactone was present only 1.1 nmol (12%) was hydrolysed.

Relative activities. UDP-glucuronyltransferase activity towards *p*-nitrothiophenol was determined colorimetrically in fresh homogenates of various tissues from the same mouse; mean results from three animals, expressed in nmol of *p*-nitrothiophenol conjugated/20 min per mg of protein were: liver, 8.4; kidney, 4.2; stomach, 4.3. In microsomal fractions activity towards *p*-nitrothiophenol was similar in all three tissues (Table 1).

Under the identical conditions of incubation employed, activities towards *p*-nitrophenol and *p*-nitrothiophenol were similar; in liver microsomal preparations the respective mean activities were 1.1:1.0 (mouse, nine animals), 1.2:1.0 (Wistar rat, four

Table 1. Intracellular location of UDP-glucuronyltransferase activity towards thiol substrates

Preparation of cellular fractions and enzyme assay were as described in the Experimental section. In each experiment, fractions compared are from one homogenate preparation; * indicates the presence of unbroken cells and debris due to omission of preliminary fractionation at 400g for 2min. Each value is the mean of duplicates differing by less than $\pm 5\%$.

Expt. no.	Species	Tissue	Substrate	Substrate conjugated (nmol/20 min per mg of protein)		
				Nuclei+ mitochondria	Microsomal fraction	High-speed supernatant
1	Chicken	Liver	<i>p</i> -Nitrothiophenol	1.1	32.0	0.9
2	Mouse	Liver	<i>p</i> -Nitrothiophenol	0	13.0	0
3	Mouse	Liver	Thiophenol	2.0	8.1	1.6
4	Mouse	Liver	Thiophenol	3.5	16.0	2.0
5	Mouse	Kidney	<i>p</i> -Nitrothiophenol	11.0	15.6	0
6	Mouse	Kidney	<i>p</i> -Nitrothiophenol	4.3	15.1	0
7	Mouse	Kidney	Thiophenol	0.7*	1.3	0.1
8	Mouse	Kidney	Thiophenol	0	3.4	0
9	Mouse	Stomach	<i>p</i> -Nitrothiophenol	0.6	16.0	0
10	Mouse	Stomach	<i>p</i> -Nitrothiophenol	6.7*	12.8	1.3

Table 2. UDP-glucuronyltransferase activities in Wistar and Gunn rats

Rat liver microsomal preparations were incubated with the required substrate and glucuronide formation was assayed as described in the text. Procedures for the determination of *p*-nitrophenol and *p*-nitrothiophenol were colorimetric, and those for *o*-aminophenol and *o*-aminothiophenol were from radioactivity of the isolated conjugate; with *o*-aminophenol, the same incubation mixture was assayed colorimetrically as a check; these results are given in parentheses. * indicates that diethylnitrosamine was present at 16mM during the incubation. Phenolic and thiolic substrates were always assayed together, by using the same microsomal preparation at the same time. In expressing the ratio (a)/(b), the radioactivity assay for *o*-aminophenol was employed.

Rat no.	Rat strain	Glucuronide formed (nmol/20 min per mg of protein)		Ratio (a)/(b)
		(a) <i>p</i> -Nitrophenol	(b) <i>p</i> -Nitrothiophenol	
1	Wistar	11.3; 12.4*	9.3; 9.3*	1.2; 1.3*
2	Wistar	21.9; 24.1*	20.0; 24.0*	1.1; 1.0*
3	Wistar	14.9; 14.9*	11.3; 11.3*	1.3; 1.3*
4	Wistar	24.0; 24.0*	23.0; 23.0*	1.0; 1.0*
5	Gunn	12.0; 12.0*	11.5; 11.5*	1.0; 1.0*
6	Gunn	23.0; 23.0*	22.7; 22.7*	1.0; 1.0*
		(a) <i>o</i> -Aminophenol	(b) <i>o</i> -Aminothiophenol	
7	Wistar	(2.0), 1.9; (3.5)*, 3.1*	3.5; 3.1*	0.5; 1.0*
8	Wistar	(2.6), 3.1; (4.3)*, 4.3*	3.4; 3.5	0.9; 1.2
9	Wistar	2.0; 3.5*	3.5; 3.1*	0.6; 1.1*
10	Gunn	(0.4), 0.5; (3.1)*, 3.0*	4.7; 3.6*	0.1; 0.9*
11	Gunn	(0.5), 0.5; (3.3)*, 3.9*	2.9; 2.8*	0.2; 1.4*
12	Gunn	(0.3), 0.5; (1.9)*, 2.0*	2.3; 2.0*	0.2; 1.0*
13	Gunn	(0.2), 0.2; (4.0)*, 3.8*	3.4; 4.3*	0.1; 0.9*
14	Gunn	0.2; 4.0*	3.1; 2.9*	0.1; 1.4*
15	Gunn	(0.2), 0.2; (4.5)*, 4.7*	3.6; 3.6*	0.1; 1.3*

animals), 1.0:1.0 (Gunn rat, two animals). Activity towards *o*-aminothiophenol was higher than towards *o*-aminophenol in both Gunn and Wistar rats (see Table 2).

Intracellular location of enzyme. Nuclear-mitochondrial, microsomal and soluble fractions of homogenates were prepared and assayed for UDP-glucuronyltransferase activity. Results (Table 1) indicate greatest activity towards *p*-nitrophenol and thiophenol in the microsomal fractions from liver, kidney and stomach tissue. These fractions are also the site of *O*-glucuronide synthesis (see Dutton, 1966a). Similarly, diethylthiocarbamoyl thioglucuronide was formed principally in the microsomal fraction of mouse liver homogenate.

Optimum pH of reaction

Buffer pH was measured by glass electrode and checked in reaction mixtures before and after incubation. Controls in which UDP-glucuronic acid was added only after incubation were run at each pH value. Results indicate pH optima in the range most commonly found for the *O*-conjugates (see Dutton, 1971).

p-Nitrothiophenol and *p*-nitrophenol were substrates in parallel experiments; the pH of the glycine buffer used in the colorimetric assay was checked in each tube. The pH curve for *p*-nitrothiophenol shows a sharp peak at pH 7.4 (Fig. 1); that for *p*-nitrophenol has a broad optimum over pH 7.3–7.8, as found by Winsnes (1969). With thiophenol as substrate, a broad optimum pH existed from pH 7.3 to 7.8.

Activation of UDP-glucuronyltransferase

UDP-glucuronyltransferase activity is increased *in vitro* by several procedures (see Dutton, 1971). The effect of some of these procedures on the enzymic activities towards phenolic and aromatic thiol substrates was compared. Results were broadly similar, but activity towards the thiol substrates was generally increased less than towards the phenolic substrates.

Effect of Mg^{2+} . $MgCl_2$, at final concentrations of 10, 20 30 and 40mM, was added to mouse liver microsomal preparations. It did not affect UDP-glucuronyltransferase activity towards either *p*-nitrothiophenol or *p*-nitrophenol, as also noted by Storey (1965) for *p*-nitrophenol.

Aging of microsomal preparations. Mouse liver microsomal preparations were stored at 4°C (Fig. 2). Activity towards *p*-nitrophenol was markedly increased on storage, confirming the finding of Winsnes (1969), but that towards *p*-nitrothiophenol was not increased. Similar storage at 37°C increased activity towards *p*-nitrophenol maximally (fourfold) after

Fig. 1. Optimum pH for UDP-glucuronyltransferase activity

Incubation mixtures for *p*-nitrophenol (○) and *p*-nitrothiophenol (●) contained mouse liver microsomal preparations and were incubated and assayed simultaneously at each pH value for both substrates as described in the text.

Fig. 2. Activation of UDP-glucuronyltransferase by storage

Mouse liver microsomal preparations were stored in 0.25M-sucrose–1mM-EDTA medium for various times at 0–4°C. Enzyme activity was assayed (see the Experimental section) simultaneously towards *p*-nitrophenol (○) and *p*-nitrothiophenol (●). Activity of fresh preparations is shown as unity. Graphical points are means of two experiments whose results did not differ by more than $\pm 9\%$; duplicates in each experiment did not differ by more than $\pm 5\%$.

Table 3. *Activation of UDP-glucuronyltransferase towards p-nitrothiophenol and p-nitrophenol*

Enzyme activity in fresh mouse liver microsomal preparations was assayed as described in the Experimental section; digitonin, Triton X-100 or UDP-*N*-acetylglucosamine was present during incubation as shown. Activation of enzyme is expressed below as a multiple of the activity in the absence of activator. Without activator, activity was similar for both substrates: 12.8 and 12.0nmol were conjugated respectively of *p*-nitrophenol and *p*-nitrothiophenol in Expt. 1, 7.5 and 7.2nmol in Expt. 2 and 12.0 and 10.0nmol in Expt. 3. The two substrates were assayed simultaneously in each experiment; duplicates did not differ by more than $\pm 2\%$.

Expt. no.	Activator	Final concn.	Activation factor		
			(a) <i>p</i> -Nitrophenol	(b) <i>p</i> -Nitrothiophenol	Ratio (a)/(b)
1	Triton X-100	0.01 % (v/v)	1.0	1.2	0.8
		0.02 % (v/v)	2.6	1.1	2.5
2	Digitonin	0.10 % (w/v)	1.4	1.4	1.0
		0.20 % (w/v)	3.4	1.2	2.5
3	UDP- <i>N</i> -acetylglucosamine	2.2mM	2.1	1.3	1.8

Table 4. *Induction of UDP-glucuronyltransferase activity*

Pooled livers from 11-day chick embryos were cultured as described in the text and assayed for UDP-glucuronyltransferase activity towards *p*-nitrophenol and *p*-nitrothiophenol; * indicates that phenobarbital (5.5mM) was present in the culture medium. Eggs were injected at 17 days as indicated, the embryo liver was removed at 20 days and enzyme activity assayed. Results (a) and (b) are from separate experiments.

Enzyme source	Glucuronide formed (nmol/20min per mg of protein)			
	<i>p</i> -Nitrophenol		<i>p</i> -Nitrothiophenol	
	(a)	(b)	(a)	(b)
11-day chick-embryo liver:				
Fresh	0	0	0	0
Cultured for 3 days	35.3; 58.1*	26.0; 39.0*	40.0; 64.9*	34.0; 76.0*
Cultured for 6 days	32.0; 96.0*	48.0; 109.0*	47.0; 140.0*	54.0; 233.0*
20-day chick-embryo liver:				
From untreated egg		0		0
From egg injected with [for (a)] 2.1 mg of phenobarbital, or [for (b)] 4.2mg of phenobarbital in 1 ml of water on day 17	0	12.0	12.0	26.0
3-day-old hatched-chick liver	21.0	19.6	20.0	28.0

3h; activity towards *p*-nitrothiophenol reached a maximum (1.8-fold) after 2–3 h. If 1mM-EDTA was omitted from the storage medium, the same pattern was observed but deactivation towards both substrates began sooner.

Effect of detergents. Triton X-100 or digitonin was added to incubation mixtures containing microsomal preparations, either fresh or aged for 3 days at 4°C. In fresh preparations, both detergents slightly increased UDP-glucuronyltransferase activity towards *p*-nitrothiophenol; 0.01 % (v/v) Triton X-100

and 0.07–0.10 % (w/v) digitonin gave maximal activation. However, activation by detergents towards *p*-nitrophenol was maximal when that towards the thiol substrate was beginning to be inhibited (Table 3). In aged preparations, enzyme activities were not increased by either detergent but progressively inhibited, as found by Winsnes (1969) with *p*-nitrophenol.

Effect of UDP-*N*-acetylglucosamine. This specific activator of UDP-glucuronyltransferase (Pogell & Leloir, 1961) increased synthesis of both *O*- and

S-glucuronides, but again with lesser effect on the latter. With 2.8 mM-UDP-*N*-acetylglucosamine, activity towards *o*-aminophenol rose 2.8 times in chick liver microsomal preparations and 2.5 times in those from mouse liver; corresponding activations towards thiophenol were 1.3-fold and 1.8-fold respectively. A similar pattern occurred with *p*-nitrophenol and *p*-nitrothiophenol (Table 3). These activations by UDP-*N*-acetylglucosamine were not evident in mouse liver microsomal preparations aged for 3 days at 4°C, confirming the observation of Winsnes (1969) on *O*-glucuronide formation.

Kinetics of reaction

Reaction velocity was linear up to 30 min; it was measured for the first 10 min and is expressed below as pmol of glucuronide formed/min per 150 µg of microsomal protein.

The first series of experiments employed 2.9 mM-UDP-glucuronic acid with various concentrations of acceptor substrate.

Fresh, unactivated UDP-glucuronyltransferase preparations display anomalous Michaelis-Menten kinetics (Winsnes, 1969, 1972; Vessey & Zakim, 1972) typical of the phenomenon of 'negative co-operativity' (Levitzki & Koshland, 1969). This effect was confirmed with *p*-nitrophenol and was almost exactly reproduced with *p*-nitrothiophenol as substrate (Fig. 3*a*). When either acceptor was below 0.25 mM, $V_{\max}$ and apparent K_m values were respectively 110–120 pmol and 0.20 mM-substrate; above 0.25 mM-substrate, the values were 500 pmol and 3.6 mM respectively.

When the enzyme was activated by 2.2 mM-UDP-*N*-acetylglucosamine, the 'negative co-operativity' disappeared, as noted by Winsnes (1969, 1972) for *p*-nitrophenol, and differences in behaviour towards

Fig. 3. Kinetic behaviour of UDP-glucuronyltransferase

The double-reciprocal plots shown were obtained under conditions described in the text, with *p*-nitrophenol (○) and *p*-nitrothiophenol (●) as acceptor substrates and UDP-glucuronic acid as donor substrate. The enzyme source was mouse liver microsomal preparations, fresh in (a) and (c), activated by 2.2 mM-UDP-*N*-acetylglucosamine in (b) and (d). Curves (a) and (b) were obtained with fixed donor and various acceptor substrate concentrations; (c) and (d) with fixed acceptor and various donor substrate concentrations. For all curves v is expressed as pmol of glucuronide formed/min per 150 µg of microsomal protein; for (a) and (b) [S] is expressed as mM-acceptor substrate, and for (c) and (d) as mM-UDP-glucuronic acid.

the two acceptor substrates became apparent (Fig. 3b). For *p*-nitrophenol, the value of $V_{\max}$ was 130 pmol and the apparent K_m value was 0.09 mM; corresponding values for *p*-nitrothiophenol were 200 pmol and 0.3 mM respectively.

The second series of experiments employed 0.20 mM-acceptor substrate and varied the amount of UDP-glucuronic acid. Again, differing behaviour towards *p*-nitrophenol and its thiol analogue appeared on activation of the enzyme. With unactivated enzyme, the apparent K_m value for UDP-glucuronic acid was 1.1 mM with either acceptor, and the $V_{\max}$ value remained similar at 80–100 pmol (Fig. 3c). On activation of the enzyme by 2.2 mM-UDP-*N*-acetylglucosamine (Fig. 3d), the value of $V_{\max}$ rose to 200–250 pmol with both substrates, but the apparent K_m value for UDP-glucuronic acid was 0.08 mM with *p*-nitrophenol and 3.0 mM with *p*-nitrothiophenol. The 'negative co-operativity' observed by Winsnes (1972) with the native enzyme at high UDP-glucuronic acid concentrations was only observed if EDTA was omitted during preparation of the microsomal fraction.

The difference in kinetic behaviour towards *p*-nitrophenol and *p*-nitrothiophenol, which is brought about by activation of the enzyme is consistent with the differences observed in Fig. 2 and Table 3.

Induction of UDP-glucuronyltransferase

Induction by natural development. Activities of UDP-glucuronyltransferase towards *p*-nitrothiophenol and *p*-nitrophenol were examined simultaneously in homogenates of mouse liver at various ages to detect any differences in rate of enzyme development. Activity towards *p*-nitrophenol developed as reported by Dutton (1966a,b) and activity towards *p*-nitrothiophenol increased in a virtually parallel manner, most obviously when enzyme development was followed through an individual litter (Fig. 4). In livers both from 18-day foetuses and from 2-day-old infants the ratio of *p*-nitrophenol conjugated/*p*-nitrothiophenol conjugated was 1.2:1.0. This value reflects the ratio of the means for the adult, which was 1.1:1.0. There is clearly greater similarity between development of these activities synthesizing

Fig. 4. Development of UDP-glucuronyltransferase activity

UDP-glucuronyltransferase activity towards *p*-nitrothiophenol (solid symbols) and *p*-nitrophenol (open symbols) in liver of mice of various ages is shown. The incubation mixture and assay were as described in the Experimental section, but with 0.1 ml of homogenate as enzyme source. UDP-glucuronic acid concentration was not significantly limiting under these conditions. Square and triangular symbols linked by broken lines represent successive determinations on members of the same litter; otherwise livers from litters of identical ages were pooled for each determination, both substrates being tested with the same enzyme preparation. The range of adult values shows the mean $\pm$ s.d. for nine mice (*p*-nitrothiophenol) and ten mice (*p*-nitrophenol).

O- and *S*-links than between those synthesizing certain *O*-links (see Dutton, 1966b).

Induction by culture and phenobarbital. UDP-glucuronyltransferase, unlike other enzymes studied, develops precociously from near-zero activity in chick-embryo liver cultured in simple media without known inducers (Ko *et al.*, 1967; Skea & Nemeth, 1969; Burchell *et al.*, 1972). Activity towards *p*-nitrothiophenol was compared with that towards *p*-nitrophenol in organ cultures of 11-day embryo liver to see if both activities were induced. Phenobarbital stimulates induction of the enzyme in cultured 11-day embryo liver (Dutton *et al.*, 1970; Burchell *et al.*, 1971, 1972) and in 17–20-day embryo liver *in ovo* (G. J. Dutton, G. Wishart & B. Burchell, unpublished work), over-riding controls developed by the embryonic cells; the drug was therefore added to parallel cultures, and also injected into eggs at 17 days.

Results (Table 4) indicate that activity towards both substrates was absent at the start of culture and developed precociously to above the values in hatched-chick liver; this development was stimulated by 5.5 mM-phenobarbital in the medium. If eggs were injected with phenobarbital at 17 days, then again both activities had been stimulated when the liver was examined at 20 days; the stimulation appeared to be dose-dependent. Induction of UDP-glucuronyltransferase activity in embryo liver by isolation or by exposure in culture or *in ovo* to phenobarbital is therefore broadly similar towards both *p*-nitrophenol and its thiol analogue.

UDP-glucuronyltransferase activity in the Gunn rat

The Gunn strain of rat is deficient in UDP-glucuronyltransferase activity towards several substrates (see Dutton, 1971). As it usually possesses 'normal' activity towards *p*-nitrophenol and defective activity towards *o*-aminophenol, conjugation of these two substrates was compared with that of their thiol analogues in Gunn and Wistar rats.

Liver microsomal preparations from both strains were incubated with *o*-aminophenol or *o*-aminothiophenol. The *o*-aminophenyl glucuronide formed was assayed colorimetrically and by radioactivity; the assays agreed. The thioglucuronide was measured by radioactivity only. The two radioactivity assays were compared. Enzyme activities towards *p*-nitrophenol and *p*-nitrothiophenol also were compared in the two strains by colorimetric assay (Table 2).

Specific activities of UDP-glucuronyltransferase towards *p*-nitrophenol and *p*-nitrothiophenol were similar in individual rats of both strains. With *o*-aminophenol and *o*-aminothiophenol, however, activity towards the thiol analogue appeared higher than towards *o*-aminophenol in Wistar rats and markedly higher in Gunn rats.

Diethylnitrosamine activates UDP-glucuronyltransferase in rat liver microsomal preparations towards *o*-aminophenol but not towards *p*-nitrophenol (Stevenson *et al.*, 1968). Diethylnitrosamine (16 mM) did not affect activity towards *p*-nitrophenol or *p*-nitrothiophenol, but it confirmed the difference already noted between the activities towards *o*-aminophenol and *o*-aminothiophenol. Diethylnitrosamine raised the activity towards *o*-aminophenol up to that already existing towards *o*-aminothiophenol, which remained unchanged; in Gunn rats this differential effect on the two UDP-glucuronyltransferase activities was striking (Table 2). The defective accessibility of *o*-aminophenol to the enzyme in Gunn-rat liver microsomal preparations depends therefore on the oxygen atom in the phenolic group; replacement of this atom by a sulphur atom allows 'normal' access of substrate.

Discussion

Because of the insolubility of UDP-glucuronyltransferase, its lipid-dependence and its activation by substrates or other compounds, studies on the kinetics, specificity and absolute amounts of the enzyme have had to be viewed with caution (Dutton, 1966a, 1971). Although recent work (Winsnes, 1972; Vessey & Zakim, 1972) promises some clarification of the problem, the difficulty of correlating such studies with the situation *in vivo* still remains.

The above results, however, indicate that the UDP-glucuronyltransferase(s) forming the thio- β -D-glucuronides investigated is generally similar in distribution, development and properties to the enzyme(s) known to form *O*- β -D-glucuronides. The differences noted between activities towards certain aromatic thiols and their phenolic analogues, e.g. in storage, activation, kinetic behaviour or in distribution among rat strains, are no greater, and are often less, than the differences already found between activities towards various phenolic substrates themselves. They may reflect lipoprotein conformation and the transport of substrates, for it is notable that differences in enzymic behaviour towards *p*-nitrophenol and its thiol analogue were most evident during and after activation. These two substrates afford a good model for the detailed kinetic investigation proposed by Vessey & Zakim (1972), particularly for examining the extent to which the different apparent K_m values for UDP-glucuronic acid depend on those for the respective acceptor substrates. A further promising model would be the behaviour of *o*-aminophenol and its thiol analogue with the enzymes from Gunn and Wistar rats.

Any separate UDP-glucuronyltransferase specificities that exist will presumably concern physiological substrates; exogenous 'foreign' substrates may often be expected to straddle the boundaries of these

physiological specificities and therefore not be reliable guides to the degree of heterogeneity of the enzyme (Dutton, 1971). We can, however, conclude that 'foreign' thioglucuronides derived from drugs, pesticides and industrial chemicals are synthesized by the UDP-glucuronyltransferase complex dealing with *O*-glucuronidation, and that such thiol substrates may compete *in vivo* with *O*-glucuronidation not only for UDP-glucuronic acid but also at times for the enzyme active centre, as suggested by Storey (1965).

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