The Heterogeneity of Uridine Diphosphate Glucuronyltransferase from Rat Liver

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1. The glucuronide conjugation of *p*-nitrophenol, phenolphthalein, *o*-aminophenol and 4-methylumbelliferone by rat liver microsomes has been studied. The detergent Triton X-100 activated UDP-glucuronyltransferase activity towards all these substrates, therefore the optimum activating concentration was added in all experiments. 2. Mg^{2+} enhanced the conjugation of the substrates. 3. With phenolphthalein substrate inhibition occurred but this could be relieved by adding albumin, which binds excess of phenolphthalein. 4. Kinetic constants of the substrates and UDP-glucuronate have been determined. Mutual inhibition was found with the substrates *p*-nitrophenol, 4-methylumbelliferone and phenolphthalein. *p*-Nitrophenol conjugation was inhibited competitively by phenolphthalein and 4-methylumbelliferone. 5. *o*-Aminophenol did not inhibit the conjugation of the other three substrates because these are conjugated preferentially to *o*-aminophenol. 6. It is concluded that the four substrates are conjugated by one enzyme at the same active site.

Different substrates have been used to determine the activity of the enzyme UDP-glucuronyltransferase (UDP-glucuronate glucuronyltransferase; EC 2.4.1.17). Differences in properties of the enzymic activity for different substrates have often led to the suggestion that there are several UDP-glucuronyltransferases, differing in substrate specificity (see Dutton, 1966, and the Discussion section). Mulder (1970) suggested that this apparent heterogeneity of the enzyme might be due, at least in part, to the microsomal localization of the enzyme, with the membrane environment playing a role in determining the specificity of the enzyme towards substrates.

Until recently nearly all the published results on the properties of UDP-glucuronyltransferase have been obtained by using enzyme preparations that were not activated, but it is now known that very marked activation is produced by treatment of the enzyme preparations with detergents (Lueders & Kuff, 1967) and dialysis against EDTA-mercaptoethanol (Halac & Reff, 1967; Halac & Scignano, 1969). Winsnes (1969) found that the addition of detergents *in vitro* activated UDP-glucuronyltransferase activity towards five substrates tested by him. This activation is not only apparent for UDP-glucuronyltransferase but is also found with other microsomal enzymes (Stetten, Malamed & Federman, 1969; Menon & Haberman, 1970).

It was suggested (Mulder, 1970) that the use of non-activated enzyme preparations might affect

the observed properties of the enzyme-substrate reaction. Since this latency of the enzyme *in vitro* may be an artifact of the homogenization procedure a re-investigation of some properties of UDP-glucuronyltransferase activity seemed indicated. The glucuronide conjugation of *p*-nitrophenol, phenolphthalein, 4-methylumbelliferone and *o*-aminophenol was studied after activation of the enzyme preparations with the detergent Triton X-100. In contrast with the findings of other investigators (Storey, 1965; Gram, Hansen & Fouts, 1968; Temple, Clement & Done, 1968), the results presented below strongly suggest the existence of only one type of enzyme and a common active site for these substrates.

MATERIALS AND METHODS

Animals. Male rats (Wistar, Organisatie voor toegepast Natuurwetenschappelijk Onderzoek, Zeist, The Netherlands) weighing 200-260 g and having free access to food and water were used. The animals were decapitated between 8 and 9 a.m.

Enzyme preparation. Unless otherwise stated the enzyme preparation used in the experiment was a post-nuclear supernatant, obtained by centrifuging a 20% (w/v) rat liver homogenate in 0.154 M-KCl for 10 min at 1470g.

Enzyme assays. UDP-glucuronyltransferase activity towards *p*-nitrophenol was measured as described by Mulder (1970) in 50 mM-sodium phosphate buffer, pH 7.3, without addition of Mg^{2+} ions.

The activity towards phenolphthalein was measured in an incubation medium containing (final concentrations) 150 μ M-phenolphthalein (E. Merck A.G. Darmstadt, Germany) and 1.5 mM-UDP-glucuronate (disodium salt; C.F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) in 50 mM-sodium phosphate buffer, pH 7.3; the total volume was 2 ml. Incubations were performed for 15 min and were terminated by the addition of 4.5 ml of 0.4 M-glycine-NaOH buffer, pH 10.4. The suspension was filtered (Blauband filterpaper, Carl Schleicher & Schull) and the decrease in extinction at 555 nm was measured against blanks that contained no UDP-glucuronate in the incubation medium but were otherwise treated identically.

After a series of experiments in which mutual inhibition of *p*-nitrophenol and phenolphthalein was studied in this phosphate buffer, 4-methylumbelliferone and *o*-aminophenol were included in the study. It was then found that the use of a 75 mM-tris-HCl buffer, pH 7.3, which was 5 mM with respect to MgCl₂ gave a considerable increase in *p*-nitrophenol and phenolphthalein glucuronide conjugation: about fourfold and threefold respectively. Therefore, in all further experiments in which mutual inhibition of the substrates was measured this tris-HCl buffer was used.

The enzymic synthesis of *o*-aminophenyl glucuronide was measured by the method of Dutton (1966). The *o*-aminophenol (E. Merck A.G.; twice sublimed) and UDP-glucuronate concentrations used were 0.5 and 1.5 mM respectively in 75 mM-tris-HCl buffer, pH 7.3, which was 5 mM with respect to MgCl₂. A calibration curve of the colour reaction was made with *o*-aminophenyl-glucuronide (Aldrich Chemical Co., Inc., Milwaukee, Wis., U.S.A.; kindly provided by Dr G. J. Dutton); the E_{360} was measured.

Biosynthesis of 4-methylumbelliferone glucuronide was measured by a modification of the method of Frezza, De Sandre, Perona & Corrocher (1968). 4-Methylumbelliferone has an absorption maximum at 365 nm whereas the glucuronide does not. Therefore the decrease in E_{365} can be measured during the incubation. This method gave the same results as measurement of the decrease in fluorescence by the method of Frezza *et al.* (1968). However, *p*-nitrophenol also absorbs strongly at 365 nm, so when *p*-nitrophenol was used as inhibitor of 4-methylumbelliferone conjugation the decrease of fluorescence was measured by the method of Frezza *et al.* (1968). The incubation medium contained 360 μ M substrate

(J.T. Baker Chemical Co., Deventer, The Netherlands) and 1.5 mM-UDP-glucuronate in 75 mM-tris-HCl buffer, pH 7.3, which also contained 5 mM-MgCl₂. The reaction was stopped by adding 0.4 M-glycine-NaOH buffer, pH 10.4. After filtration (Blauband filterpaper) the E_{365} was measured. Blanks contained no UDP-glucuronate.

In all experiments Triton X-100 (Sigma Chemical Co., St Louis, Mo., U.S.A.) was added to the undiluted enzyme preparations; to measure the UDP-glucuronyltransferase activity towards *p*-nitrophenol, phenolphthalein and 4-methylumbelliferone the concentration was 0.25% and for *o*-aminophenol the concentration was 0.2% (v/v).

Table 1 gives the amount of undiluted postnuclear supernatant in the incubation medium as a percentage of the total incubation volume (2 ml), used during incubations of 15 min at 37°C. One unit of UDP-glucuronyltransferase is defined as the amount of enzyme activity that catalyses the conversion of 1 μ mol of substrate/min in the incubation system. Activity expressed in units/ml refers to units/ml of undiluted postnuclear supernatant.

RESULTS

Effect of Triton X-100 in vitro. The effect of Triton X-100 on the glucuronide conjugation of phenolphthalein and *o*-aminophenol is shown in Fig. 1. Triton X-100 was added to the undiluted postnuclear supernatant which was diluted afterwards if necessary. The optimum activating concentrations of Triton X-100 were 0.25 and 0.20% (v/v) for the conjugation of the respective substrates; in homogenates slightly higher (0.30–0.40%) concentrations of Triton X-100 were required for full activation. 4-Methylumbelliferone conjugation could be stimulated about 20-fold by 0.30% (v/v) Triton X-100, which was the optimum activating concentration. Without Triton X-100 the measured activity was not reliable so no curve for the activity towards 4-methylumbelliferone is shown in Fig. 1.

In all further experiments UDP-glucuronyltransferase was activated by the addition of Triton X-100 (see the Materials and Methods section).

Table 1. Concentrations of postnuclear supernatant used in the glucuronide conjugation of the different substrates during incubations

Incubations were for 15 min at 37°C.

Substrate	Concn. (mM)	Buffer	%Postnuclear supernatant in incubation medium (v/v)
<i>p</i> -Nitrophenol	1.7	Phosphate	20
	1.7	Tris-HCl-MgCl ₂	5
Phenolphthalein	0.15	Phosphate	8
	0.15	Tris-HCl-MgCl ₂	4
<i>o</i> -Aminophenol	0.5	Tris-HCl-MgCl ₂	20
4-Methylumbelliferone	0.36	Tris-HCl-MgCl ₂	2

Fig. 1. Effect of Triton X-100 on glucuronide conjugation of phenolphthalein (●) and *o*-aminophenol (○) by the postnuclear supernatant in 0.154M-KCl. Triton X-100 was added to the undiluted enzyme preparation at the concentrations indicated. Thereafter the enzyme was diluted for the assay. Enzymic activity is given as a percentage of controls that contained no Triton X-100.

Effect of Mg^{2+} ions. In Fig. 2 the effect of Mg^{2+} on the glucuronide conjugation of the four substrates is shown. Mg^{2+} appeared to increase all activities but that for *o*-aminophenol was activated markedly less than that for the other substrates.

Albumin. Halac & Reff (1967) reported that albumin activates UDP-glucuronyltransferase activity for phenolphthalein and *p*-nitrophenol. However, in the present work a dual effect of albumin on phenolphthalein glucuronide conjugation was found. With a phenolphthalein concentration of $150\mu M$ the addition of albumin to the incubation medium appeared to inhibit the enzyme activity. With $500\mu M$ -phenolphthalein albumin activated the enzyme, but at higher albumin concentrations the enzyme was inhibited (Fig. 3). The addition of albumin caused a decrease in the concentration of free phenolphthalein in the incubation mixture (measured as the E_{555} of blanks) and the enzyme activity assayed correspondingly decreased with increasing albumin concentrations.

Up to 15mg of albumin/ml in the incubation medium had no effect on *p*-nitrophenol conjugation, contrary to the findings of Halac & Reff (1967).

Fig. 2. Effect of Mg^{2+} on *p*-nitrophenol (●), phenolphthalein (▲), *o*-aminophenol (○) and 4-methylumbelliferone (□) glucuronide conjugation. $MgCl_2$ was added to the incubation medium. Activities are expressed as percentages of the activities without Mg^{2+} in the incubation medium.

Kinetic properties. Phenolphthalein glucuronide synthesis is clearly inhibited at substrate concentrations higher than $100\mu M$ (Fig. 4). The product of the enzymic reaction, phenolphthalein- β -D-glucuronide (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.), did not inhibit enzyme activity when added to a concentration of $70\mu M$. This concentration is higher than that produced by enzyme action in the kinetic experiments, so this inhibition by phenolphthalein seems to be substrate inhibition. A substrate concentration of $150\mu M$ was used for the assays.

In the presence of 1.5mM-UDP-glucuronate the apparent K_m values were 0.4mM for 4-methylumbelliferone, 3.5mM for *o*-aminophenol and 0.5mM for *p*-nitrophenol. The K_m values for UDP-glucuronate were about the same for the glucuronide

Fig. 3. Effect of different concentrations of bovine serum albumin in the incubation medium on phenolphthalein glucuronide conjugation at phenolphthalein concentrations of 150 (●) and 500 μ M (▲). Enzymic activity is expressed as units/ml of undiluted postnuclear supernatant.

conjugation of the different substrates, respectively 1.2 mM (with 150 μ M-phenolphthalein), 1.5 mM (with 1.7 mM-*p*-nitrophenol), 0.8 mM (with 0.72 mM-4-methylumbelliferone) and 0.6 mM (with 0.5 mM-*o*-aminophenol).

Inhibition experiments. *o*-Aminophenol, up to 2 mM, did not inhibit the glucuronide conjugation of phenolphthalein, *p*-nitrophenol and 4-methylumbelliferone (Table 2), which were conjugated preferentially with *o*-aminophenol. Thus, when *p*-nitrophenol was present together with *o*-aminophenol, *p*-nitrophenol was conjugated first by the enzyme and only when all of it had been conjugated during the first minutes of the incubation did the conjugation of *o*-aminophenol start. In Fig. 5 the glucuronide conjugation of *o*-aminophenol is shown with and without *p*-nitrophenol present and it can be seen that there is a time lag in *o*-aminophenol glucuronide synthesis because *p*-nitrophenol is conjugated first. The duration of the time lag is dependent on the concentration of *p*-nitrophenol present. The findings with phenolphthalein and 4-methylumbelliferone were similar.

Fig. 4. Effect of phenolphthalein substrate concentration on phenolphthalein glucuronide conjugation.

p-Nitrophenol inhibited the glucuronide conjugation of phenolphthalein and, conversely, phenolphthalein inhibited *p*-nitrophenol conjugation. The latter inhibition could be analysed by means of Lineweaver-Burke plots (Fig. 6). As shown there was marked inhibition of activity towards *p*-nitrophenol in the presence of 35 μ M-phenolphthalein. Inhibition of activity towards *p*-nitrophenol substrate seems to be mixed (Leyden-Webb, 1963), with a predominant competitive component. For the UDP-glucuronate substrate this inhibition was non-competitive. The inhibition of phenolphthalein conjugation by *p*-nitrophenol is shown in Table 2. Much higher concentrations of *p*-nitrophenol were required to inhibit phenolphthalein conjugation.

Both phenolphthalein and *p*-nitrophenol glucuronide conjugation were inhibited by 4-methylumbelliferone. Table 2 shows that 4-methylumbelliferone is more inhibitory towards phenolphthalein glucuronide conjugation than *p*-nitrophenol. In a Dixon (1953) plot the inhibition by 4-methylumbelliferone of *p*-nitrophenyl glucuronide formation appeared to be competitive with a K_i of about 0.8 mM (Fig. 7). 4-Methylumbelliferone glucuronide conjugation was inhibited by both *p*-nitrophenol and phenolphthalein (Table 2). It is shown that very low concentrations of phenolphthalein were always required for inhibition of the conjugation of the other substrates.

DISCUSSION

In agreement with other findings (Winsnes, 1969; Mulder, 1970) the enzymic synthesis of both phenolphthalein and 4-methylumbelliferone glucuronides *in vitro* was very much enhanced by Triton

Table 2. *Mutual inhibition of substrates*

All experiments were performed in tris-HCl buffer, containing 5 mM-MgCl₂. The UDP-glucuronate concentration was 1.5 mM throughout.

Substrate	Concn. (mM)	Inhibitor (mM)	% Inhibition
<i>p</i> -Nitrophenol	1.7	Phenolphthalein (0.040)	39
		Phenolphthalein (0.075)	53
	0.85	4-Methylumbelliferone (0.72)	25
		4-Methylumbelliferone (1.44)	43
Phenolphthalein	0.5	<i>o</i> -Aminophenol (2.0)	0
	0.15	<i>p</i> -Nitrophenol (0.85)	16
		<i>p</i> -Nitrophenol (1.70)	24
	0.15	4-Methylumbelliferone (0.72)	31
		4-Methylumbelliferone (1.44)	50
4-Methylumbelliferone	0.15	<i>o</i> -Aminophenol (2.0)	0
		<i>p</i> -Nitrophenol (0.85)	46
	0.72	<i>p</i> -Nitrophenol (1.70)	64
		Phenolphthalein (0.040)	43
	0.72	Phenolphthalein (0.075)	52
	0.36	<i>o</i> -Aminophenol (0.5)	0

Fig. 5. Effect of the presence of *p*-nitrophenol on glucuronide conjugation of *o*-aminophenol measured as a function of time. ○, Without *p*-nitrophenol; ●, with 0.85 mM *p*-nitrophenol.

X-100. In the present work activation of *o*-aminophenyl glucuronide formation also occurred but was much smaller than that for the other substrates (Fig. 1, and Mulder, 1970). Preparations of UDP-glucuronyltransferase should be activated before study to decrease latency of the enzyme. If non-activated preparations are used effects may be ascribed to an action on the enzyme reaction, whereas in fact the effects may be caused by changes in the degree of

Fig. 6. Inhibition by phenolphthalein of *p*-nitrophenol glucuronide conjugation. Double-reciprocal plots of initial velocity; (a) various UDP-glucuronate concentrations in the absence (●) and presence (○) of 35 μM-phenolphthalein; (b) various *p*-nitrophenol concentrations in the absence (●) and presence (▲) of 35 μM-phenolphthalein. Velocity is expressed as μmol of *p*-nitrophenol converted/min per ml of undiluted postnuclear supernatant. The incubations were performed in phosphate buffer, without added Mg²⁺ (see the Materials and Methods section).

latency, which may be decreased or increased. The cause of the difference in the degree of activation of enzyme activity towards the different substrates may be that the effect of Triton X-100 depends on

Fig. 7. Inhibition by 4-methylumbelliferone of *p*-nitrophenol glucuronide conjugation. The results have been plotted by the method of Dixon (1953). Inhibition was measured in the presence of 0.5 mM (●) and 1.0 mM (○) *p*-nitrophenol.

the physicochemical properties of the substrate, which complexes with one common active site. The different degrees of activation by Mg^{2+} might have the same cause and need not be an indication for heterogeneity of the enzyme as is sometimes suggested (Frei, Birchmeier & Schmid, 1970).

Albumin exerts its effect on phenolphthalein glucuronide conjugation at the level of the substrate concentration. Because albumin can bind phenolphthalein it decreases the substrate concentration when it is added to the incubation medium. This increases enzyme activity when the substrate concentration is $500\mu M$ (as it relieves the substrate inhibition), but it is inhibitory at $150\mu M$ (when it decreases the effective substrate concentration). The substrate inhibition of phenolphthalein was also observed by Halac & Reff (1967). Because of its very high affinity for proteins or, more generally, for hydrophobic phases, phenolphthalein might bind to microsomal membranes. As Graham & Wood (1969) found that UDP-glucuronyltransferase activity is strongly dependent on the structural integrity of the microsomal membranes, such binding might inhibit enzyme activity. Tomlinson & Yaffe (1966) also found substrate inhibition in the case of the strongly hydrophobic substrate, bilirubin.

Kinetic studies of the inhibition of *o*-aminophenol glucuronide conjugation by *p*-nitrophenol suggested that inhibition by *p*-nitrophenol is non-competitive (Temple *et al.* 1968; Mowat & Arias, 1970a), but the present results indicate that kinetic measurements obtained with this system are not valid, because initial velocities are not measured. If one incubation time only had been measured in the present work (at variable *o*-aminophenol concentrations and a constant *p*-nitrophenol concentration) non-competitive inhibition would apparently have been found, which is, of course,

not correct. The difference between the K_m of *o*-aminophenol and those of the other three substrates, and the much higher enzyme concentration needed to measure *o*-aminophenol glucuronidation (Table 1), make it evident that *o*-aminophenol cannot inhibit the conjugation of the other substrates, whereas they strongly inhibit its glucuronide conjugation. This seems to be a competitive inhibition with *o*-aminophenol in a very unfavourable position.

Phenolphthalein, *p*-nitrophenol and 4-methylumbelliferone inhibit the conjugation of each other as is to be expected when one common active site is involved in the reaction. Phenolphthalein and 4-methylumbelliferone inhibit *p*-nitrophenol glucuronide conjugation competitively (Figs. 6 and 7). Hänninen & Marniemi (1971) found the same type of inhibition for *p*-nitrophenol conjugation by phenolphthalein. Halac & Reff (1967), however, found no inhibition of phenolphthalein conjugation by *p*-nitrophenol, although this may have been a consequence of the high phenolphthalein concentration ($500\mu M$) that they used. The K_m for 4-methylumbelliferone is about the same as the K_i for 4-methylumbelliferone in the inhibition of *p*-nitrophenol conjugation, in agreement with the hypothesis that 4-methylumbelliferone and *p*-nitrophenol are bound at the same active site. Moreover, the K_m values for UDP-glucuronate in the conjugation of the different substrates were about the same, again in agreement with this idea.

The present results provide evidence against the heterogeneity of UDP-glucuronyltransferase towards the substrates investigated, unless overlapping substrate specificity is assumed. The main evidence in favour of heterogeneity is the finding that an enzyme preparation that catalyses the glucuronide conjugation of one substrate may be unable to conjugate some other substrate. This is most clearly the case with enzyme preparations from the Gunn rat. However, this has been shown to be doubtful evidence: the very low *o*-aminophenol-conjugating activity of Gunn rat liver preparations could be stimulated by diethylnitrosamine to values normal for the rat (Greenwood & Stevenson, 1965; Mowat & Arias, 1970b). The case in favour of heterogeneity thus seems largely to be based on negative evidence against homogeneity.

One of the causes leading to the apparent finding of heterogeneity of UDP-glucuronyltransferase is its localization in the microsomal membrane; the use of non-activated enzyme preparations might give unreliable evidence. Moreover, very physicochemically different substrates e.g. phenolphthalein and *o*-aminophenol are compared, which may give rise to very physicochemically different enzyme-substrate complexes. Thus, the degree of latency of the enzyme activity might be a function of the physicochemical properties of the substrate used.

The present results, however, indicate one common active site for the glucuronide conjugation of the four substrates studied. It remains to be investigated whether physiological substrates e.g. bilirubin are also conjugated at this same active site.

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