Separation of Oestrone UDP-Glucuronyltransferase and *p*-Nitrophenol UDP-Glucuronyltransferase Activities

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Rabbit liver UDP-glucuronyltransferase activity was resolved into two separate fractions on DEAE-cellulose, one containing most of the transferase activity toward oestrone and the other most of the activity toward *p*-nitrophenol. These two activities were completely separated by rechromatography of each fraction on a second DEAE-cellulose column.

Hepatic microsomal UDP-glucuronosyltransferase (EC 2.4.1.17) catalyses the conjugation of UDP-glucuronic acid with numerous classes of chemicals, such as carboxylic acids (Dutton, 1956), amines (Arias, 1961), thiol derivatives (Dutton & Illing, 1972), bilirubin (Schmid *et al.*, 1957; Lathe & Walker, 1958) and certain hormones (Harri *et al.*, 1970; Miller *et al.*, 1973; Fuchs *et al.*, 1977). Since such a broad spectrum of substrates is glucuronidated, numerous studies have suggested a multiplicity of glucuronyltransferases (Dutton & Burchell, 1977). Certain examples include the lack of glucuronidation of bilirubin, but not other substrates, in the Gunn rat (Dutton, 1966), the absence of mutual product inhibition by various glucuronides (Zakim *et al.*, 1973*b*), selective induction by phenobarbital, 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin towards glucuronidation of some substrates and not others (Sanchez & Tephly, 1973; Bock *et al.*, 1973; Lucier *et al.*, 1975), and changes in the various glucuronyltransferase activities in the presence of various metals (Zakim *et al.*, 1973*a*). Developmental studies demonstrating a differential pattern of glucuronidation with age towards both xenobiotic and endogenous substrates (Lucier & McDaniel, 1977; Fuchs *et al.*, 1977; Wishart *et al.*, 1977) point toward a separate group of transferases responsible for steroid conjugation and, possibly within this group, multiple steroid glucuronyltransferase activities exist.

Our laboratory has demonstrated the probable existence of multiple glucuronyltransferases by separating and partially purifying transferases involved in the glucuronidation of morphine and *p*-nitrophenol (del Villar *et al.*, 1975, 1977). This work has been confirmed and extended by Bock *et al.* (1977). We now report that a solubilized rabbit liver microsomal preparation has been fractionated into two separate glucuronyltransferase activities; one uses oestrone and another uses *p*-nitrophenol as substrate.

Materials and Methods

Female rabbits, starved for 48 h, were killed by a sharp blow to the head and their livers were perfused with ice-cold 0.15% KCl. Microsomal fractions were prepared as previously described (Baron & Tephly, 1970).

Oestrone glucuronyltransferase activity was determined by a modification of the procedure of Rao *et al.* (1976). [4-¹⁴C]Oestrone (New England Nuclear, Boston, MA, U.S.A.), in benzene/ethanol (9:1, v/v), was added to propylene glycol and the solvent evaporated under a gentle stream of N₂. Reactions were carried out in 15 ml glass-stoppered centrifuge tubes with 10 μ l of the [¹⁴C]oestrone/propylene glycol mixture (approx. 88000 d.p.m.), 10 nmol of unlabelled oestrone in 5 μ l of dimethyl sulphoxide, 0.05 M-Tris/HCl, pH 8.0, 0.01 M-MgCl₂ and 2.0 mM-UDP-glucuronic acid. Reactions were started by adding 0.5 ml of enzyme solution to give a final volume of 1 ml and incubations were carried out at 37°C for 5 min. Under these conditions product formation was linear with time. The concentrations of propylene glycol (1%, v/v) and dimethyl sulphoxide (0.5%, v/v) did not interfere with the reaction. The reactions were stopped by adding 10 ml of water-saturated dichloromethane, the mixtures were shaken for 10 min and centrifuged at 1500 rev./min for 5 min to separate the aqueous and organic phases. The unchanged oestrone was recovered quantitatively in the dichloromethane fraction. The aqueous phase (0.5 ml) was placed in scintillation vials, 10 ml of Aquasol 2 (New England Nuclear) was added, and the radioactive glucuronide determined by using liquid-scintillation-counting techniques (del Villar *et al.*, 1974).

p-Nitrophenol glucuronyltransferase activity was also assayed by a radiochemical method. Incubations contained 0.1 M-Tris/HCl buffer, pH 8.0, 0.01 M-MgCl₂, 2.5 mM-UDP-glucuronic acid and 1 mM-

p-nitro[U-¹⁴C]phenol (California Bionuclear, Sun Valley, CA, U.S.A.; 105d.p.m./nmol). Reactions were initiated by adding 0.5ml of enzyme solution, giving a final volume of 2ml. The incubations were conducted for 10min at 37°C and terminated by the addition of 1ml of 10% (w/v) trichloroacetic acid. The precipitated protein was removed by centrifugation at 1000g for 10min and a 2ml sample of the clear supernatant was added to 1ml of 2.0M-Tris/HCl buffer, pH9.0, containing 3μmol of *p*-nitrophenol. Unchanged *p*-nitrophenol was removed quantitatively from this solution by extraction with 2×15ml of diethyl ether. The radioactivity in the aqueous phase (0.5ml) was determined by liquid-scintillation techniques as described for morphine metabolism. Control incubations were carried out under the same conditions by using all components except that UDP-glucuronic acid was omitted. Product formation was linear with time and protein concentration.

For assay of transferase activity towards both oestrone and *p*-nitrophenol the radioactivity in the aqueous phase could be readily recovered as the aglycone after incubation with β-glucuronidase and extraction with organic solvent. Protein concentrations were measured by a modification of the method of Lowry *et al.* (1951), by using the technique of Retz & Steele (1977). To remove interfering buffer components such as glycerol and detergent, the samples (0.5ml) were incubated for 30min at 4°C with 0.1% sodium dodecyl sulphate and 1ml of 10% trichloroacetic acid. The precipitated protein was collected by centrifugation (1000g, 10min), resuspended and washed with 5% trichloroacetic acid. It was then dissolved in 0.25ml of 1M-NaOH and analysed by the method of Lowry *et al.* (1951).

Standard curves were determined in the same manner by using bovine serum albumin.

Results

Rabbit liver microsomal preparation was suspended in 0.025M-Tris/HCl buffer, pH8.0, containing 10% (v/v) glycerol, 1mM-EDTA and 0.1mM-dithiothreitol (buffer A) at a final concentration of 10mg of protein/ml of buffer. Sodium cholate (10%, w/v) was slowly added to give a final concentration of 1mg of sodium cholate/mg of protein, and the mixture was stirred for 30min at 0°C. This preparation was centrifuged at 105000g for 2h, and the supernatant was removed. The supernatant fraction contained more than 90% of the *p*-nitrophenol and oestrone glucuronyl-transferase activity. It was fractionated further with solid (NH₄)₂SO₄. The precipitate that developed in the 40–70%-satd. (NH₄)₂SO₄ solution was dissolved in buffer A to which 20% (v/v) glycerol (buffer B) was added. A concentration of 10mg of protein/ml of buffer was used for further studies. This suspension was passed immediately through a column (2.5cm×35cm) of Sephadex G-25 (Pharmacia, Uppsala, Sweden) and then dialysed against 2 litres of buffer B with at least three changes over 24h. After centrifugation of the suspension at 30000g for 1h, Emulgen 911 (10%, v/v; Kao-Atlas Co., Tokyo, Japan) was added to the suspension to give a concentration of 0.1% (v/v) and 0.1mg of detergent/mg of protein. This preparation was slowly placed (50ml/h) on a column (2.5cm×35cm) of DEAE-cellulose DE-52 (Whatman, Clifton, NJ, U.S.A.) previously equilibrated with buffer B containing 0.1% Emulgen 911 (v/v) (buffer C).

Fig. 1. DEAE-cellulose column chromatography of rabbit hepatic oestrone and *p*-nitrophenol UDP-glucuronyltransferase activity

For complete details, see the text. A 40–70%-satd.-(NH₄)₂SO₄ precipitate was passed through a Sephadex G-25 column, dialysed against buffer C, and applied to a DEAE-cellulose column (2.5cm×35cm). Approx. 400ml of the buffer was passed through the column before introduction of a linear 0–0.5M-KCl gradient (—). Fractions (12ml/tube) were collected at a flow rate of 50ml/h, and assayed for UDP-glucuronyltransferase activity towards oestrone (●) and *p*-nitrophenol (○). Protein (□) was determined as described in the text.

The column was washed with 400 ml of the equilibrium buffer, which resulted in elution of 99% of the recovered oestrone glucuronyltransferase activity and 7% of the *p*-nitrophenol glucuronyltransferase activity (Fig. 1, peak A, fractions 16–18). With a linear (0–0.5 M-KCl gradient, the remaining oestrone glucuronyltransferase activity and most of the *p*-nitrophenol glucuronyltransferase activity were eluted from the column at approx. 0.15 M-KCl (Fig. 1, peak B, fractions 46–48).

To determine whether the transferase activity towards oestrone and *p*-nitrophenol that was recovered in the void volume could be separated, peak-A material was dialysed overnight against 0.015 M-Tris/HCl buffer, containing 1 mM-EDTA,

0.1 mM-dithiothreitol, 20% glycerol and 0.1% Emulgen (buffer D), and rechromatographed on a second DEAE-cellulose column (1.5 cm × 25 cm), which was equilibrated with the same buffer. As shown in Fig. 2(a), when peak-A material was rechromatographed, only the activity toward oestrone passed through the column in the void volume (fraction A), whereas the activity towards *p*-nitrophenol remained bound to the column and was eluted after introduction of a linear 0–0.2 M-KCl gradient.

Peak-B material, which contained most of the *p*-nitrophenol glucuronyltransferase activity, was also dialysed overnight against buffer D and rechromatographed on a second DEAE-cellulose column (1.5 cm × 25 cm) under conditions identical with those used

Fig. 2. Elution profiles of UDP-glucuronyltransferase activities towards oestrone and *p*-nitrophenol after rechromatography of material from peaks A and B (Fig. 1) on separate DEAE-cellulose columns

For complete details see the text. (a) Peak-A material from the first DEAE-cellulose column was dialysed against buffer D and applied to a second DEAE-cellulose column (1.5 cm × 25 cm). Approx. 200 ml of the buffer was washed through the column before introduction of a linear 0–0.2 M-KCl gradient (—). (b) Peak-B material, also from the first DEAE-cellulose column, was dialysed against buffer D and rechromatographed on a second DEAE-cellulose column (1.5 cm × 25 cm) under conditions identical with those used to rechromatograph peak-A material. Fractions (6 ml/tube) were collected at a flow rate of 50 ml/h and assayed for UDP-glucuronyltransferase activity towards oestrone (●) and *p*-nitrophenol (○). Protein (□) was assayed as described in the text.

Table 1. *Separation and purification of rabbit liver UDP-glucuronyltransferases*

An outline of the separation and purification of UDP-glucuronyltransferase activities with oestrone and *p*-nitrophenol as substrates. A unit of activity represents 1 nmol of substrate conjugated/min. —, No detectable activity.

Purification step	Total protein (mg)	Specific activity (units/mg of protein)		Total activity (units)		Percentage yield	
		Oestrone	<i>p</i> -Nitrophenol	Oestrone	<i>p</i> -Nitrophenol	Oestrone	<i>p</i> -Nitrophenol
Solubilized microsomal preparation	672	6.4	60	4300	40320	100	100
40–70%-(NH ₄) ₂ SO ₄ precipitate	188	8.9	115	1673	21620	38	54
First DEAE-cellulose chromatography							
Peak A	13.9	22.9	18.0	318	250	7.2	0.6
Peak B	6.6	0.3	520	1.8	3433	0.04	8.5
Second DEAE-cellulose rechromatography of peak A							
Fraction A	7.2	31.8	—	230	—	5.3	—
Second DEAE-cellulose rechromatography of peak B							
Fraction B	2.8	—	682	—	1909	—	4.7

to rechromatograph peak-A material. As shown in Fig. 2(b), the transferase activity towards *p*-nitrophenol was retained on the column and was eluted at approx. 0.1M-KCl (fraction B). None of the transferase activity towards oestrone that was initially present in fraction B was detectable after rechromatography. The loss of this activity is not surprising, since the oestrone glucuronyltransferase activity present in peak-B material constituted only 0.06% of the oestrone glucuronyltransferase activity that was eluted from the initial DEAE-cellulose column. The individual steps for the fractionation and the estimation of purification are outlined in Table 1.

Discussion

The present paper demonstrates that rabbit liver microsomal glucuronyltransferase can be resolved into two separate enzymes, one with activity towards *p*-nitrophenol and one with activity toward oestrone. Although indirect evidence supports glucuronyltransferase multiplicity, there has been a striking lack of success in separating these activities with the usual biochemical techniques. Isselbacher *et al.* (1962) separated an *N*-glucuronyltransferase from an *O*-glucuronyltransferase, and others have shown partial purification of glucuronyltransferases, but without definitive enzyme separation (Mowat & Arias, 1970; Lucier *et al.*, 1975). Reports by Gorski & Kasper (1977) and by Burchell (1977) have demonstrated that a *p*-nitrophenol glucuronyltransferase activity can be purified to apparent homogeneity. However, the range of substrate interaction is not clear. Our laboratory has separated *p*-nitrophenol and morphine glucuronyltransferase activity by solubilizing hepatic microsomal preparations in Emulgen 911, and resolving the activities into separate fractions by

chromatography on DEAE-cellulose (del Villar *et al.*, 1975, 1977). Using a different detergent for solubilizing the microsomal preparations, Bock *et al.* (1977) confirmed these studies and found a similar chromatographic profile for *p*-nitrophenol glucuronyltransferase and morphine glucuronyltransferase activities.

Initially, attempts were unsuccessful in separating glucuronyltransferase activity towards *p*-nitrophenol and oestrone by using the methods developed by del Villar *et al.* (1975). The activity towards oestrone was present exclusively in the peak with the activity towards *p*-nitrophenol. However, these two activities were partially resolved by solubilization of the microsomal fraction with sodium cholate, followed by precipitation with (NH₄)₂SO₄ and chromatography on DEAE-cellulose in the presence of 0.1% Emulgen 911.

The two activities were completely separated by rechromatography on DEAE-cellulose. Experiments were performed with these partially purified preparations to determine if *p*-nitrophenol or oestrone could produce mutual competitive inhibition: neither substrate was able to inhibit the conjugation of the other.

Though the final yield of these two activities is small (Table 1), this may be attributable to removal of phospholipids, which may be essential for exhibiting maximal UDP-glucuronyltransferase activity (Berry *et al.*, 1976). Addition of a microsomal lipid fraction to material from peaks A and B from the first DEAE-cellulose column increased the specific activity obtained with both oestrone and *p*-nitrophenol (R. Tukey, R. E. Billings & T. R. Tephly, unpublished work). Reconstitution of the resolved fractions with microsomal phospholipids may be required to determine the maximum activities of certain of the partially purified enzymes. Nevertheless, these results

strongly support previous studies (del Villar *et al.*, 1975, 1977; Bock *et al.*, 1977) that suggest that multiple UDP-glucuronyltransferases function catalytically in hepatic microsomal preparations.

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