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The separation and purification of rat liver UDP-glucuronyltransferase activities towards testosterone and oestrone

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1. Reconstitution of UDP-glucuronyltransferase preparations with phosphatidylcholine liposomes facilitated the purification of testosterone UDP-glucuronyltransferase. 2. Transferase activity towards testosterone co-purifies with that towards 4-nitrophenol. 3. UDP-glucuronyltransferase activity towards oestrone was separated from that towards testosterone. 4. These results suggest that testosterone and 4-nitrophenol may be glucuronidated by a different form of UDP-glucuronyltransferase from the one glucuronidating oestrone.

Liver microsomal UDP-glucuronyltransferase(s) is an important enzyme involved in the metabolism and in facilitating excretion of steroids [see Dutton & Burchell (1977) for references]. Clinically, it is important to know whether drugs or xenobiotics compete with steroids for the same active site(s) on UDP-glucuronyltransferase, to prevent therapeutically induced complications. Such information can only be obtained by defining the boundaries of specificity of isolated and purified steroid UDP-glucuronyltransferase(s) (Burchell, 1980*b*).

Rat hepatic UDP-glucuronyltransferase with activity towards phenolic substrates has been purified to apparent homogeneity in this (Burchell, 1977, 1978) and other laboratories (Gorski & Kasper, 1977; Bock *et al.*, 1979). Transferase activity towards testosterone, when looked for, was either not detectable or extremely low (Burchell, 1978; Bock *et al.*, 1979). Steroid transferase activities may depend on the presence of phospholipids (Tukey *et al.*, 1979), and the extensive delipidation of the transferase enzyme which occurs during purification (Burchell & Hallinan, 1978; Burchell, 1980*a*) may be responsible for the loss of activity towards steroids. Indeed, the reconstitution of UDP-glucuronyltransferase preparations with phosphatidylcholine liposomes has facilitated the assay and purification of bilirubin UDP-glucuronyltransferase (Burchell, 1980*a*).

An earlier series of studies on the purification of steroid UDP-glucuronyltransferase(s) has indicated that separate forms of UDP-glucuronyltransferase may be responsible for the glucuronidation of 3-hydroxy steroids, 17β-hydroxy steroids and oestriol-16α, although no direct physical separation of a mixture of these activities was achieved (Rao & Breuer, 1969; Rao *et al.*, 1970*a*,*b*). More recent purification work has indicated that multiple forms of rat liver UDP-glucuronyltransferase may exist (see Burchell, 1980*b*). Indeed, a highly purified preparation of rabbit liver oestrone UDP-glucuronyltransferase can be apparently separated from the transferase activity towards 4-nitrophenol (Tukey *et al.*, 1978; Billings *et al.*, 1978).

The present paper reports that reconstitution of UDP-glucuronyltransferase preparations with phosphatidylcholine liposomes facilitates the purification of testosterone UDP-glucuronyltransferase to apparent homogeneity and the separation of this transferase activity from a partially purified preparation of oestrone UDP-glucuronyltransferase.

Experimental

Lubrol 12A9 was obtained from ICI Organics Division, Manchester, U.K. [4-^{14}C]Testosterone and [2,4,6,7-^{3}H]oestrone were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Non-radioactive steroids and the other substrates for the assay of UDP-glucuronyltransferase activities were purchased from Sigma (London) Chemical Co. Egg-yolk phosphatidylcholine was purchased from Lipid Products, South Nutfield, Surrey, U.K. All other chemicals, where available, were analytical-reagent grade.

UDP-glucuronyltransferase activity towards various substrates was assayed by the following

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methods: 4-nitrophenol (Winsnes, 1969), testosterone and oestrone (Rao *et al.*, 1976). Samples for assay were reconstituted with dispersions of phosphatidylcholine in a 2:1 (w/w) ratio of lipid:protein (Burchell, 1980*a*). Protein concentrations were determined by the method of Bradford (1976), with bovine serum albumin as standard.

UDP-glucuronyltransferase was purified to apparent homogeneity from the livers of phenobarbital-treated Wistar rats by the method of Burchell (1978). Testosterone and oestrone UDP-glucuronyltransferase activities were separated by preparing an $(NH_4)_2SO_4$ fraction from phenobarbital-treated Wistar rat liver (Burchell, 1978). The $(NH_4)_2SO_4$ precipitate was dissolved in 0.01% (w/v) Lubrol/5mM-potassium phosphate/5mM-mercaptoethanol buffer, pH8.0, dialysed against this buffer and applied to a DEAE-cellulose column. UDP-glucuronyltransferase activities were eluted as previously described (Burchell, 1980*a*).

Polyacrylamide-gel electrophoresis was performed in 0.1% sodium dodecyl sulphate as described by Weber & Osborn (1969). The conditions used for running, staining and destaining the gels were as described by Burchell (1977).

Results

Purification of testosterone UDP-glucuronyltransferase

UDP-glucuronyltransferase purified to apparent homogeneity from phenobarbital-treated Wistar-rat liver (see the Experimental section) exhibited activity towards 4-nitrophenol (Table 1), as previously described (Burchell, 1978). However, only low transferase activity towards testosterone was detectable during purification, and this activity was not detectable in the final enzyme preparation when assayed in the absence of phospholipid (Table 1 and Burchell, 1978). Further investigation of the substrate specificity of this transferase, made possible by reconstitution with phosphatidylcholine liposomes, revealed previously undetectable UDP-glucuronyltransferase activity towards testosterone (Table 1). The considerable co-purification of UDP-glucuronyltransferase activities towards testosterone and 4-nitrophenol up to the final purification step, when assayed in the presence of phospholipid, suggests that these two substrates may be glucuronidated by a single form of UDP-glucuronyltransferase. Indeed, only one polypeptide-staining band was visible after sodium dodecyl sulphate/polyacrylamide-gel-electrophoretic analysis of the purified testosterone UDP-glucuronyltransferase, as would be predicted from the earlier physico-chemical analysis of the 4-nitrophenol UDP-glucuronyltransferase (Burchell, 1978). The difference observed in the final relative purification value may be due to a difference in the degree to which the activities are reconstituted and does not appear until the final purification step, when any remaining endogenous phospholipids are removed (Burchell & Hallinan, 1978).

UDP-glucuronyltransferase activity towards

Table 1. *Purification of testosterone UDP-glucuronyltransferase activity*

UDP-glucuronyltransferase activities were purified as described. A portion of each purification fraction was reconstituted with phosphatidylcholine (2mg/mg of protein) before the assay of transferase activities towards various substrates in the presence or absence of the phospholipid. Specific activity is expressed as nmol of glucuronide formed/min per mg of protein. Abbreviation: ND, not detectable.

		UDP-glucuronyltransferase activity					
	Substrate ...	4-Nitrophenol		Testosterone		Oestrone	
Fraction	Phosphatidylcholine ...	−	+	−	+	−	+
25–60%-satd.-$(NH_4)_2SO_4$ precipitate	Specific activity	54	63	2.3	6.0	0.4	0.54
	Yield (%)	100	100	100	100	100	100
DEAE-cellulose eluate	Specific activity	81	87	2.6	6.1	0.05	0.27
	Yield (%)	56	66	62	55	2.3	2.0
CM-cellulose eluate	Specific activity	89	109	1.8	6.7	ND	ND
	Yield (%)	27	37	22	33	—	—
DEAE-Sephadex eluate	Specific activity	262	207	4.3	11.1	ND	ND
	Yield (%)	13	10	6.6	6.0	—	—
UDP-glucuronic acid eluate from UDP-hexanolamine–Sepharose 4B	Specific activity	2928	3092	ND	92	ND	ND
	Yield (%)	2.6	2.9	—	1.2	—	—
	Relative purification	54.2	49.1	—	15.3	—	—

oestrone was gradually lost during purification; no activity was detectable after the penultimate purification step, even after reconstitution with phosphatidylcholine liposomes (Table 1).

Separation of UDP-glucuronyltransferase activities towards testosterone and oestrone

The yield of UDP-glucuronyltransferase activity towards oestrone was greatly decreased when compared with that towards testosterone after DEAE-cellulose chromatography (Table 1). Therefore it was logical to attempt to separate these two activities by DEAE-cellulose chromatography.

An $(NH_4)_2SO_4$ fraction was prepared from phenobarbital-treated Wistar-rat livers (see the Experimental section) and 30ml of this was applied to a DEAE-cellulose column previously equilibrated with 0.01% Lubrol/5mM-potassium phosphate/5mM-mercaptoethanol buffer, pH8.0 (buffer A; see the Experimental section). A portion of each eluted fraction was reconstituted with phospholipid before assay. Under these conditions some transferase activity towards testosterone was eluted from the column by buffer A; no activity towards oestrone was detected in these fraction (Fig. 1). A second peak of testosterone UDP-glucuronyltransferase activity was eluted at up to 40mM-KCl of the linear salt gradient. However, a large peak of oestrone UDP-glucuronyltransferase was eluted by approx. 120–160mM-KCl. More than 85% of UDP-glucuronyltransferase activities towards testosterone and 80% of that towards oestrone applied to the DEAE-cellulose column were recovered. Thus the glucuronidation of testosterone and oestrone is apparently catalysed by two separate entities.

UDP-glucuronyltransferase activity towards 4-nitrophenol was co-eluted with the activity towards testosterone. The total activity recovered towards 4-nitrophenol was 61%, and only 2.6% was associated with the large peak of oestrone UDP-glucuronyltransferase eluted by approx. 120–160mM-KCl. Therefore 4-nitrophenol and testosterone appear to be glucuronidated by the same form of UDP-glucuronyltransferase, which is a different form from the one that glucuronidates oestrone.

Discussion

The work reported above shows that purified testosterone UDP-glucuronyltransferase does not glucuronidate oestrone. However, this evidence alone

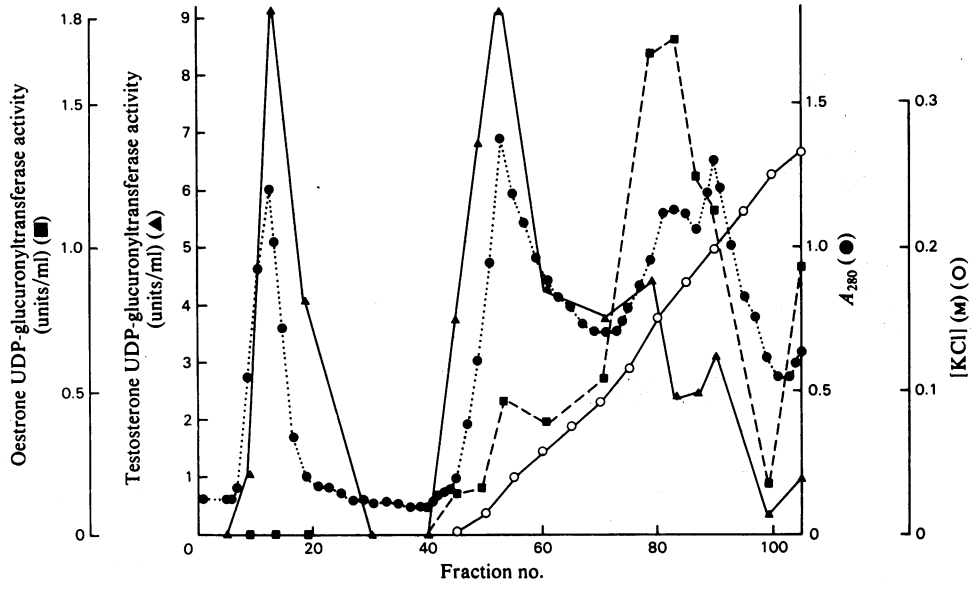

Fig. 1. *Separation of rat liver testosterone and oestrone UDP-glucuronyltransferase activities by DEAE-cellulose chromatography*

An $(NH_4)_2SO_4$ fraction was prepared, dialysed and applied to a DEAE-cellulose column (see the text). Fractions (5ml) were collected at a flow rate of 15ml/h and assayed for transferase activity towards testosterone (▲) and oestrone (■) after reconstitution with phosphatidylcholine liposomes (2mg of phosphatidylcholine/mg of protein). ●, A_{280} (protein); ○, 0–0.3M-KCl gradient in buffer A. The total transferase activities applied to the column were 626 units of testosterone UDP-glucuronyltransferase and 227 units of oestrone UDP-glucuronyltransferase: 1 unit is 1nmol of glucuronide formed/min.

is not sufficient to indicate whether oestrone UDP-glucuronyltransferase activity represents a different form of the enzyme or that loss of this activity is due to differential inactivation of the transferase activity towards oestrone. Oestrone UDP-glucuronyltransferase activity must be recovered at some stage of the purification procedure to establish whether glucuronidation of oestrone and testosterone is catalysed by two separate entities.

Rat liver testosterone UDP-glucuronyltransferase activity and oestrone UDP-glucuronyltransferase activity can be physically separated by DEAE-cellulose chromatography. UDP-glucuronyltransferase activity towards testosterone was eluted from DEAE-cellulose before that towards oestrone. Tukey *et al.* (1978) have reported the separation of rabbit liver oestrone UDP-glucuronyltransferase activity from that towards 4-nitrophenol by DEAE-cellulose chromatography, but that the oestrone UDP-glucuronyltransferase activity was eluted at low ionic strength.

As the work reported here indicates that testosterone and 4-nitrophenol may be glucuronidated by the same form of UDP-glucuronyltransferase, this separation of liver steroid UDP-glucuronyltransferases might be expected to occur with rabbit liver, despite the obvious species difference in elution pattern that has been noted.

One criticism that should be made of earlier studies (Rao & Breuer, 1969; Rao *et al.*, 1970*a*,*b*; Tukey *et al.*, 1978; Billings *et al.*, 1978) is that, since only very low yields of activity were recovered after chromatography, the suggested separation of UDP-glucuronyltransferase activities towards oestrone from that of 4-nitrophenol (Tukey *et al.*, 1978) could be due to differential inactivation of the UDP-glucuronyltransferase towards one substrate but not towards another (Jansen & Arias, 1975) because of separation and removal of phospholipids (Burchell, 1980*b*). Indeed, oestrone apparently reaches the active site of UDP-glucuronyltransferase via diffusion through the microsomal membrane (Zakim & Vessey, 1977).

Bock *et al.* (1979) detected only very low transferase activity towards testosterone in their purified preparation of 1-naphthol (or 4-nitrophenol) UDP-glucuronyltransferase, when assayed in the absence of phospholipid, and the activities were not co-purified (see Table 1). However, transferase activity towards testosterone was only moderately increased by the addition of lysophosphatidylcholine. From the work reported here, it seems that phosphatidylcholine liposomes are better agents for reconstitution of steroid transferase activities than are those containing lysophosphatidylcholine. Thus reconstitution of the purified enzyme protein with phospholipid is extremely important in the determination of the substrate specificity of UDP-glucuronyltransferase.

To establish the existence and separation of different forms of UDP-glucuronyltransferase, high yields of transferase activity towards each substrate must be recovered after the separation procedure. The experiments reported here show that, after reconstitution with phospholipids, of the transferase activities applied to DEAE-cellulose, more than 85% of the UDP-glucuronyltransferase activity towards testosterone can be recovered and approx. 26% can be completely separated from the 80% of the transferase activity towards oestrone that is also recovered. These results show that hepatic testosterone UDP-glucuronyltransferase and oestrone UDP-glucuronyltransferase are indeed separable entities.

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