Biochem. J. (1978) **173**, 749–757
Printed in Great Britain

Substrate Specificity and Properties of Uridine Diphosphate Glucuronyltransferase Purified to Apparent Homogeneity from Phenobarbital-Treated Rat Liver

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(*Received* 23 *November* 1977)

1. The purification to homogeneity of stable highly active preparations of UDP-glucuronyltransferase from liver of phenobarbital-treated rats is briefly described. 2. A single polypeptide was visible after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, of mol.wt. 57000. 3. Antiserum raised against the pure enzyme produces a single sharp precipitin line after Ouchterlony double-diffusion analysis. 4. The pure UDP-glucuronyltransferase isolated from livers of untreated and phenobarbital-pretreated rats appears to be the same enzyme. 5. The K_m (UDP-glucuronic acid) of the pure enzyme is 5.4mM. 6. The activity of the pure enzyme towards 2-aminophenol can still be activated 2–3-fold by diethylnitrosamine. 7. UDP-glucose and UDP-galacturonic acid are not substrates for the purified enzyme. 8. The final preparation catalysed the glucuronidation of 4-nitrophenol, 1-naphthol, 2-aminophenol, morphine and 2-aminobenzoate. 9. Activities towards 4-nitrophenol, 1-naphthol and 2-aminophenol were all co-purified. The proposed heterogeneity of UDP-glucuronyltransferase is discussed.

Bilirubin, steroid hormones and many xenobiotics are biologically active compounds that as a result of hepatic metabolism are rendered water-soluble by conjugation with glucuronic acid. The microsomal-membrane-bound enzyme UDP-glucuronyltransferase (EC 2.4.1.17) catalyses the conjugation of this wide variety of compounds. The water-soluble glucuronides are usually more readily excreted from the body and are less harmful than the original lipophilic substrates [see Dutton & Burchell (1977) for references].

In recent years evidence has accumulated that suggests that there may be more than one UDP-glucuronyltransferase (see Dutton, 1971; Dutton & Burchell, 1977). Evidence from developmental studies (Wishart *et al.*, 1977; Lucier & McDaniel, 1977) and glucocorticoid-inducibility (Wishart *et al.*, 1977) of UDP-glucuronyltransferase with up to 12 aglycone substrates (G. J. Wishart, personal communication) suggests that at least two functionally heterogeneous forms of the enzyme may exist. Activities towards 1-naphthol or 4-nitrophenol appear to have been separated from the activity towards morphine (Del Villar *et al.*, 1975, 1977; Bock *et al.*, 1977). Furthermore, Bock *et al.* (1973, 1977) have proposed that different forms of UDP-glucuronyltransferase may be induced in response to phenobarbital and 3-methylcholanthrene.

My approach to this problem has been slightly different. As drugs may preferentially induce one form of the enzyme, I have initially isolated homogeneously pure highly active UDP-glucuronyltransferase from untreated rat liver to obtain the enzyme from the native state (Burchell, 1977*a*,*b*). However, larger quantities of the pure enzyme protein were required for critical assessment of the substrate specificity. Thus I have now isolated the pure enzyme from liver of phenobarbital-treated rats.

The present paper describes the isolation and properties of UDP-glucuronyltransferase from liver of phenobarbital-treated rats, and the results show that this enzyme has properties very similar to, if not identical with, those displayed by the pure enzyme from untreated rat liver. Furthermore, the substrate specificity has now been determined and shows that at least three aglycone substrates are glucuronidated by the same enzyme.

While this work was in progress Gorski & Kasper (1977) independently reported the purification of UDP-glucuronyltransferase from phenobarbital-treated rat liver, although the actual specific activity of their final product is at most one-sixth of that reported here.

Materials and Methods

The radioactive substrates 1-[1-^{14}C]naphthol, [4-^{14}C]testosterone and [*N-methyl*-^{14}C]morphine hydrochloride were all purchased from The Radiochemical Centre, Amersham, Bucks, U.K. 2-

Aminophenol and 2-aminobenzoate were obtained from BDH Chemicals, Poole, Dorset, U.K. Bilirubin, 4-nitrophenol, UDP-glucuronic acid (triammonium salt), UDP-glucose (sodium salt), UDP-galacturonic acid (tripotassium salt) and UDP-*N*-acetylglucosamine (sodium salt) were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Diethylnitrosamine was obtained from Eastman Kodak Co., Rochester, NY, U.S.A. All other chemicals were the purest grade available.

Lubrol 12A9 (a condensate of dodecyl alcohol with approx. 9.5mol of ethylene oxide/mol) was a gift from I.C.I. Organics Division, Blackley, Manchester, U.K. Chromatography materials [CM-cellulose (CM52) and DEAE-cellulose (DE52)] were obtained from Whatman Biochemicals, Maidstone, Kent, U.K. DEAE-Sephadex A-50 grade was from Pharmacia Fine Chemicals, Uppsala, Sweden.

Synthesis of UDP-hexanolamine-Sepharose 4B

Ethyl trifluorothiolacetate and trifluoroacetic acid were purchased from Pierce and Warriner (U.K.), Chester, Cheshire, U.K. UMP (disodium salt) was obtained from Sigma. Tributylamine was from BDH. Crystalline phosphoric acid was purchased from Fluorochem, Glossop, Derbyshire, U.K. 1,1'-Carbonyldi-imidazole was obtained from Lancaster Synthesis, Lancaster, U.K. 6-Aminohexan-1-ol was purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Dowex resins (analytical grade) were from Sigma. CNBr-activated Sepharose 4B was obtained from Pharmacia. All other reagents were analytical grade or better from commercial sources and were used without further purification.

In earlier preparations of P^1-(6-aminohex-1-yl)-P^2-(5'-uridine)-pyrophosphate (or UDP-hexanolamine), 6-aminohexanol phosphate was kindly provided by Dr. I. P. Trayer, Department of Biochemistry, University of Birmingham, U.K. The synthesis of UDP-hexanolamine and its coupling to CNBr-activated Sepharose 4B were performed by the method previously described (Barker *et al.*, 1972). Approximately 6–7μmol of UDP-hexanolamine was bound per g of moist swollen gel.

Enzyme assays

UDP-glucuronyltransferase activity towards various substrates was assayed by the following methods: 4-nitrophenol and 2-aminophenol (Winsnes, 1969), bilirubin (Heirwegh *et al.*, 1972), 1-naphthol (Otani *et al.*, 1976), testosterone (17β-hydroxyandrost-4-en-3-one) (Rao *et al.*, 1976*a*) and morphine (Del Villar *et al.*, 1974). One unit of enzyme activity represents the formation of 1nmol of glucuronide/min. Epoxide hydratase (EC 4.2.1.63) activity was measured by the method of Oesch (1974).

Protein concentrations were determined by the biuret method (see Layne, 1957) or the method of Lowry *et al.* (1951). Very low protein concentrations were assayed by the method described by Bradford (1976). In all cases bovine serum albumin was used as standard.

Gel electrophoresis

Gel electrophoresis was performed by the method of Weber & Osborn (1969) in the presence of 0.1% sodium dodecyl sulphate as previously described (Burchell, 1977*b*).

Immunochemical analysis

Antiserum was raised in White Lop-Eared rabbits by subcutaneous injection of pure UDP-glucuronyltransferase. Equal volumes of pure protein (0.5mg/ml) and Freund's complete adjuvant (purchased from Grand Island Biological Co., Grand Island, NY, U.S.A.) were mixed into a fine emulsion. This mixture (1ml) was injected into each rabbit. After 4 weeks a second identical quantity of protein mixture was injected into the rabbits. Two weeks later 20ml of blood was collected from the ear vein and allowed to clot overnight at 4°C. Clear antiserum was obtained after removal of the clot and blood cells by centrifugation at 2000*g* for 10min.

Immunochemical analyses were performed by the Ouchterlony double-diffusion technique in 1% agar (Ouchterlony, 1949).

Amino acid analysis

The amino acid composition of pure UDP-glucuronyltransferase was determined by the method described by Taylor *et al.* (1975).

Treatment of the rats

Male Wistar rats were given 2g of sodium phenobarbital/litre in their drinking water for 7 days before the animals were killed.

Results

Purification of UDP-glucuronyltransferase

UDP-glucuronyltransferase was purified by ion-exchange chromatography and affinity chromatography on a UDP-hexanolamine–Sepharose 4B column (6cm×2cm) as previously described (Burchell, 1977*a*,*b*). Homogeneously pure enzyme was specifically eluted by using 5mM-UDP-glucuronic acid, and a detailed diagram of the affinity-chromatographic step is shown (Fig. 1). About 9mg of the 10mg of protein sample routinely applied and approx. 30% of UDP-glucuronyltransferase activity was washed straight through the column with 0.05%

Lubrol/25mM-potassium phosphate buffer, pH7.4 (buffer A). Then up to 50% of the bound enzyme was eluted with UDP-glucuronic acid in buffer A. Further washing of the column with 2M-KCl in buffer A removed all the remaining enzyme activity from the column, although this non-specific eluate was impure and contained contaminating protein. Attempts to remove this salt from this latter eluate by dialysis resulted in loss of enzyme activity.

One further advantage of the enzyme purification with affinity chromatography as the last step is that it enables the preparation of homogeneously pure enzyme in a sufficiently concentrated form for immediate use. Potentially destructive procedures such as dialysis, vacuum dialysis and further chromatography, which might be required to concentrate the enzyme preparation, were avoided. Indeed Gorski & Kasper (1977) reported a 59% loss of enzyme units by an 11h dialysis of the purified enzyme, and certainly some activity was lost during their concentration of UDP-glucuronyltransferase by adsorption on and desorption from DEAE-agarose.

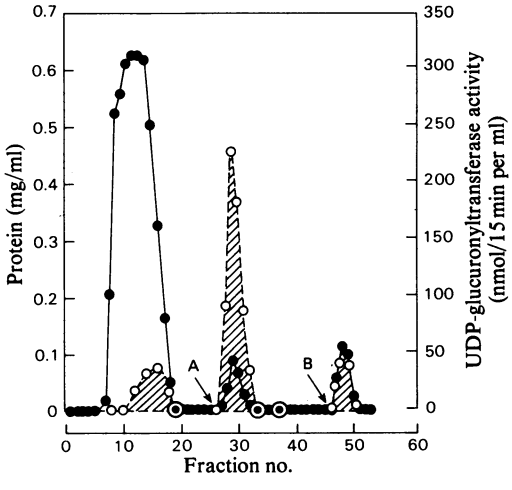

Fig. 1. *Affinity chromatography of UDP-glucuronyltransferase on UDP-hexanolamine–Sepharose 4B*
DEAE-Sephadex eluate (approx. 10mg of protein) was applied to a column (6cm×2cm). The elution of protein (●) and enzyme activity (○), assayed with 4-nitrophenol as aglycone substrate, are shown. The arrows indicate points at which changes in the composition of the elution buffer occurred: A, 5mM-UDP-glucuronic acid; B, 2M-KCl; 1.3ml fractions were collected (see the Results section for further experimental detail).

Table 1 shows the results of the whole purification procedure described above. A range of values are shown, which were obtained from four complete purification experiments. The specific activity of the Lubrol-soluble supernatant, increased 70–125% by phenobarbital pretreatment of rats, was further increased by purification some 51–88-fold, from 32.1–43.9 units/mg of protein in the Lubrol-soluble supernatant to 2261–2843 units/mg of protein in the final preparation, with 4-nitrophenol as substrate. Thus UDP-glucuronyltransferase activity was purified approx. 350-fold over the activity present in the 10000*g* supernatant (see Burchell, 1977*a*). Detergent activation of the enzyme has been taken into account in calculation of the purification values. The specific activity of the final UDP-glucuronyltransferase preparation is the best value recorded for a pure enzyme preparation, 6-fold higher than the actual value recorded by Gorski & Kasper (1977), and in agreement with their predicted final specific activity. The yield of UDP-glucuronyltransferase activity with 4-nitrophenol as substrate was 2–3% and up to 1.5mg of pure enzyme was obtained.

The pure enzyme exhibited a half-life of approx. 18–20 days when stored at 0°C in ice. Storage of the enzyme by freezing at −20°C resulted in a complete loss of enzyme activity. Pure UDP-glucuronyltransferase also proved to be thermolabile when incubated at 37°C for longer than 15min. Thus all experiments involving assay of the pure enzyme were conducted within this time limit.

Table 1. *Purification of rat liver UDP-glucuronyltransferase towards 4-nitrophenol as substrate*
One unit of activity represents the formation of 1 nmol of 4-nitrophenol glucuronide/min. A range of values is shown, representing four complete isolation experiments. Purification values are shown starting from the Lubrol-soluble supernatant. Preliminary steps up to the solubilization of the microsomal fraction have been previously described (Burchell, 1977*a*).

Purification step	Total protein (mg)	Specific activity (units/mg of protein)	Relative purification	Yield (%)
Lubrol-soluble supernatant	2337–4332	32.1–43.9	1	100
25–60%-satn.-$(NH_4)_2SO_4$ precipitate	814–1650	34.4–45.2	0.8–1.4	29.8–68.6
DEAE-cellulose eluate	256–825	50.4–94.5	1.2–2.9	21.9–46.7
CM-cellulose eluate	146–444	45.2–110.1	1.0–3.4	10.4–31.1
DEAE-Sephadex eluate	21–76	237–247	5.4–7.7	8.7–10.1
UDP-glucuronic acid eluate from UDP-hexanolamine–Sepharose 4B	0.7–1.5	2261–2843	51.5–88.5	1.9–2.9

Criteria for homogeneity of the purified UDP-glucuronyltransferase

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis in the presence of 0.1% sodium dodecyl sulphate was used to assess the purity of the UDP-glucuronyltransferase preparation. This procedure was capable of separating the polypeptides contained in the $(NH_4)_2SO_4$ fraction into more than 30 distinct staining bands. Fig. 2 is a photograph of the stained polypeptides visible after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis: gel A shows pure UDP-glucuronyltransferase from the livers of rats pretreated with phenobarbital, gel B the enzyme preparation obtained from untreated rat liver (see Burchell, 1977*b*) and gel C the polypeptides present in the DEAE-Sephadex eluate. Only one staining band of mol.wt. 57000 (as reported by Burchell, 1977*b*) was observed in the UDP-glucuronic acid eluates from the UDP-hexanolamine–Sepharose column, whether liver from untreated or phenobarbital-treated rats was used as enzyme source (see below). This value of 57000 for the subunit molecular weight of UDP-glucuronyltransferase is in agreement with the value of 59000 reported by Gorski & Kasper (1977), but larger than the values of 52000 or 48000 for species isolated by Bock *et al.* (1977).

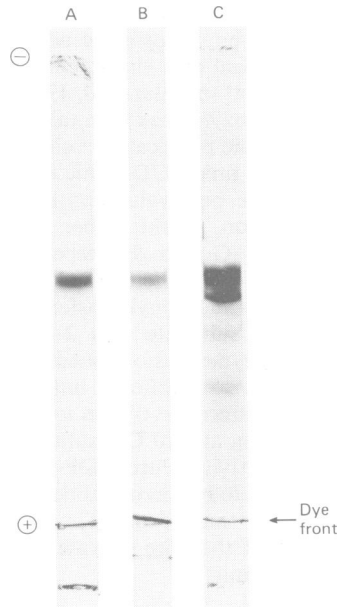

Fig. 2. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoretograms of purified UDP-glucuronyltransferase*
Disc electrophoresis was performed with cylindrical 6.5% polyacrylamide gels (7.5cm×0.6cm) in the presence of 0.1% sodium dodecyl sulphate (see the Materials and Methods section). Gel A, pure UDP-glucuronyltransferase from phenobarbital-pretreated rat liver (4μg of protein), specific activity 2843 units/mg of protein. Gel B, pure UDP-glucuronyltransferase from untreated rat liver (1μg of protein), specific activity 2747 units/mg of protein. Gel C, DEAE-Sephadex eluate (15μg of protein), specific activity 237 units/mg of protein. Gels were stained with 0.25% Coomassie Blue for 60min and destained with acetic acid/methanol/water (7:5:43, by vol.). Direction of migration is from top to bottom.

Immunochemical analysis. Antiserum was raised against pure UDP-glucuronyltransferase in rabbits (see the Materials and Methods section). Fig. 3(A) shows that fractions collected throughout the purification procedure, when allowed to react against the antiserum in the Ouchterlony double-diffusion technique, produced a single sharp continuous precipitation line with no visible spur formation, suggesting that only one species of protein had been isolated by the above purification procedure. No precipitation line could be observed when the antiserum was allowed to react with a pure preparation of the likely major contaminant protein, epoxide hydratase (see Burchell, 1977*a*), prepared by the method of Knowles & Burchell (1977). Indeed no epoxide hydratase activity can be detected in the pure preparation of UDP-glucuronyltransferase. Furthermore, when antisera raised against pure epoxide hydratase and UDP-glucuronyltransferase were mixed and the mixed antisera reacted against the pure antigens a crossed precipitin line was observed (see Fig. 3B). This experiment demonstrated that the two pure proteins and their respective antisera produce two different precipitin lines and illustrates the non-identity of the two individual enzyme preparations. Therefore examination of the UDP-glucuronyltransferase preparation by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and immunochemically, suggests that UDP-glucuronyltransferase has been purified to homogeneity.

Comparison of pure UDP-glucuronyltransferases from phenobarbital-treated and untreated rat liver

As described above, the pure enzymes obtained from phenobarbital-treated and untreated rat liver exhibit the same mol.wt. of 57000 after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The specific activities of the final preparations are similar (see Burchell, 1977*b*); indeed I have obtained preparations of pure enzyme from both sources with the same final specific activity of 2800 units/mg of protein. Immunochemical experiments similar to those reported here show that UDP-glucuronyltransferase preparations from the two liver sources are immunologically identical (B. Burchell, unpublished work). Finally the pure enzymes from both hepatic sources can be activated 2–3-fold by 10mM-diethyl-

nitrosamine (see Burchell, 1977*a*, and Table 2). Thus these pieces of evidence indicate that UDP-glucuronyltransferases isolated from untreated or from phenobarbital-treated liver appear to be the same enzyme.

Hydrophobic nature of pure UDP-glucuronyltransferase

During attempts to remove the detergent from pure UDP-glucuronyltransferase by dialysis, the enzyme was precipitated from solution, indicating the hydrophobic nature of the protein structure. This type of structure would be essential for binding of the enzyme to the microsomal membrane. However, amino acid analysis of the protein does not show any unusually high content of hydrophobic amino acid residues. The amino acid composition was determined as described in the Materials and Methods section. The preliminary results obtained were very similar to those reported by Gorski & Kasper (1977). The number of non-polar residues represents 37% of the total number of residues, which is in good agreement with the 39% of non-polar residues calculated from data of Gorski & Kasper (1977). A small difference was that I observed a higher serine content. These results suggest that the enzyme does not have any unusual hydrophobic amino acid content. Thus the hydrophobic nature of the protein

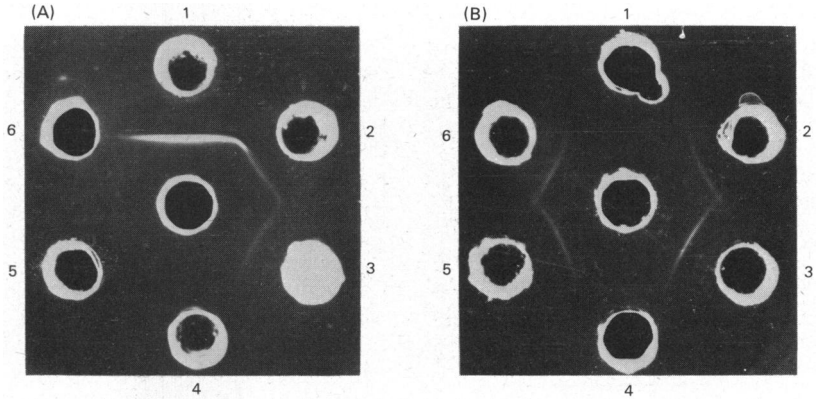

Fig. 3. *Ouchterlony double-diffusion analysis of pure UDP-glucuronyltransferase*
Experiments performed with 1% agar plates were allowed to develop for 48h at 15°C before photography. (A) Wells contained: centre, antiserum raised against pure UDP-glucuronyltransferase; 1, 25–60%-satn.-$(NH_4)_2SO_4$ precipitate; 2 and 3, identical quantities of pure UDP-glucuronyltransferase; 4, pure epoxide hydratase; 5, buffer blank; 6, empty. (B) Wells contained: centre, antiserum raised against pure UDP-glucuronyltransferase and antiserum raised against pure epoxide hydratase, mixed in equal volumes; 1, empty; 2 and 5, identical quantities of pure UDP-glucuronyltransferase; 3 and 6, identical quantities of pure epoxide hydratase; 4, buffer blank.

Table 2. *Activation of UDP-glucuronyltransferase by diethylnitrosamine*
Diethylnitrosamine (10mM final concentration) was added to the enzyme-assay mixtures immediately before incubation. Data are shown from three different isolation experiments. Each value represents an experimental result obtained at that purification step.

Purification step	UDP-glucuronyltransferase activity (nmol of 2-aminophenol glucuronide/min per mg of protein)		Activation (%)
	Diethylnitrosamine absent	Diethylnitrosamine present	
25–60%-satn.-$(NH_4)_2SO_4$ precipitate	0.32	2.5	781
	0.18	1.8	1000
DEAE-cellulose eluate	0.33	1.3	393
CM-cellulose eluate	0.41	1.6	391
DEAE-Sephadex eluate	2.04	5.0	250
	3.68	10.22	277
	2.57	7.97	317
UDP-glucuronic acid eluate from UDP-hexanolamine–Sepharose 4B	16.4	33.25	202
	14.3	46.3	323

must be conferred by the three-dimensional arrangement of the non-polar amino acid residues.

Activation of pure UDP-glucuronyltransferase

The kinetic constants of pure UDP-glucuronyltransferase were measured by using 0.5mM-4-nitrophenol as aglycone substrate and varying the concentration of UDP-glucuronic acid. Fig. 4 shows the resultant Lineweaver–Burk plot obtained; the enzyme K_m (UDP-glucuronic acid) was 5.4mM and the $V_{max.}$ (UDP-glucuronic acid) 4.35μmol of 4-nitrophenol glucuronidated/min per mg of protein. This K_m value is of the same order as the value obtained with a microsomal preparation maximally activated by a non-ionic detergent and this range of UDP-glucuronic acid concentrations (Winsnes, 1972). Thus the purified enzyme appears to be in the activated form, as would be expected, since the enzyme was exposed to detergent treatment before purification. Therefore we could perhaps predict that the purified enzyme would not be activated by the so-called physiological activator UDP-*N*-acetylglucosamine (see Dutton & Burchell, 1977), under conditions where the native microsomal fraction was activated 2.5-fold. However, more surprisingly the homogeneously pure enzyme can be activated 2–3-fold with 2-aminophenol as substrate by 10mM-diethylnitrosamine, as previously reported for the partially purified enzyme (Burchell, 1977*a*). Nakata *et al.* (1975) have claimed that diethylnitrosamine modifies the membrane protein–lipid interactions, thereby increasing the activity of the lipid-dependent UDP-glucuronyltransferase. An alternative explanation would be that diethylnitrosamine could modify the protein conformation, changing it to a more active configuration for that substrate.

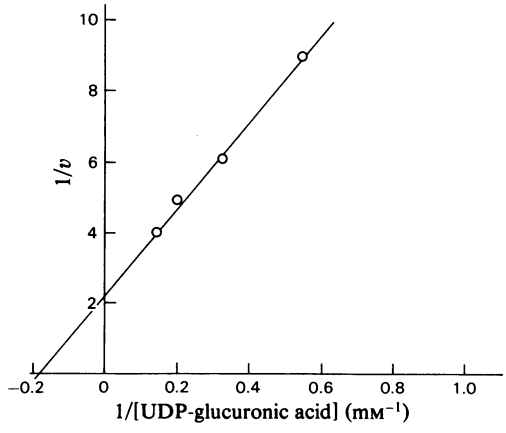

Fig. 4. *Lineweaver–Burk plot of pure UDP-glucuronyltransferase activity for a range of UDP-glucuronic acid concentrations*

Conditions of assay were as described in the Materials and Methods section with 0.5mM-4-nitrophenol as aglycone substrate. v is the rate of reaction expressed as nmol of 4-nitrophenol glucuronide formed/min per mg of protein. K_m=5.4mM. $V_{max.}$=4.35μmol/min per mg of protein.

Sugar nucleotide substrate specificity of pure UDP-glucuronyltransferase

The specificity of the pure preparation of UDP-glucuronyltransferase was examined by using 4mM-UDP-glucose and 4mM-UDP-galacturonic acid as sugar nucleotide substrates and 0.5mM-4-nitrophenol in the assay procedure. No conjugating-enzyme activity was detected when UDP-glucose and UDP-galacturonic acid were used as substrates for purified active UDP-glucuronyltransferase. Under identical assay conditions, the glucoside of 4-nitrophenol was formed by the microsomal fraction at a rate of 0.32 unit/mg of protein, compared with 0.84 unit/mg of protein for the glucuronidation of 4-nitrophenol, in agreement with earlier work (Gessner *et al.*, 1973). No conjugates of UDP-galacturonic acid were detectable in native or Lubrol-solubilized rat liver microsomal fractions. Further I have observed that a 5-fold excess of UDP-glucose (10mM) over UDP-glucuronic acid (2mM) did not inhibit UDP-glucuronyltransferase activity of Triton X-100-treated rat liver microsomal fraction or the purified enzyme.

Thus purified UDP-glucuronyltransferase appears to exhibit absolute specificity for UDP-glucuronic acid as sugar nucleotide substrate, and the glucoside conjugation of 4-nitrophenol must be catalysed by a different microsomal enzyme. The latter conclusion confirms a result obtained by Labow *et al.* (1971), where selective inhibition or destruction of the transferase in solubilized microsomal extracts together with gel filtration was used to obtain partially purified preparations of either transferase activity essentially devoid of the other.

Aglycone substrate specificity of pure UDP-glucuronyltransferase

The activity of UDP-glucuronyltransferase towards six aglycone substrates was assayed in all the purification fractions from the $(NH_4)_2SO_4$ stage through to the final preparation. The relative yield of total activity and purification values are compared at each stage (Table 3). Enzyme activities towards 4-nitrophenol, 2-aminophenol and 1-naphthol exhibited similar yields and increases of specific activity throughout the whole purification procedure. The coincidence of these two measured parameters in the pure enzyme fraction is consistent with the finding that the same enzyme protein is responsible for the glucuronidation of these three substrates, as pre-

Table 3. *Aglycone substrate specificity of UDP-glucuronyltransferase*
Specific activities are expressed as nmol of glucuronide formed/min per mg of protein.

		Purification step				
Aglycone substrate		25–60%-satn.-$(NH_4)_2SO_4$ precipitate	DEAE-cellulose	CM-cellulose	DEAE-Sephadex	UDP-glucuronic acid eluate from UDP-hexanolamine–Sepharose 4B
4-Nitrophenol	Specific activity	34.4	50.4	45.2	242	2261
	Purification	1	1.5	1.3	7	66
	Yield (%)	100	75	35	32	9
2-Aminophenol	Specific activity	0.32	0.33	0.41	3.67	16.4
	Purification	1	1	1.3	11.5	52
	Yield (%)	100	51	34	53	7.5
1-Naphthol	Specific activity	70	180	133	777	4510
	Purification	1	2.6	1.9	11	64
	Yield (%)	100	128	51	51	8.7
Morphine	Specific activity	15.3	8.4	4.6	56.8	397
	Purification	1	0.55	0.30	3.7	26
	Yield (%)	100	27	8	16	3.5
Testosterone	Specific activity	3.6	2.5	4.0	20.5	0
	Purification	1	0.7	1.1	5.7	—
	Yield (%)	100	35	30	26	0
Bilirubin	Specific activity	0.15	0.02	0.03	0	0
	Purification	1	0.13	0.2	—	—
	Yield (%)	100	7.4	5.8	0	0

viously reported by preliminary findings (Burchell, 1977*b*). Enzyme activity towards testosterone appears to be purified to a comparable degree until the final stage, when no activity was detectable. Activity towards morphine can be detected throughout the purification procedure, including the final product, although the purification value and yield of enzyme are approx. 50% of the values recorded for the three phenolic substrates. UDP-glucuronyltransferase activity towards bilirubin as substrate is difficult to estimate in the later purification steps and no activity was detected in the two final fractions. Very low activity towards bilirubin was previously detected in the DEAE-Sephadex fraction (Burchell, 1977*a*). However, the loss of bilirubin UDP-glucuronyltransferase activity is not due to the loss of ester-glucuronidating activity, as high activity towards 2-aminobenzoate was displayed by the final preparation, purified some 40-fold over the $(NH_4)_2SO_4$ stage.

Discussion

Having compared the results in the present paper with the relevant previous work, I wish in this discussion to focus attention on two interesting aspects of this work not yet discussed in the light of recent findings.

Firstly, I shall examine the UDP-glucuronyltransferase enzyme reaction mechanism. Information is presented in Fig. 1 that shows that UDP-glucuronyltransferase binds to UDP-hexanolamine–Sepharose 4B and that the presence of Mg^{2+} is not essential for enzyme binding. In this case, the UDP is pyrophosphate-linked to hexanolamine (see Barker *et al.*, 1972). However, if UDP is bound through the ribose dialdehyde derivative of UDP by the method described by Lamed *et al.* (1973) and then linked to Sepharose 4B through adipic acid dihydrazine, UDP-glucuronyltransferase does not bind to this ligand. Thus, from simple examination of the chemical structures of these ligands, I can envisage the orientation of the nucleotide in the active site. The binding of the nucleotide to the active centres may well be through interaction with the uracil base rather than the pyrophosphate group. UDP-glucuronyltransferase does not bind to Sepharose 4B–hexane-4-aminophenol, probably because the nucleotide substrate must bind to the enzyme first, as suggested by kinetic analysis, where a sequential-type reaction mechanism has been proposed of an ordered Theorell–Chance type (Vessey & Zakim, 1972; Rao *et al.*, 1976*b*).

Although Mg^{2+} is not essential for UDP binding to UDP-glucuronyltransferase, its presence certainly increases the rate of the enzyme reaction. Its possible association with and partial neutralization of the charged substrate, UDP-glucuronic acid, could in fact facilitate binding of the nucleotide sub-

strate to the enzyme by a similar mechanism to that observed when $ATPMg^{2-}$ is used as substrate for protein kinase enzymes.

Finally, I shall consider my results and other recent reports about the heterogeneity of UDP-glucuronyltransferase. Information obtained from study of the development of UDP-glucuronyltransferase (Wishart *et al.*, 1977; Lucier & McDaniel, 1977; G. J. Wishart, unpublished work) has enabled the categorization of up to 12 substrates into two groups. Enzyme activity towards six substrates develops in rat liver at the late foetal stage (foetal group of substrates) and glucuronidation of the other six substrates was observed immediately after birth (neonatal group of substrates). With the substrates in this paper as examples, bilirubin, morphine and testosterone are placed in the neonatal group of substrates, whereas 1-naphthol, 4-nitrophenol and 2-aminophenol are found in the foetal group of substrates. Likewise, Bock *et al.* (1973, 1977) and G. J. Wishart (unpublished work) have shown that the enzyme activity towards the neonatal group of substrates is preferentially induced in rat liver by administration of phenobarbital, and the activity towards the foetal group of substrates is increased by pretreatment of rats with 3-methylcholanthrene. These results suggest the existence of two functionally distinct forms of UDP-glucuronyltransferase. Thus a possible explanation of my results, obtained by measurement of the activity of pure UDP-glucuronyltransferase towards various substrates, might be that my preparation is slightly contaminated by a second UDP-glucuronyltransferase form, since morphine glucuronyltransferase activity was displayed by the pure enzyme. However, if the latter statement is indeed correct, the molecular weight (possibly subunit molecular weight) of the two forms would appear to be very similar, as they cannot be separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. An alternative explanation of the observed functional heterogeneity of UDP-glucuronyltransferase based on developmental and drug-induction studies could be formulated. The enzyme activity towards the group of substrates exemplified by the phenolic aglycones is first to appear during development; perhaps this basic foetal form of the enzyme may not be capable of glucuronidating neonatal-group substrates until further functional modification of the protein or its environment has taken place. A possible form of this type of enzyme maturation, already tentatively suggested (Winsnes, 1971; Dutton & Burchell, 1974), may be due to change of lipid binding to the enzyme causing conformational alteration and demonstrable functional heterogeneity during development (see Mulder, 1974). Furthermore, the pure UDP-glucuronyltransferase described above does not appear to have any bound lipids (B. Burchell & T. Hallinan, unpublished work). Thus activity towards bilirubin may be lost by inactivation rather than by removal of the enzyme protein. Reconstitution of the pure enzyme with a phospholipid environment may allow reconstitution of activity towards neonatal-group substrates.

Del Villar *et al.* (1975, 1977) and Bock *et al.* (1977) have reported separation of UDP-glucuronyltransferase activity towards a phenolic substrate from the enzyme activity towards morphine by chromatography on DEAE-cellulose. Indeed the results presented here confirm these previous findings in that 73% of the UDP-glucuronyltransferase activity towards morphine and more than 90% of the enzyme activity towards bilirubin is apparently bound to DEAE-cellulose, when 75% of the activity towards 4-nitrophenol is eluted from the column. The elution of the bound protein from the DEAE-cellulose with a salt gradient always released very low yields of enzyme activity towards both bilirubin and 4-nitrophenol. Thus the interpretation of the above results is still an open question. Del Villar *et al.* (1975) have shown that after chromatography on DEAE-cellulose the recovery of UDP-glucuronyltransferase activity towards 4-nitrophenol increased to 128% of the starting values whereas that towards morphine decreased to 43%. They offer no explanation for the occurrence of a protein fraction containing enzyme activity towards both substrates. Further, the question arises: does the concentration of salt (0.5 M-KCl) used by Del Villar *et al.* (1977) to elute a second peak of UDP-glucuronyltransferase activity from DEAE-cellulose selectively inhibit the enzyme activity towards one type of substrate, namely 4-nitrophenol, and not morphine? Bock *et al.* (1977) have claimed that partially purified morphine UDP-glucuronyltransferase and 1-naphthol UDP-glucuronyltransferase have different molecular weights. However, conclusive evidence of separation of two forms of UDP-glucuronyltransferase must await complete purification to homogeneity of a second form of the enzyme to enable direct comparison of two forms.

In conclusion, one might envisage the possible existence of two forms of UDP-glucuronyltransferase, although the present author has so far been able to obtain only one UDP-glucuronyltransferase enzyme in the pure state. However, clearly there is not a separate UDP-glucuronyltransferase for each substrate.

I thank Dr. G. J. Wishart for measurement of UDP-glucuronyltransferase activity with morphine as substrate, and Dr. R. L. Hill, Dr. I. P. Trayer and colleagues for their help and advice about the synthesis of UDP-hexanolamine–Sepharose. I also thank Dr. A. Burchell for stimulating discussion and advice, Professor G. J. Dutton for his critical assessment of the manuscript, Miss L. Husband for excellent technical assistance and the Medical Research Council for a grant supporting this work.

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