ISOLATION AND PURIFICATION OF BILIRUBIN UDP-GLUCURONYL-TRANSFERASE FROM RAT LIVER

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1. Introduction

Liver microsomal bilirubin UDP-glucuronyltransferase is the rate limiting enzyme in the excretion of bilirubin in neonatal jaundice [1] and Crigler-Najjar syndrome [2]. Clinically, it is important to know whether drugs or xenobiotics compete with bilirubin for the same active site on a single UDP-glucuronyltransferase, to prevent therapeutically-induced problems. Such information can only be obtained by purification of bilirubin UDP-glucuronyltransferase.

Hepatic UDP-glucuronyltransferase with activity towards phenolic substrates has been purified to apparent homogeneity in [3–7]. In these transferase preparations, when looked for, activity towards bilirubin was not found [4,6]. These transferase activities depend on the presence of phospholipids [8–12]. Transferase activity towards bilirubin can be restored to delipidated hepatic microsomal preparations by addition of lecithin [8].

This paper shows that lecithin liposomes need to be added to each isolated transferase fraction to facilitate the assay and purification of the hitherto elusive bilirubin UDP-glucuronyltransferase for the first time. The existence of multiple forms of hepatic UDP-glucuronyltransferase is also discussed.

2. Materials and methods

Egg lecithin, grade 1, was purchased from Lipid Products, South Nutfield Surrey. Bilirubin and 4-nitrophenol were from Sigma (London). [*N-methyl*-^{14}C]Morphine hydrochloride and [*methyl*-^{3}H]choline were obtained from The Radiochemical Centre, Amersham. UDP-hexanolamine was synthesised following [13] as in [4].

2.1. *Enzyme assays*

UDP-glucuronyltransferase activity towards various substrates was measured by the methods described: 4-nitrophenol [14]; morphine [15]; testosterone [16]; bilirubin [17]. One unit of enzyme activity represents 1 nmol glucuronide formed/min. Protein concentrations were determined by the method in [18].

2.2. *Purification of UDP-glucuronyltransferase*

Wistar rats were pretreated with 2 g/l phenobarbital in drinking water for 7 days. Hepatic phosphatidyl choline was labelled in vivo by intraperitoneal injection of 200 μCi [*methyl*-^{3}H]choline into each rat 35 min before the animals were killed [19]. UDP-glucuronyltransferase was solubilized and partially purified by ammonium sulphate fractionation as in [20].

The ammonium sulphate precipitate was dissolved in 0.01% (w/v) Lubrol/5 mM potassium phosphate/5 nM mercaptoethanol buffer (pH 8.0) (buffer A) to give a red solution (~40 ml). This extract was dialysed against 6 l buffer A for 72 h. The clear red non-diffusible material was applied to a DEAE-cellulose column (12 × 3.5 cm). Bilirubin UDP-glucuronyltransferase activity was eluted at 15 ml/h with 200 ml buffer A followed by 400 ml linear gradient of 0–0.3 M KCl in buffer A (see section 3).

Selected DEAE-cellulose eluate fractions were concentrated ~15-fold by vacuum dialysis and the concentrates were dialysed against 250 vol. buffer A overnight. UDP-glucuronyltransferase in these concentrates was further purified by affinity chromatography using UDP-hexanolamine–Sepharose 4B as in [2].

2.3. *Reconstitution of UDP-glucuronyltransferase with lecithin*

Egg yolk phosphatidyl choline liposomes were prepared in 0.4 M Tris/maleate/60 mM $MgCl_2$ (pH 7.7) buffer, ultrasonicated to a small size and added to enzyme fractions as in [21].

2.4. *Gel electrophoretic analysis*

SDS–polyacrylamide gel electrophoresis was done as in [3].

3. Results

3.1. *Reconstitution of the bilirubin UDP-glucuronyltransferase activity of ammonium sulphate extracts of rat liver microsomes*

More than 90% of the phosphatidylcholine originally present in the lubrol-solubilized microsomes was removed during ammonium sulphate fractionation of this preparation (table 1). After this purification procedure transferase activity towards bilirubin was decreased by 50% from the Lubrol soluble stage whereas activity towards 4-nitrophenol increased ~2-fold (cf. table 1, fig.1). Thus these two activities are apparently not copurified during ammonium sulphate fractionation; this difference could be due

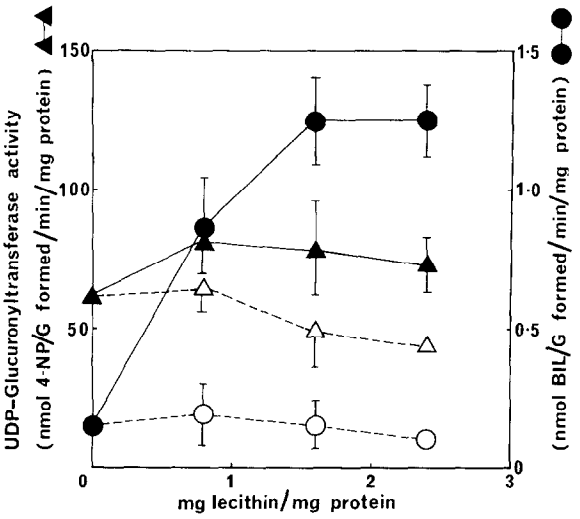

Fig.1. Effect of lecithin on UDP-glucuronyltransferase activity of ammonium sulphate fractions. Various quantities of lecithin liposomes in buffer or buffer alone were mixed with samples of ammonium sulphate fractions as shown above. These mixtures were incubated for 30 min at 20°C prior to assay for transferase activity towards bilirubin or 4-nitrophenol. Open circles or triangles linked by broken lines are control experiments and show the effect of dilution with buffer; transferase activity towards bilirubin, (○– – –○) or towards 4-nitrophenol (△– – –△). BIL/G-bilirubin glucuronide, 4-NP/G 4-nitrophenol glucuronide.

Table 1
Purification of rat liver bilirubin UDP-glucuronyltransferase

		Total protein (mg)	[^{3}H]phosphatidylcholine (dpm)	UDP-glucuronyltransferase activity					
				Bilirubin			4-nitrophenol		
				Spec. act.	Rel. purif.	Yield (%)	Spec. act.	Rel. purif.	Yield (%)
Lubrol-soluble supernatant		1404	4.0×10^6	0.29	1	100	35	1	100
25–60% Satn $(NH_4)_2SO_4$ precipitate		300	3.3×10^5	0.82	2.8	60	86	2.5	53
DEAE-cellulose eluate concentrates	A.	24.6	5.1×10^4	0	–	0	93	2.7	4.6
	B.	16.4	2.6×10^4	1.65	5.7	6.6	262	7.5	8.7
	C.	26.4	8.9×10^4	3.41	11.7	22.1	45	1.3	2.4
UDP-hexanolamine Sepharose 4B/UDPGA eluates	A.	0.7	0	0	–	0	999	28.5	1.4
	B.	1.0	0	0.51	1.7	0.13	2608	74.5	5.6
	C.	0.7	0	2.55	8.8	0.46	250	7.1	0.4

Specific activity of UDP-glucuronyltransferase is represented as 1 nmol glucuronide formed . min^{-1} . mg $protein^{-1}$. All enzyme assays were done after reconstitution of protein with lecithin (1 mg lecithin/mg protein). Results shown were obtained from 3 phenobarbital-treated male Wistar rat livers. UDPGA = UDP-glucuronic acid

to the extensive phospholipid depletion of the purified fraction. Therefore, transferase activities were determined in ammonium sulphate fractions reconstituted with various amounts of lecithin liposomes (fig.1). Bilirubin UDP-glucuronyltransferase activity was increased ~9-fold over the activity in original ammonium sulphate fraction by the addition of ⩽1.6 mg lecithin liposomes in buffer/mg protein. Dilution of the ammonium sulphate fraction up to 4-fold with buffer alone did not significantly alter the enzyme activity (fig.1). Therefore, activation of bilirubin UDP-glucuronyltransferase does not appear to be due to a possible decreased inhibition by detergent. The addition of lecithin liposomes to ammonium sulphate fractions only increased transferase activity towards 4-nitrophenol by 25% (fig.1) presumably because this activity can be maintained by the presence of Lubrol 12A9 [22].

Thus bilirubin UDP-glucuronyltransferase activity even with Lubrol present can only be measured after reconstitution with phospholipid. Using this technique, purification of bilirubin UDP-glucuronyltransferase was then attempted.

3.2. *Purification of bilirubin UDP-glucuronyltransferase*

Ammonium sulphate fraction containing UDP-glucuronyltransferase activities was applied to a DEAE-cellulose column pre-equilibrated with buffer A. A sample of each eluted fraction was tested with lecithin liposomes (mg lecithin/mg protein, 1:1) before assay of transferase activities, to overcome any differential separation of phospholipid that may have occurred during chromatography and which might lead to misinterpretation of the data. Some transferase activity towards 4-nitrophenol and morphine, but not towards bilirubin, was eluted before the salt gradient was applied to the column (fig.2.). A large peak of bilirubin UDP-glucuronyltransferase activity was then eluted at ~70 mM KCl by a linear salt gradient (fig.2); 95% of the transferase activity towards bilirubin originally applied to the DEAE-cellulose was recovered by this procedure. Additional transferase activity towards 4-nitrophenol was eluted at ~20 mM KCl by the salt gradient. Morphine UDP-glucuronyltransferase activity was present throughout, in eluted fractions.

To further purify transferase activities towards bilirubin, fractions 4–14, 43–52 and 55–70 (fig.2.) were pooled and vacuum-dialysed to give 3 concentrates A, B and C, respectively. Bilirubin UDP-glucuronyltransferase recovered in concentrate C was purified 12-fold over the activity in the Lubrol-soluble supernatant, whereas the little transferase activity towards 4-nitrophenol in concentrate C had not been significantly purified (table 1). In contrast, concentrate A exhibited UDP-glucuronyltransferase activity towards 4-nitrophenol and morphine, but not bilirubin. Transferase activities towards bilirubin, 4-nitrophenol and morphine recovered in concentrate B were all purified 6–7-fold over the activities in the Lubrol-soluble supernatant.

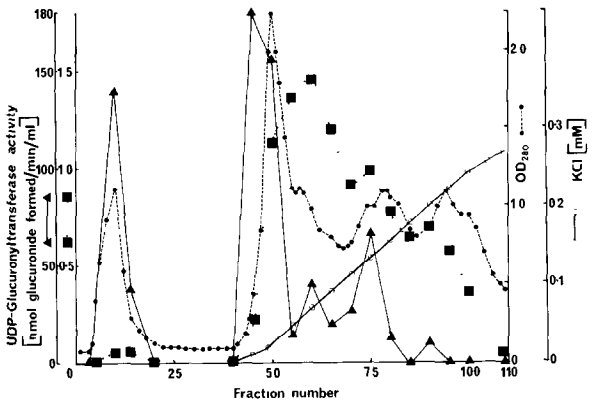

Fig.2. DEAE-cellulose column chromatography of rat hepatic bilirubin UDP-glucuronyltransferase activity. Ammonium sulphate fraction (30 ml) was applied to a DEAE-cellulose column. Buffer A (~200 ml) was passed through the column before application of a linear 0–0.3 M KCl gradient (○——○). Fractions (5 ml) were collected at 15 ml/h flowrate and assayed for transferase activity towards bilirubin (■), 4-nitrophenol (▲).

Overall, 50% of the transferase activity toward 4-nitrophenol and 95% of the activity towards bilirubin applied to the column were recovered. The distribution of these two activities throughout the column strongly suggests that they are separable entities.

UDP-glucuronyltransferase activities in concentrates A–C were further purified by affinity chromatography using UDP-hexanolamine–Sepharose 4B. Table 1 shows the results of these experiments. During affinity chromatography all of the phosphatidyl choline was separated from UDP-glucuronyltransferases, as in [14]. As a consequence of affinity chromatography of concentrate C no UDP-glucuronyltransferase activity towards bilirubin could be detected until these highly purified fractions were reconstituted with

lecithin liposomes. The highest transferase specific activities with bilirubin as substrate obtained in 4 separate purification experiments. 1.5–4.8 units . min^{-1} . mg $protein^{-1}$, after elution from the UDP-hexanolamine–Sepharose were not increased over the values obtained in DEAE-cellulose eluate concentrate C (table 1). A single polypeptide staining band was observed to be present in these preparations after analysis by SDS–gel electrophoresis, which suggests that bilirubin UDP-glucuronyltransferase has been highly purified. Transferase activity towards 4-nitrophenol in the same concentrate C was purified by affinity chromatography. Presumably simple reconstitution of purified bilirubin UDP-glucuronyltransferase with lecithin is not sufficient to restore full catalytic activity to all of the enzyme protein. Alternatively, another unknown protein may have been purified, although this possibility seems unlikely, because transferase activities towards 4-nitrophenol present in concentrate A or B was purified a further 11-fold by chromatography on UDP-hexanolamine–Sepharose.

4. Discussion

UDP-glucuronyltransferase activity towards bilirubin has been purified >50-fold over the crude liver homogenate, although optimal reconstitution of this highly purified bilirubin UDP-glucuronyltransferase has probably not yet been achieved.

The above results also suggest that transferase activities towards bilirubin and 4-nitrophenol may represent separate entities. Separation of these transferase activities may be improved by:

(i) Use of a shallower KCl gradient;
(ii) A more exhaustive phospholipid depletion prior to DEAE-cellulose chromatography, although these procedures may reduce the quantities of enzyme activities recovered.

Problems were associated with delipidation of microsomal UDP-glucuronyltransferase by gel filtration [8]; where transferase activity towards bilirubin, but not towards 4-nitrophenol was recovered after the addition of lecithin. Delipidation has apparently irreversibly inactivated transferase activity towards 4-nitrophenol [8]. Thus the separation of different transferase activities by DEAE-cellulose chromatography must be carefully evaluated because phospholipids bind to DEAE-cellulose (table 1) and may be separated from the enzyme by this method, as suggested [20]. 95% of transferase activity toward bilirubin and >50% of the activity towards 4-nitrophenol applied to the DEAE-cellulose column can be recovered, only after addition of lecithin liposomes to each eluted fraction. Therefore, it is likely that the separation, rather than differential inactivation [8] of these two entities has been achieved.

If 4-nitrophenol UDP-glucuronyltransferase activity can be representative of 'enzyme 1' [6], which glucuronidates Wishart's planar molecules [23] then bilirubin UDP-glucuronyltransferase may be 'enzyme 2' proposed [6] which glucuronidates non-planar substrates. In this case morphine with planar and non-planar portions in its molecular structure may be glucuronidated by both enzymes.

Rabbit liver oestrone UDP-glucuronyltransferase activity was reported separated from that towards 4-nitrophenol using DEAE-cellulose chromatography [24]; however, the recovery of each activity was only 8% of that applied to the column. Reconstitution of the protein in the separate fractions with phospholipids should have been attempted and then transferase activity towards 4-nitrophenol and oestrone should probably have been measured in both fractions to establish separate entities. For example, further investigation of the substrate specificity of UDP-glucuronyltransferase purified to apparent homogeneity [4] made possible by reconstitution with lecithin, revealed previously undetectable transferase activity towards testosterone; transferase activity towards bilirubin remained undetectable (P. J. Weatherill, B.B., unpublished). Indeed preliminary experiments also show that testosterone and bilirubin UDP-glucuronyltransferases are separable entities and both preparations are able to glucuronidate morphine (B.B., P. J. Weatherill, unpublished).

In vivo UDP-glucuronyltransferases were suggested to have evolved to serve endogeneous substrates, such as steroids, bilirubin, catecholamines and those endogeneous molecules routinely encountered by the species in its food [25]. Consequently drugs or laboratory xenobiotics are unlikely to be entirely satisfactory tools for tracing boundaries of transferase specificity. Overlap must occur. Reliable classification of UDP-glucuronyltransferase specificities requires the use of endogeneous substrates [26].

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