Studies on the Purification of Rat Liver Uridine Diphosphate Glucuronyltransferase

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1. A stable, more highly purified, preparation of UDP-glucuronyltransferase was obtained than previously reported. 2. Enzyme activity towards o-aminophenyl and p-nitrophenyl was increased 43- and 46-fold respectively. 3. The final preparation contains only three staining polypeptide bands visible after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. 4. The only known major accompanying protein appears to be epoxide hydratase. 5. The purified enzyme activity towards o-aminophenol can still be activated 3-fold by diethylamino benzidine. 6. On evidence from purification, o-aminophenol and p-nitrophenol appear to be glucuronidated by the same enzyme protein. The possible recognition of the UDP-glucurononyltransferase enzyme is discussed.

Hepatic microsomal UDP-glucuronyltransferase (EC 2.4.1.17) catalyses the conjugation of endogenous compounds and xenobiotics with glucuronic acid (see Dutton, 1966). Several lines of evidence suggest that there is more than one UDP-glucuronyltransferase (see Dutton, 1971; Dutton & Burchell, 1976). Part of this evidence is based on partial separation of detergent-solubilized enzyme activity towards two xenobiotic substrates by density-gradient centrifugation (Howland et al., 1971) or DEAE-cellulose chromatography (Del Villar et al., 1975). However, the existence of more than one enzyme protein could not be unequivocally demonstrated by these workers using partially purified enzyme preparations and cannot be verified until catalytically active UDP-glucuronyltransferase has been purified to homogeneity.

Several membrane-bound enzymes involved in the microsomal mixed-function oxidase system have been purified to apparent homogeneity (Bentley & Oesch, 1975; Dignam & Strobel, 1975; Haugen et al., 1975; Kawalek et al., 1975). Pharmacologically important UDP-glucuronyltransferase still has not been purified, despite a number of attempts, since the initial work of Isselbacher et al. (1962).

The present paper describes a different approach to this problem. A preparation obtained from consecutive ion-exchange-chromatographic steps contains active UDP-glucuronyltransferase and only three polypeptides.

A preliminary report of a similar purification procedure, now improved, has been published (Burchell & Burchell, 1976).

Materials and Methods

Lubrol 12A9 (a condensate of dodecyl alcohol with approx. 9.5 mol of ethylene oxide/mol) was a gift from I.C.I. Organics Division, Blackley, Manchester, U.K. UDP-glucuronic acid (triammonium salt), p-nitrophenol, bilirubin and 5-hydroxytryptamine (serotonin, as creatinine sulphate complex) were all purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. o-Aminophenol was from BDH Chemicals Ltd., Poole, Dorset, U.K., and twice resublimed before use. Diethylnitrosamine was obtained from Eastman Kodak Co., Rochester, NY, U.S.A. All other chemicals, where available, were analytical-reagent grade.

Chromatography materials CM-cellulose (CM 52) and DEAE-cellulose (DE 52) were obtained from Whatman Biochemicals, Maidstone, Kent, U.K. DEAE-Sephadex A-50 grade, was from Pharmacia Fine Chemicals, Uppsala, Sweden.

Protein standards used for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, catalase, aldolase and cytochrome c were purchased from Boehringer, Mannheim, West Germany. Bovine serum albumin and Coomassie Brilliant Blue protein stain were from Sigma (London) Chemical Co.

Enzyme assays

UDP-glucuronyltransferase activity towards various substrates was assayed by the following methods: o-aminophenol and p-nitrophenol (Winsnes, 1969), bilirubin (Heirwegh et al., 1972) and 5-hydroxytryptamine (serotonin) by a modification of the method of Airaksinen et al. (1965), whereby the glucuronide is precipitated with acetone and treated with a dimethylaminobenzaldehyde reagent to give a complex measured colorimetrically at 550 nm (J. E. A. Leakey, unpublished work). Cytochrome P-450 was determined as previously described (Burchell et al.,...
Purification solution floating viscous was phase final a pellets was were homogenized 361 g/litre than M-potassium 0.2 centrifuged (fraction 1) were using sodium silicotungstate Valentine was supernatant of the mobility of the partially purified enzyme with standard proteins of known molecular weight.

Electron microscopy
Electron microscopy was performed by the method of Valentine et al. (1968) by using uranyl acetate and sodium silicotungstate staining procedures. Routine searches were conducted at a magnification of ×40000–60000.

Purification of UDP-glucuronyltransferase
Livers from five to ten male (200–250 g) Wistar rats were homogenized in 3 vol. of ice-cold 0.25 M-sucrose by using three strokes of a Teflon/glass homogenizer. The homogenization and all subsequent steps were performed at 0–4°C. This 25% (w/v) homogenate was centrifuged for 10 min at 10000 g, and the resulting supernatant (fraction 1) was centrifuged at 105000 g for 60 min in an MSE Superspeed 65 centrifuge. The microsomal pellets were collected and the supernatant was discarded. Then the microsomal pellets were resuspended in a volume of 1% Lubrol 12A9/0.2 M-potassium phosphate buffer, pH 7.0 'equivalent to 50% of the volume of fraction 1, by gentle homogenization and mixed for 20 min at 4°C. This preparation was further centrifuged at 105000 g for 60 min. The 105000 g supernatant (fraction 2) contained more than 90% of the original microsomal transferase activity (see the Results and Discussion section).

Solid (NH₄)₂SO₄ was slowly added to fraction 2 to a final concentration of 134 g/litre (25% saturation). The solution was stirred for 20 min and then centrifuged for 15 min at 15000 g, giving three phases, a floating viscous pink layer, a clear red intermediate phase and a small pink sediment. The red intermediate phase was removed by siphon and more solid (NH₄)₂SO₄ was added to give a final concentration of 361 g/litre (60% saturation). After being stirred for 20 min the mixture was centrifuged at 15000 g for 15 min. The resultant supernatant was removed by siphon and discarded. The precipitate was redissolved in 0.05% Lubrol/25 mM-potassium phosphate/2 mM-EDTA/0.25 mM-2-mercaptoethanol buffer, pH 7.4, and dialysed against large volumes of the same buffer overnight (fraction 3).

The clear red non-diffusible material was applied to a DEAE-cellulose column (20 cm × 6 cm) previously equilibrated with 0.05% Lubrol/25 mM-potassium phosphate buffer, pH 7.4. The UDP-glucuronyltransferase activity towards p-nitrophenol was eluted in the first protein peak separated from most of the red-coloured proteins. The fractions containing transferase activity were pooled, concentrated by vacuum dialysis (fraction 4) and dialysed overnight against 0.05% Lubrol/5 mM-potassium phosphate buffer, pH 6.5. The non-diffusible material was applied to a CM-cellulose column (12 cm × 6 cm) previously equilibrated in 0.05% Lubrol/5 mM-potassium phosphate buffer, pH 6.5. Transferase activity was eluted from this column with the above buffer. Active fractions were pooled, concentrated by vacuum dialysis and dialysed against 0.05% Lubrol/25 mM-potassium phosphate buffer, pH 7.4. UDP-glucuronyltransferase activity was again washed straight through this column in the first major protein peak. The active fractions were pooled, concentrated by vacuum dialysis and dialysed against the eluting buffer (fraction 6).

Results and Discussion

Solubilization of UDP-glucuronyltransferase activity
Microsomal pellets were resuspended in 1% Lubrol (see the Materials and Methods section) and further centrifuged at 105000 g for 60 min. The pellet and supernatant obtained and a sample of the original microsomal suspension in Lubrol were assayed for UDP-glucuronyltransferase activity and protein content. Table 1 shows that over 90% of the enzyme activity present in the microsomal suspension in 1% Lubrol towards o-aminophenol and p-nitrophenol and 87% of the activity towards bilirubin was present in the 105000 g supernatant (fraction 2). However, since 82% of the original microsomal protein was also contained in this fraction, only slight purification was achieved, and was ignored in estimates of enzyme purification. The enzyme protein in fraction 2 was retarded by detergent-equilibrated Sephadex G-200 and Sepharose 6B, and no vesicles were visible by electron microscopy, thereby satisfying the criteria for solubility proposed by Razin (1972).
Table 1. Solubilization of UDP-glucuronyltransferase activity
Microsomal pellets were obtained and resuspended in 1% Lubrol/0.2 M-potassium phosphate buffer, pH 7.0, by gentle homogenization and mixed for 10 min at 4°C. A small sample was removed (a) and kept at 0°C. The remainder was centrifuged at 105 000 g for 60 min to give a clear pellet (b), which was resuspended in a small volume of 1% Lubrol buffer, and a red supernatant (c). UDP-glucuronyltransferase activity and protein content were assayed in all three fractions by methods described in the text. Results are expressed as % of protein or % of activity of fraction (a) and are the average findings from three experiments.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>% of total protein</th>
<th>With p-nitrophenol (%</th>
<th>With o-aminophenol (%)</th>
<th>With bilirubin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Microsomal suspension in 1% Lubrol</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(b) 105 000 g Lubrol pellet</td>
<td>12.3</td>
<td>5.8</td>
<td>6.9</td>
<td>3.4</td>
</tr>
<tr>
<td>(c) 105 000 g Lubrol supernatant</td>
<td>82.2</td>
<td>91.6</td>
<td>90.5</td>
<td>87.0</td>
</tr>
</tbody>
</table>

Table 2. Activation of microsomal UDP-glucuronyltransferase by Lubrol
Microsomal pellets were resuspended in 0.25 M-sucrose or in 1% Lubrol/0.2 M-potassium phosphate buffer, pH 7.0, and stored in ice for 60 min. UDP-glucuronyltransferase activity was determined by methods described in the text. Values are the averages of three experimental results.

<table>
<thead>
<tr>
<th>Sample</th>
<th>With p-nitrophenol (nmol of glucuronides formed/min per mg of protein)</th>
<th>With o-aminophenol</th>
<th>With bilirubin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal fraction</td>
<td>1.51</td>
<td>0.197</td>
<td>0.073</td>
</tr>
<tr>
<td>Microsomal fraction + 1% Lubrol</td>
<td>20.8</td>
<td>0.416</td>
<td>0.175</td>
</tr>
</tbody>
</table>

UDP-glucuronyltransferase was activated by the Lubrol concentration used in the above solubilization procedure. Data shown in Table 2 illustrate the activation phenomenon observed with three substrates. Enzyme activities towards p-nitrophenol, o-aminophenol and bilirubin were increased 13.7-, 2.1- and 2.4-fold respectively. The activation at this stage must be taken into account during estimation of UDP-glucuronyltransferase purification (see Dutton & Burchell, 1976).

Further purification of UDP-glucuronyltransferase
After solubilization, UDP-glucuronyltransferase activity was concentrated by 25–60% satd. (NH₄)₂SO₄ precipitation and subjected to three consecutive ion-exchange-chromatographic steps.
Table 3. Purification of rat liver UDP-glucuronyltransferase activity towards p-nitrophenol as substrate

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg of protein)</th>
<th>Relative purification</th>
<th>Total activity (units)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10000g supernatant (fraction 1)</td>
<td>12565</td>
<td>0.39</td>
<td>1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Microsomal fraction</td>
<td>3221</td>
<td>1.51</td>
<td>3.9</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Lubrol-soluble supernatant (fraction 2)</td>
<td>2648</td>
<td>20.8</td>
<td>3.9</td>
<td>55084</td>
<td>100</td>
</tr>
<tr>
<td>25-60% satn.- (NH₄)₂SO₄ precipitate (fraction 3)</td>
<td>1246</td>
<td>25.2</td>
<td>4.9</td>
<td>31398</td>
<td>57.0</td>
</tr>
<tr>
<td>DEAE-cellulose eluate (fraction 4)</td>
<td>355</td>
<td>46.8</td>
<td>8.7</td>
<td>16614</td>
<td>30.1</td>
</tr>
<tr>
<td>CM-cellulose eluate (fraction 5)</td>
<td>216</td>
<td>68.7</td>
<td>12.8</td>
<td>14818</td>
<td>26.9</td>
</tr>
<tr>
<td>DEAE-Sephadex (fraction 6)</td>
<td>34</td>
<td>233.6</td>
<td>43.3</td>
<td>8036</td>
<td>14.6</td>
</tr>
</tbody>
</table>

Gel electrophoresis of highly purified UDP-glucuronyltransferase

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate has not previously been used in an attempt to assess the purity of UDP-glucuronyltransferase preparations. The gel-electrophoresis system used (see the Materials and Methods section) separated the polypeptides in fraction 3 into more than 30 distinct staining bands.

A photograph of the stained polypeptides after gel electrophoresis of fraction 6 is shown on Plate 1. This gel shows only three distinct polypeptide bands, which, by comparison with standard proteins of known subunit molecular weight, exhibit mol. wts. estimated at 58000 (polypeptide A), 50000 (polypeptide B) (Fig. 1) and approx. 15000. The gel was purposely overloaded in an attempt to make any minor bands visible. Thus the distinct staining bands appear broader and their width is further exaggerated by the photography. If smaller amounts of fraction 6 (less than 3 µg of protein) are subjected to gel electrophoresis, only three narrow bands are observed. A densitometric-trace analysis of the gel in Plate 1 revealed only three distinct symmetrical peaks, suggesting the existence of only three staining bands. However, further investigation is needed to determine whether these staining bands contain only one species of polypeptide.

Analysis of fraction 6 for other enzyme proteins

Fraction 6 was examined in an attempt to identify which of the remaining polypeptides might have been UDP-glucuronyltransferase or derived from it. The presence of likely contaminating proteins was sought. For example, cytochrome P-450 content was assayed by spectrophotometric analysis. Fraction 3 contains

...
EXPLANATION OF PLATE 1

Gel electrophoresis of the final preparation (fraction 6)
Disc electrophoresis was performed with 7.5% cylindrical polyacrylamide gels (7.5 cm x 0.6 cm) in the presence of 0.1% sodium dodecyl sulphate (see the Materials and Methods section). A 10μg sample of fraction-6 protein was applied to the gel. The gel was stained with 0.25% Coomassie Blue for 60 min and de-stained with acetic acid/methanol/water (7:5:43, by vol.). The direction of migration is from the top to the bottom.
2571 nmol of cytochromes P-450 and P-420, and fraction 6 only 3.8 nmol of cytochromes P-420. The cytochrome P-450 content of fraction 6 can thus be estimated to be about 6 µg/mg (assuming that microsomal cytochrome P-450 has a subunit mol. wt. of 50000). Therefore, since only 10 µg of protein was used for gel-electrophoretic analysis, the 0.06 µg of spectrophotometrically detectable cytochrome P-450 present in this sample will not be visible after gel electrophoresis, although this estimation does not eliminate the possibility that some of the apoprotein may be present.

Although the final preparation was usually colourless, occasionally a slight yellowness suggested a trace contamination by a flavin protein. This colour is associated with a stainable polypeptide of mol. wt. 80000 in gel electrophoresis, most of which binds to DEAE-Sephadex. Removal of this band coincides with removal of the yellow colour. This protein therefore possesses similar characteristics to NADPH-cytochrome P-450 reductase, which was shown to be of mol. wt. 80000 and to be bound to DEAE-Sephadex under these conditions (Dignam & Strobel, 1975). NADPH-cytochrome P-450 reductase activity assayed with cytochrome c as substrate could in fact be detected at 0.033 nmol/min per mg of protein in fraction 3 and only 0.001 nmol/min per mg of protein in fraction 5; in fraction 6 activity was not detectable. Therefore the activity data in combination with electrophoretic analysis indicated that protein had been completely removed in the colourless final preparations.

An occasional contamination of fraction 6 by a low-mol. wt. protein of 14000-16000 daltons (see Plate 1) may be due to cytochrome b, found by Spatz & Strittmatter (1971) to have this molecular weight and usually to be bound to DEAE-cellulose.

Fraction 6 was also assayed for epoxide hydratase activity. A high activity (approx. 0.5 µmol/min per mg of protein) was detected with styrene oxide as substrate. Epoxide hydratase, purified to apparent homogeneity, exhibits a specific activity of 0.4-0.5 µmol/min per mg of protein and a subunit mol. wt. of 49000-53000 (Bentley & Oesch, 1975; Lu et al., 1975). Therefore, as this preparation contains epoxide hydratase activity similar to that of the purified preparations, the major band visible after gel electrophoresis would seem to contain mainly epoxide hydratase.

Although the UDP-glucuronyltransferase polypeptide cannot be directly recognized after this type of gel electrophoresis, the enzyme in this highly purified state appears to be contained in one of the other two staining polypeptide bands that are visible. Recent results obtained with slightly modified purification systems have shown that the UDP-glucuronyltransferase activity appears to be related to the protein-staining band of 58000 mol. wt. as the
removal of this band and no other band correlates with the removal of UDP-glucuronyltransferase activity from the preparation (B. Burchell, unpublished work). Further, in agreement with this result, J. Gorski & C. B. Kasper (unpublished work; see Blackburn et al., 1976) find that the subunit of UDP-glucuronyltransferase has a mol. wt. of 59000.

Purified UDP-glucuronyltransferase activity towards other substrates

During purification, fractions 3–6 were assayed for UDP-glucuronyltransferase activity towards p-aminophenol, 5-hydroxytryptamine and bilirubin as well as towards p-nitrophenol. The relative yields of total activity and purification values at each stage are compared in Table 4. Enzyme activities towards p-nitrophenol and o-aminophenol exhibited similar yields and increases of specific activity throughout these final four stages. This result is consistent with the same enzyme being responsible for the glucuronidation of these two phenolic substrates, although no attempt has been made to confirm the earlier reported separation of these two activities by sucrose-density-gradient centrifugation (Howland et al., 1971). Activity towards bilirubin does not appear to co-purify with these activities; at all stages with bilirubin as substrate yields are much lower (4% as opposed to 24%), and the specific activity only up to 1.6-fold, compared with 9-fold for the phenolic substrates.

Activation of highly purified UDP-glucuronyltransferase by diethylnitrosamine

Diethylnitrosamine, an organic solvent, is capable of activating rat liver UDP-glucuronyltransferase activity towards a limited number of substrates (see Dutton, 1971; Dutton & Burchell, 1976). Diethylnitrosamine acts as a membrane perturbant and modifies the membrane protein–lipid interactions, thereby enhancing the activity of the lipid-dependent UDP-glucuronyltransferase (Nakata et al., 1975). Therefore we have investigated the activation of Lubrol-solubilized UDP-glucuronyltransferase by diethylnitrosamine. Table 5 shows that the transferase activity of fractions 3 and 6 with o-aminophenol as substrate could be activated some 3.1- and 3.7-fold respectively by 10 mM-diethylnitrosamine. Thus the transferase activity can still be enhanced to the same extent in the final preparation, in the ammonium sulphate fraction and, as reported by Stevenson et al. (1968), in the untreated microsomal fraction.

Lipids are still present in the final preparation (B. Burchell, unpublished work), and as diethylnitrosamine can still activate the highly purified 'soluble' UDP-glucuronyltransferase, protein–lipid interactions may still be important to the activity of the partially purified enzyme.

Stability of highly purified UDP-glucuronyltransferase

All purification fractions 2–6, including the highly purified final preparation, were stored at 0°C in ice in sterile plastic tubes and vessels. Assay of the final preparation after 30 days storage revealed that only 11% of enzyme activity at the beginning of storage with p-nitrophenol and only 19% of enzyme activity with o-aminophenol was lost. Further, the transferase activity towards o-aminophenol remained activatable by 10 mM-diethylnitrosamine. Freezing and thawing of the final preparation caused precipitation, and enzyme activity in the mixture was decreased or lost completely.

In conclusion, I have obtained a highly purified preparation of stable UDP-glucuronyltransferase that shows only three protein-staining bands after electrophoresis. The presence of epoxide hydratase may be important to the functional activity of UDP-glucuronyltransferase, as indicated by their co-purification and seemingly close association. Their separation will be essential in the isolation of pure UDP-glucuronyltransferase.

I thank Mr. J. E. A. Leakey for measurement of UDP-glucuronyltransferase activity with 5-hydroxytryptamine as substrate, and the Medical Research Council and NATO for grants supporting this work.

Table 5. Activation of UDP-glucuronyltransferase by diethylnitrosamine

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Diethylnitrosamine absent</th>
<th>Diethylnitrosamine present</th>
<th>Activation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25–60% satn.-(NH₄)₂SO₄ precipitate</td>
<td>0.34</td>
<td>1.05</td>
<td>309</td>
</tr>
<tr>
<td>DEAE-Sephadex eluate</td>
<td>2.83</td>
<td>10.44</td>
<td>369</td>
</tr>
</tbody>
</table>

Diethylnitrosamine (final concn. 10 mM) was added to the enzyme-assay mixtures immediately before incubation. Results presented are the arithmetic means of three experimental determinations.
References


Dutton, G. J. & Burchell, B. (1976) Prog. Drug Metab. 2, in the press


