Phospholipid Content and Activity of Pure Uridine Diphosphate–Glucuronyltransferase from Rat Liver

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Rat liver phospholipids were radioactively labelled *in vivo* before purification of UDP-glucuronyltransferase to homogeneity. The pure enzyme contained very little phospholipid (approx. 0.7 mol of phospholipid/mol of protein). The solubilization detergent Lubrol 12A9 appeared to act as a phospholipid substitute, capable of supporting UDP-glucuronyltransferase activity. Phospholipase C did not inhibit the pure enzyme activity and pure UDP-glucuronyltransferase was stimulated by 40–100% by the addition of phospholipid dispersions.

The effects of phospholipids on hepatic UDP-glucuronyltransferase (EC 2.4.1.17) activity have been exhaustively studied in microsomal preparations (see Zakim & Vessey, 1976, and Dutton & Burchell, 1977, for references). Evidence indicates that UDP-glucuronyltransferase in microsomal fractions requires phospholipids to exhibit maximum demonstrable activity (Berry *et al.*, 1976, 1978; Graham *et al.*, 1977).

In the present study, the role of phospholipids was examined directly on pure homogeneous UDP-glucuronyltransferase preparations (Burchell, 1977a).

Materials and Methods

Microsomal and intermediate purification fractions and pure UDP-glucuronyltransferase were prepared from the livers of five male Wistar rats, pretreated with 2 g of phenobarbital/litre in the drinking water for 7 days, by the method previously described (Burchell, 1977a, 1978). Phospholipids were labelled *in vivo* with radioactive tracers purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Rats were injected with 200 μ Ci of [*Me*-³H]-choline and were killed 35 min later (Nagley & Hallinan, 1968) or were injected with 1 mCi of [³²P]P_i 16 h before death. Incorporation of ³²P for 16 h ensured that all of the microsomal phospholipid species including sphingomyelin would be maximally labelled (Gurr *et al.*, 1965; Holtzman *et al.*, 1970). The 35 min labelling with [*Me*-³H]-choline was chosen to ensure that this was incorporated very selectively into phosphatidylcholine with only 1–3% incorporation into sphingomyelin or protein (Nagley & Hallinan, 1968; Cater *et al.*,

1976): 60–70% of the individual molecular species of phosphatidylcholine are efficiently labelled by this procedure (Trehwella & Collins, 1973). ³H radioactivity was counted in toluene scintillant (Cater *et al.*, 1975) at an efficiency of 40%, and ³²P radioactivity was measured by Čerenkov counting at an efficiency of 30%. The specific radioactivity of the ³²P-labelled phospholipid was determined by measuring radioactivity in purified phospholipids extracted from 25% (w/v) liver homogenate and then by measuring their P_i content after wet-ashing (Cater *et al.*, 1975).

Phospholipid depletion of enzyme preparations was carried out at 5–10°C as described by Allistone *et al.* (1977), who used Ca²⁺ at a concentration of 1 mM and pure *Bacillus cereus* phospholipase C (1 unit of phospholipase liberates 1 μ mol of phosphocholine/min from ether-saturated egg phosphatidylcholine at 37°C). Removal of diacylglycerols with lipase and bovine serum albumin was omitted, since this is without effect on glucuronyltransferase activity after extensive delipidation with pure *B. cereus* phospholipase (Allistone *et al.*, 1977). Egg yolk phosphatidylcholine and lysophosphatidylcholine, purchased from Lipid Products, South Nutfield, Surrey, U.K., were ultrasonically dispersed and added to enzyme fractions as described by Cater *et al.* (1975).

UDP-glucuronyltransferase activity was assayed at 23°C by using 4-nitrophenol as described by Winsnes (1969). Protein concentrations were determined by the biuret method (see Layne, 1957), the method of Lowry *et al.* (1951) and the method of Bradford (1976), with bovine serum albumin as standard. The biuret method was used to confirm results obtained by the method of Lowry and co-workers for protein solutions of concentration

greater than 5 mg/ml. Similarly the Bradford technique was used to confirm the results obtained by the method of Lowry and co-workers for protein solutions of concentration less than 1 mg/ml.

Results and Discussion

Liver phospholipids were labelled *in vivo* by injection of [^{32}P]P_i or [$M\epsilon$ - ^3H]choline, before purification of UDP-glucuronyltransferase to homogeneity (see the Materials and Methods section). This enabled the phospholipid content of the pure transferase to be sensitively measured and allowed the detection of any phosphatidylcholine present. Table 1 shows the radioactive material contained in each purification fraction throughout the UDP-glucuronyltransferase-isolation procedure. This radioactivity, representing labelled phospholipid (with possibly some labelled phosphoproteins with [^{32}P]P_i), gradually accumulated in a similar manner from the $(\text{NH}_4)_2\text{SO}_4$ fraction up to the penultimate stage of the purification procedures, irrespective of the radioactive label used. No [^3H]phosphatidylcholine and very little [^{32}P]P_i could be detected in the final enzyme preparations. The final enzyme preparations exhibited only one polypeptide-staining band after analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (see Burchell, 1978), although in this case demonstrable enzyme specific activity was approx. 50% of the values obtained during purification of UDP-glucuronyltransferase (Burchell, 1968). Thus UDP-glucuronyltransferase purified to apparent homogeneity contains very little, if any, bound phospholipid. Calculation based on the specific radioactivity of purified phospholipids from the initial liver homogenate shows that the pure transferase binds only 0.7 mol of phospholipid/mol of enzyme. Since detergent solubilization typically tends to randomize the phospholipids associated with proteins (Mull *et al.*, 1977; Le Maire *et al.*, 1977), so that their native phospholipid-complement can be virtually completely substituted (Warren & Metcalfe, 1977), the above calculation is unlikely to be seriously biased owing to the purified transferase selectively retaining phospholipid species with specific radioactivities that differ markedly from the mean.

The results in the present paper do not agree with those of Gorski & Kasper (1977), whose pure glucuronyltransferase reportedly contained 47% by weight of phospholipid. However, Bock *et al.* (1977) obtained an active partially purified transferase preparation that was severely depleted of phospholipid, from 0.34 mg of phospholipid/mg of protein in the microsomal fraction to 0.01 mg of phospholipid/mg of protein in the DEAE-cellulose fraction.

The virtual absence of phospholipid from our pure active enzyme preparations appeared to question the idea that UDP-glucuronyltransferase is lipid-depen-

Table 1. Purification of UDP-glucuronyltransferase from rat liver containing radioactive phospholipids

Lubrol solubilization of UDP-glucuronyltransferase was achieved by the method previously described (Burchell, 1977b), before enzyme purification by the experimental procedure shown below (also see Burchell, 1977a, 1978). Data are reported for each purification fraction analysed (see the Materials and Methods section). Preparation 1: phosphatidylcholine was labelled *in vivo* with [$M\epsilon$ - ^3H]choline. Preparation 2: phospholipids were labelled *in vivo* with [^{32}P]P_i. One unit of enzyme activity represents 1 nmol of 4-nitrophenol glucuronide formed/min.

Purification step	Preparation 1			Preparation 2		
	Specific activity (units/mg of protein)	Total radioactivity (c.p.m.)	Radioactivity (c.p.m./mg of protein)	Specific activity (units/mg of protein)	Total radioactivity (c.p.m.)	Radioactivity (c.p.m./mg of protein)
25–60%-satd.-(NH_4) ₂ SO ₄ precipitate	20.1	702 000	1032	37.2	11 067 840	10248
DEAE-cellulose eluate	30.1	456 300	2829	46.6	3 756 150	9329
CM-cellulose eluate	35.9	393 154	5494	52.6	3 406 748	12 508
DEAE-Sephadex eluate	141.0	98 340	17 427	148.4	1 260 665	46 379
UDP-glucuronic acid eluate from UDP-hexanolamine-Sephadex	1128	Not detectable	0	1246	109	289

dent. To test this hypothesis we have conducted further experiments. Treatment of sonicated rat liver microsomal fractions (14 and 1.3 mg of protein) with 7.5 units of pure phospholipase C for 60 min at 5°C (Allistone *et al.*, 1977) resulted in 93–99% inhibition of UDP-glucuronyltransferase activity towards 4-nitrophenol, suggesting lipid-dependence, as is also suggested by similar studies with guinea-pig liver microsomal fractions (Berry *et al.*, 1978). Exposure of pure UDP-glucuronyltransferase to similar treatment with phospholipase C did not cause any measurable enzyme inhibition, presumably because of the virtual absence of phospholipid bound to the pure transferase. Duck-Chong (1976) suggested that membrane-bound enzyme activities may be supported by the presence of detergents acting as lipid substitutes. Furthermore, Lu *et al.* (1974) showed that the benzphetamine (*N*-benzyl-*N*^α-dimethylphenethylamine) *N*-demethylase activity of a reconstituted microsomal enzyme system was stimulated when a number of detergents were substituted for a crude liver lipid fraction in the reconstituted system: some detergents were as effective as lipid in eliciting enzyme activity. Our pure enzyme preparation may therefore remain active owing to the presence of Lubrol.

If Lubrol can indeed serve as a phospholipid substitute then it should support UDP-glucuronyltransferase activity in microsomal fractions when their phospholipids are destroyed. This appeared to be the case when a microsomal fraction (7 mg of protein), labelled with [*Me*-³H]choline, was treated for 60 min at 5°C with 2 units of phospholipase C. This caused 95% hydrolysis of the [³H]phosphatidylcholine and 65% inhibition of UDP-glucuronyltransferase activity towards 4-nitrophenol. Addition of 0.036, 0.05, 0.1 and 0.2 mg of Lubrol/mg of microsomal protein restored 72, 94, 102 and 104% of the maximum activity demonstrable for 4-nitrophenol glucuronidation. Maximum activity was determined in a microsomal control, not pretreated with phospholipase but optimally activated with Lubrol (see Burchell *et al.*, 1976). In two complementary experiments, [*Me*-³H]choline-labelled microsomal fractions (1.3 and 14 mg of protein) were pretreated with 0.1 mg of Lubrol/mg of protein before exposure for 60 min at 5°C to 7.5 units of phospholipase C. In this case, hydrolysis of 83% of the microsomal phosphatidylcholine caused only 36 and 10% inhibition of the maximum demonstrable activity for 4-nitrophenol glucuronidation, compared with 93 and 99% inhibition in phospholipase-treated ultrasonicated samples. Phosphatidylcholine hydrolysis was 90% in these controls. The ability of Lubrol to substitute substantially for phospholipids in supporting UDP-glucuronyltransferase activity suggests that phospholipids may fulfil a rather non-specific physical role for the transferase, since Lubrol and phospholipids

are chemically unrelated except for their amphipathic properties.

It seems possible that the large excess of Lubrol over protein (10 mg of detergent/mg of protein) in the pure preparations of UDP-glucuronyltransferase may partially inhibit activity. However, attempted removal of Lubrol usually resulted in precipitation of protein and complete loss of enzyme activity. Gorski & Kasper (1977) also reported a 59% loss of enzyme activity after dialysis.

Addition to the pure UDP-glucuronyltransferase of ultrasonically dispersed phospholipids in Tris/maleate buffer (see the Materials and Methods section) increased the rate of glucuronidation of 4-nitrophenol by 40–100% at a phospholipid/protein ratio of approx. 1:1 (w/w).

It appears therefore (a) that pure UDP-glucuronyltransferase contains only extremely small quantities of phospholipid and (b) that enzyme activity may be supported by bound detergent acting as a phospholipid substitute.

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