The Phospholipid-Dependence of Uridine Diphosphate Glucuronyltransferase

PHOSPHOLIPID DEPLETION AND RE-ACTIVATION OF GUINEA-PIG LIVER MICROSOMAL ENZYME

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More than 80% of the phospholipid component of guinea-pig liver microsomal membranes (prepared with 154mM-KCl) was removed by treatment with phospholipase A followed by extraction of the lysophosphatides and fatty acids produced with albumin. Delipidation strongly inactivated the highly active UDP-glucuronyltransferase of these preparations and activity was restored by mixtures of phosphatidylcholine and lysophosphatidylcholine. However, small quantities of lysophosphatides were still associated with the delipidated fractions after extraction with albumin and might have influenced the inactivation and re-activation observed. To eliminate these uncertainties, microsomal proteins and phospholipids were separated by gel filtration on Sephadex G-150 in the presence of cholate. This technique also strongly inactivated the enzyme but did not generate membrane-active phospholipid degradation products. High transferase activity was again restored to the delipidated protein by choline glycerophosphatides. These results confirm the view that the fully active form of microsomal UDP-glucuronyltransferase is phospholipid-dependent.

Studies of membrane perturbants on liver microsomal UDP-glucuronyltransferase (EC 2.4.1.17) show that membrane phospholipids modulate the enzyme's activity (Zakim & Vessey, 1974; Graham *et al.*, 1974a; Mulder, 1974). In certain microsomal preparations, especially those isolated with 0.25M-sucrose, phospholipids strongly restrict activity, and phospholipid hydrolysis or membrane disruption activates the enzyme. Zakim and co-workers (Vessey & Zakim, 1971, 1972; Zakim *et al.*, 1973) proposed that in these preparations intact phospholipids constrain the transferase in a conformation of low activity. However, other workers suggest that UDP-glucuronyltransferase molecules are located deeply within the microsomal membrane, so that phospholipids act as a hydrophobic barrier hindering access of reactants to the sites of glucuronidation (Winsnes, 1969; Mulder, 1970; Hänninen & Puukka, 1970, 1971; Högborg *et al.*, 1973; Graham & Wood, 1974). Recent evidence favours the second hypothesis (Berry *et al.*, 1975, 1976; A. B. Graham, D. T. Pechey & G. C. Wood, unpublished work). According to this, treatments that degrade phospholipids or disrupt membrane structure enhance UDP-glucuronyltransferase activity by increasing membrane permeability.

When liver microsomal membranes were isolated with 154mM-KCl and washed with this solution, the restriction or latency of UDP-glucuronyltransferase is less marked (Graham & Wood, 1973). Indeed, the transferase of guinea-pig microsomal fractions prepared in this way is highly active and almost free from latency (Graham & Wood, 1972, 1973), allowing a second modulating effect of phospholipids to be observed. Disruption of these microsomal membranes with detergents did not affect transferase activity significantly (Attwood *et al.*, 1971; Graham & Wood, 1972, 1973), but phospholipid hydrolysis with phospholipase A (EC 3.1.1.4) or phospholipase C (EC 3.1.4.3) inactivated (Graham & Wood, 1969; Attwood *et al.*, 1971). Almost full activity was restored after phospholipase A treatment by a phospholipid mixture extracted from the native microsomal fractions (Graham & Wood, 1969) or phosphatidylcholine (Attwood *et al.*, 1971), and we concluded that UDP-glucuronyltransferase, in these preparations, is phospholipid-dependent. The data of Berry *et al.* (1976) support our views on the dual effects of phospholipids on UDP-glucuronyltransferase. They showed that latency of the enzyme in guinea-pig microsomal fractions, prepared with 0.25M-sucrose, was removed by ultrasound or

phospholipase C. However, treatment of the ultrasonically activated fraction with phospholipase C destroyed the activity, and this was almost fully restored by the addition of microsomal phospholipids.

We have now examined the phospholipid-dependence of UDP-glucuronyltransferase more closely, using guinea-pig microsomal fractions prepared with 154mM-KCl where this modulation mechanism predominates. The main criteria required to demonstrate the phospholipid-dependence of a membrane-bound enzyme (Fleischer *et al.*, 1962) are that removal of phospholipid from the membrane should be correlated with loss of enzyme activity and that activity should be restored to lipid-depleted fractions by addition of the appropriate phospholipids. Although previous work strongly suggests that UDP-glucuronyltransferase is phospholipid-dependent, there are serious objections to the validity of some of the conclusions. When microsomal fractions are treated with phospholipases, the resulting inactivation of the transferase might be due to accumulation of phospholipid degradation products rather than phospholipid destruction itself; fatty acids, lysophosphatidylcholine, diglycerides and phosphorylcholine did not appreciably affect the transferase activity of intact guinea-pig microsomal fractions prepared with KCl (Graham & Wood, 1969), but their effects when generated in the membranes from microsomal phospholipids are uncertain. Moreover, previous observations of UDP-glucuronyltransferase re-activation were made by adding phospholipids to phospholipase-treated membranes containing degradation products, which might influence this process also.

Therefore we have investigated the effect on UDP-glucuronyltransferase activity of removing fatty acids and lysophosphatides from phospholipase A-treated microsomal fractions so as to delipidate the membranes substantially. We have achieved the same end by a method not involving phospholipid hydrolysis and have determined its effect on transferase activity and have examined the effects of adding phospholipids to these lipid-depleted fractions.

A very brief account of part of this work has been published (Graham *et al.*, 1974a).

Materials and Methods

Materials

Phospholipase A (proteinase-free) was purified from *Crotalus adamanteus* venom (Graham & Wood, 1969). Bovine serum albumin (crystalline), from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K., was washed with acetone as recommended by Fiehn & Hasselbach (1970). The acetone was purified by refluxing with KMnO_4

and dried with anhydrous K_2CO_3 (Vogel, 1962). Albumin (3g) was stirred with $3 \times 300\text{ml}$ of cold acetone. The protein was collected by vacuum filtration, air-dried, and the remaining solvent removed in a vacuum desiccator. The lipid-depleted albumin was stored over silica gel at 4°C *in vacuo*. Cholic acid (Sigma, 99–100% pure) was dissolved in a solution of Tris base and the pH adjusted to 8.0 with HCl. The solutions were clarified by filtration and contained 25mM-Tris base. Sephadex G-150 was from Pharmacia (G.B.) Ltd., London W5 5SS, U.K. Bio-Beads SM-2 (Bio-Rad Laboratories, Bromley, Kent, U.K.) were washed with methanol and water and stored as described by Holloway (1973). Phosphatidylcholine from egg yolk was purified (Attwood *et al.*, 1971). Lysophosphatidylcholine (98% pure) from egg yolk was from Sigma.

Microsomal fractions

Male guinea pigs (Hartley strain, 250–400g; David Hall, Newchurch, Burton-on-Trent, Staffs., U.K.) were starved overnight, killed and liver microsomal fractions prepared and washed with 154mM-KCl (Graham & Wood, 1973). The microsomal membranes were resuspended in 154mM-KCl, adjusted to 20mg of protein/ml, and either kept on ice and used on the day of preparation, or at -20°C and used within 24h.

Delipidation techniques

(a) *Phospholipase A digestion followed by albumin washing.* Microsomal membranes were treated with phospholipase A to hydrolyse their phospholipids extensively, and the fatty acids and lysophosphatides produced were removed by defatted albumin. The method is based on those used by Fiehn & Hasselbach (1970) and Meissner & Fleischer (1972) to delipidate sarcoplasmic-reticulum membranes.

Microsomal suspensions (final concn. 5mg of protein/ml) were digested at 32°C with phospholipase A (50 μg /mg of microsomal protein) in the presence of 30mM-Tris/HCl buffer (pH 8.0) and 2.5mM- CaCl_2 . After 2h, phospholipid hydrolysis was stopped by the addition of solid EDTA (final concn. 10mM) and the modified membranes were sedimented at $38000g_{av.}$ for 30min at 4°C . The membranes (90–100mg of microsomal protein) were resuspended in 15ml of albumin solution (5g/100ml of 0.1M-KCl containing 10mM-Tris/HCl buffer, pH 8.0) by using a tube homogenizer with a tightly fitting Teflon pestle and sedimented as above. They were washed with two more 15ml portions of albumin solution and then with 20ml of buffered KCl solution to remove the albumin. The delipidated fractions were resuspended in the buffered KCl, adjusted with it to 10mg of protein/ml, and stored at -20°C .

(b) *Gel filtration on Sephadex G-150 in the presence of cholate.* Microsomal components have been dissociated by dispersing the membranes with detergents and separating the protein-detergent and phospholipid-detergent complexes by gel filtration (Garland & Cori, 1972; Lu & Levin, 1974; Jansen & Arias, 1975). Microsomal membranes (final concn. 10mg of protein/ml) were stirred for 15min in a medium (pH8.0) containing 0.5% cholate, 25mM-Tris/HCl and 127mM-KCl. Insoluble material was removed by centrifuging at 100000g_{av.} for 1 h, and a 5ml portion of the supernatant fraction (containing 27–28mg of protein) was applied to a column (80cm×2.5cm; void volume 155–160ml) of Sephadex G-150 equilibrated with a solution (pH8.0) containing 0.25% cholate, 25mM-Tris/HCl and 127mM-KCl. The column was eluted with the same solution and 5ml fractions were collected. The peak protein fractions (open circles in Fig. 2) were concentrated to approx. 0.5mg of protein/ml by using an Amicon model 52 ultrafiltration cell and a 30000-mol.wt. exclusion-limit membrane. All operations were carried out at approx. 4°C.

Analytical methods

The protein contents of native microsomal suspensions and of membranes delipidated by using phospholipase A and albumin were measured with a biuret reagent (Graham & Wood, 1974). Dispersions of microsomal protein in the cholate solutions contained sufficient Tris to interfere with the biuret reaction. Their concentration was determined from the A_{280} . To relate A_{280} to protein concentration, native microsomal suspensions of known protein concentration were dispersed in the 0.5% cholate solution described above at 10mg of protein/ml. These mixtures were diluted 20-fold with column eluate (containing 0.25% cholate) and A_{280} was determined in a 1cm-light-path cell. An A_{280} value of 2.21 (corrected for the extinction of solvent) was equivalent to 1 mg of protein/ml. A similar value was obtained when microsomal membranes were dispersed in cholate solutions buffered with borate, which does not interfere with the biuret reaction.

The phospholipid contents of native, phospholipase-treated and delipidated microsomal fractions were determined by measuring P contents (Graham *et al.*, 1974b) and expressed as μg of phospholipid P/mg of protein [corrected for 1.7 μg of P/mg of protein not extractable from microsomal membranes by extensive washing with a chloroform/methanol (2:1, v/v) mixture].

The phospholipid compositions of native, phospholipase-treated and delipidated fractions were determined by extracting freeze-dried material (4–20mg of protein) with three or four 5ml portions of chloroform/methanol (2:1, v/v). The combined

extracts were washed with water (3 or 4ml) and the bottom phases dried with a stream of N_2 . The phospholipids were separated by t.l.c. and their proportions calculated as described previously (Graham *et al.*, 1974b).

Cholate present in delipidated and re-activated microsomal protein fractions was determined by a specific colorimetric method (Heaton, 1972).

Phospholipid dispersions

Phosphatidylcholine was dispersed in aqueous media (see below) by ultrasonication at 21kHz and its concentration determined as P content after drying (Attwood *et al.*, 1971). Lysophosphatidylcholine solutions were prepared by dissolving sufficient material in the appropriate media to give the required P content. The P content of lysophosphatidylcholine was 5.92% (w/w).

Re-activation conditions

(a) *Phospholipase A/albumin method.* Suspensions of delipidated membranes (0.3ml of 10mg of protein/ml) were incubated at room temperature (18–22°C) with phospholipids dispersed in 20mM-Tris/HCl buffer, pH8.0, containing 1mM-EDTA, making a final volume of 0.8ml. Delipidated membranes receiving no phospholipid were diluted with 0.5ml of the Tris/HCl/EDTA, pH8.0. After 5min, portions of the mixtures were assayed for UDP-glucuronyltransferase activity.

(b) *Gel-filtration method.* Phospholipids added to delipidated microsomal fractions did not re-activate UDP-glucuronyltransferase, and an indirect method was used. Bio-Beads remove detergents from solubilized membrane proteins (Holloway, 1973) and were used here to achieve exchange of cholate for phospholipid. To portions of the concentrated delipidated protein (2ml) were added phospholipids dispersed in 127mM-KCl/25mM-Tris/HCl buffer, pH8.0, making a final volume of 2.24ml. To protein receiving no phospholipid 0.24ml of the KCl/Tris/HCl buffer, pH8.0, was added. Bio-Beads (250mg) were added, and the mixtures were shaken gently at 4°C. After various times the beads were allowed to settle and portions of the protein dispersions assayed for UDP-glucuronyltransferase activity.

UDP-glucuronyltransferase assays

Since the enzyme's response to membrane perturbants varies slightly with the concentration of UDP-glucuronate used to determine its activity (Graham & Wood, 1973), UDP-glucuronyltransferase was assayed by using three different sets of substrate concentrations: (a) 0.17mM-*p*-nitrophenol and 0.167mM-UDP-glucuronate (Graham & Wood,

1972), (b) 0.17 mM-*p*-nitrophenol and 0.5 mM-UDP-glucuronate (Graham & Wood, 1972) and (c) 0.6 mM-*p*-nitrophenol and 4 mM-UDP-glucuronate (Graham & Wood, 1973).

Results and Discussion

In agreement with our earlier work (Graham & Wood, 1969; Attwood *et al.*, 1971), digestion with phospholipase A of guinea-pig liver microsomal fractions isolated with 154 mM-KCl inactivated their highly active UDP-glucuronyltransferase (74–88% inactivation, from the mean values given in Table 1), when this was assayed at all three sets of substrate concentrations. In a separate single experiment, this treatment hydrolysed 86% of the microsomal phospholipid (95% of the phosphatidylcholine, 91% of the phosphatidylethanolamine and 58% of the phosphatidylinositol plus phosphatidylserine). Thorough washing of the modified membranes

with albumin removed most of the lysophosphatides formed by phospholipase A (Table 2), and in seventeen microsomal preparations (Table 1) the method produced $81 \pm 1\%$ delipidation (calculated from the phospholipid P content of the fractions). Since Fiehn & Hasselbach (1970) and Meissner & Fleischer (1972) showed that albumin extracted fatty acids from phospholipase-treated microsomal fractions much more easily than it extracted lysophosphatides, we assume that they also were removed efficiently in our experiments. Removal of these large quantities of hydrolysis products increased slightly the UDP-glucuronyltransferase activity of phospholipase A-modified membranes so that the whole process was responsible for 69–82% loss of transferase activity (from the mean values given in Table 1).

In our earlier work, when no attempt was made to remove lysophosphatides or fatty acids, the low UDP-glucuronyltransferase activity of phospho-

Table 1. *Delipidation of microsomal membranes with phospholipase A and albumin and its effect on the activity of UDP-glucuronyltransferase*

Microsomal fractions were delipidated by treatment with phospholipase A followed by albumin washing as described in the Materials and Methods section. UDP-glucuronyltransferase activity was determined at various stages in the procedure by using three different sets of substrate concentrations: (a) 0.17 mM-*p*-nitrophenol and 0.167 mM-UDP-glucuronate, (b) 0.17 mM-*p*-nitrophenol and 0.5 mM-UDP-glucuronate, and (c) 0.6 mM-*p*-nitrophenol and 4 mM-UDP-glucuronate. Enzyme activity was also determined after treating the delipidated fraction with a phospholipid dispersion containing 67% phosphatidylcholine and 33% lysophosphatidylcholine at a total concentration of 60 μ g of P/mg of protein (see the text). The phospholipid contents of the various fractions were determined by measuring phospholipid (i.e. phosphatide plus lysophosphatide) P. The values quoted are means $\pm$ S.E.M. of results obtained with different microsomal preparations; the numbers of preparations are in parentheses.

Assay conditions ...	UDP-glucuronyltransferase activity (nmol/min per mg of protein)			Phospholipid content (μ g of P/mg of protein)
	(a)	(b)	(c)	
Native microsomal fraction	2.07 ± 0.08 (17)	4.15 ± 0.28 (16)	20.7 (1)	21.1 ± 0.4 (17)
Microsomal fraction incubated without phospholipase A	1.64 ± 0.15 (17)	3.36 ± 0.28 (16)	16.5 (1)	20.9 ± 0.4 (16)
Microsomal fraction incubated with phospholipase A	0.24 ± 0.05 (7)	0.62 ± 0.18 (6)	5.3 (1)	21.0 ± 0.7 (7)
Delipidated fraction	0.38 ± 0.03 (17)	0.86 ± 0.07 (16)	6.0 (1)	4.0 ± 0.2 (17)
Delipidated fraction plus phospholipids	1.65 ± 0.08 (8)	3.91 ± 0.23 (8)	18.2 (1)	—

Table 2. *Phospholipid compositions of native and delipidated microsomal fractions*

The contents of individual phospholipids were calculated from the proportions of the phospholipids and the total phospholipid P contents of the various fractions (see the Materials and Methods section). The values quoted for native membranes and membranes delipidated by using the method with phospholipase A and albumin are typical of these preparations. Those quoted for the fraction delipidated by gel filtration were obtained from the material obtained in the experiment of Fig. 2. Abbreviations: PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; LPI, lysophosphatidylinositol; LPS, lysophosphatidylserine; Sph, sphingomyelin.

	Phospholipid content (μ g of P/mg of protein)						
	PC	LPC	PE	LPE	PI+PS	LPI+LPS	Sph
Native microsomal fraction	11.8	0	5.7	0	2.9	0	0.6
Delipidated fraction (phospholipase A and albumin)	0.4	0.6	0.3	1.3	0.4	0	1.0
Delipidated fraction (Sephadex G-150/cholate)	1.6	0	1.6	0	0.7	0	0

Fig. 1. Concentration-dependence of the re-activation of UDP-glucuronyltransferase of delipidated microsomal membranes by choline glycerophosphatides

Delipidated membranes, prepared by the phospholipase A and albumin method, were added to various concentrations of a mixture containing 67% phosphatidylcholine and 33% lysophosphatidylcholine (see the Materials and Methods section) and UDP-glucuronyltransferase activities were measured by using (a) 0.17 mM *p*-nitrophenol and 0.167 mM-UDP-glucuronate and (b) 0.17 mM *p*-nitrophenol and 0.5 mM-UDP-glucuronate. A represents the activity of microsomal membranes incubated without phospholipase A or albumin washing.

lipase A-treated membranes was restored to values near those of native microsomal fractions by dispersions of microsomal phospholipids (Graham & Wood, 1969), and phosphatidylcholine was the best individual phospholipid for re-activation (Attwood *et al.*, 1971). In the present study, however, the transferase activity of the delipidated microsomal membranes was stimulated only weakly by phosphatidylcholine (Graham *et al.*, 1974a). Its effect was maximal at about 60 µg of P/mg of protein. The major phospholipid components of the phospholipase A-treated membranes are lysophosphatides, and possibly these are involved in the re-activation observed previously. This suspicion was confirmed; lysophosphatidylcholine alone at a concentration of 60 µg of P/mg of protein stimulated the transferase activity of delipidated membranes more strongly than did phosphatidylcholine, and mixtures of phosphatidylcholine and its lysophosphatide were even more effective (Graham *et al.*, 1974a). Optimum re-activation was achieved with a mixture containing 67% phosphatide and 33% lysophosphatide. When the total concentration of this 2:1 mixture was varied, maximum stimulation was observed at 45–60 µg of P/mg of protein (Fig. 1). Therefore a mixture containing 67% phosphatidylcholine and 33% lysophosphatidylcholine at a total concentration of 60 µg of P/mg of protein was adopted as a standard re-activating reagent, and with nine different preparations of delipidated

microsomal membranes it enhanced UDP-glucuronyltransferase activity to values in the range 80–94% of the activity of native microsomal fractions (from the mean values given in Table 1). The extent of re-activation is even greater (100–116%) when calculated with reference to the activity of control microsomal fractions that had been incubated at 32°C without phospholipase A (Table 1).

Thus substantial delipidation of guinea-pig microsomal preparations reversibly inactivates non-latent UDP-glucuronyltransferase. However, the delipidated fractions still contained small amounts of lysophosphatides (Table 2). To eliminate the possibility that these play a significant part in the inactivation of UDP-glucuronyltransferase or in its subsequent re-activation by phospholipids, we attempted to dissociate microsomal protein from phospholipids by a method that does not generate phospholipid degradation products.

Microsomal membranes dispersed in a medium containing 0.5% cholate retained their high UDP-glucuronyltransferase activity (Table 3). Higher concentrations of the detergent progressively inhibited the enzyme. After this dispersion was centrifuged, the supernatant fraction (containing about 55% of the microsomal protein) had only low activity (Table 3). Re-activation experiments (see below) demonstrated that the supernatant fraction also contained enzyme that had been inactivated, probably by the increased cholate/protein ratio.

Table 3. *Delipidation of microsomal membranes by gel filtration in the presence of cholate, and its effect on the activity of UDP-glucuronyltransferase*

Microsomal membranes were delipidated by gel filtration on Sephadex G-150 in the presence of 0.25% cholate (see Fig. 2). UDP-glucuronyltransferase activity was determined by using 0.6 mM-*p*-nitrophenol and 4 mM-UDP-glucuronate at various stages of the procedure. The enzyme activity of the delipidated fraction after removal of cholate also was determined, as was activity after delipidation and treatment for 3.5 h with a phospholipid dispersion containing 67% phosphatidylcholine and 33% lysophosphatidylcholine at a total concentration of 60 µg of P/mg of protein (see the Materials and Methods section and the text). The phospholipid contents of the various fractions were determined by measuring phospholipid P.

	UDP-glucuronyl- transferase activity (nmol/min per mg of protein)	Phospholipid content (µg of P/mg of protein)
Microsomal fraction dispersed in 0.5% cholate	17.1	23.4
Protein solubilized	2.8	24.7
Delipidated fraction (in 0.25% cholate)	0	3.9
Delipidated fraction (cholate removed)	4.3	—
Delipidated fraction plus phospholipids	24.3	—

Fig. 2. *Separation of microsomal protein and phospholipid by gel filtration in the presence of cholate*
Microsomal membranes were dispersed in a medium containing 0.5% cholate, and the solubilized material was applied to a column of Sephadex G-150 equilibrated with a solution containing 0.25% cholate. Protein [○ (peak protein fractions), ●] and phospholipid [■] were eluted with the 0.25% cholate solution (see the Materials and Methods section).

The supernatant material was applied to a column of Sephadex G-150, and elution with a 0.25% cholate solution separated the protein and the phospholipid. In a typical experiment (Fig. 2), 75% of the microsomal protein and 85% of the phospholipid were recovered after chromatography, and the protein in the peak fractions was substantially depleted of phospholipid (84% delipidation, from the data of Table 3). The protein-rich fractions contained no UDP-glucuronyltransferase activity (Table 3) and no lysophosphatides (Table 2). When 96% of the cholate in these fractions was removed with Bio-Beads only 11% of the transferase activity of the original microsomal dispersion reappeared (Fig. 3, see also Table 3). Thus delipidated protein fractions which had only 16–17% of the phospholipid content of native microsomal membranes had specific activities 75–80% lower than that of these membranes, and this result is very similar to those obtained when phospholipids were extracted by using phospholipase A and albumin (Table 1).

Fig. 3. *Removal of cholate and re-activation of UDP-glucuronyltransferase of microsomal proteins delipidated by gel filtration*
Microsomal proteins were delipidated (83% delipidation) by gel filtration on Sephadex G-150 in the presence of 0.25% cholate. The standard reactivating phospholipid mixture (67% phosphatidylcholine, 33% lysophosphatidylcholine) at a total concentration of 220 µg of P/mg of protein (●) and Bio-Beads were added (see the Materials and Methods section). As a control for the experiment, buffer replaced the phospholipid mixture (○). Samples of the protein dispersions were removed immediately and after various times and assayed for UDP-glucuronyltransferase activity (0.6 mM-*p*-nitrophenol and 4 mM-UDP-glucuronate) and cholate. A represents the activity of the original microsomal membranes dispersed in 0.5% cholate.

Direct addition of phospholipids did not re-activate UDP-glucuronyltransferase in the protein-rich fractions from gel filtration. This is due, presumably, to the association of microsomal proteins with detergent micelles. Removal of cholate before the addition of phospholipids caused aggregation of the proteins, and subsequent addition of phospholipid did not stimulate their low transferase activity. Substantial re-activation was achieved (Table 3) with the standard re-activation mixture (67% phosphatidylcholine, 33% lysophosphatidylcholine at a concentration of 60 μ g of P/mg of protein) when cholate was removed by Bio-Beads in the presence of the phospholipids. The rate of re-activation depended on the concentration of re-activating phospholipids; when the concentration of the standard phospholipid mixture was increased from 60 to 220 μ g of P/mg of protein, the time required for full re-activation was decreased from 3.5 to 1.5 h. It is concluded that in the inactive protein fractions prepared by chromatography the microsomal proteins and residual phospholipids are associated with cholate, and that, as detergent is removed during re-activation, cholate bound to protein is exchanged for phospholipids. During re-activation by the standard phospholipid mixture (220 μ g of P/mg of protein) there was an inverse relationship between the concentration of cholate and the UDP-glucuronyltransferase activity (Fig. 3). Extrapolation of the data to zero detergent concentration gives a high activity close to that of the original microsomal dispersion.

Since two radically different ways of delipidating microsomal membranes inactivate UDP-glucuronyltransferase, and since high activity can be restored to lipid-depleted fractions by phospholipids, we conclude that for full activity the enzyme requires a suitable phospholipid environment. In intact microsomal membranes, this is provided by membrane phospholipids. Removal of this environment inactivates the enzyme and a suitable environment can then be provided by the addition of appropriate phospholipids. These data satisfy the criteria for phospholipid-dependence (Fleischer *et al.*, 1962) and confirm our earlier conclusions (Graham & Wood, 1969; Attwood *et al.*, 1971).

The results of our experiments with cholate are similar to those of Jansen & Arias (1975), who showed that rat liver microsomal UDP-glucuronyltransferase was also phospholipid-dependent and that it was fully active when complexed with phosphatidylcholine. They dispersed rat microsomal membranes in a medium containing deoxycholate and separated protein from phospholipid by using Bio-Gel P-30 in the presence of the detergent. Their delipidated protein fractions had very low transferase activity, and phospholipids did not re-activate the enzyme when added to these fractions either before or after removal of deoxycholate. Exchange of

detergent for phospholipid by dialysing mixtures of the dispersed proteins and phospholipids against buffer for several hours resulted in a preparation that actively catalysed glucuronidation of bilirubin but not *p*-nitrophenol. When a rat liver microsomal fraction was treated with phospholipase A and albumin in exactly the same way as guinea-pig preparations, we also found that UDP-glucuronyltransferase (with *p*-nitrophenol as acceptor) in the delipidated microsomal protein had only low activity and that it could not be re-activated by the standard phospholipid mixture. This difference between rat and guinea-pig enzymes may be related to the observation (Vessey & Zakim, 1972) that treatment of microsomal membranes from certain animal species with phospholipase A produced a thermolabile form of UDP-glucuronyltransferase, whereas similar treatment of guinea-pig preparations produced a form of the enzyme that is stable.

It was surprising to find that lysophosphatidylcholine was an essential component of our re-activating phospholipid mixture and caused some re-activation on its own. Fully active intact microsomal fractions do not contain lysophosphatides (Table 2), and phospholipase A, which inactivates UDP-glucuronyltransferase, produces these compounds in the microsomal membranes. Preliminary experiments have shown that, during re-activation, both phosphatidylcholine and the lysophosphatide were bound to delipidated membranes, and we conclude that lysophosphatidylcholine plays an important part in re-activation. It is noteworthy that in microsomal preparations where UDP-glucuronyltransferase is highly latent, e.g. those from rat liver, lysophosphatidylcholine or phospholipase A activates the enzyme, and the effect has been attributed to an increase in membrane permeability facilitating access of substrates to the enzyme (Hänninen & Puukka, 1971; Graham & Wood, 1974; Graham *et al.*, 1974b). It is possible that the lysophosphatide has a similar effect in our re-activated fractions. On the other hand, delipidated microsomal proteins were extremely insoluble in detergent-free aqueous media and lysophosphatidylcholine may act as a surface-active agent allowing access of phosphatidylcholine to a rather hydrophobic microsomal protein. More detailed study of these complex multicomponent interactions is necessary to understand the nature of re-activation, and the delipidation techniques described in this paper provide suitable material for such work.

The data presented here confirm our earlier conclusion (Graham & Wood, 1969; Attwood *et al.*, 1971) that the fully active enzyme species requires intact phospholipid, perhaps phosphatidylcholine specifically. Nevertheless, phospholipid-dependence is only one of two modulating effects of phospholipids on UDP-glucuronyltransferase in native

microsomal preparations (Graham *et al.*, 1974a). In certain microsomal preparations, the transferase is highly latent, owing to the predominance of a second effect, i.e. restriction of activity. Zakim and co-workers (Vessey & Zakim, 1971, 1972; Zakim *et al.*, 1973) proposed that phospholipids restrict by constraining the enzyme in a conformation of low activity. This theory cannot be reconciled readily with the conclusions drawn from the present work; it is difficult to imagine how phospholipids simultaneously constrain and maintain high transferase activity. On the other hand, many workers believe that intact phospholipids act as a permeability barrier to the transferase's soluble substrates (see the introduction) and, like Berry *et al.* (1976), we also envisage phospholipid modification of these latent microsomal preparations to cause two superimposed and opposing effects on UDP-glucuronyltransferase activity, namely activation of latent enzyme by disruption of the permeability barrier and inactivation of enzyme released from latency by destruction of essential phospholipid.

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