Preparation and Properties of a Phospholipid-Free Form of Microsomal UDP-Glucuronyltransferase†

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ABSTRACT: Phospholipids have been removed completely from microsomal UDP-glucuronyltransferase (EC 2.4.1.17; assayed with *p*-nitrophenol as aglycone). The phospholipid-free form of the enzyme contains neutral lipids, fatty acids, and small amounts of cholate and has a small residual activity as compared with other forms prepared during removal of the phospholipids. The residual activity does not depend on the presence of the above compounds but seems to be an intrinsic property of the phospholipid-free enzyme. Activity of the phospholipid-free form of UDP-glucuronyltransferase is stimulated as much as 50-fold by addition of lysolecithin or lecithin. All other classes of phospholipids, detergents, and neutral lipids tested, except for sphingomyelin, did not increase the activity of the phospholipid-free form of UDP-glucuronyltransferase. Activation by lysolecithin or lecithin from egg yolk produced different forms of UDP-glucuronyltransferase as reflected by kinetic constants after addition of each of these lipids. Activation by a series of pure species of lecithin and lysolecithin indicated that the length and degree of unsaturation of the acyl chains had important effects on the activity of reconstituted enzyme. Within a series of lecithins or lysolecithins, differences were limited, however, to variability of activity at V_{max}. Although it was possible to reconstitute a form of UDP-glucuronyltransferase with high activity, we were not able, with any lipid or combination of lipids, to restore the regulatory properties of UDP-glucuronyltransferase as it exists in untreated microsomes.

The catalytic activities of several integral enzymes of biological membranes have a demonstrated dependence on interactions with a phospholipid environment (Beaufay & de Duve, 1954; Dutton et al., 1968; Martonosi et al., 1968; Abou-Issa & Cleland, 1969; Strobel et al., 1970; Zakim & Vessey, 1976). UDP-glucuronyltransferase of liver microsomes is perhaps one of the most interesting enzymes in this group because of the complexity of lipid-induced modulations of its kinetic properties. Thus, the affinity of this enzyme for aglycone and UDP-glucuronic acid, response to allosteric effectors, specificity for nucleotide sugars, and activity at V_{max} are altered by treatments that modify the structure of the microsomal lipids, as for example, temperature-induced phase transitions, detergents, or digestion with purified phospholipases (Zakim & Vessey, 1976). Although these data suggest that interactions with microsomal lipids are significant for the dynamic regulation of UDP-glucuronyltransferase, little is known about how the enzyme interacts with its lipid environment. Studies of the effects of temperature-induced phase transitions within the membrane lipids suggest that bulk-phase properties influence the function of UDP-glucuronyltransferase (Eletr et al., 1973; Zakim & Vessey, 1975). On the other hand, we have found no correlations between activations of UDP-glucuronyltransferase induced by a variety of detergents, including lysolecithins, and changes in the bulk-phase properties of the membrane lipids, as measured with nitroxide labeled fatty acids.[1] There are several possible explanations for these observations, including the failure of probes to sample the most intimate lipid environment of UDP-glucuronyltransferase. Further resolution of the functional significance of lipid–enzyme interactions seemed to depend, therefore, on preparing phospholipid-free forms of UDP-glucuronyltransferase as a first step in studying the properties of reconstituted systems. We report in this paper the methods used for preparing this form of the enzyme, and the kinetic properties of phospholipid-free UDP-glucuronyltransferase after addition of different types of pure phospholipids.

Materials and Methods

Materials

Dithioerythritol (DTE), Trizma Base, egg lysolecithin, egg lecithin, bovine brain sphingomyelin, fatty acid free bovine serum albumin (fraction V), lysophosphatidylethanolamine, and silica gel H were purchased from Sigma Chemical Co. (St. Louis, Mo.). Cholic acid was obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wis.) and further purified by treatment with Norit A and recrystallization from ethanol. Lysophosphatidylserine (bovine) and the series of lecithin and lysolecithin used were purchased from Applied Science Laboratories (State College, Pa.). Lyophilized snake venom (*Naja naja*, Miami Serpentarium) was used as the starting material for the purification of phospholipase A_2 (Cremona et al., 1973). Agarose A-1.5 was obtained from Bio-Rad Laboratories (Richmond, Calif.).

Methods

Delipidation of Microsomes. Livers were removed from Dunkin-Hartley guinea pigs (male, retired breeders), and the microsomal fraction isolated in 0.25 M sucrose (Zakim & Vessey, 1973). The microsomes (40 to 50 mg/mL) were frozen (−85 °C) until needed. The first step in delipidation of microsomes was adopted from the method of Kuriyama (1972) for solubilization of nucleotide diphosphatase. Microsomes were suspended in 10 mM Tris-HCl, 0.25 M sucrose, pH 7.5, to a concentration of 12 to 15 mg of protein per mL. Deoxycholate (recrystallized, 10% w/v solution, pH 7.8) was added with stirring to a final concentration of 0.05% (w/v). The pH

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[1] D. A. Vessey & D. Zakim, unpublished observations.

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of the microsomal suspension was adjusted to 10.7 with 3 M ammonium hydroxide, and maintained at this point for 5 min. The pH then was adjusted rapidly to 7.5 with 3 M acetic acid, and the microsomes centrifuged for 2 h at 80 000*g*. The microsomal pellet, which contained all of the recoverable UDP-glucuronyltransferase, was resuspended in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, and washed once by repeated centrifugation at 80 000*g* for 2 h. The pellet was resuspended in 0.25 M sucrose to a concentration of 20 to 25 mg of protein per mL. Tris-HCl, pH 8.0, was added to a final concentration of 50 mM. After addition of $CaCl_2$ to a concentration of 1 mM, the microsomes were treated for 30 min with purified phospholipase A_2 (*Naja naja*, 1 mg per 300 mg of microsomal protein). EDTA (5 mM) was added to inhibit phospholipase A_2. The concentration of Tris-HCl, pH 8.0, was increased to 100 mM, and dithioerythritol was added to 2 mM. The mixture was cooled to 4 °C and centrifuged at 100 000*g* for 2 h. The resulting pellet was discarded. UDP-glucuronyltransferase activity was found only in the supernatant and was stable for several months at −85 °C.

Solid $MgCl_2$ was added to the above supernatant to a final concentration of 50 mM. The protein concentration was adjusted to 10 mg per mL by adding a mixture of 0.1 M Tris-HCl, 0.25 M sucrose, 50 mM $MgCl_2$, 2 mM DTE, pH 8.0, and a solution of 20% cholic acid (pH 7.8) added to a final concentration of 10 mg of cholate per mL. After stirring on ice for 10 min, this mixture was centrifuged at 80 000*g* for 90 min in order to remove precipitated material, which was discarded. The supernatant was fractionated with solid ammonium sulfate between 0–40% and 40–60% saturation. UDP-glucuronyltransferase was recovered in the 40–60% fraction.

The pellet from this fraction was taken up in 0.1 M Tris-HCl, 0.25 M sucrose, 50 mM $MgCl_2$, and 2 mM DTE, pH 8.0 (5 mL, 30 mg per mL), and dialyzed for 5 h against two 250-mL changes of 0.1 M Tris-HCl, 50 mM $MgCl_2$, and 2 mM DTE, pH 8.0. The protein concentration was adjusted to 10 mg and a solution of cholate (20% w/v recrystallized, pH 8.0) was added to bring the final concentration of cholate to 10 mg per mL. Solid ammonium sulfate was added to 40% saturation, and the mixture stirred on ice for 20 min. The solution was centrifuged for 20 min at 20 000*g*, and the pellet taken up in 100 mM Tris-HCl, 30% (w/v) glycerol, 50 mM $MgCl_2$, 2 mM DTE, pH 8.0, to a concentration of 30–50 mg of protein per mL. Ninety-five percent of the recovered activity of UDP-glucuronyltransferase was in this fraction.

Chemical Analyses. Phospholipids were extracted from aqueous samples of microsomes with chloroform–methanol (Folch et al., 1957). Precipitated protein was removed by filtering. The extract was washed with 0.33 vol of aqueous, 1% sodium chloride (w/v) followed by two washes with an equal volume of a 1/1 mixture of 1% sodium chloride and methanol. The final chloroform phase was taken to dryness under a stream of nitrogen. The residue was dissolved in a small amount of chloroform and stored at −20 °C. Lipid samples, stored in chloroform, were dried under a stream of nitrogen, and redissolved in toluene. Samples of 10 μL were spotted on glass plates, coated with silica gel H. Chromatograms were developed in a mixture of chloroform:methanol:water (65:25:4, v/v/v). Individual lipids were located by spraying with 50% sulfuric acid, followed by charring for 20 to 30 min at 120 °C. The phosphorus content of lipid samples was determined by the method of Sumner (1941), after digestion with 10 N sulfuric acid. Analysis of phospholipid phosphorus in concentrated $CHCl_3$–CH_3OH extracts of microsomal preparations would have detected less than 1 nmol per mg of microsomal protein. Proteins were measured with the biuret reaction (Gornall et al., 1949).

Enzyme Assay. The rate of glucuronidation of *p*-nitrophenol was measured at 30 °C by monitoring the change in absorbance at 400 nm in the presence of *p*-nitrophenol, UDP-glucuronic acid, and 50 mM Tris-HCl, pH 8.0 (30 °C). The concentrations of *p*-nitrophenol and UDP-glucuronic acid are indicated in legends to figures, tables, and in the text. Phospholipids, when used to activate the enzyme, were dried under a stream of nitrogen and sonicated for 15 min (Heat Systems, Ultrasonics, Inc., Model W 185 F, power setting 2) at a concentration of 10 mg lipid per mL of 2.5 mM Tris-HCl, pH 7.4. The mixture of buffer and lipid was saturated with nitrogen prior to sonication, and a stream of nitrogen was passed over the surface of the mixture during sonication. The sonicated suspension of lipid was centrifuged at 100 000*g* for 10 min, and the supernatant used for activation studies. Preparations of lysophosphatides were dissolved in water and used without further treatment. The enzyme preparation was preincubated with the indicated phospholipids at 0 °C for 2 min prior to addition to the standard reaction mixture. Activities are expressed as nmol of *p*-nitrophenol glucuronidated per min per mg of protein.

Results

Relationship between Enzyme Activity and Phospholipid Content. The initial step in removing phospholipids from UDP-glucuronyltransferase involved treatment of microsomes at pH 10.7 for 5 min. The pH then was decreased to 7.5. These manipulations removed a large amount of microsomal protein and enhanced the activity of UDP-glucuronyltransferase. They did not diminish the amount of phospholipid associated with the enzyme, however, when the data were expressed as mg of phospholipid phosphorus per mg of residual protein. Treatment at alkaline pH was followed by complete digestion of phospholipids with a preparation of phospholipase A_2 that was purified to homogeneity from *Naja naja* venom. Digestion of microsomal phospholipids with phospholipase A was essential for subsequent complete removal of phospholipid. Cholate (1.0%) and Mg^{2+} (50 mM) were added to the microsomes after completion of the digestion by phospholipase A. This mixture was fractionated with ammonium sulfate. An active preparation of UDP-glucuronyltransferase was obtained in the protein precipitated between 40 and 60% saturation. The preparation of UDP-glucuronyltransferase precipitated between 40 and 60% ammonium sulfate contained 6% of the original phospholipids, expressed as mg of phospholipid phosphorus per mg of protein (Table I). Removal of 94% of phospholipids decreased the specific activity of UDP-glucuronyltransferase by about 30% (Table I). Total activity recovered, however, was diminished by about 90%. Insignificant amounts of activity were recovered at lesser or greater concentrations of ammonium sulfate. Specific activity and total activity fell nearly to zero when the remaining phospholipids were removed completely by a second fractionation with ammonium sulfate in 1.0% cholate. All recoverable activity of UDP-glucuronyltransferase was precipitated between 0 and 40% saturation with ammonium sulfate.

The final preparation of UDP-glucuronyltransferase (Table I) contained no detectable phospholipids. It did contain small amounts of cholate, neutral lipids, and fatty acids. Cholate was removed by gel filtration of the enzyme without changing the activity of the phospholipid-free preparation. Extraction of neutral lipids also did not alter residual activity. Attempts to extract fatty acids completely with dry acetone, ether, and mixtures of chloroform and methanol were unsuccessful, however. We felt it important to assess the significance of the

TABLE I: Activity and Phospholipid Content of Different Preparations of UDP-Glucuronyltransferase.[a]

preparation	percent phospho-lipid	spec act.	total protein	total act. $\times 10^{-3}$
untreated microsomes	100	12	3240	38.8
pH treated	100	44	1850	81.4
phospholipase A treated	100	32.4	1840	59.6
1st ammonium sulfate treatment	6	20.0	334	6.7
2nd ammonium sulfate treatment	0	4.8	202	1.0

[a] Preparations of different forms of UDP-glucuronyltransferase are described in Methods. Percent phospholipid is expressed as phospholipid phosphorous per mg of protein as a percent of that ratio in untreated microsomes. Activities are given as activity at V_{max} at 30 °C determined by graphical analysis of bisubstrate kinetic data (Cleland, 1970). Assays were carried out as in Methods. Units of activity are nmol of *p*-nitrophenol conjugated per min per mg of protein.

TABLE II: Effect of Lipids and Detergents on the Activity of Phospholipid-Free UDP-Glucuronyltransferase.[a]

addition to assay	activity
none	2.0
lysophosphatidylcholine	15.6
phosphatidylcholine	20.6
phosphatidylethanolamine	2.7
lysophosphatidylethanolamine	2.2
phosphatidylserine	1.6
lysophosphatidylserine	2.0
sphingomyelin	4.8
microsomal lipids	16.1

[a] Enzyme (240 μg) was incubated with lipid (100 μg) or detergent (100 μg) and activity assayed as in Methods. The concentration of UDP-glucuronic acid was 1.0 mM and that for *p*-nitrophenol, 0.1 mM in all assays. Units of activity are nmol of *p*-nitrophenol glucuronidated per min per mg of protein.

remaining fatty acids in supporting the residual activity. Quantitation of the amount remaining could not resolve this question. We decided, therefore, to measure activity of the phospholipid-free form of UDP-glucuronyltransferase after addition of fatty acids. Added fatty acids inhibited the residual activity. Although they do not constitute absolute proof, these data suggest that the residual activity of the phospholipid-free enzyme is not related to remaining fatty acids. The most reasonable explanation for this activity, therefore, is aggregation of membrane proteins in order to satisfy the requirements of their hydrophobic portions that normally are in contact with the acyl chains of phospholipids. This aggregation appears to stabilize a form of the enzyme that has a small residual activity. Gel filtration of phospholipid-free UDP-glucuronyltransferase confirmed that it was aggregated since its approximate molecular weight was 2×10^6.

In addition to data on residual activity and phospholipid content, Table I lists the yield of protein and total enzyme activity at each step of the delipidation procedure. These data indicate that large amounts of protein are removed from the enzyme during delipidation, and that there is a large loss of total activity when phospholipids are removed completely. The significance of the loss of activity is considered below in the section on reconstitution of activity.

Effect of Phospholipids on the Activity of the Phospholipid-Free Form of UDP-Glucuronyltransferase. In view of the loss of activity of UDP-glucuronyltransferase on removal of phospholipids, we attempted to regenerate catalytic function by adding phospholipids to the phospholipid-free form. The activities of mixtures of phospholipid-free enzyme and phospholipids were measured at several time points over a period of several hours in each experiment. In addition, activity was assessed as a function of the amounts of phospholipid added. These experiments revealed that phospholipids with specific structural features were essential for regenerating the activity of phospholipid-free UDP-glucuronyltransferase (Table II). Thus, only those lipids with a phosphorylcholine head group reconstituted activity. The data in Table II were obtained using the minimum duration of incubation and amounts of phospholipid required for maximum activation under the conditions of assay that were employed. The data in Table II indicate that phosphatidylcholine or a derivative is essential for conferring catalytic activity on the phospholipid-free form of UDP-glucuronyltransferase. However, enzyme activity was variable after reconstitution with three different types of phosphorylcholine containing phosphatides. Since treatment of untreated microsomes with pure phospholipase A alters the kinetic properties of UDP-glucuronyltransferase (Vessey & Zakim, 1971; Zakim et al., 1973; Zakim & Vessey, 1976), we wondered whether interactions between phospholipid-free enzyme and phosphatidylcholine produced an enzyme with kinetic functions different from those after reconstitution of enzyme with lysophosphatidylcholine and sphingomyelin. This problem was investigated by determining the kinetic constants for maximally activated forms of phospholipid-free UDP-glucuronyltransferase after addition of lysophosphatidylcholine, phosphatidylcholine, and sphingomyelin. The data in Table III reveal that activation by these phospholipids had variable effects on the kinetic properties of UDP-glucuronyltransferase.

The kinetic mechanism of UDP-glucuronyltransferase is random order, rapid equilibrium (Vessey & Zakim, 1972). The K terms in Table IV thus reflect the binding affinity of either substrate in the absence of the second substrate, and the K' terms reflect the binding affinity of either substrate in the presence of saturating amounts of the second substrate (Cleland, 1970). Activation by lysolecithin has a significant effect on the affinity of UDP-glucuronyltransferase for UDP-glucuronic acid as compared with activation by lecithin or sphingomyelin. In addition, activation by lysolecithin, but not lecithin and sphingomyelin, decreased the affinity of the enzyme for *p*-nitrophenol. This second effect was especially marked for enzyme previously saturated with UDP-glucuronic acid, in which case *p*-nitrophenol is the second substrate to bind. These data establish that it is possible to produce at least two kinetically different forms of UDP-glucuronyltransferase by addition of different phospholipids to the phospholipid-free enzyme.

The data in Table III suggest that the lysolecithin-reconstituted UDP-glucuronyltransferase is a more efficient form of the enzyme as compared with lecithin-reconstituted enzyme. Depending on the conditions of assay, however, the activity of the lecithin-activated enzyme can be substantially greater than that for activation with lysolecithin. This point is illustrated in Figure 1. For assays carried out at 0.025 mM *p*-nitrophenol, the activity of the lysolecithin–UDP-glucuronyltransferase complex becomes smaller as compared with the lecithin complex, when the concentration of UDP-glucuronic acid is increased. The kinetic explanation for this is that prior binding of UDP-glucuronic acid to the lysolecithin form of reconstituted UDP-glucuronyltransferase decreases the affinity of the

TABLE III: Kinetic Constants of Phospholipid-Free and Phospholipid-Activated Forms of UDP-Glucuronyltransferase.[a]

form of UDP-glucuronyltransferase	kinetic constant				
	V_{max}	K_{UDPGA}	K'_{UDPGA}	$K_{p\text{-NP}}$	$K_{p\text{-NP}}$
phospholipid-free	4.8	0.36	0.36	0.12	0.14
activated with egg lysophosphatidylcholine	80.0	0.071	0.20	0.36	1.0
activated with egg phosphatidylcholine	53.2	0.29	0.29	0.18	0.18
activated with sphingomyelin	12.0	0.35	0.35	0.15	0.18

[a] The phospholipid-free form of UDP-glucuronyltransferase was incubated with phospholipids as in Methods. The amount of added phospholipid gave maximal activations in each case. Kinetic constants were determined by graphical analysis of rate data according to Cleland (1970). Units of activity are nmol per min per mg of protein. Units for K and K' terms are mM.

TABLE IV: Effects of Detergents on the Activity of Mixtures of Phospholipid-Free Enzyme and Lysophosphatidylcholine or Phosphatidylcholine.[a]

addition to assay	activated form of phospholipid-free UDP-glucuronyltransferase	
	activation with lysophosphatidylcholine	activation with phosphatidylcholine
none	16.7	14.1
cholate (100 μg)	11.1	10.5
cholate (200 μg)	8.7	8.0
Triton X-100 (100 μg)	6.0	5.7
Triton X-100 (200 μg)	4.1	
Tween 80 (100 μg)	5.3	5.7
Tween 80 (200 μg)	2.0	2.3

[a] Phospholipid-free enzyme (260 μg) was activated by addition of either 100 μg of lysophosphatidylcholine or phosphatidylcholine, as in Methods. Assays were started by adding UDP-glucuronic acid to an assay mixture containing the preincubated enzyme and phospholipid. The concentration of UDP-glucuronic acid was 2.0 mM and that for *p*-nitrophenol was 0.1 mM in all assays. Detergents in the amounts indicated then were added sequentially to the reaction. Activities are nmol of *p*-nitrophenol glucuronidated per min per mg of protein.

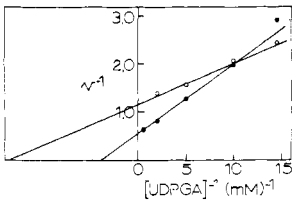

FIGURE 1: The activity of phospholipid-free UDP-glucuronyltransferase was measured, as in Methods, after reconstitution with egg lecithin (●) or egg lysolecithin (○). Assays were carried out at 30 °C in 0.025 mM *p*-nitrophenol and the indicated concentrations of UDP-glucuronic acid. Data are plotted in double-reciprocal form. Units of activity are nmol of *p*-nitrophenol conjugated per min per mg of protein.

enzyme for *p*-nitrophenol. This does not take place when UDP-glucuronic acid binds to the lecithin-reconstituted form (Table III). Thus, despite a higher activity at V_{max} and a greater affinity for UDP-glucuronic acid, UDP-glucuronyltransferase activated with lysolecithin can be a less efficient form of the enzyme, as compared with the lecithin-activated form. Data such as those in Table III and Figure 1 indicate the potential for kinetic complexity and subtlety in the regulation of enzymes by phospholipids.

The Effects of Detergents on the Activity of Phospholipid-Free UDP-Glucuronyltransferase. One of the interesting properties of UDP-glucuronyltransferase in untreated microsomes is activation on addition of a number of detergents (Vessey & Zakim, 1971). It is unclear whether this effect is due to direct interactions between the enzyme and detergents, or whether detergents modify the phospholipid environment of the enzyme. Availability of a phospholipid-free form of the enzyme provides an opportunity to examine this question. Addition of detergents to phospholipid-free enzyme inhibited activity slightly (data not shown). In no case did detergents activate the phospholipid-free form of UDP-glucuronyltransferase. Moreover, detergents had a marked inhibitory effect on phospholipid-induced reactivations of phospholipid-free enzyme (Table IV). This was true whether they were added to phospholipid-free enzyme prior to or subsequent to addition of phospholipids (Table IV). Detergent-induced inhibition of reconstitution was reversed, however, by adding excess lecithin or lysolecithin. Although the data are not shown, added fatty acids had an effect on activity that was identical with that for detergents. Fatty acid induced inhibition also was reversed by excess lecithin or lysolecithin. These results suggest that detergents bind to UDP-glucuronyltransferase, thereby interfering with interaction between enzyme and phospholipids. Addition of large amounts of phospholipid appears to provide a phase for sequestering detergent, and hence removing them from the enzyme. The extent to which phospholipids reversed detergent-induced inhibitions of activation depended on the type of detergent. The effect of cholate was reversed completely, whereas inhibition due to Triton X-100 and Tween 80 was more difficult to overcome by adding an excess of phospholipids.

Influence of Length and Unsaturation of Acyl Chains of Lecithin and Lysophosphatidylcholine on the Properties of Phospholipid-Free UDP-Glucuronyltransferase. The reconstitution experiments reported in Tables II and IV above were carried out with mixed species of lecithin or lysolecithin derived from egg yolk and sphingomyelin from brain. The variability of kinetic constants in Table IV for enzyme reconstituted with different lipids could reflect the effects of different chain lengths and unsaturations of the acyl chains of each type of phospholipid. We undertook, therefore, kinetic analyses after activation with lipids of defined composition. The specific purpose of these experiments was to determine whether the chain length and degree of unsaturation of the acyl groups of phospholipids had an effect on the activity of reconstituted forms of UDP-glucuronyltransferase.

Differences in kinetic properties were limited, within each series of phospholipids, to activities at V_{max}. Binding constants after reconstitution with the series of lecithins were not different significantly from those in Table IV for egg lecithin. Similarly, kinetic constants for activation with the series of lysolecithins, other than activities at V_{max}, were nearly identical with those in Table IV for activation with egg lysolecithin. The relationship between activity at V_{max} and the acyl chain in symmetrical lecithins is shown in Figure 2. For saturated fatty acids, there was a clear optimum chain length for maximal activation in that activation was maximal with dipalmitoyllecithin. Lecithins containing longer, saturated acyl chains

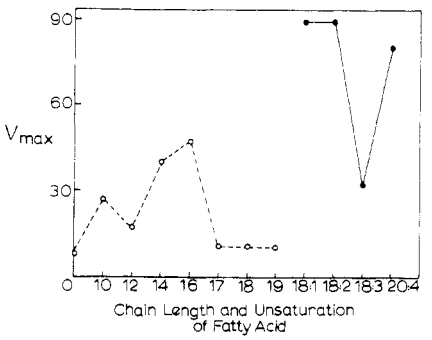

FIGURE 2: The effect of different pure species of lecithin on the activity at V_{max} of phospholipid-free UDP-glucuronyltransferase was determined as in Methods, after mixture of phospholipid-free enzyme with sonicated dispersions of symmetric lecithins of indicated acyl chain composition. In each case, conditions were determined that gave maximal activities after adding phospholipid to enzyme. Activities are at V_{max}, as in Table I. Units of activity are nmol of *p*-nitrophenol glucuronidated per min per mg of protein.

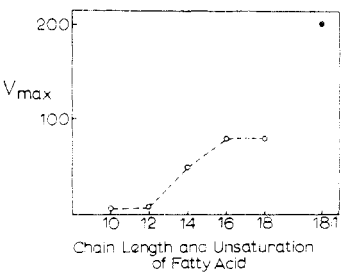

FIGURE 3: The effect of different species of pure lysolecithin on activities at V_{max} of phospholipid-free UDP-glucuronyltransferase was determined as in Figure 2.

were inactive in reconstituting activity. The degree of unsaturation also was an important determinant of reconstitution. Activation with dioleolyllecithin, for example, gave the highest activity of any lecithin. Dipalmitoleoyllecithin was not available for testing, so we were unable to investigate precise interactions between chain length and unsaturation. Nevertheless, the limited data obtained do suggest that chain length and unsaturation interact. For example, di-18:3-lecithin was a less effective activator as compared with di-18:1- and di-18:2-lecithins. There hence may be an optimum number of double bonds for maximal activation. On the other hand, di-20:4-lecithin yielded maximal activation.

Length of the acyl chain was less important in the series of lysolecithins as compared with lecithins in that activity increased with increasing chain length up to the stearoyl derivative, the largest chain length tested (Figure 3). Introduction of a single double bond, as in oleoyllysolecithin, produced a more active enzyme than any other preparation. Activation by oleoyllysolecithin was approximately 50-fold as compared with phospholipid-free enzyme.

A systematic study with sphingomyelins has not been carried out because of the absence of a commercial source of pure sphingomyelin with a defined composition of the acyl chain. It is known, however, that sphingomyelin from beef brain contains predominantly long chain, highly saturated fatty acids. We feel, therefore, that the relatively poor activation by sphingomyelin (Table IV), as compared with other choline-containing phosphatides, is due to the properties of the acyl chains of the sphingomyelin we used.

Recovery and Regulatory Properties of Reconstituted UDP-Glucuronyltransferase as Compared with Enzyme in Untreated Microsomes. We cannot estimate the total recovery of activity in the reconstituted preparations because activities at V_{max} were not measured in the presence of oleoyllysolecithin with a preparation other than phospholipid-free enzyme. Total recovery of reconstituted activity vs. pH-treated enzyme (Table I) was about 30%, however, for assays in the presence of optimal amounts of egg lysolecithin.

Perhaps a more significant question than total recovery of activity is the relationship between the kinetic properties of reconstituted species of UDP-glucuronyltransferase and the enzyme in untreated microsomes. We examined this question in the following way. Untreated UDP-glucuronyltransferase in native microsomes has absolute specificity for binding of UDP-glucuronic acid at the sugar nucleotide site. In addition, this form of the enzyme is sensitive to allosteric activation by UDP-*N*-acetylglucosamine and does not fit the Michaelis-Menten equation with respect to variation of activity as a function of the concentration of UDP-glucuronic acid (Vessey et al., 1973). None of the phospholipid-activated forms of UDP-glucuronyltransferase had these characteristics. For example, UDP-glucose and UDP-*N*-acetylglucosamine inhibited the phospholipid-free form of UDP-glucuronyltransferase in all mixtures of enzyme and pure phospholipids tested, including phospholipids from guinea pig liver microsomes. Similarly, the kinetics of all reconstituted forms fit the Michaelis-Menten equation. Finally, the kinetic constants of untreated enzyme are different from those of the reconstituted enzyme. For UDP-glucuronyltransferase in untreated microsomes K_{UDPGA} is 16 mM, $K_{p\text{-nitrophenol}}$ is 0.1 mM, and activity at V_{max} is 12 nmol per min per mg of protein (assays at 30 °C). We considered the possibility that components essential for maintaining the kinetic properties of the untreated UDP-glucuronyltransferase were removed during preparation of the phospholipid-free enzyme. We thus saved protein and lipid fractions that contained no UDP-glucuronyltransferase activity. Addition of these fractions, together with phospholipids, to phospholipid-free enzyme did not restore an enzyme with kinetic properties similar to those in untreated microsomes.

Discussion

The UDP-glucuronyltransferase used in the above experiments was not a pure enzyme. There are, we believe, two possible theoretical objections to using impure enzymes in studies such as those reported. The first is that phospholipids interact nonspecifically with proteins other than the enzyme of interest. The second is that the phospholipid-free enzyme may be inhibited by another protein that is removed from it on addition of phospholipids. We are aware, however, of no precedent for this with integral proteins of membranes. We believe the data exclude both of these possibilities. Thus, the studies were done at concentrations of phospholipid that yielded maximal effects on activity. The experimental design excludes effects of nonspecific binding of phospholipids to proteins other than UDP-glucuronyltransferase. The specificity of phospholipid-induced activations, the differential effects due to phospholipids containing acyl chains of varying length and unsaturation, the significant kinetic differences of lecithin vs. lysolecithin activation make it highly unlikely that the activations reflect simple reversal of an inhibitor. Moreover, more recent studies with a highly purified form of UDP-glucuronyltransferase indicate that extensive removal of nonenzymatic protein does not alter the specificity of activation by certain types of phospholipids, nor is the residual activity of the enzyme enhanced as nonactive proteins are removed. We conclude, therefore, that the experimental results presented provide substantial new information on the interrelation between the

activity of UDP-glucuronyltransferase and the interaction of the enzyme with its lipid environment. Thus, intimate interaction with a small amount of specific lipid, rather than a simple requirement for a general type of bulk phase, is the basis for determination of functional status by interactions with phospholipids. In view of the data indicating that activity is reconstituted only by phospholipids with a phosphorylcholine head group and the modulating effect of acyl chain length and unsaturations on activities at V_{max}, we believe it is reasonable to consider that UDP-glucuronyltransferase has two regions that interact with phospholipids. One of these is a specific site for phosphorylcholine. Binding at this site is essential for efficient catalytic activity. A second region of the enzyme interacts with the acyl chains of phospholipids in a manner that modulates phosphorylcholine binding to UDP-glucuronyltransferase.

Another major point elucidated by the present experiments is the demonstration that reconstitution of UDP-glucuronyltransferase is not a simple all-or-none phenomenon. Interactions with variable lipid environments produce different kinetic forms of the enzyme. This explains, in part, why treatments that alter the membrane lipids change the kinetic properties of UDP-glucuronyltransferase. Thus, the kinetic properties of all reconstituted forms generated by adding lysolecithin to phospholipid-free enzyme differ from those generated by mixture of phospholipid-free enzyme with lecithin. Most significant is that variability of the activity of different enzyme–lipid mixtures extends to differences beyond activation at V_{max}. The data in Table IV and Figure 1 begin to indicate, in fact, the subtlety of dynamic regulation of UDP-glucuronyltransferase by its lipid environment. Of interest in this regard is the clear separation between the kinetic properties, other than activity at V_{max}, of enzyme reconstituted by lecithin or lysolecithin. This separation may reflect the sensitivity of the enzyme to a physical property of its environment since lysolecithin forms a micellar array whereas lecithin forms bilayers.

Most reconstitution studies with lipid-sensitive enzymes have elicited primarily the effects of phospholipids on rates, without reference to determining specific kinetic constants (Martonosi et al., 1968; Strobel et al., 1970; Gazzotti et al., 1975; Grover et al., 1975). Little attention has been given to such questions as the substrate specificity and regulatory properties of reconstituted, lipid-sensitive enzymes. Our results, therefore, are significant in showing that this is an important problem to consider in reconstitution studies of integral membrane proteins with kinetic properties that are sensitive to environmental perturbations. For example, although we could recover the catalytic activity of UDP-glucuronyltransferase after removal of phospholipids, we were not able to regenerate an enzyme with regulatory properties like those in untreated microsomes. We cannot attribute the failure to accomplish this type of reconstitution to the lipids used because addition of microsomal phospholipids from guinea pig liver to phospholipid-free enzyme did not restore the regulatory properties of the untreated form of the enzyme. It is likely that the process of delipidation alters UDP-glucuronyltransferase irreversibly with respect to its regulatory properties. On the other hand, we find it is important to consider the possibility that there is a level of organization in intact, untreated microsomes that cannot be accounted for by phospholipid–protein interactions in reconstitution studies. It is significant, in this regard, that the regulatory properties of UDP-glucuronyltransferase which we could not restore are the type that depend on protein–protein interactions. Hence the phospholipids of the membrane may not only interact directly with UDP-glucuronyltransferase in a manner that affects activity, but also promote interactions between subunits of the enzyme or between it and other proteins.

Other laboratories have reported phospholipid-induced activation of preparations of UDP-glucuronyltransferase from which phospholipids have been removed (Jansen & Arias, 1975; Graham et al., 1977). Substantial amounts of phospholipids of unknown compositions (from 6 to 17%) remained attached to microsomal proteins in these experiments, making it difficult to interpret the exact effects of added phospholipids. In addition, other workers have reported a loss of activity when 95% of phospholipids were removed. Differences between our work and that already reported may reflect variations in the methods used for removing microsomal lipids. The glucuronidation of *p*-nitrophenol could not be reconstituted, for example, in one reported study after removal of 95% of phospholipids. Another major difference between our results and those reported earlier is the duration of incubation required to activate UDP-glucuronyltransferase that has been delipidated (Jansen & Arias, 1975). Reactivation in our experiments was complete within 2 min whereas others have found that several hours were required. The basis for this discrepancy is unclear, but may reflect the complete absence of phospholipids in our preparation.

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