The Phospholipid-Dependence of Uridine Diphosphate Glucuronyltransferase

EFFECT OF PROTEIN DEFICIENCY ON THE PHOSPHOLIPID COMPOSITION AND ENZYME ACTIVITY OF RAT LIVER MICROSOMAL FRACTION

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(Received 5 November 1973)

After force-feeding a protein-free diet to male rats for 5-7 days a substantial (2.4-fold) increase in the specific activity of the liver microsomal enzyme UDP-glucuronyltransferase (EC 2.4.1.17) was observed. A similar activation of the enzyme occurred when rats were fed on a low-protein (5%, w/w, casein) diet for 60 days. Although both the short- and long-term protein-deficient diets decreased the contents of microsomal protein and phospholipid in liver tissue they did not significantly alter the ratio of these major membrane components. Protein deficiency profoundly altered the phospholipid composition of microsomal membranes. The most striking difference in microsomal phospholipid composition between control and protein-deficient rats was their content of lysophosphatides. Whereas microsomal membranes from protein-deficient rats contained significant proportions of lysophosphatidylcholine and lysophosphatidylethanolamine very little or no lysophosphatides were detected in control preparations. Pretreatment of microsomal fractions from normal rats with phospholipase A markedly increased their UDP-glucuronyltransferase activity as did their pretreatment with lysophosphatidylcholine. It is concluded that the quantities of lysophosphatides present in microsomal membranes from protein-deficient rats were sufficient to have caused the increased UDP-glucuronyltransferase activities of these preparations. Evidence is presented suggesting that these changes in microsomal phospholipid composition and UDP-glucuronyltransferase activity caused by protein deficiency reflect changes that occur *in vivo*. The possible physiological significance of these findings is discussed.

When immature and young adult male rats were fed on a protein-free diet for 7 days a marked increase in the specific activity of liver microsomal UDP-glucuronyltransferase (UDP-glucuronate glucuronyltransferase, acceptor unspecific, EC 2.4.1.17) was observed with *p*-nitrophenol and *o*-aminophenol as acceptors (Wood & Woodcock, 1970; Woodcock & Wood, 1971).

The reasons for this activation of the enzyme in protein-deficient animals are obscure. Protein deficiency might alter the activities of the closely related microsomal hydrolases UDP-glucuronate pyrophosphatase (nucleoside diphosphate sugar pyrophosphatase) and β -glucuronidase (EC 3.2.1.31) to such an extent that they interfere in the assay of UDP-glucuronyltransferase and cause merely an apparent stimulation of its activity (Dutton, 1971; Graham & Wood, 1972). This theory, however, was discounted since D-glucaro-(1 $\rightarrow$ 4)-lactone, a specific inhibitor of β -glucuronidase, had no significant

effect on the UDP-glucuronyltransferase activities of microsomal fractions from normal and protein-deficient rats (Woodcock & Wood, 1971) and since the activation of UDP-glucuronyltransferase in protein-deficient animals was not affected when activities were measured at a much higher concentration of UDP-glucuronate, which greatly decreased competition between the transferase and pyrophosphatase for the sugar nucleotide substrate (Woodcock & Wood, 1971). Supporting these conclusions, Graham & Wood (1972) found that changes in the activities of these closely related hydrolases were not responsible for the activation of rat liver UDP-glucuronyltransferase by detergents. Thus we concluded that protein deficiency caused a real activation of microsomal UDP glucuronyltransferase.

There would appear to be two further mechanisms by which the increased activities of microsomal UDP-glucuronyltransferase might occur in protein-deficient rat liver. (1) In spite of the general decrease in protein synthesis accompanying protein deficiency (Munro, 1964), there might be a specific induction of the synthesis of UDP-glucuronyltransferase, as has been reported for rat liver cytosol fraction 'malic

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enzyme' (malate-NADP⁺ oxidoreductase, decarboxylating, EC 1.1.1.40) (Frenkel *et al.*, 1972), 3-phosphoglycerate dehydrogenase (EC 1.1.1.x) and phosphoserine phosphatase (EC 3.1.3.3) (Fallon *et al.*, 1966). (2) Since the activity of UDP-glucuronyltransferase is markedly dependent on the structure and phospholipid composition of the microsomal membrane (Graham & Wood, 1969; Attwood *et al.*, 1971) protein deficiency, as we have suggested (Woodcock & Wood, 1971), might increase the enzyme's specific activity by altering the structure of the microsomal membrane.

The present paper is concerned with investigating the second possibility. This study of microsomal preparations from normal and protein-deficient rats concentrates mainly on their phospholipid compositions since it has been shown that the enzyme in normal rat liver microsomal preparations is powerfully enhanced by reagents, like detergents and phospholipases, which perturb membrane structure and phospholipid composition (Leuders & Kuff, 1967; Winsnes, 1969; Mulder, 1970; Hänninen & Puukka, 1971; Graham & Wood, 1972; Vessey & Zakim, 1972).

In addition, since our earlier work (Wood & Woodcock, 1970; Woodcock & Wood, 1971) utilized rats with an acute, short-term protein-deficiency condition it was also decided to investigate the effects on UDP-glucuronyltransferase activity with animals maintained on a less severe long-term protein-deficient dietary regimen. The phospholipid contents and compositions of liver microsomal membranes isolated from these animals are also reported.

A preliminary account of part of this work has been published (Graham *et al.*, 1972).

Experimental

Materials

Male Wistar rats were purchased from Fisons Pharmaceuticals, Loughborough, Leics., U.K. Phospholipase A (EC 3.1.1.4) was purified from *Crotalus adamanteus* venom (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.) as described by Graham & Wood (1969). Lysophosphatidylcholine was purchased from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. and used as a 3 mM solution in water.

Methods

Dietary regimens. Protein deficiency was produced in male rats by two different methods. (1) Rats (initially 150–180 g) were force-fed at 8 h intervals on a protein-free diet (Woodcock & Wood, 1971) for 5–7 days. The diet was given as a slurry in water at

approximately 100 g of solid/day per kg body wt. Control rats were force-fed on a normal diet containing 18% (w/w) casein which was of equal energy value to the protein-free diet. (2) Rats (initially 80–100 g) were fed *ad libitum* on a 5% casein diet for 60 days (Kirsh *et al.*, 1968). Control animals were fed on an 18% (w/w) casein diet in restricted amounts so that the dietary intakes of test and control rats were of equal energy value. Over the 60-day period in each of two separate experiments both groups received on average 8.7 g/day per animal (Expt. a) and 11.4 g/day per animal (Expt. b).

In both methods rats were kept in individual cages in an animal house maintained at 22–24°C and the protein omitted from the deficient diets was replaced by starch.

Liver homogenate preparations. Control and protein deficient animals were killed by a sharp blow and their livers rapidly removed. A portion (0.25 g) of the liver from each animal was immediately homogenized in 1 ml of ice-cold 0.15 M-KCl containing EDTA (4 mM) and the homogenate freeze-dried.

Liver microsomal preparations. Microsomal fractions from the individual livers of control and protein-deficient rats were prepared in 0.15 M-KCl by the method of Woodcock & Wood (1971). In one experiment (b) the KCl solution contained EDTA (4 mM). To study the effect of lysophosphatidylcholine and phospholipase A on UDP-glucuronyltransferase activity, normal rats (150–200 g) were starved overnight and microsomal fractions prepared in 0.154 M-KCl as described by Graham & Wood (1969). These fractions were adjusted to a protein content of 20 mg/ml and used within 1 h of preparation.

UDP-glucuronyltransferase assays. The activities of liver microsomal fractions from control and protein-deficient rats were measured with *p*-nitrophenol (0.22 mM) or *o*-aminophenol (0.15 mM) as acceptors with 0.49 mM-UDP-glucuronate, as described by Woodcock & Wood (1971). When the effects of lysophosphatidylcholine and phospholipase A were studied, *p*-nitrophenol UDP-glucuronyltransferase activities were measured by the method of Graham & Wood (1972) with 0.50 mM-UDP-glucuronate.

Pretreatments of microsomal fractions. (1) Microsomal suspensions (5 mg of protein/ml, final concn.) were shaken at 20°C with phospholipase A (10 µg/mg of microsomal protein) in a medium containing 12.5 mM-Tris-HCl buffer, pH 8.0 and 2.5 mM-CaCl₂. Portions (0.1 ml) of the digest were assayed for UDP-glucuronyltransferase activity in the presence of 3.3 mM-EDTA. (2) The time-course of the phospholipase A-catalysed hydrolysis of microsomal phospholipid at pH 8.0 was followed by using a similar medium with no buffer. The reaction mixture was stirred under a stream of N₂ and NaOH (25 mM)

was added from an Agla micrometer syringe to maintain it at pH 8.0 (Radiometer TTTI pH meter with scale expansion attachment). (3) A microsomal fraction (5 mg of protein/ml) was incubated at 20°C for 10 min with 25 mM-Tris-HCl buffer, pH 8.0, and various concentrations of lysophosphatidylcholine in the range 0–0.3 μ mol/mg of microsomal protein. Portions (0.1 ml) of these mixtures were assayed for UDP-glucuronyltransferase activity.

Analytical methods. The protein contents of microsomal fractions from control and protein-deficient rats were determined with the Folin-Ciocalteu reagent (Woodcock & Wood, 1971). Those of microsomal fractions pretreated with lysophosphatidylcholine and phospholipase A were determined by the biuret method of Layne (1957) standardized with bovine serum albumin.

Microsomal phospholipid contents were estimated from their contents of total phosphorus by assuming that 1 g of phosphorus is derived from 25 g of phospholipid. Portions (0.1 ml) of the microsomal suspensions were dried in a vacuum oven at 55–60°C and total phosphorus was measured after digestion of the residual material with H₂SO₄ and HClO₄ by the method of Chen *et al.* (1956).

Microsomal phospholipid compositions were determined by extracting the phospholipids and separating them by t.l.c. Portions (0.5 ml) of microsomal suspensions were dried (vacuum oven, 35°C) and the residues extracted with three 5 ml portions of chloroform-methanol (2:1, v/v). The combined extracts were washed with water (3 ml) and the bottom phases dried *in vacuo*. The residues of lipid were dissolved in a small volume of chloroform-methanol and the phospholipids separated by Morrison's (1968) two-dimensional t.l.c. system. After the spots had been located, removed from the plates and digested with H₂SO₄ and H₂O₂, their phosphorus contents were measured by the method of Morrison (1964). In a single experiment, recovery of phospholipid P from this t.l.c. system was found to be 102% in

approximate agreement with Morrison's (1968) quoted recoveries of 90–101%. The proportions of individual phospholipids are given by the relationship:

Phospholipid P in spot $\times$ 100 / total phospholipid P
recovered after chromatography

as recommended by Rouser *et al.* (1968). The phospholipid compositions of the dried liver homogenates were determined by the same procedure except that the preparations were extracted with four 5 ml portions of chloroform-methanol and the combined extracts were washed with 4 ml of water.

Analysis of data. Results are quoted as mean values of results obtained with different animals $\pm$ s.e.m. After the variances had been compared with an *F* test, mean values were compared by using Student's *t* test and the equations described by Bailey (1961). Values of means and variances were considered to be significantly different when *P* < 0.05.

Results and Discussion

In agreement with our previous results (Wood & Woodcock, 1970; Woodcock & Wood, 1971), protein deficiency, produced by feeding rats on a protein-free diet for 5–7 days enhanced about 2.4-fold the UDP-glucuronyltransferase activity of liver microsomal fraction with *p*-nitrophenol as acceptor (Table 1). In addition, our earlier work showed that the activity of *o*-aminophenol UDP-glucuronyltransferase was also strongly stimulated under these acute conditions. A more progressive chronic protein-deficient condition produced by feeding rats on a low-protein (5% w/w casein) diet for 60 days caused an activation (at least two-fold) of *p*-nitrophenol UDP-glucuronyltransferase, similar to that observed in acutely protein-deficient animals (Table 1). *o*-Aminophenol UDP-glucuronyltransferase was also powerfully enhanced under these chronic protein-deficiency conditions (Table 1).

Table 1. Liver microsomal UDP-glucuronyltransferase activities of control and protein-deficient rats

Acute protein deficiency was produced by feeding animals on a protein-free diet for 5–7 days. The chronic condition was produced in two separate experiments by feeding animals on a 5% (w/w) casein diet for 60 days, Expt. (a) microsomal fractions prepared in the absence of EDTA and Expt. (b) in the presence of EDTA, see the text. UDP-glucuronyltransferase activities are quoted as nmol of substrate glucuronidated/h per mg of protein.

Animals ...	Acute protein deficiency		Chronic protein deficiency (Expt. a)		Chronic protein deficiency (Expt. b)	
	Control	Deficient	Control	Deficient	Control	Deficient
No. of animals	6	6	4	4	3	3
Acceptor substrate <i>p</i> -nitrophenol	63.2 $\pm$ 6.8	148.7 $\pm$ 13.4 (<i>P</i> < 0.001)	66.6 $\pm$ 6.0	185.7 $\pm$ 18.1 (<i>P</i> < 0.002)	67.9 $\pm$ 4.5	126.5 $\pm$ 13.0 (<i>P</i> < 0.02)
<i>o</i> -Aminophenol	not determined		9.03 $\pm$ 0.77	41.34 $\pm$ 5.93 (<i>P</i> < 0.02)	not determined	

Table 2. *Effect of protein deficiency on the contents of microsomal protein and phospholipid in rat liver*

Acute protein deficiency was produced by feeding animals on a protein-free diet for 5–7 days. The chronic condition was produced in two separate experiments by feeding animals on a 5% (w/w) casein diet for 60 days, Expt. (a) microsomal fractions prepared in the absence of EDTA. Expt. (b) in the presence of EDTA, see the text.

Animals ...	Acute protein deficiency		Chronic protein deficiency (Expt. a)		Chronic protein deficiency (Expt. b)	
	Control	Deficient	Control	Deficient	Control	Deficient
No. of animals	6	6	4	4	3	3
Protein (mg/g of liver)	17.16 ± 0.58	10.96 ± 1.20	22.46 ± 1.36	15.32 ± 0.81	23.03 ± 2.42	15.71 ± 1.86
		(<i>P</i> < 0.005)		(<i>P</i> < 0.01)		(<i>P</i> < 0.05)
Phospholipid (mg/g of liver)	17.18 ± 0.58	11.86 ± 1.42	15.30 ± 0.73	10.06 ± 0.30	14.79 ± 2.10	10.67 ± 1.30
		(<i>P</i> < 0.01)		(<i>P</i> < 0.02)		(<i>P</i> > 0.10)
Phospholipid/protein ratio (g/g)	1.00 ± 0.01	1.07 ± 0.03	0.68 ± 0.03	0.66 ± 0.02	0.64 ± 0.02	0.68 ± 0.01
		(<i>P</i> > 0.10)		(<i>P</i> > 0.50)		(<i>P</i> > 0.10)

Acute protein deficiency and chronic protein deficiency (Expt. a) were both accompanied by greatly decreased weights of microsomal protein and phospholipid in the liver (Table 2). In chronic protein deficiency (Expt. b) failure to detect a statistically significant difference between control and protein-deficient animals was probably due to the smaller number of rats used and the unexpectedly larger spread of individual values. The ratio of phospholipid/protein was markedly different in the chronic and acute experiments, probably as a result of the different dietary intake of the animals (Table 2). Nevertheless, the ratio was not significantly different in the control and deficient rats of each type of experiment. We conclude that the increased UDP-glucuronyltransferase activity of protein-deficient rat liver cannot be explained by an alteration in the relative proportions of protein and phospholipid in microsomal membranes.

The phospholipid compositions of liver microsomal fractions from our control animals (Table 3) agree fairly well with those reported for male rats (Duttera *et al.*, 1968; Schulze & Staudinger, 1970; Rogers, 1971). These authors, however, prepared their microsomal fractions in sucrose-based media and quoted slightly lower values for phosphatidylcholine and slightly higher ones for phosphatidylethanolamine. In addition, Duttera *et al.* (1968) reported the presence of a trace of lysophosphatidylcholine which we observed in only one set of animals and Rogers (1971) reported a trace of phosphatidic acid which we could not detect. These workers utilized one-dimensional t.l.c. techniques and were unable to separate phosphatidylinositol and phosphatidylserine, and Rogers (1971) also could not separate sphingomyelin from lysophosphatidylcholine. In the present work we have adopted a two-dimensional t.l.c. method (Morrison, 1968) which allowed a good separation of the phospholipid components. Nevertheless phosphatidylinositol and

phosphatidylserine ran very closely together in both solvent systems, and sometimes did not separate completely.

The phospholipid compositions of microsomal fractions from acutely protein-deficient rats and control animals were similar (Table 3). There were, however, significant differences in the contents of some of the phospholipids. The content of phosphatidylinositol was slightly decreased by protein deficiency whereas that of sphingomyelin was slightly increased. Moreover, lysophosphatidylcholine and lysophosphatidylethanolamine were found in microsomal membranes from protein-deficient rats whereas no lysophosphatides were detected in control preparations.

Alterations in the phospholipid compositions of rat liver microsomal fraction caused by chronic protein deficiency were similar to those observed in acutely deficient animals. Although phosphatidylinositol and phosphatidylserine could not be completely separated during fractionation of the microsomal phospholipids from long-term protein-deficient animals, the sum of the contents of phosphatidylinositol and phosphatidylserine was significantly lower than in normal rats. Since the content of phosphatidylserine was unaltered from its normal value in the short-term protein-deficient animals, it is assumed that the result represents a more marked decrease in the phosphatidylinositol content caused by the chronic condition. The sphingomyelin content was significantly increased in one experiment but not in the other. Unlike the acutely protein-deficient rats, microsomal fractions from chronically protein-deficient animals had significantly lower phosphatidylethanolamine contents than the corresponding controls. The most striking difference between the microsomal fractions of control and protein-deficient animals, in both acute and chronic experiments, is their content of lysophosphatidylcholine and lysophosphatidylethanolamine. In control preparations

Table 3. *Effects of protein deficiency on the phospholipid composition of rat liver microsomal fraction*

Values quoted are the percentages of total phospholipid P. Those quoted for total choline and ethanolamine phosphatides are the sums of the percentages of phosphatide and corresponding lysophosphatide. On occasions phosphatidylinositol could not be separated from phosphatidylserine. Thus the sums of these phospholipids are also quoted. The chronic protein-deficiency condition was produced in two separate experiments, (a) microsomal fractions prepared in the absence of EDTA, (b) in the presence of EDTA, see the text.

Animals ...	Acute protein deficiency		Chronic protein deficiency (Expt. a)		Chronic protein deficiency (Expt. b)	
	Control	Deficient	Control	Deficient	Control	Deficient
No. of animals	6	6	4	4	3	3
Total choline phosphatides	66.5 ± 0.9 (<i>P</i> > 0.50)	66.3 ± 0.6	65.4 ± 1.2 (<i>P</i> > 0.25)	63.9 ± 0.5	67.5 ± 0.5 (<i>P</i> < 0.01)	71.5 ± 0.6
Total ethanolamine phosphatides	17.8 ± 0.5 (<i>P</i> > 0.50)	17.7 ± 0.7	17.8 ± 0.9 (<i>P</i> < 0.005)	12.1 ± 0.8	14.9 ± 0.8 (<i>P</i> < 0.05)	11.9 ± 0.3
Phosphatidylinositol	8.7 ± 0.3 (<i>P</i> < 0.001)	6.1 ± 0.5	7.1 ± 0.4	*	*	*
Phosphatidylserine	2.8 ± 0.3 (<i>P</i> > 0.50)	3.0 ± 0.6	3.2 ± 0.2	*	*	*
Phosphatidylinositol + phosphatidylserine	11.5 ± 0.5 (<i>P</i> < 0.05)	9.1 ± 0.8	10.2 ± 0.5 (<i>P</i> < 0.005)	7.0 ± 0.4	13.4 ± 0.6 (<i>P</i> < 0.02)	10.8 ± 0.4
Sphingomyelin	4.2 ± 0.3 (<i>P</i> < 0.002)	7.0 ± 0.6	6.7 ± 0.3 (<i>P</i> < 0.001)	17.1 ± 0.2	4.2 ± 0.5 (<i>P</i> > 0.10)	5.7 ± 0.6
Lysophosphatidylcholine	0	7.4 ± 0.5	0	11.4 ± 1.0	2.7 ± 0.2 (<i>P</i> < 0.001)	7.9 ± 0.4
Lysophosphatidylethanolamine	0	3.1 ± 0.9	0	2.5 ± 0.3	0	1.2 ± 0.2

* On these occasions phosphatidylinositol was not completely separated from phosphatidylserine.

the former was only observed in one set of animals and then only in low concentrations, whereas the latter was not detected in any control preparation. Quite significant concentrations of both lysophosphatides were, however, observed in preparations from all protein-deficient animals.

The alterations in microsomal phospholipid composition caused by protein deficiency reported here do not agree with those observed by Rogers (1971). Rogers fed rats on a 4% (w/w) casein diet for 42 days and found that, compared with normal, the contents of phosphatidylethanolamine, sphingomyelin plus lysophosphatidylcholine and phosphatidic acid were very slightly increased and that the content of phosphatidylcholine was decreased. An explanation for the discrepancy between these results and ours (for both long- and short-term feeding periods) is not clear. Perhaps the discrepancy results from Rogers' 42 day feeding period being too short to cause full onset of the protein-deficient condition. Had full onset of protein deficiency been produced in Rogers' experiments, it would still have been difficult to compare results owing to the inability of his one-dimensional t.l.c. system to separate sphingomyelin and lysophosphatidylcholine.

The presence of lysophosphatides in liver microsomal fractions of protein-deficient rats is important since it may explain the increased UDP-glucuronyltransferase activity which accompanies protein

deficiency. Hänninen & Puukka (1971) observed that addition of lysophosphatidylcholine to liver microsomal preparations from normal rats greatly stimulated UDP-glucuronyltransferase activity. Their treatment with phospholipase A₂, which generates lysophosphatides, had a similar effect (Hänninen & Puukka, 1971; Vessey & Zakim, 1972). Results obtained in this laboratory agree with these findings. Treatment of washed microsomal fractions from normal rats with a purified phospholipase A₂ preparation increased *p*-nitrophenol UDP-glucuronyltransferase activity two- to three-fold (Fig. 1). Under these experimental conditions 0.3 μmol of titratable fatty acid was released/mg of microsomal protein in about 30 min. On the basis of a 1:1 stoichiometry between phospholipid and products this would mean that 0.3 μmol of lysophosphatide/mg of microsomal protein was also produced. When a microsomal fraction from a normal rat was pretreated with various concentrations of lysophosphatidylcholine within this concentration range (0–0.3 μmol/mg of microsomal protein) *p*-nitrophenol UDP-glucuronyltransferase activity was substantially enhanced (Fig. 2). More than two-fold activation of the enzyme was observed at concentrations of lysophosphatidylcholine greater than 0.12 μmol/mg of microsomal protein. By assuming a molecular weight of 550 for lysophosphatidylcholine we calculated that two-fold activation of the enzyme would occur when 7–10%

Fig. 1. Effect on UDP-glucuronyltransferase activity of pretreating microsomal fractions from normal rats with phospholipase A

Microsomal fractions were pretreated with phospholipase A and UDP-glucuronyltransferase activities assayed as described in the Experimental section. Enzyme specific activity is expressed relative to that of untreated microsomal fraction. The quantities of titratable fatty acid released during phospholipase A-digestion are also indicated. (A), 0.12 μmol of fatty acid/mg of protein; (B) 0.30 μmol of fatty acid/mg of protein.

Fig. 2. Effect of UDP-glucuronyltransferase activity of pretreating microsomal fractions from normal rats with lysophosphatidylcholine

Microsomal fractions were pretreated with various concentrations of lysophosphatidylcholine and UDP-glucuronyltransferase activities assayed as described in the Experimental section. Enzyme specific activity is expressed relative to that of untreated microsomal fraction.

of the microsomal phospholipid was lysophosphatide. The data of Table 3 show that lysophosphatidylcholine plus lysophosphatidylethanolamine made up 9–14% of the microsomal phospholipid of protein-deficient animals. Moreover, the increase in enzyme activity in microsomal fractions from the three sets of protein-deficient animals is approximately proportional to their total lysophosphatide content (Fig. 3). Thus these lysophosphatides could well account for the increased UDP-glucuronyltransferase activities we have observed in liver microsomal fractions from protein-deficient rats. However, the involvement of the small changes in the content of microsomal phosphatidylinositol and phosphatidylethanolamine in the activation of the enzyme cannot be excluded.

The significant lysophosphatide content of microsomal fractions from protein-deficient rats might reflect an alteration in membrane composition which occurs *in vivo* or might be a result of hydrolysis of phospholipids by an endogenous phospholipase after death but before analysis of the extracted phospholipids. For a number of reasons we consider the latter possibility to be unlikely. (a) The pH of the KCl solutions used to prepare microsomal fractions (pH 5.5–6.0) is not conducive to the action of intracellular phospholipases. The membrane-bound phospholipases A of mitochondrial, microsomal, plasma membrane and lysosomal fractions have pH-optima between 8.5 and 9.0, and the soluble phospholipase contained within the lysosomal membrane

Fig. 3. Variation of *p*-nitrophenol UDP-glucuronyltransferase activity with the content of lysophosphatides in liver microsomal fractions from control and protein-deficient rats

The results plotted (mean values $\pm$ S.E.M.) were taken from Tables 1 and 3.

Table 4. *Effect of chronic protein deficiency on the phospholipid composition of rat liver homogenates*

Chronic protein deficiency was produced by feeding rats on 11.4 g of a 5% (w/w) casein diet/day per animal. Values quoted are the percentages of total phospholipid P. Those quoted for total choline phosphatides are the sums of the percentages of phosphatidylcholine and lysophosphatidylcholine. In these experiments phosphatidylinositol was not completely separated from phosphatidylserine. The control and protein-deficient groups each contained three animals. For comparison the table includes the composition of normal rat liver reported by Rouser *et al.* (1968).

Phospholipid	Results of Rouser <i>et al.</i> (1968)	Control rats	Protein deficient rats
Total choline phosphatides	52.2	54.4 ± 0.4	53.0 ± 0.6
Phosphatidylethanolamine	25.3	23.5 ± 0.3 (<i>P</i> > 0.10)	25.1 ± 0.8
Phosphatidylinositol + phosphatidylserine	12.7	12.1 ± 0.2 (<i>P</i> > 0.10)	11.3 ± 0.2
Diphosphatidylglycerol	4.9	6.1 ± <0.1 (<i>P</i> > 0.05)	6.4 ± 0.3
Sphingomyelin	4.5	3.8 ± <0.1 (<i>P</i> > 0.25)	3.9 ± 0.3
Phosphatidic acid	0.3	<0.1 (trace)	0.3 ± <0.1
Lysophosphatidylcholine		0.5 ± <0.1 (<i>P</i> < 0.05)	1.3 ± 0.3
Lysophosphatidylethanolamine		0	0

has a pH optimum near 4.5. All of these enzymes show only a small fraction of their optimum activity in the pH range 5.5–6.0 (Nachbaur *et al.*, 1972; Newkirk & Waite, 1973). (b) Although the activities of the phospholipases A of microsomal and lysosomal membranes are stimulated by Ca²⁺ ions (Nachbaur *et al.*, 1972; Newkirk & Waite, 1973), the activities of the mitochondrial and plasma membrane phospholipases show an absolute requirement for Ca²⁺ and are inhibited by EDTA (Vignais *et al.*, 1971; Nachbaur *et al.*, 1972). When, in one experiment, microsomal fractions from groups of control and chronically protein-deficient rats were prepared in the presence of EDTA to minimize the effect of any phospholipase A activity which might persist at pH 5.5–6.0, the enhanced microsomal lysophosphatide content of the deficient group was similar to that observed in separate experiments in which EDTA was not used (Table 3). Indeed the use of EDTA had little effect on either phospholipid content (Table 2) or phospholipid composition (Table 3) whereas the UDP-glucuronyltransferase activity (Table 1) was consistent with the microsomal content of lysophosphatides (Fig. 3). The minor differences in phospholipid composition between the two groups of chronically protein-deficient rats and between the two corresponding control groups might be attributed to slight differences in nutritional status (see dietary regimens). (c) Portions of microsomal suspensions prepared in the presence of EDTA from livers of chronically protein-deficient rats and corresponding control animals were incubated at 37°C for 30 min at pH 7.4. The phospholipid compositions of these microsomal fractions were closely similar to those of

portions of the same preparations which had not been incubated. The lysophosphatide contents of incubated controls (2.3 ± 0.2%) and incubated protein-deficient preparations (4.9 ± 0.3%) were somewhat lower than those of unincubated suspensions (2.7 ± 0.2% and 9.1 ± 0.6% respectively). These results do not indicate the presence of a phospholipase A (not inhibited by EDTA) in either of the microsomal preparations. (d) When whole liver homogenates were prepared from control rats under conditions which minimize exposure of the material to conditions favouring the action of intracellular phospholipases, the phospholipid composition (Table 4) of the dried homogenate was in good agreement with that reported by Rouser *et al.* (1968). The phospholipid composition of homogenates from chronically protein-deficient rats was indistinguishable from that of the controls except for the significantly increased content of lysophosphatidylcholine (Table 4). The magnitude of the increase is consistent with the lysophosphatide contents of the microsomal fractions (Table 3).

Our data strongly suggest that the diet-induced changes in microsomal membrane phospholipid composition and the closely associated changes in UDP-glucuronyltransferase activity observed *in vitro* reflect changes that occur *in vivo*. Some support for this view is provided by the results of preliminary perfusion experiments (B. G. Woodcock, unpublished work) which indicate that the rate of *p*-nitrophenol glucuronidation/g of liver is increased in the livers of protein-deficient rats. Moreover, Eakins & Slater (1973) found that protein deficiency in rats was accompanied by an enhanced rate of biliary excretion of bilirubin, presumably as the glucuronide.

The activities of many enzymes are regulated by dietary protein intake (Aebi, 1969). The specific activity of rat liver cytosol fraction 'malic enzyme' was greatly enhanced by protein deficiency. Frenkel *et al.* (1972) interpreted this effect as being due to specific induction of the enzyme's synthesis. The specific activities of liver cytosol fraction 3-phosphoglycerate dehydrogenase and phosphoserine phosphatase were also increased well above normal values when rats were fed on a 2% (w/w) casein diet for 7 days (Fallon *et al.*, 1966). Data obtained with inhibitors of protein synthesis suggested that this activation was due to increased concentrations of the enzymes in liver cytosol fraction during protein deficiency, caused by altered rates of enzyme synthesis. Although the increased activities of these soluble enzymes would appear to be due to increased synthesis of enzymic protein, our work would suggest that this reason for the increased activity of UDP-glucuronyltransferase in microsomal fractions from protein-deficient rats is unlikely. From our results we have accounted satisfactorily for the increase in the enzyme's activity by the presence of stimulatory lysophosphatides. The concentrations of these lysophosphatides present in microsomal fractions from protein-deficient rats were sufficient to have caused the activations of UDP-glucuronyltransferase.

Winsnes (1972) has speculated that the activation *in vitro* of rat liver microsomal UDP-glucuronyltransferase by membrane-perturbing reagents may reflect a regulatory mechanism of importance in the intact cell even though he could find no direct evidence in the literature for such a mechanism of regulation. Evidence presented in the present paper clearly establishes a regulatory mechanism based on membrane phospholipid perturbation which might exist in the cell. Thus lysophosphatides within endoplasmic reticulum membranes could serve to regulate the amount of cellular UDP-glucuronyltransferase activity. Winsnes (1972) also considered that activation of the enzyme *in vitro* by membrane perturbing agents was unspecific and 'unphysiological'. In contrast, our results show that studies of the membrane-dependence of UDP-glucuronyltransferase activity with detergents and phospholipases might yield results of physiological significance. These reagents *in vitro* generate different forms of the enzyme with altered activities, and these may simulate similar enzyme forms occurring within the cell which might be involved with the regulation of biological glucuronidation.

We are indebted to the Science Research Council and the Medical Research Council for financial support, and to Mrs. Carolyn Hibberd and Mr. T. McBride for technical assistance.

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