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KINETIC PROPERTIES OF DIFFERENT FORMS OF HEPATIC UDPGLUCURONYLTRANSFERASE

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SUMMARY

1. Liver microsomes from mouse, rat and guinea pig were used for the enzyme source in the study of *p*-nitrophenol and *o*-aminophenol glucuronidation at varying UDPglucuronate concentrations and a fixed concentration of acceptor substrate.

2. In the case of *p*-nitrophenol glucuronyltransferase (UDPglucuronate glucuronyltransferase (acceptor-unspecific), EC 2.4.1.17) abrupt transitions in the Lineweaver–Burk plots (indicating negative cooperativity) were obtained with "native", frozen, sonicated, phospholipase C (EC 3.1.4.3) digested and detergent-treated microsomes from mouse and rat liver. With guinea pig microsomes most double-reciprocal plots were linear. The UDP-*N*-acetylglucosamine-activated enzyme also exhibited typical linear Lineweaver–Burk plots with mouse liver microsomes.

3. When *o*-aminophenol served as acceptor, linear Lineweaver–Burk plots were obtained in all instances except with frozen mouse liver microsomes as enzyme source.

4. UDP-*N*-acetylglucosamine treatment, freezing, sonication, phospholipase C digestion and detergent treatment of microsomes resulted in the activation of glucuronyltransferase at V as well as substantial increases in apparent $K_{\text{UDPglucuronate}}$ values.

5. The apparent $K_{\text{UDPglucuronate}}$ values increased 7–20-fold as a result of phospholipase C treatment of microsomes. Addition of lecithin micelles to such preparations lowered the apparent $K_{\text{UDPglucuronate}}$ markedly, but did not reverse the activation at V. It is concluded that lecithin may have an important function in promoting a conformational change towards an enzyme form with better substrate affinity. As a constituent of the microsomal membrane, however, lecithin also seems important for the postulated masking of active sites of glucuronyltransferase.

INTRODUCTION

Hepatic UDPglucuronyltransferase (UDPglucuronate glucuronyltransferase (acceptor-unspecific), EC 2.4.1.17) is tightly bound to microsomal membranes and has probably not yet been truly solubilized[1]. In liver homogenates most glucuronyltransferase is present in a latent form which can be activated by detergents[2–7],

ageing[2,3,6], sonication[7,8] and alkaline buffers[7,9,10]. These agents and treatments probably affect the integrity of the microsomal membranes. A more specific activator, UDP-*N*-acetylglucosamine[11], strongly activates the "native" enzyme, but not the enzyme already activated by detergents[3].

Some earlier studies have indicated no change in apparent substrate affinity on activation[5,6], whereas others noted increased[7,8] as well as decreased[7] apparent $K_{\text{UDPglucuronate}}$ values as a result of activation. In the literature[5,7,12] the apparent $K_{\text{UDPglucuronate}}$ values with *p*-nitrophenol as acceptor range from 0.06 mM to 1.3 mM for enzyme treated with detergent. These discrepancies can partly be explained by the fact that *p*-nitrophenol glucuronyltransferase does not exhibit Michaelis–Menten kinetics, but shows abrupt transitions in Lineweaver–Burk plots indicative of the negative cooperativity described by Levitzki and Koshland[13]. Two sets of kinetic parameters could thus be obtained with the same enzyme preparation, depending on the range of substrate concentrations employed. However, with *o*-aminophenol as acceptor, typical Michaelis–Menten kinetics were found with most enzyme preparations; a fact consistent with the suggestion that different enzymes are involved with *o*-aminophenol and *p*-nitrophenol[14,15].

As phospholipids are important constituents of microsomal membranes, the effect on enzyme activity of their specific degradation by phospholipases is important in the study of membrane-bound enzymes. By this means several microsomal enzymes have been found to require phospholipids for activity[16–22]. Similar results are reported for glucuronyltransferase by Graham and Wood[23] and Attwood *et al.*[24], though Vessey and Zakim[7] and Hänninen and Puukka[25] found that phospholipase digestion activated this enzyme. No explanation for these discrepancies has been offered. The present report may help to clarify this problem since inhibition or activation of glucuronyltransferase can occur, depending on the substrate concentration in the enzyme assay; this is because the apparent $K_{\text{UDPglucuronate}}$ towards both acceptor and donor substrates is strikingly increased by digestion with phospholipase C (EC 3.1.4.3). Lecithin micelles can largely reverse the change in $K_{\text{UDPglucuronate}}$ but not in $K_{p\text{-nitrophenol}}$.

MATERIALS AND METHODS

Female NMRI/BOM mice (20–30 g), female Wistar rats (150–250 g) and male guinea pigs of a local strain (400–500 g) were used. Liver homogenates (in 0.154 M KCl solution) were centrifuged at 2000 × *g* for 15 min. The supernatant was spun at 40 000 × *g* for 90 min and the resulting microsomal pellet was resuspended in isotonic KCl solution and used as the enzyme source. Ultrasonicated microsomes were prepared according to Henderson[8]. Phospholipase C treatment of microsomes and the preparation of lecithin micelles were performed essentially as described by Graham and Wood[23].

The enzyme assay technique was as described elsewhere[3] except that the incubation time was 6–15 min depending on the enzyme activity. To ensure linearity of the reaction with time, pilot incubations were performed to determine a suitable incubation time so that not more than 25–30% of the acceptor substrate would be consumed at the highest concentration of UDPglucuronate.

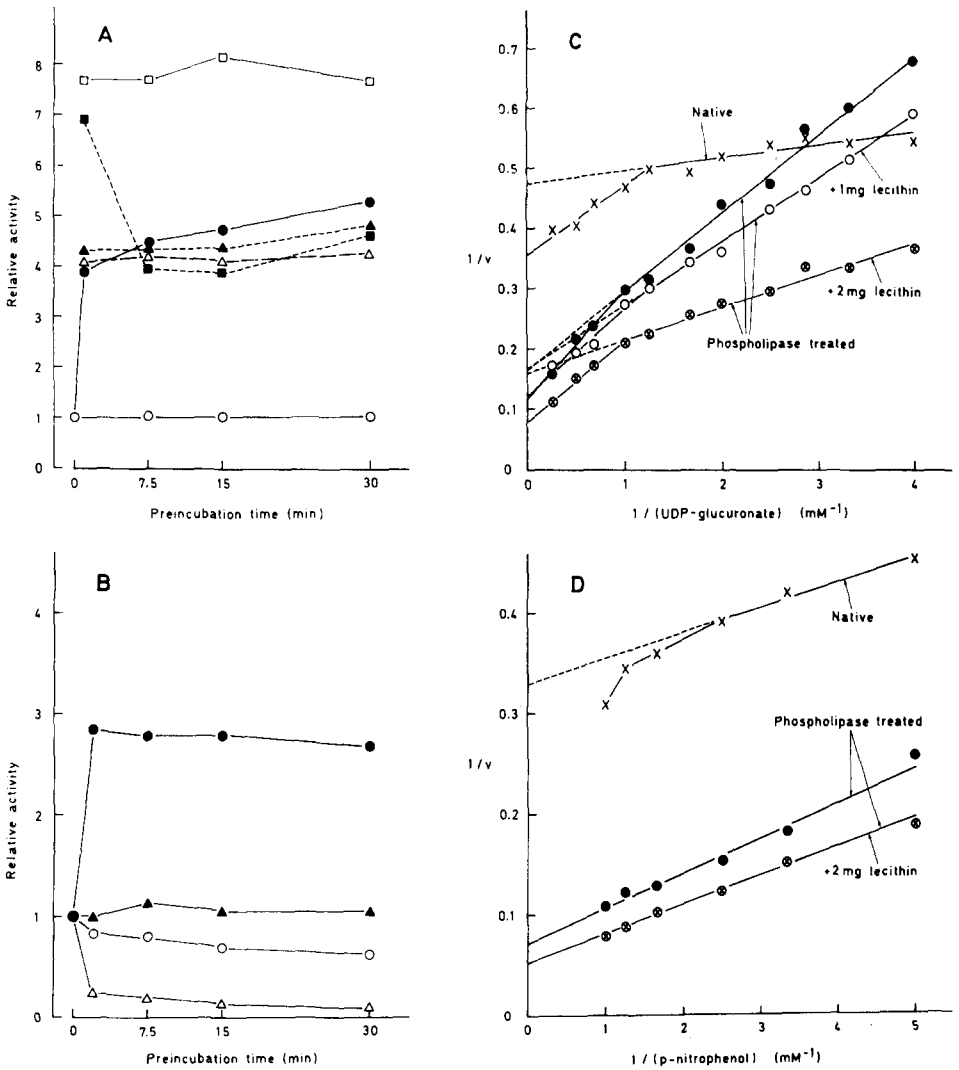

Fig. 1. Effects of phospholipase C treatment on glucuronyltransferase. Mouse liver microsomes resuspended in 15 mM Tris–maleate buffer, pH 8.0, were preincubated at 20–22 °C with phospholipase C (from *Cl. perfringens*, Koch-Light Laboratories) for various lengths of time as indicated in A and B, while in the experiments shown in C and D the preincubation time was 15 min. The microsomal protein to crude phospholipase C protein ratio was approx. 10/1. 2.7 mM $CaCl_2$ was present during the phospholipase preincubation, and the reaction was stopped by addition of 4.4 mM EDTA. In A closed symbols represent enzyme treated with phospholipase, whereas open symbols represent controls treated in a similar manner except that phospholipase was omitted. The enzyme assays were performed with 0.5 mM *p*-nitrophenol and 2.0 mM UDPglucuronate without additions to standard incubation mixtures (○, ●), with 2 mM UDP-*N*-acetylglucosamine (△, ▲) and with 0.05 (w/v) Triton X-100 present in the incubation mixtures (□, ■). In B open symbols represent assays at 0.2 mM UDPglucuronate with 0.2 mM *p*-nitrophenol (○) and 0.2 mM *o*-aminophenol (△), whereas closed symbols represent assays at 2.0 mM UDPglucuronate with 0.5 mM *p*-nitrophenol (●) and 0.5 mM *o*-aminophenol (▲). C represents Lineweaver–Burk plots at fixed concentration of *p*-nitrophenol (0.5 mM) and UDPglucuronate concentrations varying between 0.25 and 4.0 mM. D represents Lineweaver–Burk plots at fixed concentration of UDPglucuronate (2.0 mM) and *p*-nitrophenol concentrations varying between 0.20 and 1.0 mM. Incubations were performed with fresh microsomes (native) as well as phospholipase C-pretreated microsomes without and with lecithin added. The lecithin (Commercial grade from egg yolk, Sigma Chemical Co) suspension was prepared as described by Graham and Wood[23]. Approx. 2.5 mg microsomal protein per incubation mixture was pretreated with 1 and 2 mg lecithin, respectively, at 37 °C for 15 min before enzyme assay.

RESULTS

Effects of phospholipase C on glucuronyltransferase

At relatively high substrate concentrations phospholipase C treatment results in an almost immediate 3–4-fold activation of *p*-nitrophenol glucuronyltransferase (Fig. 1A). Further treatment for 30 min had little effect. Triton increased the enzyme activity further when added after 0.5 min phospholipase pretreatment, but resulted in a slight inhibition when added after more prolonged phospholipase treatment of microsomes (Fig. 1A). UDP-*N*-acetylglucosamine had little effect when added to phospholipase-pretreated enzyme. However, addition of this nucleotide to fresh microsomes activated the enzyme to a degree similar to that of phospholipase C (Fig. 1A).

At low concentrations of substrates (Fig. 1B) the *p*-nitrophenol glucuronyltransferase activity declined to about 60% of the initial activity whereas the activity towards *o*-aminophenol decreased almost to zero after 30 min preincubation with phospholipase C (Fig. 1B). At higher concentrations of substrates *o*-aminophenol glucuronyltransferase activity was little affected by phospholipase C (Fig. 1B).

These different effects of phospholipase pretreatment at low and high substrate concentrations are due to the decreased apparent substrate affinity of the phospholipase-treated enzyme compared to the "native" one as seen in Fig. 1 C and 1 D and Tables I and II.

The Lineweaver–Burk double reciprocal plots with respect to varying UDP-glucuronate concentrations did not produce the expected linear results with either "native" or phospholipase C-pretreated *p*-nitrophenol glucuronyltransferase in mouse-liver microsomes (Fig. 1C). Two sets of kinetic parameters could thus be calculated, and phospholipase C seemed to affect both (Tables I and II). The apparent $K_{\text{UDP-glucuronate}}$ values increased 7–20-fold as a result of phospholipase C pretreatment (Table I) when *p*-nitrophenol was acceptor, whereas the increase was 60-fold or higher in the case of *o*-aminophenol glucuronyltransferase (Table II).

p-Nitrophenol glucuronyltransferase in guinea pig microsomes was affected in a similar manner to the mouse-liver enzyme by phospholipase C as seen in Fig. 2 and Table I. There is considerable inhibition at low UDPglucuronate concentrations, in agreement with Graham and Wood[23], though the activation at V is equally evident.

When phospholipase C-pretreated microsomes were treated with lecithin micelles, there was a considerable increase in glucuronyltransferase activity at lower concentrations of UDPglucuronate. The apparent $K_{\text{UDPglucuronate}}$ values were decreased from 0.80 and 2.22 mM to 0.34 and 1.20 mM by treatment with 2 mg lecithin (Table I). The activation at V was not reversed by lecithin at the concentrations used here.

Lecithin micelles did not, however, decrease the apparent $K_{p\text{-nitrophenol}}$ of phospholipase C-treated enzyme, but increased it from 0.49 mM to 0.64 mM, a value which is similar to that of Triton-activated enzyme[3]. The Lineweaver–Burk plot of "native" enzyme with respect to *p*-nitrophenol was atypical, indicating negative cooperativity as also reported earlier[3].

These observations, therefore, explain the apparent contradictory findings of Graham and Wood[23], Attwood *et al.*[24], Hänninen and Puukka[25] and Vessey and Zakim[7], since the first two studies[23,24] were performed at low substrate concentrations only. Vessey and Zakim[7] who performed extensive kinetic analyses observed only a

TABLE I

APPARENT KINETIC PARAMETERS OF VARIOUS FORMS OF *p*-NITROPHENOL UDPGLUCURONYLTRANSFERASE

The kinetic constants were determined graphically according to the method of Lineweaver and Burk. A fixed concentration of *p*-nitrophenol (0.5 mM) was used, and the concentrations of UDP-glucuronate were varied between 0.25 and 4.0 mM. Where two values are given for $K_{UDPglucuronate}$ and V, this indicates that bent curves were obtained in the Lineweaver–Burk plot (see Figs 2 and 3). In the assays with rat-liver microsomes, 10 mM EDTA was included to inhibit the pyrophosphatase activity. "Solubilized" enzyme was obtained by treatment of microsomes with 0.25% (w/v) Triton X-100 for 1 h before centrifugation at 40 000 × *g* for 90 min. The pellet (non-solubilized fraction) was resuspended in 0.154 M KCl solution without* and with** Triton X-100 giving a final detergent concentration of 0.05% in the incubation mixture. The values are the mean of a number of experiments given in parentheses.

Animal species	*Treatment of microsomes or addition to standard assay mixture*	$K_{UDPglucuronate}$ *(mM)*			*V (μmoles/g wet wt liver per h)*		
Mouse	None	0.04	0.29	(7)	2.1		2.8
	Frozen and thawed	0.33	1.08	(3)	5.9		10.0
	Sonicated	0.23	0.91	(1)	18.2		30.0
	UDP-*N*-acetylglucosamine	0.27		(5)		21.8	
	Phospholipase C	0.80	2.22	(2)	6.0		10.5
	Phospholipase C + 1 mg lecithin	0.63	1.05	(1)	6.0		7.7
	Phospholipase C + 2 mg lecithin	0.34	1.20	(1)	6.2		10.9
	Triton X-100	0.33	1.23	(8)	23.9		43.0
	"Solubilized" by Triton X-100	0.38	1.04	(2)	3.9		6.1
	Non-solubilized fraction*	0.25	1.22	(2)	9.4		21.4
	Non-solubilized fraction**	0.32	1.59	(1)	15.4		32.2
	Digitonin	0.35	1.16	(3)	22.2		37.9
	Nonidet	0.36	1.65	(1)	23.8		46.0
Rat	None	0.03	1.45	(2)	0.9		2.6
	Triton X-100	0.36	1.80	(4)	15.7		32.8
	Digitonin	0.28	1.03	(2)	21.0		46.3
Guinea pig	None	0.12	0.29	(3)	3.3		4.0
	Frozen and thawed	1.15		(1)		8.0	
	UDP-*N*-acetylglucosamine	0.32		(2)		39.5	
	Phospholipase C	2.94		(1)		20.0	
	Triton X-100	1.21		(3)		101.0	

TABLE II

APPARENT KINETIC PARAMETERS OF VARIOUS FORMS OF *o*-AMINOPHENOL UDPGLUCURONYLTRANSFERASE

Kinetic constants were determined graphically according to the method of Lineweaver and Burk. A fixed concentration of *o*-aminophenol (0.5 mM) was used and the concentrations of UDP-glucuronate was varied between 0.25 and 4.0 mM. Two values are given for $K_{UDPglucuronate}$ and V in the case of frozen and thawed mouse-liver microsomes indicating that an abrupt transition was obtained in the Lineweaver–Burk plot (Fig. 2). The values are the mean of a number of experiments given in parentheses. In the assay with rat-liver microsomes, 10 mM EDTA was included to inhibit the pyrophosphatase activity.

Animals species	*Treatment of microsomes or addition to standard assay mixture*	$K_{UDPglucuronate}$ *(mM)*			*V (μmoles/g wet wt liver per h)*		
Mouse	None	≦0.125		(5)		1.1	
	Frozen and thawed	0.31	1.28	(2)	1.6		3.0
	UDP-*N*-acetylglucosamine	0.49		(3)		6.9	
	Phospholipase C	8.30		(1)		2.0	
	Triton X-100	1.26		(2)		3.3	
	Digitonin	1.52		(5)		9.3	
	Nonidet	1.65		(2)		3.4	
Rat	Digitonin + diethylnitrosamine	2.30		(1)		4.6	
Guinea pig	Digitonin	0.67		(1)		10.2	

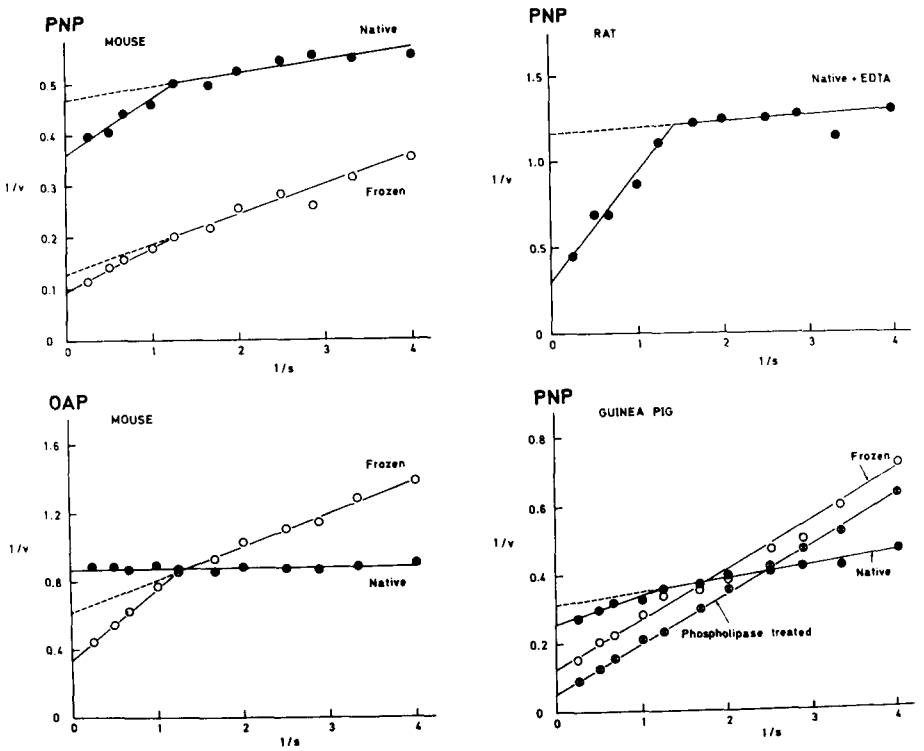

Fig. 2. Double-reciprocal plots with respect to varying UDPglucuronate concentrations of different forms of glucuronyltransferase. Mouse, rat and guinea pig liver microsomes were assayed for glucuronyltransferase activity at 0.5 mM fixed concentration of *p*-nitrophenol (PNP) and *o*-aminophenol (OAP) at UDPglucuronate concentrations varying between 0.25 and 4.0 mM. Fresh microsomes (native) as well as microsomes frozen at −15 °C for 24 h (mouse liver) and 5 days (guinea pig liver) were used. An experiment with phospholipose C-pretreated guinea pig microsomes is also presented. The microsomal protein to phospholipase C protein ratio was approx. 10:1 and the preincubation time was 15 min. 10 mM EDTA was present in the case of rat-liver microsomes to inhibit the nucleotide pyrophosphatase. The enzyme activity (v) was calculated as μmoles/g wet weight liver per h.

slight or no decrease in apparent affinity for UDPglucuronate of phospholipase A (EC 3.1.1.4)-pretreated enzyme compared with their "native" enzyme preparation though $K_{p\text{-nitrophenol}}$ was substantially increased. This might be due to the fact that they used frozen microsomes in their studies (see next section) and omitted studies at the lower concentrations of UDPglucuronate. Hänninen and Puukka[25] reported similar increases in $K_{\text{UDPglucuronate}}$ to those found here.

Kinetics of glucuronyltransferase in fresh and frozen microsomes

Kinetic studies at varying UDPglucuronate concentrations with fresh liver microsomes as enzyme source are complicated by low activities, resulting in poor accuracy. The present experiments were repeated 3–7 times with the mean presented in Fig. 2. With *p*-nitrophenol as acceptor the Lineweaver–Burk plots of "native" glucuronyltransferase were atypical, exhibiting abrupt transitions in the UDPglucuronate concentration range 0.25–4.0 mM. This change in slope was great especially with rat liver microsomes, whereas in guinea pig liver it was insignificant.

When *o*-aminophenol served as acceptor, no change in activity was observed in the substrate concentration range used. Consequently no $K_{UDPglucuronate}$ value could be calculated from these experiments, though it probably would have been equal to or less than 0.125 mM (half the lowest UDPglucuronate concentration used).

After storage of mouse-liver microsomal suspension at −15 °C for 24 h a considerable increase was observed in both $K_{UDPglucuronate}$ and V values with both *p*-nitrophenol and *o*-aminophenol as acceptors (Fig. 2). With the last acceptor the straight line obtained with fresh microsomes was changed to a bent curve similar to that obtained with *p*-nitrophenol as substrate. The change was also considerable in guinea pig microsomes stored in a frozen state for 5 days (Fig. 2). Microsomal suspensions kept frozen cannot, therefore, be used as source of "native" glucuronyltransferase. Other microsomal enzymes have been activated *in vitro* by freezing and thawing[26–28] though no change in apparent K_m was observed[27].

Kinetics of glucuronyltransferase activated by detergents and sonication

With *o*-aminophenol as acceptor the Lineweaver–Burk plots were typical with digitonin, Triton and Nonidet as activators in mouse and with digitonin as activator in guinea pig microsomes (Fig. 3). Rat liver microsomes without added EDTA resulted in curves which were bent as described earlier[29]. This was suggested[29] to be due to the rather high nucleotide pyrophosphatase (EC 3.6.1.9) activity in the rat species[30]. When 10 mM EDTA was present during the enzyme assay the curve was straight indicating that UDPglucuronate breakdown disturbs the kinetics at the lower concentrations of substrate. That EDTA inhibits UDPglucuronic acid pyrophosphatase has been shown by several investigators[11,30,31], though others failed to inhibit the hydrolysis of UDPglucuronate by EDTA[7].

As seen from Table II the apparent $K_{UDPglucuronate}$ values for detergent-treated *o*-aminophenol glucuronyltransferase was rather high though much less than the value found after phospholipase C pretreatment. V was increased by all treatments, but to different degrees, with digitonin being most effective.

When *p*-nitrophenol served as acceptor, the double reciprocal plots exhibited abrupt transitions when mouse or rat liver was used as enzyme source, whereas a straight curve was obtained with guinea pig microsomes. The pyrophosphatase activity is rather low in mouse liver[30] and no stimulating effect of EDTA was noted; on the contrary, inhibition was revealed at all the concentrations of UDPglucuronate used (Fig. 3).

The two sets of $K_{UDPglucuronate}$ values were rather similar, irrespective of what detergent was used, the range being 0.28–0.36 mM and 1.03–1.80 mM with mouse and rat-liver microsomes (Table I). The values were similar for sonicated enzyme, whereas the Triton-treated guinea pig liver enzyme revealed a $K_{UDPglucuronate}$ value of the high type only. The two sets of V values were also rather similar with mouse and rat liver as enzyme source whereas the activity was considerably higher with the guinea pig liver enzyme.

The apparent $K_{UDPglucuronate}$ for *p*-nitrophenol glucuronyltransferase was analysed in the high speed supernatant of mouse-liver microsomes after treatment with 0.25% Triton for 1 h ("solubilized enzyme", see ref. 3) as well as in the resulting pellet resuspended in isotonic KCl solution (non-solubilized enzyme). Both enzyme preparations exhibited the same kind of bent Lineweaver–Burk plots with $K_{UDPglucuronate}$

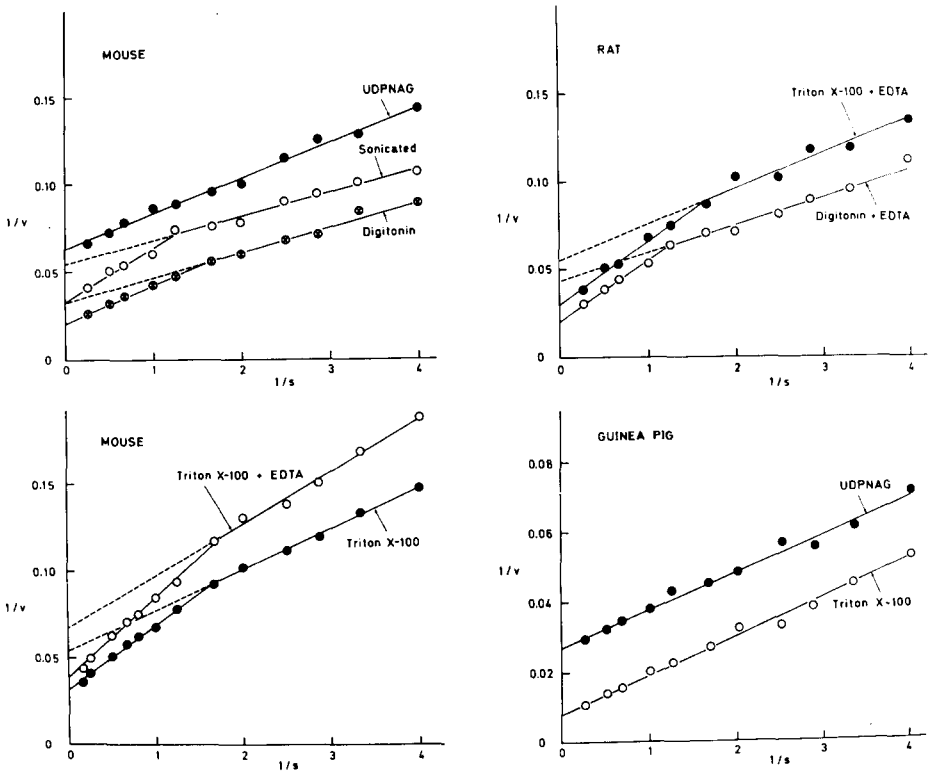

Fig. 3. Double-reciprocal plots with respect to varying UDPglucuronate concentrations of different forms of *p*-nitrophenol glucuronyltransferase. Mouse, rat and guinea pig liver microsomes were assayed for glucuronyltransferase activity at a fixed concentration of 0.5 mM *p*-nitrophenol and UDPglucuronate concentrations in the range 0.25 to 4.0 mM. Microsomes were assayed with either 2 mM UDP-*N*-acetylglucosamine (UDPNAG), 0.05% (w/v) Triton X-100 or 0.2% (w/v) digitonin added in the incubation mixture. One curve represents an experiment with an enzyme preparation treated with ultrasonic oscillation for 1 min. 10 mM EDTA was present where indicated in the figure. The enzyme activity (*v*) was calculated as μmoles/g wet weight liver per h.

values similar to that of detergent-treated microsomes (Table I). Therefore no separation of an enzyme form with low and high $K_{\text{UDPglucuronate}}$ values was achieved by the "solubilization" procedure.

Kinetics of UDP-N-acetylglucosamine-activated glucuronyltransferase

With both *p*-nitrophenol and *o*-aminophenol as acceptors and with mouse and guinea pig microsomes, typical Lineweaver–Burk plots were obtained using UDP-*N*-acetylglucosamine as activator (Figs 3 and 4). The apparent $K_{\text{UDPglucuronate}}$ with *p*-nitrophenol as acceptor was similar to the high value found for "native" enzyme and similar to the low value found for detergent-treated enzyme as seen from Table I.

When *o*-aminophenol served as acceptor the apparent $K_{\text{UDPglucuronate}}$ was 0.49 mM which is considerably lower than that found for detergent-treated enzyme, but definitely higher than the value for "native" enzyme which probably is equal to or lower than 0.125 mM (*cf.* Fig. 2).

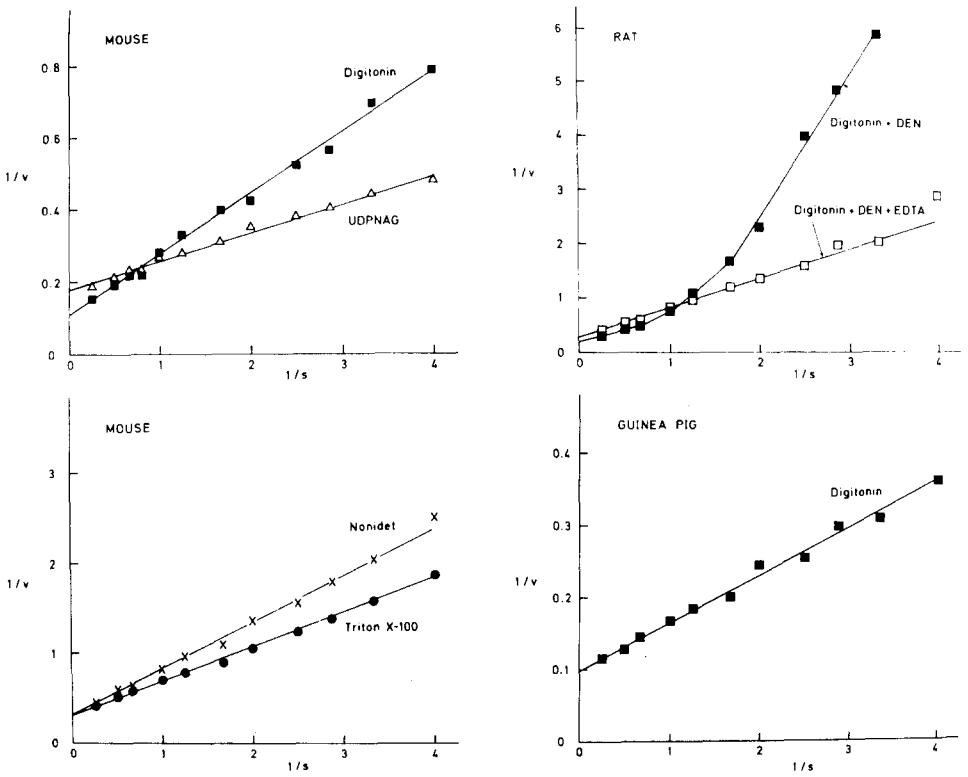

Fig. 4. Double-reciprocal plots with respect to varying UDPglucuronate concentrations of different forms of *o*-aminophenol glucuronyltransferase. Mouse, rat and guinea pig liver microsomes were assayed for glucuronyltransferase activity at a fixed concentration of 0.5 mM *o*-aminophenol and UDPglucuronate concentrations in the range 0.25 to 4.0 mM. Microsomes were assayed with either 2 mM UDP-*N*-acetylglucosamine (UDPNAG), 0.2% (w/v) digitonin, 0.1 (w/v) Nonidet (Nonidet P.42, British Drug Houses Ltd), 0.05% (w/v) Triton X-100, 15 mM diethylnitrosamine (DEN) or 10 mM EDTA added in the incubation mixture. The enzyme activity (v) was calculated as μmoles/g wet weight liver per h.

V was increased 6–7-fold with both acceptors compared to the "native" enzyme. This shows without doubt that UDP-*N*-acetylglucosamine unmasks latent glucuronyltransferase activity as suggested earlier[3]. The inhibition of nucleotide pyrophosphatase by UDP-*N*-acetylglucosamine causes a significant activation of rat liver glucuronyltransferase at low levels of UDPglucuronate[11,29,32], but cannot explain the great increases in V values observed here.

DISCUSSION

Though several reports on the apparent kinetic parameters of glucuronyltransferase have appeared, bent Lineweaver–Burk plots have not yet been described. In some cases such a tendency towards abrupt transitions can be deduced from the data [33,34]. Failure to demonstrate such atypical kinetics at varying UDPglucuronate concentrations might be due to differences in species or acceptor substrates (see Figs 2–4). Again, the great discrepancies reported between $K_{\text{UDPglucuronate}}$ values of *p*-nitro-

phenol glucuronyltransferase may be partly explained by these atypical kinetics, since some investigators used low substrate concentrations and others high concentrations.

Similar atypical double-reciprocal plots have been reported for CTP synthetase (EC 6.3.4.2)[13], cyclic nucleotide phosphodiesterase[35] and other enzymes, as discussed by Levitzki and Koshland[13]. Several theoretical models[13,36] could explain such negative homotropic kinetics. In the case of CTP synthetase the most likely explanation was that a ligand induced a conformational change affecting the interactions of enzyme subunits[13]. For cyclic nucleotide phosphodiesterase[35] the existence of two different enzyme forms with different K_m values appeared the more likely. With glucuronyltransferase one could not, at present, discriminate between the various models. It is interesting to note that both UDPglucuronate and *p*-nitrophenol (in the case of "native" enzyme (Fig. 1 and ref. 4)) are responsible for negative cooperativity effects. When glucuronyltransferase is treated with sulfhydryl-blocking agents a positive cooperative effect of UDPglucuronate seemed to be present[37].

The significance of kinetic studies with intact microsomes as the enzyme source is limited both because of possible side-reactions, inhibitors *etc.* and because of membrane transport of substrates being rate-limiting. With mouse-liver microsomes the β-glucuronidase (EC 3.2.1.31), nucleotide pyrophosphatase or reversal of the glucuronyltransferase reaction (see ref. 38) did not interfere significantly in the enzyme assay system used in this study. Probably of greater importance here is the limitation due to membrane transport phenomena.

A further limitation is that all kinetic experiments with varying UDPglucuronate concentrations were performed at only one fixed concentration (0.5 mM) of acceptor substrate. Though an earlier study[39] on *p*-nitrophenol glucuronyltransferase had indicated that $K_{\text{UDPglucuronate}}$ for one substrate was independent of the concentration of the other substrate, Vessey and Zakim[7] recently showed that this need not always be the case. The $K_{\text{UDPglucuronate}}$ values given above are therefore valid only at the particular acceptor concentrations used. At lower concentrations of acceptor, higher $K_{\text{UDPglucuronate}}$ values would be expected; slightly lower values might be obtained by increasing the acceptor concentration further, although the difference in $K_{\text{UDPglucuronate}}$ values obtained with 0.4 and with 0.8 mM *p*-nitrophenol as acceptor was insignificant[7]. Probably, therefore, the $K_{\text{UDPglucuronate}}$ values given in Table I approach their lower limits.

The kinetic differences shown above, whether negative cooperativity was exhibited or not, and the different $K_{\text{UDPglucuronate}}$ values between different forms of enzyme, all suggest that conformational changes may be induced by the activators. Possibly some of these changes in the enzyme are secondary to changes in its membrane environment. The high $K_{\text{UDPglucuronate}}$ values (1–2 mM, and even up to 8 mM when *o*-aminophenol was acceptor) caused by detergents, sonication and phospholipase C treatment could be due to the breaking of protein–phospholipid bonds, resulting in a less active enzyme form. In support of this, lecithin micelles lowered the $K_{\text{UDPglucuronate}}$ values of phospholipase C treated enzyme markedly. Such an effect of lecithin might be important in the cell.

Zhivkov[40] and Keppler *et al.*[41] found UDPglucuronate present at 0.12–0.41 μmole/g wet weight of rat and guinea pig liver. These concentrations are similar to the apparent $K_{\text{UDPglucuronote}}$ value of UDP-*N*-acetylglucosamine-activated enzyme

reported above as well as to the higher $K_{UDPglucuronate}$ value of "native" and the lower value of detergent-activated enzyme (with *p*-nitrophenol as acceptor). Thus, if there is no compartmentation of UDPglucuronate in the cell, the enzyme forms with the high $K_{UDPglucuronate}$ might be of comparatively little importance, if indeed they exist *in vivo*.

It has been suggested that glucuronyltransferase is situated within or on the inside of microsomal membranes, which constitute a permeability barrier towards the substrate(s). Recent studies[42] indicate that charged substances of molecular weight above 90 do not penetrate microsomes. UDPglucuronic acid, which is synthesized in the cytosol[43], would thus need to be actively transported to the active site of glucuronyltransferase. UDP-*N*-acetylglucosamine could possibly stimulate such transport. The membrane transport model, however, seems inconsistent with the lack of effect of Triton on the activity of glucuronyltransferase in the reverse direction[7]. It is, however, possible that the $K_{UDPglucuronate}$ values obtained above, especially with fresh (,,native") microsomes, reflect to a great extent the substrate saturation characteristics of a transport site on the microsomal membrane.

We have seen above that lecithin may reverse the lowered substrate affinity of phospholipase C-treated glucuronyltransferase. However, since phospholipase C treatment activates glucuronyltransferase at V this indicates that lecithin also may constrain the enzyme activity. This effect could, however, not be simulated *in vitro* by addition of lecithin micelles (in the concentrations tested) to phospholipase C-pretreated microsomes since V was not significantly changed (Fig. 1). Phenobarbital treatment of rats and mice, on the other hand, increased the latency of glucuronyltransferase considerably[4,44]. This is of interest in view of the fact that the microsomal lecithin-to-protein ratio increased by a factor of 1.74 as a result of phenobarbital treatment of rats[45]. It seems, therefore, that lecithin, as part of the intact microsomal membrane, is important for the postulated masking of active sites of glucuronyltransferase[3].

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