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GUINEA PIG LIVER MICROSOMAL UDP-GLUCURONYLTRANSFERASE: COMPARTMENTED OR PHOSPHOLIPID-CONSTRAINED?

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Summary

1. Three mechanical methods for disrupting membranes substantially stimulated two forward reactions and a reverse reaction of UDPglucuronyltransferase (EC 2.4.1.17) in guinea-pig liver microsomes. Stimulation of glucuronyltransferase was highly significant and at least as extensive as that of nucleoside diphosphatase, reportedly a marker intracisternal enzyme.

2. Stimulation of glucuronyltransferase did not appear to be caused by induction of lipid peroxidation or by lipid hydrolysis during membrane disruption.

3. Addition of phospholipid dispersions failed to significantly re-constrain glucuronyltransferase stimulated by mechanical disruption and did not markedly inhibit the enzyme in untreated control microsomes.

4. Compartmentation appears at least as feasible an explanation for the latency of glucuronyltransferase in guinea-pig liver microsomes as is its conformational constraint by membrane phospholipids. Compartmentation of glucuronyltransferase is functionally attractive since it would ensure its effective interaction with nucleoside diphosphatase on the cisternal side of the endoplasmic reticulum. This would tend to make conjugation reactions unidirectional.

Introduction

Of the latent enzyme activities of freshly prepared liver microsomes [1—7], at least two i.e. UDPglucuronyltransferase (EC 2.4.1.17), and glucose-6-phosphatase (EC 3.1.3.9), are tightly associated with membrane elements. Thus, whereas both are substantially activated in vitro by a wide range of treatments, which includes surfactants [1,2], dilute alkali [2,8] and ultrasonication [9,10], attempts to free these enzymes from membrane lipids, or from

the detergents employed in preparation of 'soluble' fractions, results in partial or complete loss of activity [11,12]. In contrast, microsomal nucleoside diphosphatase (EC 3.6.1.6), while highly latent [3,13,14], appears only weakly membrane-associated and is released from liver microsomes in soluble form by Ultra-Turrax blending [13]. This behaviour, which is reminiscent of the 'soluble' lysosomal hydrolases [15], and studies with an antibody raised to the enzyme, has led to the view that nucleoside diphosphatase is structurally latent [3]. However, a similar explanation for glucuronyltransferase latency has been specifically rejected [8], and instead the view has been consistently advocated that latency results from interactions of glucuronyltransferase with its lipid environment, which constrain it in a conformation of low catalytic activity: stimulation by a wide range of treatments is attributed to a relief of constraint by perturbation of lipid-protein interactions (e.g. refs 8, 16, 17 and 18).

Since nucleoside diphosphatase is highly active towards uridine diphosphate, a product of glucuronidation reactions, and is concentrated in the same microsomal subfraction as glucuronyltransferase [19], and because the parallel occurrence of the two enzymes in microsomes from various tissues has long been noted [20], there is reason for thinking that the enzymes may be functionally related [21]. This was reinforced by the recent demonstration that nucleoside diphosphatase may limit the rate of a reverse glucuronyltransferase reaction in vitro [21]. In this communication, we report the response of the two enzymes in guinea-pig liver microsomes to mechanical activating treatments. It was hoped that these would stimulate without introducing or generating in situ amphipathic perturbants, which are thought to account for the stimulation induced by detergents [8], or by phospholipase A_2 [22]. Drawing on reports that glucuronyltransferase is stimulated by ultrasonication [9], and nucleoside diphosphatase by Ultra-Turrax blending [13], the effect of these, and of grinding frozen microsomal pellets with glass, was studied. The effect of added phospholipid dispersions on the activity of glucuronyltransferase was also studied, since these are reported to inhibit glucose-6-phosphatase in microsomes and this has been adduced as evidence for its phospholipid constraint [23].

Materials and Methods

Preparation of microsomes

Male guinea pigs (approximately 400 g) and fasted overnight, were used. Livers were homogenized in 0.25 M sucrose, and microsomes were sedimented in 60 min at 104 000 × *g* from a supernatant, previously centrifuged for 10 min at 10 000 × *g*. Pellets were stored for up to 2 weeks at −10°C and were resuspended in 0.25 M sucrose immediately before use. Some 'spontaneous activation' of glucuronyltransferase was seen [1], but this was incomplete even after 4 weeks storage. Nevertheless, experimental findings reported in this communication have all been reproduced on freshly prepared microsomes.

Mechanical disruption of microsomes

Microsome suspensions (5—10 mg protein/ml) were ultrasonicated at 0°C for periods of 30 s, with 30-s intervals for cooling, using a 150 watt MSE

Ultrasonic Disintegrator on medium power (4—5 μm peak to peak). Ultra-Turrax blending was performed at 0°C for periods of 4 × 10 s, each interspersed with 5—10 s for defoaming, and followed by 30-s intervals for cooling. After the indicated summated times of actual disruption, samples were withdrawn for assay. Frozen microsome pellets were ground for 3—4 min to a homogeneous appearance with pre-cooled glass powder in a mortar and pestle, pre-cooled in a bath of acetone/solid CO_2.

Preparation of phospholipid dispersions

Washed total lipid was extracted from guinea-pig liver homogenate [24]. Total phospholipids were acetone precipitated in the presence of $MgCl_2$, essentially as described by Kates [25]. The phospholipids were then subjected to a Folch partition [24], using 60 mM sodium EDTA, pH 7.1 as the aqueous component and were then washed with an 'EDTA upper phase', (made by equilibrating 20 volumes of EDTA solution with 100 volumes of 2 : 1 chloroform/methanol), until Mg^{2+} was no longer detected by an 8-hydroxyquinoline fluorescence spot test. Liver phospholipids and pure egg phosphatidylcholine (a generous gift from Dr W. Tampion, R.F.H.S.M.) were dispersed at 0°C (sometimes under N_2) using a 150 watt MSE Ultrasonic Disintegrator for a total of 3—5 min, sonicating for 30 s at a time and then cooling.

All enzyme assays were performed at 37°C in 100 mM maleate buffer pH 7.1, and enzyme activities are expressed as nmol/min/mg microsomal protein. Phospholipid phosphorus [26], long chain fatty acids [27], calibrated with palmitic acid, and protein [28], were assayed by standard methods. Unpaired t-Tests were used to evaluate statistical significance.

Results

Effects upon UDPglucuronyltransferase of mechanically disrupting microsomes

Fig. 1 illustrates the effect of ultrasonication and Ultra-Turrax blending of microsome suspensions upon the latencies of UDPglucuronyltransferase (*o*-aminophenol acceptor) (Fig. 1A) and upon uridine diphosphatase (Fig. 1B): both methods stimulate UDPglucuronyltransferase substantially, maximum stimulation in Fig. 1A being 450—580%. Activation by ultrasonication confirmed the findings of others [8,9,29] and as shown in Expt 2 ultrasonication can stimulate as efficiently as UDP-*N*-acetylglucosamine and Mg^{2+} [30,31], though it does not always do so. Ultrasonication had a biphasic effect upon UDPglucuronyltransferase activity, causing a peak stimulation at about 4 min, with the stimulated rate substantially reduced by 10 min. Addition at 10 min of UDP-*N*-acetylglucosamine and Mg^{2+} (Fig. 1A, Expt 2) did not restore the lost activity. Though stimulating substantially, blending was considerably less effective than ultrasonication in paired experiments. However, no inactivation by blending was seen over the time period studied, though in one experiment UDP-*N*-acetylglucosamine and Mg^{2+} further stimulated slightly (39%) over the activity already seen after 8 min blending.

Ultrasonication also stimulated the conjugation of *p*-nitrophenol 525% and stimulated the UDP-dependent hydrolysis of *p*-nitrophenyl-β-D-glucuronoside (reverse reaction) 360%: blending stimulated *p*-nitrophenol conjugation

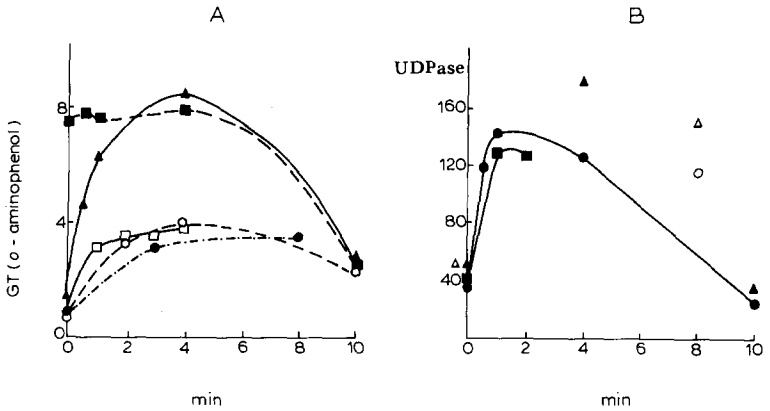

Fig. 1. Effect of ultrasonication or blending upon the latencies of glucuronyltransferase and nucleoside diphosphatase. Conjugation of *o*-aminophenol was assayed at 37°C with 1—2 mg microsomal protein using 0.4 mM *o*-aminophenol (resublimed), 4 mM UDP-glucuronate and 4 mM EDTA, pH 7.1. Controls without UDP-glucuronate were run. When UDP-*N*-acetylglucosamine was present, its concentration was 2 mM; when $MgCl_2$ was present its concentration was 4 mM and EDTA was omitted. Apparent initial rates were measured over 0—30 min by the method of Dutton and Storey [49]. Nucleoside disphosphatase was assayed for 5 min with 0.25—0.75 mg microsomal protein using 2 mM UDP and 4 mM $MgCl_2$. A. Ortho-aminophenol conjugation: (□———□) sonication Expt 1; (▲———▲) sonication Expt 2, here all assays were performed at 0.004 M Mg^{2+}; (■- - - - -■) microsomes from Expt 2 assayed in the presence of 0.004 M Mg^{2+} plus 0.002 M UDP-*N*-acetylglucosamine. (○- - - - -○) sonication Expt 3; (●— · — · —●) microsomes from Expt 1 disrupted by Ultra-Turrax blending. B. Nucleoside diphosphatase assayed with UDP: (●———●) sonication Expt 1; (■———■) sonication Expt 2; (▲) sonication Expt 3; microsomes from Expt 1 (○) and Expt 3 (△) disrupted by Ultra-Turrax blending. All activities are expressed as nmol aglycone conjugated or P_i formed/min/mg microsomal protein.

TABLE I

STIMULATION OF GLUCURONYLTRANSFERASE AND NUCLEOSIDE DIPHOSPHATASE BY ULTRASONICATION, BLENDING OR GRINDING IN THE FROZEN STATE

Apparent initial rates of conjugation of *p*-nitrophenol were assayed from 0—10 min at 37°C with 0.5—1.0 mg microsomal protein, using 0.4 mM *p*-nitrophenol, 4 mM UDP-glucuronate and 4 mM EDTA, pH 7.1. The reverse reaction was assayed from 0—15 min at pH 7.1 and 37°C with 0.5—1.0 mg protein using 4 mM *p*-nitrophenylglucuronide and 2 mM UDP in the presence of 10 mM glucaro-1,4-lactone and 4 mM EDTA. Reactions were stopped by the addition of 2% trichloroacetic acid, and the appearance or disappearance of *p*-nitrophenol was measured at 400 nm after addition of 10 M KOH to the protein-free supernatant. Hydrolysis of UDP and conjugation of *o*-aminophenol were assayed as in Fig. 1, the latter activities being reported as the means ± standard deviations with the number of experiments bracketed. Effects of sonication upon the conjugation of *p*-nitrophenol and of sonication and grinding upon the hydrolysis of UDP were measured in three experiments; the range and means are reported. Effects of mechanical disruption upon the reverse reaction and of blending and grinding upon the conjugation of *p*-nitrophenol were each examined in single experiments.

Treatment	*o*-Aminophenol conjugation (nmol/min/mg)	*p*-Nitrophenol conjugation (nmol/min/mg)	Reverse reaction (*p*-nitrophenol) (nmol/min/mg)	Nucleoside diphosphatase (nmol/min/mg)
Non-sonicated control	0.85 ± 0.24 (12)	2.24 (1.9— 2.5)	3.22	54 (35— 76)
Sonicated	4.20 ± 0.92 (12)	10.90 (9.8—11.8)	11.6	167 (130—200)
Unblended control	0.67 ± 0.11 (4)	1.87	3.22	54 (35— 76)
Blended	3.75 ± 0.3 (4)	7.60	8.40	155 (110--200)
Unground control	0.70 ± 0.12 (4)	1.90	3.20	54 (35— 76)
Ground	5.37 ± 0.46 (4)	11.2	14.20	198 (115—380)

400% and the reverse reaction 260% (Table I). Fig. 1 shows substantial variation in glucuronyltransferase activity, due in part to individual variation between different microsomal preparations, a phenomenon commented upon recently by others [32]. It is also partly due in Expt 2 (Fig. 1) to assaying in the presence of 0.004 M Mg^{2+}, to obtain an optimum UDP-*N*-acetylglucosamine effect [3]: the divalent metal increased both the basal and stimulated activities. Despite these variations (Fig. 1; Table I), 12 microsome samples which had been ultrasonicated for 4 min and assayed without Mg^{2+} showed highly significant stimulation of *o*-aminophenol conjugation ($p < 0.001$). Similarly, for 4 samples of microsomes, blending for 8 min also stimulated highly significantly ($p < 0.001$).

As shown in Fig. 1B ultrasonication and Ultra-Turrax blending also stimulated nucleoside diphosphatase. Stimulation (270—320%) was generally less than with glucuronyltransferase but prolonged ultrasonication appears to inactivate nucleoside diphosphatase even more markedly.

As shown in Table I, extensive stimulation of glucuronyltransferase assayed ($\pm Mg^{2+}$) with *o*-aminophenol (significant at $p < 0.001$ without Mg^{2+}) or with *p*-nitrophenol (forward or reverse reactions), or of nucleoside diphosphatase, was obtained when frozen microsome pellets were ground with glass (445—1050% stimulations of glucuronyltransferase).

Is the stimulation of UDPglucuronyltransferase by ultrasonication due to lipid breakdown products?

The ability of ultrasonication to inactivate enzymes on prolonged exposure, in which a role for free radicals has been suggested [33] made it essential to exclude chemical degradation of membrane lipids as the mechanism of activation of glucuronyltransferase. Extensive peroxidation of microsomal phospholipids, induced in vitro with pro-oxidants, is reported to stimulate the enzyme [34].

Microsome suspensions were ultrasonicated for 4 min in the presence of 100 μg/ml α-tocopherol, dispersed in a variety of ways: (i) by previous ultrasonication in sucrose, by injecting an ethanol solution into sucrose from a Hamilton syringe and then (ii) shaking or (iii) ultrasonicating, (iv) as equimolar phosphatidylcholine: α-tocopherol liposomes. In some experiments, also, the

TABLE II

α-TOCOPHEROL OR BOVINE SERUM ALBUMIN FAIL TO PREVENT STIMULATION OF GLUCURONYLTRANSFERASE BY ULTRASONICATION

Assays of aglycone conjugation were performed and results are expressed as in Table I.

Protective agent and activity examined	Control activity	Control + agent	Sonicated activity	Sonicated + agent
α-Tocopherol (*o*-Aminophenol)	1.02 ± 0.23 (5)	0.95 ± 0.23 (4)	3.25 ± 0.37 (5)	3.20 ± 0.50 (4)
Albumin (*o*-aminophenol)	0.51; 0.88	0.54; 0.95	5.7; 3.53	4.6; 4.39
Albumin (*p*-nitrophenol)	2.49	1.85	11.0	12.3

microsome suspension and anti-oxidant were gassed with N_2 prior to ultrasonication, and N_2 was passed into the vessel throughout the procedure.

Comparison between microsomes, ultrasonicated with or without anti-oxidant, shows that α-tocopherol did not impair the stimulation of *o*-aminophenol conjugation (Table II).

Several types of experiment were also performed to explore the possibility that ultrasonication might facilitate lipid hydrolysis allowing stimulation of enzyme activity by the liberated long chain fatty acids or partial glycerides [35]. Negligible increase was seen in the long chain fatty acid content of microsomes ultrasonicated for 4 min i.e. from 8.2 to 10.4 nmol/mg microsomal protein in one experiment, and from 16.5 to 18.4 nmol in a second experiment. Ultrasonication of microsomes in the presence of excess bovine serum albumin still resulted in large activations of glucuronyltransferase activity, with small variable effects of albumin on basal activity in non-sonicated control microsomes (Table II).

Effect of phospholipid dispersions upon glucuronyltransferase activities in native and disrupted microsomes

Addition of dispersions of phosphatidylcholine (up to 1.4 mg/mg microsomal protein) or of total guinea pig liver phospholipids (up to 1.75 or 3.0 mg/mg microsomal protein) to native microsomes, caused no loss of glucuronyltransferase activity, assayed with *o*-aminophenol (Fig. 2A, Table III) or

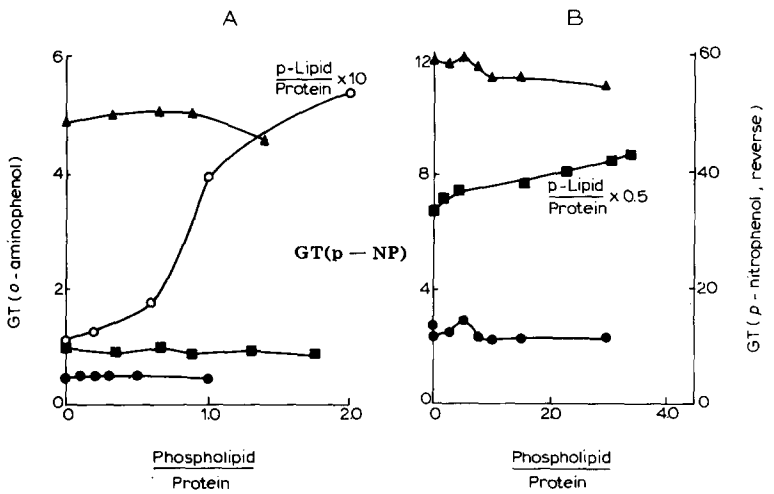

Fig. 2. Phospholipid dispersions do not inhibit glucuronyltransferase in native or ultrasonicated microsomes. Microsome suspensions (5 mg protein/ml) were exposed for 10—30 min at 0° C to phospholipid dispersions in amounts indicated by the phospholipid: protein ratio. Glucuronyltransferase activity was then assayed as in Fig. 1 and Table I. A. Conjugation of *o*-aminophenol: (●——●) untreated microsomes exposed to 0.1-1.0 mg of phosphatidylcholine/mg protein; (■——■) untreated microsomes and (▲——▲) ultrasonicated microsomes exposed to 0.33—1.75 mg total liver phospholipid/mg protein; (○——○) untreated microsomes exposed to 0.02—0.2 mg (note change in phospholipid/protein scale) lysophosphatidylcholine/mg protein. B. Untreated microsomes (●——●) and ultrasonicated microsomes (▲——▲) exposed to 0.25—3.0 mg phospholipid/mg protein and the conjugation of *p*-nitrophenol assayed; (■——■) ultrasonicated microsomes exposed to 0.55—7.7 mg phospholipid/mg protein and the reverse *p*-nitrophenol reaction assayed.

TABLE III

PHOSPHATIDYLCHOLINE DISPERSIONS DO NOT INHIBIT *o*-AMINOPHENOL CONJUGATION IN CONTROL OR DISRUPTED MICROSOMES

Enzyme activity was assayed as in Fig. 1. The asterisked control activity represents microsomes, disrupted by the 3 mechanical procedures in the day of preparation. Phosphatidylcholine/microsomal protein ratios are bracketed.

Disruption method	Control activity	Control + phosphatidylcholine	Disrupted activity	Disrupted + phosphatidylcholine
Sonication	0.57*	0.67 (0.7)	5.5	5.5 (0.7)
		0.69 (1.4)		5.3 (1.4)
	0.75	0.88 (1.0)	3.8	5.0 (1.0)
Ultra-Turrax	0.57*		3.9	3.9 (0.7)
				3.9 (1.4)
Ground	0.57*		5.4	5.7 (0.7)
				5.6 (1.4)

p-nitrophenol (Fig. 2B). Neither were the stimulated rates in microsomes which had been subjected to three mechanical treatments significantly reduced by dispersions of phosphatidylcholine (Table III), but a small inhibition (<10%) of rates of *o*-aminophenol (Fig. 2A) and *p*-nitrophenol conjugation (Fig. 2B) was seen at high concentrations of total liver phospholipids. However, a reverse reaction with ultrasonicated microsomes was significantly enhanced (Fig. 2B) by even very large amounts of total phospholipids (up to 15 times greater than the phospholipid content of microsomes).

The maximum phosphatidylcholine/microsomal protein employed in these experiments represented about 5 times the normal content in microsomes. Much smaller amounts of lysophosphatidylcholine caused marked stimulation of *o*-aminophenol conjugation in native microsomes (Fig. 2A), as reported for *p*-nitrophenol conjugation [35], and consistent with the well known detergency action of this compound.

These experiments were based on the report by Zakim [23], that phospholipid constraint of glucose-6-phosphatase could be detected because diacylphospholipid dispersions enhanced constraint in untreated control microsomes and can reimpose constraint after lipid-perturbation. By this criterion, which is perhaps conjectural, mechanical disruption does not stimulate glucuronyltransferase by relieving phospholipid constraint. Since there has been no precise mechanism yet described for phospholipid constraint it is not yet clear how it may be more rigorously tested.

Discussion

These findings seem in accord with previous proposals that glucuronyltransferase is compartmented (e.g. refs 35—39). The simplest explanation for extensive stimulation of glucuronyltransferase by mechanical treatments is that this is predominantly due to relief of structural latency, since these treatments concomitantly removed the latency of nucleoside diphosphatase, which is apparently localized intravesicularly in microsomes [3,13,14]. However, it

must be considered whether compartmentation of glucuronyltransferase is a unique interpretation of these mechanical effects, even though no evidence was found that lipid peroxidation or liberated amphipaths contributed.

Vessey and Zakim [8] apparently included ultrasonication with phospholipase A_2 and Triton X-100 as a treatment which stimulated glucuronyltransferase by relieving its conformational constraint by phospholipids, this being curiously exerted only on the forward (conjugation) reactions of the system. Principally, because of the apparently unidirectional nature of latency (constraint) and because kinetic parameters varied with different modes of stimulation, they rejected the possibility that glucuronyltransferase could be structurally latent. However, data in this paper and elsewhere [21,40], now clearly shows that both forward and reverse reactions of glucuronyltransferase are latent. Furthermore all of the treatments interpreted as relieving phospholipid constraint on a glucuronyltransferase would also disrupt microsomal vesicles and therefore do not uniquely support a constraint model.

Mechanical treatments might enhance membrane fluidity in microsomes, due to the increased curvature as vesicles are progressively reduced in size [41]. Increased membrane fluidity might then contribute to the stimulation of glucuronyltransferase. Discontinuous Arrhenius plots [42], not seen however by others [43], suggest that membrane fluidity may indeed influence glucuronyltransferase activity [42], but probably only slightly [42,44]. Their detection on initial rates [42], would seem to indicate that anomalous thermal effects (phase transitions) must be virtually instantaneous. Therefore, they cannot account for thermal removal of latency [43]. This is time-dependent and also much less efficient than mechanical methods for removing latency, causing only about 80% stimulation of glucuronyltransferase. A major role for membrane fluidity in mechanical effects also poorly fits the retention of Mg^{2+}-stimulation of *o*-aminophenol conjugation, after mechanical treatments, since divalent cations seem to typically reduce fluidity (e.g. refs 45 and 46).

Nevertheless, important secondary effects do apparently accompany disruption of microsomes by ultrasonication. Thus Fig. 1 suggests that inactivation of nucleoside diphosphatase (B) and to a lesser extent of glucuronyltransferase (A) is superimposed upon their stimulation by ultrasonication. Therefore, after 1—2 min, ultrasonication appears to inactivate more nucleoside diphosphatase than it expresses, so by 10 min more than 80% of the stimulated activity is destroyed. Glucuronyltransferase is more resistant to inactivation than nucleoside diphosphatase and this is probably the major reason why ultrasonication continues to stimulate it for up to 4 min. However differences in permeability to substrates could also contribute to the earlier peak activation of nucleoside diphosphatase, since its latent activity in microsomes exceeeds that of any glucuronyltransferase activities by at least 10-fold. Ultra-Turrax blending seems less destructive and appeared to cause little inactivation of either enzyme at 8 min. An important implication of inactivation superimposed upon stimulation is the uncertainty it confers on values for 'total enzyme activity'. It is unclear whether any treatments stimulating glucuronyltransferase avoid this difficulty and give a true value for 'total enzyme activity'.

In conclusion, the substantial stimulation by mechanical treatments of glucuronyltransferase in parallel with nucleoside diphosphatase, is consistent

with the structural latency of most or all of the glucuronyltransferase activities examined. However, because of uncertainty about whether a suspected increase in membrane fluidity might contribute (though its effect is likely to be small), disruption of vesicles cannot be assumed to wholly and uniquely account for the observed stimulations. Compartmentation of glucuronyltransferase with nucleoside diphosphatase in the endoplasmic reticular cisternae is functionally attractive, however, since it should ensure their optimal interaction. Nucleoside diphosphatase, by hydrolysing the UDP product of conjugation reactions, would then tend to make these unidirectional. Nevertheless, glucuronyltransferase and nucleoside diphosphatase may not wholly account for all of the observed regulatory behaviour of conjugation reactions, though a recent model attempts to do this on the basis of glucuronyltransferase alone [18]. A postulated permease for UDP-glucuronate has been considered to explain stimulation by UDP-*N*-acetylglucosamine [29], though this may occur instead by allosteric activation of glucuronyltransferase, not involving alteration of lipid-protein interactions [18,47]. Finally, no mechanism is yet postulated for the effect of diethylnitrosamine. This stimulates some conjugation reactions additively with digitonin [48], though the latter removes latency and might also be expected to relieve any phospholipid constraint.

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