Diazobenzenesulphonate selectively abolishes stimulation of glucuronidation by UDP-*N*-acetylglucosamine

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1. Basal rates of glucuronidation of oestrone (guinea pig) or of 4-nitrophenol (rat or guinea pig) were not significantly altered in sealed liver microsomal vesicles, treated with the membrane-impermeant protein-modifying agent diazobenzenesulphonate at 0.5–1.0 mM. 2. Contrarily, diazobenzenesulphonate abolished the normal stimulation of glucuronidation by UDP-*N*-acetylglucosamine. 3. Ultrasonication to increase microsomal permeability activated glucuronidation by 680–750% and permitted significant inhibition by diazobenzenesulphonate. 4. These findings are consistent with a model wherein glucuronyltransferases are embedded in the luminal leaflet of the endoplasmic reticulum and access of UDP-glucuronic acid to the transferases is facilitated by transmembrane carriers, which are stimulated by UDP-*N*-acetylglucosamine and are available to diazobenzenesulphonate; ultrasonication serves to permit access of diazobenzenesulphonate to glucuronyltransferases themselves, resulting in inhibition of their activity.

UDP-glucuronyltransferase is an intrinsic enzyme embedded in the endoplasmic-reticular membranes of several cell types. The rate of conjugation by the transferase of all aglycones tested is markedly stimulated in sealed microsomal vesicles by addition of UDP-*N*-acetylglucosamine to assays (Dutton & Burchell, 1977), although the latter agent probably does not interact directly with glucuronyltransferase to cause this stimulation. This is suggested by the ability of *N*-ethylmaleimide virtually to abolish stimulation by UDP-*N*-acetylglucosamine without significant binding of [¹⁴C]*N*-ethylmaleimide to glucuronyltransferase purified from the treated vesicles (Berry, 1978). Activation by UDP-*N*-acetylglucosamine occurs under conditions where it acts neither as a transferase substrate nor by preventing catabolism of UDP-glucuronic acid (Dutton & Burchell, 1977). With oestrogens, at physiological concentrations of UDP-glucuronic acid and UDP-*N*-acetylglucosamine, stimulation is about 2.5-fold (de Brito & Hallinan, 1979) and the work of Zakim & Vessey (1976), Bock *et al.* (1976) and Otani *et al.* (1976) indicates that the action of UDP-*N*-acetylglucosamine is essential for efficient glucuronidation of xenobiotics *in vivo*.

Theories of UDP-*N*-acetylglucosamine action are bound up with views on why UDP-glucuronyltransferases are highly latent in native microsomal

vesicles. Here, procedures that lyse and/or perturb membranes can activate glucuronidation as much as 40-fold (Dutton & Burchell, 1977). Pronounced transferase latency, inability of proteinases to inhibit glucuronidation in sealed microsomal vesicles while inhibiting in leaky preparations, as well as other evidence, leads some workers to conclude that glucuronyltransferases are embedded in the luminal microsomal compartment (Hallinan, 1978). Others maintain that the transferases are on the cytoplasmic microsomal face, but are either conformationally constrained or activated there by phospholipids and other effectors (Zakim & Vessey, 1976). In 'conformationalist' terms, UDP-*N*-acetylglucosamine serves as a K-type allosteric activator of glucuronyltransferase, whereas a 'compartmentalist' theory postulates that this nucleotide activates a UDP-glucuronic acid permease, which facilitates donor-substrate access to the lumenally embedded transferases (Berry & Hallinan, 1976). In the present study, the poorly membrane-permeant inhibitor diazobenzenesulphonate is used to probe further the transverse membrane topology of glucuronyltransferases and to study UDP-*N*-acetylglucosamine stimulation of glucuronidation.

Materials and methods

The preparation of microsomal fractions from

starved male Duncan–Hartley guinea pigs or Wistar rats, ultrasonication of these fractions for 8 min in the cold and the assay of 4-nitrophenol glucuronidation with 4 mM-UDP-glucuronic acid, 2 mM-UDP-*N*-acetylglucosamine and about 1 mg of microsomal protein are described by Hallinan *et al.* (1979). Oestrone glucuronidation was assayed at pH 7.4 essentially as described by Rao *et al.* (1977), but with physiological concentrations of 0.4 mM-UDP-glucuronic acid and 0.2 mM-UDP-*N*-acetylglucosamine and omitting EDTA; about 100 µg of microsomal protein was used. Diazobenzenesulphonate was synthesized by the method of Nilsson *et al.* (1978a). Protein was assayed by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Results and discussion

The rate of oestrone glucuronidation in native sealed guinea-pig liver microsomal fractions, treated with 0.5–1.0 mM concentrations of the membrane-impermeant reagent diazobenzenesulphonate, was almost identical with the basal rate of oestrone conjugation assayed in untreated control microsomal fractions (Table 1). In a single experiment a range of concentrations of diazobenzenesulphonate (0, 0.25, 0.5, 1.0, 1.5, 2.0 and 4.0 mM) was used and the corresponding rates of oestrone glucuronidation were 1.2, 1.2, 1.5, 1.4, 2.5, 2.1 and 2.4 nmol/min per mg of microsomal protein. Hence diazobenzenesulphonate does not inhibit oestrone glucuronidation under these conditions.

Similarly, treating native guinea-pig or rat liver microsomal fractions with 0.5–1.0 mM-diazobenzenesulphonate also failed to inhibit 4-nitrophenol glucuronidation. Instead it caused a small but consistent activation, which was just non-significant (Table 1). Nilsson *et al.* (1978a) observed likewise that 0.75 mM-diazobenzenesulphonate did not inhibit 4-nitrophenol glucuronidation in undisrupted rat liver microsomal fractions.

As has been frequently observed (Dutton & Burchell, 1977), UDP-*N*-acetylglucosamine markedly and significantly stimulated the glucuronidation of oestrone or 4-nitrophenol (Table 1). Oestrone conjugation was increased by 2.3-fold ($P = 0.0002$), whereas 4-nitrophenol glucuronidation was increased 3- and 3.5-fold in rat and guinea pig respectively ($P = 0.002$ and 0.02). However, when added to diazobenzenesulphonate-treated microsomal fractions, UDP-*N*-acetylglucosamine now was without significant effect (Table 1). Its apparent stimulation of 4-nitrophenol conjugation in guinea-pig fractions is an artefact, since almost all of the observed increase is caused by diazobenzenesulphonate itself. This agent increased 4-nitrophenol glucuronidation from 4.3 to 6.7 nmol/

Table 1. Glucuronidation rates in control and treated liver microsomal fractions

Before measuring their glucuronidating activity, control and ultrasonicated microsomal fractions were exposed at 0°C to 250 mM-sucrose/50 mM-KCl/50 mM-phosphate, pH 7.5. They were aggregated in 250 mM-sucrose/13 mM-CaCl₂/8 mM-MgCl₂, sedimented at 25 000 *g* for 15 min and then resuspended and washed by re-centrifugation as described above from 250 mM-sucrose/50 mM-KCl/50 mM-Tris/HCl, pH 7.5. Diazobenzenesulphonate treatment also involved these procedures (Nilsson *et al.*, 1978a), so their use with both control and treated fractions compensated for any effects that they might have exerted on glucuronidation rates. P_a is the probability that the rate is identical with the basal rate in control microsomal fraction; P_b is the probability that the rate is identical with the rate in ultrasonicated microsomal fraction.

Substrate	Species	Glucuronidation rate (nmol/min per mg of microsomal protein)				
		Basal rate in control microsomal fraction	Control + diazobenzenesulphonate	UDP- <i>N</i> -Acetylglucosamine	UDP- <i>N</i> -Acetylglucosamine + diazobenzenesulphonate	Rate in ultrasonicated microsomal fraction
Oestrone	Guinea pig	1.8 ± 0.49 (26)	1.7 ± 0.19 (11) $P_a = 0.7$	4.2 ± 1.4 (7) $P_a = 0.0002$	2.0 ± 0.5 (12) $P_a = 0.99$	14.0, 16.5 $P_a = 0.02$
4-Nitrophenol	Guinea pig	4.3 ± 0.9 (3)	6.7 ± 1.2 (3) $P_a = 0.07$	15.0 ± 4.2 (3) $P_a = 0.02$	7.4 ± 0.9 (3) $P_a = 0.01^*$	33.4 ± 5.0 (3) $P_a = 0.002$
4-Nitrophenol	Rat	2.2 ± 0.8 (4)	3.6 ± 0.6 (6) $P_a = 0.06$	6.5 ± 1.3 (4) $P_a = 0.002$	3.4 ± 1.6 (6) $P_a = 0.14$	18.5, 17.4 $P_a = 0.0004$
						6.8, 6.5 $P_b = 0.02$
						21.2 ± 2.4 (3) $P_b = 0.03$
						5.6, 8.1 $P_b = 0.01$

* However, UDP-*N*-acetylglucosamine does not stimulate the glucuronidation rate above that in diazobenzenesulphonate-treated control microsomal fraction ($P = 0.5$).

min per mg of microsomal protein. In agreement with Wilkinson & Hallinan (1977a), UDP-*N*-acetylglucosamine did not itself serve to conjugate either aglycone.

Ultrasonication of microsomal vesicles to increase their permeability greatly enhanced glucuronidation. The efficiency of this and of numerous other membranolytic procedures for enhancing glucuronidation (Dutton & Burchell, 1977) appears most consistent with structural latency of the isoenzymes of UDP-glucuronyltransferase, with these integral membrane proteins being embedded in the luminal face of microsomal membranes (Hallinan, 1978). In support of this interpretation, ultrasonication also markedly activates two other structurally latent liver microsomal enzymes, nucleoside diphosphatase (Berry *et al.*, 1975) and mannose 6-phosphatase. Ultrasonication significantly stimulated the latter enzyme by $331 \pm 25\%$ ($P = 0.0001$) in four experiments with rat liver microsomal fractions (P. Trivedi & T. Hallinan, unpublished work).

If assignment of glucuronyltransferase to the luminal microsomal compartment is correct, ultrasonication should also make the transferase more freely available to the protein-modifying agent diazobenzenesulphonate, which is poorly membrane-permeant and is shown above not to inhibit transferase activity in sealed control microsomal vesicles. Table 1 shows that diazobenzenesulphonate treatment of ultrasonicated microsomal fractions did indeed significantly inhibit glucuronidation of both oestrone and 4-nitrophenol, the latter in both rat and guinea-pig liver fractions. The above finding is consistent with the observations of Nilsson *et al.* (1978a) that, although it is non-inhibitory in 'intact' control rat liver microsomal vesicles, diazobenzenesulphonate inhibited 4-nitrophenol glucuronidation in microsomal fractions disrupted with Triton X-100.

However, despite this finding, Nilsson *et al.* (1978a) state that their experimental data indicate that glucuronyltransferase is located in the outer cytoplasmic face of microsomal vesicles, rather than in the luminal face. The only evidence for this appears to be that proteolysis of sealed microsomal vesicles increased 4-nitrophenol glucuronidation by about 85%, a modest stimulation compared with the increase of about 700% observed after ultrasonication in the present study (see also Dutton & Burchell, 1977). Nilsson *et al.* (1978a) attribute this proteolytic activation of glucuronyltransferase to the destruction of some other membrane component that 'covers' glucuronyltransferase on the cytoplasmic face of microsomal vesicles. Others, who saw comparable modest activations of glucuronyltransferase by proteinase treatment of sealed microsomal vesicles, instead attributed it to limited impairment of the membrane permeability barrier,

thus improving substrate access to the lumenally located transferases (for review see Wilkinson & Hallinan, 1977b). Elsewhere, Nilsson *et al.* (1978b) also comment that over-extensive treatment of sealed microsomal vesicles with proteinases or diazobenzenesulphonate may break down their permeability barrier, so it is quite unclear why they reject a limited permeability change to explain the small proteolytic activation of glucuronyltransferase.

The data below show that proteolysis of sealed hepatic microsomal vesicles does indeed impair their permeability barrier, activating the classical luminal enzyme mannose 6-phosphatase (Arion *et al.*, 1976). This is shown in Fig. 1, which illustrates the effect of trypsin treatment of initially sealed guinea-pig liver microsomal vesicles on the activity of mannose 6-phosphatase assayed at a range of substrate concentrations. As others have repeatedly shown, trypsin treatment fails to stimulate this activity at a mannose 6-phosphate concentration of 2 mM, but activation is discernible at 3 mM and increases to almost 300% at 20 mM. Similar results were obtained in three other experiments. Since the latency of mannose 6-phosphatase is extensively used as a standard test of liver microsomal-membrane integrity, it is highly questionable whether an analogous proteolytic activation of glucuronyltransferase provides adequate evidence that this enzyme is located

Fig. 1. Effect on mannose 6-phosphatase activity of trypsin-treated sealed guinea-pig liver microsomal vesicles

Vesicles were preincubated for 40 min at 20°C with 500 μg of bovine trypsin (Sigma, type 1)/mg of microsomal protein. Phosphatase activity was then assayed at 37°C (Cater *et al.*, 1975) in the presence of soya-bean trypsin inhibitor (Sigma, type 1s) at twice the concentration of trypsin. Trypsin inhibitor was added at the same time as trypsin to controls. ○, Control; ●, trypsin-treated.

on the cytoplasmic face of microsomal vesicles, but 'covered' by other membrane components.

The salient finding in the present study is that treatment of sealed microsomal vesicles with diazobenzenesulphonate prevents UDP-*N*-acetylglucosamine from causing its normal marked stimulation of glucuronidation, although this membrane-impermeant agent clearly does not decrease the basal glucuronidation rate under these conditions. Therefore UDP-*N*-acetylglucosamine must stimulate glucuronidation via membrane components that are readily available in sealed microsomal vesicles to diazobenzenesulphonate, and hence are presumably located on their cytoplasmic leaflet. This finding fits a model described previously (Berry & Hallinan, 1976; Hallinan, 1978), wherein UDP-*N*-acetylglucosamine increases glucuronidation by activating transmembrane carriers that facilitate access of UDP-glucuronic acid to isoenzymes of glucuronyltransferase, which are themselves embedded in the luminal face of microsomal membranes. Parts of these putative carriers would be expected to be exposed on the cytoplasmic face of microsomal membranes. Finally, assignment of glucuronyltransferases to the luminal microsomal face is in accordance with the inability of diazobenzenesulphonate to inhibit glucuronidation, in the absence of UDP-*N*-acetylglucosamine except in ultrasonicated leaky microsomal fractions.

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