Evidence for an UDP-glucuronic acid/phenol glucuronide antiport in rat liver microsomal vesicles

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The transport of glucuronides synthesized in the luminal compartment of the endoplasmic reticulum by UDP-glucuronosyltransferase isoenzymes was studied in rat liver microsomal vesicles. Microsomal vesicles were loaded with *p*-nitrophenol glucuronide (5 mM), phenolphthalein glucuronide or UDP-glucuronic acid, by a freeze–thawing method. It was shown that: (i) the loading procedure resulted in millimolar intravesicular concentrations of the different loading compounds; (ii) addition of UDP-glucuronic acid (5 mM) to the vesicles released both intravesicular glucuronides within 1 min; (iii) glucuronides stimulated the release of UDP-glucuronic acid from UDP-glucuronic acid-loaded microsomal vesicles; (iv) *trans*-stimu-

lation of UDP-glucuronic acid entry by loading of microsomal vesicles with *p*-nitrophenol glucuronide, phenolphthalein glucuronide, UDP-glucuronic acid and UDP-*N*-acetylglucosamine almost completely abolished the latency of UDP-glucuronosyltransferase, although mannose 6-phosphatase latency remained unaltered; (v) the loading compounds by themselves did not stimulate UDP-glucuronosyltransferase activity. This study indicates that glucuronides synthesized in the lumen of endoplasmic reticulum can leave by an antiport, which concurrently transports UDP-glucuronic acid into the lumen of the endoplasmic reticulum.

INTRODUCTION

Transport events across the endoplasmic reticulum (ER) are vital for the function and the regulation of intraluminal pathways involved in numerous cell processes such as post-translational modification of proteins, glucose secretion and biotransformation. Examples of transport processes previously investigated are ATP (see, for example, [1]), glutathione [2], glucose 6-phosphate (see, for example, [3]), UDP-glucose [4] and UDP-glucuronic acid [5–8]. The aim of the present study was to investigate the transport of glucuronides synthesized by UDP-glucuronosyltransferase (UDPGT; EC 2.4.1.17) in the lumen of the liver ER.

Glucuronidation is one of the most important reactions in phase II of biotransformation. It is catalysed by UDPGTs; these are integral proteins of the endoplasmic reticulum [9]. The most characteristic feature of these enzymes is their latency: the activity is low in native microsomes compared with the maximal activity elicited after elimination of the membrane barrier [10]. According to the compartmentation hypothesis of glucuronidation [11], the active site of UDPGTs is intraluminal. Therefore transporter(s) might be expected to exist both for UDP-glucuronic acid (UDPGA) synthesized in the cytosol and for the aglycone glucuronide produced intraluminally; both compounds are also charged and water soluble [12]. However, the microsomal transport of UDPGA, and especially that of glucuronides, has been little characterized. UDPGA is thought to be transported by an antiport jointly with UMP generated in the lumen of ER [11]. Recent findings indicate a more complex mechanism with the participation of two asymmetric transport pathways: UDPGA influx is coupled to UDP-*N*-acetylglucosamine efflux, and UDP-*N*-acetylglucosamine influx is coupled to UMP efflux

[6]. UDPGA transport has been reported to be dependent on time and temperature, saturable, selective, carrier-mediated and capable of *trans*-stimulation [7]. Glucuronide transport has not yet been investigated in detail, but early results indicate that most glucuronides appear in the cytosol after their synthesis [13,14].

In the present study the transport of glucuronides was investigated in rat liver microsomal vesicles by two different experimental approaches: efflux was measured from vesicles loaded with glucuronides, and UDPGA influx was estimated indirectly on the basis of UDPGT activity under various circumstances. Two model glucuronides, one (*p*-nitrophenol glucuronide) of low molecular mass and one (phenolphthalein glucuronide) of high molecular mass, were chosen because their excretory routes are different in the liver [15].

EXPERIMENTAL

Materials

UDPGA, UDP-*N*-acetylglucosamine, UDP, UMP, *p*-nitrophenol glucuronide, phenolphthalein glucuronide, D-saccharic acid 1,4-lactone, polyethylene glycol (PEG; molecular mass 8000 Da), mannose 6-phosphate and alamethicin were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade.

Preparation of rat liver microsomes

Rat liver microsomes were prepared from male Sprague–Dawley rats (180–230 g) as previously described [16] and resuspended (60–80 mg of protein/ml) in a medium with the following com-

Abbreviations used: ER, endoplasmic reticulum; PEG, polyethylene glycol; UDPGA, UDP-glucuronic acid; UDPGT, UDP-glucuronosyltransferase. ‡ To whom correspondence should be addressed.

position (mM): KCl, 100; NaCl, 20; MgCl₂, 3; Mops, 20, pH 7.2. The suspensions were frozen and maintained under liquid nitrogen until used. Intactness of the microsomal membrane was ascertained by measuring the latency of mannose 6-phosphatase activity [17]. It was higher than 92% in all the preparations used.

Loading of microsomal vesicles

Aliquots of microsomal suspension (250 µl) stored in liquid nitrogen were allowed to thaw and the different loading compounds (dissolved in a small volume of water and brought to pH 7.2) were added to give final concentrations of 5 or 10 mM. Microsomes were frozen and thawed twice, then three volumes of potassium phosphate buffer (50 mM, pH 7.4) containing 5 mM MgCl₂ and 5% (w/v) PEG were added to allow rapid low-speed sedimentation of microsomes. Suspensions were centrifuged (1800 *g* for 30 s at room temperature) and the supernatant was removed immediately. In parallel experiments, we verified that the rapid low-speed sedimentation of PEG-aggregated microsomes yielded more than 95% recovery of microsomal protein, as compared with conventional microsomal ultracentrifugation (i.e. 100000 *g* for 60 min at room temperature); moreover, microsomes fully retained membrane intactness (measured as above). For the measurement of the efflux of compounds previously loaded, microsomal pellets were washed twice with the PEG/potassium phosphate buffer to remove the extravascular compounds. For the measurement of UDPGT activity the microsomal pellet was resuspended (5–10 mg/ml protein) in 50 mM potassium phosphate buffer (pH 7.4) containing 5 mM MgCl₂, 5 mM UDPGA and 0.5 mM *p*-nitrophenol. In the experiments with glucuronides, 10 mM D-saccharic acid 1,4-lactone was continuously present in the media to inhibit microsomal β -glucuronidase activity. At this inhibitor concentration no measurable amount of free aglycones was formed during the loading procedure and the subsequent efflux measurements (see below).

Measurement of efflux of UDPGA, *p*-nitrophenol glucuronide and phenolphthalein glucuronide

Loaded microsomes (1–2 mg/ml protein) were incubated at 37 °C in potassium phosphate buffer (50 mM, pH 7.4) containing 5 mM MgCl₂, 5% (w/v) PEG and the various compounds to release intravesicular glucuronides or UDPGA. Parallel samples also included alamethicin (0.05 mg per mg of microsomal protein) to render microsomal vesicles fully permeable to the loading compounds [18]. After incubation (usually 1 min) PEG-aggregated microsomes were sedimented, the supernatant was rapidly removed and the pellet was extracted with methanol (phenolphthalein glucuronide loading) or 5% (w/v) trichloroacetic acid (*p*-nitrophenol glucuronide or UDPGA loading). The extracted loading compounds were measured by HPLC essentially by the method of [19]. A C18 Bondapak column (Waters, Millipore) was used, and the mobile phase was 50 mM KH₂PO₄ for *p*-nitrophenol glucuronide and UDPGA, or H₂O/methanol/acetic acid (54:45:1, by vol.) for phenolphthalein glucuronide. The HPLC detector wavelength was 280 nm for *p*-nitrophenol glucuronide and UDPGA, or 230 nm for phenolphthalein glucuronide. The alamethicin-releasable part of the microsome-associated loading compounds was regarded as intravesicular. Because no significant decrease in the millimolar concentration of intravesicular UDPGA was observed during a 1 min control incubation (i.e. in the absence of releasing compounds), we avoided the application of microsomal nucleotide pyrophosphatase inhibitors (e.g. NAD⁺ [7]).

Measurement of *p*-nitrophenol UDPGT activity

Reaction mixtures contained 0.5–10 mg/ml microsomal protein (depending on the experiment), 50 mM potassium phosphate buffer, pH 7.4, 5 mM MgCl₂, 10 mM D-saccharic acid 1,4-lactone, 0.5 mM aglycone and 5 mM UDPGA. Maximal enzyme activity (0% latency) was obtained by the addition of alamethicin at 0.05 mg per mg of microsomal protein [18]. *p*-Nitrophenol UDPGT activity was measured spectrophotometrically on the basis of aglycone disappearance [20].

Other assays

The intravesicular water space of microsomal vesicles was measured with [³H]inulin and ³H₂O as previously reported [21]. Mannose 6-phosphatase activity was measured as reported [17]. The permeability of microsomal vesicles to UDPGA, *p*-nitrophenol glucuronide and phenolphthalein glucuronide was investigated by a light-scattering technique [23] as described elsewhere [3], except that for *p*-nitrophenol glucuronide the excitation and emission wavelength was 500 nm. Protein concentration was determined by the biuret reaction with BSA as the standard.

RESULTS

Efflux of glucuronides from glucuronide-loaded microsomal vesicles

Liver microsomes were pre-loaded with *p*-nitrophenol glucuronide (5 or 10 mM), phenolphthalein glucuronide (5 or 10 mM) or UDPGA (5 mM) by the freeze-thawing procedure described in the Experimental section. The amounts of the various loading compounds associated with the microsomal vesicles after the loading procedure are shown in Table 1. The major portion of the various loading compounds associated with microsomes (55–70%) was released by treating (1 min at 37 °C) microsomes with alamethicin. Alamethicin treatments longer than 1 min did not result in an increase of the alamethicin-releasable portion. The alamethicin-releasable portion should correspond to the intravesicular loading compound, whereas the remainder is probably accounted for by any loading compound bound to the microsomal membrane and/or eventually still present in the extravascular water space (microsomes were currently washed twice after loading). Previous data [18] indicate that the pore-forming oligopeptide alamethicin [24] does permeabilize microsomal membrane to various non-permeant compounds but without destroying the vesicular structure of microsomes. To verify the efficiency of the loading procedure, the intravesicular concentrations of the different loading compounds were calculated on the basis of the measured intravesicular (microsomal) water space (3.08 ± 0.47 µl per mg of protein, mean ± S.D., *n* = 3). As shown in Table 1, we obtained intravesicular concentrations values approx. 20–50% lower than those used in the loading procedure.

The incubation (1 min at 37 °C) of *p*-nitrophenol glucuronide- or phenolphthalein glucuronide-loaded microsomes in the presence of UDPGA (5 mM) resulted in a release of intravesicular glucuronides (Table 2); this was comparable to that induced by alamethicin. Both in *p*-nitrophenol glucuronide- and phenolphthalein glucuronide-loaded microsomes, incubation times longer than 1 min (in the presence of UDPGA) did not result in any greater release effect; a shorter treatment of vesicles (30 s) with UDPGA released approx. 80% of the maximal releasable amount. However, because the centrifugation procedure (to separate vesicles from external media) employed here required times of 15 s or more, the (rapid) kinetics of UDPGA-induced

Table 1 Total and alamethicin-releasable content of different loading compounds in microsomal vesicles

Liver microsomes were loaded by freeze-thawing with UDPGA (5 mM), *p*-nitrophenol glucuronide (5 or 10 mM) and phenolphthalein glucuronide (5 or 10 mM), and the amount of the various loading compounds associated with microsomes were measured before and after treatment with alamethicin as reported in the Experimental section. The intravesicular concentrations of the various loading compounds were calculated on the basis of an intravesicular water space of microsomes of 3.08 μ l per mg of protein, as detailed in the Results section. Data are means $\pm$ S.E.M. for three, or the means of two, independent experiments.

Loading	Loading compound associated with microsomes (nmol/mg of protein)		Intravesicular concentration (mM)
	Total	Alamethicin-releasable	
<i>p</i> -Nitrophenol glucuronide (5 mM)	12.7 $\pm$ 2.3	7.0 $\pm$ 1.4	2.3
<i>p</i> -Nitrophenol glucuronide (10 mM)	26.0	17.1	5.6
Phenolphthalein glucuronide (5 mM)	14.3 $\pm$ 2.0	9.7 $\pm$ 0.8	3.1
Phenolphthalein glucuronide (10 mM)	35.2	25.0	8.1
UDPGA (5 mM)	18.8 $\pm$ 2.0	12.2 $\pm$ 1.1	4.0

Table 2 Stimulation of UDPGA, *p*-nitrophenol glucuronide and phenolphthalein glucuronide efflux from rat liver loaded vesicles

Rat liver microsomes were loaded with UDPGA, *p*-nitrophenol glucuronide and phenolphthalein glucuronide (5 mM; see the Experimental section) and incubated (10 mg/ml protein) for 1 min at 37 °C in the presence or absence of UDPGA, *p*-nitrophenol glucuronide, phenolphthalein glucuronide or UMP; each compound was present at 5 mM. Efflux of the loading compounds was calculated as the difference in intravesicular alamethicin-releasable contents before and after the incubation. Data are means $\pm$ S.E.M. for three, or the means of two, independent experiments. Abbreviation used: NM, not measured.

Addition	Efflux (nmol per mg of protein)		
	UDP-glucuronic acid loading	<i>p</i> -Nitrophenol glucuronide loading	Phenolphthalein glucuronide loading
None	0.2	2.2 $\pm$ 0.3	0.3 $\pm$ 0.1
UDP-glucuronic acid	NM	6.6 $\pm$ 0.6*	8.7 $\pm$ 0.9
<i>p</i> -Nitrophenol glucuronide	10.6 $\pm$ 0.8	NM	7.7
Phenolphthalein glucuronide	9.2	7.9	NM
UMP	10.8	7.7	8.8 $\pm$ 0.22
Alamethicin	12.2 $\pm$ 1.1	7.0 $\pm$ 1.0	9.7 $\pm$ 0.8

* UDPGA influx measured simultaneously was 8.3 $\pm$ 2.3 nmol per mg of protein; non-measurable influx of UDPGA was present in control (unloaded) microsomes.

release of intravesicular glucuronides was not further investigated.

Glucuronides also were able to stimulate their efflux, mutually (Table 2). This *trans*-stimulatory mechanism also worked in the opposite direction: glucuronides released UDPGA from UDPGA-loaded microsomal vesicles (Table 2). UMP also released intraluminal glucuronides or UDPGA (Table 2).

The release of *p*-nitrophenol glucuronide by UDPGA was accompanied by the entry into microsomal lumen of a roughly equimolar amount of UDPGA (6.6 $\pm$ 0.5 compared with 8.0 $\pm$ 2.0 nmol per mg of protein; see Table 2).

The incubation (1 min at 37 °C) of *p*-nitrophenol glucuronide-loaded microsomes in the absence of any releasing agent resulted in the spontaneous release of a (minor) part of the intravesicular glucuronide, but no spontaneous release was present in phenolphthalein glucuronide- or UDPGA-loaded vesicles (Table 2). Microsomal membranes seemed to be somewhat permeable to *p*-nitrophenol glucuronide whereas they were apparently impermeable to phenolphthalein glucuronide. In fact, prolonged incubation of microsomes (10 min at 37 °C) resulted in the loss of approx. 85% of the intravesicular content of *p*-nitrophenol glucuronide but with no appreciable loss of phenolphthalein glucuronide. Additionally, we observed that 30–50% of the alamethicin-releasable part of microsomal *p*-nitrophenol glucuronide was lost during the second wash of

loaded vesicles (in the efflux experiments of Table 2, microsomal vesicles were washed twice after the freeze-thawing loading procedure).

In parallel experiments (results not shown), the permeability of liver microsomal vesicles towards both (extravesicular) glucuronides was also assessed by analysing their osmotic behaviour by a light-scattering technique [3,23]. Phenolphthalein glucuronide (15 mM) caused a rapid and long-lasting (up to 10 min) shrinking of microsomal vesicles, whereas *p*-nitrophenol glucuronide (15 mM) resulted in a transient shrinking followed by a swelling phase ($t_{1/2} \approx 38$ s), in accordance with the spontaneous efflux observed in *p*-nitrophenol glucuronide-loaded microsomes. We have previously observed [18] that millimolar concentrations of UDPGA cause a sustained shrinkage of microsomal vesicles that can be rapidly reversed after membrane permeabilization by alamethicin.

Effect of intravesicular loading on UDPGT activity

Because UDPGA transport is rate-limiting in the glucuronidation reaction both in microsomes and in isolated hepatocytes, its rate can be estimated by measuring UDPGT activity. In subsequent experiments, therefore, UDPGA entry was *trans*-stimulated by loading of microsomal vesicles with different compounds and *p*-nitrophenol UDPGT activity was measured.

Table 3 Effect of microsomal loading with UDPGA, *p*-nitrophenol glucuronide, phenolphthalein glucuronide, UMP or UDP-*N*-acetylglucosamine on *p*-nitrophenol UDPGT latency and activity

p-Nitrophenol UDPGT activity was measured in microsomal vesicles (5–10 mg/ml protein) preloaded with the different compounds, and incubated for 1 min at 37 °C in the presence of 0.5 mM *p*-nitrophenol and 5 mM UDPGA (see the Experimental section). Latency is expressed as the difference between the activities in native and permeabilized microsomes divided by the full activity obtained in alamethicin-permeabilized vesicles and is expressed as a percentage. For comparison the latency of mannose 6-phosphatase is also shown. Results are means $\pm$ S.E.M. for four independent experiments.

Loading	UDPGT activity (nmol/min per mg of protein)	UDPGT latency (%)	Mannose 6-phosphatase latency (%)
None	4.3 $\pm$ 0.2	90.2	94.3
UDP-glucuronic acid	43.8 $\pm$ 1.6	0.5	94.2
<i>p</i> -Nitrophenol glucuronide	41.4 $\pm$ 1.9	5.9	93.5
Phenolphthalein glucuronide	46.6 $\pm$ 1.3	0	92.8
UMP	18.2 $\pm$ 2.6	58.6	91.8
UDP- <i>N</i> -acetylglucosamine	42.9 $\pm$ 0.9	2.5	93.0
<i>p</i> -Nitrophenol glucuronide + UMP	25.9 $\pm$ 2.2	41.1	93.5
None (+ alamethicin)	44.0 $\pm$ 2.4	0.0	0.0

Table 4 Effect of the different compounds used for microsomal loading on *p*-nitrophenol UDPGT activity in native and alamethicin-permeabilized microsomal vesicles

p-Nitrophenol UDPGT activity was measured in native and alamethicin-permeabilized (unloaded) microsomes (2 and 0.5 mg/ml protein respectively) for 10 min at 37 °C in the presence of 0.5 mM *p*-nitrophenol, and of 5 or 20 mM UDPGA as described in the Experimental section. Data are means $\pm$ S.E.M. for four to six independent experiments. Values marked * and ** are significantly different from the corresponding control values: $P < 0.05$ and $P < 0.01$ respectively on the basis of a paired Student's *t*-test.

Addition	<i>p</i> -Nitrophenol UDPGT activity (nmol/min per mg of protein)			
	Native microsomes		Permeabilized microsomes	
	+ 5 mM UDPGA	+ 20 mM UDPGA	+ 5 mM UDPGA	+ 20 mM UDPGA
None	2.23 $\pm$ 0.12	3.20 $\pm$ 0.24	42.9 $\pm$ 1.5	62.6 $\pm$ 8.6
Phenolphthalein glucuronide (0.1 mM)	2.44 $\pm$ 0.28	—	34.9 $\pm$ 7.3	—
Phenolphthalein glucuronide (1 mM)	2.20 $\pm$ 0.08	—	30.3 $\pm$ 2.7**	—
Phenolphthalein glucuronide (10 mM)	1.43 $\pm$ 0.27**	2.69 $\pm$ 0.39	5.0 $\pm$ 1.7**	8.2 $\pm$ 1.5**
UMP (10 mM)	2.25 $\pm$ 0.12	—	34.8 $\pm$ 3.5*	—
UDP- <i>N</i> -acetylglucosamine (10 mM)	6.33 $\pm$ 0.01**	8.58 $\pm$ 0.02**	36.5 $\pm$ 0.7*	46.8 $\pm$ 0.5
<i>p</i> -Nitrophenol glucuronide (5 mM)	1.60 $\pm$ 0.06**	—	35.8 $\pm$ 1.2*	—
<i>p</i> -Nitrophenol glucuronide (20 mM)	0.28 $\pm$ 0.06**	—	3.6 $\pm$ 0.3*	—

In these experiments, microsomes loaded by freeze–thawing in the presence of the various compounds were pelleted and resuspended in the reaction buffer without washing (see the Experimental section), to avoid their leakage particularly in the case of *p*-nitrophenol glucuronide (see above). Extravesicular loading compounds still present in the microsomal pellet, however, were largely diluted in the reaction mixture. We also verified that none of the loading compounds used at the concentration that was presumably present in the diluted samples (not more than 0.1 mM) affected the microsomal *p*-nitrophenol UDP-glucuronosyltransferase activity.

Loading of microsomal vesicles with *p*-nitrophenol or phenolphthalein glucuronides (5 mM) resulted in a huge increase of UDPGT activity within the first minute (Table 3), which was comparable to the effect of alamethicin permeabilization. A similar effect was obtained with UDP-*N*-acetylglucosamine or UDPGA, whereas UMP loading was less effective. Moreover, UMP caused an inhibition of the *p*-nitrophenol glucuronide-stimulated UDPGT activity (Table 3). At longer incubation times (3, 5 and 10 min) the stimulatory effect due to the various loading compounds was progressively diminished (results not shown). This was most probably due to the consumption of the substrate available for the *p*-nitrophenol glucuronidation re-

action. To measure the *p*-nitrophenol UDPGT activity with confidence within 1 min of incubation, in fact, high microsomal protein concentrations (5–10 mg/ml; see legend to Table 3) were present in the incubation mixture.

The method used did not alter the intactness of the microsomal membrane as revealed by the maintenance of the mannose 6-phosphatase latency (Table 3). Moreover, no stimulatory activity was present in microsomes, loaded with *p*-nitrophenol glucuronide, that had been washed and pre-incubated (20 min at 37 °C) to allow complete spontaneous efflux of the loading glucuronide. As expected, and because of the absence of spontaneous efflux of intravesicular phenolphthalein glucuronide, the stimulatory effect was retained by microsomes loaded with phenolphthalein glucuronide even after 30 min pre-incubation.

Effect of loading compounds on UDPGT activity

To investigate the possibility that loading substances could stimulate directly UDPGT enzyme(s), these were added to both intact and permeabilized (unloaded) microsomes and UDPGT activity was then measured. In intact microsomes UMP did not modify the activity, whereas phenolphthalein- and *p*-nitrophenol-glucuronide were inhibitory at high concentration (more than

10 mM; Table 4). UDP-*N*-acetylglucosamine, as is known (see, for example, [18]), stimulated UDPGT activity (Table 4). In alamethicin-permeabilized microsomes UDP-*N*-acetylglucosamine, UDP, UMP and *p*-nitrophenol glucuronide slightly, and phenolphthalein glucuronide strongly, inhibited the glucuronidation of *p*-nitrophenol (Table 4). The inhibitory effect of phenolphthalein glucuronide could be suppressed by elevating UDPGA concentration in native, but not in permeabilized, vesicles (Table 4).

DISCUSSION

The aim of the present study was to investigate the mechanism of microsomal glucuronide transport. It is difficult to modify the composition of the microsomal intravesicular space methodologically. In previous studies, prolonged incubation in the presence of the loading compound(s) was used for this purpose [4,6,7]. However, the loading efficiency is rather unpredictable and far from equilibrium (particularly for highly impermeant compounds) and their metabolism during long-term incubations could cause further complications. The freeze-thawing method applied in our study resulted in an efficient, rapid loading of microsomal vesicles (Table 1). We also took advantage of the aggregation of microsomes by PEG to separate vesicles and extravesicular medium rapidly.

Glucuronides formed in the lumen of the ER are charged, water-soluble compounds of relatively high molecular mass (300–1000 Da). Their export from hepatocytes should presumably involve transport processes at both the ER and plasma membrane. Glucuronides can be actively transported to the bile through the canalicular membrane by multiple organic anion transporters [25,26], or by passive transport through the sinusoidal membrane to the blood [27–31]. Their transport through the ER membrane, however, has been poorly characterized. Some observations indicate that they do not accumulate in the microsomal fraction but appear in the cytosol soon after their synthesis [13,14]. We observed that a high-molecular-mass glucuronide (phenolphthalein glucuronide) does not, whereas a low-molecular-mass one (*p*-nitrophenol glucuronide) does slowly permeate through microsomal membranes. Despite this, the intravesicular accumulation of glucuronides has never been observed during glucuronidation. To explain this apparent contradiction, an antiport can be assumed to exist for exchanging glucuronides and/or other reaction products (e.g. UMP) with UDPGA. The present data indicate that the addition of UDPGA (or glucuronides) to glucuronide-loaded vesicles results in a prompt release of the luminal compound. The effectiveness of *trans*-stimulation of glucuronide efflux by UDPGA is comparable to that of alamethicin, a pore-forming agent [24], because they completely released glucuronides within 1 min (Table 2). These results suggest that the rate-limiting step of glucuronidation is not the capacity of the transporter itself but rather the intraluminal concentrations of anions to be exchanged/transported. The transport process seems to be bi-directional: glucuronides can release each other and UDPGA from the intravesicular space. The exchange seems to be equimolar: during the stimulated efflux of *p*-nitrophenol a similar amount of UDPGA enters the lumen.

As UDPGA accessibility is rate-limiting for glucuronidation in intact microsomes as well as in isolated hepatocytes [32], the measurement of the rate of glucuronidation can be confidently assumed to reflect UDPGA transport. Using this approach we have found that loading of microsomes with glucuronides or uridine derivatives (UMP, UDP-*N*-acetylglucosamine or

UDPGA) *trans*-stimulated UDPGA entry. UMP, which itself caused the weakest stimulation, inhibited the *p*-nitrophenol glucuronide-stimulated UDPGA entry (Table 3). The stimulation of UDPGA entry almost abolished in most cases the latency of (*p*-nitrophenol) UDPGT. This phenomenon cannot be brought about either by damage to the membrane, as the mannose 6-phosphatase latency was unmodified (Table 3), or by the direct effect of the loading compounds on UDPGT (Table 4). In intact microsomes the inhibition of UDPGT activity by the membrane-impermeable phenolphthalein glucuronide, which was overcome by elevating the UDPGA concentration, can be explained by competition for the binding site of the transporter. *p*-Nitrophenol glucuronide, which is able to cross the membrane, caused a similar inhibition both in native and in permeabilized vesicles (Table 4). Although a stimulatory effect of glucuronides on the glucuronidation of their own aglycones has been also reported in guinea-pig microsomes at submillimolar UDPGA concentrations [33], in our own experiments and those of others [34] at higher UDPGA levels glucuronides (as end products) inhibited UDPGT activity.

On the basis of these observations more than one microsomal UDPGA transporter may exist: as well as the UDP-*N*-acetylglucosamine-UDPGA antiport characterized by other authors [6,7] another transporter for the exchange of UDPGA and glucuronides. However, the *cis*-inhibition of glucuronide transport by UMP raises the possibility that the two processes are identical, or their ligand specificity is overlapping.

Besides the characterization of microsomal glucuronide transport, our observations may contribute to the understanding of the phenomenon of latency. In intact microsomes the very low initial rate of glucuronidation, and therefore of UDPGA entry, can be determined by the intraluminal amount of anions, e.g. uridine derivatives, likely to be retained in microsomal vesicles during microsomal preparation. This assumption is supported by the observations that long-term pre-incubation [35] increases the latency, probably by depleting the luminal substances. Glucuronidation having started, glucuronides (and UMP) synthesized intraluminally facilitate the entry of further UDPGA molecules. Consequently the initial rate of UDPGA entry defines the further rate of glucuronidation and, in turn, the rate of glucuronidation defines UDPGA transport. This mechanism can explain the observations that in microsomal vesicles the latencies of different UDPGTs are similar despite their very different activities.

As mentioned, glucuronides can leave hepatocytes through the sinusoidal or canalicular membrane. Low-molecular-mass compounds (less than 350 Da) are excreted preferentially towards blood, and compounds of higher molecular mass to the bile [15]. The mechanism of discrimination is unknown. However, the possibility that selective ER transporters for different glucuronides are present in different regions of the ER network (close to the blood or bile hepatocellular pole) seems unlikely. Present data suggest that ER membranes contain a high-capacity antiport rather unspecific for the transported substrates. Moreover, no microsomal heterogeneity apparently exists as, independently of the loading compound, UDPGA entry is maximal (comparable to the effect of complete microsomal permeabilization). The discrimination between the two routes of excretion is therefore likely to be due to (non-reticular) cytosolic events.

In summary, it is concluded that glucuronides can leave the lumen of endoplasmic reticulum by an antiport. The transport is bi-directional, can be *trans*-stimulated by UDPGA and glucuronides, and can be *cis*-inhibited by UMP. The outward transport of glucuronides, under optimal conditions, is so efficient

that the latency of UDPGT becomes completely absent owing to the stimulated inward transport of UDPGA.

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