# Carrier-mediated transport of uridine diphosphoglucuronic acid across the endoplasmic reticulum membrane is a prerequisite for UDP-glucuronosyltransferase activity in rat liver

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UDP-glucuronosyltransferases (EC 2.4.1.17) is an isoenzyme family located primarily in the hepatic endoplasmic reticulum (ER) that displays latency of activity both *in vitro* and *in vivo*, as assessed respectively in microsomes and in isolated liver. The postulated luminal location of the active site of UDP-glucuronosyltransferases (UGTs) creates a permeability barrier to aglycone and UDP-GlcA access to the enzyme and implies a requirement for the transport of substrates across the ER membrane. The present study shows that the recently demonstrated carrier-mediated transport of UDP-GlcA across the ER membrane is required and rate-limiting for glucuronidation in sealed microsomal vesicles as well as in the intact ER of permeabilized hepatocytes. We found that in both microsomes and permeabilized hepatocytes a gradual inhibition by *N*-ethylmaleimide (NEM) of UDP-GlcA transport into the ER produced a correspondingly increasing inhibition of 4-methylumbelliferone glucuronidation. That NEM selectively inhibited the UDP-GlcA transporter, without affecting intrinsic UGT activity, was demonstrated by showing that NEM had no effect on glucuronidation in microsomes or hepatocytes with permeabilized ER membrane. Additional evidence that UDP-GlcA transport is rate-limiting for glucuronidation in sealed microsomal vesicles as well as in the intact ER of permeabilized hepatocytes was obtained by showing that gradual selective *trans*-stimulation of UDP-GlcA transport by UDP-GlcNAc, UDP-Xyl or UDP-Glc in each case produced correspondingly enhanced glucuronidation. Such stimulation of transport and glucuronidation was inhibited completely by NEM, which selectively inhibited UDP-GlcA transport.

## INTRODUCTION

Conjugation with glucuronic acid enhances the water-solubility of a vast array of endogenous compounds and xenobiotics, thereby facilitating their excretion into bile and urine [1–4]. Glucuronide formation is catalysed by the UDP-glucuronosyltransferase (UGT) system, a supergene family of isoenzymes located primarily in the hepatic endoplasmic reticulum (ER), where they are deeply embedded in the membrane [5]. Two functional properties apply to each of the UGT isoenzymes in native microsomal preparations: first, UGT activity is markedly latent in native microsomes [6]; secondly, UDP-GlcNAc [7–10] and UDP-Xyl [11] enhance UGT activity severalfold in native microsomes.

These properties also seem to apply to UGT in the intact hepatocyte [8]. It has been postulated, but remains unproved, that these properties are related to compartmentation. The catalytic centre of the UGTs has a luminal location [1,9,12–18]. Such topology creates a permeability barrier for substrate access to the active site of the UGT and necessitates translocation across the ER membrane of the hydrophilic donor substrate, UDP-GlcA, which is synthesized in the cytosol [19]. There is evidence for carrier-mediated transport of both UDP-GlcA and UDP-GlcNAc across the ER membrane [20–23]. It has also been demonstrated that UDP-GlcA transport is markedly stimulated by UDP-GlcNAc by a *trans*-stimulation mechanism, whereby UDP-GlcNAc shuttles back and forth across the membrane [24]. Collectively, these findings are consistent with the so-called compartmentation model for regulation of UGT activity. This model postulates that transmembrane transport of UDP-GlcA is rate-limiting for UGT activity in the ER, thereby explaining UGT latency in native microsomes and *in vivo*, as shown in the isolated liver. By taking into account that UDP-GlcNAc enhances the transport of UDP-GlcA into the ER, this hypothesis can also explain the stimulatory effect of UDP-GlcNAc on glucuronidation. However, direct evidence to support this model is still lacking because there have been no reports on the comparison of UGT activities with UDP-GlcA transport rates measured under the same conditions in microsomal vesicles. Moreover, the direct dependence of glucuronidation by intact ER membranes, either in native well-sealed microsomes or in isolated hepatocytes, on recently identified carrier-mediated transport of UDP-GlcA has not been demonstrated.

The present study was undertaken to elucidate the role of carrier-mediated transport of UDP-GlcA across the ER membrane in UGT activity. We examined (1) whether the glucuronidation rate specifically attributable to sealed microsomal vesicles quantitatively corresponds to the microsomal UDP-GlcA uptake rate, (2) whether selective and gradual inhibition or stimulation of UDP-GlcA uptake results in equal impairment or enhancement of glucuronidation by correctly sealed ER vesicles, and (3) whether glucuronidation in hepatocytes depends on, and is determined by, UDP-GlcA transport across the ER membrane.

## EXPERIMENTAL

### Chemicals

UDP-[$^{14}$C]GlcA (11.1 GBq/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). 4-Methylumbelliferone (4-MU) was from Janssen Chimica (Beerse, Belgium). BSA

Abbreviations used: 4-MU, 4-methylumbelliferone; ER, endoplasmic reticulum; NEM, *N*-ethylmaleimide; UGT, UDP-glucuronosyltransferase.

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fraction V, Hepes and all unlabelled nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *Staphylococcus aureus* $\alpha$-toxin (from strain Wood 46) was purified by a pore glass adsorption procedure [25]. All other chemicals were of analytical reagent grade quality. Aqueous solutions were prepared with deionized water.

### Preparation, characterization and treatment of microsomes

Microsomes were prepared from male Wistar rat liver and characterized as described [20]. The structural intactness of the microsomes was verified by determination of mannose-6-phosphatase latency [26], which was more than 95% in all preparations used.

When labile phosphate esters (e.g. ATP) were present in the incubation medium, we used a radioactive assay for measurement of mannose-6-phosphatase activity [27]. The microsomal vesicles were completely disrupted by incubation with the detergent Chapso for 60 min at 0 °C, as previously described [26]. Stable permeabilization of the microsomal membrane by using *S. aureus* $\alpha$-toxin (125 $\mu$g of toxin/mg of protein) was produced as reported elsewhere [28]. Loading microsomes with nucleotide sugars was done as follows. The vesicles were first incubated for 150 min at 37 °C. This preincubation was performed to dissipate a luminal pool of endogenous nucleotides, which when present inside the vesicles *trans*-stimulates the microsomal uptake of nucleotide sugars [16,20,23]. Thereafter the vesicles were incubated at 37 °C in the absence (control vesicles) or in the presence of 5 mM UDP-GlcNAc, 5 mM UDP-Xyl or 5 mM UDP-Glc. This was followed by re-isolation and washing (twice) of the vesicles by ultracentrifugation (104000 ***g*** for 30 min at 4 °C) [29].

### Isolation and permeabilization of rat hepatocytes

Rat hepatocytes were isolated by the collagenase perfusion method [30]. The cells were maintained in Krebs–Henseleit medium supplemented with 1.2 mM $CaCl_2$ and saturated with carbogen. Cell viability was estimated by determining the exclusion of Trypan Blue and the leakage of LDH. Only cell suspensions with a cell viability higher than 90% were used. Selective permeabilization of the plasma membrane was performed by incubating 10 mg of protein hepatocytes with 50 $\mu$g of *S. aureus* $\alpha$-toxin for 8 min at 37 °C. To measure the total UGT activity in the hepatocytes, the cells were sonicated twice for 10 min with an exponential probe sonicator (20 kHz, amplitude 10 $\mu$m). Thereafter the preparation was incubated for 5 min in 2.6 mM Chapso.

### Various assays

Microsomal uptake of radiolabelled nucleotide was assessed by a previously reported rapid filtration technique [20]. 4-MU glucuronidation was assayed as follows. The enzymic incubation mixture consisted of the so-called transport assay incubation medium [20] supplemented with 1 mM 4-MU. This 4-MU was added as an 8 mM solution, prepared by diluting a stock solution of 1.5 M 4-MU in DMSO with 1 mM BSA. The glucuronidation assay was started by the addition of 25 $\mu$M UDP-GlcA and stopped by transferring 100 $\mu$l portions into 1 ml of 1 mM trichloroacetic acid. Extraction of unreacted substrate and determination of the glucuronide were done as described [31]. Protein was determined by the protein–Coomassie Blue dye binding method with BSA as calibration standard [32].

## RESULTS AND DISCUSSION

### Determination of UGT activities selectively in the correctly sealed ER vesicles of native microsomal preparations

During the preparation of native microsomes, some vesicles become disrupted or leaky. We describe the well-sealed, right-side-out vesicles as correctly sealed ER vesicles and the remaining ER structures as perturbed microsomes. In our microsomal preparations, perturbed microsomes amounted to less than 5% of the total microsomes, as assessed by the assay of mannose-6-phosphatase latency [26]. Although perturbed microsomes form only a minor fraction of the total membranes in native microsomal preparations, they contribute substantially to the basal glucuronidation activity. To determine the specific activity of UGT selectively in the fraction of perturbed microsomes, we permeabilized all ER membranes in the microsomal preparation by using the pore-forming $\alpha$-toxin from *S. aureus*. The 4-MU-UGT activity (mean $\pm$ S.D.) in these fully permeabilized microsomes amounted to $8773 \pm 440$ ($n = 3$) pmol/min per mg of protein, which averages 31 times the glucuronidation activity [$254 \pm 73$ ($n = 3$) pmol/min per mg of protein] found in our native microsomal preparations. The glucuronidation rate in correctly sealed microsomes was then estimated by subtracting from the basal rate the glucuronidation activity contributed by the perturbed microsomes [33] as assessed by the mannose-6-phosphatase latency. For the above-mentioned case of 4-MU glucuronidation, we calculated that if 1.4% of the native microsomal vesicles were disrupted, then these broken vesicles accounted for 50% of the basal glucuronidation activity. This demonstrates that the contribution by broken vesicles to the glucuronidation activity in native microsomal preparations must be taken into account when UDP-GlcA transport activities are compared with glucuronidation activities. Moreover, a small error in the estimation of the degree of membrane disruption can lead to a considerable error in estimating the contribution of the disrupted microsomes to the basal glucuronidation activity.

### Selective inhibition of microsomal UDP-GlcA transport inhibits glucuronidation by correctly sealed microsomes

We examined the effects of variable degrees of inhibition of microsomal UDP-GlcA transport on UGT activity in native microsomes. *N*-Ethylmaleimide (NEM) was used to inhibit UDP-GlcA transport selectively. This compound effectively inhibits microsomal UDP-GlcA transport [20] and only minimally affects the intrinsic UGT activity [34]. In the absence and the presence of 2.5 mM NEM, the 4-MU glucuronidation activity (mean $\pm$ S.D.) in $\alpha$-toxin-permeabilized microsomes was respectively $8773 \pm 440$ ($n = 3$) and $7853 \pm 574$ ($n = 3$) pmol/min per mg of protein. A spectrum of degrees of impairment of UDP-GlcA transport was produced by pretreating the microsomes with different concentrations of NEM. Figure 1 shows that there existed a remarkably close parallelism between the changes in transport activity and the alterations in activity of UGT in native microsomes. At high concentrations of NEM, UDP-GlcA uptake was almost completely restrained, whereas a rest activity of UGT remained, reflecting the enzyme activity attributable to the perturbed microsomes. By subtraction of the UGT activity attributed to the perturbed vesicles from the basal UGT activity found in native preparations, we calculated the UGT activity originating specifically from correctly sealed vesicles. Figure 1 (broken line) shows that (1) such corrected values for enzyme activity were correlated with UDP-GlcA transport rates and (2) the gradually increasing inactivation of

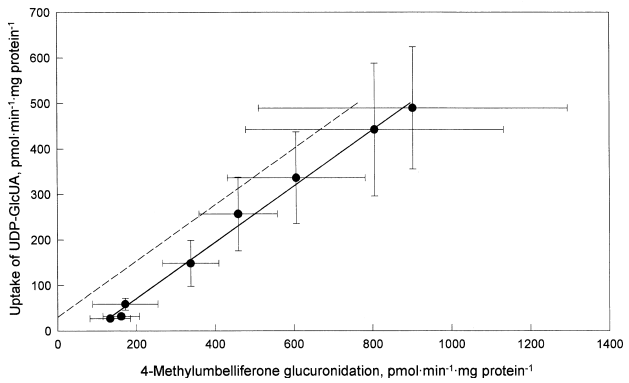


**Figure 1 Relationship between UDP-GlcA uptake and 4-MU glucuronidation in microsomes**

Microsomes were pretreated for 15 min at 4 °C with various concentrations (between 0 and 10 mM) of NEM. Thereafter, initial rates of UDP-GlcA uptake and of glucuronidation of 4-MU were determined (see the Experimental section). The figure illustrates the relationship between uptake and glucuronidation rates. Values for glucuronidation in correctly sealed vesicles (broken line) were computed by subtracting from the basal conjugation rate the glucuronidation rate found in the presence of 10 mM NEM. The data points represent the means ± S.E.M. for five independent experiments.

UDP-GlcA transport resulted in a gradual abolition of the glucuronidation reaction. These results imply that UDP-GlcA transport is a prerequisite for glucuronidation by correctly sealed vesicles.

### Stimulation of microsomal UDP-GlcA transport produces a correspondingly large enhancement of UGT activity

Another approach to verifying our working hypothesis that UDP-GlcA is necessary for glucuronidation was to assay UGT activity under conditions whereby various degrees of stimulation of UDP-GlcA transport were produced. Microsomal uptake of UDP-GlcA can be stimulated by preloading the microsomes with UDP-GlcNAc, UDP-Glc or UDP-Xyl [20,21,24]. UDP-GlcNAc-preloaded vesicles displayed very high UDP-GlcA uptake rates when compared with control, non-preloaded vesicles (Table 1). UDP-GlcA uptake was also markedly stimulated by preloading the vesicles with UDP-Xyl and, to a lesser extent, by preloading the vesicles with UDP-Glc. In each case the stimulation of uptake of UDP-GlcA was inhibited by NEM (Table 1). The residual radiolabel associated with the microsomal vesicles after treatment with NEM was due to the incorporation of GlcA into endogenous acceptors [20]. Approximately 35 % of the total vesicle-associated radiolabel was precipitable with trichloroacetic acid (results not shown), indicating that the radiolabel was incorporated into lipid and/or protein. As expected, *trans*-stimulation of uptake of UDP-GlcA by preloading the microsomal vesicles with UDP-Glc, UDP-Xyl and UDP-GlcNAc also resulted in an NEM-inhibitable stimulation of 4-MU glucuronidation (Table 1). The stimulation was most pronounced when the vesicles had been preloaded with UDP-GlcNAc; the next most pronounced stimulation, in decreasing order, was when the microsomes had been preloaded with UDP-Xyl and with UDP-Glc. The residual glucuronidation found after treatment with NEM was the same for the various experimental conditions (Table 1) and reflected the glucuronidation activity evoked by the perturbed microsomes. The UGT activity that is postulated to be dependent on UDP-GlcA transport, i.e. the NEM-inhibitable glucuronidation activity, approximated the UDP-GlcA transport rate for each of the experimental conditions ($P$ 0.05 for control, UDP-Glc and UDP-GlcNAc). These results illustrate that experimental conditions that stimulate UDP-GlcA transport also enhance glucuronidation to the same extent; this therefore underscores the notion that transport of UDP-GlcA is necessary and rate-limiting for glucuronidation in correctly sealed ER vesicles.

### Importance of UDP-GlcA transport for glucuronidation by permeabilized hepatocytes

To assess the importance of UDP-GlcA transport for glucuronidation in intact, unfragmented ER, we determined the glucuronidation of 4-MU under various conditions in permeabilized hepatocytes. The plasma membrane of hepatocytes was permeabilized for small-molecular-mass molecules with *S. aureus* $\alpha$-toxin. As shown in Figure 2, NEM inhibited the glucuronidation of 4-MU in isolated hepatocytes in a concentration-dependent manner. In contrast, NEM did not affect the glucuronidation of 4-MU by sonicated hepatocytes in which the ER membranes were fully disrupted. The total glucuronidation of 4-MU in sonicated hepatocytes amounted to 2.3 nmol/min per mg of protein in the absence of NEM and 2.4 nmol/min per

**Table 1 Effect of preloading microsomes with UDP-GlcNAc, UDP-Xyl or UDP-Glc on UDP-GlcA uptake and on 4-MU glucuronidation**

Control microsomes (non-preloaded microsomes) and microsomes preloaded with UDP-Glc, UDP-Xyl or UDP-GlcNAc (see the Experimental section) were used to study the uptake of UDP-GlcA and the glucuronidation of 4-MU. Uptake and glucuronidation were measured in the absence (−NEM) or the presence (+NEM) of 2.5 mM NEM, which was added 1 min before the reaction was started. Glucuronidation and UDP-GlcA transport rates postulated to depend specifically on the NEM-inhibitable carrier for UDP-GlcA in the ER membrane were calculated by subtracting the rates found in the presence of NEM from the rates in the absence of NEM. These calculated values are shown under 'carrier-dependent'. Results represent the means ± S.D. for three determinations in different microsomal preparations.

| | 4-MU glucuronidation (pmol/min per mg of protein) | | | UDP-GlcA uptake (pmol/min per mg of protein) | | |
|---|---|---|---|---|---|---|
| Loading condition | −NEM | +NEM | Carrier-dependent | −NEM | +NEM | Carrier-dependent |
| Control | 304 ± 21 | 301 ± 10 | 3 ± 31 | 91 ± 16 | 45 ± 10 | 46 ± 26 |
| UDP-Glc | 408 ± 82 | 290 ± 64 | 118 ± 146 | 167 ± 25 | 47 ± 5 | 120 ± 30 |
| UDP-Xyl | 1283 ± 100 | 267 ± 20 | 1016 ± 120 | 775 ± 78 | 50 ± 10 | 725 ± 88 |
| UDP-GlcNAc | 1513 ± 141 | 272 ± 10 | 1241 ± 161 | 1095 ± 104 | 63 ± 18 | 1032 ± 122 |

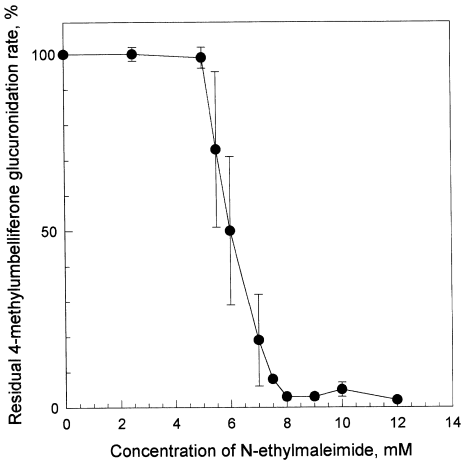


**Figure 2 Effect of NEM on 4-MU glucuronidation by permeabilized hepatocytes**

*S. aureus* $\alpha$-toxin-permeabilized hepatocytes (7 mg/ml protein) were preincubated at 37 °C for 3 min with various concentrations of NEM. Thereafter the glucuronidation of 4-MU was determined. The figure shows, as a function of the NEM concentration in the medium, the residual glucuronidation rate expressed as a percentage of the UGT activity found in the absence of NEM. Each data point represents the mean $\pm$ S.E.M. for five independent experiments.

mg of protein in the presence of 10 mM NEM. Together these results indicate that UDP-GlcA transport is a necessary and rate-limiting step in the overall glucuronidation by the intact, unfragmented ER.

As observed with microsomes, UGT activity could be stimulated by preincubating the permeabilized hepatocytes with UDP-GlcNAc. The glucuronidation of 4-MU by permeabilized hepatocytes amounted to 1.3 nmol/min per mg of protein in the absence of UDP-GlcNAc and 2.2 nmol/min per mg of protein in the presence of 2 mM UDP-GlcNAc. This UDP-GlcNAc-induced stimulation was totally abolished by NEM (results not shown), indicating that UDP-GlcNAc activated glucuronidation by activating UDP-GlcA transport.

The observation that UDP-GlcNAc is able to elicit 97 % of the total UGT activity in permeabilized hepatocytes illustrates the potential physiological role of UDP-GlcNAc in the regulation of UGT.

It is unlikely that the low latency of 4-MU-UGT activity is due to a disrupted ER environment in permeabilized hepatocytes because mannose-6-phosphatase latency was $96.8 \pm 0.1$ % and 4-MU-UGT activity was completely blocked by NEM. The latency calculated from the 4-MU-UGT activity found after inhibition of UDP-GlcA transport with NEM was 99 % and thus comparable to mannose-6-phosphatase latency.

In summary, the selective inhibition or stimulation of UDP-GlcA transport by NEM or UDP-GlcNAc resulted in inhibition or stimulation respectively of glucuronidation by permeabilized hepatocytes.

Collectively, our findings with microsomal preparations and isolated hepatocytes support the hypothesis that the recently identified carrier-mediated transport of the co-substrate UDP-GlcA across the ER plays a pivotal and regulatory role in overall hepatic glucuronidation *in vivo*.

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