ATP-dependent transport of unconjugated bilirubin by rat liver canalicular plasma membrane vesicles

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The transport of highly purified ³H-labelled unconjugated bilirubin (UCB) was investigated in rat liver plasma membrane vesicles enriched in the canalicular domain and found to be stimulated (more than 5-fold) by the addition of ATP. Other nucleotides, such as AMP, ADP, GTP and a non-hydrolysable ATP analogue (adenosine 5'-[α,β -methylene] triphosphate), did not stimulate [³H]UCB transport, indicating that ATP hydrolysis was necessary for the stimulatory effect. [³H]UCB uptake occurred into an osmotically sensitive space. At an unbound bilirubin concentration ([Bf]) below saturation of the aqueous phase (no more than 70 nM UCB), the ATP-dependent transport followed saturation kinetics with respect to [Bf], with a K_m of 26 ± 8 nM and a V_{max} of 117 ± 11 pmol per 15 s per mg of protein. Unlabelled UCB inhibited the uptake of [³H]UCB, indicating

that UCB was the transported species. Inhibitors of ATPase activity such as vanadate or diethyl pyrocarbonate decreased the ATP effect ($59 \pm 11\%$ and 100% respectively). Daunomycin, a known substrate for multidrug resistance protein-1, and taurocholate did not inhibit the ATP-dependent [³H]UCB transport, suggesting that neither *mdr-1* nor the canalicular bile acid transporter is involved in the canalicular transport of UCB. [³H]UCB uptake (both with and without ATP) in canalicular vesicles obtained from TR⁻ rats was comparable to that in vesicles obtained from Wistar rats, indicating that the canalicular multispecific organic anion transporter, cMOAT, does not account for UCB transport. These results indicate that UCB is transported across the canalicular membrane of the liver cell by an ATP-dependent mechanism involving an as yet unidentified transporter.

INTRODUCTION

A large number of organic anions are transported across the canalicular plasma membrane by active, ATP-dependent mechanisms. These transport activities are mediated by various carrier proteins belonging to the so-called ATP binding cassette (ABC) proteins [1–3], which have in common two ATP-binding domains situated in the cytoplasmic domain. ATP hydrolysis is required for the transport function, although the exact mechanism by which ATP mediates the transport activity is not fully understood.

Many ABC proteins have been functionally identified and some of them have been cloned [1,4]. An important class of ABC proteins is those belonging to the multidrug resistance protein (*mdr*) family, which mediate the biliary excretion of many xenobiotic and endogenous substrates such as drugs and lipids [4]. Another ABC protein, the so-called canalicular multispecific organic anion transporter (cMOAT), also known as *mrp-2*, has been characterized and shown to mediate the canalicular secretion of a variety of organic acids, including glutathione-conjugated substrates [5] and bilirubin conjugates [6]. cMOAT has been shown to be defective in both animals (the TR⁻/GY rat [7] and humans (Dubin–Johnson syndrome [8]) with hereditary chronic conjugated hyperbilirubinaemia.

In contrast with conjugated bilirubins, unconjugated bilirubin (UCB) is found in bile in only very low concentrations, where its appearance is believed to arise from the hydrolysis of secreted conjugates and from the excretion of the small quantity of UCB photoisomers formed under natural lighting [9]. However, significant concentrations of UCB (up to $0.5 \mu\text{M}$) have been found in the bile of the Gunn rat [10], an animal in which the hydrolysis

mechanism cannot be operative because it cannot conjugate bilirubin. Interestingly, in the Gunn rat, the biliary secretion of UCB increases linearly with bile acid output [10].

In this study we report the transport of [³H]UCB by canalicular rat liver plasma membrane vesicles (cLPMV). This transport is ATP-dependent and does not involve the canalicular cMOAT or bile acid (cBAT) transporters.

MATERIALS AND METHODS

Preparations of cLPMV

Liver plasma membrane vesicles highly enriched in the canalicular domain (cLPMV) were prepared from female Wistar rats (60–90 days old, 200–250 g) by the method of Meier et al. [11]. Animals were allowed free access to a standard laboratory chow (Nissan, Milan, Italy) and tap water, and were housed in an environment of constant temperature and humidity with alternating 12 h cycles of light and darkness. Livers were harvested at 08:00h. Enrichment in the canalicular domain was assessed by measuring canalicular marker enzymes (5'-nucleotidase and Mg-ATPase) by the methods of Emmelot et al. [12] and Schoner et al. [13] respectively. These enzyme activities were increased more than 60-fold over those of the starting homogenate. In agreement with previous reports [14], contamination by basolateral plasma membranes, as assessed by the activity of Na/K-ATPase, was negligible, cLPMV were stored in liquid nitrogen until use (within 4 weeks), thawed at 37 °C and revesiculated by passage 20 times through a 25-gauge needle [15]. Protein concentration was measured by the bicinchoninic acid protein assay [16].

Abbreviations used: UCB: unconjugated bilirubin; [Bf]: unbound bilirubin concentration; cLPMV: canalicular liver plasma membrane vesicles; HSA: human serum albumin; DEPC: diethyl pyrocarbonate; ABC proteins: ATP binding cassette proteins; AMP-PCP, adenosine 5'-[α,β -methylene] triphosphate; cMOAT, canalicular multispecific organic anion transporter; *mdr*, multidrug resistance protein.

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Preparation and purification of [³H]UCB

[³H]UCB, specific radioactivity 46000 d.p.m./ μ g, was prepared as described previously [17,18]. Labelled polar impurities were removed by dissolving the UCB in chloroform (0.9 mM) and extracting five times with 20 vol. of 0.1 M phosphate buffer, pH 7.0. After dehydration of the chloroform by filtration through a chloroform-presaturated Whatman no. 40 paper and determination of the amount and specific activity of the UCB, the solution was distributed among vials, evaporated to dryness, sealed under argon and stored in the dark at -20°C until use. Every 4 weeks, UCB was dissolved in DMSO, and the quantity and specific radioactivity of UCB were determined by diazo assay [19] and radioassay. All these and subsequent procedures involving UCB were performed under dim red light to minimize the photodegradation of UCB [18].

Preparation of [³H]UCB human serum albumin (HSA) solutions

[³H]UCB (5–10 μ g) was dissolved in 30 μ l of DMSO and added to 100 vol. of buffered 10 μ M HSA (fatty acid-free, catalogue no. A3782, lot no. 93H9345; Sigma, St. Louis, MO, U.S.A.) in 250 mM sucrose/20 mM Hepes/Tris buffer (pH 7.0). The UCB-to-HSA ratio varied from 0.1 to 0.65. Some experiments were also performed at 20 μ M HSA with virtually identical results. The concentration of unbound UCB ([Bf], nM) was calculated from UCB/HSA binding constants previously determined by ultrafiltration in the same medium [17].

UCB uptake studies in cLPMV

Uptake of [³H]UCB was determined by rapid filtration after 15 s as previously described for basolateral LPMV [17]. For each uptake experiment, 25 μ l of revesiculated cLPMV (70–100 μ g of protein) were added to 100 μ l of the UCB/HSA solution in 250 mM sucrose/20 mM Hepes/Tris buffer (pH 7.0). The final free [Bf], ranging from 7.5 to 70 nM, was varied by changing the UCB-to-HSA ratio at a constant [HSA] of 10 μ M. To evaluate the effect of supersaturation of the aqueous phase with unbound UCB, uptake was also determined at a [Bf] of between 80 and 215 nM and UCB-to-HSA ratios higher than 0.50 [17]. After 15 s of incubation at either 25 or 4 $^{\circ}\text{C}$, 1.0 ml of ice-cold stop solution (medium lacking UCB and HSA) was added and the sample was rapidly filtered through nitrocellulose membranes with a pore size of 0.4 μ m (HAWP 0.24; Millipore, Bedford, MA, U.S.A.) previously soaked in the stop solution. The filters were washed three times with 2.5 ml of ice-cold stop solution and dried, then 10 ml of scintillation medium (Filtercount; Packard, Groningen, The Netherlands) was added. Radioactivity was measured in a liquid-scintillation spectrometer (Beta V; Kontron, Milan, Italy) with automatic quench corrections with external reference standards. Preliminary experiments, in which the vesicles were incubated for different periods (5–30 s) at different [Bf] (10–70 nM) indicated that uptake was linear up to 15 s, as reported for basolateral LPMV [17]. Accordingly, the 15 s sampling time was chosen for determining the initial uptake rate. Non-specific binding of UCB to cLPMV was determined from a paired measurement of UCB uptake by cLPMV incubated at 4 $^{\circ}\text{C}$. The difference between the 25 and 4 $^{\circ}\text{C}$ values was considered as the specific uptake of UCB.

To determine whether uptake occurred into an osmotically sensitive space, the intravesicular volume was changed by varying the sucrose concentration in the medium from 250 mM to 1 M.

All the determinations were performed at least in triplicate with three or more separate membrane preparations. The co-

efficient of variation was 3 % for a given vesicle preparation and 8 % between different vesicle preparations.

Effect of ATP and other nucleotides

ATP and other nucleotides [AMP, ADP, the non-hydrolysable ATP analogue adenosine 5'-[α,β -methylene] triphosphate (AMP-PCP) and GTP], obtained from Sigma, were added as their sodium salts to final concentrations ranging from 0.5 to 5 mM. In some experiments the magnesium salt of ATP was used. The nucleotides were dissolved in water, the pH of the solution was adjusted to 7.0 with Tris, and the nucleotides were added to the medium immediately before addition of the vesicles. Uptake was then measured at 15 s, and the effect induced by the nucleotides was calculated as the difference between the specific initial uptake rates (rate obtained at 25 $^{\circ}\text{C}$ minus the rate at 4 $^{\circ}\text{C}$) observed in the presence and in the absence of the nucleotide. UCB uptake was also measured in the presence of a inside-positive membrane potential generated by the addition of valinomycin (50 mg/mg of protein) and iso-osmotic replacement of sucrose with 50 mM KCl. UCB uptake rates were virtually identical in the presence and in the absence of membrane potential.

Statistical analysis of the data

Results are expressed as means $\pm$ S.D. for at least three experiments. Kinetic analyses (K_m and V_{max}) were performed with a computer-assisted program (SIGMAPLOT; Landel Scientific, San Rafael, CA, U.S.A.) in accordance with the Michaelis-Menten equation. The significance of differences was assessed with a two-tailed Student's *t* test for unpaired data.

RESULTS

Figure 1(A) shows the specific initial uptake rates of UCB at various [Bf] in the presence or absence of 1.5 mM ATP. In the absence of ATP the uptake rates were linearly related to [Bf] from 0 to 210 nM. Addition of ATP engendered a steep increase in UCB uptake with increases in [Bf] up to 70 nM. When [Bf] exceeded aqueous phase supersaturation (at least 70 nM), the ATP-dependent UCB uptake became linear, with a slope (0.36) that was virtually identical to that (0.35) obtained in the absence of ATP. When the curve obtained in the absence of ATP was subtracted from the curve in its presence, a saturative function was obtained representing ATP-dependent uptake with a K_m of 26 ± 8 nM and a V_{max} of 117 ± 11 pmol per 15 s per mg of protein (Figure 1B).

To check whether the ATP-dependent initial uptake occurred into an osmotically sensitive space, the effects of variations in the intravesicular space were studied at a fixed [Bf] of 50 nM in the presence and in the absence of 1.5 mM ATP. Figure 2 shows that the specific, ATP-dependent rate of UCB uptake was linearly correlated with the reciprocal of the sucrose concentration ($y = 18x + 8$, $r^2 = 0.999$, $P < 0.001$), indicating that the ATP-dependent UCB uptake occurred into an osmotically sensitive space. By extrapolating the value to infinite osmolality ($1/[\text{sucrose}] = 0$), theoretically representing zero intravesicular space, it could be calculated that about 10 % of UCB was passively absorbed to vesicles, whereas the remaining 90 % was taken up by the vesicles.

Table 1 demonstrates that the stimulation of UCB transport by ATP was specific for this nucleotide. At a comparable nucleotide concentration (1.5 mM), ATP induced a 524 ± 64 % stimulation of UCB uptake ($P < 0.001$), whereas AMP, ADP or

Figure 1 Dependence of specific [^3H]UCB uptake by cLPMV on unbound UCB concentration in the presence and in the absence of 1.5 mM ATP

[^3H]UCB uptake was determined in cLPMV in the presence and in the absence of 1.5 mM ATP (sodium salt). (A) Initial (15 s) uptake rates of [^3H]UCB (specific radioactivity 46 000 d.p.m./ μg) obtained with (■) or without ATP (□). For each uptake experiment, 25 μl of revesiculated cLPMV (70–100 μg of protein) was added to 100 μl of the [^3H]UCB/HSA solution [250 mM sucrose/20 mM Hepes/Tris buffer (pH 7.0)]. The uptake rate was calculated as the difference between the values obtained at 25 $^{\circ}\text{C}$ and at 4 $^{\circ}\text{C}$. [Bf] was varied by changing the UCB-to-HSA ratio at a constant [HSA] of 10 μM , uptake measured at [Bf] either below (no more than 70 nM) or above (more than 70 nM) supersaturation of the aqueous phase of unbound UCB. When [Bf] was higher than 80 nM, the UCB-to-HSA ratio was higher than 0.50. After 15 s of incubation either at 25 $^{\circ}\text{C}$ or at 4 $^{\circ}\text{C}$, 1.0 ml of ice-cold stop solution (medium lacking UCB and HSA) was added and the sample was rapidly filtered through nitrocellulose membranes with a pore size of 0.45 μm . The filters were washed three times with 2.5 ml of ice-cold stop solution and dried; 10 ml of scintillation medium was then added. Radioactivity was measured in a liquid-scintillation spectrometer (Beta V; Kontron, Milan, Italy) with automatic quench correction with external reference standards. In the absence of ATP, transport is linearly correlated with [Bf]. In the presence of ATP, uptake kinetic is compatible with a saturation curve up to a [Bf] of 70 nM. At [Bf] above saturation (70 nM), the ATP-dependent uptake is linear, with a slope (0.35) virtually identical with that (0.36) obtained in the absence of ATP. (B) Specific [^3H]UCB transport (●) obtained when the specific uptake rates observed in the absence of ATP were subtracted from those observed in the presence of ATP [see (A)]. The kinetics follows a saturation behaviour with an apparent K_m of 28 ± 8 nM and a V_{max} of 117 ± 11 pmol per 15 s per mg of protein. Each point represents the mean $\pm$ S.D. for at least three determinations.

Figure 2 Effect of the intravesicular space on ATP-dependent UCB uptake

The effects of variations of the intravesicular space were studied at a fixed [Bf] of 50 nM and [HSA] of 10 μM in the presence of 1.5 mM ATP. The intravesicular volume was changed by varying the sucrose concentration in the medium from 250 mM to 1 M. The specific ATP-dependent rate of UCB uptake was linearly correlated with the reciprocal of the sucrose concentration ($y = 18x + 8$, $r^2 = 0.999$, $P < 0.01$), indicating that ATP-dependent UCB uptake by the cLPMV occurred into an osmotically sensitive space.

a non-hydrolysable ATP analogue (AMP-PCP) did not. GTP also did not increase UCB uptake, whereas the addition of MgATP stimulated UCB transport by an additional 47% ($824 \pm 72\%$) compared with an equimolar concentration of the sodium salt of ATP ($P < 0.01$).

When ATP (1.5 mM) was added to cLPMV in the absence of UCB and the vesicles were incubated at 25 $^{\circ}\text{C}$ for 60 min, the

Table 1 Effect of the addition of different nucleotides (1.5 mM) and oligomycin on specific [^3H]UCB uptake in cLPMV

Nucleotides were sodium salts, with the exception of MgATP when indicated. Specific UCB transport was measured at a [Bf] of 53 nM at pH 7.0 in 250 mM sucrose/20 mM Hepes/Tris buffer as the difference between the values observed at 25 $^{\circ}\text{C}$ and at 4 $^{\circ}\text{C}$. Results are expressed as means $\pm$ S.D. for three experiments for each nucleotide. Significance levels: * $P < 0.001$ compared with control; † $P < 0.01$ compared with ATP (sodium salt).

	Specific UCB uptake rate (pmol per 15 s per mg of protein)	Uptake (% of control)
None (control)	20.4 ± 3.8	100
ATP (Na^+ salt)	107.5 ± 13.5	$524 \pm 64^*$
ATP (Mg^{2+} salt)	168.1 ± 18.9	$824 \pm 72^{\dagger}$
GTP	21.4 ± 4.2	100 ± 17
AMP	18.4 ± 3.0	90 ± 10
ADP	17.1 ± 4.9	85 ± 11
AMP-PCP	20.5 ± 4.0	100 ± 18

concentration of ATP at the end of incubation, measured by the method of Trauttschold et al. [20], was found to be virtually zero, indicating total hydrolysis of the nucleotide. If UCB at a [Bf] of 65 nM was then added to these vesicles preincubated with ATP, the UCB uptake was identical with that found in the absence of ATP (45 ± 12 compared with 46 ± 13 pmol per 15 s per mg of protein; n.s.). These results rule out a role of the phosphate ions generated by ATP hydrolysis in the stimulatory effect of added ATP.

As shown in Figure 3, on increasing the ATP concentration from 0.5 to 5.0 mM the specific, ATP-dependent transport of UCB increased in a saturable fashion with an apparent K_m of ATP of 1.6 ± 0.4 mM ATP.

Figure 3 ATP dependence of $[^3\text{H}]$ UCB transport in canalicular plasma membrane vesicles

$[^3\text{H}]$ UCB transport was studied at a fixed $[\text{Bf}]$ (30 nM) in the presence of increasing concentrations of ATP (0.5–10 mM). From the curve, an apparent K_m for ATP of 1.6 ± 0.4 mM was derived. Each point represents the mean $\pm$ S.D. for at least three determinations.

Table 2 Effect of inhibitors of ATPase activity, daunomycin and taurocholate on ATP-induced UCB uptake in cLPMV

Specific UCB transport was measured at a $[\text{Bf}]$ of 66 nM at pH 7.0 in 250 mM sucrose/20 mM Hepes/Tris buffer as the difference between the values observed at 25 °C and at 4 °C. Results are expressed as means $\pm$ S.D. for three experiments for each nucleotide. Significance levels compared with control: * $P < 0.001$; ** $P < 0.01$.

Inhibitor added	Specific UCB uptake rate (pmol per 15 s per mg of protein)	Inhibition (%)
None (control)	100.5 $\pm$ 22.1	0
Vanadate (100 μM)	41.2 $\pm$ 8.0	59 $\pm$ 11**
DEPC (100 μM)	2.0 $\pm$ 1.3	100 $\pm$ 10*
Oligomycin (10 μM)	100.7 $\pm$ 14.9	0
Taurocholate (50 μM)	97.2 $\pm$ 13.6	3 $\pm$ 1
Daunomycin (25 μM)	93.3 $\pm$ 20.8	7 $\pm$ 2

To check whether UCB was indeed the transported species, unlabelled unconjugated UCB, purified by the method of McDonagh and Assisi [21], was added in the presence of 10 μM HSA at a final $[\text{Bf}]$ of 35 nM to a solution containing an equal concentration of $[^3\text{H}]$ UCB. The addition of unlabelled UCB decreased the transport of $[^3\text{H}]$ UCB by 50%, indicating that UCB was the transported species.

Table 2 shows the effect of known ATPase inhibitors on the ATP-induced uptake of UCB in the presence of 1.5 mM ATP. The addition of 100 μM vanadate inhibited the ATP-induced UCB uptake by 59 $\pm$ 11%, whereas diethyl pyrocarbonate (DEPC), which reportedly inhibits mainly the activity of ecto-ATPases in the canalicular membrane [22], fully abolished ATP-dependent UCB transport. In contrast, either 25 μM daunomycin, a substrate for the *mdr-1* ABC transporter [23], or 50 μM taurocholate had no effect on ATP-dependent $[^3\text{H}]$ UCB transport. ATP-dependent UCB transport was also unaffected by the addition of 10 μM oligomycin, excluding transport by contaminating mitochondrial ATPases.

Both basal (no ATP) and specific, ATP-dependent transport of UCB were the same in cLPMV obtained from mutant TR^- rats, which do not express cMOAT [7], as in cLPMV from

Table 3 ATP-dependent UCB uptake by cLPMV from Wistar rats (normal) and TR^- rats (deficient in cMOAT)

cLPMV were prepared from control Wistar rats and from the TR^- as described in the Materials and methods section. $[^3\text{H}]$ UCB uptake was measured in the presence and in the absence of 1.5 mM ATP at a $[\text{Bf}]$ of 50 nM. No significant difference in ATP-dependent UCB transport was observed between the two strains of rats. Results are expressed as means $\pm$ S.D. for three experiments.

Rat strain	Specific UCB uptake rate (pmol per 15 s per mg of protein)	
	Without ATP	With ATP
Wistar (NO ATP)	27.4 $\pm$ 5.3	101.3 $\pm$ 9.8
TR^- (NO ATP)	25.8 $\pm$ 4.9	98.2 $\pm$ 11.3

normal Wistar rats (Table 3). This indicates that cMOAT is not responsible for ATP-dependent UCB transport by cLPMV. Moreover, experiments performed by using cLPMV obtained from Gunn rats yielded results comparable to those obtained with Wistar rats, indicating that UCB conjugation is not involved in the observed phenomenon.

DISCUSSION

Our results indicate that UCB is transported by rat cLPMV via an ATP-dependent mechanism. The effect of ATP is specific, as indicated by the lack of stimulation when other nucleotides such as ADP, AMP or GTP were added. The failure of the non-hydrolysable ATP analogue AMP-PCP to stimulate UCB transport indicates that ATP hydrolysis is required. This is also supported by the experiment in which UCB transport was not enhanced when ATP was added but allowed to hydrolyse before the UCB was added. That experiment also shows that the effect is not mediated by the phosphate ions released by ATP hydrolysis. Further evidence for the importance of ATP hydrolysis comes from the effects of different inhibitors of ATPase activity. The stimulatory effect of added ATP was decreased by 60% by vanadate, a known inhibitor of P-ATPases [24], and completely abolished by DEPC, a known inhibitor of ecto-ATPases [22,25].

Several aspects of our experimental design require comment. All the uptake studies were performed at 15 s in the absence of an ATP-regenerating system, which includes Mg^{2+} ions and creatine kinase [26]. This was done for two reasons: the addition of MgCl_2 led to precipitation of magnesium salts of UCB; and added creatine kinase might have interacted with UCB, thus altering $[\text{Bf}]$. Nevertheless, despite the absence of the ATP-regenerating system, 80% of the initial 1.5 mM ATP remained at the end of the 15 s incubation, indicating that ATP had been consumed but not severely depleted. When Mg^{2+} salts were present in the system, owing to the replacement of the sodium salt with the magnesium salt of the nucleotide, the stimulatory effect was significantly increased, indicating that the transporting system is Mg^{2+} -sensitive as described for several ABC proteins related to the activity of endo-ATPases [27].

As with our studies of $[^3\text{H}]$ UCB transport by basolateral LPMV [17], the present studies were performed at pH 7.0, rather than the physiological pH of 7.4, to minimize $[^3\text{H}]$ exchange and oxidation of the $[^3\text{H}]$ UCB. Finally, the linear increase in UCB transport with increasing $[\text{Bf}]$, which occurred at $[\text{Bf}] \geq 70$ nM in the presence of ATP, paralleled the non-specific adsorption of UCB that occurred in the absence of ATP. This suggests that the microsuspended UCB that was present above saturation was not

transported by, but only adsorbed on, the vesicles. It is noteworthy that the K_m for UCB for the ATP-mediated process (26 ± 8 nM) was well below the saturating aqueous solubility of unbound UCB (more than 70 nM), and that the K_m for ATP was in the physiologically relevant range of ATP concentrations in the hepatocyte.

Altogether, these results indicate that UCB is transported by an ATP-dependent mechanism, and that the stimulatory effect of ATP is dependent on ATP hydrolysis but not mediated by phosphate ions released by the hydrolysis. Moreover, the possibility that ATP hydrolysis might stimulate UCB uptake by changing membrane potential can be ruled out on the basis of the observation that the UCB uptake in cLPMV is insensitive to the creation of a membrane potential, as indicated by experiments performed by the addition of valinomycin in the presence of an inwardly directed K^+ gradient.

ATP-dependent mechanisms have been described for the biliary secretion of a variety of endogenous and exogenous compounds, including various cationic and neutral anti-cancer agents, as well as organic acids such as conjugated bilirubin [5–7], bile salts [28], leukotrienes [29] and glutathione [30]. These ATP-dependent transporters all belong to the family of phosphoglycoproteins (ABC proteins), which mediate concentrative canalicular secretion via energy derived from the hydrolysis of ATP. It must therefore be considered whether the observed ATP-dependent transport of UCB was mediated by any of these other known ABC proteins.

That this is unlikely is suggested by the known failure of UCB to compete with any of the known substrates for the described ABC transporters [1–4]. The effect of DEPC suggests, however, that an ecto-ATPase in the cLPMV might have been responsible for the ATP-dependence of UCB canalicular transport. Both vanadate and DEPC have also been reported to inhibit the ATP-dependent transport of sulphobromophthalein in canalicular vesicles, which is mediated by cMOAT [22]. Our observation of comparable UCB transport in cLPMV from Wistar and TR⁻ rats, both in the presence and in the absence of ATP, rules out the involvement of cMOAT in the canalicular transport of UCB. Vanadate has also been shown to decrease the ATP-dependent transport of daunomycin, a process mediated by several *mdr* gene products, especially *mdr-1* [23,31]. The failure of daunomycin to inhibit ATP-dependent UCB transport in our study renders unlikely the involvement of *mdr-1*. Another possibility is that cBAT, the ecto-ATPase putatively involved in the canalicular excretion of bile acids [32] might also be involved in the observed UCB transport. The lack of inhibition observed when taurocholate was added seems to exclude this possibility.

Collectively, these considerations suggest that ATP-dependent UCB transport by cLPMV is most probably mediated by an as yet unidentified ABC-protein.

It remains to explain why, with such a high-capacity transporter present, the concentration of UCB in bile is relatively low. This might be explained by the normally very low concentration of UCB within the hepatocyte and/or the possible reabsorption of the UCB by the biliary epithelium, for example by a cholehepatic shunt mechanism, which involves the passive ductular absorption of protonated cholephilic organic anions [33]. At the pH of hepatic bile in the rat (7.2–8.0), 90 % and 50 % respectively of unbound UCB would be in the protonated, diacid form [34]. This and other possibilities need to be tested experimentally.

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