CANALICULAR MEMBRANE TRANSPORT IS PRIMARILY RESPONSIBLE FOR THE DIFFERENCE IN HEPATOBILIARY EXCRETION OF TRIETHYLMETHYLAMMONIUM AND TRIBUTYLMETHYLAMMONIUM IN RATS

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ABSTRACT:

Two structurally similar quaternary ammonium compounds, triethylmethylammonium (TEMA, \(M_r 116\)) and tributylmethylammonium (TBuMA, \(M_r 200\)) were used as model compounds to identify the unit process of hepatobiliary excretion that is responsible for markedly different biliary excretion of organic cations (OCs). Cumulative biliary excretion (in percentage of dose; i.v., 12 \(\mu\)mol/kg) was 0.17 for TEMA and 34.5 for TBuMA. In vivo uptake clearance into the liver was 0.686 ± 0.020 ml/min for TEMA and 0.421 ± 0.028 ml/min for TBuMA. When the uptake clearance was examined in an isolated hepatocyte system, comparable clearance between TEMA and TBuMA was obtained, consistent with the in vivo result. These observations suggest that uptake into the liver is not the major determinant for the difference in biliary excretion of the OCs. Co-administration of colchicine, an inhibitor of microtubule formation, had no effect on biliary excretion of the model compounds, and the primary site of subcellular distribution of the OCs appears to be the cytosol, suggesting that intracellular movement does not play a major role in the markedly different biliary excretion of the OCs. In contrast, in vivo excretion clearance across the canalicular membrane for TBuMA was 180-fold greater than that for TEMA, and in vitro efflux clearance of TBuMA was smaller than that of TEMA (\(p < .01\)).

The objective of the present study is to identify the unit transport process(es) responsible for the markedly different biliary excretion of TEMA and TBuMA.

The bile/plasma concentration ratio of a significant number of biologically important amines (e.g., atropine, propranolol, neostigmine, and imipramine) is significantly greater than unity (Klaassen et al., 1981). A number of published reports have suggested that carrier-mediated transport processes may be responsible for such high concentrations of such amine salts (i.e., organic cations, OCs\(^1\)) in the bile (Meijer et al., 1990; Steen et al., 1992; Nakamura et al., 1994). The carrier-mediated transport processes involved in the biliary excretion of OCs include secondarily active transport systems for sinusoidal efflux, intracellular transport, binding to intracellular proteins, and canalicular excretion of hepatobiliary transport (Moseley et al., 1992a, 1996). Despite similarities in chemical structure and mechanism of hepatobiliary transport, however, the extent of biliary excretion of OCs differs significantly. That is, the high molecular weight analog, TBuMA, is primarily excreted (>40% of the dose) in bile, whereas the extent of biliary excretion for TEMA is negligible (i.e., <1%; Neef et al., 1984). Because a high molecular weight (i.e., >200 ± 50) OC is known to be preferentially excreted in the bile, the unit process that is responsible for the markedly different biliary excretion for TEMA and TBuMA may also be involved in the molecular weight-dependent biliary excretion for OCs. Unfortunately, mechanisms for the molecular weight-dependent biliary excretion is presently unknown, and the unit process(es) responsible for the markedly different extent of biliary excretion for TEMA and TBuMA have not been identified.

The bile/plasma concentration-time curve from time zero to infinity.

\(^1\) Abbreviations used are: OC, organic cation; TEMA, triethylmethylammonium; TBuMA, tributylmethylammonium; TEA, tetraethylammonium; AUC, area under the plasma concentration-time curve from time zero to infinity.

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The objective of the present study is to identify the unit transport process(es) that is responsible for these differences in biliary excretion for TEMA and TBuMA. Because these OCs are not protein bound in either plasma or liver cytosol and are not metabolized (Neef et al., 1984), a considerable simplification in the comparison can be made. In addition, because the comparison should be carried out, not only between the model cations but between the sequential unit transports, it is necessary to carry out this study in an experimental system that...
contains all relevant unit transports involved in hepatobiliary transport, such as an isolated hepatocyte preparation or an in vivo experimental system. Our findings show that the excretion process across the canalicular membrane is primarily responsible for the large differences in biliary excretion of TEMA and TBuMA into the bile.

**Experimental Procedures**

**Materials.** \[^{3}H\]TEMA (specific activity, 3.20 \muCi/\mumol) and \[^{3}H\]TBuMA (specific activity, 5.17 \muCi/\mumol) were synthesized (Neef et al., 1984; Neef and Meijer, 1984) by reaction of an excess of the corresponding tertiary amines with \[^{3}H\]methyliodide (Amersham, Arlington Heights, IL, specific activity, 85 \muCi/\mumol). The product of the reaction, performed using unlabeled methyl-iodide, had thin-layer chromatography, infrared, and nuclear magnetic resonance spectral characteristics identical with authentic TEMA and TBuMA. The radiochemical purities of synthesized \[^{3}H\]TEMA and \[^{3}H\]TBuMA were >99.0\%, as determined by thin-layer chromatography (data not shown). Unlabeled methyliodide, triethylamine, tributylamine, and silicone oil were purchased from Aldrich Chemicals (Milwaukee, WI). Collagenase (type IV), colchicine, mannitol, mineral oil (light), BSA, and HEPES were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents used were of the highest grade commercially available.

**In Vivo Study.** *Systemic pharmacokinetic study.* Under light ether anesthesia, the left femoral vein and artery of adult male Wistar rats (250–300 g) were cannulated with polyethylene tubing (PE-50). After the abdomen was opened, the bile duct and both ureters were cannulated with polyethylene tubing (PE-10). After the animal had recovered from the surgery (2 h), a 3% mannitol solution was infused through the femoral vein cannula at a rate of 10 ml/kg to obtain a constant urine flow. The animals received a bolus dose of 12 \mumol of \[^{3}H\]TEMA or \[^{3}H\]TBuMA per kilogram of body weight (0.6 \muCi/ml) via the femoral vein.

Bile was collected at 5-min intervals up to 30 min after injection, and urine was collected every 10 min. Bile and urine samples were then collected at 10-min intervals from 30 min to 3 h and at 30-min intervals from 3 to 5 h. Blood samples (250 \muL) were collected nine times at appropriate intervals during the 5-h period. The total volume of blood withdrawal was 2.5 ml, and fluid loss was compensated by an injection of saline via the i.v. catheter. Body temperature was maintained with a heat lamp. The volumes of bile and urine were determined gravimetrically, assuming a density of 1.0 g/ml. In a preliminary study, densities of bile and urine were very close to unity (i.e., 0.971–0.983 g/ml) during the collection period of 5 h, indicating that the approximation was reasonable. The concentrations of \[^{3}H\]TEMA and \[^{3}H\]TBuMA in plasma, bile, and urine were quantified by liquid scintillation counting (LSC System 1400; Wallac) in 10 ml of scintillation fluid (Ultima Gold; Packard, Downers Grove, IL). Quenching was corrected by use of an external standard.

The area under the plasma concentration-time curve from time zero to infinity (AUC) and the area under the first moment-time curve from time zero to infinity (AUMC) were calculated by a trapezoidal rule, followed by extrapolation. Standard methods (Gibaldi and Perrier, 1982) were used to calculate the following parameters: the time-averaged total body clearance (CL), mean residence time (MRT), apparent volume of distribution at steady-state (Vss), urinary clearance (CLu), and biliary clearance (CLb) were calculated by the equations shown in the Appendix (eqs. 1–5).

**Estimation of in vivo hepatic uptake clearance.** To evaluate the in vivo clearance of hepatic uptake, the rat received a dose of 12 \mumol/kg of \[^{3}H\]TEMA or \[^{3}H\]TBuMA via the femoral vein catheter, and blood samples were collected every 30 s. Animals were sacrificed at 0.5, 1, 2, 3, and 4 min for TEMA and 0.5, 1, 2, 3, 4, and 5 min for TBuMA. The liver was immediately dissected, and liver samples were collected and weighed. The samples were solubilized in Soluene-350 (Packard) and counted for radioactivity. The content of the OCs per gram of liver was then expressed as the mean content per gram of liver sample. Radioactivity in an aliquot of blood and liver sample was determined by a liquid scintillation counting. In vivo uptake clearance was estimated by the equation in the Appendix (eq. 8).

**Intracellular transport study.** To estimate intracellular transport of the model substrates in the liver, the effect of colchicine, an inhibitor of microtubule formation, on biliary clearance was examined. An i.v. dose of 1.2 mg/kg of colchicine was administered to a group of rats, whereas the control group received vehicle (i.e., saline). The dosing solution of colchicine was prepared by dissolving the inhibitor in saline just before administration. At the dose used in this study, Kacich et al. (1983) showed that pretreatment with colchicine inhibits the microtubule-dependent transport of taurocholate in rats without a significant alteration in bile flow. Three hours after the administration of inhibitor, TEMA or TBuMA was administered via an i.v. bolus at a dose of 12 \mumol/kg. Blood and bile samples were collected for 2 h after the administration, and radioactivity associated with the biological fluids was determined. The biliary excretion rate and apparent clearance of the OCs were then calculated and compared.

**Subcellular distribution of the OCs in hepatocytes.** To further compare the intracellular transport of the OCs in hepatocytes, the subcellular distribution of the substrates in hepatocytes was examined. TEMA and TBuMA (12 \mumol/kg) were administered via an i.v. bolus injection. Ten minutes after the administration, rats were decapitated, and subcellular fractionation of the liver was carried out according to the method of Chung et al. (1990). A 20% w/v homogenate was prepared, and differential centrifugation (at 4°C) was carried out to sequentially obtain 600g pellet (centrifuged for 10 min), 6,800g pellet (centrifuged for 9 min), 28,000g pellet (centrifuged for 10 min), 100,000g pellet (centrifuged for 90 min), and 100,000g supernatant (centrifuged for 90 min). The radioactivity associated with these subcellular fractions was determined after solubilization of corresponding fractions in 1% Triton X-100.

**Biliary excretion study.** After the rats were lightly anesthetized with ether, the femoral vein, artery, and bile duct were cannulated as described earlier. For an estimation of biliary excretion clearance of TEMA, rats were divided into four groups that received an i.v. bolus injection, followed by infusion, to obtain four steady-state concentrations in the liver. For each group, TEMA was infused at a rate of 1.7, 3, 12, and 48 \mumol/kg after a bolus administration of 0.75, 1.5, 6.0, and 24 \mumol/kg. Similarly, TBuMA was given i.v. at a dose of (i.v. bolus dose/i.v. infusion rate, in \mumol/kg and \mumol/kg, respectively) 0.3/0.15, 1.5/0.75, 3.0/1.5, 12.0/6.0, and 48.0/24.0. Bile was collected at 30-min intervals up to 4 h; blood and liver samples were immediately collected upon sacrifice for 4 h for a determination of the hepatic concentration of the substrates. Radioactivity in liver, plasma, and bile was counted, and the in vivo excretion clearance was calculated by dividing the rate of excretion by liver substrate concentration.

**In Vitro Hepatocyte Studies.** *Preparation of hepatocytes.* Livers from male Wistar rats (200–250 g) were used for the experiment. Hepatocytes were freshly prepared using modifications of the procedure described by Baur et al. (1975). Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.). After catheterization of the portal vein, the liver was removed and perfused for 5 min with an oxygenated, calcium-free buffer (137 mM NaCl, 5.4 mM KCl, 0.5 mM NaH2PO4, 0.4 mM NaHCO3, 4.2 mM NaHCO3, 5 mM glucose, 0.5 mM EGTA, and 10 mM HEPES, pH 7.2, at 37°C) at a flow rate of 30 ml/min. Subsequently, perfusion was switched to a recirculating system with calcium and magnesium containing buffer (137 mM NaCl, 5.4 mM KCl, 5 mM CaCl2, 0.5 mM NaH2PO4, 0.4 mM NaHPO4, 4.2 mM NaHCO3, 10 mM HEPES, 0.05% collagenase, and 0.005% trypsin inhibitor, oxygenated, pH 7.2, at 37°C). The perfusion was continued for 15 min, after which time the liver was transferred into a beaker filled with the perfusion buffer (80 ml) and gently disrupted. Vascular and connective tissues were discarded, and enzymatic treatment was continued for another 9 min in a rotary evaporator (Eyela, Japan; Drochmans et al., 1975). After filtration through nylon filter (50 \mum) and cooling on ice, the resulting cell preparation was washed by adding ice-cold incubation medium, followed by centrifugation (50g for 2 min at 4°C). This procedure was repeated three times. The pellet was resuspended to give a typical concentration of 2.5 to 3.5 mg protein/ml for the “Estimation of Efflux Clearance” study, a protein concentration of 10 mg/ml was used in ice-cold HEPES supplemented Hanks buffer (137 mM NaCl, 5.4 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 0.8 mM MgSO4, 0.5 mM NaH2PO4, 0.4 mM NaHPO4, 4.2 mM NaHCO3, 5 mM glucose, and 10 mM HEPES, oxygenated, pH 7.4). The viability of hepatocytes was in excess of 90% as measured by trypan blue exclusion. Protein concentration was determined by the Lowry method using bovine serum albumin as a standard. The resuspended hepatocyte preparation was used in subsequent experiments.

**Estimation of uptake clearance.** Each hepatocyte suspension (2 ml, 2.5–3.5 mg protein/ml) was preincubated for 5 min at 37°C. An aliquot of TEMA or TBuMA (30 \muL) solution of various initial concentrations was added to the
The OCs were i.v. injected at the dose of 12 μmol/kg in rats. A, temporal profile of OC concentrations in plasma. B, temporal profile of the rate of biliary excretion for TEMA and TBuMA. ●, TEMA; ○, TBuMA. Each data point represents the mean ± S.D. of plasma concentrations or rate of biliary excretion from three different rats.

Suspension to give a medium concentration of 1 to 500 μM for TEMA and 0.5 to 300 μM for TBuMA. An aliquot (200 μl) of the suspension was added at 20, 40, 60, 90, and 120 s and placed in a centrifuge tube (0.4 ml) containing KOH (50 μl, 3 M) and silicone/mineral oil (100 μl, density, 1.015). Subsequent centrifugation at 50g for 5 s resulted in the sedimentation of hepatocytes through the oil layer into the KOH layer. The bottom KOH layer, containing hepatocytes, was collected, and the radioactivity was determined. The amount of the substrates in hepatocytes (expressed in nmol/mg protein) was plotted against time. The initial uptake rate of the OCs into the hepatocyte was calculated from a linear portion (i.e., generally up to 1 min) of the plot using linear regression analysis. The rate of initial uptake was then plotted against the initial concentration of the substrate in the medium, and the uptake kinetic was characterized as described in the Appendix using eq. 9.

In addition, control experiments were carried out to correct for the amount of substrate associated with the surface of hepatocytes by measuring the volume of medium adherent to sedimented hepatocyte (using 3H2O and the aqueous volume of hepatocyte (using 3H2O). We also examined release of the preloaded substrate from hepatocytes to estimate efflux (i.e., transport back into the aqueous volume of hepatocyte (using 3H2O). In addition, control experiments were carried out to correct for the amount of substrate associated with the surface of hepatocytes by measuring the volume of medium adherent to sedimented hepatocyte (using 3H2O and the aqueous volume of hepatocyte (using 3H2O). Estimation of efflux clearance. We also examined release of the preloaded substrate from hepatocytes to estimate efflux (i.e., transport back into the medium from the hepatocytes via the sinusoidal membrane) clearance by the method of Chung et al. (1990). A hepatocyte suspension (4 ml, 10 mg/ml) was preincubated at 37°C for 5 min, and an aliquot (60 μl) of TEMA or TBuMA solution of varying concentrations was added to obtain a final concentration (18–700 μM) of the substrates in the medium. The mixture was then incubated for 30 min to allow the accumulation of substrates in hepatocytes. The mixture was centrifuged (50g for 2 min at 4°C), and the resulting pellet (the preloaded cell) was washed with ice-cold buffer (30 ml). After the process was repeated once, the pellet was resuspended in ice-cold buffer (2 ml). An aliquot (0.5 μl) of the resuspension was diluted in 2 ml of a prewarmed incubation buffer (37°C). The purpose of the two-step resuspension was to rapidly achieve incubation temperature (37°C). Aliquots (200 μl) of the cell suspension were taken at various times from 0 (immediately sampled after the addition of the prewarmed buffer) to 15 min, and radioactivity remaining in the hepatocyte suspension was estimated as described in Estimation of Uptake Clearance. The intracellular concentration of the OCs at 0 time ranged from 18 to 700 μM for TEMA and from 14 to 440 μM for TBuMA. The OC content associated with the cell was plotted against time, and the slope of the linear portion (up to 2 min) of the plot was estimated by linear regression. The rate of release (i.e., the slope) was then plotted against the initial intracellular concentration of the substrates, and the kinetic was characterized as described in the Appendix using eq. 10.

To estimate nonspecific binding of the OCs on the hepatocyte surface and subsequent release, control efflux studies for both cations were carried out at 4°C. Under these conditions, the efflux of TEMA and TBuMA was negligible (data not shown), indicating that nonspecific association of the OCs from the hepatocyte surface was minimal.

Results

Disposition of TEMA and TBuMA after i.v. Administration. When TEMA and TBuMA were i.v. administered to rats at a dose of 12 μmol/kg, temporal profiles of plasma concentration followed a triexponential decline (Fig. 1A). Using model-independent methods, the initial plasma concentrations of TEMA and TBuMA, 31.8 and 96.8 μM, respectively, were estimated. Terminal half-lives of TEMA and TBuMA were 59.4 and 24.0 min, respectively, indicating that the decline of plasma concentration for TBuMA was more rapid. Temporal profiles of biliary excretion for TEMA and TBuMA are also shown in Fig. 1B. The cumulative biliary excretion of TEMA and TBuMA was 0.17 and 34.5% of the dose, respectively (Table 1). The cumulative urinary excretion of TEMA and TBuMA was 83.0 and 48.4% of dose, respectively (Table 1). Calculated pharmacokinetic parameters are shown in Table 1.

In Vivo and In Vitro Estimation of Hepatic Uptake of TEMA and TBuMA. In vivo hepatic uptake of TEMA and TBuMA was examined after an i.v. administration of each compound. As shown in Fig. 2A, the slope of the integration plot was linear up to 4 and 5 min for TEMA and TBuMA, respectively. From the initial slope, the estimated CLuptake in vivo for TEMA and TBuMA were 0.686 ± 0.020 and 0.421 ± 0.028 ml/min/g liver, respectively. The estimated CLuptake in vivo for TEMA was larger than that for TBuMA, indicating that TEMA is readily transported into the liver.

Table 1

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>TEMA</th>
<th>TBuMA</th>
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<tbody>
<tr>
<td>AUC (μM × min)</td>
<td>427 ± 36.1</td>
<td>276 ± 6.98</td>
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<tr>
<td>MRT (min)</td>
<td>78.8 ± 12.5</td>
<td>17.8 ± 1.44</td>
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<tr>
<td>CL (ml/min/kg)</td>
<td>31.2 ± 2.63</td>
<td>48.3 ± 1.23</td>
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<tr>
<td>Vss (ml/kg)</td>
<td>2460 ± 243</td>
<td>863 ± 62.7</td>
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<tr>
<td>Cumulative urinary excretion (% of dose)</td>
<td>83.0 ± 1.28</td>
<td>48.4 ± 3.23</td>
</tr>
<tr>
<td>Cumulative biliary excretion (% of dose)</td>
<td>0.17 ± 0.03</td>
<td>34.5 ± 1.60</td>
</tr>
<tr>
<td>Urinary clearance (ml/min/g)</td>
<td>27.3 ± 0.93</td>
<td>23.4 ± 1.34</td>
</tr>
<tr>
<td>Biliary clearance (ml/min/g)</td>
<td>0.049 ± 0.006</td>
<td>16.5 ± 1.23</td>
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Temporal profiles of uptake for TEMA and TBuMA into the hepatocyte were apparently linear for 1 min (data not shown). The rate versus initial substrate concentration plot (Fig. 2B) showed a curvature relationship, indicating that the uptake of the cations was mediated by both saturable and nonsaturable processes. An Eadie-Hofstee plot was also consistent with the mixed kinetic process (data not shown). As a result, a nonlinear regression of the concentration dependence was carried out, assuming a simple Michaelis-Menten component and a linear uptake term (eq. 9). Estimated kinetic parameters were: for TEMA, $687 \pm 50.8$ pmol/min/mg protein, $V_{\text{max, uptake}}$; $46.2 \pm 6.58$ μM, $K_{\text{m, uptake}}$; $0.878 \pm 0.150$ μl/min/mg protein, $CL_{\text{linear uptake, in vitro}}$; and for TBuMA, $72.7 \pm 7.32$ pmol/min/mg protein, $V_{\text{max, uptake}}$; $1.09 \pm 0.360$ μM, $K_{\text{m, uptake}}$; $0.830 \pm 0.07$ μl/min/mg protein. When compared at the same initial concentration, uptake rate for TEMA was larger than that of TBuMA (for all concentrations above 10 μM, $p < .001$), indicating that the uptake of TEMA into the hepatocyte was faster than that of TBuMA. This observation is consistent with the in vivo data.

**In Vitro Determination of Hepatic Efflux for TEMA and TBuMA.** Efflux of TEMA and TBuMA from isolated hepatocytes was also investigated at various intracellular concentrations. The efflux of TEMA and TBuMA was apparently linear up to 1.5 min. Initial efflux velocity was thus calculated, and initial efflux velocity versus intracellular concentration was plotted (Fig. 3A). The rate of efflux for TEMA appears to be linear with respect to the estimated intracellular concentration (up to 700 μM), whereas the rate of efflux

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**Fig. 2.** Uptake of TEMA and TBuMA into rat liver and hepatocyte preparations.

A, integration plot analysis (see Appendix) of hepatic uptake in vivo after i.v. administration of 12 μmol/kg dose in rats. B, plot of uptake rate into isolated hepatocyte preparation versus OC concentration in the medium. ●, TEMA; ○, TBuMA. Each data point in (B) represents mean ± S.D. of at least three separate experiments. The curves represented by a thick line in (B) were generated by a nonlinear regression fitting of the data to an uptake model (eq. 9) involving saturable and nonsaturable processes (see Results). The curves and lines represented by a thin line in B were theoretically calculated by a simple Michaelis-Menten kinetics (curves) and a linear kinetics (lines).
for TBuMA versus the initial concentration plot was apparently curvilinear. An Eadie-Hofstee plot (Fig. 3B) shows a biphasic decline for both substrates with respect to the efflux rate, indicating that single linear kinetics or simple Michaelis-Menten kinetics cannot adequately explain these results. Accordingly, the data were fitted to an equation that assumes saturable and nonsaturable efflux mechanisms (eq. 10). The kinetic parameters of the OCs for efflux process were estimated to be: for TEMA, $562 \pm 101$ pmol/min/mg protein, $V_{\text{max, efflux}}$; $666 \pm 91.4$ μM, $K_m, \text{efflux}$; and $0.96 \pm 0.14$ μl/min/mg protein, $CL_{\text{linear, efflux}}$; and for TBuMA, $123 \pm 33.6$ pmol/min/mg protein, $V_{\text{max, efflux}}$; $88.2 \pm 30.0$ μM, $K_m, \text{efflux}$; and $0.20 \pm 0.07$ μl/min/mg protein, $CL_{\text{linear, efflux}}$. The efflux rate of TEMA was larger than that of TBuMA at a comparable intracellular concentration ($p < .01$, for the entire concentration range studied).

Intracellular Transport Study. To determine whether intracellular transport plays a major role in overall hepatobiliary excretion of OCs, profiles of biliary excretion for the cations were investigated after a pretreatment with colchicine, an inhibitor of microtubule formation. The extent of biliary excretion for both OCs and bile flow was unaffected by pretreatment with colchicine (Fig. 4). Constant bile flow was consistent with results obtained by Kacich et al. (1983), where the pretreatment condition reduced the intracellular transport of taurocholate without significant alteration in bile flow.

TEMA and TBuMA were distributed primarily in 100,000 g supernatant, and the amounts in other subcellular fractions were quantitatively insignificant (Fig. 5). In general, the subcellular distribution of the OCs was quite comparable. These distribution data demonstrate that the transport pathways for the cations are not different from one another.

Excretion of TEMA and TBuMA across Bile Canalicular Membrane. To determine the in vivo excretion rate across the bile canalicular membrane, we examined biliary excretion of the model cations when various concentrations of substrate in the liver were maintained. Loading doses were, therefore, given as a bolus injection, and subse-
three separate experiments. Subsequently, the cationic drugs were infused to obtain hepatic concentrations of TEMA and TBuMA in the range of 100 to 550 and 1 to 300 nmol/g liver, respectively. Under these infusion conditions, plasma concentration, liver concentration, and excretion rate reached a steady state (data not shown). Excretion clearance of TBuMA (i.e., 0.245 g/min/rat) was greater, by 2 orders of magnitude, than that of TEMA (i.e., 0.0013 g/min/rat; Fig. 6).

Discussion

The purpose of this study was to identify the unit process(es) for hepatobiliary transport that explains the markedly different biliary excretion of structurally similar OCs, TEMA and TBuMA. Systemic pharmacokinetic studies of TEMA and TBuMA, at an i.v. dose of 12 μmol/kg each, were first carried out to verify the differences in biliary clearance in our experimental system. Cumulative biliary excretion of the cations was 164-fold higher for TBuMA (0.17% for TEMA and 34.5% for TBuMA; Table 1). In contrast, cumulative urinary excretion was higher for TEMA by only a factor of 1.71 (Table 1).

The average hepatic concentration of TEMA at 1 min was found to be 61.8 nmol/g liver whereas that of TBuMA was 48.4 nmol/g liver, indicating that ~24.7% of TEMA and 19.4% of TBuMA were distributed in the liver within 1 min (i.e., 250 g of body weight; 12 g of liver weight). It is interesting to note that TEMA, an OC with a low biliary clearance value, was readily distributed into the liver initially. This observation is consistent with the fact that uptake clearances for TEMA and TBuMA were comparable in vivo (Fig. 2A) and in vitro (Fig. 2B) experiments.

In this study, we attempted to evaluate unit clearances for TEMA and TBuMA in vivo. However, efflux clearance in vivo is extremely difficult to estimate; thus, in vivo efflux clearance was estimated using a kinetic model (Scheme 1). This calculation was carried out assuming 250 g rat with 12 g liver was applicable (i.e., typical weight of the rat and the rat liver used in this study). For TEMA, the in vivo estimation of uptake clearance was 0.686 ml/min/g liver. The uptake clearance was calculated to be 32.9 ml/min/kg. Using the equation shown in Scheme 1, the in vivo estimation of efflux clearance was 3.49 ml/min/kg. Similarly, for TBuMA, in vivo uptake clearance and excretion clearance were 20.2 and 0.98 ml/min/kg, respectively. Thus, in vivo efflux clearance was estimated to be 0.220 ml/min/kg. A comparison of the calculated in vivo efflux clearance indicated that TEMA is transported out of the hepatocyte faster than TBuMA by a factor of 15.9. However, this figure alone does not sufficiently explain the 337-fold difference in biliary clearance of TEMA and TBuMA (Table 1).

In general, a negligible amount in the cytosol would be expected for drugs that are intracellularly transported via a microtubule-dependent pathway (e.g., rose bengal, 17%; Wang et al., 1992). In this study, 100,000g supernatant, most likely the cytosolic fraction, contained the highest concentration of the substrates, whereas negligible amounts were associated with other cellular organelles. This profile of subcellular distribution is consistent with microtubule-independent transport, similar to that of DBSP (Iga and Klaassen, 1979). In addition, no difference in intracellular distribution is observed between TEMA and TBuMA. Taken together with the study involving inhibition of microtubule formation, the intracellular transport of TEMA and TBuMA was comparable and appears to be independent of a microtubule-based system. Therefore, intracellular transport is unlikely to be responsible for the difference in biliary excretion of TEMA and TBuMA.

When the same doses of TEMA and TBuMA are infused, a higher liver concentration is obtained for TEMA than for TBuMA. The extremely low bile:liver concentration ratio for TEMA in this study demonstrates that the transport of TEMA across the canalicular membrane is an unfavorable process, whereas TBuMA appears to be transported efficiently through this membrane. This difference is reflected in biliary excretion clearance. The biliary excretion clearance for TBuMA is ~337-fold greater than that for TEMA (Table 1). Consistent with the differences, the estimated excretion clearance for the cations in vivo reveals that TBuMA penetrated the canalicular membrane ~180 times faster than TEMA (Fig. 6). In contrast, comparative differences of the clearance for the cations in uptake and intracellular transport are not likely to explain the marked difference in the biliary excretion between TEMA and TBuMA. Secondary to the excretion clearance, more rapid efflux of TEMA from the hepatocyte to systemic circulation may also be involved in the marked differences in biliary clearances between TEMA and TBuMA.

Recently, pH-dependent OC transport systems have been cloned from fetal human liver (Tamai et al., 1997) and human placental...
trophoblast cell lines (Wu et al., 1998). Therefore, in the rat liver, functional analogs of these transporters may exist. Indeed, Moseley et al. (1996) recently reported the presence of a \(H^+\)-exchange mechanism for TBuMA uptake in rat liver canalicular membrane vesicles. In addition, the \(H^+\)-exchange mechanism has been described for canalicular transport of tetraethylammonium (TEA, \(M_r 130\); Moseley et al., 1992a), an OC that closely resembles TEMA in molecular weight and chemical structure. The cross inhibition study indicated that TEA and TBuMA share a canalicular transport mechanism (Moseley et al., 1992a, 1996). Interestingly, however, these authors reported that carrier-mediated canalicular uptake clearance is comparable for TEA and TBuMA (i.e., 0.938 \(\text{ml/mg protein/5 s}\) for TEA and 1 \(\text{ml/mg protein/5 s}\) for TBuMA). Therefore, it is unlikely that the transport mechanism is involved in the marked difference of the biliary excretion for TEMA and TBuMA. Johan et al. (1998) demonstrated recently that overexpression of P-glycoprotein (MDR1) in LLC-PK1 cells enhanced TBuMA uptake. Because the subcellular location of the primarily active transport system is the bile-canalicular membrane, it is tempting to speculate that the P-gp, not the \(H^+\)-exchange mechanism, may be involved in the preferential transport of TBuMA across the bile-canalicular membrane. This aspect of canalicular transport of OC is presently under investigation in our laboratory. In general, there are at least four distinct classes of primary active transporters in addition to P-glycoprotein that transport xenobiotics and endogenous compounds into bile: on the bile-canalicular membrane; canalicular bile acid transporter for bile acids; \(mdr2\) gene product for phospholipids; canalicular multispecific organic anion transporter for organic anions (Bateman and Arias, 1995; Yamazaki et al., 1996). However, these transporters are not generally thought to be involved in the transport of OCs across the bile-canalicular membrane (Bateman and Arias, 1995; Yamazaki et al., 1996).

In summary, the rate of unit transport processes of vectorial transport from blood to bile was separately examined and compared to identify the process that governs the marked difference in the biliary excretion between TEMA and TBuMA. The results obtained in this study strongly indicate that a significantly higher biliary excretion of TBuMA is primarily mediated by a higher transport through the canalicular membrane. Secondary to the canalicular excretion process, the efflux of the OCs from the hepatocyte into the systemic circulation appears to be partly involved. These processes may also play a role, at least in part, in the molecular weight-dependent hepatobiliary transport of OCs.

### Appendix

When it is necessary to calculate the systemic pharmacokinetic parameter, the following equations were used:

\[
\begin{align*}
\text{CL} &= \frac{\text{Dose}}{\text{AUC}} \\
\text{MRT} &= \frac{\text{AUMC}}{\text{AUC}} \\
\text{V}_{\text{SS}} &= \text{CL} \times \text{MRT}
\end{align*}
\]

\[
\begin{align*}
\text{CL}_b &= \frac{\text{Cumulative amount of OC in bile up to 5 h}}{\text{AUC}} \\
\text{CL}_u &= \frac{\text{Cumulative amount of OC in urine up to 5 h}}{\text{AUC}}
\end{align*}
\]

To estimate in vivo uptake clearance for OCs, the following rationalization was made. The uptake clearance was estimated within an initial period, during which time efflux or excretion clearance from the liver is negligible. During the initial period, the liver uptake rates for the OCs can be expressed by:

\[
\frac{dX}{dt} = \text{CL}_{\text{uptake}} \times C_P
\]
where $X_t$ represents the content of the OCs in the liver at time $t$, $C_P$ represents the plasma concentration of the OCs, and $CL_{uptake}$ in vivo represents the uptake clearance in vivo. An integration of the above equation with respect to time 0 to $t$ yields:

$$X_t = CL_{uptake} \cdot \int_0^t C_P \cdot dt \quad (7)$$

Dividing this equation with respect to $C_P$ gives:

$$\frac{X_t}{C_P} = CL_{uptake} \cdot \int_0^t C_P \cdot dt / C_P \quad (8)$$

Thus, the uptake clearance can be determined from the initial slope of the plot of $X_t / C_P$ versus $\int_0^t C_P \cdot dt / C_P$.

(Kim et al., 1988).

When it was necessary to estimate parameters of mixed uptake processes involving saturable and nonsaturable kinetics, the following equation was used in a nonlinear regression analysis using PCNONLIN (version 4.0; SCI Software, Lexington, KY):

$$\text{Initial uptake rate in vitro} = \frac{V_{\text{max, uptake}} \cdot C_{\text{medium}}}{K_{m, \text{uptake}} + C_{\text{medium}}} + CL_{\text{linear, uptake}} \cdot C_{\text{medium}} \quad (9)$$

where $V_{\text{max, uptake}}$ and $K_{m, \text{uptake}}$ represent the maximal rate of uptake and the medium concentration at half-maximal rate, respectively. $CL_{\text{in vivo}}$ represents the linear uptake clearance.

When it was necessary to estimate parameters of mixed efflux processes involving saturable and nonsaturable kinetics, the following equation was used in the nonlinear regression analysis as described earlier.

$$\text{Initial efflux rate in vitro} = \frac{V_{\text{max, efflux}} \cdot C_{\text{hepatocyte}}}{K_{m, \text{efflux}} + C_{\text{hepatocyte}}} + CL_{\text{linear, efflux}} \cdot C_{\text{hepatocyte}} \quad (10)$$

where $V_{\text{max, efflux}}$ and $K_{m, \text{efflux}}$ represent the maximal rate of efflux with the concentration of the hepatocytes at half-maximal rate, respectively. $CL_{\text{linear, efflux}}$ represents the linear efflux clearance term $K_{m, \text{efflux}} \gg C_{\text{hepatocyte}}$, in vitro efflux uptake clearance ($CL_{\text{efflux}}^{\text{in vitro}}$) can be estimated by:

$$CL_{\text{efflux}}^{\text{in vitro}} = \frac{V_{\text{max, efflux}}}{K_{m, \text{efflux}}} + CL_{\text{linear, efflux}} \quad (11)$$

References


