Functional Impairment of Sinusoidal Membrane Transport of Organic Cations in Rats with CCl_4-Induced Hepatic Failure

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Purpose. The effect of CCl_4-induced experimental hepatic failure (EHF) on the sequential hepatobiliary transport of model organic cations (OCs), triethylmethylammonium (TEMA), and tributylmethylammonium (TBuMA), was investigated in rats.

Methods. EHF was induced by an i.p. injection of CCl_4 at a dose of 1 ml/kg 24 hr prior to the transport study. The cumulative *in vivo* biliary excretion, *in vitro* hepatic uptake by isolated hepatocytes, *in vitro* efflux (i.e., release) from hepatocytes, and *in vivo* hepatobiliary excretion clearance were measured for normal and CCl_4-EHF rats.

Results. The CCl_4-EHF decreased the apparent *in vivo* biliary clearance (CL_b) and the *in vitro* maximum uptake rate ($V_{max,\ uptake}$) of TBuMA by 66 and 48%, respectively. The CCl_4-EHF had no effect on the CL_b of TEMA, but decreased both the $V_{max,\ uptake}$ (59%) and the *in vitro* maximum hepatic efflux rate ($V_{max,\ efflux}$) of TEMA (80%). On the contrary, the CCl_4-EHF had no influence on the *in vivo* hepatobiliary excretion clearance (CL_{exc}) of both OCs.

Conclusions. Transport systems for the OCs on the sinusoidal membrane (uptake and/or efflux), rather than those on the bile canalicular membrane (excretion) appear to be prone to damage by the CCl_4-EHF.

KEY WORDS: CCl_4; organic cations (OCs); hepatocytes; uptake; efflux; excretion.

INTRODUCTION

The liver plays a critical role in the hepatobiliary excretion of many endogenous and exogenous substances from the body (1). As might be expected, various liver diseases have an influence on this excretion. Experimental hepatic failure (EHF) induced by a single administration of carbon tetrachloride (CCl_4) has been widely used as a pathological model for liver diseases, since it is known that CCl_4 produces acute hepatocellular injury with centrilobular necrosis and steatosis (2). In fact, the effects of CCl_4-EHF on the apparent phamacokinetics or hepatobiliary excretion of xenobiotics have been widely studied (3–6). However, considerably less information is available on the effects of CCl_4-EHF on sequential unit transport (e.g., sinusoidal uptake into hepatocytes, sinusoidal efflux, intracellular transport, and canalicular excretion) of xenobiotics.

Recent work in this laboratory has focused on the molecular weight dependent hepatobiliary excretion of type I organic cations (OCs) including triethylmethylammonium (TEMA, Mol. Wt: 116) and tributylmethylammonium (TBuMA, Mol. Wt: 200) (7). It is generally thought that these compounds share common unit transport processes relative to hepatobiliary transport (7). However, TEMA and TBuMA exhibit dramatically different *in vivo* hepatobiliary excretion characteristics, i.e., more than 30% of dosed TBuMA, while less than 1% of dosed TEMA, is excreted into the bile (7). Hepatic uptake of type I cations is mediated electrogenically by an organic cation transporter 1 (OCT1), which is expressed in the sinusoidal plasma membrane of hepatocytes (8). Saturable and linear transports are involved in the efflux process of these OCs from hepatocytes to the blood stream (7). For the case of canalicular membrane transport, which is primarily responsible for the difference in the hepatobiliary excretion of these type I OCs (7), an ATP-dependent transport, probably via P-gp, and a proton driven transport are involved (9).

Despite the widespread acceptance of CCl_4-EHF as a pathophysiological model of liver diseases, its utility in the prediction of changes in transport processes in the diseased states has not been adequately studied. Thus, the purpose of the present study was to investigate the effect of the CCl_4-EHF on unit processes for the hepatobiliary transport of type I OCs, which has not yet been investigated. TEMA and TBuMA were selected as model type I OCs because they are not protein bound in either plasma or liver cytosol and are not metabolized (10). The study was performed using an isolated hepatocyte preparation as well as *in vivo* experimental systems.

MATERIALS AND METHODS

Chemicals

[^{3}H]TEMA (0.2 Ci/mmol) and [^{3}H]TBuMA (0.2 Ci/mmol) were synthesized according to the method of Neef *et al.* (10). All other reagents employed here were of the highest grade commercially available.

Induction of Experimental Hepatic Failure by CCl_4

Male Sprague-Dawley rats, 7 to 8 weeks of age, were given a single i.p. injection of CCl_4 (1 ml/kg) as a 50% (v/v) solution in olive oil 24 hr prior to the experiment. Following the administration of CCl_4 or olive oil, the rats had free access to food and water.

Systemic *In Vivo* Pharmacokinetic Study

Under light ether anesthesia, the femoral artery and vein of normal and CCl_4-pretreated rats were cannulated with polyethylene tubing (PE-50) for blood sampling and OC administration, respectively. After the abdomen was opened, the common bile duct was cannulated with PE-10, in order to collect bile specimens. After recovery from the surgery, the animals received [^{3}H]TEMA or [^{3}H]TBuMA at a bolus dose of 6.6 μmole (13.2 μCi) per kilogram of body weight (0.6 ml/kg) via the femoral vein.

Blood samples (250 μl) and bile were collected at appropriate intervals over a 3 hr 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 concentrations of [^{3}H]TEMA

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and [^{3}H]TBuMA in plasma and bile were quantified by liquid scintillation counting (LSC System 1409, Wallac) as described previously (7). The area under the plasma concentration-time curve from time zero to 3 hr (AUC) was calculated by a trapezoidal rule. The apparent biliary clearance (CL_b) was obtained by dividing the amount of OCs excreted into the bile during a 3 hr period by the AUC up to 3 hr.

In Vivo Biliary Excretion Across Canalicular Membrane

After the rats had been lightly anesthetized with ether, the femoral artery and vein were cannulated with PE-50, and bile duct with PE-10. For an estimation of the biliary excretion clearance of OCs, rats received an i.v. bolus injection, followed by infusion, in order to obtain the steady state concentration in the liver. [^{3}H]TEMA or [^{3}H]TBuMA was infused to normal or CCl_4-pretreated rats at a rate of 1.5 μmole (13.2 μCi)/hr/kg after a bolus administration of 1.5 μmole (13.2 μCi)/kg. Plasma and bile were collected at 30 min intervals up to 3 hr and blood and liver samples were immediately collected at the time of sacrifice at the steady state (3 hr) for a determination of the hepatic concentration of the substrates. Radioactivity in the liver, plasma and bile was determined, and the *in vivo* excretion clearance (CL_{exc}) was calculated by dividing the rate of excretion by the liver substrate concentration.

In Vitro Uptake into Hepatocytes

Hepatocytes were obtained according to the procedure described by Han *et al.* (7). The cell suspension (2 ml, 2.5–3.0 $\times$ 10^6 cells/ml) was pre-incubated in the medium for 5 min at 37°C. An aliquot of a [^{3}H]TEMA or [^{3}H]TBuMA (20 μl) solution at various initial radioactivity was added to the suspension to give a final medium concentration of 5–500 μM (0.02 $\sim$ 2 μCi) for TEMA and 1–100 μM (0.004 $\sim$ 0.4 μCi) for TBuMA. An aliquot (200 μl) of the suspension was sampled at 20, 40, 60, 90, and 120 sec, and the level of radioactivity in the hepatocytes was determined as described previously (7). The amount of substrates in the hepatocytes (expressed in pmole/10^6cells) was then plotted against time. The initial uptake rate of the OCs into the hepatocyte, which was calculated from the linear portion (i.e., generally up to 1 min) of the plot, was then plotted against the initial concentration of the substrate in the medium. A nonlinear regression analysis was performed in the fitting of the plot to following equation using WINNONLIN (version 1.0; SCI Software, Lexington, KY).

$$V_o = V_{max,\ uptake} \cdot S/(K_{m,\ uptake} + S) + CL_{linear,\ uptake} \cdot S \quad (1)$$

Where V_o is the initial uptake rate of the OCs (pmole/min/10^6cells), S is the concentration of OCs in the medium (μM). $V_{max,\ uptake}$ and $K_{m,\ uptake}$ are the maximum uptake rate and the medium concentration at half maximal rate, respectively, and $CL_{linear,\ uptake}$ represents the linear uptake clearance.

Control experiments were also carried out to correct for the amount of substrate associated with the surface of hepatocytes by measuring the volume of medium which adhered to sedimented hepatocytes (using ^{14}C-inulin) and the aqueous volume of hepatocyte (using ^3H_2O). The adherent volume and intracellular space (i.e., aqueous volume-adherent volume) were calculated to be 1.76 μl/10^6cells and 6.21 μl/10^6cells, respectively.

In Vitro Efflux from Hepatocytes

A freshly isolated hepatocyte suspension (2 ml, 10 $\times$ 10^6 cells/ml) was pre-incubated at 37°C for 5 min and an aliquot (20 μl) of a [^{3}H]TEMA or [^{3}H]TBuMA solution at varying concentrations was added to the suspension to give a final medium concentration of 1, 5, 20, 50, 100 and 200 μM (0.004 $\sim$ 0.8 μCi). The mixture was then incubated to allow the accumulation of substrates in the hepatocytes for 20 min, which was confirmed to be sufficient time to reach steady state accumulation. After the incubation, the concentration of the OCs in the hepatocytes was measured using a 200 μl aliquot of the cell suspension. Control experiments were also carried out to correct for the amount of substrate associated with the surface of hepatocytes as described in "in vitro Uptake into Hepatocytes." Subsequently an aliquot (100 μl) of this concentrated cell suspension was added to 1.9 ml fresh incubation medium (Hank's buffer, pH 7.4). Aliquots (200 μl) of the cell suspension were removed at appropriate intervals, and the efflux velocity (rate of release) of TEMA or TBuMA was estimated as described previously (7).

The rate of release (i.e., the slope) was then plotted against the initial intracellular concentration of the substrates, and the data were fitted to Eq. 2, using a nonlinear regression analysis.

$$V_o = V_{max,\ efflux} \cdot S/(K_{m,\ efflux} + S) + CL_{linear,\ efflux} \cdot S \quad (2)$$

Where V_o is the initial efflux rate of OCs (pmole/min/10^6 cells), $V_{max,\ efflux}$ and $K_{m,\ efflux}$ represent the maximal rate of efflux and the medium concentration of substrate in the hepatocytes at half-maximal rate, respectively. $CL_{linear,\ efflux}$ and S represent the linear efflux clearance term and the measured concentration of OCs in the hepatocytes, respectively.

Data Analysis

All data are expressed as mean $\pm$ S.E. The Student's unpaired t-test was used to test the difference between treatments. In all cases, $p < 0.05$ was accepted as representing a statistical difference.

RESULTS AND DISCUSSION

Disposition of TEMA and TBuMA

Temporal profiles of the plasma concentrations of TEMA and TBuMA following their intravenous administration to rats at a dose of 6.6 μmole/kg for each are shown in Fig. 1. The mean half-lives of both compounds at the terminal phase in normal rats were extremely long (i.e., 300 and 211 min for TEMA and TBuMA, respectively), in agreement with the conclusion of Neef *et al.* (11). Plasma concentrations of both compounds at each sampling time, and consequently, the trapezoidal AUCs up to 3 hr were increased slightly (i.e., 14% for TEMA and 44% for TBuMA, Table I) by the CCl_4-EHF, although no significance was observed for the increase.

Cumulative biliary excretion in the normal rats reached 33.3% of the dose for TBuMA, but was less than 1% of the dose for TEMA (Table I). These levels were decreased by the CCl_4-EHF to 13.2% (60% decrease, $p < 0.05$) for TBuMA, but not for TEMA (Table I). As a consequence, a 66% decrease ($p < 0.05$) in the CL_b of TBuMA, but not for TEMA, was

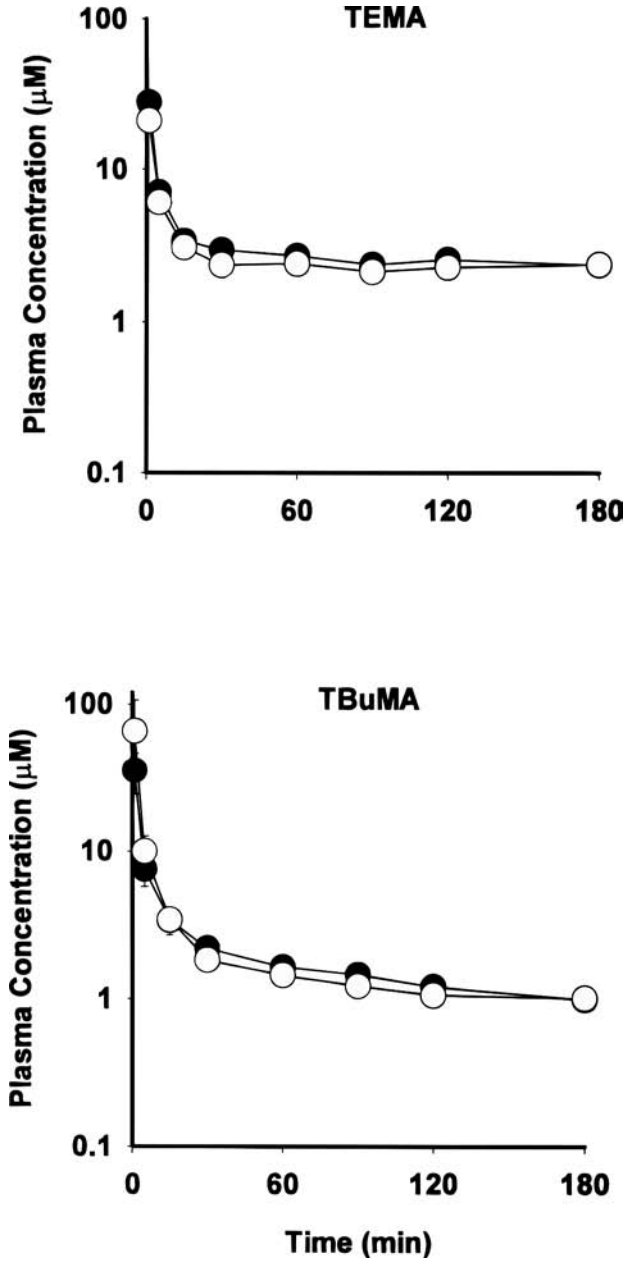

Fig. 1. Temporal profiles for the plasma concentration of TEMA and TBuMA in normal (○) and CCl_4-EHF rats (●) following intravenous administration of each compound at a dose of 6.6 μmole (13.2 μCi)/kg rat (0.6 ml/kg rat). Each data point represents the mean ± S.E. of three animals.

observed by the CCl_4-EHF. The decrease in CL_b for TBuMA appears to be consistent with the 43.6% increase in the AUC of TBuMA (Table I). The EHF was confirmed, in separate experiments, not to significantly affect the renal clearance of TBuMA (i.e., 7.2 ± 0.5 ml/min/kg for normal rats, and 7.5 ± 1.5 ml/min/kg, mean ± SE, n = 4).

A decreased biliary excretion by the CCl_4-EHF has been reported for some drugs (3,4), but not for other drugs (6). Thus, CCl_4-EHF appears to influence the biliary excretion of xenobiotics in a different manner probably depending on excretion characteristics of the xenobiotics.

In Vitro Hepatic Uptake of TEMA and TBuMA

The temporal uptake profiles were apparently linear for at least 1 min (data not shown) for hepatocytes from both normal and CCl_4-pretreated rats, where the slopes of the lines represent the initial uptake rate (velocity, V_o). The rate vs. the initial substrate concentration (S) plots for TEMA and TBuMA exhibited curvi-linear relationships for hepatocytes from the normal and CCl_4-EHF rats (Fig. 3), indicating that the uptake is mediated by both saturable and non-saturable processes. Eadie-Hofstee plots for the uptake were consistent with the mixed kinetic processes (data not shown). It is particularly noteworthy that the apparent uptake rates for TEMA into hepatocytes from the normal and CCl_4-EHF rats were more than 3-fold larger than those for TBuMA, despite the negligible biliary excretion of TEMA compared to TBuMA (Table I). An apparent decrease in the uptake rate by the CCl_4-EHF was observed for both compounds. Considering the minor contribution (approximately 13%) of the canalicular membrane to the total surface area of the hepatocyte plasma membrane (12), the uptake appears to represent mostly the penetration across the sinusoidal membrane.

Table II (upper row) summarizes the results of the fitting of the data in Fig. 3 to Eq. 1. The contribution of the carrier-mediated uptake to the total uptake was estimated to be more than 92%. Thus, both OCs seem to be taken up by the liver mainly by the carrier-mediated mechanism. A significant decrease in $V_{max,\ uptake}$ for both OCs was observed by the CCl_4-EHF (a 58.5% decrease for TEMA, a 48.4% decrease for TBuMA). However, for $K_{m,\ uptake}$ and $CL_{linear,\ uptake}$, no change by the CCl_4-EHF was observed for both OCs. These data suggest that the quantity of carriers responsible for the hepatic uptake of the OCs (OCT1, 8) across the sinusoidal membrane is decreased by the CCl_4-EHF, but not for the affinity of the carrier, $1/K_{m,\ uptake}$, for the OCs. The data also suggest that the nonsaturable uptake of the OCs, probably passive diffusion, across the sinusoidal membrane is not influenced by the CCl_4-EHF.

In Vitro Hepatic Efflux of TEMA and TBuMA

The accumulation of the OCs in the hepatocytes from normal and CCl_4-EHF rats under the given incubation condition (see in vitro Uptake into Hepatocytes) was generally higher for TEMA than for TBuMA (compare x axes for both OCs in Fig. 4). The efflux of the OCs into fresh incubation medium was highly variable (Fig. 4) compared to the uptake into the hepatocytes (Fig. 3). The efflux may represent mostly the release across the sinusoidal membrane considering the minor contribution of canalicular membrane to total surface area of the hepatocyte plasma membrane (12). However, the release across the canalicular membrane (i.e., excretion) might contribute, at least in part, to the high variation of the efflux. Despite of the variation, the efflux was apparently linear up to 1.5 min for both hepatocytes (data not shown), where the slopes of the lines represent the initial efflux velocity (V_o). The V_o was plotted against the initial intracellular concentration of each OC (Fig. 4). An apparently larger efflux rate was observed for TEMA compared to TBuMA from the normal and EHF heptocytes.

The results of the regression analysis of the data (Fig. 4) are summarized in Table II (middle row). Generally, the contribution of saturable component to the overall efflux of the

Table I. Summary of the Effects of CCl_4 Pretreatment on the Pharmacokinetic Parameters of TEMA and TBuMA after i.v. Administration (6.6 μmol/kg)[a]

Pharmacokinetic parameters	TEMA		TBuMA	
	Normal	Failure	Normal	Failure
AUC (μM × min)[b]	509 ± 26	584 ± 26	436 ± 38	626 ± 167
Cumulative biliary excretion (% of dose)[b]	0.71 ± 0.07	0.63 ± 0.08	33.30 ± 2.78	13.20 ± 1.81*
CL_b (ml/min/kg)[b]	0.090 ± 0.006	0.068 ± 0.006	4.886 ± 0.593	1.661 ± 0.366*

[a] Each data is expressed as mean ± S.E. of three separate experiments.
[b] Calculated up to 3 hr.
* $p < 0.05$ from the normal group by the unpaired Student's t-test.

OCs was smaller(94 and 59% for normal hepatocytes, 55 and 68% for EHF hepatocytes, for TEMA and TBuMA, respectively) compared to the case for the uptake (>92% for all the cases). By the CCl_4-EHF, the $V_{max,\ efflux}$, but not the $K_{m,\ efflux}$ or $CL_{linear,\ efflux}$, of TEMA was decreased significantly (81.9% decrease). These data suggest that the quantity of carriers which are responsible for the efflux of TEMA across the sinusoidal membrane, but not the affinity of the carrier to the TEMA, $1/K_{m,\ efflux}$, is decreased significantly by the CCl_4-EHF. On the other hand, the CCl_4-EHF had no significant effect on any of the kinetic constants for the efflux of TBuMA from hepatocytes. It is noteworthy that the CCl_4-EHF selectively damaged the efflux of TEMA. Although the mechanism could not be explained adequately in the present study, the CCl_4-EHF appears to have quite different effects on the efflux of xenobiotics, depending on their characteristics. The mechanism of the substrate-specific damage of the CCl_4-EHF is under investigation in our laboratory using hepatic sinusoidal membrane vesicles.

In Vivo Excretion of TEMA and TBuMA across Bile Canalicular Membrane

Under the given experimental conditions, the concentrations of OCs in the plasma and liver reached a steady state by 120 min after the start of the infusion in both normal and CCl_4-EHF rats. In the normal rats, the steady state liver concentration of TEMA was 2.6-fold higher than that of TBuMA (17.4 ± 1.2 vs. 6.6 ± 1.3 nmole/g liver), while the CL_{exc} of TBuMA was almost 25-fold higher than that of TEMA (196.0 ± 36.3 vs. 8.0 ± 0.4 μl/min/kg rat) (Table II, bottom row), consistent with our previous study (7). TEMA was taken up at a higher rate (Table II, upper row), but excreted at much lower rate compared to TBuMA in normal rats (Table II bottom row). The discrepancy in the mass balance would be explained by the much larger efflux rate for TEMA compared to TBuMA (Table II, middle row).

By the CCl_4-EHF, the liver concentration of TEMA at the steady state was decreased almost one-half (i.e., 17.4 ± 1.2 to 9.9 ± 2.6 nmole/g liver), but a parallel decrease in the biliary excretion rate of the compound (data not shown) was also observed. As a result, the CL_{exc} of TEMA remained unchanged by the CCl_4-EHF (Table II, bottom row). For the case of TBuMA, no changes in the liver concentration (i.e., 6.6 ± 1.3 to 5.7 ± 0.5 nmole/g liver) and biliary excretion rate (data not shown) were observed by the CCl_4-EHF, again resulting in an unchanged CL_{exc} (Table II, bottom row). In summary, the transport of both OCs across the bile canalicular membrane

Table II. Effects of CCl_4-induced Hepatic Failure on the Kinetic Constants for TEMA and TBuMA in Terms of *in vitro* Uptake into Isolated Hepatocytes, *in vitro* Efflux from Hepatocytes, and *in vivo* Bile Canalicular Excretion[a]

Kinetic constants	TEMA		TBuMA	
	Normal	Failure	Normal	Failure
Uptake				
$V_{max,\ uptake}$ (pmole/min/10^6 cells)	849 ± 70	352 ± 105*	52.3 ± 4.5	27.0 ± 3.8*
$K_{m,\ uptake}$(μM)	49.0 ± 0.5	49.7 ± 11.8	1.85 ± 0.85	1.34 ± 0.46
$CL_{linear,\ uptake}$(μl/min/10^6 cells)	0.84 ± 0.23	0.65 ± 0.18	1.25 ± 0.04	1.19 ± 0.05
Efflux				
$V_{max,\ efflux}$(pmole/min/10^6 cells)	486 ± 150	98 ± 23*	129 ± 44	312 ± 154
$K_{m,\ efflux}$(μM)	445 ± 210	218 ± 97	663 ± 430	324 ± 115
$CL_{linear,\ efflux}$(μl/min/10^6 cells)	0.30 ± 0.18	0.45 ± 0.06	0.46 ± 0.18	0.81 ± 0.48
Excretion				
CL_{exc} (μl/min/kg)	8.0 ± 0.4	14.2 ± 4.2	196 ± 36	211 ± 32

[a] Each data is expressed as mean ± S.E. of three separate experiments for uptake and excretion studies, and of six separate experiments for efflux studies.
* $p < 0.05$ from the normal group by the unpaired Student's t-test.

was not influenced by the CCl_4-EHF, which is contrary to the case for the sinusoidal membrane (i.e., uptake and efflux).

In this study, it can be concluded that the decrease in the *in vivo* hepatobiliary excretion of TBuMA by the CCl_4-EHF (Fig. 2 and Table I) represents a reflection of the decreased hepatic uptake of this compound. On the other hand, the *in vivo* hepatobiliary excretion of TEMA remained unchanged by the CCl_4-EHF (Fig. 2 and Table I). However, these facts do not necessarily indicate that the CCl_4-EHF does not influence the unit transport processes involved in the hepatobiliary excretion of TEMA. Rather, it appears that no change for the case of TEMA is a reflection of a balanced parallel decrease in both uptake and efflux across the sinusoidal membrane (Table II, upper and middle rows).

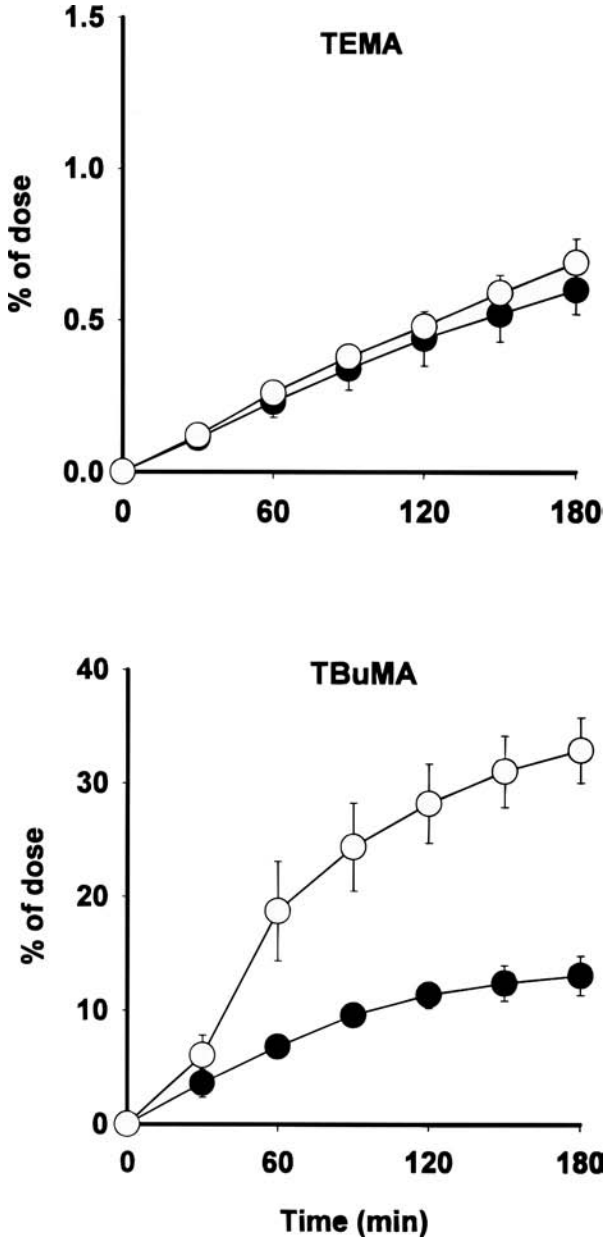

Fig. 2. Cumulative biliary excretion of TEMA and TBuMA in normal (○) and CCl_4-EHF rats (●) following intravenous administration of each compound at a dose of 6.6 μmole (13.2 μCi)/kg rat (0.6 ml/kg rat). Each data point represents the mean ± S.E. of three animals.

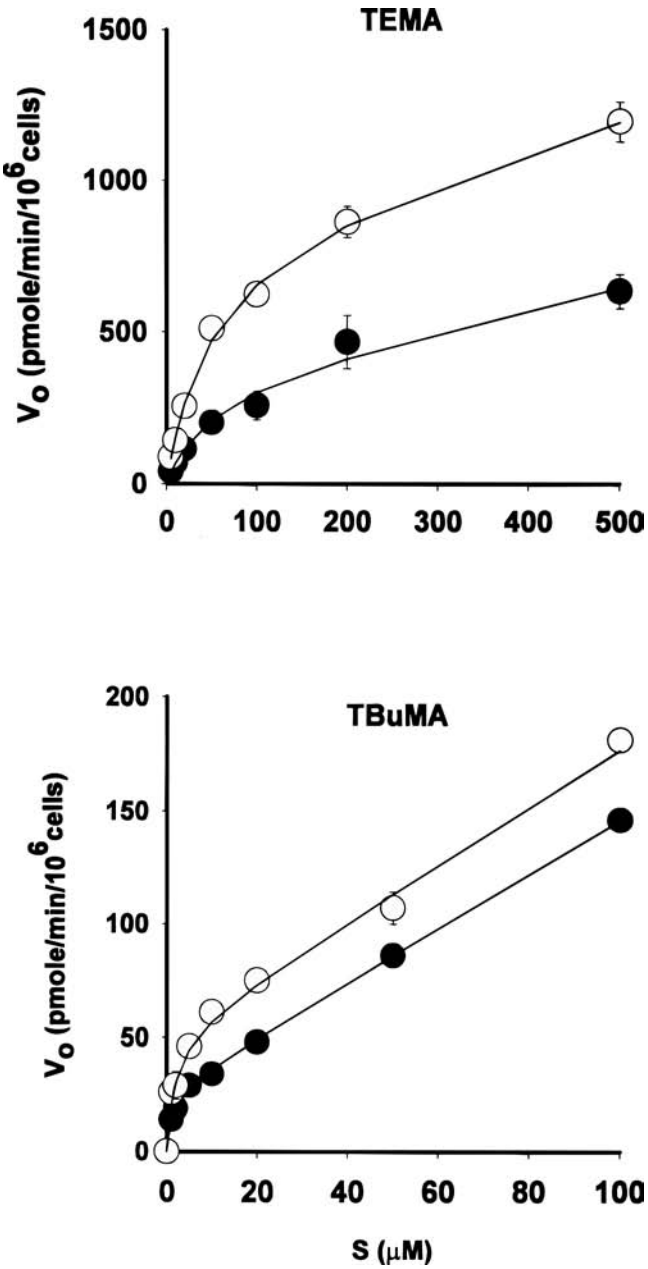

Fig. 3. Concentration dependence for the uptake of TEMA and TBuMA by isolated hepatocytes from normal (○) and CCl_4-EHF rats (●). Each point and vertical bar represents the mean ± S.E. of three different preparations. Solid curves were generated using mean uptake parameters in Table II (upper row).

It is interesting to note that damage by the CCl_4-EHF exhibits both membrane and substrate selectivity in terms of transport; the CCl_4-EHF preferentially affected the sinusoidal uptake rather than the canalicular excretion of both OCs, and the sinusoidal efflux of TEMA rather than that of TBuMA. Considering the fact that canalicular excretion is the last and rate-determining process in the overall hepatobiliary transport of the OCs, apparently intact function of the canalicular membrane transport during the CCl_4-EHF might be related to the

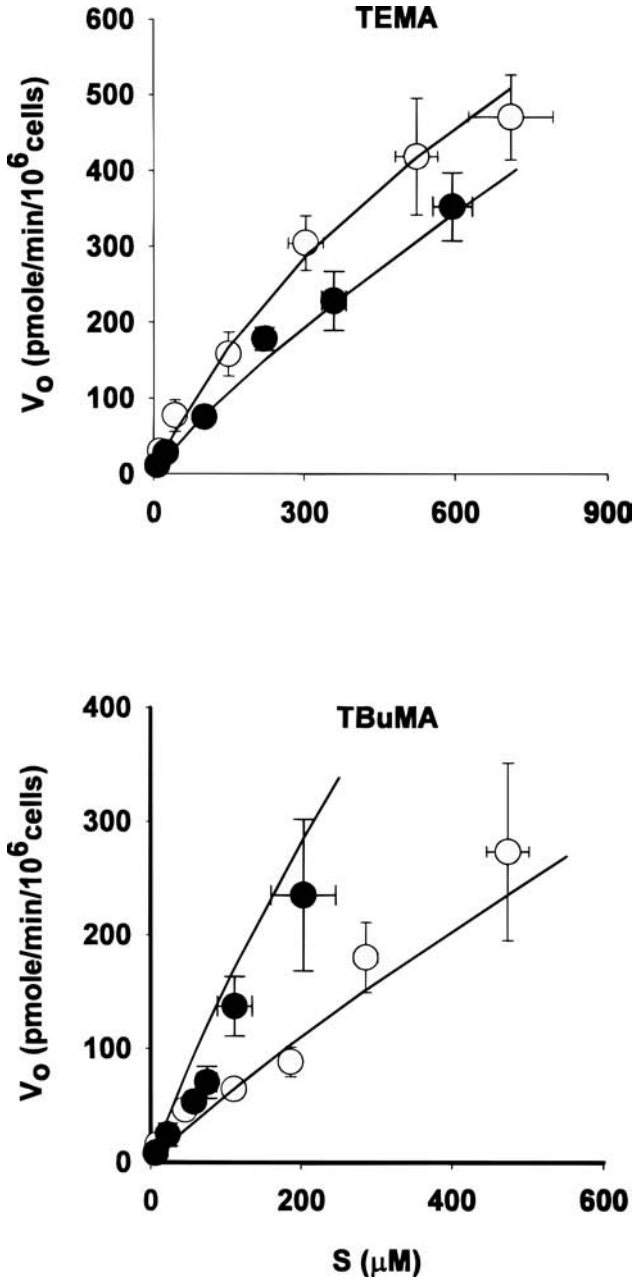

Fig. 4. Concentration dependence for the efflux of TEMA and TBuMA from isolated hepatocytes from normal (❍) and CCl_4-EHF rats (●). Each point and bar represents the mean ± S.E. of six different preparations. Solid curves were generated using mean efflux parameters in Table II (middle row).

protective mechanism of the liver against xenobiotics in the diseased state. This hypothesis is supported by a 3∼5-fold increase in the multidrug resistance (MDR) genes in the liver by the CCl_4-EHF (13). However, the increase in MDR genes is not consistent with the present study which shows an unchanged bile canalicular transport, CL_{exc}, of TBuMA, a potential substrate for the gene products (P-gp) (9). Thus, pathophysiological factors other than the expression of P-gp appear to be involved in the inconsistency. The changes in the formation of bile and its flow by the CCl_4-EHF, for example, might be associated with the pathophysiological factors. This aspect is under investigation in our laboratory using liver plasma membrane vesicle systems. In conclusion, the membrane- and substrate selective damage should be kept in mind in utilizing the CCl_4-EHF as a model for the liver diseases.

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