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Review

Recent Advances in Carrier-mediated Hepatic Uptake and Biliary Excretion of Xenobiotics

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Purpose. Besides renal excretion, hepatic metabolism and biliary excretion are the major pathways involved in the removal of xenobiotics. Recently, for many endogenous and exogenous compounds (including drugs), it has been reported that carrier-mediated transport contributes to hepatic uptake and/or biliary excretion. In particular, primary active transport mechanisms have been shown to be responsible for the biliary excretion of anticancer drugs, endogenous bile acids and organic anions including glutathione and glucuronic acid conjugates. Primary active excretion into bile means the positive removal of xenobiotics from the body, and this elimination process is now designated as "Phase III" (T. Ishikawa, Trends Biochem. Sci., 17, 1992) in the detoxification mechanisms for xenobiotics in addition to Phase I by P-450 and Phase II by conjugation.

Methods. The transporters, which have been called P-glycoprotein (MDR), multidrug resistance related protein (MRP) and GS-X pump and which are believed to be involved in the primary active pumping of xenobiotics from the cells, are now known as the ATP-binding cassette (ABC) transporters. In this review, we first describe the HMG-CoA reductase inhibitor, pravastatin, as a typical case of a carrier-mediated active transport system that contributes to the liver-specific distribution in the body.

Results. Regarding biliary excretion, we have summarized recent results suggesting the possible contribution of the ABC transporters to the biliary excretion of xenobiotics. We also focus on the multiplicities in both hepatic uptake and biliary excretion mechanisms. Analyzing these multiplicities in transport is necessary not only from a biochemical point of view, but also for our understanding of the physiological adaptability of the living body in terms of the removal (detoxification) of xenobiotics.

Conclusions. Clarification of these transport mechanism may provide important information for studying the pharmacokinetics of new therapeutic drugs and furthermore, leads to the development of the drug delivery systems.

KEY WORDS: organic anion; hepatic clearance; primary active transport; bile acid; HMG-CoA reductase; TR^- rat; Eisai hyperbilirubinemic rat; GS-X-pump; P-glycoprotein; multidrug resistance related protein.

INTRODUCTION

Clearance concepts (1, 2) which have been used widely in the field of pharmacokinetics play a major role in predicting the plasma disappearance of drugs. Besides kidney, liver is the major organ involved in the detoxification of xenobiotics. Evaluating accurately hepatic clearance is very important for predicting the pharmacological effect and/or side-effects of drugs, as well as changes in drug disposition during disease. Recently, for many endogenous and exogenous compounds (including drugs), it has been reported that carrier-mediated transport contributes to hepatic uptake and/or biliary excretion (3, 4). In particular, primary active transport mechanisms have been shown to be responsible for the biliary excretion of anticancer drugs, endogenous bile acids and organic anions including glutathione and glucuronic acid conjugates, and the elimination process including biliary excretion is now designated as "Phase III" detoxification (5) in addition to Phase I by P-450 and Phase II by conjugation. Saturation of the membrane transport processes is one of the factors that induce the non-linearity of not only hepatic clearance but also disposition in the whole body.

In this review, we first introduce our quantitative approaches to clarify the rate-limiting processes in hepatic clearance. Next, we describe the HMG-CoA reductase inhibitor, pravastatin, as a typical case in which carrier-mediated active transport system contributes to the liver specific distribution in a whole body. Regarding biliary excretion process, we focus on the multiplicity of organic anion excretion mechanisms which exhibit primary active transport under various experimental systems. Furthermore, we explain the experimental finding that P-glycoprotein, expressed on the liver canalicular membrane of normal rats, is actually responsible for the biliary excretion of drugs. Finally, we discuss a model at the molecular level for this multiple excretion mechanism as well as the physiological significance of these complex transport systems in the body.

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This review is mainly based on the recent results of us and others from studies of the hepatobiliary transport mechanisms of organic anions and the biliary excretion mediated by primary active transporter. Therefore, this is not a comprehensive review, and thus this does not aim to include all information on this topic. As far as the hepatic transport of organic compounds is concerned, excellent and comprehensive reviews have been recently published (4, 6).

KINETIC CONSIDERATION

Rate-limiting Process of Hepatic Elimination

Clearance concepts (1, 2), which have been used extensively in pharmacokinetics, are generally based on a rapid equilibrium for the distribution of drugs between blood and cells. According to this, organ clearance is expressed in terms of blood flow, blood unbound fraction (fb) and intrinsic organ clearance (CLint,all). Hepatic clearance (CLh), is described by the following equations, according to the venous equilibrium model (well-stirred model) (7), sinusoidal perfusion model (parallel-tube model) (8) and dispersion model (9), respectively,

$$CLh = Qh \cdot fb \cdot CLint,all/(Qh + fb \cdot CLint,all) \quad (1)$$

$$CLh = Qh \cdot \{1 - \exp(-fb \cdot CLint,all/Qh)\} \quad (2)$$

$$CLh = Qh \cdot [1 - 4a/[(1 + a)^2 \cdot \exp\{(a - 1)/2 \cdot D_N\} - (1 - a)^2 \cdot \exp\{-(a + 1)/2 \cdot D_N\}] \quad (3)$$

where,

$$a = (1 + 4\, R_N \cdot D_N)^{1/2} \quad (4)$$

$$R_N = fb \cdot CLint,all/Qh \quad (5)$$

Qh is the hepatic blood flow and D_N is the dispersion number. CLint,all represents the overall intrinsic clearance which includes not only metabolism and/or biliary excretion but also the membrane permeability as described by the following equation (10, 11):

$$CLint,all = PSu,influx \cdot \{CLint/(PSu,efflux + CLint)\} \quad (6)$$

where, PSu,influx and PSu,efflux represent the membrane permeability clearance of unbound ligand for the influx and efflux processes, respectively, and CLint represents the "exact" intrinsic clearance for metabolism and/or biliary excretion of the unbound ligand. We would like to emphasize here that CLint, not CLint,all is the parameter comparable with that evaluated in *in vitro* experiments using microsomes, *etc.*

As shown in Eq. 6, the rate-limiting step in CLint,all depends on the relative values of PSu,efflux and CLint. If PSu,efflux is much smaller than CLint (PSu,efflux << CLint), Eq. 6 gives

$$CLint,all = PSu,influx \quad (7)$$

Equation 7 indicates that only the influx process influences CLint,all. In this case, if one applied the metabolic clearance evaluated in *in vitro* experiments to the CLint,all in Eqs. 1–3 in order to predict the *in vivo* metabolic ability, then overestimation would occur. If PSu,efflux is much larger than CLint (PSu,efflux >> CLint), Eq. 6 gives

$$CLint,all = (PSu,influx/PSu,efflux) \cdot CLint \quad (8)$$

Equation 8 shows that CLint,all reflects both the asymmetry of the membrane permeability (influx/efflux) and the intrinsic metabolic and/or biliary excretion ability (CLint). If we assume that there is no asymmetry associated with the membrane transport process (PSu,influx = PSu,efflux), CLint,all becomes

$$CLint,all = CLint \quad (9)$$

Only in this case, CLint,all in Eqs. 1-3 becomes identical with CLint.

Furthermore, with the venous equilibrium model, if we assume symmetrical membrane transport (PSu,influx = PSu,efflux = PSu), Eq. 1 can be transformed (taking Eq. 6 into consideration) as follows:

$$1/CLh = 1/Qh + 1/(fb \cdot PSu) + 1/(fb \cdot CLint) \quad (10)$$

The inverse of CLh can be described by the sum of the inverses of Qh, membrane permeability, metabolism and/or biliary excretion, respectively, and the rate-limiting step is dependent on the relative values of those three processes. Thus, we cannot evaluate the hepatic clearance of drugs without consideration of these three processes (Qh, membrane permeability, and metabolism/excretion). However, quantitative studies are scarce with regard to the rate-limiting process of CLint,all for the hepatic clearance of drugs. We have attempted to address this point by using the multiple indicator dilution method (MID method) developed by Goresky (13, 14). The MID method has a great advantage in the individual determination of the influx, efflux, and sequestration processes of a ligand with the hepatic spatial architecture being maintained (12–14). We will now give some examples.

Dose-dependent Change in the Rate-limiting Process of Hepatic Elimination

The hepatic elimination of 4-methylumbelliferone (4-MU) was studied by means of an MID method (10). When the dose was increased from a low dose (50 μg/rat liver) to a high dose (3000 μg/rat liver), the hepatic availability of 4-MU increased from 0.11 to 0.73. To determine which process (influx, efflux or sequestration) caused the nonlinearity, we calculated the permeability for influx and efflux (PSinflux, PSefflux, respectively) and sequestration clearance (CLint) for total drug based on the mathematical model (10). As shown in Table 1, with

Table 1. Rate-Determining Step of 4-MU Elimination in Rat Liver Evaluated by the MID Method.[10]

	low dose (50 μg)	high dose (3000 μg)
CLint,all (ml/min/g liver)	6.24 ± 0.62	2.08 ± 1.16
PSinflux (ml/min/g liver)	12.5 ± 1.3	13.3 ± 2.1
PSefflux (ml/min/g liver)	2.68 ± 0.48	4.45 ± 0.63
CLint (ml/min/g liver)	2.68 ± 0.29	0.887 ± 0.104
CLint/(PSefflux + CLint)	0.524 ± 0.048	0.176 ± 0.025
rate-determining step of CLint,all	both membrane transport and sequestration	sequestration
rate-determining step of CLh	hepatic blood flow	sequestration

increasing dose, the PSefflux increased approx. two fold, while the CLint value decreased to approx. one-third. The remarkable dose-dependence of hepatic availability was due to nonlinearity in both the PSefflux and CLint values. However, the PSinflux was almost independent of dose. The dose-dependent change in CLint might be explained by the saturation of the conjugative metabolism of 4-MU, while the increase in PSefflux value with increasing dose might be partly explained by nonlinear tissue binding of 4-MU, since the tissue unbound fraction determined by ultrafiltration using liver homogenate increased approx. 1.5-fold at higher concentrations of 4-MU compared to that at lower concentrations. In addition, based on a comparison of the individual intrinsic parameters, *i.e.*, PSinflux, PSefflux, and CLint the major determining process for the CLint,all of 4-MU is thought to be sequestration at high dose. However, at the low dose, membrane transport (influx and efflux processes) as well as sequestration determine the CLint,all. Viewed in the light of CLh, the rate-determining factor is Qh at the low dose and CLint at the high dose. Further study (15) has confirmed that the saturation of the sequestration process observed using the MID method reflects that of conjugative metabolism.

Furthermore, we also examined the hepatic elimination of *l*-propranolol (*l*-PR) using the MID technique. *l*-PR has been assumed to be rapidly distributed between blood and hepatocytes, because of its high lipophilicity. Our study (16) has demonstrated that this assumption of a rapid equilibrium does not hold even for such a lipophilic drug. With increasing *l*-PR concentration in the perfusate, PSefflux increased approx. two-fold, whereas CLint decreased by approx. one-half. In contrast, PSinflux was independent of perfusate concentration. We came to the conclusion similar to the case for 4-MU based on a comparison of the kinetic parameters representing the three processes. That is, the rate-determining process for CLint,all of *l*-PR is a combination of membrane transport and sequestration at low concentrations, while at high concentration, the rate-determining process is "sequestration". Such a concentration-dependent shift in the rate-determining process can be attributed to saturation of the sequestration process as well as the tissue binding.

HEPATIC UPTAKE

HMG-CoA Reductase Inhibitor, Pravastatin; Contribution of a Carrier-Mediated Active Transport System to the Liver Specific Distribution

The HMG-CoA reductase inhibitors decrease serum cholesterol by competitive inhibition of the rate-limiting enzyme of cholesterol biosynthesis, HMG-CoA reductase, and are now widely used to treat patients with hypercholesterolemia. Among these inhibitors, pravastatin (Prav) which is hydrophilic in nature, has been shown to exhibit relatively specific inhibition of cholesterol synthesis in the liver (17). One of the reasons for this relatively specific pharmacological activity, we hypothesized, is that the tissue distribution of Prav is limited because of its high hydrophilicity, while hepatic uptake by some particular mechanism may take place at the liver surface. Initially, we examined the initial uptake clearance for Prav *in vivo* using the integration plot analysis to estimate the uptake ability of each tissue (18).

After *i.v.* bolus administration of [^{14}C] Prav, arterial blood samples were collected at intervals. After a certain time, the rats were killed and several tissues were excised, and a portion of each weighed and the radioactivity counted. When the tissue uptake was measured within a short period during which the efflux and elimination of radioactivity from the tissues were negligible, the degree of tissue uptake at time t was proportional to the AUC from time 0 to t (AUC(0-t)). Both the drug amount per g tissue at time t (Ctissue (t)) and AUC (0-t) are divided by the plasma concentration at time t (Cp(t)) and, if we plot the Ctissue (t)/Cp(t) (*i.e.*, Kp (t) (ml/g tissue); tissue-to-plasma concentration ratio) as an ordinate and the AUC(0-t)/Cp(t) as an abscissa, the plot is a the straight-line with the slope giving the early-phase uptake clearance for the tissue (CLuptake). This plot is called an "integration plot". The integration plot analysis revealed that the largest clearance was observed for the liver, suggesting that the difference in the early-phase tissue distribution is one of the reasons for the liver-specific pharmacological effect of Prav (M. Yamazaki, T. Tokui, M. Ishigami, Y. Sugiyama; *Biopharm. Drug Disposit., in press.*). Furthermore, using isolated rat hepatocytes, the uptake mechanism for Prav was examined. The initial uptake velocity (v_o) exhibited a clear concentration-dependence, inhibited by hypothermia, metabolic inhibitors and sulfhydryl-modifying reagents. These experimental results together with the observation of the competitive inhibition by the more lipophilic structural analog, simvastatin (Fig. 1(B)) confirmed that Prav uptake was carrier-mediated (18).

Next, we attempted to quantitatively evaluate the contribution of this carrier-mediated uptake system which we had demonstrated *in vitro* to the initial uptake of this drug *in vivo*. With the calculated CLuptake value for Prav *in vivo* (estimated by the integration plot analysis), the hepatic blood flow rate and plasma unbound fraction of Prav, the values of CLint,all were calculated based on the respective kinetic models shown in Eqs. 1–3. In this experiment (integration plot analysis) the initial uptake was investigated and, therefore we can substitute CLh in Eqs. 1–3 for CLuptake, and also CLint,all in Eq. 6 for PSu,influx, respectively. PSu,influx calculated in this way can be compared with the PSu,influx obtained by *in vitro* experiments. Under linear conditions, PSu,influx is described by the parameters obtained in *in vitro* experiments as follows:

$$\text{PSu,influx} = \text{Vmax/Km} + \text{Pdif} \qquad (11)$$

As shown in Table 1, the PSu,influx value in Eq. 11, after correcting for the cell number per g liver, was comparable with that obtained *in vivo*. This result indicates that the carrier-mediated active transport system we demonstrated *in vitro* is responsible for the hepatic uptake *in vivo*.

The PSu,influx of Prav evaluated in the MID study was comparable with that reported in isolated rat hepatocytes and *in vivo* (integration plot analysis) as shown in Table 2 (19). This result was consistent with our previous findings that the values of PSu,influx evaluated with isolated hepatocytes were similar to those determined by the MID method for ligands with uptake clearances below approx. 1 ml/min/g liver (Fig. 2) (20). We also examined the uptake clearance of Prav using primary cultured hepatocytes, and found that the uptake ability decreased with time in culture; however, for a short time in culture (~ 6 hr), uptake was comparable with that in the other three different experimental systems (19) (Table 2). This is the

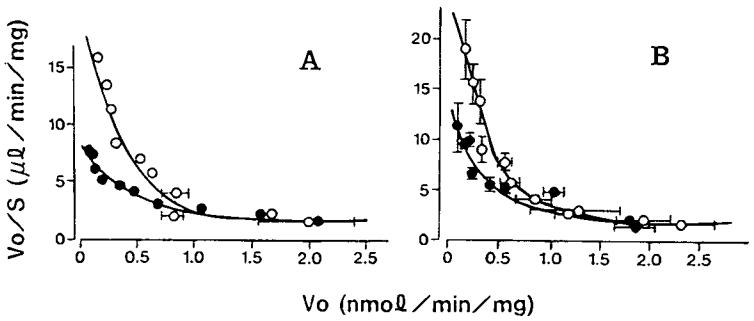


Fig. 1. Eadie-Hofstee plots of pravastatin uptake in the presence (closed circles) or absence (open circles) of other compounds. (18) A. dibromosulfophthalein (10 μM), B. simvastatin (open acid form, 3.5 μM). Lines were "best fit" of the data to the equation describing competitive-type inhibition.

first case in which PSu,influx values evaluated in 4 different experimental systems (*in vivo,* MID method, isolated cells and primary cultured cells (for short time culture)) for one ligand exhibited good agreement.

Prav is a typical drug whose clearance in the liver, which is the target organ in terms of its pharmacological effect as well as the major organ for its pharmacokinetics in the whole body, is governed mainly by an uptake process mediated by active transport.

Hepatic Uptake Mechanisms for Organic Anions: Relationship with the Bile Acid Uptake System

We have examined the uptake mechanism of Prav using isolated rat hepatocytes in an attempt to investigate the relationship with that for other organic anions and bile acids. The initial uptake velocity exhibited no Na^+-dependence and competitive inhibition was clearly observed for dibromosulfophthalein (DBSP), which is extensively taken up by the liver *via* a carrier-mediated active transport mechanism (21) (Fig. 1 (A)) and taurocholate (TCA) and cholate (CA), and the calculated inhibition constants (Ki) for each inhibitor were comparable with their Km values. Combined with the finding that Prav inhibited competitively the Na^+-independent uptake of taurocholate with a Ki comparable with the Km of Prav, the hepatic uptake of Prav occurs *via* a carrier-mediated active transport mechanism involving the so-called multispecific anion transporter (22), which is shared by the Na^+-independent bile acid uptake system (18).

Table 2. Comparison of the Permeability-Surface Area Product for the Influx of Unbound Pravastatin (PSu,influx) Determined in Different Experimental Systems (*in Vivo,* Perfused Liver, Isolated Cells, and Primary Cultured Cells)[19]

	in vivo	perfused liver	isolated cells	primary cultured cells	
				6 hr	24hr
ml/min/g liver	1.1[a]–1.9[b]	2.1	2.6–2.9	2.0	1.0
μl/min/mg protein	—	—	20.4–23.0	16.2	8.3

[a] Calculated using the sinusoidal perfusion model[8].
[b] Calculated using the venous equilibrium model[7].

An interesting experimental result was obtained in an inhibition study with Prav. As described above, Prav inhibited competitively the Na^+-independent uptake of TCA and the Na^+-dependent uptake of TCA was also inhibited partially (18). On the other hand, both the Na^+-dependent and -independent uptake of cholate, which are believed to be shared with TCA, were inhibited completely by Prav (23). Based on this result, the Na^+-dependent uptake mechanism for TCA and CA may at least partially differ.

The effects of cellular ATP content on the initial uptake velocity of the organic anions, DBSP and benzylpenicillin (PCG), whose uptake was shown to be by active transport in our previous experiments (24), were examined by measuring their uptake by isolated rat hepatocytes under conditions of reduced ATP. The uptake of organic anions fell in parallel with the decrease in cellular ATP, and the initial uptake velocity was shown to be saturable with respect to the to cellular ATP content, irrespective of the exposure time to metabolic inhibitors.

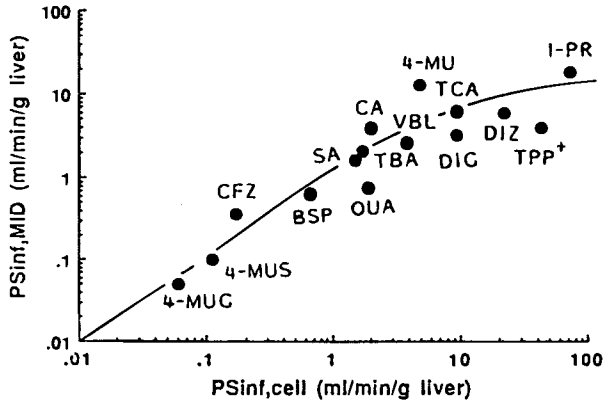


Fig. 2. Comparison of the hepatic influx clearances determined using perfused livers (MID technique) and isolated rat hepatocytes (20). Abscissa: the influx clearance determined using isolated hepatocytes (PSinf, cell; ml/min/g liver). Ordinate: the influx clearance determined using isolated perfused livers (PSinf, MID; ml/min/g liver). *l*-PR, *l*-propranol; TPP^+, tetraphenylphosphonium; DIZ, diazepam; DIG, digoxin; TCA, taurocholate, VBL, vinblastine; 4-MU, 4-methylumbelliferone; TBA, tolubutamide; CA, cholate; OUA, ouabain; SA, salycylic acid; BSP, bromosulfophthalein; CFZ, cefodizime; 4-MUS; 4-methylumbelliferone sulfate; 4-MUG, 4-methylumbelliferone glucuronide.

A similar result was obtained with CA under the same experimental conditions (measured in the presence of Na^+), however for TCA, the initial uptake remained virtually unchanged after a 5-min incubation with metabolic inhibitors; a significant decrease in uptake was observed only after longer incubation periods. This unequivocal dissociation between changes in cellular ATP and changes in the transport of TCA can be explained as follows: there was no marked change in intracellular Na^+ concentration during the short period of time (< 5 min) after the addition of metabolic inhibitors, i.e., an inwardly directed Na^+ gradient, the driving force for the Na^+-dependent uptake of TCA is maintained for a certain time, even under conditions of reduced ATP. On the other hand, uptake of CA exhibited a pattern similar to that for organic anions, suggesting that multiple transport mechanisms are responsible for the Na^+-dependent uptake for bile acids (23).

Regarding the multiplicity in bile acid uptake mechanism, Honscha et al. (25) obtained interesting findings by functional expressing study using *xenopus laevis oocytes.* The mRNA-fraction coding for the uptake of the loop diuretic bumetanide, which exhibits both Na^+-dependent and Na^+-independent uptake and is a competitive inhibitor of carrier-mediated bile acid uptake in liver cells, is clearly different from the mRNA-fraction coding for the taurocholate transport protein. Furthermore, uptake of cholate was induced by both mRNA fractions with almost 2.5 fold greater expression by the bumetanide fraction. There was no increase in bumetanide uptake in oocytes which expressed the cloned taurocholate transporter gene (sinusoidal Na^+/bile acid cotransporter (26)). Further biochemical and molecular biological studies are required to characterize the carrier-proteins with regard to multiplicity, regulation of expression, *etc.*

Driving Force for Hepatic Uptake of Organic Anions

Based on the results described above, we hypothesized that there was a contribution from either primary active transport or secondary active transport in which an unidentified driving force dissipates much more rapidly than the Na^+-gradient for the hepatic uptake of organic anions. Wolkoff et al. (27, 28) have reported Cl^--dependence in BSP uptake, followed by the finding that BSP uptake is not coupled directly to Cl^-, since there was no evidence of a dependence on Cl^- ion gradient. We found that uptake of the organic anion, 1-anilino-8-naphthalein sulfonate (ANS), whose uptake is mediated by active transport (24), was competitively inhibited by BSP and Rose Bengal, which are taken up by facilitated diffusion (not by active transport) (29). The comprehensive mechanisms for the uptake of organic anions which fit these observations have not yet been clarified.

As shown in Table 3, using expression cloning in *Xenopus laevis oocytes,* a cDNA encoding a rat liver organic anion-transporting polypeptide (oatp) has been isolated (30). In addition, several distinct proteins have been identified as candidate BSP transporters (31–33) and, among these, it has been suggested that "bilitranslocase" is responsible for the electrogenic uptake and "sulfobromophthalein/bilirubin-binding protein" for the electroneutral uptake of BSP, respectively (34). Further experiments at a molecular level are required to identify the multiplicities in the transport proteins as well as the exact driving force for the uptake.

BILIARY EXCRETION

Primary Active Transport

Regarding biliary excretion mechanisms of xenobiotics, although the existence of a transport maximum (Tmax) and mutual inhibition has been indirect evidence for the involvement of carrier protein(s) on the bile canalicular membranes, the precise mechanism has been clarified only recently. This comes from the difficulty in directly measuring the intracellular pool of ligands, which is virtually responsible for the transport from intracellular compartment to the bile. Owing to this difficulty, kinetic studies has been a major approach to investigating biliary excretion.

In these situations, many experimental observations have suggested that the biliary excretion mechanism(s) involve a primary active transport for several compounds such as (i) amphipathic organic cations including anticancer drugs, (ii) organic anions and drug conjugates such as glutathione conjugates and glucuronides (this transport route is designated as a "multispecific organic anion transporter (MOAT) (22)), (iii) conjugated bile acids and (iv) phospholipids; the biochemical identification of these carrier proteins is also in progress (4) (Fig. 3, Table 3). Such remarkable progress of this research area has been brought by, 1) the development of a preparation of bile canalicular membrane vesicles (CMV) (35, 36), 2) the discovery of the mutant rats such as TR^- (37) and the Eisai hyperbilirubinemic rat (EHBR) (38) with an inherited deficiency of biliary excretion of organic anions. In the following sections, we introduce (1) the multiplicity of organic anion biliary excretion mechanisms, (2) the experimental finding that P-glycoprotein, expressed on the liver canalicular membrane of normal rats, is responsible for the biliary excretion of drugs. Finally, we discuss a molecular model for this multiple excretion mechanism as well as the physiological significance of these complex transport systems in the body.

Biliary Excretion of Organic Anions

The precise mechanism for the biliary excretion of organic anions has not yet been clarified, although involvement of carrier-mediated transport has been suggested. In a series of *in vivo* studies using the Wistar-derived transport mutant rat strain (TR^-) Jansen et al. (37) made an important breakthrough. In this mutant rat, biliary excretion of LTC4, the glutathione-conjugate of LTA4, was dramatically reduced after i.v. administration (less than 2 % of dose at 60 min after administration compared to 77 % of dose in normal rats) (39) and further evidence has shown that the biliary excretion of DBSP, conjugated bilirubins *etc.* is also impaired. The mutant rats with conjugated hyperbilirubinemia possessing an autosomal recessive trait inheritance (EHBR) discovered in Japan were bred from Sprague-Dawley rats and also exhibited a decrease in their biliary excretion of organic anions (40). Furthermore, by using CMV, it has been demonstrated directly that both TR^- and EHBR lack the primary active transport system for organic anions which is directly coupled with ATP-hydrolysis, and that both mutant rats possess similar characteristics in terms of the hepatobiliary excretion of ligands. Ishikawa et al. (41) clearly demonstrated that LTC4 uptake by CMV from normal rats was stimulated by ATP, however, no enhancement was observed in CMV from TR^-. In addition, neither glutathione (GSH) itself

Table 3. Characterization of the Carrier-Protein for Organic Anions (Including Their Conjugates)

	Uptake[30–33]			Excretion[67, 68, 77]
MW (subunit)	37, 35.5 kD	55 kD	55 kD	
MW (native)	110 kD			90 kD
protein isolation	+	+	+	+
photoaffinity labeling	NR	+	NR	+
pI	9.0	7.0	3.5	NR
carbohydrate component	—	+	+	+
organ distribution (immunofluorescence)	liver, kidney	ubiquitous except for erythrocytes	liver, intestine not kidney and spleen	NR
inhibition of uptake by antiprotein antibodies	+	+	+	NR
reconstitution	+	NR	NR	NR
typical substrates	BSP	BSP	BSP	S-(2,4-dinitrophenyl)glutathione, LTC4, GSSG
driving force (or cofactors)	H^+	Cl^-	NR	ATP-hydrolysis
cloning	NR	+	NR	NR
cross reactivity (Northern blot analysis)	kidney, brain, lung, skeletal muscle, proximal colon liver from mouse and rabbit			NR

NR: not reported.

nor LTB4, which is a structural analog of LTC4, but not the glutathione conjugate, was a substrate for this transport system. Kobayashi et al. (42) prepared CMV and sinusoidal membrane vesicles (SMV) simultaneously, and showed that CMV not SMV exhibited ATP-dependent uptake of the glutathione-conjugate, S-(2,4-dinitrophenyl)-glutathione (DNP-SG). A primary active transport system for glutathione-conjugate was also shown in erythrocytes (43), heart sarcolemma (44) and mastocytoma cells (45), and this has been called a "GS-X-pump" (41), however at present, the molecular structure of the GS-X pump is not known. Many organic anions and their conjugates have been shown to be excreted into bile by a primary active transport mechanism, which is deficient in mutant rats, and at present, some experimental results suggest multiplicity in substrate recognition for this primary active transport system and also differential impairment for several organic anions in mutant rats. We now wish to summarize these topics.

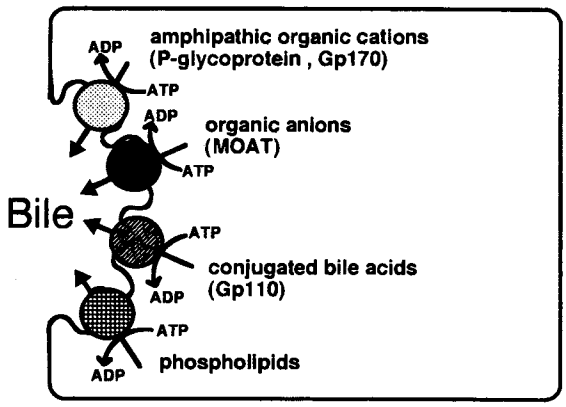


Fig. 3. ATP-dependent primary active transport carriers in the canalicular membrane involved in the biliary excretion of amphipathic organic cations, organic anions, conjugated bile acids and phospholipids.

Decrease in Biliary Excretion of Organic Anions in Mutant Rats

As described above, studies with mutant rats provided a lot of important information on the biliary excretion mechanism for organic anions. It has been suggested that the hereditary defect might originate from a mutation of the gene encoding the transport protein on the canalicular membrane (37, 38), however, other secondary factors may affect the impairment of biliary excretion in mutant rats. Possible factors which may influence the decrease in biliary excretion are considered below.

After being taken up by the liver through the sinusoidal membrane, organic anions diffuse into the intracellular space (*i.e.* cytosol) and eventually binds to the transport protein on the bile canalicular membrane. We tried to clarify whether the impairment in biliary excretion of organic anions can be accounted for only by a defect in bile canalicular membrane transport or by some other mechanisms(s) (46).

After i.v. bolus injection, the biliary excretion of nonmetabolizable organic anions, DBSP, indocyanine green (ICG) and cefodizime (CFDZ), a novel third generation cephalosporin antibiotic with two carboxyl groups, was markedly impaired (Fig. 4). The kinetic analysis of the disposition of these ligands revealed 1) that the transport rate via bile canalicular membrane was severely impaired in EHBR for CFDZ and DBSP and 2) that the intracellular transport rate of ICG was decreased in EHBR, which contributed more than the decrease in the canalicular membrane transport to the net reduction of the ICG excretion rate in EHBR. The latter finding was also supported by *in vitro* results (47): the binding of ICG to ligandin(s) in EHBR was less compared with that in normal rat, resulting in a higher distribution of ICG to intracellular organelles. The reduction in the ICG binding to ligandin(s) in EHBR was explained by the increase in the concentrations of intracellular bilirubin and its glucuronide. Because the ligand molecules bound to organelles diffuse within the cells much more slowly than the molecules in the cytosol, the higher distribution of ICG to organelles in EHBR results in a reduction in the intracellular transport

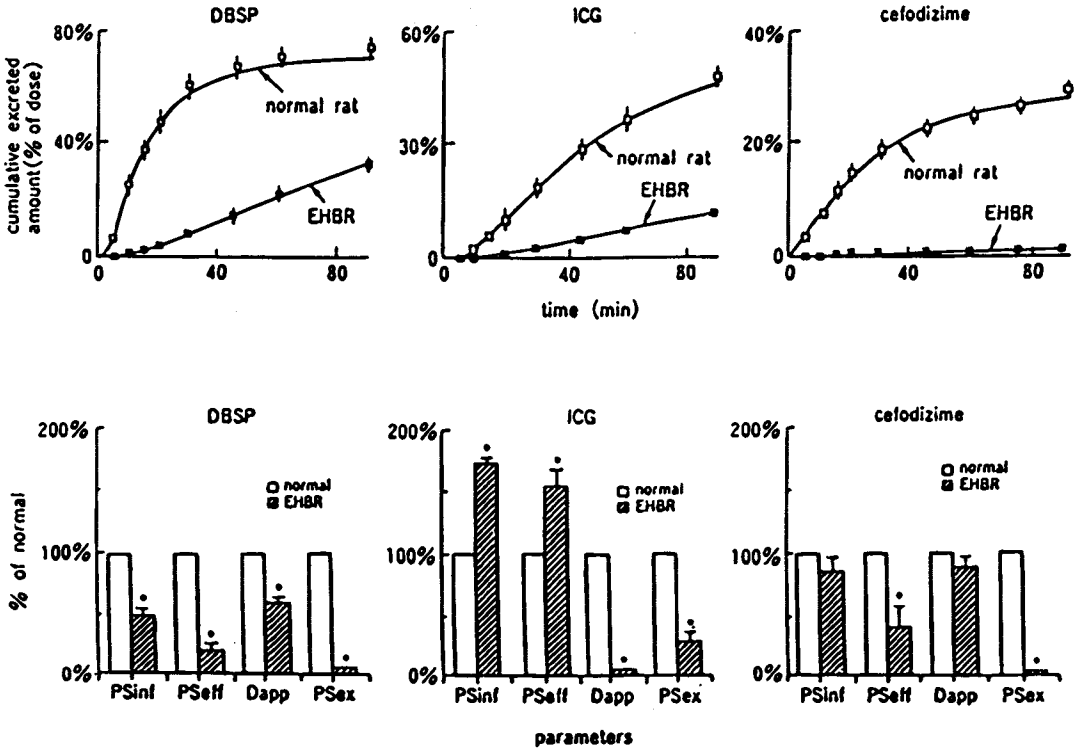


Fig. 4. Biliary excretion of DBSP, ICG and CFDZ in normal and EHBR (46). Upper panel: Cumulative biliary excretion of each ligand (DBSP (10 μmol/kg), ICG (5 μmol/kg), CFDZ (23.8 μmol/kg including 10 μCi/kg [^{14}C]CFDZ) administered intravenously as a bolus dose to normal rats (○) and EHBR (●). Lower panel: Calculated parameters. PSinf: membrane permeability clearance from blood to liver, PSeff: membrane permeability clearance from liver to blood, Dapp: intracellular diffusion coefficient, PSex: membrane permeability clearance from liver to bile.

rate. These results indicate that differential impairment mechanisms can be proposed for the excretion of organic anions: one being impaired transport across the canalicular membrane, the other being impaired intracellular transport due to the inhibition of cytosolic protein binding caused by the accumulation of intracellular bilirubin glucuronides.

As described above, for several organic anions, transport across the canalicular membrane is actually impaired, however, is this transport route unique one or not? In the following section, we would like to discuss this point.

Multiplicity of Biliary Excretion Mechanisms for Organic Anions

We have obtained results which suggest a difference in the biliary excretion mechanism for conjugated metabolites, glucuronide and sulfate, from studies of liver perfusion and isolated CMV (48, 49). As a model compound, 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole (E3040), a novel dual inhibitor of 5-lipoxygenase and thromboxane A2 synthetase, was used, since its major metabolites are glucuronide and sulfate which are predominantly excreted into the bile. The biliary excretion of the glucuronide formed in the liver, measured by the liver perfusion method, was severely impaired in EHBR, whereas for sulfate, there was no significant decrease. This result was confirmed by the uptake study by CMV (Fig. 5). The uptake of E3040 glucuronide by CMV prepared from SD rats exhibited ATP-dependence, whereas ATP had a minimal effect on the uptake of the glucuronide into CMV from EHBR (49). In contrast, the uptake of E3040 sulfate was comparable for SD rats and EHBR. Furthermore, ATP did not stimulate the uptake of sulfate into the CMV. The CMVs we used exhibited a clear ATP-dependent TCA uptake for both SD rats and EHBR, while for DNP-SG uptake we confirmed that only the CMV from SD rats showed ATP-dependence (Fig. 5). Based on these results, we suggest (1) that the excretion of E3040 glucuronide across the bile canalicular membrane is mediated by a primary active transporter which is defective in EHBR and (2) that the bile canalicular transport system for E3040 sulfate is different from that of the glucuronide in that the former remains normal in EHBR.

Next, we investigated the multiplicity of the transport of organic anions across the bile canalicular membrane both *in vivo* and *in vitro* (50). *In vivo*, a high concentration of DBSP in plasma reduces the biliary excretion of CFDZ and LTC4 radioactivity to about 15 and 35% of their control values, respectively, but does not affect the excretion of ICG. A high plasma concentration of ICG exerts a minimal effect on the excretion of LTC4 radioactivity and DBSP. *In vitro*, the ATP-dependent uptake of LTC4 into the CMV was reduced by DBSP, CFDZ and ICG in a dose-dependent manner, with approx. IC50 values of 0.1, 10 and 1 μM, respectively. The hepatic unbound concentration of DBSP sufficient to reduce the excretion of CFDZ, leukotriene radioactivity and DBSP itself *in vivo* was calculated to be approx. 10 μM, a concentration which was also sufficient

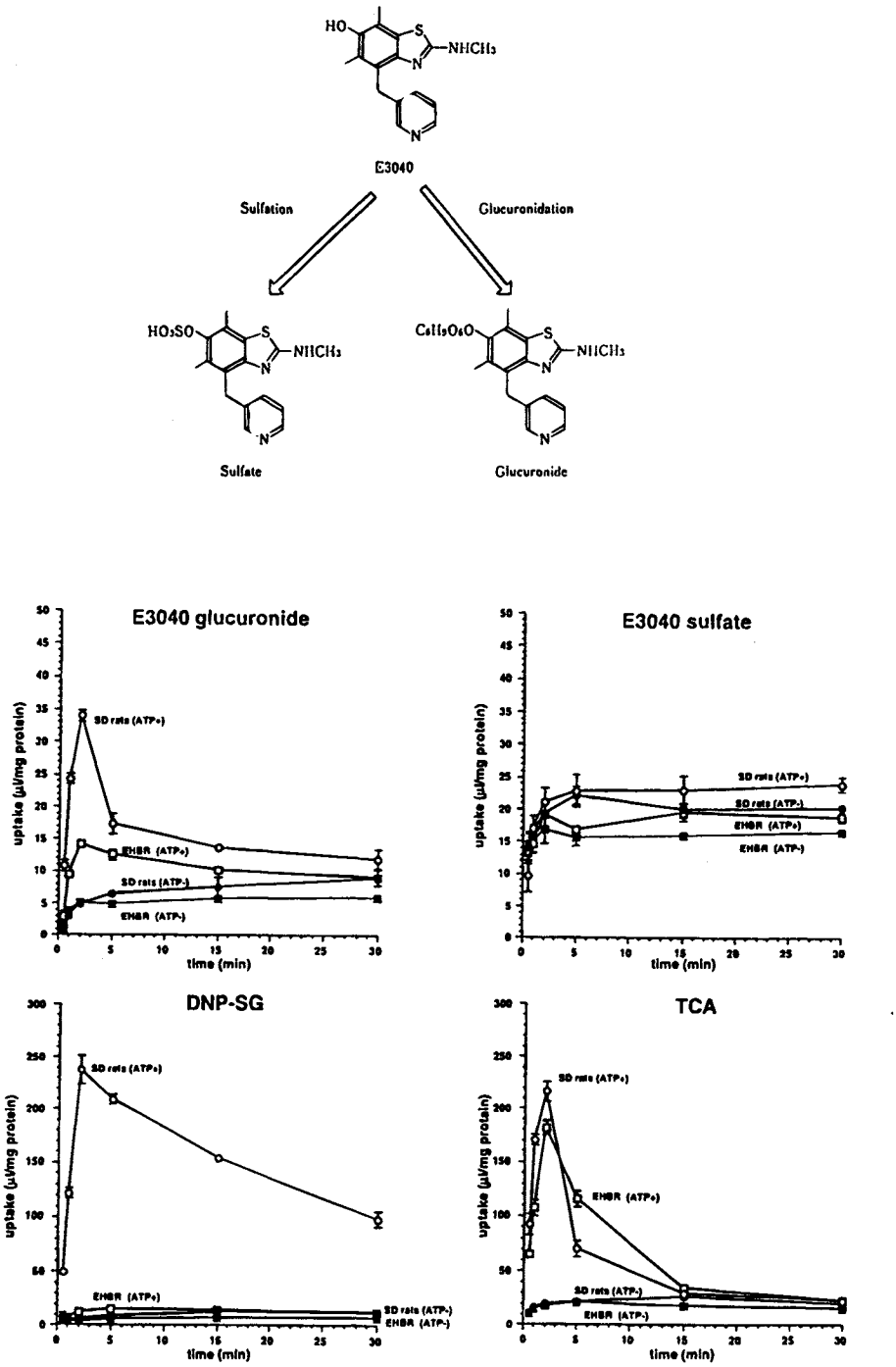


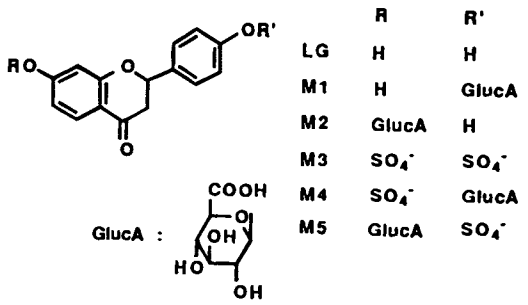
Fig. 5. Upper: Structure of E-3040 and its conjugated metabolites. **Lower:** ATP-dependent uptake of organic compounds by CMV prepared from normal rats and EHBR (49). CMV from normal rats (left) and EHBR (right) were incubated with 25 μM [^{14}C]E-3040 glucuronide, 25μM [^{14}C]E-3040 sulfate, 1.4 μM [^{3}H]TCA or 1 μM [^{3}H]DNP-SG in the presence (●) and absence (○) of 5 mM ATP and an ATP-regenerating system (10 mM creatine-phosphate and 100 μg/ml creatinephosphokinase).

to reduce the transport of LTC4 to CMV *in vitro*. In contrast, the hepatic unbound concentration of ICG that saturated the excretion of ICG *in vivo* was calculated to be less than 0.1 μM, which was more than ten times smaller than the IC50 of ICG for the transport of LTC4 to CMV *in vitro*. The inhibitory effect of ICG on the ATP-dependent uptake of LTC4 at concentrations higher than 1 μM may be accounted for by some nonspecific effect caused by such a hydrophobic reagent. These results also support our conclusion obtained in an *in vivo* study that the major transport system for DBSP and CFDZ is primary active transporter (MOAT) shared with a typical endogenous substrate, LTC4. Furthermore, the decrease in the biliary excretion of ICG

in EHBR is mainly caused by the impairment of intracellular diffusion as a secondary effect.

We further investigated multiplicity for the biliary excretion of conjugates *in vivo* using liquiritigenin (LG, 2,3-dihydro-7-hydroxy-2-(4-hydroxyphenyl)-(S)4H-1-benzopyran-4-1) as a model compound (51). LG, which is contained in licorice root, contains two carboxyl groups and is metabolized to five different conjugates: M1-M5 (Fig. 6), which are predominantly excreted into the bile. LG was administered i.v. as a bolus to SD rats receiving i.v. infusions of the inhibitors (DBSP, ICG and glycyrrhizin (GR), the ether type glucuronide of the triterpenoid contained also in licorice root, which is predominantly excreted into the bile in the rat) (Fig. 6). A high plasma concentration of GR and DBSP reduced significantly the biliary excretion of all the conjugates except M3 (disulfate), whereas that of ICG did not affect the excretion of any of the conjugates. In EHBR, the biliary excretion of M1, M2, M4, and M5 was markedly reduced, whereas that of M3 was comparable with that in normal rats. These results, together with those described above, lead us to suggest the presence of at least three different systems for the transport of organic anions across the bile canalicular membrane: the system for glucuronides of LG (M1, M2, M4 and M5) that is inhibited by GR and DBSP (defective in EHBR); the system for the disulfate of LG (M3) that is not inhibited by GR and DBSP (present in EHBR) and the system for ICG that is different from the other two (present in EHBR).

We have summarized the multiplicity in the biliary excretion of organic anions in Table 4. Further studies using CMV are required to examine whether the transport mechanisms (for LG(M3), E3040 sulfate, and Prav (M. Yamazaki, K. Kobayashi,



	R	R'
LG	H	H
M1	H	GlucA
M2	GlucA	H
M3	SO_4^-	SO_4^-
M4	SO_4^-	GlucA
M5	GlucA	SO_4^-

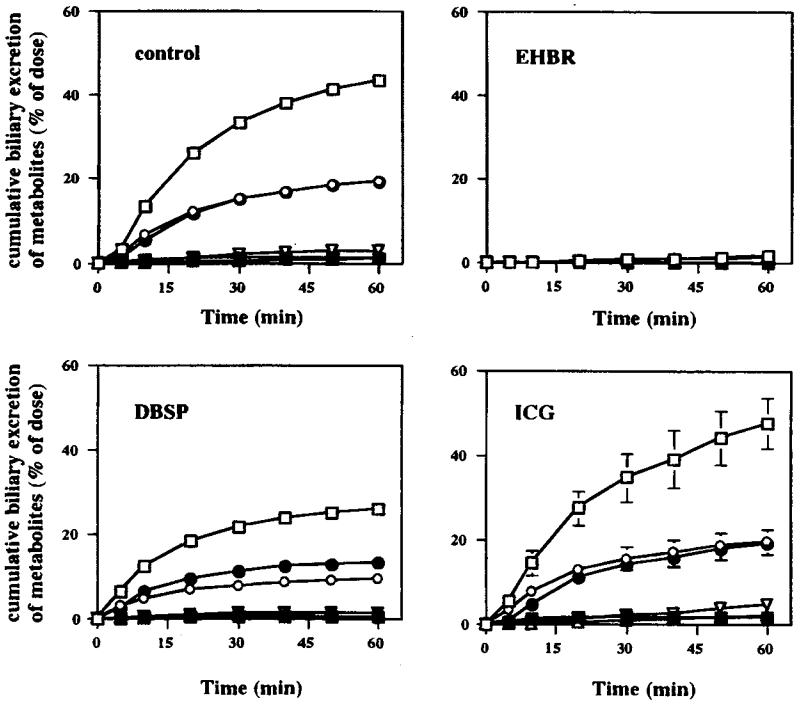


Fig. 6. Top: Structure of liquiritigenin (LG) and its conjugated metabolites. **Middle:** Biliary excretion of LG metabolites in normal rat (left) or EHBR (right) after i.v. bolus administration of LG (5 mg/kg) (51). **Bottom:** Effect of DBSP and ICG on the cumulative amount of LG metabolites excreted into the bile in normal rats (51). Each inhibitor (DBSP, 3.0 μmol/min/kg; ICG, 0.15 μmol/min/kg) was infused i.v. 60 min before i.v. bolus injection of LG (5 mg/kg). ■, M1; ○, M2; △M3; ●, M4; ▽, M5; and □, Total.

Table 4. Multiplicity of Biliary Excretion Mechanism for Organic Anions in Terms of the Transport Deficiency in EHBR and Their Mutual Inhibitors

Ligand	Co-infused Inhibitor			EHBR
	DBSP	ICG	GR	
DBSP (46, 50)	—	→	nd	defective
cefodizime (46, 50)	↓	→	nd	defective
LTC4 (50)	↓	→	nd	defective
LG-glucuronides (51) (M1, M2, M4, M5)	↓	→	↓	defective
GR (51)	↓	→	—	defective
E-3040-glucuronide (48, 49)	nd	nd	nd	defective
DNP-SG (49)	nd	nd	nd	defective
LG-disulfate (M3) (51)	→	→	→	maintained
E-3040-sulfate (48, 49)	nd	nd	nd	maintained
ICG (46, 50)	→	—	nd	maintained (decrease to some extent)
Pravastatin*	nd	nd	nd	maintained (decrease to some extent)

→: No change in the biliary excretion of ligand by inhibitors.
↓: Decreased in the biliary excretion of ligand by inhibitors.
nd: not determined
*: M. Yamazaki, K. Kobayashi, and Y. Sugiyama; *Biopharm. Drug Disposit.*, in press.

and Y. Sugiyama; *Biopharm. Drug Disposit., in press.*)), which are present in EHBR are common or not.

Possible Other Mechanism(s) for Biliary Excretion of Organic Anions

Tamai et al. (52) investigated the biliary excretion mechanism of cephalosporin antibiotics, cefpiramide (monovalent anion at physiological pH) using CMV and suggested that cefpiramide is excreted into bile *via* a carrier-mediated mechanism which depends on a membrane potential difference across the bile canalicular membrane. However, it has not yet been clarified whether the transport mechanism for cefpiramide is identical with the ATP-dependent transport mechanism for organic anions described above. Furthermore, recent studies using CMV have demonstrated that both ATP- and membrane potential-dependent transport mechanisms are responsible for the biliary excretion of bilirubin glucuronide (53) and DNP-SG (54), and that a membrane potential-dependent transport system is maintained in mutant rats (53). Further study is required to clarify the relationship between membrane potential dependent and ATP-dependent transport systems.

The tripeptide GSH is the main nonprotein thiol in cells and plays an important role in a variety of cell functions including detoxification of electrophilic xenobiotics and conjugation of endogenous compounds leading to synthesis of prostaglandins and leukotrienes. Both mutant rat strains, TR^- and EHBR, do not secrete GSH into bile *in vivo* (55, 56) or in the perfused liver, while there was no significant difference in the efflux of GSH from liver to perfusate compared with that of normal rats (55). In addition, GSH-conjugates inhibited GSH transport in CMV (57), suggesting a possibility of shared transport with GSH-conjugates *via* a primary active transport system. In CMV from EHBR, however, the characteristics and kinetics of GSH transport were the same as in the controls, and there was no stimulation by ATP of the transport of GSH into CMV from both normal rats and EHBR (40). How can these apparently inconsistent results be explained? Fernandex-Checa et al. (40) suggested a possible mechanism for GSH transport *via* the canalicular membrane according to their experimental results. They suggested that the near absence of GSH in the bile of mutant rats can be best explained as a secondary defect due to *cis*-inhibition from retained endogenous organic anions for the defective carrier and/or loss of *trans*-stimulation by these same substrates which are normally concentratively transported into bile. Further studies are required to demonstrate this hypothesis and to identify which endogenous organic anion(s) might be responsible.

A Possible Molecular Model for the Multiple Transporters of Organic Anions Which Are Deficient in Mutant Rats

Based on the results described above, it is obvious that multiple systems play a role in the biliary excretion of organic anions. However, the multiplicity of transport proteins and/or molecular mechanisms for the deficiency in biliary excretion of mutant rats have not yet been clarified. We would like to discuss here the case of UDP-glucuronosyltransferase (UDPGT), which is very helpful as an example of a molecular model for enzyme multiplicity with broad substrate specificity.

The Gunn rat is a mutant strain of Wistar rat which has unconjugated hyperbilirubinemia as a result of the absence of hepatic UDP-glucuronosyltransferase (UDPGT) activity towards bilirubin (58). They also have a markedly decreased hepatic UDPGT activity for phenol and digitoxigenin-monodigitoxiside substrates (58). Iyanagi et al. (59, 60) proposed the molecular basis of multiple UDPGT isoenzyme deficiencies in the Gunn rat as described below.

They found that the defect of the methylchoranthrene (MC)-inducible isoenzyme in Gunn rats arises from a −1 frameshift mutation that removes 115 amino acids from the

COOH terminus and further, they isolated and sequenced cDNAs from a Gunn rat liver library. Three novel cDNAs were identified that had an identical 3′-region of 1362 base pairs containing a single-base deletion in the same position as that of the mutant cDNA. However, their 5′-regions, encoding the substrate-binding domain, showed no more than a 40 % homology with that of other UDPGTs. The variety of amino-terminal domains may provide the aglycone-binding site whereas the conserved carboxyl domain provides the binding site for the common cosubstrate, UDP-glucuronic acid (UDPGA). According to these results, the most plausible mechanism they proposed was that all the mRNAs are transcribed from a single gene. The exons encoding the variable 5′-portions of the mature transcripts (which provides the substrate binding site) are transcribed independently and differentially spliced to common exon(s) encoding the conserved 3′-portion (which provides the binding site for the common cosubstrate, UDPGA). The defects in some UDPGT isoenzymes in the Gunn rat are caused by a single mutation that results in the formation of a common truncated COOH terminus and, consequently, binding to UDPGA cannot occur leading to the lack of activity for several substrates with different specificities. We may apply this hypothesis to the defect in primary active transport activity of mutant rats (TR^- and EHBR) involving more than two kinds of substrates. The exons encoding the binding sites for different kinds of substrates and the exon(s) for encoding the ATP-binding site may be in a single gene and, for mutant rats, a mutation may occur in the region of the ATP-binding site (Fig. 7).

This hypothesis may be supported by our recent findings (61). We fixed our eyes upon the fact that the substrate specificity of multidrug resistance associated protein (MRP), a member of the ABC-transporter family (62), is similar to that of MOAT in that both transporters can accept DNP-SG and LTC4 as the substrates (63–65; Fig. 8) and hypothesized that MRP and/or MRP-related transporter might be involved in the biliary excretion of organic anions across the bile canalicular membrane. To examine this hypothesis, we prepared the degenerate primers for PCR based on the conserved sequence in the COOH-terminal ABC region of human MRP. PCR with these primers resulted in the amplification of a 421 bp cDNA fragment from SD rat liver cDNA (61). The amplified fragment exhibited homology with a human MRP and the human MRP-like fragment (yp75a11, L. Hillier et al. (1995) Genebank accession No. H47977) with homology score of 66.3% and 83.0% at the

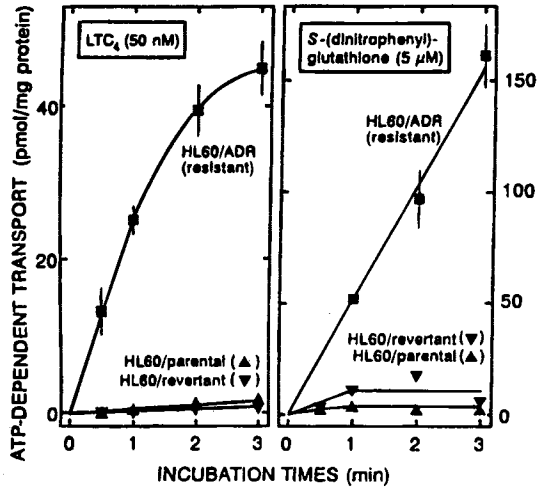


Fig. 8. ATP-dependent transport of [^{3}H]LTC4 and [^{3}H]DNP-SG in membrane vesicles of MRP-overexpressing and control HL60 cells. Data from Jedlitschky et al. (63).

cDNA level, and 73.3% and 84.7% at the deduced amino acid level, respectively (Table 5; Ref. 61). Northern blot analysis of poly(A)$^+$ RNA prepared from SD rat liver revealed the presence of approx. 5 kb and 8.6 kb mRNA species which hybridized to this fragment (61). In contrast, poly(A)$^+$ RNA from EHBR did not hybridize to this fragment (61). These results suggest (1) that the impaired expression to this particular region might be related to the pathogenesis of hyperbilirubinemia in EHBR and (2) that this region might encode part of MOAT (61). The defect in the expression of two mRNA species in EHBR could be accounted for by assuming that the mRNA for the transporter(s) is generated by alternative splicing, as discussed above in detail (61).

Independently, Mayer et al. (66) took the same strategy as that of ours, and found that the putative COOH-terminal ABC region (347 bp) was amplified from the Wistar rat, but not from the TR^- rat. The results obtained by Mayer et al. (66) are summarized in Table 6. The sequence of the amplified region was completely the same as that amplified in our laboratory (61, 66). Furthermore, they succeeded in the amplification of NH_2-terminal ABC region in both Wistar and TR^- rats by RT-PCR (66). Mayer et al. (66) also used an antibody against human MRP and found the selective loss of MRP from the canalicular

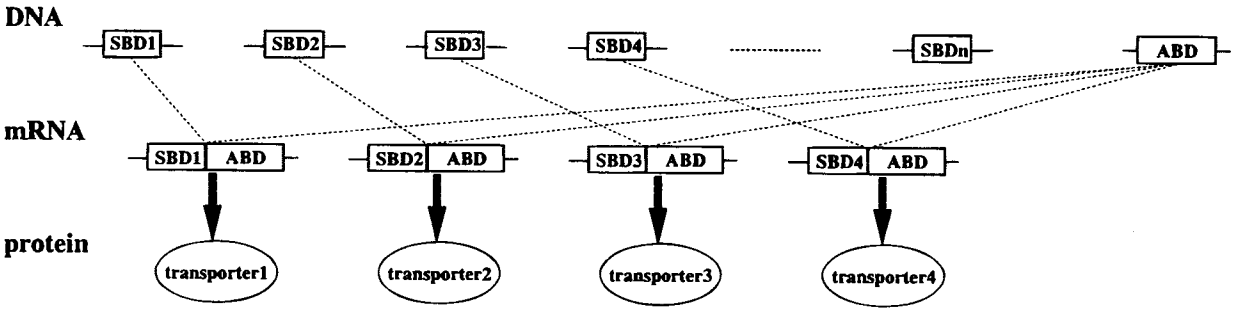


Fig. 7. Proposed scheme of the formation mechanism of an organic anion transporting protein with multiple substrate specificity. This scheme is based on the model for generation of diverse mRNAs of UDPGT family 1 proposed by Iyanagi (60). SBD: substrate binding domain, ABD: ATP binding domain.

Table 5. Alignment of the cDNA and the Deduced Amino Acid Sequence of the PCR Product, Human MRP, and Human MRP-like Protein (yp75a11) (61)

```
    cDNA sequence

Human MRP] 4185' GAGCTGGGAAGTCGTCCCTGACCCTGGGCTTATTTCGGATCAACGAGTCTGCCGAAGGAGAGATCATCAT
PCR fragment]28  GGGCTGGGAAATCATCCCTCACAAACTGCCTCTTCAGAATCTTAGAGTCTGCGGGGGGCCAGATCATCAT
yp75a11]     26" GAGCTGGAAAGTCATCCCTCACAAACTGCCTCTTCAGAATCTTAGAGGCTGCCGGTGGTCAGATTATCAT

             4255' CGATGGCATCAACATCGCCAAGATCGGCCTGCACGACCTCCGCTTCAAGATCACCATCATCCCCCAGGAC
               98  TGATGGGATAGATGTTGCCTCCATTGGACTGCACGACCTTCGAGAGAGGCTGACCATCATTCCCCAGGAC
               96" TGATGGAGTAGATATTGCTTCCATTGGGCTCCACGACCTCCGAGAGAAGCTGACCATCATCCCCCAGGAC

             4325' CCTGTTTTGTTTTCGGGTTCCCTCCGAATGAACCTGGACCCATTCAGCCAGTACTCGGATGAAGAAGTCT
              168  CCCATTTTGTTCTCGGGGAGTCTGAGGATGAATCTCGACCCTTTCAACAAATATTCAGATGAGGAGGTTT
              166" CCCATNCTGTTCTCTGGAAGCCTGAGGATGAATCTCGACCCTTTCAACAACTACTCAGATGAGGAGATTT

             4395' GGACGTCCCTGGAGCTGGCCCACCTGAAGGACTTCGTGTCAGCCCTTCCTGACAAGCTAGACCATGAATG
              238  GGAGGGCCCTGGAGTTGGCTCACCTCAGATCCTTTGTGTCTGGCCTACAGCTTGGGTTGTTATCCGAAGT
              236" GGAAGGCCTTGGAGCTGGCTCACCTCAAGTCTTTTGTGGCCAGCCTGCAACTTGGGTTATCCCACGAAGT

             4465' TGCAGAAGGCGGG-GAGAACCT-CA-GTG-TCGGG-CAGCGCCAG--CTTGTGTGCCT-AGCCC-GGGCC
              308  GACAGAGGGTGGT-GACAACCT-GA-GCA-TAGGG-CAGAGGCAG--CTCCTATGCCT-GGGCA-GGGCT
              306" TACAGAGGCTGGTGGGCAACCTGGAGGCATTAGGGCCAGAGGAAGNCTTGCTGTGGCTGGGGCAGGGGCT

             4526' CTGC-TGA--GGAAGACGAAGATCCTTGTGTTG
              369  NTGC-TTC--GAAAATCCAAAAT-CTGGTCCTG
              376" CTGCTTTCGGGAAATTCCAAG

    Amino acid sequence

Human MRP] 1396" AGKSSLTLGLFRINESAEGEIIIDGINIAKIGLHDLRFKITIIPQDPVLFSGSLRMNLDPFSQYSDEEVW
PCR fragment]10  AGKSSLTNCLFRILESAGGQIIIDGIDVASIGLHDLRERLTIIPQDPILFSGSLRMNLDPFNKYSDEEVW
yp75a11]     10" AGKSSLTNCLFRILEAAGGQIIIDGVDIASIGLHDLREKLTIIPQDPXLFSGSLRMNLDPFNNYSDEEIW

             1466" TSLELAHLKDFVSALPDKLDHECAEGGENLSVGQRQLVCLARALLRKTKILV
               80  RALELAHLRSFVSGLQLGLLSEVTEGGDNLSIGQRQLLCLGRAXLRKSKIWS
               80" KALELAHLKSFVASLQLGLSHEVTEAGG
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The cDNA fragment (421 bp) was amplified from Sprague-Dawley rat liver cDNA with the degenerate primers which were prepared from the conserved sequence in the ATP-binding cassette region of human multidrug resistance associated protein (61). The sequence between the forward and reverse primers (370 bp) is listed in this table. The deduced amino acid sequence is also listed. Asterisks represent the consensus sequence.

Table 6. Characteristics of MRP Gene Expression in Wistar and TR⁻ Rat Liver

	Wistar rat	TR⁻ rat
Amplification by RT-PCR		
aminoterminal ABC region	182bp	182bp
carboxyterminal ABC region	347bp	no amplification
Western blot		
canalicular membrane	170kDa	weak band at 170 kDa
basolateral membrane	170kDa	170kDa, 180kDa
Immunohisto-chemical staining	lateral and canalicular membranes	lateral membrane

Note: The results reported by Mayer et al. (66) are summarized in this table. Mayer et al. (66) could amplify the carboxyterminal ABC region from the Wistar rat but not from TR⁻ rat. Using the polyclonal antibody against carboxyterminus of human MRP (QRGLFYSMAKDAGLV, amino acids 1517–1531), Mayer et al. (66) found the absence of MRP (170 kDa) on the canalicular membrane of TR⁻ rat.

membrane of TR⁻ rats, providing the molecular basis for the pathogenesis of TR⁻ rat. Collectively, the hereditary defect in EHBR and TR⁻ can be related to the defective expression of COOH-terminal ABC region of putative MOAT, which has homology with MRP (61, 66).

In addition, several pieces of information are available on the biochemical properties of putative MOAT(s). Previously, a 90 kDa glycoprotein was isolated from rat liver as an organic anion-transporting ATPase and a reconstitution study with liposomes has all been successfully performed (67, 68). Furthermore, Ishikawa et al. (69) have found functional overexpression of GS-X pump, whose substrate specificity is similar to that of MOAT, in *cis*-Diamminedichloro-platinum (II)-resistant human leukemia HL-60 (HL-60/R-CP) cells. In HL-60/R-CP cells, three membrane proteins with apparent molecular masses of 200, 110 and 70 kDa, respectively, were found to be overexpressed. Using a polyclonal antibody against human MRP, Mayer et al. (66) clarified the presence of 190 and 170 kDa proteins on bile canalicular membrane isolated from human and Wistar rats, respectively.

As mentioned above, further progress in the study of biliary excretion is expected by applying a molecular biological approach in combination with a quantitative kinetic approach to evaluate the true contribution of each transport system to the biliary excretion under physiological conditions.

Transport Mechanism Involving P-glycoprotein (P-gp)

At the beginning of this section, we would like to summarize the recent findings regarding the contribution of several P-gps on the transport of organic compounds. P-gps belong to a class of vectorial transport proteins known as the ATP-binding cassette (ABC) transporters (62). The mammalian P-gps are encoded by small families of linked genes, two in human, three in rodents (70). The human MDR1 gene, the mdr1 (or mdr1b) and mdr3 (or mdr1a) genes of mice, and the pgp1 and pgp2 genes of hamster encode related proteins that transport hydrophobic drugs. The MDR3 P-gp is the most conserved of the mammalian P-gps and it shares more than 90% identity at the amino acid level with the P-gps encoded by the mdr 2 gene in mice and the pgp3 gene in hamsters. MDR1 P-gp has been known to confer MDR in mammalian tumor cells by actively extruding a wide range of structurally unrelated amphiphilic hydrophobic drugs from the cell. In addition, disruption of the mouse mdr1a P-gp gene actually leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs such as ivermectine and vinblastine (71). These experimental results clearly indicate that MDR1 P-gp plays an important role in the blood-brain barrier as a drug efflux pump.

The localization of MDR1 P-gp at the bile canalicular surface of hepatocytes suggests that it has a physiological role in the excretion of xenobiotics into bile. Kamimoto et al. (72) characterized the transport of daunomycin into inside-out CMV as being: 1) ATP-dependent and having saturable transport; 2) inhibited by vinca alkaloid and verapamil, known to be a substrate and inhibitor of P-gp- related transport, respectively. These features have also been found in tumor cells with MDR, suggesting that hepatic P-gp is involved in the excretion of xenobiotics such as anthracyclines and vinca alkaloids.

Membrane vesicles are a useful tool for investigating the precise mechanism of transport, however, this is an artificial experimental system and thus a quantitative assessment is hardly possible. Furthermore, hepatobiliary excretion consists of an uptake process through the sinusoidal membrane, intracellular-diffusion, and/or -binding, and excretion through the bile canalicular membrane. Consequently to investigate the contribution of P-gp to the biliary excretion of xenobiotics, studies should be performed with other experimental systems which are more physiological than membrane vesicles, *e.g.* liver-perfusion system as well as *in vivo* where the lobular architecture of the liver is maintained. Therefore, we attempted to examine the role of P-glycoprotein in the hepatobiliary transport of vincristine (VCR), a substrate for P-gp, using *in vivo,* perfused liver and CMV. Firstly, we examined the effect of hepatic P-gp induction on the biliary excretion of VCR in rats (73). Immunoblot analysis using C219, a monoclonal antibody to P-gp, demonstrated that phenothiazine (PTZ) treatment increased the P-gp level in CMV approx. 6.5-fold. Transport of [^{3}H]VCR to CMVs from the PTZ-treated and control rats revealed ATP-dependence, with an overshoot. The cumulative biliary excretion 3 hr after i.v. bolus administration of a tracer amount of [^{3}H]VCR was 69% of the dose for PTZ-treated rats and 45% for control rats. Further studies using an MID method revealed that the ratios of the cumulative amount of [^{3}H]VCR taken up by the liver at 15 and 30 min were significantly increased in the PTZ-treated rats by 60 and 45%, respectively, compared with the control rats. These results suggest that P-gp induced by PTZ on the bile canalicular membrane functions as a transporter not only in the isolated membrane but also in a more physiological system. Furthermore, we attempted to examine the contribution of P-glycoprotein to the biliary excretion *per se* of VCR using a single-pass liver perfusion system (74). The bile/liver unbound concentration ratio at steady-state obtained after correction for intracellular binding and the inside-negative membrane potentials and/or pH difference between the inside and outside of the cells was 160–280, suggesting a highly concentrated process, *i.e.* active biliary excretion of VCR. In addition to these results, selective inhibition by moderate concentrations of verapamil in the liver perfusion study confirmed that the hepatic P-gp

functions under physiological conditions, excreting VCR concentratively into the bile *via* an active transport mechanism.

Attempts to identify a function for the human MDR3 gene and its mouse homolog (mdr 2) have long been unsuccessful. However, using mdr 2 gene knock-out mice, Smit et al. (75) demonstrated that the mdr 2 P-gp has an essential role in the secretion of phosphatidylcholine into bile (Fig. 9), leading to liver disease, and hypothesized that it may be a phospholipid transport protein or phospholipid flippase. This function cannot be provided by the mdr1 and mdr3 genes, expressed at elevated levels in mice homozygous for a disruption of the mdr 2 gene, suggesting that mdr 2 P-gp has a quite distinct function. Berr et al. (76) produced evidence for the presence of a phosphatidylcholine translocator in CMV from normal rats. As suggested by Smit et al. (75), an attractive possibility is that mdr 2 could catalyze phosphatidylcholine translocator activity.

PHYSIOLOGICAL SIGNIFICANCE OF MULTIPLICITY IN THE BILIARY EXCRETION PROCESS

In general, multiplicity is involved in proteins (enzymes, carrier-proteins) which are responsible for detoxification (removal of xenobiotics), an important function for the living body. For example, many isozymes are involved in P-450, a typical enzyme for the metabolism of a wide variety of xenobiotics. In addition, it is now being shown that inter-individual differences are reflected by P-450 polymorphism and that this polymorphism is associated with the side-effects of some drugs. The involvement of polymorphism in drug metabolism is confirmed not only in "Phase I" by P-450, but also in "Phase II" by conjugation and, further, in the "Phase III (5)" step by biliary excretion, as described in this review. This multiplicity and polymorphism in detoxification mechanisms reflect the "self-defense" ability of a living organism against a wide variety of xenobiotics acquired through adaptation and/or evolution. We hope that molecular and biochemical investigation of the detailed mechanisms of each process, important factors determining the detoxification activity towards drugs, and identification of carrier-proteins and genes, as well as kinetic analysis of these detoxification reactions and their relationship to physiological conditions may provide new understanding of the detoxification and its regulation mechanisms. Furthermore, this could also provide more important information for studying the pharmacokinetics of new drugs and, finally, allow the development of novel and effective drug delivery systems.

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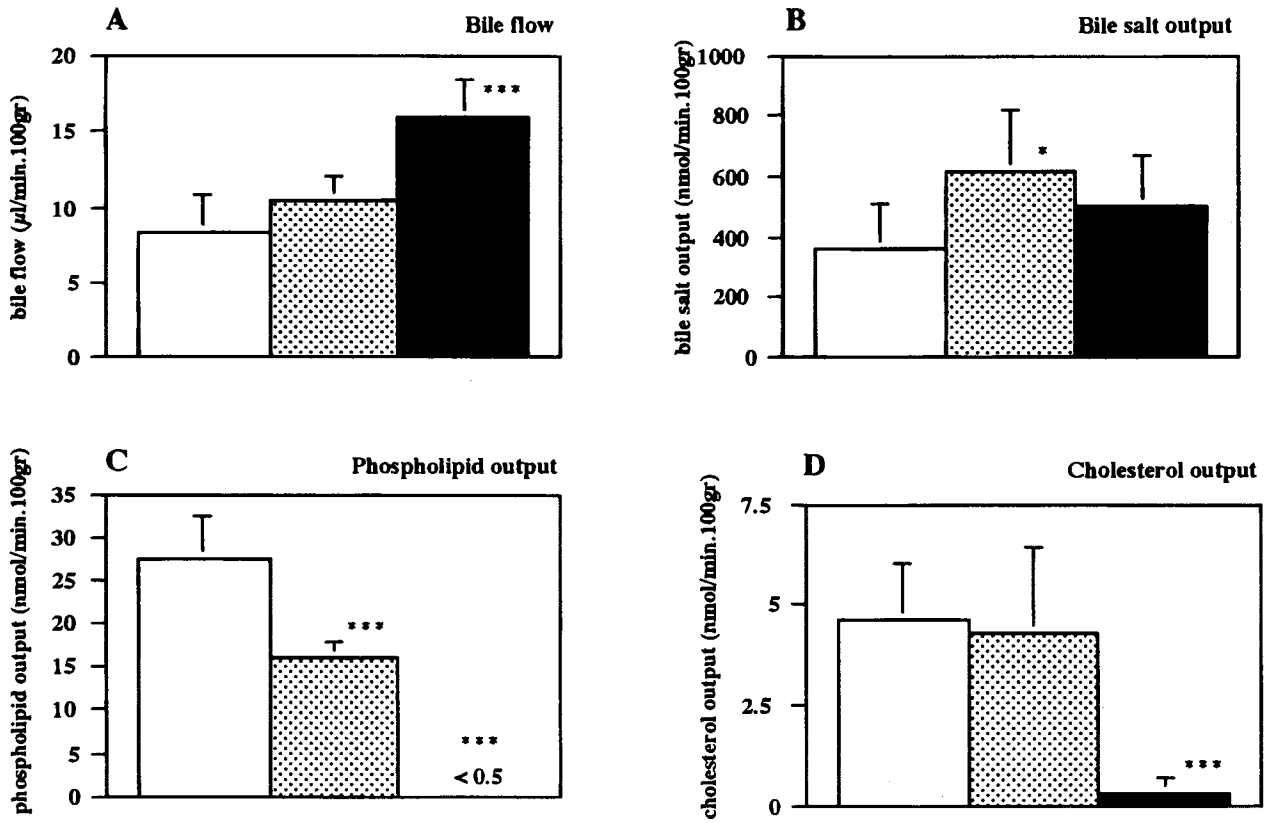

Fig. 9. Bile analysis of the normal and mdr2 knock out mouse. Data from Smit et al. (75). (A) bile flow, (B) bile salt output, (C) phospholipid output, (D) cholesterol output. Open bars, control mice; hatched bars, mdr2 heterozygous disrupted mice; closed bars, mdr2 homozygous disrupted mice.

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