# Biliary Excretion of Glycyrrhizin in Rats: Kinetic Basis for Multiplicity in Bile Canalicular Transport of Organic Anions

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***Purpose.*** To examine the presence of multiplicity for the biliary excretion of xenobiotic conjugates, we studied the disposition of glycyrrhizin (GR), which has glucuronide within its molecular structure and has the ability to inhibit the biliary excretion of liquiritigenin (LG) glucuronides.

***Methods.*** GR was administered intravenously as a bolus to Sprague-Dawley (SD) rats which received an i.v. infusion of inhibitors (dibromosulfophthalein (DBSP) and indocyanine green (ICG)) at their transport maximum rates. Biliary excretion of GR was also examined in Eisai hyperbilirubinemic rats (EHBR), which have a hereditary defect in the canalicular transport system of several organic anions.

***Results.*** Infusion of ICG did not affect the biliary excretion of GR, whereas infusion of DBSP reduced it significantly. The plasma concentration of GR was increased by DBSP but not by ICG. In EHBR, the biliary excretion of GR was severely impaired, resulting in an increase in the plasma concentration of GR.

***Conclusions.*** These findings suggest (1) that the biliary excretion of GR is mediated by the system which is shared by DBSP and LG glucuronides but not by ICG and (2) that this system is hereditarily defective in EHBR. Together with our previous findings, the multiplicity for the biliary excretion of organic anions is shown.

**KEY WORDS:** biliary excretion; glycyrrhizin (GR); Eisai hyperbilirubinemic rat (EHBR); primary active transport; bile canalicular membrane; canalicular multispecific organic anion transporter (cMOAT).

## INTRODUCTION

Recent studies have revealed that the mechanism by which a number of organic anions are transported across the bile canalicular membrane (BCM), *i.e.*, the uptake of anions such as glutathione conjugates (such as S-(2,4-dinitrophenyl)-glutathione, glutathione disulfate and leukotriene $C_4$) is mediated by a primary active transporter which has been defined as cMOAT (canalicular multispecific organic anion transporter, 1–3). In addition, this hypothesis has been supported by the *in vivo* finding that the biliary excretion of these organic anions is reduced in the mutant rats such as $TR^-$ and Eisai hyperbilirubinemic rats (EHBR) that have a hereditary defection in the expression of cMOAT (1–3).

However, no systematic study has been performed to clarify the multiplicity for the biliary excretion of non-bile acid organic anions. Based on kinetic analysis, we previously suggested the presence of multiple systems for the transport of organic anions across the BCM, *i.e.*, canalicular transport of dibromosulfophthalein (DBSP) is mediated by cMOAT, whereas that of indocyanine green (ICG) is mediated predominantly by another transporter that is present in EHBR (4,5). We also found that the biliary excretion of liquiritigenin (LG) glucuronides after an i.v. administration of LG is inhibited by DBSP and glycyrrhizin (GR) but not by ICG (6). Moreover, the biliary excretion of these glucuronide conjugates was severely impaired in EHBR (6). These findings, together with the fact that GR has an ether-type of glucuronide within its molecular structure, suggests that the biliary excretion of the glucuronides of these compounds is mediated by cMOAT.

Herein, we further examined the multiplicity for the biliary excretion of glucuronide-conjugates, using GR as a model compound. GR is excreted predominantly into the bile. More than 90% of the intravenously administered dose (5 or 10 mg/kg) is excreted into the bile without further metabolic conversion (7). Furthermore, Ishida et al. (7) reported the saturable biliary excretion of GR; clearance for the biliary excretion of GR decreased as the administered dose increased up to 20 or 50 mg/kg.

## MATERIALS AND METHODS

### Materials

GR (monoammonium salt) was obtained from a methanol extract of licorice root after column chromatography on a silica gel (8). DBSP and ICG were purchased from Societe d'Etudes et de Recherches Biologiques (S.E.R.B., Paris, France) and Daiichi Pure Chemicals Co. Ltd. (Tokyo, Japan), respectively. All other chemicals were commercial products and of reagent grade.

Male EHBR (each weighing 280–310 g) were supplied by Eisai Laboratories (Gifu, Japan). In the inhibition studies, male Sprague Dawley (SD) rats (each weighing 280–350 g) that were purchased from SLC Co., Ltd. (Shizuoka, Japan), were used.

### Animal Experiments

Cannulation was performed as described (6). The rats were allowed to recover from anesthesia prior to the experiment. Their body temperature was kept at 37°C throughout the experiments by the use of a heat lamp.

DBSP, or ICG (dissolved in a physiological saline), was infused at a constant rate (DBSP; 3.0 μmol/min/kg, ICG; 0.15

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**ABBREVIATIONS:** SD rats, Sprague Dawley rats; EHBR, Eisai hyperbilirubinemic rats; GR, glycyrrhizin; LG, liquiritigenin [2,3-dihydro-7-hydroxy-2-(4-hydroxyphenyl)-(S)-4H-1-benzopyran-4-one]; DBSP, dibromosulfophthalein; ICG, indocyanine green; cMOAT, canalicular multispecific organic anion transporter; BCM, bile canalicular membrane; $AUC_{plasma(0-120\ min)}$, area under the plasma concentration time profile from 0 to 120 min; $AUC_{plasma(0-\infty)}$, area under the plasma concentration time profile from 0 to infinite time; $Ai_{bile}$, amount of conjugate excreted into the bile; $CL_{tot}$, total body clearance; $CL_{tot,u}$, total body clearance defined for the unbound plasma concentration of GR; $Cl_{bile}$, biliary excretion clearance.

μmol/min/kg) into the femoral vein through a catheter. In the experiments for the control rats and EHBR, physiological saline was infused at the same rate (60–75 μl/min/kg). At 60 min after initiation of the infusion, GR (10 mg/kg), that was dissolved in a 5% glucose solution (10 mg/ml), was injected as a bolus into the same catheter. Bile and arterial blood specimens were collected at specified time intervals. The experiments were conducted up to 120 min after an i.v. injection of GR.

### Determination of Ligand Concentration in the Specimens

For a quantitative analysis of GR, each blood specimen was immediately centrifuged, and then 0.05 ml of phosphate buffer (0.3 M, pH 4.4) and 0.5 ml of methanol were added to 0.05 ml of the plasma specimen. After centrifugation, the supernatant was evaporated until dry with a $N_2$ gas. The residue was dissolved in 0.05 ml of distilled water for the analysis. Each bile specimen was diluted with the appropriate volumes of 90% methanol in acetate buffer (0.3 M, pH 5.0), and was then centrifuged. The supernatant was used for analysis. For GR, recovery from the plasma and bile specimens was more than 90%. The concentrations of GR were determined using an HPLC method on a reversed phase column (A312-ODS, YMC Co. Ltd., Kyoto, Japan), at a wavelength of 254 nm. The solvent system used was a 0.05 M phosphate buffer (pH 2.1)-methanol (1:2) for GR (9). HPLC equipment is described in Ref. 6. The detection limit of GR was approximately 2 μg/ml and 20 μg/ml from plasma and bile specimens, respectively.

The concentration of DBSP and ICG was determined according to the previously described method (4).

### Protein Binding of GR in Serum

Binding of GR to serum proteins was determined by means of an ultrafiltration technique. Serum was obtained by the centrifugation from 10 SD rats or 5 EHBR. DBSP (100 nmol) or ICG (3 nmol) was added to each 0.2 ml of serum that contained GR (50 mg/ml). These specimens were incubated at 37°C for 5 min and then were centrifuged (1000 × $g$) for 15 min through the membrane (MPS-3; Amicon, W.R. Grace Co. Beverly, MA). The absorption of the drugs on the membrane was negligible.

### Data Analysis

For individual animals, the area under the plasma concentration-time profile ($AUC_{plasma(0-120\ min)}$) was determined by means of the trapezoidal method (10). $AUC_{plasma(0-\infty)}$ was calculated by extrapolating the concentration profiles at the last three points. The total body clearance ($CL_{tot}$) for GR was calculated by dividing the dose by the $AUC_{plasma(0-120\ min)}$ value of GR. The biliary excretion clearance ($CL_{bile}$) for GR was calculated by dividing the cumulative amount of GR ($Ai_{bile}$) excreted into the bile up to 120 min by the respective $AUC_{plasma(0-120\ min)}$ value. The results are shown as mean ±S.E. with the number of determinations. The clearance value that were defined for the unbound GR concentration ($CL_{tot,u}$ and $CL_{bile,u}$) were determined by dividing the corresponding clearances ($CL_{tot}$ and $CL_{bile}$) by fu. The S.E. of $CL_{tot,u}$ and $CL_{bile,u}$ were calculated according to the law of propagation of errors. The Dunnett's test was used to determine the significance of the differences.

## RESULTS

### Disposition of Inhibitors

The plasma concentration of each inhibitor (ICG and DBSP) is shown in Fig. 1. The biliary excretion rate for ICG and DBSP was 90 and 1800 nmol/min/kg at 30–90 min after initiation of the infusion, respectively (Fig. 2), which were comparable to the maximum biliary excretion rate previously reported (90 nmol/min/kg for ICG and 1600 nmol/min/kg for DBSP; Ref. 5).

### Serum Protein Binding of GR

In the control experiments, the unbound fraction (fu) of GR in rat serum was 0.0046 ± 0.0002, suggesting a high affinity binding of GR to the serum proteins. The fu value of GR was not affected by 15 μM of ICG (0.0047 ± 0.0003), whereas 500 μM of DBSP increased the fu value of GR to 0.0073 ± 0.0005 ($p < 0.01$). In EHBR plasma, the fu value of GR was significantly higher than that in SD rats (0.0118 ± 0.0003, $p < 0.01$).

### Effects of Inhibitors on the Disposition of GR

Figure 3 shows the plasma concentration time profiles for GR after an i.v. administration to the SD rats that received the inhibitor infusion. The kinetic analysis revealed that the $V_d$ of GR was not significantly affected by the inhibitors (Table 1). DBSP (but not ICG), significantly raised the $CL_{tot}$ values of GR (Table 1). Since the fu of GR increased in the presence of DBSP, the profound effect of DBSP was shown when it was compared to $CL_{tot,u}$ (Table 1).

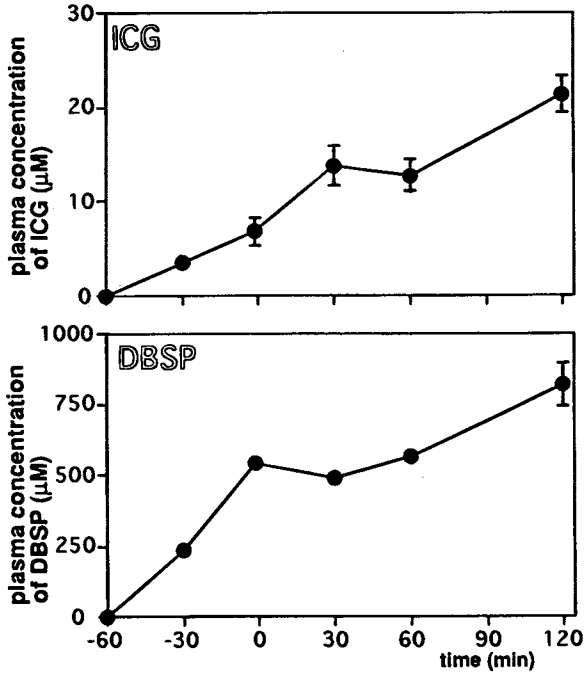


**Fig. 1.** Time profiles for the plasma concentration of ICG and DBSP in SD rats. Each inhibitor (ICG; 0.15 μmol/min/kg or DBSP; 3.0 μmol/min/kg) was infused intravenously 60 min prior to the i.v. bolus injection of GR (10 mg/kg). Each point and vertical bar represent the mean ± S.E. of 3 different experiments.

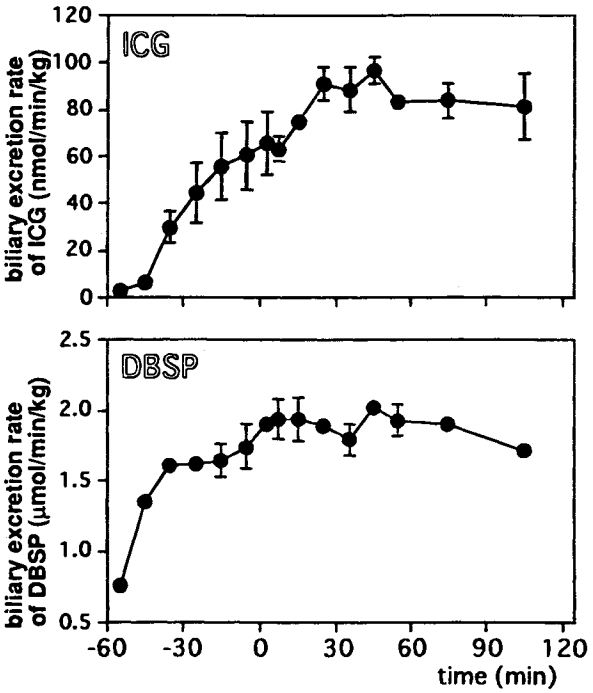


**Fig. 2.** Time profiles for the biliary excretion rate of ICG and DBSP in SD rats. Each inhibitor (ICG; 0.15 μmol/min/kg or DBSP; 3.0 μmol/min/kg) was infused intravenously 60 min prior to the i.v. bolus injection of GR (10 mg/kg). Each point and vertical bar represent the mean ± S.E. of 3 different experiments.

Figure 4 shows the time profiles for the biliary excretion of GR. In the control rats, approximately 70% of the administered GR was excreted into the bile (Fig. 4 and Table 1). The biliary excretion of GR was not affected by ICG (Fig. 4), whereas the infusion of DBSP significantly reduced the amount of GR excreted into the bile to 33% of the control value (Table 1). The infusion of DBSP reduced the $CL_{bile}$ and $CL_{bile,u}$ values of GR (Table 1).

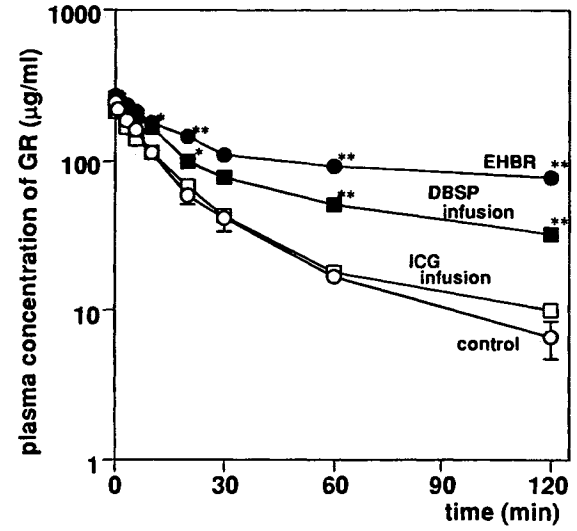


**Fig. 3.** Time profiles for the plasma concentration of GR. Each inhibitor (ICG; 0.15 μmol/min/kg or DBSP; 3.0 μmol/min/kg) was infused intravenously 60 min prior to the i.v. bolus injection of GR (10 mg/kg) to SD rats. Control SD rats and EHBR received an i.v. bolus administration of GR (10 mg/kg). Each point and vertical bar represent the mean ± S.E. of 3 different experiments. Keys: ○, control; ●, EHBR. □, ICG infusion; ■, DBSP infusion. *,**: Significantly different from control ($p < 0.05$, 0.01, respectively), by Dunnett's test.

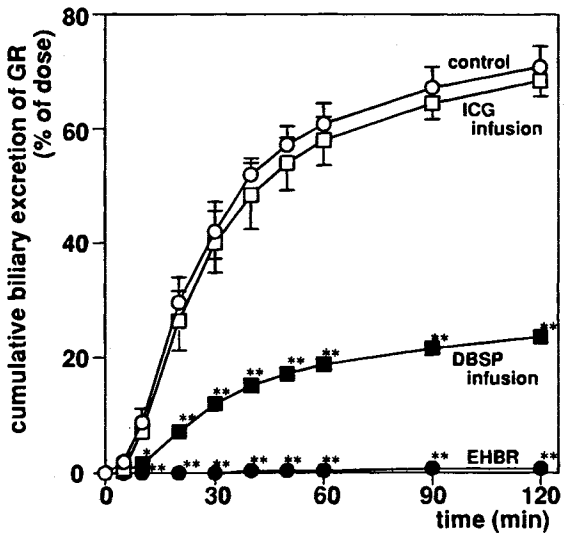


**Fig. 4.** Time profiles for the biliary excretion of GR. Each inhibitor (ICG; 0.15 μmol/min/kg or DBSP; 3.0 μmol/min/kg) was infused intravenously 60 min prior to the i.v. bolus injection of GR (10 mg/kg) to SD rats. Control SD rats and EHBR received an i.v. bolus administration of GR (10 mg/kg). Each point and vertical bar represent the mean ± S.E. of 3 different experiments. Keys: ○, control; ●, EHBR. □, ICG infusion; ■, DBSP infusion. *,**: Significantly different from control ($p < 0.05$, 0.01, respectively), by Dunnett's test.

## Disposition of GR in EHBR

Figure 3 also shows the plasma concentration time profiles of GR after its i.v. administration to EHBR. The plasma concentrations of GR significantly increased in EHBR. The $AUC_{plasma}$ values of GR in EHBR were 3 times higher than the control values (Table 1).

The bile flow rate in EHBR was not different form that in the control rats (data not shown), whereas in EHBR, biliary excretion of GR obviously decreased (Fig. 4, Table 1). Based on these results, the $CL_{bile}$ values for GR in EHBR was only 0.5% of that in the control rats (Table 1).

## DISCUSSION

In the present study, we systematically examined the mechanisms for the biliary excretion of glucuronide by performing an inhibition experiment in normal rats and a biliary excretion experiment on EHBR.

The biliary excretion of GR was not affected by ICG infusion (Fig. 4, Table 1). Since the biliary excretion of ICG is saturated under this experimental condition (Fig. 2; Ref. 4), the excretion of GR is mediated by a system different from that for the excretion of ICG.

By contrast, a constant infusion of DBSP significantly reduced the biliary excretion of GR (Fig. 4 and Table 1). As a result of this inhibition, the $AUC_{plasma}$ of GR increased in rats which received a DBSP infusion (Fig. 3 and Table 1). This result is consistent with the hypothesis that DBSP inhibits the

**Table I.** Kinetic Parameters for the Disposition of GR

| | Control | ICG Infusion | DBSP Infusion | EHBR | |
|---|---|---|---|---|---|
| Vd | 42.2 ± 2.9 | 49.6 ± 8.3 | 38.1 ± 1.6 | 37.6 ± 2.9 | (ml/kg) |
| $AUC_{plasma(0-120min)}$ | 4.63 ± 0.10 | 4.76 ± 0.66 | 8.74 ± 0.52[a] | 13.27 ± 0.39[a] | (μg · min/ml) |
| $AUC_{plasma(0-\infty)}$ | 4.86 ± 0.14 | 5.80 ± 0.73 | 16.03 ± 1.98[a] | 54.49 ± 9.47[a] | (μg · min/ml) |
| $Ai_{bile(0-120min)}$ | 70.8 ± 3.8 | 68.3 ± 2.6 | 23.5 ± 1.8[a] | 0.87 ± 0.17[a] | (% of dose) |
| $CL_{tot}$ | 2.06 ± 0.06 | 1.78 ± 0.21 | 0.64 ± 0.08[a] | 0.19 ± 0.03[a] | (ml/min/kg) |
| $CL_{tot,u}$ | 444.1 ± 21.6 | 366.5 ± 53.9 | 85.5 ± 17.4[a] | 15.5 ± 3.2[a] | (ml/min/kg) |
| $CL_{bile(0-120min)}$ | 1.53 ± 0.09 | 1.48 ± 0.18 | 0.27 ± 0.04[a] | 0.007 ± 0.001[a] | (ml/min/kg) |
| $CL_{bile,u(0-120min)}$ | 329.8 ± 16.3 | 304.7 ± 47.2 | 36.1 ± 9.4[a] | 0.57 ± 0.09[a] | (ml/min/kg) |

Note: DBSP (3.0 μmol/min/kg) or ICG (0.15 μmol/min/kg) was infused to SD rats through the catheter inserted into the femoral vein. In the experiment for the control rats and EHBR, physiological saline was infused at a constant rate. At 60 min after initiation of the infusion, GR (10 mg/kg) was injected as a bolus through the same catheter. Bile, urine and arterial blood specimens were collected at the specified time. The experiments were conducted up to 120 min after i.v. injection of GR. The kinetic parameters were calculated from the values in Figs. 3 and 4. Each value represents the mean ± S.E. of three different determinations.

[a] Significantly different from control ($p < 0.01$, by Dunnett's test); Vd, the volume of distribution of GR; $AUC_{plasma}$, the area under the plasma concentration-time curve of GR; $Ai_{bile}$, the amount of biliary excretion of GR; $CL_{tot}$, the total body clearance of GR calculated as dose divided by $AUC_{plasma(0-120min)}$; $CL_{tot,u}$, the total body clearance defined for the unbound plasma concentration of GR; $CL_{bile}$, the biliary excretion clearance of GR; $CL_{bile,u}$, the biliary excretion clearance defined for the unbound plasma concentration of GR.

uptake of GR into the liver and/or the excretion of GR into the bile. The former hypothesis may be supported by the previous results by Ishida *et al.* (7) who indicated that the saturable uptake of GR by isolated hepatocytes was inhibited by BSP ($K_i$ = 9.2 μM) and ICG ($K_i$ = 13.5 μM) in a non-competitive manner. Since the serum unbound concentration of DBSP is calculated as less than 2 μM throughout the present study (Fig. 1, Ref. 4), it might be possible that the uptake of GR is inhibited to some extent by DBSP. Although a direct demonstration of the effect of DBSP on GR uptake by isolated hepatocytes is required in order to examine this hypothesis, the results in mutant rats strongly support the latter hypothesis, which is discussed as follows.

As shown in Fig. 4, the biliary excretion of GR in EHBR was almost completely defective, irrespective of the increased serum unbound fraction. As a consequence, the plasma disappearance of GR was also reduced in EHBR (Fig. 3 and Table 1). This result suggests that the biliary excretion of GR is mediated by cMOAT.

Previously, we performed a kinetic analysis for the biliary excretion of the conjugative metabolites of LG (11). We indicated that the biliary excretion of four kinds of LG-glucuronides (M1 (4′-*O*-glucuronide), M2 (7-*O*-glucuronide), M4 (4′-*O*-glucuronide, 7-*O*-sulfate) and M5 (7-*O*-glucuronide, 4′-*O*-sulfate) which are formed in the hepatocytes after the administration of the parent compound (11), were reduced by DBSP or GR infusion, but were not affected by ICG infusion (6). In addition, the biliary excretion of these glucuronides and that of DBSP had almost completely disappeared in EHBR (Fig. 5; Refs. 4, 6). Together with the present findings (4,5), we suggested that GR, DBSP and LG glucuronides are excreted into the bile by cMOAT, whereas the excretion of ICG is mediated by another transporter which is present in EHBR (Fig. 5).

The hypothesis that the xenobiotic glucuronides can be the substrate for cMOAT was also consistent with our previous results that the biliary excretion of E3040 (6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl)-benzothiazole)-glucuronide is mediated by cMOAT (12,13). Since the transport of E3040-sulfate and that of LG-disulfate (4′-*O*,7-*O*-sulfate)

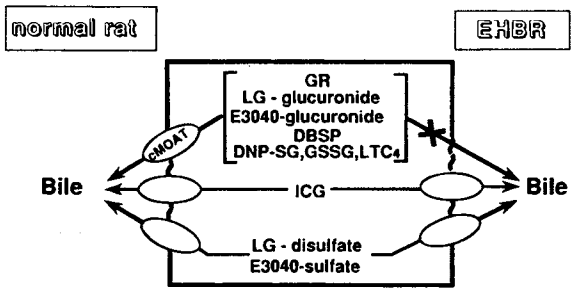


**Fig. 5.** A proposed scheme for the biliary excretion systems by the primary active transporter in the hepatocyte of normal rat and EHBR. The present and previous findings (3) suggested that GR is excreted into the bile by cMOAT, shared with the glucuronides of LG, E3040-glucuronide, DBSP, glutathione conjugates ((S-(2,4)-dinitrophenyl)-glutathione; DNP-SG), oxidized glutathione (GSSG) and leukotriene $C_4$ ($LTC_4$). cMOAT is hereditarily defective in EHBR. Previous findings suggested that ICG is excreted into the bile by another mechanism present in EHBR (3). Furthermore, recent findings suggested that the di-sulfate of LG and E3040-sulfate are excreted into the bile by a system which is present in EHBR and is different from two systems described previously (3).

across the bile canalicular membrane was maintained in EHBR, another transporter(s) may be responsible for the excretion of these sulfated compounds (11–13).

In conclusion, the biliary excretion of GR was reduced by DBSP but not by ICG. The biliary excretion of GR was also impaired in EHBR. Together with our previous studies (4–6), at least three kinds of transport systems may be involved in the biliary excretion of organic anions in normal rats (Fig. 5). Although cMOAT molecule was recently identified (14–17) and the mechanism for the impaired expression in EHBR was clarified (17), the precise molecular basis for this multiplicity still remains to be completely understood.

## REFERENCES

1. E. Petzinger. *Physiol. Biochem. Pharmacol.* **123**:47–211 (1994).
2. R. P. J. Oude Elferink, D. K. F. Meijer, F. Kuipers, P. L. M.

Jansen, A. K. Groen, and G. M. M. Groothuis. *Biochim. Biophys. Acta.* **1241**:215–268 (1995).
3. M. Yamazaki, H. Suzuki, and Y. Sugiyama. *Pharm. Res.* **13**:495–511 (1996).
4. K. Sathirakul, H. Suzuki, K. Yasuda, M. Hanano, O. Tagaya, T. Horie, and Y. Sugiyama. *J. Pharmacol. Exp. Ther.* **265**:1301–1312 (1993).
5. K. Sathirakul, H. Suzuki, T. Yamada, M. Hanano, and Y. Sugiyama. *J. Pharmacol. Exp. Ther.* **268**:65–73 (1994).
6. H. Shimamura, H. Suzuki, M. Hanano, A. Suzuki, O. Tagaya, T. Horie, and Y. Sugiyama. *J. Pharmacol. Exp. Ther.* **271**:370–378 (1994).
7. S. Ishida, Y. Sakiya, T. Ichikawa, and Z. Taira. *Biol. Pharm. Bull.* **16**:293–297 (1993).
8. P. Karrer, W. Karrer, and J. C. Chao. *Helv. Chim. Acta* **4**:100–108 (1921).
9. T. Ichikawa, S. Ishida, Y. Sakiya, and Y. Sawada. *Chem. Pharm. Bull.* **33**:3031–3033 (1985).
10. K. Yamaoka, Y. Tanigawara, T. Nakagawa, and T. Uno. *J. Pharmacobio-Dyn.* **4**:879–885 (1981).
11. H. Shimamura, H. Suzuki, M. Hanano, A. Suzuki, and Y. Sugiyama. *Biol. Pharm. Bull.* **16**:899–907 (1993).
12. O. Takenaka, T. Horie, H. Suzuki, and Y. Sugiyama. *J. Pharmacol. Exp. Ther.* **274**:1362–1369 (1995).
13. O. Takenaka, T. Horie, K. Kobayashi, H. Suzuki, and Y. Sugiyama. *Pharm. Res.* **12**:1746–1755 (1995).
14. R. Mayer, J. Kartenbeck, M. Buchler, G. Jedlitschky, I. Leier, and D. Keppler. *J. Cell. Biol.* **131**:137–150 (1995).
15. K. Ito, H. Suzuki, T. Hirohashi, K. Kume, Y. Shimizu, and Y. Sugiyama. *International Hepatology Communications*, **4**:292–299 (1996).
16. C. C. Paulusma, P. J. Bosma, G. J. R. Zaman, C. T. M. Bakker, M. Otter, G. L. Scheffer, R. J. Scheper, P. Borst, and R. P. J. Oude Elferink. *Science*, **271**:1126–1128 (1996).
17. K. Ito, H. Suzuki, T. Hirohashi, K. Kume, Y. Shimizu, and Y. Sugiyama. *Am. J. Physiol.*, in press.