Kinetic Analysis of Hepatobiliary Transport for Conjugated Metabolites in the Perfused Liver of Mutant Rats (EHBR) with Hereditary Conjugated Hyperbilirubinemia

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Purpose. Previously, we found that the biliary excretion of the 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole (E3040) glucuronide is severely impaired in Eisai hyperbilirubinemic rats (EHBR), while that of sulfate remains normal (Takenaka et al., J. Pharmacol. Exp. Ther., 274: 1362–1369, 1995). The purpose of the present study is to clarify the mechanisms for impairment of the biliary excretion of E3040 glucuronide in EHBR.

Methods. We kinetically analyzed the disposition of the conjugates in the perfused liver at steady state. The uptake of the conjugates into the isolated canalicular membrane vesicles (CMVs) was also examined.

Results. At steady state, the bile/liver unbound concentration ratios of the conjugates were 40-400 in both rat strains, indicating a highly concentrated process. The biliary excretion clearance ($CL_{u,bile}$) of the glucuronide, defined for the unbound concentration in the liver, was decreased in EHBR to 1/30 of that in normal rats, whereas the $CL_{u,bile}$ of the sulfate was comparable between the two rat strains. *In vitro*, the transport of E3040 glucuronide into CMV prepared from SD rats exhibited the ATP dependency, whereas minimal effect of ATP was observed on the uptake of the glucuronide into CMV from EHBR. In contrast, the uptake of E3040 sulfate was comparable between SD rats and EHBR. Furthermore, ATP did not stimulate the uptake of sulfate into the CMVs.

Conclusions. It was suggested (1) that the excretion of E3040 glucuronide across the bile canalicular membrane is mediated by the primary active transporter which is defective in EHBR and (2) that the bile canalicular transport system for E3040 sulfate is different from that for the glucuronide in that the former remains normal in EHBR.

KEY WORDS: Eisai hyperbilirubinemic rats (EHBR); conjugated metabolites; hepatobiliary transport; 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole (E3040); liver perfusion.

INTRODUCTION

Recent progress in the study of hepatobiliary transport systems for organic anions has been made possible predominantly by the utilization of bile canalicular membrane vesicles (CMV) and by the discovery of mutant rats with a hereditary defect in the biliary excretion of these ligands. Three different types of mutant rats, TR$^-$ (1) and GY (2) derived from the Wister strain as well as EHBR derived from Sprague-Dawley strain have been established. These rats may be good animal models for the Dubin-Johnson syndrome found in human. Based on the experiments with CMV (3,4), bile acids, non-bile acid organic anions, and some anticancer drugs have been shown to be transported into the bile via ATP-dependent primary active transport systems on the bile canalicular membrane. The ATP-dependent uptake of non-bile acid organic anions into CMV prepared from the liver of TR$^-$ is impaired, although that of taurocholate and anticancer drugs is maintained (5). The ATP-driven canalicular transport system for non-bile acid organic anions is also defective in EHBR (6). These results indicate that the transport mechanism for non-bile acid organic anions is different from that for bile acids and anticancer drugs, and that only the transport system(s) for non-bile acid organic anions is impaired in mutant rats.

The mechanism for the biliary excretion of conjugates such as glucuronide and sulfate, however, has not yet been clarified. It had been suggested that the glucuronide and sulfate conjugates of various bile acids are excreted into the bile via ATP-dependent transport system(s) that might be shared by non-bile acid organic anions (7). However, comparison of the behavior of liquiritigenin conjugates in SD rats and EHBR suggested that the glucuronides and disulfate are excreted into the bile via different transport system (8). Furthermore, we investigated the disposition of the glucuronide and sulfate of 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole (E3040, Fig. 1) *in vivo* and in the indicator dilution (MID) method (9). We found that the biliary excretion of the E3040 glucuronide was severely decreased in EHBR, while the corresponding sulfate excretion was unchanged between the two strains. Furthermore, no significant difference in the conjugating activities between EHBR and SD rats was observed (9). These results support the hypothesis that the biliary excretion system for the glucuronide may differ from that of the sulfate.

Since such reduction in the biliary excretion in EHBR can be also observed as the secondary effect of increased bilirubin level in the hepatocytes (10), comparison of the intrinsic clearance for the transport across the bile canalicular membrane between SD rats and EHBR is essential to obtain the final conclusion. Actually, we had clarified that the reduced biliary excretion of ICG in EHBR is predominantly accounted for by the reduced intracellular transport process rather than the impaired transport activity across the bile canalicular membrane (10).

In order to directly demonstrate the presence of the multiplicity for the biliary excretion of the conjugates, we also examined the ATP-dependent uptake of the conjugates into the CMV isolated from SD rats and EHBR.

MATERIALS AND METHODS

Materials

Unlabeled and [^{14}C]-labeled E3040 ([2-^{14}C]-6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothia-

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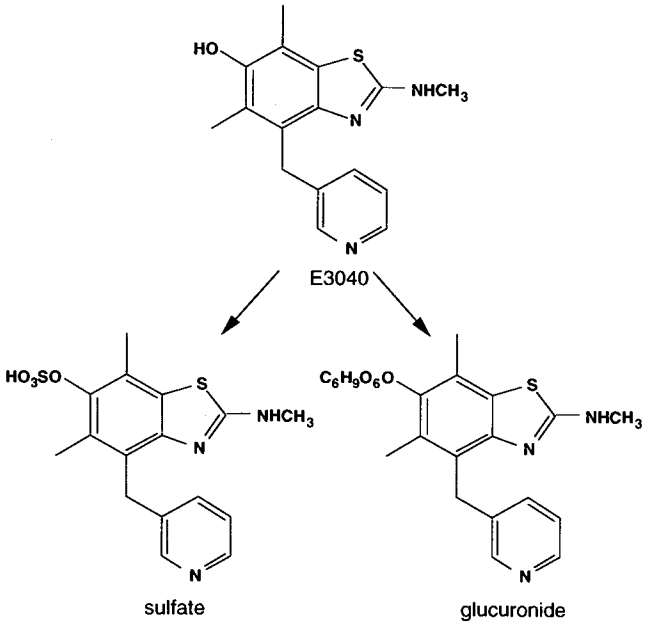

Fig. 1. Chemical structure of E3040 and its conjugates (glucuronide and sulfate).

zole dihydrochloride) was synthesized in our laboratories (Tsukuba, Japan) (9). The radiochemical purity of [^{14}C]E3040 determined by HPLC was 98.7%, and the specific activity was 58.0 μCi/μmol. BSA (fraction V) and glutathione S-transferase from rat liver were purchased from Sigma Chemical Co., (St Louis, MO). [^{3}H]S-(2,4-dinitrophenyl) glutathione ([^{3}H]DNP-SG, 50.0 μCi/nmol) was synthesized according to the method described previously (11). [^{3}H]Taurocholate ([^{3}H]TCA, 2.0 μCi/nmol) were purchased from New England Nuclear (Boston, MA). The glucuronide and sulfate of [^{14}C]E3040 were prepared by incubating [^{14}C]E3040 with rat liver microsomes and cytosol, respectively (9). Each conjugate was purified from the microsome and the cytosol preparations by HPLC (9). The purity of conjugates labeled by ^{14}C was checked by HPLC and was confirmed to be more than 99% pure (9). Other chemicals used were commercially available and were reagent grade products.

Male EHBR of 270-350 g body weight from Eisai laboratories (Gifu, Japan) and male SD rats (280-310 g) from Japan Laboratory Animals Inc. (Tokyo, Japan) were used.

Liver Perfusion Study

Liver perfusion was performed by the method reported previously (12). The perfusate consisted of 3% BSA in Krebs-Ringer bicarbonate buffer (pH 7.4) and the flow rate of the perfusate was 28-32 ml/min. After a stabilization period of 15 min, a tracer dose of [^{14}C]E3040 (0.25 μM) was perfused continuously and the outflow from the hepatic vein was collected at given times. Bile was also collected at 10-min intervals for 60 min. After each perfusion, liver homogenates (33% w/v) in 1/15 M phosphate buffer containing 250 mM sucrose (pH 7.4) were prepared. The concentration of [^{14}C]E3040 and its conjugates in outflow, liver homogenate and bile was determined as follows: CH_3CN corresponding to double the volume of the outflow samples or liver homogenates was added to each outflow sample or liver homogenate. After centrifuging the mixture for 5 min in a table-top microfuge (Beckman Instruments, Fullerton, CA), the supernatant was analyzed by silica-gel thin-layer chromatography in butanol-water-acetic acid (3:1:1). The concentration of [^{14}C]E3040 and its conjugates was determined using a Bio-Image Analyzer (Bas 2000, Fuji Photo Film Co., Ltd. Tokyo, Japan). The bile specimens were diluted with CH_3CN-water (1:1), and the concentration of ligands in the bile was determined in the same way as the outflow samples. The value of the hepatic extraction ratio of E3040 (Eh) was calculated as:

$$E_h = (C_{in} - C_{out}) / C_{in} \quad (1)$$

where C_{in} and C_{out} denote the perfusate concentration of [^{14}C]E3040 in the portal inflow and hepatic venous outflow, respectively. The availability of E3040 (F) was obtained from the following equation:

$$F = C_{out} / C_{in} = 1 - E_h \quad (2)$$

The biliary excretion ratio and the ratio of efflux into the outflow were determined as follows:

$$\text{The biliary excretion ratio} = V_{bile}/I \quad (3)$$

$$\text{The ratio of efflux into the outflow} = Q \cdot C_{outflow}/I \quad (4)$$

where V_{bile} and $C_{outflow}$ denote the biliary excretion rate and perfusate concentration in the outflow of [^{14}C]E3040 conjugates, respectively. I denotes the infusion rate of [^{14}C]E3040 which was given by the following equation:

$$I = Q \cdot C_{in} \quad (5)$$

where Q denotes the flow rate.

Binding of E3040 Conjugates to Liver Homogenates, Liver Cytosol and 3% BSA in Perfusate

After each perfusion, liver homogenates (33% w/v) in 250 mM sucrose and 50 mM Tris-HCl buffer (pH 7.4) were separated into two fractions. One was further centrifuged for 60 min at 105000 × g (4°C) to obtain cytosol while the other was used as homogenate. To determine the binding of E3040 conjugates by ultrafiltration, both specimens were centrifugated through an MPS-3 membrane (Amicon Division, W.R. Grace & Co., Beverly, MA) at 3000 rpm for 30-60 min. The buffer solution containing the [^{14}C]E3040 conjugates was also centrifuged (control experiment) to determine the recovery of the ligands through the membrane, which was more than 90%. In addition, CH_3CN corresponding to double the volume of homogenate or cytosol was added to the liver homogenate and to cytosol specimens. After centrifugation, the supernatant was lyophilized, and was dissolved in MeOH / H_2O (1 : 1). The filtrates of liver homogenate and cytosol were also lyophilized and were then dissolved in MeOH / H_2O (1 : 1). The concentration of [^{14}C]E3040 conjugates in each sample was determined by TLC as described previously. The unbound fraction (f_u) was calculated as follows:

$$f_u = C_{filtrate} / C_{sample} / R \quad (6)$$

where $C_{filtrate}$ and C_{sample} denote the concentration in the filtrate after ultrafiltration of the liver cytosol or homogenate and that in the liver cytosol or homogenate, respectively. R denotes the recovery determined in the control experiment which was determined to be 0.9. The value of the unbound fraction in 100% cytosol ($f_{u,cyt}$) was extrapolated from the observed f_u values ($f_{u,cyt(33\%)}$) obtained as follows (10):

$$f_{u,cyt} = f_{u,cyt(33\%)} / (d \cdot (1 - f_{u,cyt(33\%)}) + f_{u,cyt(33\%)}) \quad (7)$$

where d denotes the dilution factor of the cytosol. In the present study, d equals three.

The binding of [^{14}C]E3040 conjugates to 3% BSA in the perfusate was also determined by Ultrafiltration using an MPS-3 membrane. Various amounts of [^{14}C]E3040 conjugates were added to the Krebs-Ringer buffer containing 3% BSA and then the mixture was incubated for 5 min at 37°C. Ultrafiltration of the mixture was accomplished by centrifugation (1000 × g) at 37°C for 10min. The ^{14}C-radioactivity in the filtrate was determined in a liquid scintillation spectrophotometer (LSC-3500, Aroka CO., Tokyo, Japan).

Estimation of the Fraction of [^{14}C]E3040 Conjugates Distributed in the Perfused Liver Cytosol

The fraction of [^{14}C]E3040 conjugates distributed in the perfused liver cytosol ($Fract_{,cyt}$) was calculated as (10):

$$Fract_{,cyt} = \{1 + d \cdot (1 / f_{u,cyt(33\%)} - 1)\} / \{1 + d \cdot (1 / f_{u,homo(33\%)} - 1)\} \quad (8)$$

where $f_{u,homo(33\%)}$ denotes the unbound fraction in the 33% liver homogenate specimen.

Binding to Liver Cytosolic Protein(s)

To determine cytosolic binding, the cytosol specimen was prepared as follows: liver homogenates (33% w/v) in 250 mM sucrose and 50 mM Tris-HCl buffer (pH 7.4) were prepared from three SD rats and three EHBR animals, and then were pooled for the preparation of a 10,5000 × g cytosol fraction. To 0.75 ml of the cytosolic specimen, [^{14}C]E3040 glucuronide or [^{14}C]E3040 sulfate (final concentration 31 μM) was added. After 15 min incubation at 37°C, 0.5 ml of the mixture was analyzed by HPLC whose equipment was consisted of a Waters 712 WISP (Millipore Corporation, Milford, MA), TOSOH CCPM pump, a variable wavelength detector, TOSOH UV-8020 and a controller, TOSOH PX-8010 (Tosoh Co,. Ltd., Tokyo, Japan) with a gel filtration column (Asahipak GS-510, 50 cm × 7.6 mm i.d., Asahikasei Kogyo Co., Ltd., Kawasaki, Japan). The solvent system used was 50 mM potassium phosphate buffer (pH 7.4) at a flow rate of 0.5 ml/min and eluate samples were collected (1.0 ml). The protein concentration was measured spectrophotometrically at 280 nm and the radioactivity of [^{14}C]E3040 conjugates was determined in a liquid scintillation spectrophotometer (LSC-3500, Aroka Co., Tokyo, Japan). Glutathione S-transferase (GST) activity in the eluted fractions of liver cytosol obtained by HPLC was measured as reported elsewhere (13). GST activity with respect to 1-chloro-2,4-dinitrobenzene was determined by monitoring changes in the absorbance at 340 nm. The specific activity is defined as the formation of products per min per fraction. GST from rat liver was also applied to this HPLC system to confirm its retention time.

Calculation of Bile/Liver Concentration Ratios of [^{14}C]E3040 Conjugates

The steady-state concentration of [^{14}C]E3040 conjugates in the liver (C_{liver}) were measured after the single-pass continuous perfusion of a tracer concentration of [^{14}C]E3040. The intracellular volume of the liver was assumed to be 0.7 ml/g liver (14). The unbound concentration of [^{14}C]E3040 conjugates in the cytosol fraction ($C_{u,cyt}$) was calculated as:

$$C_{u,cyt} = C_{liver} \cdot Fract_{,cyt} \cdot f_{u,cyt} \quad (9)$$

The bile to liver (C_{bile} / C_{liver}) and the bile to liver unbound concentration ratios ($C_{bile} / C_{u,cyt}$) were calculated.

Calculation of Biliary Excretion Clearance and Efflux Clearance into the Outflow of [^{14}C]E3040 Conjugates

After each perfusion, the biliary excretion clearance (CLbile) and the efflux clearance into the outflow ($CL_{outflow}$) of [^{14}C]E3040 conjugates were calculated as follows:

$$CL_{bile} = V_{bile}/C_{liver} \quad (10)$$

$$CL_{outflow} = Q \cdot C_{outflow}/C_{liver} \quad (11)$$

The value of unbound clearance was determined as follows:

$$CL_{u,bile} = V_{bile}/C_{u,cyt} \quad (12)$$

$$CL_{u,outflow} = Q \cdot C_{outflow}/C_{u,cyt} \quad (13)$$

Transport Study in Canalicular Membrane Vesicle (CMV)

CMV were prepared from male SD rats and EHBR (250-280g) liver according to the method of Kobayashi et al. (11), and were suspended in 50 mM Tris buffer (pH 7.4) containing 250 mM sucrose. The transport study of [^{14}C]E3040 conjugates (25 μM), [^{3}H]DNP-SG (1.0 μM) and [^{3}H]TCA (1.4 μM) was performed by the method reported previously(15). The transport medium (10 mM Tris, 250 mM sucrose and 10 mM $MgCl_2 \cdot 6H_2O$, pH7.4) contained the ligands, 5 mM ATP and an ATP-regenerating system (10 mM creatine phosphate and 100 μg/ml of creatine phosphokinase) (15). An aliquot of transport medium (17-18 μl) was mixed rapidly with the vesicle suspension (8 μg of protein in 2-3 μl) (15). The transport reaction was stopped by the addition of 1 ml of ice-cold buffer containing 250 mM sucrose, 0.1 M NaCl and 10 mM Tris-HCl (pH 7.4) (15). The stopped reaction mixture was filtered through a 0.22-μm GVWP filter (Millipore Corp., Bedford, MA), and then was washed twice with 5 ml of the stop-solution (15). Radioactivity retained on the filter was determined using a liquid scintillation counter (LSC-3500, Aroka Co., Tokyo, Japan). Uptake of ligands was normalized with respect to the amount of membrane protein.

Statistical Method

The results are shown as means ± S.E.. The Mann-Whitney U test was used to determine the significance of differences between the two groups.

RESULTS

The Disposition of Conjugates in a Single-pass Continuous Liver Perfusion of a Trace Concentration of [^{14}C]E3040

The time profiles for the C_{out} / C_{in} ratio of unchanged E3040 and the biliary excretion rate of the conjugates normalized for the infusion rate of [^{14}C]E3040 ($Q \cdot C_{outflow} / I$ and V_{bile} / I, respectively) are shown in Fig. 2. The C_{out} / C_{in} value of E3040 in EHBR and SD rats reached the steady-state at 30 min. The efflux rate into the outflow and the biliary excretion rate of the conjugates formed in the liver also reached the steady-state at 30 min. The extraction ratio (E_n) of E3040 was 0.975 for EHBR and 0.961 for SD rats. The biliary excretion rate of the glucuronide was markedly decreased in EHBR, and consequently, the efflux rate into the outflow was increased. On the other hand, the biliary excretion rate and efflux rate of the sulfate were comparable between the two rat strains (Table I).

Unbound Fraction and Cytosol Distribution of E3040 Conjugates in the Perfused Rat Liver

After a single-pass perfusion of [^{14}C]E3040 for 60 min, $Fract_{,cyt}$ and f_u of the conjugates in liver cytosol of EHBR and SD rats were determined (Table II). No significant difference was observed in $Fract_{,cyt}$ of the conjugates between the rat strains. The absolute values of $Fract_{,cyt}$ for both conjugates were 0.64 to 0.83, suggesting that the conjugates are predominantly distributed in the liver cytosol. The $f_{u,cyt}$ was also not significantly different between the two rat strains (Table II).

To identify the cytosolic binding protein for the conjugates, a mixture of the conjugates and liver cytosol prepared from EHBR and SD rats was applied to HPLC using a gel filtration column. The elution pattern of the cytosolic protein and that of the radioactivity was similar in both rat strains (Fig. 3). The determination of the glutathione S-transferase (GST) activity in each HPLC effluent fraction suggests that the protein responsible for the binding the conjugates in the liver cytosol may be ligandin(s) (GST), such the peak of GST activity coincided with that of the radioactivity. Furthermore, that authentic GST from rat liver had almost the same retention time.

The Bile/Liver Concentration Ratio of E3040 Conjugates

To examine whether the concentrative excretion of the conjugates is observed, the bile / liver concentration ratio (C_{bile} / C_{liver}) and the bile to the unbound concentration in liver cytosol (C_{bile} / $C_{u,cyt}$) were determined for the conjugates. The results are shown in Table. III. The C_{bile} / $C_{u,cyt}$ values in SD rats and EHBR for the glucuronide were approximately 400 and 40, respectively. In the same manner, the corresponding values for the sulfate were 40 and 200, respectively. These results suggest that the biliary excretion of the conjugates was a highly concentrative process in the two rat strains. The C_{bile} / $C_{u,cyt}$ value of the glucuronide in EHBR, however, was reduced to approximately 1/10 of that in SD rats (Table III). In contrast, the C_{bile} / $C_{u,cyt}$ of the sulfate was increased approximately 5-fold (Table III).

The Biliary Excretion Clearance and Efflux Clearance into the Outflow of E3040 Conjugates

The CL_{bile} and the $CL_{outflow}$ of the conjugates, defined for the total concentration of each conjugate in the liver after a single-pass perfusion of [^{14}C]E3040, are shown in Table IV. The $CL_{u,bile}$ and the $CL_{u,outflow}$, defined for the unbound concentration of each conjugate in perfused liver cytosol are also shown in Table. IV. The CL_{bile} and $CL_{u,bile}$ values of the glucuronide in EHBR decreased to 1/30 of those in SD rats, whereas the $CL_{outflow}$ and $CL_{u,outflow}$ in EHBR were 4 times greater than those in SD rats. In contrast, no difference was observed in the sulfate clearances between EHBR and SD rats.

Comparison of $CL_{u,bile}$ and $CL_{u,outflow}$ values (Table IV) in normal rats suggest that the intracellularly formed glucuronide undergoes biliary excretion in preference to the efflux into the blood, whereas the sulfate has a higher preference for the efflux system compared to the biliary excretion system.

Transport Study in Canalicular Membrane Vesicles (CMV)

As shown in Fig. 4, the transport of glucuronide into CMV prepared from SD rats exhibited the ATP dependency, whereas no stimulatory effect of ATP was observed on the uptake of glucuronide into CMV from EHBR. In contrast, the uptake of sulfate was comparable between SD rats and EHBR. Furthermore, ATP did not stimulate the uptake of sulfate into the CMVs. As the control experiment, we also examined the uptake of taurocholate and DNP-SG. The ATP-dependent uptake of taurocholate was observed in the CMV prepared from both SD rats and EHBR, which was consistent with the previous observation by Adachi et al. (16). In contrast, the ATP-dependent uptake of DNP-SG was defective in EHBR, which was consistent with the previous observation by Kobayashi et al. (11).

DISCUSSION

Transport properties of organic anions across the bile canalicular membrane have been characterized by using CMV. ATP-dependent uptake of non-bile acid organic anions such as sulfobromophthalein (BSP) (4) and glutathione conjugates such as leucotriene C_4 (LTC_4) into the CMV (15, 17) indicate the presence of a primary active transport system(s) on the bile canalicular membrane. In addition, mutant rats with conjugated hyperbilirubinemia (EHBR, TR^-, GY) exhibit imparied biliary excretion only for non-bile acid organic anions (6, 10). It is generally believed that these *in vivo* observations are caused by a defect in the ATP-dependent transport carrier protein(s) on the bile canalicular membrane, although membrane potential-dependent transport of non-bile acid organic anions into CMV is present in CMV prepared from TR^- (5). Previously, we examined the hepatobiliary transport of glucuronide and sulfate conjugate in EHBR (8, 9). Based on the findings that the biliary excretion of the glucuronide of E3040 and that of liquiritigenin (LG) were impaired in EHBR whereas those of sulfates remains normal, we hypothesized that the biliary excretion system for glucuronide was distinct from that of sulfate (8,9). In addition, we hypothesized that the biliary excretion of LG

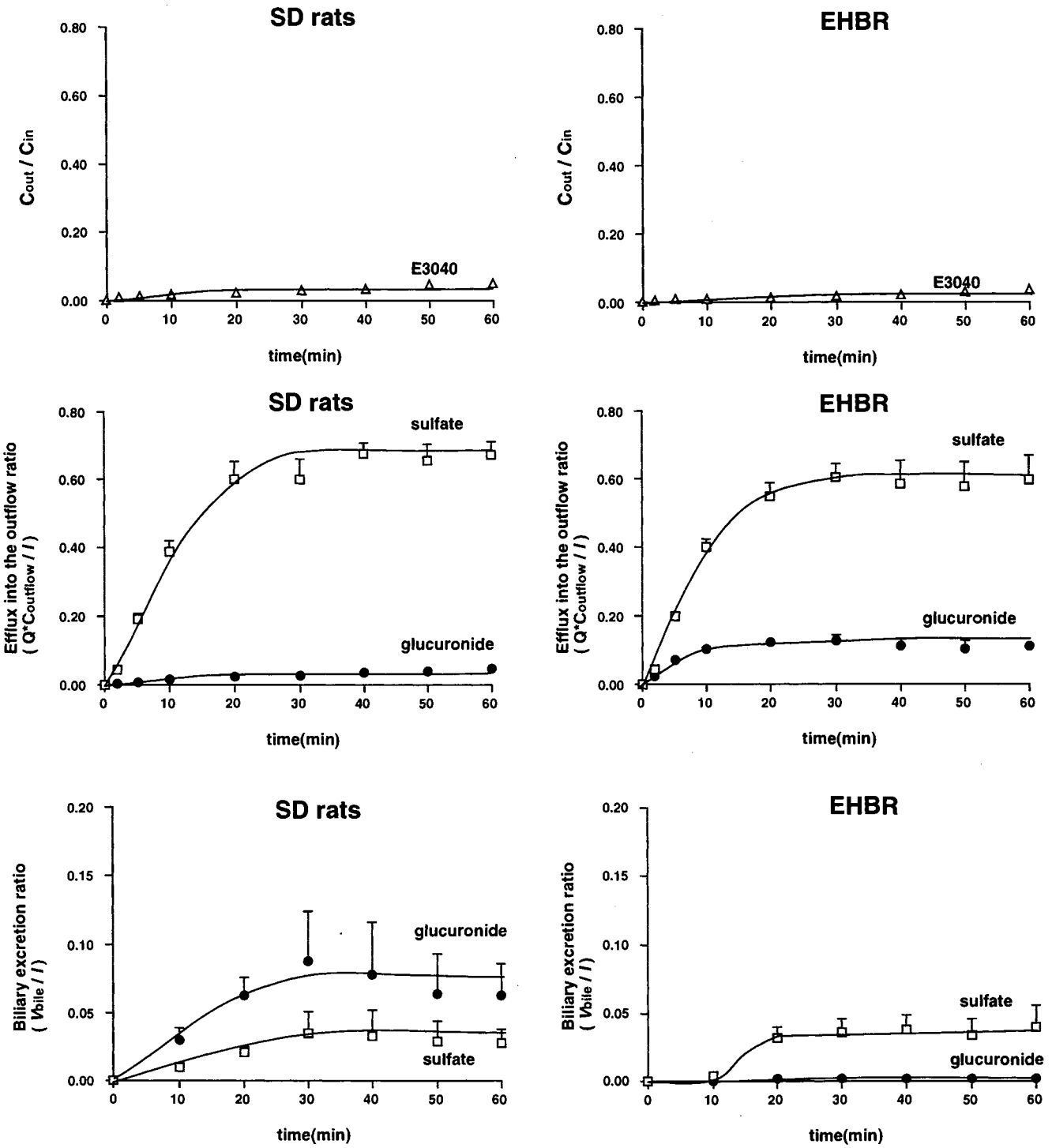

Fig. 2. Single-pass steady-state perfusion of [^{14}C]E3040. The time profile of the C_{out} / C_{in} value of E3040 (top), the Q · $C_{outflow}$ / I value (middle) and the V_{bile} / I value (bottom) are shown. C_{in} and C_{out} denote the perfusate concentration of [^{14}C]E3040 in the portal inflow and the hepatic venous outflow, respectively. V_{bile} and $C_{outflow}$ denote the biliary excretion rate and the perfusate concentration in the hepatic outflow of [^{14}C]E3040 conjugates, respectively. I denotes the infusion rate obtained from the equation : I = Q · C_{in}, where Q denotes the flow rate. Each point and vertical bar represent the mean ± S.E. of 4 different experiments. right panel : EHBR, left panel : SD rats.

glucuronides in SD rats may be mediated by an ATP-dependent active transport system which is defective in EHBR (4, 8). It was considered that the impairment of the transport system from hepatocytes into bile causes the reduced biliary excretion of glucuronides in EHBR. The decrease in the biliary excretion in EHBR, however, may be observed if the efflux capacity for the conjugates into the blood increases and/or if the increased bilirubin level in the hepatocytes induces some secondary effects (10).

In the present study, we attempted to reveal the cause(s)

Table I. Kinetic Parameters for the Hepatic Handling of [^{14}C]E3040 and Its Conjugates at Steady-State in the Single-Pass Perfused Rat Liver

	SD rats			EHBR		
	E3040	Glucuronide	Sulfate	E3040	Glucuronide	Sulfate
Extraction ratio	0.961 ± 0.004	—	—	0.975 ± 0.005	—	—
Efflux into outflow ratio ($Q^*C_{outflow}/I$)	—	0.039 ± 0.008	0.651 ± 0.044	—	0.115 ± 0.017*	0.590 ± 0.060
Biliary excretion ratio (V_{bile}/I)	n.d.	0.073 ± 0.031	0.031 ± 0.015	n.d.	0.002 ± 0.001*	0.037 ± 0.012
Bile flow (μl/min/g liver)		0.67 ± 0.19			0.19 ± 0.05*	

* $p < 0.05$ (by Mann-Whitney U test).
n.d. Not detectable.
The values were determined by averaging each point from 30 min to 60 min. The efflux rate into outflow ($Q^*C_{outflow}$) and the biliary excretion rate (V_{bile}) of [^{14}C]E3040 conjugates were normalized for the infusion rate (I) of [^{14}C]E3040. All values are given as the mean ± S.E. of 4 different experiments.

for the impairment of the biliary excretion of E3040 glucuronide in EHBR. For this purpose, we performed *in situ* liver perfusion experiments to determine the intrinsic clearance for the efflux of the conjguates across the bile canalicular membrane. Furthermore, we also measured the intracellular distribution and protein binding of the conjugates in liver cytosol, in order to indicate that the impairment of the biliary excretion EHBR is not caused by the impariment of intracellular transport (10). Finally, we also examined the uptake of the conjugates into the CMV isolated from SD rats and EHBR.

As shown in Fig. 2 and Table. I, the hepatic extraction ratio of E3040 at steady-state was high (0.96-0.97) in both rat strains. This result was comparable to that observed in a single bolus injection experiment in the perfused liver which indicated that the hepatic clearance of E3040 is limited by hepatic blood flow (9). In SD rats, the C_{bile} / $C_{u,cyt}$ of the glucuronide and sulfate was approximately 400 and 40, respectively (Table III). Such highly concentrative excretion suggest that active transport plays an important role in the biliary excretion of these conjugates. The biliary excretion rate (V_{bile} / I) of the glucuronide formed in the liver was severely decreased in EHBR compared with that in SD rats (Table I). In contrast, the efflux rate into the outflow ($Q \cdot C_{outflow}$ / I) was increased to some extent for the glucuronide (Table I). No significant diference was observed in the disposition of the sulfae between EHBR and SD rats.

To examine the mechanism for the decrease in biliary excretion and the increase in efflux into the outflow for the glucuronide, we calculated the CL_{bile} and $CL_{outflow}$ for the conjugates (Table. IV). The $CL_{u,bile}$ of the glucuronide in EHBR decreased to approximately 1/33 of that in SD rats, whereas the $CL_{u,outflow}$ of the glucuronide was approximately 4.5 times greater than that in SD rats. This result indicates that marked impairment of the biliary excretion capacity of the glucuronide is predominantly responsible for the decreased biliary excretion in EHBR discussed as follows: the ratio of biliary excretion rate to efflux rate into the perfusate of the glucuronide is determined by that of $CL_{u,bile}$ to $CL_{u,outflow}$ under the steady-state condition. This value for the glucuronide was approximately 2 : 1 in SD rats (Table I and IV). Assuming the 4.5-fold increased in $CL_{u,outflow}$ of glucuronide, the ratio of $CL_{u,bile}$ to $CL_{u,outflow}$ as well as that of biliary excretion rate to efflux rate into the perfusate are expected to be approximately 1 : 2. However, this ratio was 1 : 50 in EHBR (Fig. 2 and Table I), indicating that the reduced biliary excretion of the glucuronide in EHBR can not be absolutely accounted for by the increase in $CL_{u,outflow}$. If we also consider the 1/30-fold reduction in $CL_{u,bile}$ (Table IV), the ratio of biliary excretion rate to efflux rate into the perfusate can be extimated to be 1 : 20, which is comparable to that observed in EHBR (1 : 50, Table I/Fig. 2). Based on these findings, reduction on the biliary excretion rate of the glucuronide is predominantly caused by the reduced $CL_{u,bile}$ in EHBR. At present time, we don't have any good reason to account for the increase in $CL_{u,outflow}$ in

Table II. Unbound Fraction and Cytosolic Distribution of [^{14}C]E3040 Conjugates

	Glucuronide		Sulfate	
	SD rats	EHBR	SD rats	EHBR
$f_{u,outflow}$	0.286 ± 0.057		0.065 ± 0.002	
$Fract_{,cyt}$	0.730 ± 0.247	0.681 ± 0.081	0.825 ± 0.068	0.638 ± 0.153
$f_{u,cyt(33\%)}$	0.618 ± 0.055	0.624 ± 0.012	0.164 ± 0.010	0.177 ± 0.012
$f_{u,cyt}$	0.360 ± 0.051	0.357 ± 0.012	0.062 ± 0.004	0.067 ± 0.005

$Fract_{,cyt}$ value and $f_{u,cyt}$ value were calculated from equations (8) and (7), respectively. The $f_{u,cyt(33\%)}$, $f_{u,homo(33\%)}$ and $f_{u,outflow}$ value were determined by ultrafiltration.

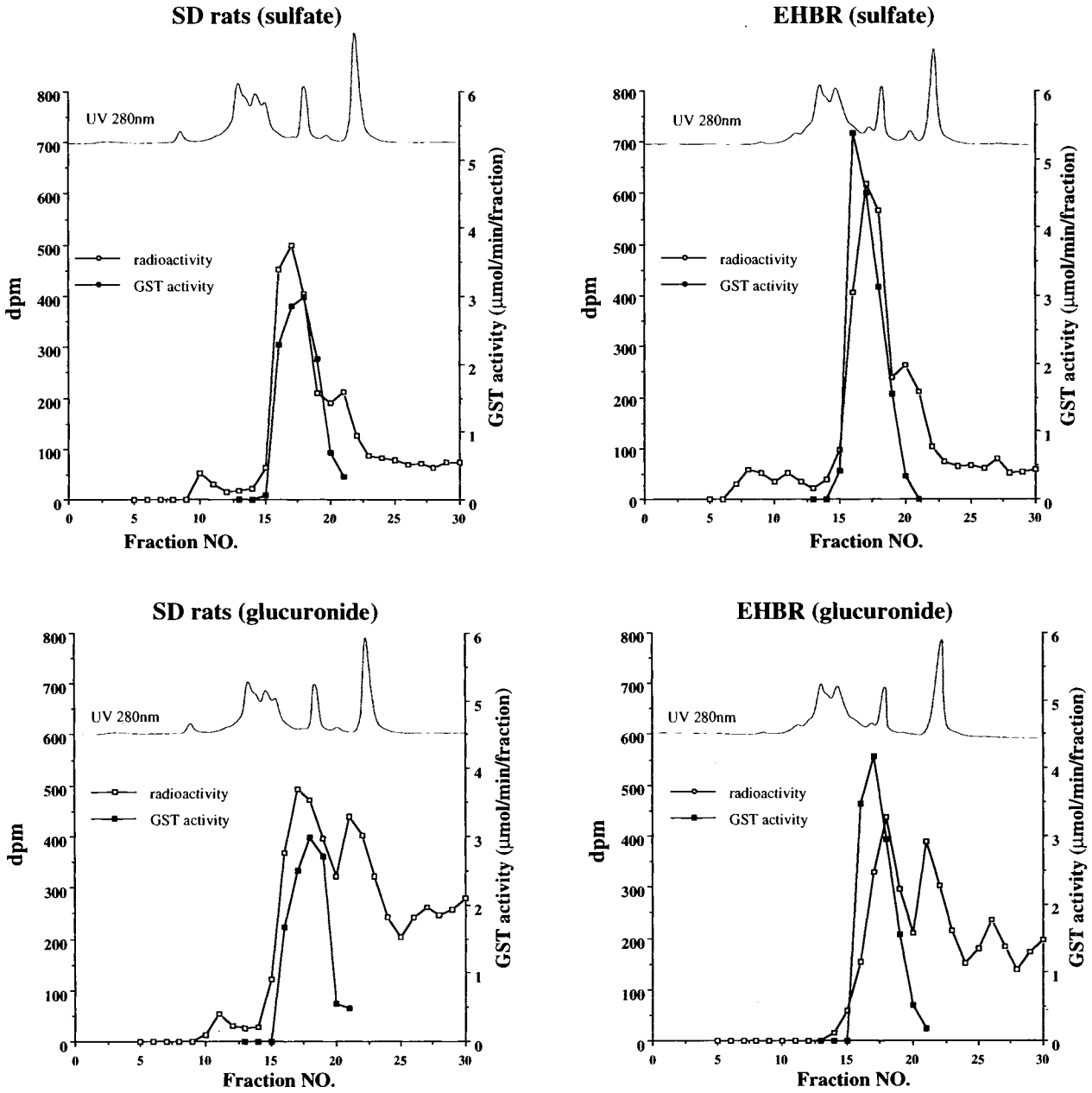

Fig. 3. Elution patterns for the binding of E3040 conjugates to the liver cytosol of EHBR and SD rats. [^{14}C]E3040 glucuronide or sulfate was added to 0.75 ml of each liver cytosol specimen from EHBR (right) or SD rats (left) to produce a final concentration of 31 μM. After addition of [^{14}C]E3040 conjugates, specimens were incubated at 37°C for 15 min and then applied to HPLC using a gel filtration column (Asahipak GS-510, 50 cm × 7.6 mm i.d.). The solvent system used was 50 mM potassium phosphate buffer (pH 7.4) at a flow rate of 0.5 ml / min. Solid line ; protein absorbance (UV 280 nm), -○-, -□-; radioactivity of [^{14}C]E3040 conjugates, -●-, -■-; GST activity. top ; sulfate, bottom ; glucuronide, left panel ; SD rats, right panel ; EHBR.

EHBR. If we assume the presence of carrier-mediated efflux of E3040 glucuronide into the outflow as suggested for DBSP (18), an increase in the $CL_{u,outflow}$ of glucuronide in EHBR might result from the alteration in the characteristics of cariers on the sinusoidal membrane, e. g., an alteration in capacity (Vmax) and/or affinity (Km), and/or that in the distribution of carriers among the sinusoidal and canalicular membranes. This latter hypothesis is also plausible since such alteration in the distribution of proteins in rat hepatocytes is observed by colchicine and phalloidin treatment or bile duct ligation (19). We can not clarify the mechanism for the increase in $CL_{u,outflow}$, however, since the carrier protein(s) responsible for the sinusoidal efflux has not been identified. The biliary excretion capacity and efflux capacity of the sulfate, however, was similar in both rat strains.

Comparison of the $CL_{u,bile}$ and $CL_{u,outflow}$ values in the normal rats indicated that the intracellularly formed glucuronide and sulfate may have higher preference for biliary excretion system and efflux system into the blood, respectively (Table IV). Furthermore, $CL_{u,outflow}$ of sulfate was 4 times higher compared to that of glucuronide (Table IV). Since $CL_{u,outflow}$ is the net efflux clearance which is influenced by

Table III. Bile/Liver Concentration Ratio of [^{14}C]E3040 Conjugates in EHBR and SD Rats

	Glucuronide		Sulfate	
	SD rats	EHBR	SD rats	EHBR
C_{bile}/C_{liver} [a]	95.6 ± 3.3	11.7 ± 2.4*	2.0 ± 0.3	9.4 ± 3.2
$C_{bile}/C_{u,cyt}$ [b]	434.0 ± 51.8	46.3 ± 10.3*	40.2 ± 5.5	224.9 ± 72.3*

[a] The C_{liver} value was measured after single-pass continuous perfusion, assuming the intracellular volume to be 0.7 ml/g liver.
[b] The $C_{u,cyt}$ value was calculated from equation (9). The $f_{u,cyt}$ and $Fract_{,cyt}$ values were determined by ultrafiltration.
* $p < 0.05$ (by Mann-Whitney U test).

the intrinsic efflux clearance, the intrinsic clearance for the re-uptake, the protein binding and the flow rate, it is possible that the higher protein binding of sulfate prevents the re-uptake, resulting in the observed higher $CL_{u,outflow}$ of sulfate. However, we found that the uptake clearance of glucuronide which was defined for the total glucuronide concentration in the perfusate was 1.6 times greater than that of sulfate (manuscript in preparation); we also performed the multiple indicator dilution (MID) study in which the conjugates were injected as a bolus to the perfusate and then the outflow concentration was analyzed as described previously (12). In this MID study, the BSA concentration and the flow rate of the perfusate was the same as that used in the present study. Based on these observations, we suggest that the higher $CL_{u,outflow}$ of sulfate represents not only the lower re-uptake capacity of this conjugates, but also the higher intrinsic clearance for the efflux across the sinusoidal membrane. The $CL_{u,bile}$ for glucuronide was approximately ten times higher compared to that for sulfate. This may be one of the major reasons for the much greater biliary excretion of the glucuronide than that of the sulfate *in vivo* (9).

In the present study, we also examined the intracellular disposition of the conjugates. Previously, we reported that the biliary excretion of ICG is severly imparied in EHBR. Pharmacokinetic analysis suggested that the impariment of the biliary excretion of ICG was caused predominantly by reduced intracellular transport, but not by a defect in transport ability across the bile canalicular membrane (10). This phenomenon in EHBR was caused by a decrease in the binding of ICG to cytosolic protein (10). The cause of this decrease in binding was associated with an increase in hepatic bilirubin concentration, and this decrease in binding to cytosolic protein resulted in the increase in the distribution of ICG to cellular organelles in hepatocytes (10). Since the same phenomenon may be hold for E3040 glucuronide, we examined the intracellular distribution (Table II) and protein binding (Fig. 3 and Table II) of the conjugates in liver cytosol. No difference in the $f_{u,cyt}$ and in $Fract_{,cyt}$ was observed between the two strains. This result shows that the impaired biliary excretion of the glucuronide in EHBR can not be accounted for by the alterations in the intracellular transport. In addition, the cytosolic binding protein of the conjugates was identified as ligandin (GSTs) in the two rat strains (Fig. 3).

These results support the hypothesis that the biliary excretion system for the glucuronide, but not that for sulfate, is impaired in EHBR. Since the ATP-dependent primary active transport on the bile canalicular membrane is hereditarily defective in EHBR, it was also hypothesized that the glucuronide can be the ligand for this transporter. In order to examine this hypothesis, we studied the uptake of the conjugates into CMV isolated from SD rats and EHBR. Since DNP-SG, as well as LTC_4, is the typical ligand for this active transporter (11), we examined the ATP-dependent uptake of this compound as a positive control. As shown in Fig. 4, the uptake of both E3040 glucuronide and DNP-SG into CMV from SD rats was stimulated in the presence of ATP, suggesting the contribution of primary active transporter for the biliary excretion of these ligands. Furthermore, the stimulatory effect of ATP on the uptake of the glucuronide and DNP-SG into CMV from EHBR was minimal (Fig. 4). These results suggest that E3040 glucuronide is transported across

Table IV. The Biliary Excretion Clearance (CL_{bile}) and Efflux Clearance into Outflow ($CL_{outflow}$) of [^{14}C]E3040 Conjugates in EHBR and SD Rats

	Glucuronide		Sulfate	
	SD rats	EHBR	SD rats	EHBR
CL_{bile} [a] (ml/min/g liver)	0.096 ± 0.051	0.003 ± 0.001*	0.002 ± 0.001	0.002 ± 0.001
$CL_{u,bile}$ [b] (ml/min/g liver)	0.366 ± 0.135	0.011 ± 0.006*	0.041 ± 0.023	0.053 ± 0.022
$CL_{outflow}$ [a] (ml/min/g liver)	0.043 ± 0.007	0.195 ± 0.039*	0.040 ± 0.007	0.033 ± 0.009
$CL_{u,outflow}$ [b] (ml/min/g liver)	0.209 ± 0.052	0.880 ± 0.258*	0.795 ± 0.117	0.789 ± 0.160

[a] CL_{bile} and $CL_{outflow}$ were calculated by dividing the biliary excretion rate and the efflux rate into outflow of E3040 conjugates by their respective concentrations in the perfused liver.
[b] $CL_{u,bile}$ and $CL_{u,outflow}$ are the clearances of unbound E3040 conjugates in the liver.
* $p < 0.05$ (by Mann-Whitney U test).

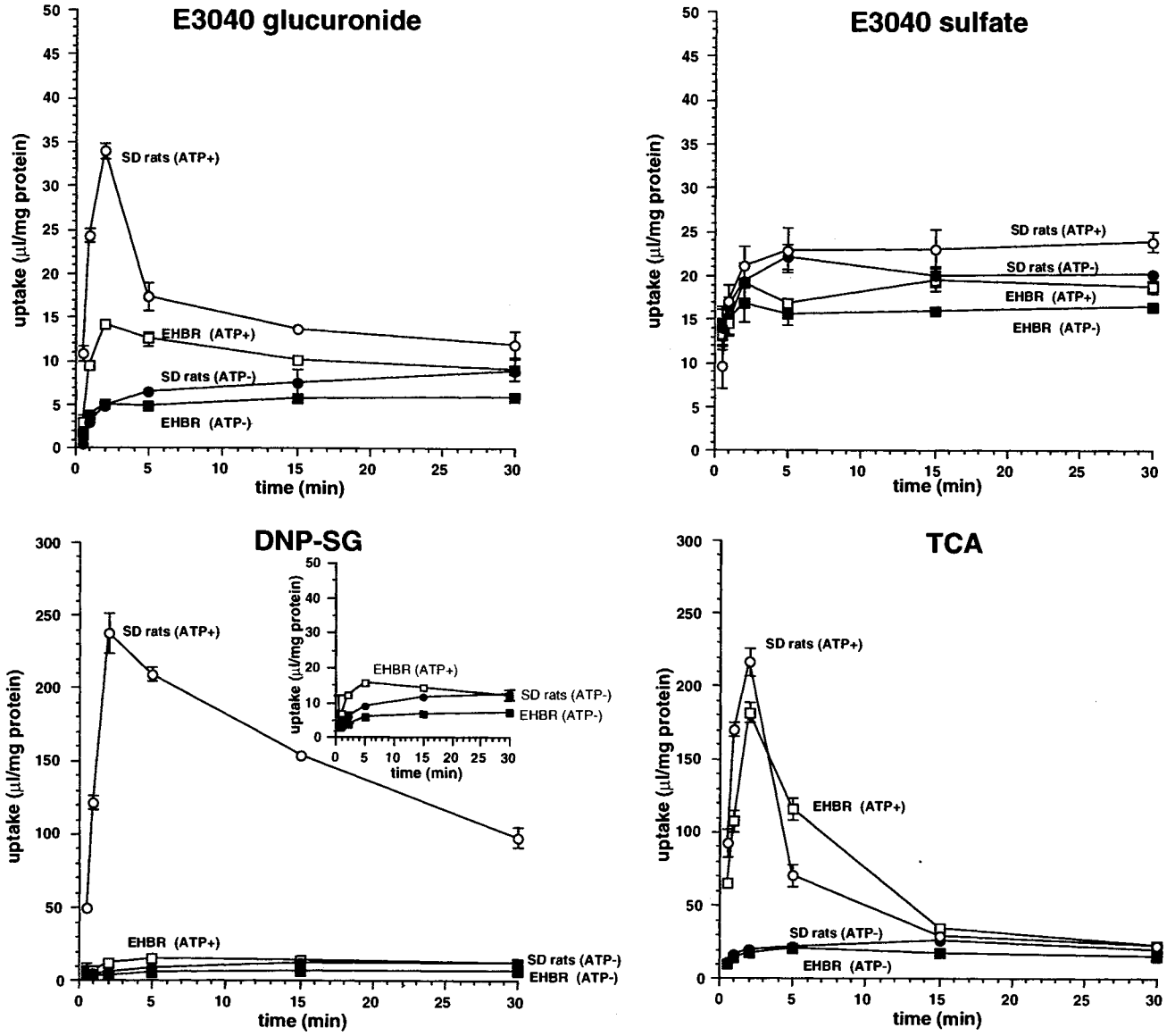

Fig. 4. Time profiles for the uptake of [^{14}C]E3040 conjugates, [^{3}H]Taurocholate and [^{3}H]S-(2,4-dinitrophenyl) glutathione by the CMV prepared from SD rats and EHBR. CMV (8 μg of protein) were incubated with (open) or without (closed) ATP (5 mM) and ATP regenerating system (10 mM creatine phosphate and 100 μg/ml of creatine phosphokinase) in the medium. The concentration of [^{14}C]E3040 conjugates, [^{3}H]Taurocholate (TCA) and [^{3}H]S-(2,4-dinitrophenyl) glutathione (DNP-SG) were 25 μM, 1.4 μM and 1.0 μM, respectively. Each point and vertical bar represent the mean ± S.E. of 3 different experiments. -○-, -●- ; SD rats, -□- -■- ; EHBR.

the bile canalicular membrane *via* the primary active transporter which is defective in EHBR. In contrast, the uptake of E3040 sulfate into CMV was comparable between SD rats and EHBR and was not stimulated in the presence of ATP (Fig. 4). This result supports the *in situ* experimental data in that the biliary excretion of the sulfate remains normal in EHBR. Collectively, these *in vitro* data support the hypothesis that the transport system for the glucuronide across the bile canalicular membrane is different from that for the sulfate.

In conclusion, collectively, it was suggested (1) that the excretion of E3040 glucuronide across the bile canalicular membrane is mediated by the primary active transporter which is defective in EHBR and (2) that the bile canalicular transport system for E3040 sulfate is different from that for the glucuronide in that the former remains normal in EHBR. We could thus suggest the presence of multiplicity in the biliary excretion of conjugative metabolites.

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