Uptake of Glycyrrhizin by Isolated Rat Hepatocytes

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The mechanism of uptake of glycyrrhizin (GLZ) by isolated rat liver cells was studied. The uptake rate was dependent on the unbound GLZ concentation. The initial uptake rate with respect to the unbound GLZ concentration reflected the operation of both saturable and nonsaturable processes, which followed Michaelis-Menten type kinetics; the process involves a K_m of 11.3 μM, V_{max} of 0.112 nmol/min/10^6 cells, and a first-order rate constant (K_d) of 0.195 nmol/min/10^6 cells/mM. GLZ adsorption on the cell membrane occurs at two types of binding sites with a linear adsorption coefficient = 2.81 nmol/10^6 cells/mM and a dissociation constant = 18.3 μM and its adsorption capacity = 0.12 nmol/10^6 cells describing specific adsorption. GLZ uptake did not require the presence of Na^+ in the incubation medium and was not significantly inhibited by ouabain. The Arrhenius plot of uptake of 10 μM GLZ presented a single straight line in the range of 4—37 °C, with an activation energy of 15.9 kcal/mol. An energy requirement was also demonstrated, as all metabolic inhibitors studied (rotenone, antimycin A, 2,4-dinitrophenol, and KCN) significantly reduced the uptake of 10 μM GLZ ($p<0.01$). The uptake was competitively inhibited by glycyrrhetinic acid (GLA), taurocholate (TCA), and probenecid (PBC) with inhibition constants, K_i, of 13.7, 48.5, and 115.9 μM, respectively, and it was noncompetitively inhibited by bromosulfophthalein (K_i 9.2 μM) and indocyanine green (K_i 13.5 μM) only at low GLZ concentrations (5 and 10 μM).

It was concluded that a carrier-mediated transport system participates in the uptake of GLZ into isolated rat hepatocytes and the affinity site of the transport carrier commonly binds GLA, TCA, and PBC.

Keywords glycyrrhizin; hepatic uptake; isolated rat hepatocyte; carrier-mediated uptake

Introduction

Glycyrrhizin (GLZ) is an anionic drug used frequently in the treatment of chronic hepatitis.[1] Yamamura *et al.*[2] showed that GLZ is predominantly excreted in the bile (*ca.* 80%) after 80 mg/kg i.v. administration to patients with liver failure. We also showed that biliary excretion of GLZ is the major route in rats (*ca.* 86—98%) after an i.v. administration at the dose range of 5—50 mg/kg.[3] We established that the biliary excretion of the drug in rats follows Michaelis–Menten kinetics ($K_{m,B}$ 1.83 μg/ml and $V_{max,B}$ 98.77 μg/min) and the dose dependency of biliary clearance determines that of the total body clearance.[3] Such observations emphasize the importance of investigating the mechanism of hepatic uptake of GLZ.

The mechanisms of hepatic uptake of anionic drugs such as bromosulfophthalein (BSP), indocyanine green (ICG), and bile acids have been studied using isolated rat hepatocytes,[4-10] isolated perfused rat liver,[11-13] and whole animals.[13-15] These studies suggested that these organic anions are taken up by a carrier-mediated system. Isolated liver cells are particularly advantageous in the study of the hepatic uptake process, as the uptake can be studied independently of unspecific binding to plasma proteins, different distributional compartments, and hemodynamic factors.

The purpose of the present study is to elucidate the mechanism of hepatic uptake of GLZ by using isolated rat liver cells, and to determine whether the transport system is shared by other organic anions such as BSP, ICG, and bile acid.

Experimental

Chemicals GLZ monoammonium salt and glycyrrhetinate (GLA) sodium salt were kindly supplied by Minophagen Pharmaceutical Co. (Tokyo, Japan). BSP sodium salt, probenecid (PBC), taurocholate (TCA) sodium salt, *p*-aminohippurate (PAH) sodium salt, antimycin A, ouabain, rotenone, bovine serum albumin (fraction V) (BSA), fluorescein isothiocyanate dextran (avg. mol. wt. 64200), and collagenase (type II) were purchased from Sigma Chemical Co. (St. Louis, MO). Silicone oil ($d=1.02$) and KCN, LiCl, and trypan blue were purchased from Aldrich Chemical Company Co. (Milwaukee, WI) and Wako Pure Chemical Ind., Ltd. (Osaka, Japan), respectively. ICG was obtained from Daiichi Pharmaceutical Co. (Tokyo, Japan). All other reagents were commercial products of analytical grade.

Preparation of Isolated Hepatocytes Isolated liver cells from male Wistar rats weighing 250—300 g, fed on standard chow, were prepared according to the method of Lin *et al.*,[16] which was slightly modified from that of Baur *et al.*[17] Cell viability was estimated by trypan blue exclusion. All experiments were carried out on cell suspensions with at least 90% viability.

Uptake Studies The centrifugal filtration method was used to measure the uptake of GLZ by isolated hepatocytes.[17] Hepatocytes were suspended in the incubation medium containing 131 mM NaCl, 5.2 mM KCl, 0.9 mM $MgSO_4$, 0.12 mM $CaCl_2$, 3 mM Na_2HPO_4, 5 mM glucose, and 15 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), pH 7.4. After preincubation of 5.0 ml of the cell suspension containing 8×10^6 cells/ml for 5 min at 37 °C, the reaction was started by the addition of 20 μl of the incubation medium containing GLZ preincubated for 5 min at 37 °C. After incubation for a suitable time at 37 °C, 200 μl of the reaction mixture was placed on top of an upper layer of silicone oil (50 μl) with an under layer (50 μl) of 3 M KOH solution, which was added in order to dissolve the cells, in a polyethylene tube, and the whole was centrifuged for 5 s in a Beckman microfuge (Beckman Instrument Inc., CA) to stop the reaction by separating the cells from the incubation medium. To 50 μl of the KOH layer was added 40 μl of 20% $HClO_4$ solution to bring the medium pH to approximately 7, and then 300 μl of MeOH was added. The solution was centrifuged and the obtained supernatant solution was used for the determination of GLZ taken up by the cells. Upon centrifugation in a Beckman microfuge, the cells together with a small amount of adherent medium passed through the silicone oil. The volume of adherent medium was determined by the method of Mizuma *et al.*[10] using impermeable fluorescein isothiocyanate dextran (final concentration: 5 μM). The volume of adherent fluid obtained was 1.03 ± 0.02 μl/10^6 cells (mean ± S.E., $n=7$). All the uptake data were corrected for the amount of GLZ in this volume of adherent fluid.

Measurement of Unbound GLZ Concentration The unbound GLZ concentration in the cell suspension was determined by an ultrafiltration technique using an ultrafiltration membrane (Amicon Micropartition System, MPS-1, Danvers, MA). GLZ in the incubation medium preincubated for 5 min at 37°C was added to the stirred cell suspension preincubated similarly, and the mixture was immediately centrifuged for

3 s at 16000 rpm. The obtained supernatant solution was applied to the membrane. The filtrate was subjected to the determination of GLZ.

Determination of GLZ The supernatant solution and filtrate (50 μl each) were each directly injected into a high performance liquid chromatograph (HPLC), Hitachi type L, with a Wakosil-II 5C18-HG column (250 × 4.6 mm i.d.) and an ultraviolet detector operating at 254 nm. Other conditions were the same as those described previously.[18]

Data Analysis Uptake of GLZ was measured at 1, 2, 3, 4, and 5 min. Efflux was assumed to be negligible at these sampling points. Since the uptake at various GLZ concentrations proceeded linearly for 5 min in all cases, the initial uptake rate was taken to be the slope of the linear regression line. The amount of GLZ adsorbed on the cell membrane was inferred from the y-intercept at time zero, obtained by extrapolating the linear regression line. The kinetic parameters of uptake were estimated by a least-squares regression analysis program (MULTI[19]) using a digital computer (NEC PC-9801 RA).

Results

Uptake of GLZ by Isolated Hepatocytes The time course of uptake of GLZ into isolated hepatocytes is shown in Fig. 1 at various GLZ concentrations ranging from 5.0 μM to 1.0 mM. The uptake proceeded linearly over the 5-min incubation period. GLZ uptake in the presence of 2% BSA was also linear over the 5-min incubation period at various GLZ concentrations ranging from 0.1 to 2.4 mM (results not shown). Therefore, the initial uptake rate was taken to be the slope of the regression line in both cases. The initial uptake rate in the absence (A) and presence (B) of 2% BSA analyzed as a function of the unbound GLZ concentration is illustrated in Fig. 2. The results suggest that GLZ uptake consists of two components, *i.e.*, the saturable and nonsaturable processes at lower and higher unbound GLZ concentrations than 0.1 mM, respectively. Thus, the initial uptake rate (V_0) can be related to the unbound GLZ concentration (C_f) as follows:

$$V_0 = \frac{V_{max} \cdot C_f}{K_m + C_f} + K_d \cdot C_f \quad (1)$$

where V_{max} is the maximum uptake rate for a carrier-mediated process, K_m is the Michaelis constant, and K_d is the first-order uptake rate constant. Table I lists the kinetic parameters estimated from Eq. 1 by MULTI analysis.[19] None of parameters was significantly changed in the presence of BSA.

The adsorption data of GLZ on the cells were further analyzed by means of a Scatchard plot (Fig. 3). From the steep part of the curve, a dissociation constant of 18.3 ± 7.3 μM (± computer calculated S.D.) was obtained. The corresponding adsorption capacity was 0.12 ± 0.02 nmol/10^6 cells. The linear part of the curve had a linear adsorption coefficient of 2.81 ± 0.05 nmol/10^6 cells/mM.

Effect of Na^+ on Initial Uptake Rate To study the Na^+ dependency of the GLZ uptake process, NaCl in the medium was replaced by LiCl (131 mM), so that 99.8% of Na^+ was substituted. The replacement did not significantly change the initial uptake rate of 10 μM GLZ (102.5 ± 4.8% of control, $n=4$). Also, the Na^+, K^+-dependent ATPase

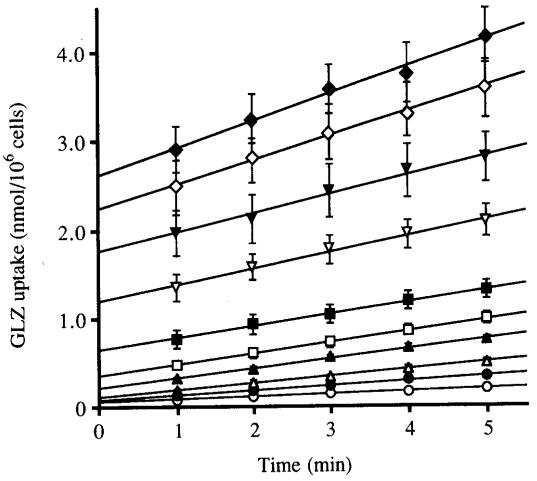

Fig. 1. Time Course of Uptake of GLZ by Isolated Rat Hepatocytes in the Absence of BSA at 37 °C

Total concentration of GLZ: ○, 5 μM; ●, 10 μM; △, 20 μM; ▲, 50 μM; □, 0.1 mM; ■, 0.2 mM; ▽, 0.4 mM; ▼, 0.6 mM; ◇, 0.8 mM; ◆, 1.0 mM. Each point is the mean ± S.E. of nine independent experiments. The solid lines were drawn by linear regression analysis.

TABLE I. Kinetic Parameters of GLZ Uptake by Isolated Rat Hepatocytes in the Absence and Presence of 2% BSA

BSA	K_m [a] (μM)	V_{max} [b] (nmol/min/10^6 cells)	K_d [c] (nmol/min/10^6 cells/mM)
Absence	11.3 ± 2.3	0.112 ± 0.013	0.195 ± 0.032
Presence	15.4 ± 2.3	0.124 ± 0.016	0.188 ± 0.049

Data are presented as the mean ± S.E. of seven or nine independent experiments. *a*) The Michaelis constant. *b*) The maximum uptake rate. *c*) The first-order uptake rate constant.

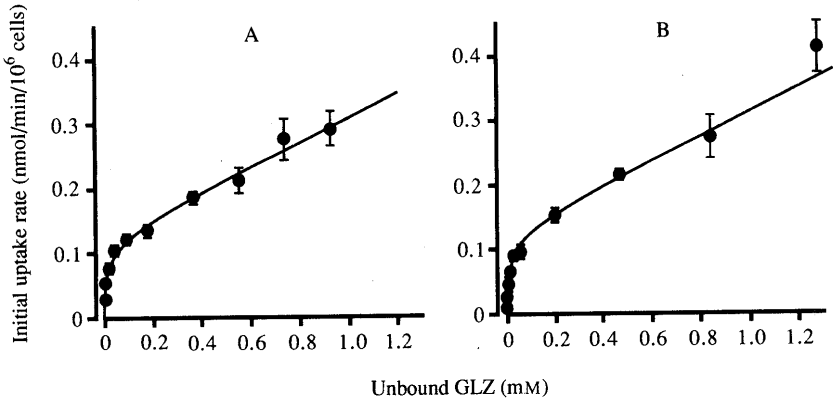

Fig. 2. The Initial Uptake Rate of GLZ by Isolated Rat Hepatocytes in the Absence (A) and Presence (B) of 2% BSA at 37 °C as a Function of the Unbound GLZ Concentration

Each point is the mean ± S.E. of seven or nine independent experiments. The solid lines were generated from Eq. 1 using the MULTI-fitted parameters listed in Table I. For the determination of the unbound GLZ concentration, see the text.

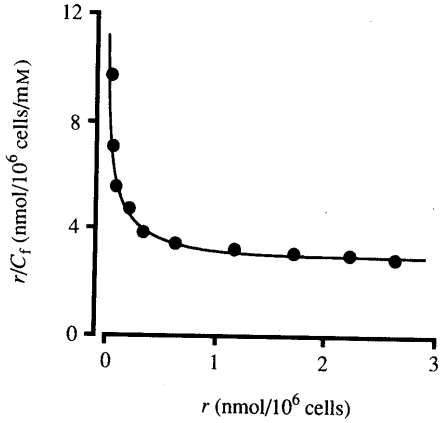

Fig. 3. Scatchard Plot of Adsorption of GLZ on the Cells

r, the adsorption amount of GLZ per 10^6 cells, which was inferred from the intercept of the regression line at time zero in Fig. 1; C_f, the unbound GLZ concentration. The solid line was generated from the following equation using the MULTI-fitted parameters (the adsorption capacity of specific affinity sites, $N_s = 0.12$ nmol/10^6 cells; the dissociation constant corresponding to N_s, $K_s = 18.3$ μM; the adsorption coefficient of nonspecific affinity sites, $K_n = 2.81$ nmol/10^6 cells/mM).

$$r = N_s \cdot C_f/(K_s + C_f) + K_n \cdot C_f$$

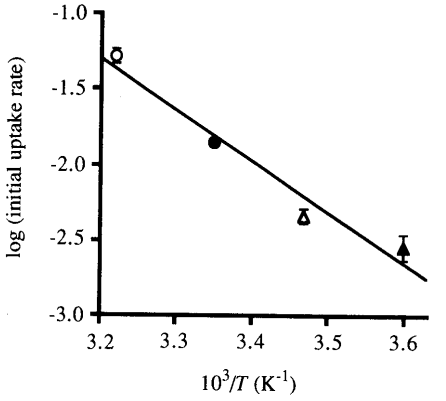

Fig. 4. Arrhenius Plot for the Initial Uptake Rate of GLZ

The initial uptake rate of 10 μM GLZ was determined at 4 (○), 15 (●), 25 (△), and 37 °C (▲). Each point is the mean ± S.E. of six to nine experiments. The solid lines were drawn by linear regression analysis. An activation energy of 15.9 kcal/mol was calculated from the slope of the straight line.

inhibitor ouabain (1 mM) did not significantly alter the uptake rate (86.0 ± 4.3% of control, $n = 4$).

Temperature Dependence of Initial Uptake Rate Uptake of 10 μM GLZ into isolated hepatocytes was determined at four different temperatures, 4, 15, 25, and 37°C. Figure 4 shows an Arrhenius plot for the initial uptake rate. The plot exhibited a single straight line corresponding to an activation energy of 15.9 kcal/mol (66.5 kJ).

Effects of Metabolic Inhibitors on Initial Uptake Rate The respiratory chain inhibitors rotenone (50 μM), antimycin A (10 μM), and KCN (1 mM), and the uncoupler 2,4-dinitrophenol (1 mM), added 10 min prior to GLZ to the cells, significantly ($p < 0.01$) diminished the initial uptake rate of 10 μM GLZ by about 44, 58, 60, and 57%, respectively (Fig. 5).

Inhibition of Initial Uptake Rate by Other Organic Anions To determine the specificity of the GLZ uptake process, the influence of several organic anions on the initial uptake rate was studied. BSP, GLA, ICG, TCA, and PBC (50 μM each), added 10 s prior to GLZ to the cells, significantly ($p < 0.01$) reduced the initial uptake rate of 10 μM GLZ by about 11, 25, 34, 63, and 73%, respectively, but PAH

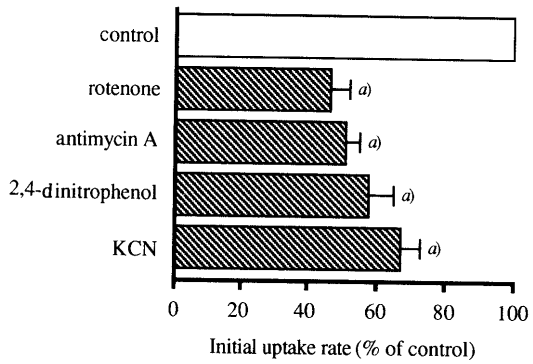

Fig. 5. Effect of Metabolic Inhibitors on the Initial Uptake Rate of 10 μM GLZ at 37 °C

The inhibitor concentrations: rotenone, 50 μM; antimycin A, 10 μM; 2,4-dinitrophenol, 1 mM; KCN. The inhibitor was added 10 min before the addition of GLZ. Each bar is the mean ± S.E. of four experiments. *a*) Significant difference from the control ($p < 0.01$).

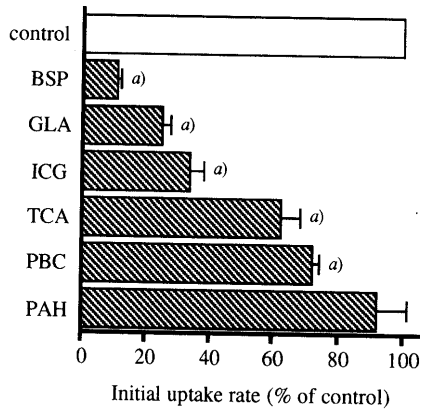

Fig. 6. Effect of Organic Anions on the Initial Uptake Rate of 10 μM GLZ at 37 °C

Each organic anion (50 μM) was added 10 s before the addition of GLZ. Each bar is the mean ± S.E. of four experiments. *a*) Significant difference from the control ($p < 0.01$). BSP, bromosulfophthalein; GLA, glycyrrhetinic acid; ICG, indocyanine green; TCA, taurocholate; PBC, probenecid; PAH, *p*-aminohippurate.

TABLE II. Evaluation of Inhibition Type of Organic Anions for GLZ Uptake into Isolated Rat Hepatocytes

Organic anion	Competitive		Noncompetitive	
	K_i (μM)	AIC[a)]	K_i (μM)	AIC[a)]
GLA	13.7	12.5	26.6	20.8
TCA	48.5	10.6	65.4	16.4
PBC	115.9	6.7	201.2	11.1
BSP	9.5	35.7	9.2	17.5
ICG[b)]	11.8	−10.1	13.5	−18.5

The values were calculated by fitting the data to Eqs. 2 and 3 that correspond to competitive and noncompetitive inhibitions, respectively, using the MULTI program.[19)]

$$V_0 = \frac{V_{max} \cdot C_f}{K_m\left(1 + \frac{C_i}{K_i}\right) + C_f} \quad (2)$$

$$V_0 = \frac{V_{max} \cdot C_f}{K_m\left(1 + \frac{C_i}{K_i}\right) + C_f\left(1 + \frac{C_i}{K_i}\right)} \quad (3)$$

Where K_i is the inhibition constant and C_i is the concentration of inhibitor. *a*) The Akaike's information criteria.[20)] *b*) The uptake data at 5 and 10 μM except 20 μM GLZ were used for the calculation.

(50 μM) was ineffective (Fig. 6). A Dixon plot of GLZ showing inhibition by organic anions is presented in Fig. 7. As can be seen in Figs. 7A—C, the straight lines

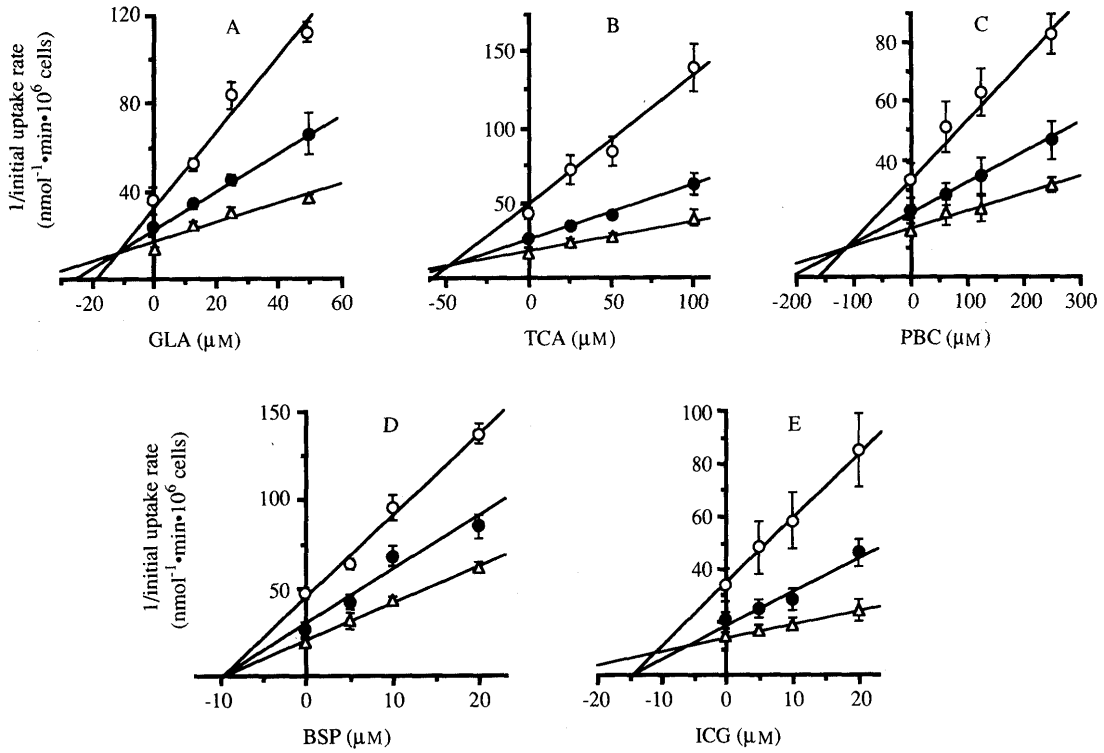

Fig. 7. Dixon Plots of GLZ Uptake Showing Inhibition by GLA (A), TCA (B), PBC (C), BSP (D), and ICG (E)

GLZ concentrations: ○, 5 μM; ●, 10 μM; △, 20 μM. Each point is the mean ± S.E. of four or five independent experiments. The solid lines represent computer-fitted lines taken from Eq. 2 for A, B, and C, and Eq. 3 for D and E, except the line of 20 μM in E. For abbreviations, see the legend to Fig. 6.

converged to a point above the abscissa, showing competitive inhibition by GLA, TCA, and PBC. In Fig. 7D, the straight lines converged to a point on the abscissa, showing noncompetitive inhibition by BSP. The noncompetitive inhibition by ICG was observed only for low GLZ concentrations (5 and 10 μM) and the straight line at high GLZ concentration (20 μM) did not pass through the convergence point (Fig. 7E). The inhibition type was evaluated from the Akaike's information criteria values[20] listed in Table II, together with the inhibition constant (K_i) values.

Discussion

The hepatic uptake of organic anions (*e.g.* BSP, TCA, and rose bengal) by albumin-mediated transport has been reported by several groups.[10,21–24] In this study, the binding of GLZ to BSA was observed, because the sum of GLZ binding to BSA and GLZ adsorption on the cells in the incubation medium with 2% BSA was 53—98%, and GLZ adsorption on the cells in the medium without BSA was 2.8—7.6%. Therefore, the effect of BSA on GLZ uptake was examined. The total GLZ concentration in the medium with BSA was chosen such that the unbound GLZ concentration was similar to that in the case without BSA (Fig. 2). In both cases, the initial uptake rate with respect to the unbound GLZ concentration reflected the operation of both saturable and nonsaturable uptake processes (Fig. 2), and none of the parameters was significantly altered in the presence of BSA (Table I). This suggests that albumin does not participate to GLZ uptake, but that the unbound GLZ concentration determines the uptake rate.

The evidence of a saturable process suggests that the uptake of GLZ into hepatocytes is carrier-mediated. The nonsaturable process could be explained by passive diffusion with the participation of a very low affinity system. The unbound GLZ concentration at the rat plasma levels (< 1.1 mM) after 50 mg/kg i.v. dose is estimated to be smaller than 30 μM by using the unbound plasma fraction (0.006—0.026).[3] Further, from the kinetic parameters (Table I), the $V_{max} \cdot C_f/(K_m + C_f)$ value obtained is 17 times or more greater than the $K_d \cdot C_f$ value. It is suggested that a carrier-mediated transport system is the dominant process for the hepatic intake process of GLZ at plasma levels lower than 1.1 mM. Assuming 1.14×10^8 cells/g of liver,[25] the V_{max} in Table I gives a predicted value of 12.8 nmol/min/g of liver. This value is close to the $V_{max,B}$ value 12.0 nmol/min/g of liver,[3] suggesting that the saturable uptake of GLZ into liver is rate-limiting in biliary excretion. However, the $K_{m,B}$ value of 2.2 μM is about 5 times smaller than the K_m value (11.3 μM). Such a low affinity of GLZ to carrier on the isolated cells might be due to changes in membrane composition by the isolation. The Scatchard plot of the adsorption data reveals the existence of two types of binding sites, of which the one with specific affinity for GLZ has a dissociation constant (18.3 μM) of the same order of magnitude as the K_m value for the uptake process. As shown in Fig. 4, the reduction of the incubation temperature from 37 to 4°C decreased the initial uptake rate, with an activation energy of 15.9 kcal/mol, a value which is in the normal range for carrier-mediated transport.[26] An energy requirement for the transport of GLZ was also apparent from the significant decrease of the initial uptake rate by all metabolic inhibitors examined (rotenone, antimycin A, 2,4-dinitrophenol, and KCN) (Fig. 5).

As shown in Fig. 6, GLA, BSP, ICG, TCA, PBC, and PAH used as inhibitors for GLZ uptake have following characteristics. GLA is a major metabolite of GLZ[27] and after an i.p. dose of ^{3}H-GLA (25 mg/kg) to rats, all the

drug is excreted in the bile as metabolites.[28,29] The uptake of BSP,[5,6] ICG,[8] and TCA[4,7,9] into isolated rat hepatocytes is a carrier-mediated process, as described already. PBC is taken up into rat liver slices *via* the organic acid transport mechanism.[30] PAH is transported by a carrier-mediated transport system through the renal basolateral membrane[31,32] and is actively secreted into bile,[33] but GLZ uptake was not significantly inhibited by PAH. Kinetic analysis demonstrated a fully competitive inhibition of GLZ uptake by GLA, TCA, and PBC (Figs. 7A—C). This suggests a common carrier-mediated transport system for GLZ and these three drugs. Anwer and Hegner[7] showed that TCA is transported into isolated rat liver cells by both Na^+-independent and Na^+-dependent systems. The K_m value (57 μM) for the Na^+-independent transport process was of the same order of magnitude as the K_i value for TCA (Table II), suggesting that GLZ and TCA have a common Na^+-independent transport system. The Na^+-independent process for GLZ uptake was also indicated from the results that the uptake did not require the presence of Na^+ in the incubation medium and was not significantly inhibited by 1 mM ouabain. Boyer and Reno[34] showed that 1 mM ouabain in isolated rat liver cells completely blocks Na^+, K^+-ATPase activity. The affinity to the putative site was in the order of GLZ > GLA > TCA > PBC, as the values of K_m for GLZ and K_i for GLA, TCA, and PBC were 11.3, 13.7, 48.5, and 115.9 μM, respectively. Noncompetitive inhibition by BSP (Fig. 7D) suggests that the affinity site of the transport carrier for GLZ is different from that for BSP. It has been reported that the Dixon plot for the uptake of 5 μM BSP inhibited by ICG shows a straight line that deviated from those of 1 and 2 μM but converged at a point above the abscissa.[6] Such a pattern is similar to that of Fig. 7 E. It was found that the different inhibitions by ICG at high and low GLZ concentrations are similar to the patterns in the case of BSP uptake.

In conclusion, a carrier-mediated transport system participates in the uptake of GLZ into isolated rat hepatocytes and requires metabolic energy. The GLZ affinity site of the transport carrier in hepatocytes is common to GLA, TCA, and PBC, which act as competitive inhibitors of GLZ uptake. Further, it is suggested that the saturable biliary excretion rate may be attributed to a saturable transport of GLZ from plasma into liver.

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