The Conjugative Metabolism of 4-Methylumbelliferone and Deconjugation to the Parent Drug Examined by Isolated Perfused Liver and *in Vitro* Liver Homogenate of Rats

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We examined the disposition of 4-methylumbelliferone (4-MU) and its conjugative metabolites, glucuronide (4-MUG) and sulfate (4-MUS), using a single-pass rat liver perfusion system. When 4-MU was delivered, the steady-state hepatic extraction ratio for 4-MU was very high (approximately 1.0) and its conjugative metabolites, 4-MUG and 4-MUS, appeared to a large extent in the effluent perfusate. The biliary excretion rate of the 4-MUG conjugated from 4-MU was 44% of the infusion rate at the steady-state, whereas those of 4-MU and 4-MUS were less than 1% of the infusion rate. When 4-MUG was delivered, the steady-state hepatic extraction ratio for 4-MUG was very low (<0.05) and the removal rate of 4-MUG from the perfusate was almost identical to the excretion rate of 4-MUG into the bile, while 4-MU and 4-MUS were slightly excreted into the bile (1% of the total biliary excretion rate), suggesting that a little deconjugation of 4-MUG to 4-MU occurred in the liver. Similarly, 4-MU and 4-MUS were not detectable in the effluent perfusate. The apparent extraction ratio (E_{app}) for the intracellularly conjugated 4-MUG was approximately twenty times higher than that for the pre-conjugated 4-MUG. This discrepancy between the values of E_{app} for the intracellularly conjugated and pre-conjugated 4-MUG might be attributed mainly to the diffusional barrier for the metabolite between the blood and hepatocytes, as suggested in the previous simulation (*J. Pharmacokin. Biopharm.*, 15, 399 (1987)). On the other hand, when 4-MUS was delivered, 4-MUG appeared in the effluent perfusate. The biliary excretion rate of 4-MUG was approximately two times higher than that of 4-MUS (30% of the total biliary excretion rate), whereas the biliary excretion rate of 4-MU was negligible, suggesting that the deconjugation of 4-MUS to 4-MU occurred to a large extent in the liver. We also confirmed that the deconjugation of 4-MUG and 4-MUS occurred in the liver, using rat liver homogenate and its 9000 × *g* fraction (pH 7.4). On the assumption that the deconjugation was an apparent first-order process, the intrinsic clearance for the sulfatase was approximately eight times higher than that for glucuronidase, being consistent with the results of a single-pass perfusion.

Keywords 4-methylumbelliferone; diffusional barrier; metabolite; deconjugation; sulfatase; glucuronidase

Introduction

Conjugation with glucuronic acid and sulfate is an important pathway in the elimination and excretion of many xenobiotics, to which man is exposed by therapeutic administration or environment contact. The liver is a major site of sulfation and glucuronidation.[1-4] After synthesis, these conjugates may be excreted into the bile or may diffuse out into the plasma. Furthermore, it is known that the liver also has the ability of deconjugation, that is, the opposite reaction that converts the conjugates to their parent drugs. Aryl sulfatase[5-9] and glucuronidase,[10-13] which catalyze the hydrolysis of aryl sulfate ester and glucuronide, respectively have been detected in most animal tissues, such as the kidney, gastrointestinal tract and liver. These hydratases are located in the microsomes and lysosomes. When conjugated drugs have pharmacological and/or toxic effects, it is important to determine their kinetics. However, although the disposition of parent drugs has been extensively studied by the *in vivo* methods[14-16] and the isolated liver perfusion system,[17-19] little is known about the actual mechanisms of the disposition of conjugated drugs. The purpose of the present study was to clarify the determining factors for the disposition of intracellularly conjugated and pre-conjugated drugs.

4-Methylumbelliferone (4-MU), which has been used therapeutically as a cholagogoue in several European countries, is well known to be conjugated to glucuronide (4-MUG) and sulfate (4-MUS).[16,20,21] Recently, we demonstrated that the diffusional clearances of these conjugates between the blood and hepatocytes were much smaller than the hepatic blood flow, suggesting that the diffusional barrier for these conjugates may cause a discrepancy between the hepatic elimination of the intracellularly conjugated and pre-conjugated drugs.[22,23] In the present study, we examined the disposition of 4-MU and its conjugative metabolites (4-MUG and 4-MUS), using a single-pass perfused rat liver. To examine further the deconjugation of 4-MUG and 4-MUS, we investigated the *in vitro* enzymic kinetics of the deconjugation by using rat liver homogenate and its 9000 × *g* fraction.

Experimental

Materials 4-MU, 4-MUG and 4-MUS and bovine serum albumin (BSA) (fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO). Beta-glucuronidase/arylsulfatase was purchased from Boehringer Mannheim GmbH (Mannheim, West Germany).

Single-Pass Liver Perfusion Study Male Wistar rats (Nihon Ikagaku Doubutsu) weighing 250—300 g were maintained on a normal laboratory diet without fasting. Under light ether anesthesia, the bile duct was cannulated with PE-10 polyethylene tubing and the portal vein was rapidly catheterized with a cannula (2.0 mm o.d.), which was attached to the perfusion system. The portal circulation was interrupted within 10 s. The inferior *vena cava* was catheterized through the right atrium with PE-240 polyethylene tubing, then the inferior *vena cava* was ligated right above the renal vein. The liver was perfused *in situ* in a temperature controlled cabinet at 37 °C (single-pass system). The perfusate consisted of 20% (v/v) washed bovine erythrocytes in Krebs–Ringer bicarbonate buffer (pH 7.4) containing 100 mg/dl glucose and 3 g/dl BSA. The perfusion rate was maintained at 16 ml/min. After a stabilization period of 30 min, the perfusate without the drug was replaced by that containing 4-MU (15 and 35 μM) or 4-MUG (50 and 100 μM) or 4-MUS (50 and 100 μM). The perfusate samples were taken from the effluent at 30 min intervals, and centrifuged; the supernatant was kept frozen at −40 °C until assay. Bile was collected at 20 min intervals and was kept frozen at −40 °C until assay. Two viability tests were routinely done in every experiment and the experiments were considered acceptable when the following criteria were

fulfilled: 1) the bile flow rate was greater than 1 μl/min/g liver throughout the perfusion experiment; 2) the glutamic oxaloxacetic transaminase (S-GOT) in the effluent was less than 10 Karmen's units. The volume of bile was determined gravimetrically assuming its specific gravity to be 1.0. The S-GOT was determined in the effluent perfusate by using a GOT assay kit (GOT-UV Test), which was purchased from Wako Chemical Co.

Each perfusate or bile sample (20 μl) was applied to precoated thin layer chromatography (TLC) plate (Silicagel 60 F_{254}, with fluorescent indicator; 0.25 mm in thickness; E. Merck Darmstadt, West Germany). The TLC plate was developed in a mixture of acetone–chloroform–methanol–glacial acetic acid (12:12:1:1). 4-MU, 4-MUG and 4-MUS were visualized by ultraviolet irradiation. The *Rf* values of 4-MU, 4-MUG and 4-MUS were approximately 0.85, 0.03 and 0.40, respectively. The spots attributable to 4-MU were scraped off and extracted with 3 ml of 1.6 M glycine–NaOH buffer (pH 10.5), and those attributable to 4-MUG and 4-MUS were scraped off and extracted with 1 ml of 40 mM acetic acid–sodium acetate buffer (pH 5.0) for 1 h. 4-MUG and 4-MUS were then enzymatically converted to 4-MU by beta-glucuronidase (84 Fishman units)–arylsulfatase (670 Roy units) at 37 °C for 5 h. The concentration of 4-MU in the samples was fluorometrically determined at 450 nm (excitation at 390 nm) after dilution with 1.6 M glycine–NaOH buffer (pH 10.5) in a Hitachi MPF-4 spectrofluorometer. The recoveries of 4-MU, 4-MUG and 4-MUS from perfusate were 0.83 ± 0.03, 0.87 ± 0.04 and 0.95 ± 0.04, respectively, and those from bile were 0.73 ± 0.01, 0.67 ± 0.01 and 0.92 ± 0.03, respectively. The recoveries from perfusate and bile were linear up to 200 μM and 5 mM, respectively. The assay was performed under conditions such that the linearity of recovery held. The correlation coefficient of the calibration curve was more than 0.90. The limit of determination in the perfusate and bile was 150 nM. Since the sample in the effluent perfusate was diluted, especially in the case of low infusion of 4-MUS (650 nmól/min), 150 μl of the effluent perfusate sample was concentrated by freeze-drying and redissolved in 50 μl of distilled water. In this case, the limit of determination was 30 nM. The rates out into the effluent perfusate and bile were normalized with respect to the infusion rate. We defined the apparent extraction ratio (E_{app}), according to the following equation.

$$E_{app}=\frac{\text{(rate out in the bile)}}{\text{(rate out in the bile)}+\text{(rate out in the effluent perfusate)}} \quad (1)$$

where the denominator represents the formation rate of the metabolite, in the absence of deconjugation. This parameter represents the true extraction ratio for the intracellularly conjugated 4-MUG and 4-MUS only when deconjugation of these conjugates to 4-MU does not occur in the hepatocytes.

After a perfusion study, the portal vein was rapidly cut off and the whole liver tissue was excised. Immediately after the excision, the whole tissue was frozen in a bath of acetone cooled to −70 °C with dry ice and kept at −40 °C until assay. The amounts of drugs (4-MU, 4-MUG and 4-MUS) in the liver at the steady-state were fluorometrically determined by using thin layer chromatography as described in detail previously.[23] The recoveries of 4-MU, 4-MUG and 4-MUS from the liver homogenate were 0.80 ± 0.03, 0.85 ± 0.04 and 0.94 ± 0.04, respectively. Linearity of recovery from the homogenate was observed up to 200 μM. The limit of determination of molecules in the liver was 200 nM.

***In Vitro* Deconjugation Studies** Rat livers (50 g) were partially thawed, cut into small pieces and rapidly homogenized (30% w/v) in a motor-driven Potter homogenizer with 25 mM Tris–HCl buffer (pH 7.5), containing 0.3 M NaCl. A part of the homogenate was centrifuged at 1000 × *g* at 4 °C for 15 min and the precipitate was discarded. The supernatant fluid was recentrifuged at 9000 × *g* at 4 °C for 80 min and the precipitate was discarded. The supernatant fluid was designated as 9000 × *g* fraction preparation. To minimize the reconjugation of the liberated 4-MU, the 9000 × *g* fraction and liver homogenate (30% w/v) preparations (volume: 30 ml) were dialyzed against the enzymic assay buffer (volume: 5 l) (described below) for 48 h at 4 °C using SPECTRA/POR (Spectrum Medical Ind.), since this treatment depletes the co-factors (PAPS and UDPGA) for sulfo- and glucuronyl-transferases, respectively. The volumes of homogenate and 9000 × *g* fraction increased by approximately 20% during dialysis.

The enzyme kinetics of deconjugation were examined as follows: 50 μl of homogenate or 9000 × *g* fraction was added to 0.5 ml of enzyme assay solution incubated at 37 °C, which consists of 100 mM Tris–HCl, 225 mM NaCl and drugs of various concentrations (4-MUG: 1—1500 μM, 4-MUS: 1—3000 μM). After incubating at 37 °C for 15 min, then the reaction was stopped by the addition of 3 ml of 1.6 N glycine–NaOH buffer (pH 10.5) and the amount of liberated 4-MU was determined fluorometrically as described above (see "Single-pass perfusion study"). The sulfatase and glucuronidase activities were linear with time (up to 15 min) and proportional to the amount of the added protein in the range of 0.05—0.20 mg protein. Similarly, the pH of enzyme assay solution (4-MUG and 4-MUS: 50 μM) was varied from 6.5 to 7.5, and the pH dependency of the activity of deconjugation was also examined.

The enzymic kinetic data were analyzed by means of Eadie–Hofstee plots.[24] The Eadie–Hofstee plot for sulfatase was linear, while that for glucuronidase bent toward the horizontal axis as the deconjugation rate of the conjugative metabolite increased. Consequently, we fitted these data to the following Michaelis–Menten equations, using the nonlinear least-squares method.

$$\text{sulfatase:}\quad v_0=\frac{V_{max}(s)}{K_m+(s)} \quad (2)$$

$$\text{glucuronidase:}\quad v_0=\frac{V_{max,1}(s)}{K_{m,1}+(s)}+\frac{V_{max,2}(s)}{K_{m,2}+(s)} \quad (3)$$

where v_0 and (s) represent the initial velocity of hydratase and the substrate concentration, respectively. $V_{max,i}$ and $K_{m,i}$ represent the maximum velocity and Michaelis constant of the i-th component, respectively.

Tissue Binding of Drugs of Liver Homogenate Livers obtained from three rats were homogenized (50% w/v) in Krebs–Ringer bicarbonate buffer (pH 7.4) and the homogenate (volume: 30 ml) was dialyzed against the same bicarbonate buffer (volume: 5 l) for 24 h at 4 °C (see "*In vitro* deconjugation studies"). Various amounts of drug (final concentration: 86—760 μM) were added to the dialyzed homogenate, and the mixture was incubated for 10 min at room temperature. Subsequently, the homogenate was ultrafiltered by centrifugation for 10 min at 1000 × *g* using an MPS-1 type micropartition system (Amicon Co., Lexington, MA). The concentrations of drugs (4-MU, 4-MUG and 4-MUS) in the filtrate were determined fluorometrically as described above. Furthermore, to determine whether drug metabolism might occur during the filtration, we added 0.5 ml of the residue to 2 ml of methanol and determined the concentrations of drugs individually, as described above (see "Single-pass perfusion study").

Results

Tissue Binding of Drugs to Liver Homogenate Unbound fractions of drugs with 50% liver homogenate and those extrapolated to the whole liver tissue are summarized in Table I. Little concentration dependence (86—760 μM) was observed in the 4-MU or 4-MUG binding to 50% liver homogenate. The mean unbound fractions of 4-MU and 4-MUG corrected for the whole liver were 0.0989 ± 0.0069 and 0.369 ± 0.007, respectively. On the other hand, we could not accurately determine the unbound fraction of 4-MUS because of metabolism of 4-MUS (deconjugation to 4-MU).

Table I. Tissue Binding of Drugs

Drug	Unbound fraction measured using 50% liver homogenate	Unbound fraction corrected[a] for the whole liver tissue
4-MU	0.180 (0.014)[b]	0.0989
4-MUG	0.546 (0.011)[b]	0.369
4-MUS	—[c]	—[c]

a) In the linear range, the unbound fraction (f_T) is given by $f_T=\frac{1}{1+(nP/K_d)}$ where nP and K_d represent the binding site concentration and dissociation constant, respectively. According to this equation, the ratio of nP/K_d was determined in the 50% liver homogenate. Subsequently, this value was multiplied by two, and substituted into the equation. We thus calculated the unbound fraction corrected for the whole liver tissue. *b*) The values in parentheses represent standard error ($n=5$). *c*) The f_T value could not be determined because of metabolism.

TABLE II. The Disposition of 4-MU in Single-Pass Liver Preparation at Steady State and under Constant Hepatic Blood Flow Rate (16 ml/min)

	Infusion rate (nmol/min)					
	High 560			Low 240		
	4-MU	4-MUG	4-MUS	4-MU	4-MUG	4-MUS
Rate out in the perfusate[a]	u.d.[b]	0.298 (0.027)	0.308 (0.017)	u.d.[b]	0.250 (0.053)	0.411[d] (0.016)
Rate out in the bile[a]	0.0007 (0.0001)	0.376 (0.016)	0.0091 (0.0022)	0.0018[d] (0.0005)	0.302[d] (0.012)	0.0256[d] (0.0054)
E_{app}[c]	1.0	0.560 (0.030)	0.0029 (0.0007)	1.0	0.563 (0.068)	0.0059 (0.0012)

a) The values are normalized with respect to the infusion rate. *b*) Undetectable under the present determination conditions (the concentration is less than 150 nM). *c*) E_{app} represents the apparent extraction ratio. The value of E_{app} is calculated by dividing the rate out of a ligand in the bile by the sum of the rates out in the effluent perfusate and bile (Eq. 1). *d*) $p<0.05$. The values in parentheses represent standard error ($n=3$).

TABLE III. The Disposition of 4-MUG in Single-Pass Liver Preparation at Steady State and under Constant Hepatic Blood Flow Rate (16 ml/min)

	Infusion rate (nmol/min)					
	High 1680			Low 710		
	4-MU	4-MUG	4-MUS	4-MU	4-MUG	4-MUS
Rate out in the perfusate[a]	u.d.[b]	0.965 (0.058)	u.d.[b]	u.d.[b]	0.942 (0.020)	u.d.[b]
Rate out in the bile[a]	0.0002 (0.0001)	0.0284 (0.0030)	0.0004 (0.0001)	0.0002 (0.0001)	0.0045 (0.0093)	0.0005 (0.0002)
E_{app}[c]	unable[d]	0.0284 (0.0030)	unable[d]	unable[d]	0.0445 (0.0093)	unable[d]

a) The values are normalized with respect to the infusion rate. *b*) Undetectable under the present determination condition (the concentration is less than 150 nM). *c*) E_{app} represents the apparent extraction ratio. The value of E_{app} was calculated by dividing the rate out of a ligand in the bile by the sum of the rates out in the effluent perfusate and bile (Eq. 1). *d*) We were unable to calculate the parameter E_{app}, since the rate out in the effluent perfusate was undetectable (below the limit of determination). *e*) $p<0.05$. The values in parentheses represent standard error ($n=3$).

Single-Pass Liver Perfusion Judging from the absence of change in the rates out into the effluent perfusate and bile flow, we found that a steady-state condition was obtained within 120 and 60 min after infusion of 4-MU and the conjugates (4-MUG, 4-MUS), respectively. The rates out of 4-MU, 4-MUG and 4-MUS into the effluent perfusate and bile were determined under steady-state conditions, and these values are summarized in Tables II, III and IV. When 4-MU was delivered, the steady-state hepatic extraction ratio for 4-MU was very high (approximately 1.0) and its conjugative metabolites, 4-MUG and 4-MUS, appeared to a large extent in the effluent perfusate (Table II). The biliary excretion rate of 4-MUG conjugated from 4-MU was approximately 30—38% of the infusion rate, whereas those of 4-MU and 4-MUS were less that 3% of the infusion rate. A slight dose-dependence was observed in the rate out into the bile. By contrast, little dose-dependence was observed in the rate out into the effluent perfusate except for that of 4-MUS. On the other hand, when 4-MUG was delivered, the steady-state hepatic extraction ratio for 4-MUG was very low (less than 5%) and the extent of biliary excretion rate

TABLE IV. The Disposition of 4-MUS in Single-Pass Liver Preparation at Steady State and under Constant Hepatic Blood Flow Rate (16 ml/min)

	Infusion rate (nmol/min)					
	High 1400			Low 650		
	4-MU	4-MUG	4-MUS	4-MU	4-MUG	4-MUS
Rate out in the perfusate[a]	u.d.[b]	u.d.[b]	0.972 (0.056)	u.d.[b]	0.048[c] (0.008)	0.946 (0.039)
Rate out in the bile[a]	0.0001 (0.00001)	0.017 (0.001)	0.0040 (0.0007)	0.0002 (0.0001)	0.0245[f] (0.0018)	0.0138[f] (0.0020)
E_{app}[d]	unable[e]	unable[e]	0.0041 (0.0007)	unable[e]	0.338 (0.009)	0.014[f] (0.002)

a) The values are normalized with respect to the infusion rate. *b*) Undetectable under the present determination condition (the concentration is less than 150 nM). *c*) Since the sample was diluted, the low-dose effluent perfusate sample was concentrated. However, we did not perform concentration of the sample at the high dose. *d*) E_{app} represents extraction ratio. The value of E_{app} was calculated by dividing the rate out of a ligand in the bile by the sum of the rates out in the effluent perfusate and bile (Eq. 1). *e*) We were unable to calculate the parameter of E_{app}, since the rate out in the effluent perfusate was undetectable (below the limit of determination). *f*) $p<0.05$. The values in the parentheses represent standard error ($n=3$).

for 4-MUG was more than 99% of the total biliary excretion rate, whereas the rates out of 4-MU and 4-MUS into the bile were less than approximately 1%. A slight dose-dependence was observed in the rate out of 4-MUG into the bile. However, the difference of the rates out between at the low- and high-dose infusions was not significant. In contrast, when 4-MUS was delivered, 4-MUG appeared in the effluent perfusate and the rate out of 4-MUG into the bile was approximately 2—4 times higher than that of 4-MUS. Furthermore, in the case of the low-dose infusion of 4-MUS, we concentrated the effluent samples by freeze-drying. We could detect 4-MUG in the effluent perfusate. These results suggested that deconjugation from 4-MUS to 4-MU occurred to a large extent in the liver. A slight dose-dependence was observed in the rates out of 4-MUS into the bile.

The biliary excretion clearance (CL_{bile}) and the drug concentration in the liver at the steady-state are summarized in Table V. CL_{bile} was calculated by dividing the biliary excretion rate by the steady-state concentration in the liver. When 4-MU was delivered, the liver concentration of 4-MUG was much higher than those of 4-MU and 4-MUS. When 4-MUS was delivered, the concentrations of 4-MU and 4-MUG were higher than that of 4-MUS, suggesting that deconjugation from 4-MUS to 4-MU in the liver occurred to a large extent. In contrast, when 4-MUG was delivered, only 4-MUG was detected in the liver, indicating that little deconjugation from 4-MUG to 4-MU occurred in the liver. The biliary clearance for the unbound ligand ($CL_{bile,free}$) was obtained by correcting for the tissue unbound fraction. The value of $CL_{bile,free}$ for 4-MUG was the highest of all (mean: 5.25 ml/min), whereas the value of $CL_{bile,free}$ for 4-MU was the lowest of all (mean: 0.072 ml/min), although the values of $CL_{bile,free}$ were different between infusions.

***In Vitro* Deconjugation Study** Figures 1 and 2 show Eadie–Hofstee plots of sulfatase and glucuronidase in the liver homogenate preparation, respectively. The Eadie–Hofstee plot of sulfatase was linear (Fig. 1), whereas that of

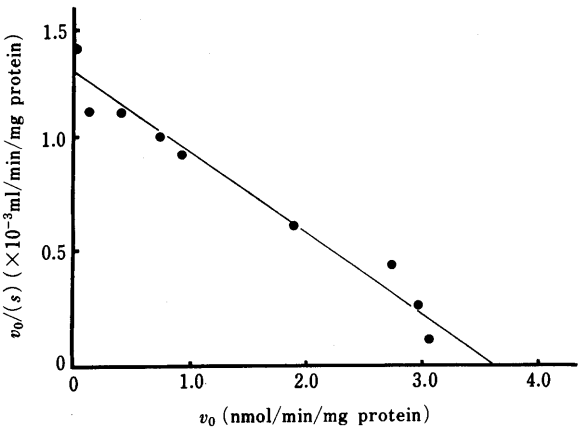

Fig. 1. Eadie–Hofstee Plot of Sulfatase (4-MUS Deconjugation) in Rat Liver Homogenate

Incubation mixtures contained substrates at various concentrations (4-MUS: 1—3000 μM) in 0.5 ml of Tris–HCl buffer (pH 7.4) and enzyme equivalent to 0.10 mg of protein. Samples were incubated for 15 min. The v_0 and $v_0/(s)$ represent the initial velocity of sulfatase and the initial velocity of sulfatase divided by the substrate concentration, respectively. The data were fitted to a one-term Michaelis–Menten equation (Eq. 2) by a nonlinear least-squares method ($K_m = 282\,\mu$M, $V_{max} = 3.6$ nmol/min/mg protein). The data are averages of three experiments.

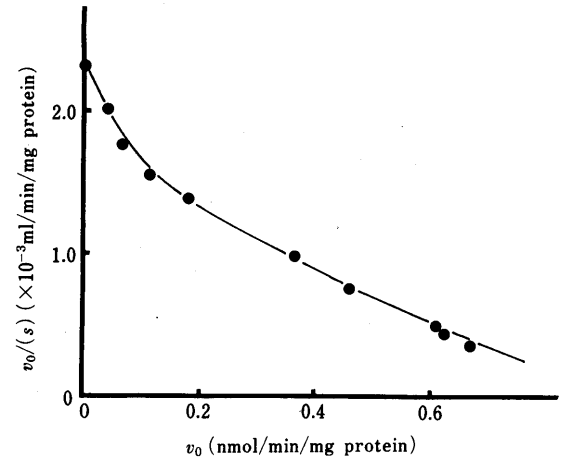

Fig. 2. Eadie–Hofstee Plot of Glucuronidase (4-MUG Deconjugation) in Rat Liver Homogenate

Incubation mixtures contained substrate at various concentrations (4-MUG: 1—1500 μM) in 0.5 ml of Tris–HCl buffer (pH 7.4) and enzyme equivalent to 0.10 mg of protein. Samples were incubated for 15 min. The v_0 and $v_0/(s)$ represent the initial velocity of glucuronidase and the initial velocity of glucuronidase divided by the substrate concentration, respectively. The data were fitted to a two-term Michaelis–Menten equation (Eq. 3) by a nonlinear least-squares method ($K_{m,1} = 6.26\,\mu$M, $K_{m,2} = 517\,\mu$M, $V_{max} = 0.00936$, $V_{max,2} = 0.84$ nmol/min/mg protein). The data are averages of three experiments.

glucuronidase bent toward the horizontal axis (Fig. 2). We could obtain similar results in the $9000 \times g$ fraction (data not shown). Consequently, sulfatase and glucuronidase were fitted to one-term and two-term Michaelis–Menten equations (Eqs. 2 and 3, respectively) as described in Experimental, using a nonlinear least-squares method. The enzymic activity of deconjugation was designated as the sum of the ratio of $V_{max,i}$ to $K_{m,i}$ ($\sum_i V_{max,i}/K_{m,i}$). The enzymic activity of sulfatase was approximately eight times higher than that of glucuronidase. We also determined the pH-dependence of sulfatase and glucuronidase activities (Fig. 3). It was observed that two hydratases have pH-dependent activities, that is, the enzymic activities increase with decrease of pH from 7.4 to 6.6. When the pH was 7.4,

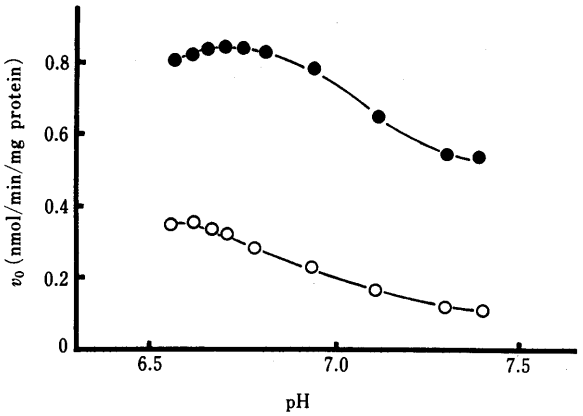

Fig. 3. pH-Dependence of Sulfatase and Glucuronidase Activities

Incubation mixtures contained constant substrate concentration (4-MUG and 4-MUS: 10 μM) in 0.5 ml of Tris–HCl buffer (pH 6.5—7.4) and enzyme equivalent to 0.10 mg of protein. Samples were incubated for 15 min. The data are averages of three experiments. ●, sulfatase activity; ○, glucuronidase activity.

the sulfatase activity was much higher than the glucuronidase activity. In contrast, when the pH was decreased to 6.7 (acidification), the sulfatase activity was approximately two-fold higher than the glucuronidase activity.

Discussion

The kinetics of intracellularly conjugated and pre-conjugated drugs are determined by various factors; 1) the diffusional clearances for the parent and the conjugated drugs between the blood and hepatocytes, 2) the distribution of the enzymic activities for the sequential metabolism and/or biliary excretion along the hepatic blood flow path and 3) the contributions of conjugation and deconjugation.

Recently, by uisng the centrifugal filtration method with isolated hepatocytes, it was demonstrated that the permeabilities of hepatocytes to 4-MUG and 4-MUS between the blood and hepatocytes were very small in relation to the hepatic blood flow,[23] suggesting that the diffusional barrier for these conjugates may cause a discrepancy between the hepatic elimination (biliary excretion) of intracellularly conjugated and pre-conjugated metabolites.[22] Indeed, as shown in Tables II and III, we found that the apparent extraction ratio (E_{app}) for the conjugated 4-MUG (Table II) was 20 times higher than that for the pre-conjugated 4-MUG (Table III), although a little deconjugation of 4-MUG to 4-MU occurred. This phenomenon may be explained as follows in terms of the two opposite effects of the diffusional barrier to 4-MUG on its kinetics. One effect is that the diffusional barrier hampers the efflux of intracellularly conjugated 4-MUG into the circulation, leading to an effective biliary excretion of conjugated 4-MUG. The other effect is that the diffusional barrier decelerates the influx of pre-conjugated 4-MUG into the hepatocytes, leading to little extraction to pre-conjugated 4-MUG. Consequently, there was a large difference of the removal between conjugated and pre-conjugated 4-MUG. However, one should be cautious in giving a quantitative interpretation to the elimination of conjugated and pre-conjugated 4-MUG, since deconjugation of 4-MUG to 4-MU occurred in the hepatocytes to a slight extent. In contrast, there was little discrepancy between the conjugated and pre-conjugated

TABLE V. Biliary Clearances and Concentrations of Drugs in the Liver at the Steady-State

Infusion rate (nmol/min)	Biliary excretion (nmol/min)			Liver concentration (μM)			Biliary clearance[a] (CL_{bile}) (ml/min)			Biliary clearance for unbound[b] ligand ($CL_{bile,free}$) (ml/min)		
	4-MU	4-MUG	4-MUS	4-MU	4-MUG	4-MUS	4-MU	4-MUG	4-MUS	4-MU	4-MUG	4-MUS
4-MU	0.50	245	5.7	49	117	9.8	0.010	2.31	0.610	0.100	6.26	u.c.[c]
560	(0.03)	(10)	(1.3)	(6)	(20)	(1.2)	(0.002)	(0.55)	(0.143)	(0.030)	(1.22)	
4-MUG	0.12	34	0.35	u.d.[d]	20	u.d.[d]	—	1.57	—	—	4.25	—
710	(0.07)	(7)	(0.15)		(1)			(0.28)			(0.53)	
4-MUS	0.16	16	8.80	37	21	9.9	0.0044	0.804	0.948	0.044	2.17	u.c.[c]
650	(0.09)	(1)	(1.26)	(1)	(3)	(1.2)	(0.0024)	(0.169)	(0.190)	(0.015)	(0.43)	

a) CL_{bile} was calculated by dividing the biliary excretion rate by the steady-state concentration in the liver. *b*) $CL_{bile,free}$ was calculated by dividing the value of CL_{bile} by the unbound fraction in the liver tissue. *c*) The unbound fraction of 4-MUS with liver homogenate (50%) could not be obtained because of the metabolism of 4-MUS. Consequently, the biliary clearance for the unbound ligand could not be calculated. *d*) Undetectable under the present determination conditions (the concentration is less than 200 nM). The values in parentheses represent the standard error ($n=3$).

4-MUS in biliary excretion, in spite of the existence of the diffusional barrier for 4-MUS between the blood and hepatocytes.[23] As shown in Tables IV and V, the deconjugation of 4-MUS to 4-MU occurred to a large extent. The deconjugation process of 4-MUS competed with the sequential biliary excretion process and decreased the intracellular concentration of 4-MUS, which can explain why there was little discrepancy between the biliary excretions of conjugated and pre-conjugated 4-MUS, in contrast to the case of 4-MUG.

To clarify whether the deconjugations of glucuronide and sulfate actually occurred in the liver, we determined the activities of sulfatase and glucuronidase, using the rat liver homogenate and $9000 \times g$ fraction. It was found that the sulfatase reaction was within the linear range in the perfused experiment, since the dissociation constant (K_m) for the sulfatase was much higher than the intracellular concentration of 4-MUS at the steady-state (at most, approximately 10 μM (Table V)). Similarly, the glucuronidase reaction was found to be almost linear, since the K_m value for high-capacity glucuronidase was also much higher than the intracellular concentration of 4-MUG at the steady-state (at most, approximately 100 μM (Table V)), although the low-capacity site was saturated. The enzymic activity ($\sum_i V_{max,i}/K_{m,i}$) of sulfatase was approximately eight times higher than that of glucuronidase. This is consistent with the results of the isolated liver perfusion experiments; deconjugation from 4-MUS to 4-MU in the liver occurred to a large extent, while the deconjugation from 4-MUG to 4-MU was slight.

Very recently, Thurman *et al.*[25] also demonstrated the deconjugation of 4-MUS using the isolated perfused rat liver. In their experiment, sulfate ion was removed from the perfusate so as to accelerate the deconjugation of 4-MUS to 4-MU. In fact, they found remarkable deconjugation of 4-MUS, and the rates out of 4-MU and 4-MUG in the effluent perfusate were 0.09 and 0.01 (normalized with respect to the infusion rate: 11 μmol/min), respectively. In contrast, in the present study, the perfusate contains sulfate ion and probably the net deconjugation process of 4-MUS might be smaller than that in their study, since the 4-MU liberated from 4-MUS underwent reconjugation in our study. In our study, the rates out of 4-MU and 4-MUG were 0.05 and less than 0.001 (normalized with respect to the infusion rate: 650 nmol/min), respectively. Comparing our study with their study, the rate out of 4-MUG in the effluent perfusate in their study was two times higher than that in our study, due to the removal of sulfate ion from perfusate. In our study, 4-MU coud not be detected below the limit of determination, since the infusion rate was much smaller than that in their experiment and the perfusate contained sulfate ion. Consequently, the discrepancy of results between our and their experiments might be caused by the difference of experimental conditions.

TABLE VI. Kinetic Parameters (K_m and V_{max}) for Homogenate and $9000 \times g$ Fraction

Preparation	Sulfatase		Glucuronidase			
	K_m (μM)	V_{max} (nmol/min/g protein)	$K_{m,1}$[a] (μM)	$K_{m,2}$[a] (μM)	$V_{max,1}$[b] (nmol/min/g protein)	$V_{max,2}$[b] (nmol/min/g protein)
Homogenate	282	3.60	6.26	517	0.00936	0.84
	(14)	(0.18)	(0.19)	(10)	(0.00028)	(0.02)
$9000 \times g$	309	2.73	1.84	558	0.00892	0.505
fraction	(15)	(0.14)	(0.06)	(22)	(0.00027)	(0.020)

a) $K_{m,1}$ and $K_{m,2}$ represent the Michaelis constants of the primary and secondary components, respectively. *b*) $V_{max,1}$ and $V_{max,2}$ represent the maximum velocities of the primary and secondary components, respectively. The values in the parentheses represent standard error ($n=3$).

Furthermore, we determined the optimum pH (approximately pH 6.6), and these two enzymic activities increased remarkably as the pH decreased from 7.4 to 6.5. When intracellular pH is within the normal range (pH 7.0—7.2), the enzymic activity for glucuronidase was much lower than that for sulfatase. Therefore, the extent of glucuronidation might be larger than that of sulfation under normal conditions, since the sulfatase hampers the net conjugation process. In fact, when 4-MU was delivered, the intracellular concentration of 4-MUG was more than 60% of the total molecules in the liver. In contrast, when the pH was decreased, the glucuronidase activity was a half of the sulfatase activity and both enzymic activities were increased. Under these conditions, the increase of deconjugation activities might cause the decrease of elimination of 4-MU and the increase of the ratio of sulfation to glucuronidation. From the viewpoint of elimination of 4-MU, the deconjugation might cause underestimation of the sulfo- and glucuronyl-transferase activities determined with isolated perfused liver or whole animal. Therefore, we

should be cautious about quantitative interpretation of the pharmacokinetic data of 4-MU.

From the steady-state parameters (bile excretion rates and liver concentrations of ligands at the steady state), we also obtained the biliary excretion clearance for an unbound ligand ($CL_{bile,free}$). The value of $CL_{bile,free}$ for 4-MUG was much higher than that for 4-MU, although the absolute values were a little different between each infusion (4-MU, 4-MUG and 4-MUS infusions). In the case of 4-MUS, the biliary clearance (CL_{bile}) of 4-MUS was also much higher than that of 4-MU. Although we could not obtain the exact value of $CL_{bile,free}$ for 4-MUS on account of its remarkable deconjugation, the value of $CL_{bile,free}$ for 4-MUS might be much larger than that for 4-MU. In general, the conjugated drug, which is more hydrophilic, is excreted into the bile to a larger extent.[26] It seems that the biliary excretion of 4-MU and its metabolites may follow this rule.

In conclusion, we demonstrated that the biliary excretion of intracellularly conjugated 4-MUG was much higher than that of pre-conjugated 4-MUG, due to the diffusional barrier between the blood and hepatocytes. In contrast, the discrepancy of the biliary excretion between the intracellularly conjugated and pre-conjugated 4-MUS was minimal, although a diffusional barrier also exists for 4-MUS.[23] This may result from the extensive enzymic activities which convert 4-MUS to 4-MU. We also determined that the biliary clearance of the conjugates (4-MUG and 4-MUS) was much higher than that of the parent drug (4-MU).

References and Notes

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