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In Vivo and *In Vitro* Fates of 8-Hydroxyquinoline Derivatives in Rat[1]

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The *in vivo* and *in vitro* fates of 8-hydroxyquinoline (8-OH), 5-chloro-8-hydroxyquinoline (MC), 5,7-dichloro-8-hydroxyquinoline (DC), and 5-chloro-8-hydroxy-7-iodoquinoline (CF) in rat was studied. The results obtained were as follows. (1) These compounds were metabolized to glucuronides and sulfates after intravenous administration. Glucuronides were excreted in bile and urine, but sulfates were excreted exclusively in urine. Unmetabolized forms were almost not excreted. (2) Glucuronides were more excreted in bile as the molecular weights increased by halogen substituents. And glucuronides having the higher affinities to plasma protein and 15% liver 100000 *g* supernatant fraction were more excreted in bile. (3) Although sulfates were well hydrolyzed as glucuronides in the body, sulfates (especially, CF sulfate) resisted enzymatic hydrolysis *in vitro* by mitochondria-lysosomal and microsomal enzymes in both liver and kidney in contrast to susceptibilities of glucuronides to them. (4) The conjugates were hydrolyzed by Cu^{2+}, Fe^{3+}, Zn^{2+}, and Mg^{2+}. Especially in the presence of Cu^{2+} or Fe^{3+}, DC sulfate and CF sulfate which were extremely stable in the enzymatic hydrolysis were unstable. Accordingly, the effects of the metal ions as well as the hydrolytic enzymes were suggested for the hydrolysis of the conjugates in the body.

Keywords——8-hydroxyquinoline; iodochlorhydroxyquin; 5-chloro-8-hydroxyquinoline; 5,7-dichloro-8-hydroxyquinoline; substituent effect on metabolism; metal ion effect on hydrolysis; glucuronide; sulfate

The absorption and metabolism of 5-chloro-8-hydroxy-7-iodoquinoline (Chinoform, Iodochlorhydroxyquin: CF),[3] which has been remarked to induce SMON (subacute myelo-optico-neuropathy) in Japan, were studied in rat by Hayashi, *et al.*[4] And further, the metabolism of its parent compound, *i.e.*, 8-hydroxyquinoline (oxine: 8-OH) in rat was also studied pharmacokinetically by Kiwada, *et al.*[5] From these studies, it was found that CFG was more excretable in bile than 8-OHG and that CFG and CFS were hydrolyzed and reconjugated *in vivo*, while the conjugates of 8-OH were not hydrolyzed. In this paper, the metabolism of MC and DC was studied in rat to discuss halogen substitution effect on *in vivo* and *in vitro* behaviors. And further, some factors which produced the hydrolysis *in vivo* of CFG and CFS were studied, since the hydrolysis of conjugates *in vivo* is scarcely reported and such biological instability of the detoxicated form of CF was considered to have some relation to SMON occurrence.

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2) Location: *Hongo, Bunkyo-ku, Tokyo, 113, Japan.*
3) The following abbreviations were used for 8-hydroxyquinoline derivatives and their conjugates: CF (chinoform), CFG (CF-glucuronide), CFS (CF-sulfate), DC (5,7-dichloro-8-hydroxyquinoline), DCG (DC-glucuronide), DCS (DC-sulfate), MC (5-chloro-8-hydroxyquinoline), MCG (MC-glucuronide), MCS (MC-sulfate), 8-OH (8-hydroxyquinoline), 8-OHG (8-OH-glucuronide), 8-OHS (8-OH-sulfate).
4) *a*) M. Hayashi, T. Fuwa, S. Awazu, and M. Hanano, *Chem. Pharm. Bull.* (Tokyo), **24**, 2589 (1976); *b*) M. Hayashi, T. Fuwa, S. Awazu, and M. Hanano, *ibid.*, **24**, 2603 (1976).
5) H. Kiwada, M. Hayashi, T. Fuwa, S. Awazu, and M. Hanano, *Chem. Pharm. Bull.* (Tokyo), **25**, 1566 (1977).

Experimental

Material——a) 8-OHG: It was isolated from urine of rabbits to which 8-OH had been given orally by the method roported previously.[5]

b) MCG, DCG, and CFG: They were all synthesized by the method of Matsunaga, *et al.*[6] MCG was obtained as the sodium salt and the others as the free acid forms.

c) 8-OHS: It was synthesized by the method of Czapet,[7] and obtained as the potassium salt.

d) MCS, DCS and CFS: They were synthesized by the method of Chen, *et al.*[8] and obtained as the sodium salts.

Procedures——a) Sample Collection: The male albino rat of Donryu (260—280 g) to which two polyethylene cannulae had been set into bladder and bile duct respectively was fasted overnight in a restraint cage. The following morning, 3 mg of MC or its conjugates (3 mg equivalent to MC), or 3 mg of DC or its conjugates (3 mg equivalent to DC) was administered intravenously to the rat. The intravenous dosage forms were aqueous solution for MCG, MCS and DCS, 0.0467 M $NaHCO_3$ for DCG, 0.1 N HCl for MC and 0.1 N NaOH for DC. During experiment, rat was kept in a restraint cage allowed to take only water *ad libitum*. Urine and bile samples were collected at an appropriate time interval until 8 hr through polyethylene cannulae set into bladder and bile duct, respectively.

b) Determination of Conjugates: Immediately after collection, urine and bile samples were adjusted to 10 ml with distilled water. They were heated for 2 min in boiling water to inactivate native enzymes. Then to 2 ml aliquot of the diluted samples was added 5 ml of ethylene dichloride (EDC), shaken for 15 min and centrifuged for 15 min at 3000 rpm. EDC layer was used for free (unconjugated) form determination as described later, and aqueous layer was treated as the following to determine conjugates. To 1 ml aliquot of aqueous layer was added 1 ml of acetate buffer (0.2 M CH_3COOH–0.2 M CH_3COONa, pH 4.5) containing 740 Fishman units of β-glucuronidase (Boehringer Mannheim, catalogue number 15472). The mixture was incubated at 38° for 2 hr to hydrolyze glucuronide completely. Then 5 ml of EDC was added to the mixture, shaken for 15 min and centrifuged for 15 min at 3000 rpm. Glucuronide was determined as free form from EDC layer. To 1 ml aliquot of the residual buffer layer was added 0.2 ml of 6 N HCl and incubated for 40 hr at 38° to hydrolyze sulfate. Sulfate was determined as liberated free form after extracted in EDC as above. To determine free form extracted in EDC, 4 ml of 1 N NaOH was added to 4 ml of the EDC layer, shaken for 15 min and centrifuged for 15 min at 3000 rpm. MC and DC extracted in NaOH layer was determined with Hitachi 124 Spectrophotometer at 255 and 258 nm, respectively.

c) Determination of β-Glucuronidase and Arylsulfatase Activity in Rat. i) Liver and Kidney Fractionation: Five rats (280 g) were fasted for 16 hr and sacrificed by depletion of blood from carotid artery. Their livers were perfused through portal vein with cold saline solution to remove residual blood and rapidly excised.[9] Fifteen g of chopped liver pieces were mixed with 85 ml of ice-cold 0.05 M tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA, and homogenized by five strokes in a teflon homogenizer (Takashima Shoten, Tokyo). The cell debris and nuclei were removed by centrifugation at 700 ***g*** for 10 min with refrigerated centrifuge (Kubota KR-6P). The supernatant was successively centrifuged at 12000 ***g*** for 20 min by the refrigerated centrifuge and then, at 105000 ***g*** for 60 min with ultracentrifuge (Hitachi 65P) to isolate mitochondria-lysosomal fraction and microsomal fraction, respectively. Kidney was treated in the same manner with liver, except that cell and nuclei were removed by centrifugation at 650 ***g*** for 2 min and the supernatant was centrifuged at 10000 ***g*** for 20 min.[10] Both of the mitochondria-lysosomal and microsomal fractions were resuspended in 32 ml of ice-cold 0.25 M sucrose solution.

ii) Measurements of Enzymatic Activity: β-Glucuronidase activity was measured using 8-OHG, MCG, DCG and CFG as substrate. A half ml of the resuspended solution, 1.0 ml of 40 mM acetate buffer (pH 4.5) containing 0.25 M sucrose and 1.0 ml of aqueous suspension of substrate (28—143 μM at the final concentration) were incubated at 38° for 5 min. Arylsulfatase activity was measured using 8-OHS, MCS, DCS, CFS as substrate. A half ml of the resuspended solution, 1.0 ml of 40 mM acetate buffer (pH 6.0) containing 0.25 M sucrose and 0.5 ml of aqueous sulfate solution were incubated at 38° for 60 min. Both reactions were terminated by heating for 30 sec in boiling water. The liberated free forms were extracted in EDC and determined as before. All reactions proceeded linearly during the incubation period.

d) Binding of the Conjugates to Rat Plasma Protein and the 100000 ***g*** Supernatant of 15% Liver Homogenate: The binding was measured by equilibrium dialysis method. Dialysis cell composed of two 2 ml chambers (Kokugo Gomu, Tokyo) was divided with cellulose membrane (#8/32, Visking Co.). One ml of conjugate solution of isotonic tris-HCl buffer (pH 7.4) was put in one chamber and 1 ml of plasma or the 100000 ***g*** supernatant fraction which had been dialyzed overnight previously before the experiment put

6) I. Matsunaga and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **19**, 1056 (1971).
7) E. Czapet, *Monatsh. Chem.*, **35**, 641 (1914).
8) C.T. Chen, K. Samejima, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **21**, 911 (1973).
9) R. Comolli and M.E. Ferioli, *Experimentia*, **29**, 795 (1973).
10) K. Kato, I. Hirohata, W.H. Fishman, and H. Tsukamoto, *Biochem. J.*, **127**, 425 (1972).

in the other chamber. After equilibrated for 70 hr at 4°, the residual conjugate in the buffer chamber was determined spectrophotometrically. The wave lengths were 236, 227, 240, 233, 239, 237, 245, and 242 nm for 8-OHG, 8-OHS, MCG, MCS, DCG, DCS, CFG, and CFS, respectively.

e) Hydrolysis of Conjugate by Metal Ion: A half ml of 10^{-3} M aqueous conjugate solution and 0.5 ml of 0.1 M metal nitrate except 0.02 M for $Cu(NO_3)_2$ were put together and incubated at 38°. After 16 min except 2 min when $Cu(NO_3)_2$ was used, 8 μl was removed from the reaction mixture and concentration of the remaining conjugate was determined by high pressure liquid chromatography (Hitachi 634A). The operation conditions were given as follows, column length; 500 × 2.1 mm, column packing; Hitachi Gel 3020, column temperature; 40°, eluent; 20% (v/v) MeOH in 0.1 M Na_2HPO_4 aqueous solution, flow rate; 1.0 ml/min, detector; UV photometer of 254 nm and at 0.04 or 0.08 absorbance unit full scale.

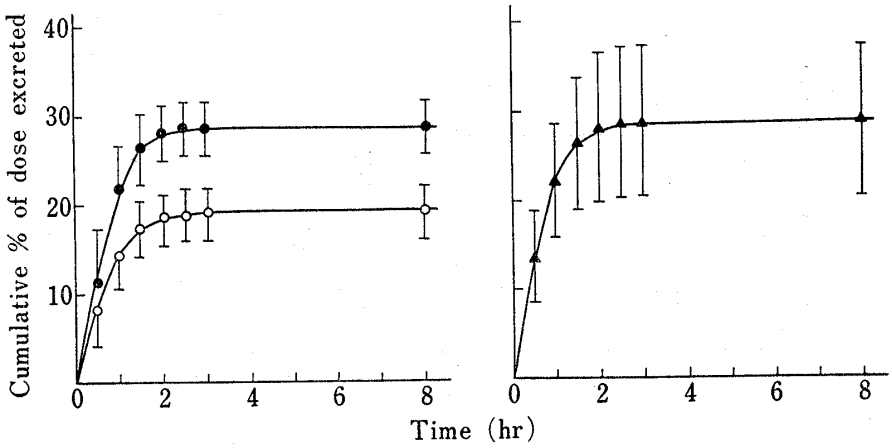

Fig. 1. Urinary (Left Hand) and Biliary (Right Hand) Excretion Time Courses of Conjugated MC after Intravenous Administration of MC in Rat

—●— glucuronide in urine,
—○— sulfate in urine,
—▲— glucuronide in bile.
Intravenous dose: 3 mg/head.
Values shown are means ± S.D. of 5 rats.

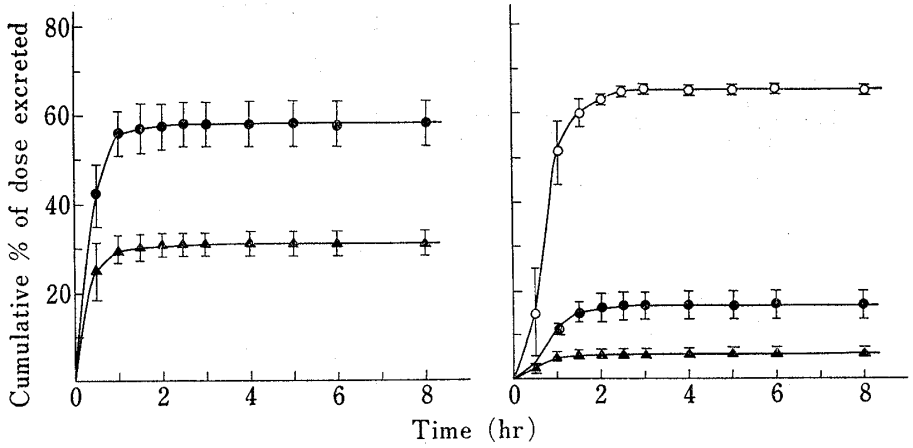

Fig. 2. Urinary and Biliary Excretion Time Courses of Conjugated MC after Intravenous Administration of MCG (Left Hand) and MCS (Right Hand) in Rat

—●— glucuronide in urine,
—○— sulfate in une,
—▲— glucuronide in bile.
Intravenous dose: 3 mg equivalent to free MC/head.
Values shown are means ± S.D of 3 rats.

Results and Discussion

Excretion Time Course and Ratio

After respective intravenous administrations of MC, DC, and their conjugates, they were excreted as glucuronides in urine and bile, and as sulfates exclusively in urine, while free forms were hardly detected both in urine and bile. Although their excretion almost ceased until 8 hr (Fig. 1—4), some of their excretion ratios to the doses in 8 hr listed together with the cases of 8-OH[5] and CF[4] in Table I were less than 100%. This implies depositing tendency in a body storage and/or other metabolism than conjugation as discussed for CF previously,[4] but their precesses were not clarified.

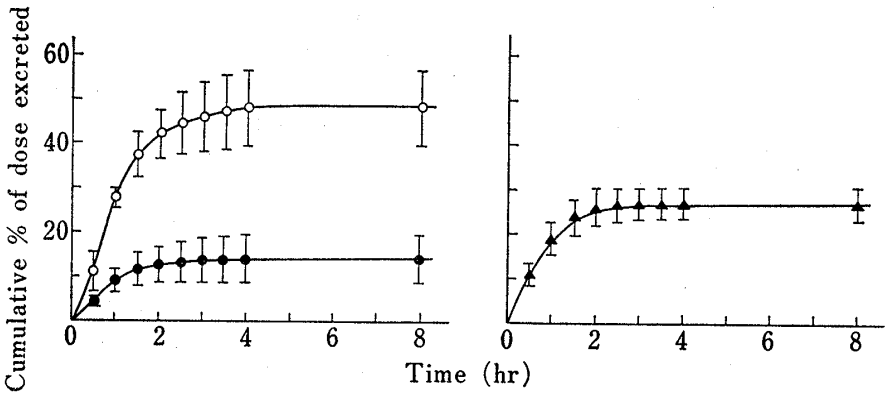

Fig. 3. Urinary (Left Hand) and Biliary (Right Hand) Excretion Time Courses of Conjugated DC after Intravenous Administration of DC in Rat

—●— glucuronide in urine,
—○— sulfate in urine,
—▲— glucuronide in bile.
Intravenous dose: 3 mg/head.
Values shown are means ± S.D. of 3 rats.

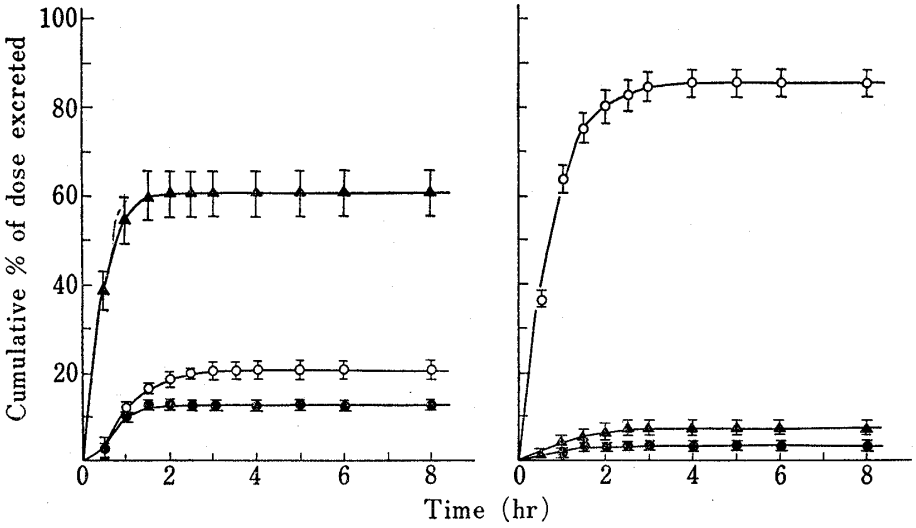

Fig. 4. Urinary and Biliary Excretion Time Courses of Conjugated DC after Intravenous Administration of DCG (Left Hand) and DCS (Right Hand) in Rat

—●— glucuronide in urine,
—○— sulfate in urine,
—▲— glucuronide in bile.
Intravenous dose: 3 mg equivalent to free DC/head,
Values shown are means ± S.D. of 3 rats.

Biliary Excretion of Glucuronide

As the ratio of the excreted amount of glucuronide in bile to that in urine after intravenous administration of the glucuronide increased in the order of 8-OHG<MCG<DCG<CFG (Table I), it can be seen that the increase of molecular weight by halogen substituents favors biliary excretion of glucuronide. This tendency is well consistent with the findings for xanthene dyes.[11]

Since protein binding of drugs has been considered as one of controlling factors for drug transport phenomena,[12] the binding activities of conjugates, *i.e.* glucuronide and sulfate to

Table I. Cumulative Excretion Ratio of Conjugates (Glucuronides and Sulfates) of 8-OH[5], MC, DC, and CF[4] to Doses after Intravenous Administration

I.V. Dosage Form	Excreted Form	8-OH (%)		MC (%)		DC (%)		CF (%)	
		Urine	Bile	Urine	Bile	Urine	Bile	Urine	Bile
Free	Glu	59.9± 1.9	8.7± 0.2	28.4± 2.7	28.8± 7.6	13.8± 4.3	26.9± 2.7	6.4± 2.1	32.3± 0.5
	Sul	22.9± 2.1	—	18.8± 2.7	—	47.9± 6.9	—	34.6± 2.4	—
Glu	Glu	91.2± 4.9	10.5± 1.0	57.9± 4.5	31.1± 2.1	13.0± 0.3	61.2± 3.8	1.7± 1.1	37.6± 8.1
	Sul	—	—	—	—	20.6± 1.4	—	9.9± 1.8	—
Sul	Glu	—	—	16.4± 2.6	6.0± 0.4	3.5± 0.3	7.9± 1.3	2.2± 0.9	24.9± 6.0
	Sul	96.7± 5.4	—	64.8± 0.6	—	86.2± 2.8	—	44.4± 4.3	—

Glu: glucuronide, Sul: sulfate.
Unmetabolized form was almost not excreted.
Values represent mean ±S.D. of 3 rats except 5 rats in the case of free MC administration.
Excretion ratios to doses until 8 hr for 8-OH, MC, and DC and until 10 hr for CF are expressed as per cent of dose.

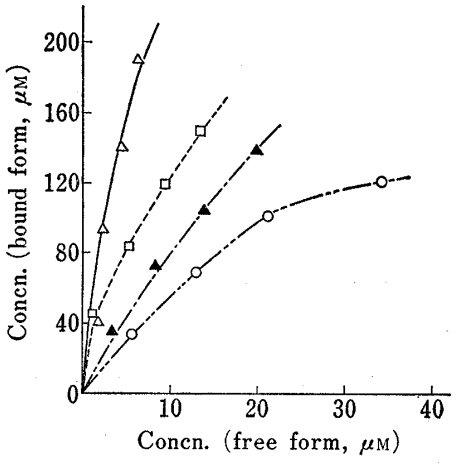

Fig. 5. Binding of Glucuronides to Plasma Protein at 4° (pH 7.4)
○ 8-OHG, ▲ MCG, □ DCG, △ CFG.

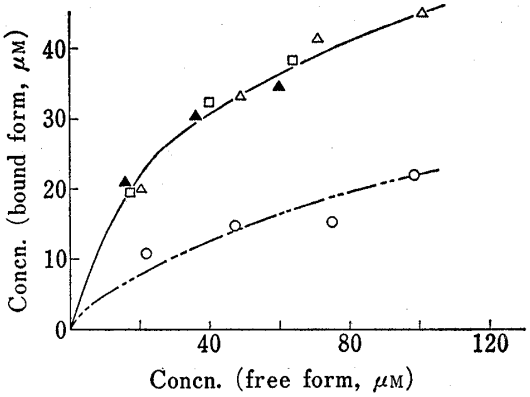

Fig. 6. Binding of Glucuronides to 100000 *g* Supernatant Fraction of 15% Liver Homogenate at 4° (pH 7.4)
○ 8-OHG, ▲ MCG, □ DCG, △ CFG.

11) J.N. Webb, M. Fonda, and E.A. Brouwer, *J. Pharmacol. Exp. Therap.*, **137**, 141 (1962).
12) *a*) J.R. Gillette, *Ann. N.Y. Acad. Sci.*, **226**, 6 (1973); *b*) H. Reyes, A.J. Levi, Z. Gatmaitan, and I.M. Arias, *J. Clin. Invest.*, **50**, 2242 (1971).

plasma protein and to the 100000 *g* supernatant of liver homogenate were determined. And the order of glucuronide binding tendency to plasma protein was 8-OHG<MCG<DCG<CFG (Fig. 5), which was the same order for the biliary excretion tendency. And to the 100000 *g* supernatant bound 8-OHG the lowest while the other three glucuronides bound at similar extent (Fig. 6). These findings seemingly suggested that a property which favors protein binding, such as hydrophobicity, plays a role for biliary excretion. But the sulfates which were not excreted in bile bound also and stronger to plasma protein than the glucuronides (Fig. 7), and bound to the 100000 *g* supernatant at similar extent to the glucuronides (Data were not shown.). Consequently, the factor which influences the partition between biliary and urinary excretion process was not clear.

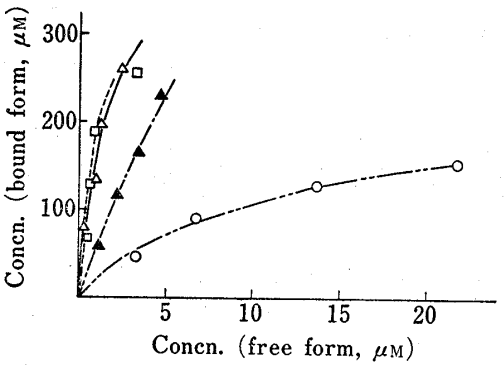

Fig. 7. Binding of Sulfates to Plasma Protein at 4° (pH 7.4)

○ 8-OHS, ▲ MCS, □ DCS, △ CFS.

Hydrolysis of Glucuronide and Sulfate *in Vivo* and *in Vitro*

As can be seen from Table I, the respective sulfates were excreted in urine after intravenous administration of DCG and CFG. And the respective glucuronides were also excreted in urine after MCS, DCS and CFS administration. This implies that both kinds of conjugates are hydrolyzed *in vivo*. Although such hydrolysis can be expected for the conjugates probably by glucuronidase and sulfatase which exist widely in a living organism, it has been scarcely reported. Therefore the detailed study on the hydrolysis was carried out.

The hydrolytic enzyme activities in the rat liver and kidney were determined by using the mitochondria-lysosomal and microsomal fractions, respectively, and the results are listed in Table II. The similar magnitude of enzyme activities were found both in the mitochondria-lysosomal and microsomal fractions. As the glucuronidase activities were about 5—10 times stronger in liver than in kidney, the hydrolysis of glucuronide was considered to proceed mainly in liver. In contrast to this, the sulfatase activities were not so different between liver and kidney that the hydrolysis by enzyme in kidney as well as in liver possibly proceeded.

Although liver had the hydrolytic activities for 8-OHG and MCG, their hydrolysis *in vivo* were not observed. But the *in vivo* hydrolysis of DCG and CFG for which the hydrolytic

Table II. Hydrolysis Rate Constants catalyzed by β-Glucuronidase and Arylsulfatese in Rat Liver and Kidney

Fraction		Tissue			
		Liver		Kidney	
		Mitochondria –Lysosome	Microsome	Mitochondria –Lysosome	Microsome
β-Glucuronidase	8-OHG	0.149	0.176	0.027	0.016
	MCG	0.271	0.270	0.040	0.026
	DCG	0.520	0.400	0.046	0.034
	CFG	0.520	0.400	0.035	0.032
Arylsulfatase	8-OHS	0.010	0.019	0.007	0.008
	MCS	0.056	0.068	0.021	0.032
	DCS	0.014	0.024	0.009	0.008
	CFS	0.002	0.001	0.003	0.003

The incubation systems were shown in experimental part.
Since the initial velocities were linear against the substrate concentrations (28—143 μM), the slopes of these lines were taken as the rate constants (min^{-1} g liver or kidney wet weight^{-1}).

activities *in vitro* were 2—3 times stronger than those for 8-OHG and MCG was observed. Accordingly, it could be said qualitatively that the hydrolytic activity for the glucuronide observed *in vivo* parallels that *in vitro*. And enhancement of susceptibility to glucuronidase by halogen substituent is consistent with the substituent effect which was found in substituted phenyl-D-glucuronic acids.[13]

The sulfatase activities were much smaller than those of glucuronidase especially in liver (Table II), but the sulfates were hydrolyzed *in vivo* to a similar extent to the glucuronides (Table I). And moreover, the hydrolysis of CFS *in vivo* proceeded to much larger extent than that of 8-OHS of which hydrolysis was not detected *in vivo*, while the suceptibility of CFS to the sulfatase was the smallest. These results suggest that the hydrolytic activity for the sulfate *in vivo* does not necessarily parallel that *in vitro*, or that some factors other than sulfatase affect the hydrolysis of sulfate *in vivo*.

Metal Ion as a Possible Factor

There have been various reports that metal ions interact with 8-hydroxyquinoline derivatives and their conjugates. For instance, the local concentration of metal in liver or sciatic nerve was influenced by CF administration.[14a] The green substance excreted in urine of SMON patients was found to be a chelate complex between CF and Fe^{3+}, which gave substantial support for the chinoform theory as the cause of SMON.[14b] Metal ions such as Cu^{2+}, Fe^{3+}, Zn^{2+}, and Mg^{2+} catalyzed the hydrolysis of 8-hydroxyquinoline β-D-glucoside[14c], 8-OHS[14d], and CFS.[14e] And recently, Hayakawa, *et al.*[14f] reported that CFG and CFS were hydrolyzed catalytically by metal ions and the order of the catalytic activities was the same with that of the stability constants between 8-OH and metal ion ($Cu^{2+}>Ni^{2+}>Zn^{2+}>Mg^{2+}>Ca^{2+}$). Based on these reports, the effects of metal ions on the hydrolysis of glucuronides and sulfates were examined, and the results are listed in Table III. Through the eight conjugates of the present study the extent of the metal ion effect was in the order of $Cu^{2+}>Fe^{3+}\approx Zn^{2+}>Mg^{2+}$, which is the same with what Hayakawa, *et al.* reported for CFG and CFS hydrolysis. And it is an interesting point that CFS is very susceptible for metal ion effect. Although the concentration of metal ion examined in the present study does not correspond to that in biological fluid, such a metal ion suggests a possible explanation for the unexpectedly high hydrolytic rate of CFS *in vivo*.

TABLE III. Degree of Hydrolysis of Conjugates in the Presence of Metal Ions

Metal ions added (incubation time)	Cu^{2+} (2 min)	Fe^{3+} (16 min)	Zn^{2+} (16 min)	Mg^{2+} (16 min)
CFG	40.6	32.6	17.9	8.8
CFS	100.0	16.4	17.5	2.7
DCG	86.1	32.0	10.0	2.3
DCS	100.0	16.4	7.6	3.0
MCG	15.2	6.1	11.1	2.2
MCS	22.3	13.1	12.3	4.5
8-OHG	12.8	5.2	9.6	4.9
8-OHS	9.6	0.1	6.3	1.4

The incubation systems were shown in the experimental part.
Values represent the hydrolysis ratio (%) of conjugates.

13) *a*) T. Watabe and K. Suzuki, *Chem. Pharm. Bull.* (Tokyo), **18**, 414 (1970); *b*) C.C. Wand and O. Touster, *J. Biol. Chem.*, **247**, 2650 (1972).

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