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Biopharmaceutical Study of the Hepato-biliary Transport of Drugs. VIII.[1] Investigation of Hepatic Uptake of Organic Cations by Portal Infusion

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The liver/plasma (L/P) ratios of acetyl procainamide ethobromide (APAEB), procainamide ethobromide (PAEB) and quinine, which were transported from plasma into liver against a concentration gradient, were greatly decreased by infusing sulfhydryl reagents, *p*-chloromercuribenzoic acid (PCMB), *p*-chloromercury phenyl sulfonic acid (PCMBS) and iodoacetamide (IAA), into portal vein on account of the increase in the plasma level and the decrease in the liver level. The metabolism of PAEB and quinine was not affected by the infusion of the reagents. The binding of APAEB, PAEB and quinine was not depressed and that to plasma was not changed in the presence of the reagents. Consequently it was deduced that the hepatic uptake of the organic cations was blocked by the treatment with the sulfhydryl reagents, which acted on the plasma membrane involved in the concentrative uptake of the organic cations. In comparison with the previous results obtained by the intrabiliary retrograde infusion, PCMB and IAA were inhibitory only on the hepatic uptake process and N-ethylmaleimide (NEM) only on the excretory process, whereas PCMBS was effective on both steps. The finding of the specific reagents which are inhibitory only on one process would be of use in the study of the hepato-biliary transport of organic cations.

Keywords——organic cation; bromphenol blue; hepatic uptake; active transport; portal infusion; sulfhydryl reagent; plasma membrane

It has previously been reported[3] that the active biliary excretion of acetyl procainamide ethobromide (APAEB) and quinine was inhibited by infusion of surfactants, sulfhydryl reagents and other reagents retrogradely into bile ducts. As the bile/liver (B/L) concentration ratio was intensively decreased by this treatment, these reagents were considered to influence the active excretory process from liver cells to bile.

The uptake process of organic cations from plasma into liver is also supposed an active process in contrast with organic anions. Accordingly in the present investigation several reagents were infused into portal vein before administration of organic cations to see the effect of the reagents on the hepatic uptake of organic cations and the results were compared with those obtained by the retrograde infusion in the previous report.[3]

Experimental

Animal Experiment——Male Wistar rats, weighing 220—250 g, were anesthetized with sodium pentobarbital. The abdomen was opened, renal pedicles ligated and the bile duct cannulated. A reagent dissolved in pH 7.4 isotonic buffer was infused into vena mesenterica superior over 2—3 min. Immediately after the injection the vein was ligated to stop bleeding. Drugs were injected into femoral vein 5 min after the portal infusion of the reagent. Bile sample was collected for two 15 min periods. Blood sample was taken from abdominal aorta at 30 min and the liver was rapidly removed.

Chemical Analysis——Determination of APAEB, procainamide ethobromide (PAEB) and quinine in plasma, liver and bile samples was performed by the same procedures as described before.[4]

1) Part VII: K. Takada, H. Mikami, S. Asada, K. Tatsuo, and S. Muranishi, *Chem. Pharm. Bull.* (Tokyo), **26**, 19 (1978).
2) Location: *Yoshida-shimoadachi-cho, Sakyo-ku, Kyoto*; *a*) Present address: *Kobe Women's College of Pharmacy*.
3) H. Nakae, H. Okamoto, K. Takada, and S. Muranishi, *Chem. Pharm. Bull.* (Tokyo), **25**, 427 (1977).
4) H. Nakae, R. Sakata, and S. Muranishi, *Chem. Pharm. Bull.* (Tokyo), **24**, 886 (1976).

Bromphenol bule was estimated according to the method of Takada with a slight modification in the determination of the liver level.[5] A 5ml of liver homogenate was mixed with 6 ml of acetone, shaken for 15 min and centrifuged at 3000 rpm for 20 min. To 2 ml of the supernate, 4 ml of the mixture of acetone and water (1: 1) was added and the absorbance at 600 nm was measured. Plasma and bile samples were treated as described before.[5]

Sulfanilamide was determined spectrophotometrically by diazocoupling method. Plasma: One ml of plasma sample was mixed with 4 ml of water and 1 ml of 30% TCA. After centrifugation, 4 ml of the supernate was taken and acidified with 1 ml of 5 N HCl, diazotized and coupled with Tsuda reagent. The absorbance of the reactant was measured at 550 nm for sulfanilamide. For determination of total sulfanilamide the acid solution was heated for 30 min at 100° before diazotization. Liver: Liver sample was homogenized with 2 volumes of 0.15 M KCl solution. To 5 ml of the homogenate 1.5 ml of 30% TCA was added and centrifuged at 3000 rpm for 30 min. A 4 ml of the supernate was taken and estimated by the same procedure as in plasma. Bile: To 0.1 ml of bile sample 4 ml of 1N HCl was added and the mixture was used for determination for free and total sulfanilamide.

Binding to Liver Homogenates and Plasma——The binding of drugs to liver homogenates and plasma was estimated by equilibrium dialysis according to the method of Takada.[6]

Male Wistar rats weighing 220—250 g were anesthetized with sodium pentobarbital. Blood sample was drawn from abdominal aorta and the liver was removed. The liver sample was homogenized with 0.15 M KCl solution to give 40% homogenates. A 2 ml of the homogenate in sacs of Visking dialysis tubing was dialyzed at 4° against 5 ml of the drug solution in the presence or in the absence of reagents. APAEB, PAEB and bromphenol blue were dissolved in isotonic buffer (pH 7.4) and quinine in saline. After two days the concentrations of drugs in the outer solution were measured and the binding percentages were calculated by correcting the blank value.

Blood sample was centrifuged at 3000 rpm for 20 min and 1 ml of the supernatant plasma was dialyzed against 4 ml of the drug solution by the same procedures described above.

Materials——PAEB was kindly provided by Squibb Institute for Medical Research. APAEB was synthesized in our laboratory according to the method of Hwang.[7] *p*-Chloromercury phenyl sulfonic acid (PCMBS) was obtained from Sigma Chemical Company, other chemicals from Nakarai Chemicals Company.

Results

Effect of Several Reagents on Hepato-biliary Transport of APAEB

Concentrations of APAEB, a non-metabolizable organic cation, in plasma, liver and bile and their concentration ratios after injection of 8.8 μmol/300 g rat body weight of APAEB are shown in Table I. When no solution was infused, the liver/plasma (L/P) ratio of APAEB

Table I. Effect of Several Reagents by Portal Infusion on Hepato-biliary Transport of APAEB

Infused solution	Plasma level[a] (nmol/ml)	Liver level[a] (nmol/g)	Bile level 0—15min (μmol/ml)	Bile level 15—30min (μmol/ml)	L/P ratio	B/L ratio[b]
No treatment	78±9	135±45	1.24±0.37	1.70±0.47	1.7 ±0.6	13.4±3.8
Isotonic buffer	97±10	178±29	1.15±0.51	1.71±0.47	1.8 ±0.1	10.0±3.9
PCMB 5μmol/100g	114±27	80±24	0.31±0.08	0.56±0.11	0.71±0.04	7.2±1.2
PCMBS 5μmol/100g	120±21	88±35	0.31±0.10	0.81±0.15	0.84±0.22	6.8±1.1
NEM 37.5μmol/100g	88±13	168±20	0.87±0.53	1.71±1.02	1.9 ±0.4	10.0±5.9
Succinic anhydride 500μmol/100g	98±10	135±12	0.41±0.11	1.17±0.27	1.4 ±0.1	8.7±1.9
DNP 10μmol/100g	106±13	180±20	0.54±0.49	1.26±0.72	1.7 ±0.03	6.9±3.2

a) Plasma and liver levels are values at 30 min.
b) Bile levels during 15—30 min were used in calculation of B/L ratios.
Results are expressed as mean±S.D. of more than three experiments.

5) K. Takada, Y. Mizobuchi, and S. Muranishi, *Chem. Pharm. Bull.* (Tokyo), **22**, 922 (1974).
6) K. Takada, O. Narumiya, and S. Muranishi, *Chem. Pharm. Bull.* (Tokyo), **23**, 729 (1975).
7) S.W. Hwang, R.H. Reuning, and L.S. Schanker, *XENOBIOTICA*, **1**, 265 (1971).

was 1.7 and the B/L ratio 13.4, which showed the concentrative transport from plasma into liver and from liver into bile. Infusion of 1 ml/100 g rat body weight of pH 7.4 isotonic buffer into portal vein raised concentrations in plasma and liver but the bile level during 15—30 min was not affected. Consequently the L/P ratio was scarcely affected and the B/L ratio decreased a little.

The pretreatment with 5 μmol/100 g of *p*-chloromercuribenzoic acid (PCMB), a sulfhydryl reagent, slightly raised the concentration in plasma and extremely lowered those in liver and bile as compared with the sham operation. Therefore the L/P ratio was decreased from 1.8 in the sham operation to 0.71. APAEB was not transported uphill from plasma into liver anymore. PCMBS, 5 μmol/100 g, also had a similar effect, namely a slight increase in concentration in plasma and an intensive decrease in those in liver and bile. But another type of sulfhydryl reagent, N-ethylmaleimide (NEM), 37.5 μmol/100 g, caused no change in the hepato-biliary transport of APAEB. Succinic anhydride, an amino-reactive agent, 500 nmol/100 g, lowered the concentrations in liver and bile and the L/P ratio was decreased to 1.4. When dinitrophenol, 10 μmol/100 g, was infused, the concentrations in plasma, liver and bile were not significantly changed. Experiments with higher doses could not be performed because of its toxicity.

Table II. Effect of Sulfhydryl Reagents on Concentration Ratios of PAEB

Compound	Concentration ratio[a]	
	L/P	B/L[b]
Isotonic buffer	7.9±2.0	10.4±2.5
PCMB	2.8±2.1	9.3±4.4
PCMBS	2.4±0.4	9.1±3.6
NEM	7.5±1.7	6.3±2.2

a) Concentration ratios were calculated from the data shown in Fig. 1.
b) Bile levels during 15—30 min were used in calculation of B/L ratios.
Results are expressed as mean±S.D.

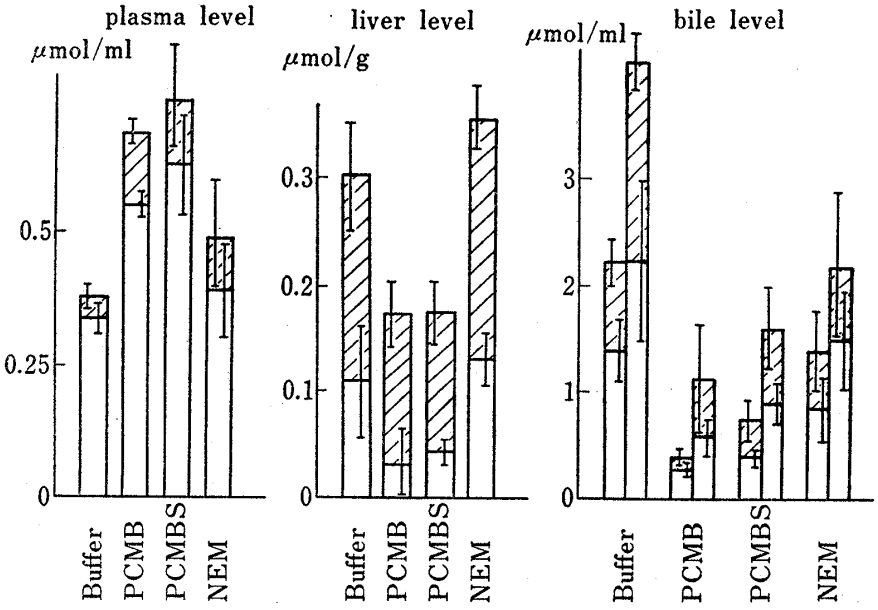

Fig. 1. Effect of Sulfhydryl Reagents on Hepato-biliary Transport of PAEB

Plasma and liver levels are values at 30 min after administration of 10 μ mol/300 g of PAEB. The left column in each pair of bile levels represents the value during 0—15 min and the right column the value during 15—30 min. Hatched areas indicate the metabolite of PAEB. Each value represents the mean±S.D. of more than three experiments.

Among the infused reagents PCMB, PCMBS and succinic anhydride lowered the L/P ratios, indicating that these reagents depressed the hepatic uptake of APAEB. But the effect of succinic anhydride was milder than the others.

Effect of Sulfhydryl Reagents on Hepato-biliary Transport of PAEB

PAEB is a desacetylated form of APAEB and metabolized to APAEB in liver. The L/P ratio after the sham operation is 7.9, which is much higher than that of APAEB, as shown in Table II. This high value might clarify the effect of the portal infused reagent on the hepatic uptake observed for APAEB. When 5 μmol/100 g of PCMB or PCMBS was infused, the concentration in plasma was greatly increased and those in liver and bile were markedly decreased (Fig. 1). Hence, the L/P ratio was decreased from 7.9 to 2.8 or 2.4, respectively. NEM did not affect the L/P ratio. These results are the same as in the case of APAEB.

The proportion of APAEB to PAEB in the concentrations in plasma, liver and bile was not changed by the infusion of these reagents. This shows that the treatment with these reagents does not affect the metabolism of PAEB irrespective of the inhibition of the heptatic uptake.

Effect of Sulfhydryl Reagents on Hepato-biliary Transport of Quinine

Next to APAEB and PAEB, the effect of the sulfhydryl reagents on the hepato-biliary transport of quinine was examined. Quinine is a tertiary amine actively transported from plasma to bile but partly accumulated by the lipid-binding like mechanism.[4]

When PCMB, 5 μmol/100 g, or iodoacetamide (IAA), 75 μmol/100 g, was infused, the concentration in plasma was increased and the liver and bile levels were decreased (Fig. 2).

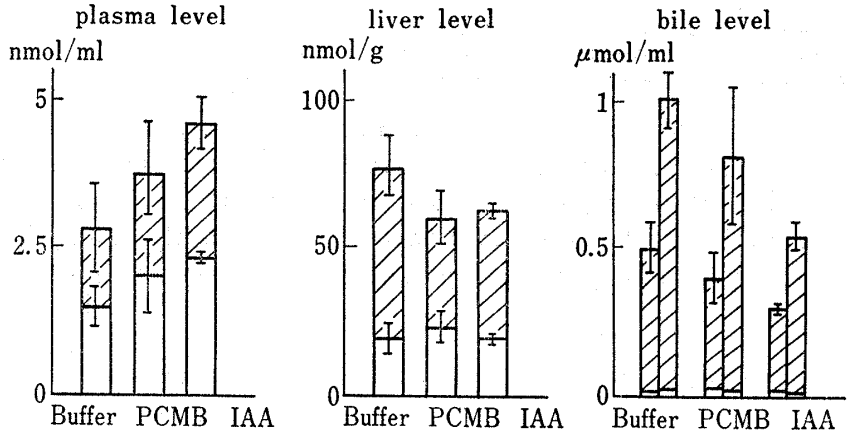

Fig. 2. Effect of Sulfhydryl Reagents on Hepato-biliary Transport of Quinine

Plasma and liver levels are values at 30 min after administration of 5 μmol/300 g of quinine. The left column in each pair of bile levels represents the value during 0—15 min and the right column the value during 15—30 min. Hatched areas indicate the metabolites of quinine. Each value represents the mean±S.D. of more than three experiments.

TABLE III. Effect of Sulfhydryl Reagents on Concentration Ratios of Quinine

Compound	Concentration ratio[a]	
	L/P	B/L[b]
Isotonic buffer	13.9±2.5	1.1±0.2
PCMB	9.9±1.8	1.0±0.2
IAA	8.3±1.6	0.7±0.1

a) Concentration ratios were calculated from the data shown in Fig. 2.
b) Bile levels during 15—30 min were used in calculation of B/L ratios. Results are expressed as mean±S.D.

Consequently the L/P ratios were considerably lowered (Table III). This shows that the hepatic uptake of quinine was also depressed by the treatment with PCMB or IAA.

IAA was not used in the case of APAEB or PAEB because the analysis of these quaternary ammonium compounds went wrong in the presence of IAA.

The metabolism of quinine was not affected by these reagents judging from the proportion of the metabolites to the intact quinine.

Effect of the Reagents on Hepato-biliary Transport of Bromphenol Blue

From the results above, the hepatic uptake of organic cations which were actively transported from plasma into bile was shown to be blocked by the portal infusion of PCMB, PCMBS, IAA and succinic anhydride. As for organic anions, which are transported uphill from plasma to liver like bromphenol blue, the roles of the liver cytoplasmic binding proteins in the hepatic uptake are mentioned but it is not known precisely whether the concentrative transport is dependent on energy or not.

TABLE IV. Effect of the Reagents on Hepato-biliary Transport of Bromphenol Blue

Infused solution	Plasma level[a] (nmol/ml)	Liver level[a] (nmol/g)	Bile level 0—10min (μmol/ml)	Bile level 10—20min (μmol/ml)	Bile level 20—30min (μmol/ml)	L/P ratio	B/L ratio[b]
Isotonic buffer	13.1± 4.5	51.7± 9.9	3.42±2.12	8.58±2.04	7.87±0.63	3.8±1.6	133±39
PCMB 5μmol/100g	34.8±11.1	48.4±19.4	0.74±0.40	4.65±1.48	5.07±1.05	1.7±0.7	116±61
Succinic anhydride 500μmol/100g	34.3± 8.3	60.1±11.4	0.83±0.50	9.52±1.72	9.80±0.55	1.3±0.5	179±30

a) Plasma and liver levels are values at 30 min.
b) Bile levels during 20—30 min were used in calculation of B/L ratios.
Results are expressed as mean±S.D. of more than three experiments.

Table IV shows the concentrations of bromphenol blue, a non-metabolizable organic anion, in plasma, liver and bile and their concentration ratios after administration of 10 μmol/300 g. Infusion of PCMB, 5 μmol/100 g, or succinic anhydride, 500 nmol/100 g, remarkably raised the plasma level but the liver level was not significantly changed. Therefore the L/P ratios were greatly reduced, indicating that the treatment was also effective to the organic anion.

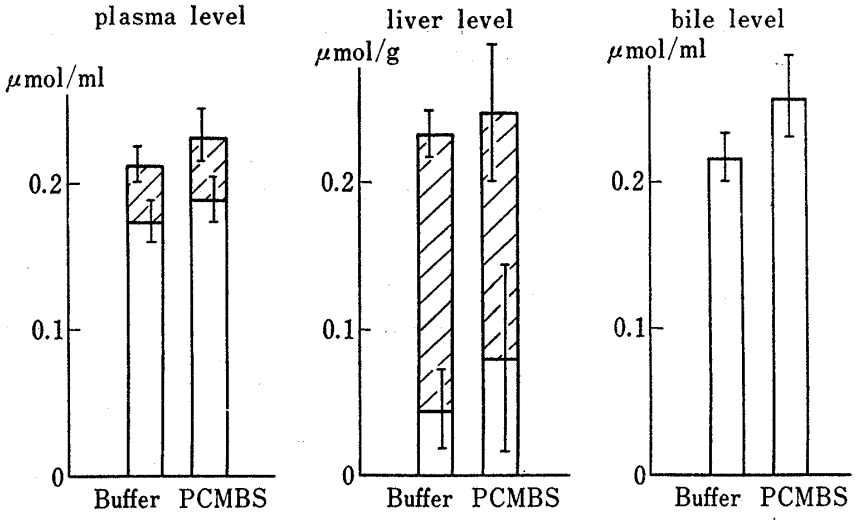

Fig. 3. Effect of PCMBS on Hepato-biliary Transport of Sulfanilamide

Plasma and liver levels are values at 30 min after administration of 60 μmol/300 g of sulfanilamide. Bile levels are at 15—30 min. Hatched areas indicate the metabolite of sulfanilamide. Each value represents the mean±S.D. of four experiments.

Effect of PCMBS on Hepato-biliary Transport of Sulfanilamide

As sulfanilamide is known to be passively transported from plasma into bile,[8] the effect of the portal infusion of PCMBS on its transport was examined. When 5 μmol/100 g of PCMBS was infused, the concentrations in plasma, liver and bile were not changed significantly as shown in Fig. 3 and their concentration ratios were about 1 not only in the sham operation but after the treatment with PCMBS (Table V). From this result, it is shown that

Table V. Effect of PCMBS on Concentration Ratios of Sulfanilamide

Compound	Concentration ratio[a]	
	L/P	B/L[b]
Isotonic buffer	1.1 ±0.03	1.0±0.2
PCMBS	0.98±0.19	1.3±0.4

a) Concentration ratios were calculated from the data shown in Fig. 3.
b) Bile levels during 15—30 min were used in calculation of B/L ratios.
Results are expressed as mean±S.D.

the inhibitory effect of the infused reagent on the hepatic uptake of drugs transported against a concentration gradient is not operative to the drug passively transferred.

Effect of the Reagents on the Binding to the Liver Homogenates

One possible mechanism of the hepatic concentrative uptake of drugs is tissue binding. Especially quinine and bromphenol blue are known to bind to liver tissues to a great extent. In order to make sure whether the inhibitory effect of the reagents on the hepatic uptake is due to the change in tissue binding of these organic compounds, the binding percentage to liver homogenates was estimated. In absence of any reagent APAEB is bound to liver homogenate by 26.9% as shown in Table VI. In the presence of PCMB or PCMBS which

Table VI. Effect of Several Reagents on Binding to Liver Homogenates

Compound	Reagent	% bound
APAEB 420 mM	Control	26.9±2.3
	PCMB 5 mM	31.1±2.4
	PCMBS 5 mM	33.5±0.9
	NEM 37.5 mM	24.4±1.6
	Succinic anhydride 500 mM	16.2±2.6
PAEB 200 mM	Control	23.7±4.0
	PCMB 5 mM	25.6±3.6
Quinine 100 mM	Control	81.7±1.5
	PCMB 5 mM	88.0±0.4
	IAA 75 mM	84.5±1.2
Bromphenol blue 50 mM	Control	96.5±3.1
	PCMB 5 mM	97.4±3.2
	Succinic anhydride 500 mM	98.8±3.2

Results are expressed as mean ± S.D. of four experiments.

blocked the uptake of APAEB *in vivo* the degree of binding was not depressed but rather slightly increased, while NEM did not affect and succinic anhydride depressed the binding. PCMB also did not inhibit the binding of PAEB, quinine and bromphenol blue. The binding of quinine and bromphenol blue was not depressed, either, by IAA and succinic anhydride,

8) S.C. Kalser, E.J. Kelvington, and M.M. Randolph, *J. Pharmacol. Exptl. Therap.*, **159**, 389 (1967).

respectively. Thus, the inhibition of the hepatic uptake can not be explained by the depression of the tissue binding except for succinic anhydride in the case of APAEB because the reagents which had the inhibitory effect *in vivo* like PCMB and PCMBS did not decrease the binding.

Effect of Sulfhydryl Reagents on Binding of APAEB to Plasma

As the mechanism of the inhibitory effect of the reagents might be alteration in the plasma binding of drugs, the binding of APAEB was determined in the presence of PCMBS and NEM. Table VII shows that the degree of the binding of APAEB was quite low and

Table VII. Effect of Sulfhydryl Reagents on Binding of APAEB to Plasma

Reagent	% bound
Control	4.2±3.1
PCMBS 1.6 mM	5.4±7.7
NEM 11.7 mM	8.5±8.6

A concentration of APAEB was 125 mM
Results are expressed as mean S.D. of four experiments.

both reagents did not affect the binding significantly. Therefore the effect of PCMBS *in vivo* is not attributed to the change in plasma binding.

Discussion

When infused into portal vein, sulfhydryl reagents, PCMB, PCMBS and IAA, and an amino-reactive agent succinic anhydride, lowered the L/P ratios of the organic cations, APAEB, PAEB and quinine, which were actively transported from plasma into bile. But NEM and dinitrophenol did not affect the L/P ratios of APAEB and PAEB. The L/P ratio of an organic anion, bromphenol blue, which was taken up by liver against a concentration gradient, was also depressed by the treatment with PCMB and succinic anhydride. However, sulfanilamide which was passively transported was not influenced by PCMBS. As the metabolism of PAEB, quinine and sulfanilamide was not affected by any reagents, the effect of the reagents is considered to be that on the transport process from plasma into liver. Namely the hepatic uptake of the organic cations was blocked by the treatment with PCMB, PCMBS, IAA and succinic anhydride but not with NEM and DNP. The organic anion was also depressed in hepatic uptake by PCMB and succinic anhydride. The inhibitory effect of the sulfhydryl reagents on the organic cations can not be explained by the change of their binding to liver homogenates or plasma. For the binding to liver homogenates was not reduced and that to plasma was not affected in the presence of the reagents. Furthermore contribution of decrease in hepatic blood flow is excluded because the bile flow was not changed after the treatment in spite of its high sensitivity to blood flow. Therefore the effect is supposed to be caused by the reaction of the reagents with the functional groups of the plasma membrane which are involved in the concentrative uptake of the organic cations. When the reagents were retrogradely infused into biliary trees as shown in the previous report,[3] PCMBS and NEM lowered the B/L ratios of APAEB and quinine but PCMB, IAA and dinitrophenol did not. Namely PCMBS and NEM inhibited the active excretion of organic cation from hepatocytes into bile, while PCMB, IAA and dinitrophenol did not. In Fig. 4, the scheme of the effect of the sulfhydryl reagents on the hepatobiliary transport of organic cations are given according to the results obtained from the previous and this reports. It is noteworthy that PCMB and IAA had an inhibitory effect only on the uptake process and NEM only on the excretory process. Accordingly these reagents are effective

only on one process of the two important steps of the biliary excretion. This specificity of the reagents suggests that separate mechanisms operate on the uptake and the excretory processes and the two processes could be discriminated by using these reagents.

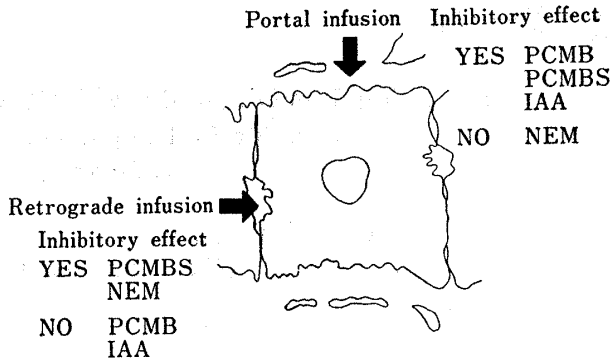

Fig. 4. Scheme of the Effect of Sulfhydryl Reagents on Hepato-biliary Transport of Organic Cations

The effect of succinic anhydride on APAEB was milder than that of PCMB or PCMBS and the binding to liver homogenate was decreased only by this reagent. Therefore its effect might be attributed to change in binding.

As for organic anions the effect of PCMB and succinic anhydride, which did not depress the binding to liver homogenate, might suggest the existence of the carrier for hepatic uptake of organic anions assumed.[9]

In respect to the energy dependency, no definite result was obtained by this experiment because dinitrophenol did not have the depressive effect on APAEB and the entanglement of sulfhydryl reagents with energy was indistinct.

Acknowledgement The authors thank professor Hitoshi Sezaki for his helpful discussions.

9) M. Frezza, C. Tiribell, E. Panfili, and G. Sandri, *FEBS letters*, **38**, 125 (1974).