

## Biopharmaceutical Study of the Hepato-biliary Transport of Drugs. V.<sup>1)</sup> Hepatic Uptake and Biliary Excretion of Organic Cations

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The hepato-biliary transport characteristics *in vivo* and the uptake process by liver slices and by liver cell suspensions were studied in rats using procaineamide ethobromide, its N-acetyl compound and quinine. These organic cations were transported from plasma to liver and from plasma to bile against a concentration gradient, and their biliary excretion was depressed by an administration of the other organic cations in accordance with earlier studies. However the hepatic uptake of quinine was extremely high, 39 of the liver/plasma concentration ratio, as compared with 8.6 of PAEB and 1.7 of APAEB. When taken up by liver slices or liver cell suspensions, PAEB and quinine showed completely different patterns. Slice/medium ratio of PAEB obtained by liver slices attained 3.6 after 4 hours and cell/medium ratio obtained by liver cell suspensions did not attain one. On the other hand, slice/medium and cell/medium ratios of quinine attained about 20 and the high slice/medium ratio was only slightly decreased in an atmosphere of nitrogen or in the presence of DNP. The hepatic high uptake of quinine observed both *in vivo* and *in vitro* is mostly explainable by lipid-binding mechanism as the binding to liver lipids showed quite a high value, 77%.

Among organic cations a number of quaternary ammonium compounds and tertiary amines have been shown to be actively transported from blood into bile.<sup>3)</sup> Although these organic cations are thought to be secreted into bile *via* a pathway different from that of organic anions, the biliary excretion of organic cations has not been so extensively studied as organic anions. The transport pathway of those compounds comprises several steps, *e.g.* uptake by the liver cell, passage through the cell and active excretion into bile, but the mechanism of each step remains quite obscure.

In this study hepato-biliary transport characteristics of certain organic cations which are known to be actively excreted into bile are investigated and *in vivo* and *in vitro* methods are utilized in order to elucidate the mechanism by which these compounds are transported from blood to liver. For this purpose procaineamide ethobromide (PAEB), N-acetylprocaineamide ethobromide (APAEB) and quinine were chosen as model compounds which were actively excreted into bile.

### Experimental

**Materials**—PAEB was provided by Squibb Institute for Medical Research; isopropamide by Sumitomo Chemical Co. APAEB was synthesized in our laboratory according to the method of Hwang, *et al.*<sup>4)</sup> Quinine hydrochloride and dinitrophenol (DNP) were obtained from a commercial source.

***In Vivo* Experiments in Rats**—Male Wistar rats weighing 250–300g were anesthetized with pentobarbital. The renal pedicles were ligated and the bile duct was cannulated with a polyethylene tube. Compounds were injected into a femoral vein and bile was collected for two 15 min periods. At the end of an experiment blood samples were withdrawn from the abdominal aorta in a heparinized syringe and the liver was rapidly removed.

**Analytical Methods**—PAEB in Plasma: After a blood specimen was centrifuged at 3000 rpm for 30 min, 1 ml of 30% trichloroacetic acid and 4 ml of distilled water were added to 2 ml of the resulting super-

1) Part IV: K. Takada, Y. Tokunaga, and S. Muranishi, *Chem. Pharm. Bull.* (Tokyo), **24**, 871 (1976).

2) Location: *Yoshida shimoadachi-cho, Sakyo-ku, Kyoto.*

3) a) L.S. Schanker and H.M. Solomon, *Am. J. Physiol.*, **204**, 829 (1963); b) P.K. Nayak and L.S. Schanker, *Am. J. Physiol.*, **217**, 1639 (1969).

natant and their mixture was centrifuged at 3000 rpm for 30 min after shaking. To 4 ml of the supernatant, 1 ml of 5N HCl was added and the color reaction was performed for free PAEB by diazo-coupling method with Tsuda reagent. Absorbance of the resulting color was measured at 550 m $\mu$ . Total PAEB was estimated by the same method after heating the acid solution at 100° for 30 min and cooling to room temperature.

**PAEB in Liver:** Liver was homogenized with 2 volumes of 0.15 M KCl solution. Three ml of 30% trichloroacetic acid was added to 4 ml of the supernatant and the color reaction was performed for free and total PAEB as described in determination in plasma.

**PAEB in bile:** 5 ml of 1N HCl was added to 0.1 ml of the bile sample and the resultant acid solution was used for free and total PAEB determination as described above.

Quinine was measured fluorometrically at 350 m $\mu$  and 450 m $\mu$  as excitation and emission wavelengths. Unchanged quinine was estimated by a modification of the method of Nayak,<sup>3b)</sup> and metabolites by a modification of the methods of Saggars<sup>5)</sup> and Brodie.<sup>6)</sup>

**Quinine in Plasma:** 0.4 ml of 5 N NaOH was added to 1 ml of plasma and the mixture was shaken for 20 min with 8 ml of benzene. After centrifugation 6 ml of the benzene layer was transferred to a tube containing 4 ml of 1 N H<sub>2</sub>SO<sub>4</sub>. The resulting mixture was shaken for 15 min and centrifuged. Fluorescence of the acid layer was measured at the wavelengths described above.

**Quinine in Liver:** Liver was homogenized with 2 volumes of 0.15 M KCl solution. Two ml of 5 N NaOH was added to 5 ml of the homogenate and the mixture was shaken for 20 min with 15 ml of benzene. After centrifugation 10 ml of the benzene layer was shaken for 15 min with 6 ml of 1 N H<sub>2</sub>SO<sub>4</sub>. The mixture was centrifuged and fluorescence of the acid layer was measured.

**Quinine in Bile:** 0.5 ml of 2 N NaOH was added to 0.1 ml of the bile sample and the mixture was shaken for 20 min with 8 ml of benzene. After centrifugation quinine in the benzene layer was reextracted with 1 N H<sub>2</sub>SO<sub>4</sub> and measured as described in determination in plasma.

**Quinine Metabolites in Plasma, Liver and Bile:** Alkaline solution of samples was extracted with isoamyl alcohol instead of benzene. After reextracted with 1 N H<sub>2</sub>SO<sub>4</sub>, fluorescence of the acid layer was measured. The concentrations were estimated from the standard curve prepared by adding known amounts of quinine. Concentration of quinine metabolites was calculated by subtracting intact quinine concentration from total concentration thus obtained. Therefore metabolites in this determination are regarded to have the same extinction coefficient as quinine as in ref. 5.

**Uptake by Liver Slices**—Uptake experiment was mostly performed by the method of Solomon.<sup>7)</sup>

Male Wistar rats weighing 250–300 g were anesthetized and the liver was removed. Slices of liver were prepared with a Stadie-Riggs microtome.

**PAEB:** 2.5 g of slices were incubated in 20 ml of Krebs-Ringer phosphate solution containing 1 g glucose/liter and 47.0 nmoles/ml (20  $\mu$ g/ml) PAEB at 37° in an atmosphere of 95% O<sub>2</sub>–5% CO<sub>2</sub>. At various times 2 ml of the medium was removed and centrifuged. To 1 ml of the supernatant 5 ml of 1 N HCl was added and was heated at 100° for 30 min. After cooling color reaction was performed. The resultant colored compound was extracted with isoamyl alcohol by adding NaCl into the water layer and absorbance at 550 m $\mu$  was measured. Concentrations of total PAEB in slices were calculated from the initial medium concentration and individual medium concentrations at each sampling time. Results were expressed as slice/medium (S/M) concentration ratios.

**Quinine:** 2.5 g of slices were incubated in 20 ml of Krebs-Ringer phosphate solution containing 1 g glucose/liter and 2.5 nmoles/ml (1  $\mu$ g/ml) quinine at 37° in 95% O<sub>2</sub>–5% CO<sub>2</sub>. At the end of the incubation period, the slices were blotted and homogenized with 2 volumes of 0.15 M KCl solution. Concentration in homogenate of liver slices was measured as described above. The medium was centrifuged immediately and 1 ml of the supernatant was used to determine medium concentration.

**Uptake by Liver Cell Suspensions**—Liver cell suspensions were prepared according to the method of Takeda.<sup>8)</sup>

**PAEB:** 5 g of liver cell suspensions were incubated in 20 ml of 47.0 nmoles/ml (20  $\mu$ g/ml) PAEB solution at 37° in an atmosphere of 95% O<sub>2</sub>–5% CO<sub>2</sub>. At various times 2 ml of the suspensions were removed and the cells were immediately separated by centrifugation at 2500 rpm for 3 min. The determination of PAEB in medium was done by the same method as in the experiment by liver slices and the concentrations in cells were calculated as well. Results were expressed as cell/medium (C/M) concentration ratios.

**Quinine:** 15 g of liver cell suspensions were incubated in 120 ml of 2.5 nmoles/ml (1  $\mu$ g/ml) quinine solution at 37° in an atmosphere of 95% O<sub>2</sub>–5% CO<sub>2</sub>. At various times 30 ml of the suspensions were removed and centrifuged at 3000 rpm for 5 min. Quinine in cells and medium was determined by the same method as in the experiment by liver slices.

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5) V.H. Saggars, N. Hariratnajoithi, and A.E. McLean, *Biochem. Pharmacol.*, **19**, 499 (1970).

6) B.B. Brodie, J.E. Baer, and L.C. Craig, *J. Biol. Chem.*, **188**, 567 (1951).

7) H.M. Solomon and L.S. Schanker, *Biochem. Pharmacol.*, **12**, 621 (1963).

8) Y. Takeda, A. Ichihara, H. Tanioka, and H. Inoue, *J. Biol. Chem.*, **239**, 3590 (1964).

**Binding to the Liver Compounds**—Rat liver cytoplasmic fraction was obtained by the method of Takada.<sup>9)</sup>

Liver lipids were prepared by Folch's method.<sup>10)</sup> Male Wistar rats were anesthetized. The liver was perfused through the portal vein with saline, removed and homogenized with an equal volume of chloroform-methanol (2:1). The homogenate was immersed in 200 ml of chloroform-methanol at 25–28° for 2 hours and successively in a refrigerator over night. After filtration the residue was again immersed in 150 ml of the chloroform-methanol for 3–4 hours and filtered. The first and the second filtrate were put together and washed with 1/5 volume of 1% NaCl solution. The chloroform-methanol layer was taken and evaporated at 40°. The resultant lipids were dispersed in 0.01 M phosphate buffer pH 7.4 (7 mg/ml).

The binding of various compounds to the liver components was studied with equilibrium dialysis according to the method of Takada,<sup>9)</sup> using about 50 µg/ml solution of each compound.

In order to investigate the effect of enzyme treatment on the binding of PAEB to the liver cytoplasmic fraction, 1 ml of the liver cytoplasmic fraction was incubated with 0.5 ml of one of the following enzyme solutions in 0.01 M phosphate buffer pH 7.4, trypsin (0.6 mg/ml, 0.3 mg/ml), papain (0.3 mg/ml), lipase (0.3 mg/ml), deoxyribonuclease (0.3 mg/ml) and ribonuclease (0.3 mg/ml), for 2 hours at 4°. After incubation with the enzyme, the cytoplasmic fraction was subjected to the equilibrium dialysis against PAEB solution as before.

**Gel Chromatography**—A 10 µmoles/300 g PAEB was administered to an anesthetized rat intravenously. After 30 min the liver was perfused with saline and removed. The liver cytoplasmic fraction was prepared by the same method previously described and eluted from a Sephadex column with 0.01 M phosphate buffer pH 7.4. Approximately 5 ml of fractions were collected and monitored for protein and PAEB at 280 mµ and 550 mµ after the color reaction, respectively.

**Ultrafiltration**—The liver cytoplasmic fraction containing PAEB was obtained by the same preparation as that in gel chromatography. Half of the cytoplasmic fraction was subjected to ultrafiltration with Diafilter® G01T and the filtrate was colored to detect PAEB and measured at 550 mµ. The rest of the cytoplasmic fraction was directly colored and the absorbance at 550 mµ was measured.

## Results

### Hepato-biliary Transport Characteristics *in Vivo*

Although the hepato-biliary transport of PAEB and APAEB has been already investigated,<sup>11)</sup> the characteristics of both organic cations *in vivo* were studied under our experimental condition in rats.

Concentrations of PAEB and its metabolite APAEB in plasma, liver and bile after an intravenous injection of 10 µmoles/300 g PAEB are shown in Fig. 1 and the liver/plasma (L/P), the bile/liver (B/L) and the bile/plasma (B/P) concentration ratios calculated from the concentrations are shown in Table I. The L/P concentration ratio of total PAEB was 8.6. The B/L ratios were 36 for free PAEB and 8.1 for the conjugated form. Thus it was confirmed that PAEB was taken up against a concentration gradient from plasma to liver and the excretion of free and conjugated PAEB from liver to bile also occurred by a concentrative transfer.

When 20 µmoles/300 g quinine, the organic tertiary amine, was injected at 5 min before PAEB administration, the biliary excretion of PAEB was markedly depressed. As the total PAEB concentrations in plasma and liver were increased by administration of quinine, its B/L ratio was exceedingly decreased from 13.6 to 1.1 and the B/P ratio from 118 to 6.7. But the L/P ratio was decreased only from 8.6 to 6.4. This result seems to indicate that quinine mainly inhibited the transport process from liver to bile rather than from blood to liver.

Twenty µmoles/300 g isopropamide, organic quaternary ammonium compound, also depressed the biliary excretion of PAEB. Although the concentration in plasma was increased, the concentration in liver was not increased in this case. The fact that the L/P ratio was decreased from 8.6 to 3.6 suggests that isopropamide seems to inhibit the uptake process of PAEB into liver stronger than quinine.

9) K. Takada, M. Ohno, and S. Muranishi, *Chem. Pharm. Bull.* (Tokyo), **22**, 1477 (1974).

10) J. Folch, M. Lees, and G.H. Sloane-Stanley, *J. Biol. Chem.*, **226**, 497 (1957).

11) a) D.K.F. Meijer, E.S. Bos, and K.J. van der Laan, *Europ. J. Pharmacol.*, **11**, 371 (1970); b) S.W. Hwang and L.S. Schanker, *Am. J. Physiol.*, **225**, 1437 (1973).

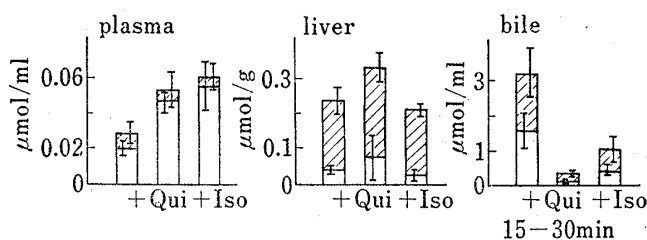


Fig. 1. Concentrations of PAEB in Plasma, Liver and Bile after Administration of 10  $\mu$ moles/300 g PAEB

□: free form, ▨: conjugated form, +Qui: 20  $\mu$ moles/300 g quinine was administered before PAEB injection. +Iso: 20  $\mu$ moles/300 g isopropamide was administered before PAEB injection. Concentrations in plasma and liver are at 30 min after PAEB administration. Results are expressed as mean  $\pm$  S.D. of at least 3 animals.

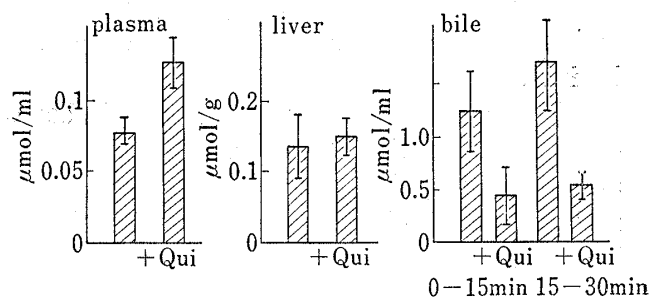


Fig. 2. Concentrations of APAEB in Plasma, Liver and Bile after Administration of 8.8  $\mu$ moles/300 g APAEB

+Qui: 20  $\mu$ moles/300 g quinine was administered before APAEB injection. Concentrations in plasma and liver are at 30 min after APAEB administration. Results are expressed as mean  $\pm$  S.D. of at least 3 animals.

TABLE I. Concentration Ratios of PAEB

	Liver/Plasma ratio	Bile/Liver ratio	Bile/Plasma ratio
PAEB (total)	8.6 $\pm$ 2.6	13.6 $\pm$ 4.2	118 $\pm$ 48
+quinine	6.4 $\pm$ 1.2	1.1 $\pm$ 0.1	6.7 $\pm$ 0.8
+isopropamide	3.6 $\pm$ 0.4	4.9 $\pm$ 1.0	17.8 $\pm$ 5.4

Results were calculated from the same data as shown in Fig. 1 and expressed as mean  $\pm$  S.D.

TABLE II. Concentration Ratios of APAEB

	Liver/Plasma ratio	Bile/Liver ratio	Bile/Plasma ratio
APAEB	1.7 $\pm$ 0.6	13.4 $\pm$ 3.8	21.6 $\pm$ 0.6
+quinine	1.2 $\pm$ 0.1	3.6 $\pm$ 0.4	4.2 $\pm$ 0.5

Results were calculated from the same data as shown in Fig. 2 and expressed as mean  $\pm$  S.D.

Hepato-biliary transport characteristics of APAEB, non-metabolizing organic quaternary cation, were examined in the same manner. Fig. 2 and Table II show the concentrations in plasma, liver and bile and the concentration ratios after administration of 8.8  $\mu$ moles/300 g APAEB. The small L/P ratio, 1.7, was obtained, while the B/L ratio was 13.4, comparable value with PAEB. The L/P ratio of APAEB was far smaller than that of PAEB, 8.6. Therefore APAEB seems to be more difficult to enter liver cells than PAEB.

Twenty  $\mu$ moles/300 g quinine sufficiently inhibited the biliary excretion of APAEB as shown in Fig. 2. The inhibition resulted in a marked decrease in the bile concentration of APAEB but an increase in the plasma concentration. As the decrease in the B/L ratio, from 13.4 to 3.6, was great as compared with that in the L/P ratio, from 1.7 to 1.2, quinine also seems to inhibit mainly the excretory process from liver to bile.

Fig. 3 and Table III are hepato-biliary transport characteristics of quinine and its metabolites after administration of 5  $\mu$ moles/300 g quinine. Although the concentration in bile was lower than that in liver, quinine showed a concentrative transport from plasma into bile as previously mentioned.<sup>3b)</sup> The B/P ratio of quinine, about 20, was not so high but the L/P ratio, 38.5, was extremely high compared with PAEB and APAEB. On the other hand, as for metabolites, although some part diffused back into plasma, most of them was excreted into bile against a concentration gradient, the B/L ratio, 39.8.

During 30 min, about 8% of the administered quinine was recovered in bile, most of which was in metabolized form, as shown in Fig. 3, in consequence of the intense metabolism in liver.

When 15  $\mu\text{mol}/300\text{ g}$  PAEB was injected before administration of quinine, the biliary excretion of quinine was inhibited. The B/P ratio of quinine was decreased from 19.7 to 14.1 and the B/L ratio of metabolites was decreased from 49 to 14. This decrease in the B/L ratio of metabolites suggests that quinine metabolites share the excretory pathway with PAEB, nevertheless their structures are unknown.

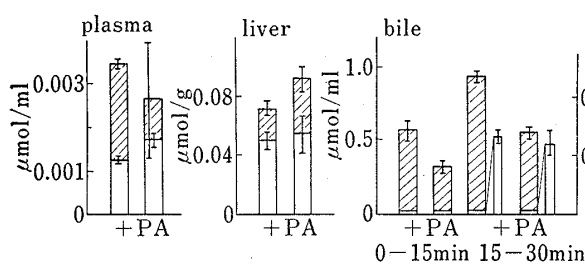


Fig. 3. Concentrations of Quinine in Plasma, Liver and Bile after Administration of 5  $\mu\text{moles}/300\text{ g}$  Quinine

□: quinine, ▨: quinine metabolites, +PA: 15  $\mu\text{moles}/300\text{ g}$  PAEB was administered before quinine injection. Results are expressed as mean  $\pm$  S.D. of at least 3 animals.

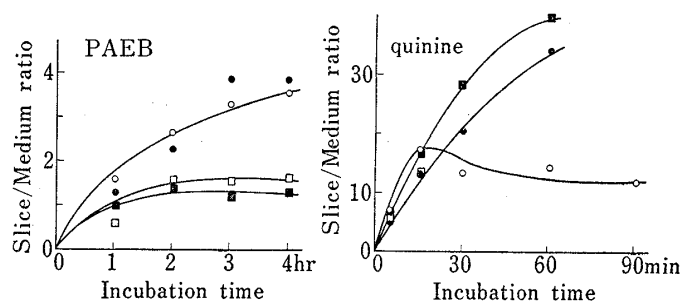


Fig. 4. Uptake of PAEB and Quinine by Liver Slices

Left: Uptake of PAEB (47.0 nmoles/ml) at 37° in Krebs-Ringer phosphate solution ○ control, ●, □, ■ Quinine was added to the incubation medium, 50.3 nmoles/ml, 126 nmoles/ml, 252 nmoles/ml, respectively.

Right: Uptake of quinine (2.5 nmoles/ml) at 37° in Krebs-Ringer phosphate solution ○ control, □ 118 nmoles/ml PAEB was added to the incubation medium. ● 1 mM DNP was added to the incubation medium. ■ Incubation was performed under 100% nitrogen.

TABLE III. Concentration Ratios of Quinine

	Liver/Plasma ratio	Bile/Liver ratio	Bile/Plasma ratio
Quinine	38.5 $\pm$ 3.7	0.52 $\pm$ 0.07	19.7 $\pm$ 2.8
+PAEB	22.4 $\pm$ 5.6	0.65 $\pm$ 0.09	14.1 $\pm$ 4.0
Metabolites		39.8 $\pm$ 15.6	
+PAEB		14.1 $\pm$ 1.39	

Results were calculated from the data shown in Fig. 3 and expressed as mean  $\pm$  S.D.

### Uptake by Liver Slices

When liver slices were incubated with PAEB, a concentrative uptake was observed as shown in Fig. 4. A S/M ratio attained more than 1 after 1 hour and 3.6 after 4 hours. Accumulation of PAEB was not depressed by 50.3 nmoles/ml (20  $\mu\text{g}/\text{ml}$ ) quinine, but 126 nmoles/ml (50  $\mu\text{g}/\text{ml}$ ) and 252 nmoles/ml (100  $\mu\text{g}/\text{ml}$ ) quinine markedly depressed 4 hour S/M ratio of PAEB from a value of 3.6 to 1.5.

In contrast to PAEB, uptake of quinine by liver slices was very large as shown in Fig. 4. A S/M ratio attained already 7 after 5 min and 17 after 15 min. After then a S/M ratio began to decrease due to the metabolism of quinine. Although 118 nmoles/ml (50  $\mu\text{g}/\text{ml}$ ) PAEB slightly depressed the S/M ratio of quinine after 15 min, the S/M ratio still show more than a value of 10. In an atmosphere of 100% nitrogen the accumulation of quinine was not affected after 5 and 15 min and the S/M ratio went on rising thereafter because of lack of the metabolism. A 1 mM DNP may slightly affect the S/M ratios after 5 and 15 min, but thereafter a S/M ratio continued to increase as well.

The uptake of PAEB by liver slices has been shown to be depressed under 100% nitrogen or in the presence of DNP and to involve at least partly an active transport process.<sup>7)</sup> From

the fact that quinine depressed the uptake of PAEB to the S/M ratio of about 1.5, it is presumed that quinine is also taken up by an active transport process. Quinine, however, seems to be taken up mainly by a different process which is energy independent, because there exists a large part in which uptake of quinine by liver slices is not affected by PAEB, an atmosphere of 100% nitrogen and DNP.

### Uptake by liver Cell Suspensions

To study uptake process from blood to the liver, another *in vitro* technique, liver cell suspensions, was employed. Uptake of PAEB by liver cell suspensions was very small as expressed as C/M concentration ratio as shown in Fig. 5. The C/M ratio gradually increased with time but did not attain one even after 4 hours.

Comparing with PAEB, quinine was taken up to a large extent (Fig. 5). The C/M ratio instantaneously attained 20 and increased still further. The different uptake patterns obtained by liver cell suspensions between PAEB and quinine also indicate that quinine is taken up into liver cells mainly by the different process from PAEB.

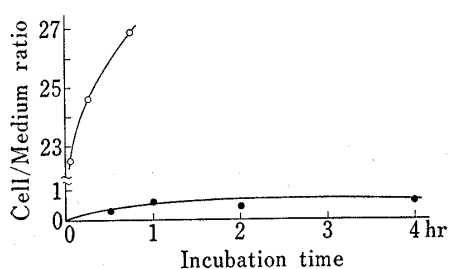


Fig. 5. Uptake of PAEB and Quinine by Liver Cell Suspensions

- : PAEB Initial concentration was 47.0 nmoles/ml.
- : Quinine Initial concentration was 2.5 nmoles/ml.

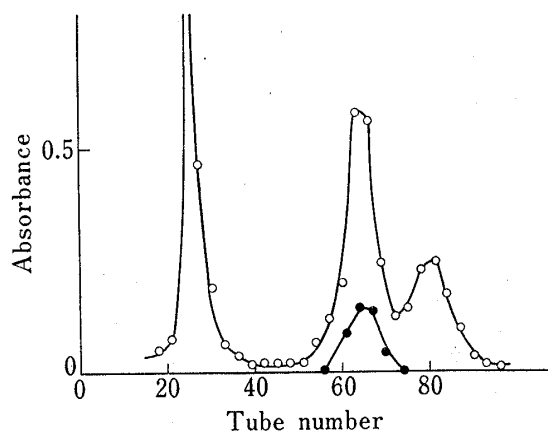


Fig. 6. Elution Pattern of PAEB from the Rat Liver Cytoplasmic Fraction

At 30 min after *i.v.* administration of 10  $\mu$ moles/300 g PAEB, the liver was perfused with saline, removed and homogenized. After centrifugation of homogenate at 100000  $g$  for 2 hours, 5 ml of the resultant supernatant was applied to a Sephadex G-50 column and eluted. Proteins were monitored at 280  $m\mu$  and PAEB at 550  $m\mu$  after the color reaction.

○—○: 280  $m\mu$ , ●—●: 550  $m\mu$

TABLE IV. Binding to the Liver Cytoplasmic Fraction and the Extracted Lipids

Compounds	% bound	
	The cytoplasmic fraction	Lipids
Bromthymol blue	77.0 $\pm$ 0.4 <sup>a)</sup>	80.2 $\pm$ 5.8
Bromphenol blue	64.1 $\pm$ 1.0 <sup>a)</sup>	28.4 $\pm$ 5.3
Amaranth	52.9 $\pm$ 1.8 <sup>a)</sup>	0
Tartrazine	35.9 $\pm$ 1.6 <sup>a)</sup>	6.0 $\pm$ 1.3
PAAH	15.8 $\pm$ 4.9 <sup>a)</sup>	0
PAEB	36.3 $\pm$ 2.3	7.1 $\pm$ 2.5
Quinine	14.5	77.4 $\pm$ 3.4

a) Results are borrowed from ref. 9.

The binding percentage of quinine to the cytoplasmic fraction is the mean of 2 experiments. The others are expressed as mean  $\pm$  S.D. of 3—4 experiments.

### Binding to Liver Components

In studies of organic anions which are actively transported from blood into bile, it has been shown that the binding to the liver cytoplasmic fraction plays important roles in the hepatic uptake process of organic anions like bromphenol blue (BPB).<sup>9)</sup> Therefore the binding percentage of PAEB and quinine to the liver cytoplasmic fraction and whole liver lipids were compared with organic anions as shown in Table IV. The 36% of PAEB and 15% of quinine were bound to the cytoplasmic fraction. These values seem rather low as compared with organic anions. Concerning binding to liver lipids, quinine showed extremely high binding percentage, 77%, while PAEB only 7%. From this high binding of quinine to lipids, it is suggested that in the case of quinine, lipid binding may be responsible for hepatic high uptake.

### Gel Filtration

When the liver cytoplasmic fraction after administration of PAEB *in vivo* was eluted through a Sephadex G-50 column, PAEB was recovered at fraction numbers 56—74, where a peak of 280 m $\mu$  absorbance was also observed (Fig. 6). Since PAEB itself shows absorbance at 280 m $\mu$ , in order to examine whether PAEB interacts with any protein in the cytoplasmic fraction, only PAEB was eluted through this column. Consequently PAEB by itself appeared just at the same fractions as that in the cytoplasmic fraction. Accordingly it is uncertain from this experiment whether PAEB is bound to any component in the cytoplasmic fraction or not, but will be discussed later.

### Discussion

From the gel filtration of cytoplasmic fraction an obvious result was not obtained whether PAEB was bound to macromolecules in it. To make clear that point, the effect of enzyme treatment on the binding of PAEB to the cytoplasmic fraction was examined. After incubation with trypsin, papain, lipase, deoxyribonuclease and ribonuclease, the binding of PAEB to the cytoplasmic fraction examined with equilibrium dialysis was not affected significantly by any of these enzymes. In an experiment of ultrafiltration of the cytoplasmic fraction, the absorbance at 550 m $\mu$  of the filtrate was almost identical with that of non-filtered one. If PAEB had been bound to macromolecules in the cytoplasmic fraction, the absorbance of the filtrate would have been smaller than that of non-filtered one. Furthermore when the cytoplasmic fraction from a PAEB administered rat was eluted through a Sephadex G-25 column, peaks of PAEB and total PAEB appeared a little separately, indicating that PAEB and APAEB were eluted separately according to the slight difference of their molecular weights. From these results, it is not considered, consistently with other report,<sup>12)</sup> that PAEB is bound to organic anion binding macromolecular components in the cytoplasmic fraction.

PAEB, APAEB and quinine should be classified into B/P ratio=10—100 type compound, which is actively excreted into bile. Compounds in this type have been further classified into the following two categories according to their L/P ratios by Takada.<sup>13)</sup>

- (i) bile level > liver level > plasma level (BPB type)
- (ii) bile level > plasma level > liver level (PAAH type)

This distinction may be due to the hepatic uptake process by the liver parenchymal cells. PAEB and APAEB belong to type (i) and quinine including metabolites also the category of type (i). Unmetabolized quinine, however, was observed to be very high value of L/P ratio *in vivo*, exceeding over bile level. Such figures have not been seen in the biliary excretion of organic anions. The reason for such a high hepatic uptake from plasma was investigated.

12) D.K.F. Meijer, J. Wester, and M. Gunnink, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **273**, 179 (1972).

13) K. Takada, Y. Mizobuchi, and S. Muranishi, *Chem. Pharm. Bull.* (Tokyo), **22**, 922 (1974).

Quinine was taken up both by liver slices and liver cell suspensions to a large extent and attained to a value of about 20 of S/M or C/M ratio. S/M ratio of quinine decreased notably neither in an atmosphere of nitrogen nor in the presence of DNP. Organic anions are known to be taken up in a similar manner and the binding to proteins in the cytoplasmic fraction is considered a factor participating in the uptake process of organic anions. Accordingly tissue binding could be a reasonable uptake factor of quinine. However the degree of binding to the cytoplasmic fraction was 15% and binding to X, Y and Z proteins was not observed by Sephadex gel filtration, either, though data are not shown. On the other hand, the degree of binding to liver lipids was very high, 77%, indicating that lipid-binding like mechanism seems to largely contribute to the uptake process of quinine.

As for PAEB, a concentrative uptake by liver cell suspensions prepared by a chelator perfusion technique was not observed. Although the same uptake experiment was also performed by cell suspensions prepared by an enzymatic digestion technique, the same result was obtained. On the contrary uptake of PAEB by liver slices occurred against a concentration gradient. If tissue binding to any components is responsible for uptake process of PAEB, possibly a concentrative uptake by cell suspensions would be observed like BPB and quinine, and intensive decrease of S/M value to about one by anoxia or metabolic inhibitors, which has been shown to occur by Solomon,<sup>7)</sup> would not be observed by liver slices. Therefore it is thought that a mechanism other than tissue binding *e.g.* an energy-dependent accumulation, which was impaired on account of manipulation in preparing cell suspensions, operates in the concentrative uptake of PAEB.

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