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Biopharmaceutical Study of the Hepato-biliary Transport of Drugs. II.[1] Roles of the Liver Cytoplasmic Y and Z Binding Proteins and T Binder on the Hepato-biliary Transport of Organic Anionic Compounds

Kanji Takada, Masanori Ueda, Mariko Ohno and Shozo Muranishi

Faculty of Pharmaceutical Sciences, Kyoto University[2]

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The roles of the rat liver cytoplasmic Y and Z binding proteins and T binder on the hepato-biliary transport of some organic anionic compounds, four non-metabolizing sulfonic acid dyes, bromphenol blue (BPB), bromthymol blue (BTB), amaranth (AM) and tartrazine (TZ) and a non-metabolizing carboxylic acid, *p*-acetylaminohippuric acid (PAAH) which are excreted into bile by an active transport system were investigated. No free form of these compounds was detected in the rat liver cytoplasmic fraction by means of the gel filtration of the liver 100000 g (av.) supernatant using a column of Sephadex G-75 following the intravenous administration of these compounds, and these compounds were in common bound to Y binding protein. In addition BPB and BTB of which the liver/plasma concentration ratios were greater than one were bound to Z binding protein but AM, TZ and PAAH of which the liver/plasma concentration ratios were equal or less than one were not bound to Z binding protein. When sulfobromophthalein, 5 times molar ratio to BPB, was pre-administered to rats, the binding of BPB to Y binding protein was decreased 65% but the binding to Z binding protein was reversely increased 27%. Kinetical experiment showed that the binding of BPB to Y binding protein followed a hyperbolic curve and reached maximum at 15 min after the administration whereas the binding to Z binding protein was more slowly increased.

In our previous report, basic principles for the hepato-biliary transport of organic anionic compounds were studied using bromphenol blue (BPB) and *p*-acetylaminohippuric acid (PAAH) which were not metabolized in the rat liver as model compounds and it was suggested that there exist at least two steps in the hepato-biliary transport of these compounds, namely the uptake by the liver from the blood stream and the secretion from the liver into bile and that the former appears to be due to the protein binding-like mechanism. Although the mechanism for the hepatic uptake of organic anionic compounds has not perfectly become clear, recently many reports have been published about the liver cytoplasmic binding proteins which are believed to play important roles on the hepatic uptake of organic anions. In 1967 Ketterer, *et al.* isolated two hepatic azo-carcinogen binding proteins, basic azo-dyebinding protein and small molecular one.[3] Next, Litwack, *et al.* isolated and studied the properties of two cortisol metabolites binding proteins, large binder and small binder.[4] And it was identified that the basic azo-dyebinding protein and the large cortisol metabolites binder were the same protein.[5] On the other hand, Arias, *et al.* studied the roles of the liver cytoplasmic organic anion binding protein fractions, X, Y and Z, on the hepatic uptake of bilirubin and BSP, *etc.*[6] After that they actively studied about this problem, phylogenetic study of

1) Part I: K. Takada, Y. Mizobuchi and S. Muranishi, *Chem. Pharm. Bull.* (Tokyo), **22**, 922 (1974).
2) Location: *Yoshida-shimoadachi-cho, Sakyo-ku, Kyoto.*
3) B. Ketterer, P. Ross-Mansell and J.K. Whitehead, *Biochem. J.*, **103**, 316 (1967).
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organic anion transfer from plasma into the liver,[7] effects of drugs, chemicals, hormones and cholestatics on Y and Z binding proteins[8] and the hormonal control of hepatic bilirubin transport and conjugation.[9] In 1971 it was demonstrated that the three binding proteins, basic azo-dyebinding protein, large cortisol binder and Y binding protein, were the same protein, which was named as "Ligandin" by Litwack, Ketterer and Arias.[10] Though these liver cytoplasmic binding proteins were made clear partly in their physico-chemical properties, their functions have not been so clarified yet. About Y binding protein, it was suggested by Arias that it acts as a hepatic pool in the liver parenchymal cells when a number of exogeneous organic anions are entrapped by the liver.[6] With respect to the roles of the other liver cytoplasmic binding proteins on the hepato-biliary transport of drugs, no paper has appeared up to date. Furthermore, recently, it has become clear that the liver cytoplasm play an important role when the metabolites of hepato-carcinogen, 3,4-benzpyrene and 3-methylcholanthrene[11] and unchanged and conjugated procaine amide ethobromide[12] are excreted into bile.

The purpose of this report is to show that the liver cytoplasm play an important role on the hepato-biliary transport of some organic anions that are actively excreted into bile and to clarify the roles of the liver cytoplasmic binding proteins on the hepato-biliary transport of these compounds.

Experimental

Materials——Bromphenol blue (BPB), bromthymol blue (BTB), tartrazine (TZ) and amaranth (AM) were purchased from Nakarai chemicals Co., Ltd. and brilliant blue (BB), new coccine (NC), and light green SF (LG) were purchased from Tokyo-kasei chemical industries Co., Ltd.

Analytical Methods——Bromthymol Blue in Plasma: A blood sample was diluted with an equal volume of saline and was centrifuged for 20 min at 3000rpm. To 0.5 ml of the resulting supernatant, 3 ml of pH 11.5 phosphate buffer solution, 5 ml of isoamylalcohol and 1.5 g of NaCl were added and the mixture was shaken for 10 min and was centrifuged for 10 min at 3000 rpm. To the resulting isoamylalcohol phase, one piece of sodium hydroxide was added and the mixture was shaken for 10 min and was centrifuged for 10 min at 3000 rpm and immediately the optical density of the resulting isoamylalcohol phase was measured at 620 mμ.

Bromthymol Blue in the Liver: The liver being homogenized in twice its weight of saline, 6 ml of acetone was added to this homogenate and the mixture was shaken for 15 min and was centrifuged for 20 min at 3000 rpm. To 6 ml of the resulting supernatant, 2 ml of 0.1N NaOH solution was added and the mixture was shaken for 15 min and was centrifuged for 20 min at 3000 rpm. The resulting supernatant was recentrifuged for 20 min at 3000 rpm and the optical density of the resulting supernatant was measured at 620 mμ.

Bromthymol Blue in Bile: The dye content of a bile sample was determined by diluting the sample with 0.1N NaOH solution to a suitable volume and reading the optical density at 620 mμ.

Tartrazine in Plasma: A blood sample was diluted with an equal volume of saline and was centrifuged for 20 min at 3000 rpm. To the resulting supernatant, 3 ml of distilled water, 1 ml of 40% zinc sulphate solution and 1 ml of 11.2% potassium hydroxide solution were added according to the method of O'Reilly, *et al.*,[13] and the mixture was centrifuged for 20 min at 3000 rpm after shaken for 15 min. The optical density of the resulting supernatant was measured at 427 mμ.

Tartrazine in the Liver: After the liver was homogenized in twice its weight of saline, 6 ml of acetone was added to 5 ml of the homogenate and the mixture was centrifuged for 20 min at 3000 rpm after shaken for 15 min. To the resulting supernatant, 1 ml of 40% zinc sulphate solution and 1 ml of 11.2% potassium hydroxide solution were added, and after shaking for 15 min the mixture was centrifuged for 20 min at 3000 rpm. The optical density of the resulting supernatant was measured at 427 mμ.

Tartrazine in Bile: A bile sample was diluted with distilled water to a suitable volume and the optical density was measured at 427 mμ.

7) R.I. Levine, H. Reyes, A.J. Levi, Z. Gatmaitan and I.M. Arias, *Nature New Biology*, **231**, 277 (1971).
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Amaranth in Plasma and in the Liver: According to the method of O'Reilly, *et al.*,[14] amaranth contents in plasma and in the liver were measured.

Amaranth in Bile: The liver was homogenized in twice its weight of phosphate buffer solution (pH 7.4) and the homogenate was centrifuged for 20 min at 3000 rpm. The optical density of the resulting supernatant was measured at 520 mμ.

Hepato-biliary Transport Experiments——At 30 min after the intravenously administration of these compounds, the liver and blood of rats, male Wistar rats 250—350 g, were removed and bile samples were collected for three 10 min periods. Plasma level, liver level and bile level were measured with the methods mentioned above. (Details are shown in our previous report.[1])

Operational Methods for obtaining the Rat Liver Cytoplasmic Fraction——Male Wistar rat, 200—300 g, was anesthetized by the intraperitoneal injection of sodium pentobarbital. Mostly according to the methods of Arias, *et al.*,[6] operations were performed. After the liver was perfused with saline through the portal vein to remove the blood which was trapped in the liver, the liver was removed, homogenized to a 25% homogenate with 0.25M sucrose–0.01M phosphate buffer (pH 7.4) and the resulting homogenate was centrifuged at 100000 g (av.) for 2 hr in a Hitachi 55P ultra-centrifuge at 2°. After the surface lipids were removed, the supernatant fraction was carefully collected, and was used for the binding and the gel filtration studies.

Binding to the Liver Cytoplasmic Fraction——Using cells for equilibrium dialysis, the binding of various organic anions to the rat liver cytoplasmic fraction was measured according to the method which was described in our previous report.[1] BPB, BTB, TZ and AM were measured spectrophotometrically and PAAH was measured after colored by the same method described in our previous report.[1] In this case, the calibration curve of each compound was made in each experiment.

Gel Filtration Method with Sephadex G-75 (*in Vitro*)——To 5 ml of the liver cytoplasmic fraction, 0.1 ml of the BPB solution, 10 μmole/ml, was added and the mixture was incubated at 4° for 2 hr. After that it was applied to a column of Sephadex G-75 equilibrated with 0.01M phosphate buffer (pH 7.4) at room temperature. The column of which size was 2.5×45 cm was purchased from Pharmacia Fine Chemicals Co., Ltd. With upward flow system, elution was performed with the same buffer and the flow rate was 30—40 ml/hr. The following elution pattern was monitored spectrophotometrically: protein at 280 mμ and BPB at 600 mμ.

***In Vivo* Gel Filtration Experiment**——At 15 min after the compounds were administered to rat from femoral vein, the liver which had been perfused with saline was removed and was homogenized to a 25% homogenate. The liver cytoplasmic fraction was collected after centrifugation of the resulting homogenate at 100000 g (av.) for 2 hr and 5 ml of the liver cytoplasmic fraction was applied to a Sephadex G-75 column and was eluted as the same method mentioned above. The elution pattern was monitored spectrophotometrically: protein at 280 mμ, BPB at 600 mμ, TZ at 427 mμ, AM at 535 mμ, BSP at 580 mμ, BB at 630 mμ, NC at 535 mμ, BTB at 615 mμ (as the color of BTB bound to Y binding protein was yellow, optical density was measured at 410 mμ for Y binding protein fraction) and PAAH at 550 mμ after it was colored.

Results

In Fig. 1, the chemical structures of the compounds used in this report are presented. All these compounds have commonly one or more sulfonic acid groups and their molecular weights are greater than 325 which has been thought to be the minimum limit for the organic anionic compounds to be excreted into rat bile by Smith.[15] Though AM and NC have the same molecular weight, 604.6, their hepato-biliary transport characteristics are extremely different, namely the percentage recovery in bile for AM was about 72% for 2 hr[13] and that of NC was 12.3% for 4 hr.[16] In the same manner, BB has a large affinity to the hepato-biliary transport system than LG in spite of the same molecular weight (792.9); the percentage recovery of BB and LG in bile for 4 hr was 96% and 21%, respectively.[16] About the hepatic metabolism, AM[17] and TZ[18] are known to be excreted into rat bile as unchanged forms and BTB is also a non-metabolizing compound, because no metabolite was detected in rat bile

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15) R.D. Hughes, P. Millburn and R.L. Smith, *Biochem. J.*, **128**, 144p (1972).
16) T. Iga, S. Awazu, M. Hanano and H. Nogami, *Chem. Pharm. Bull.* (Tokyo), **18**, 2431 (1970).
17) A.J. Ryan and S.E. Wright, *J. Pharm. Pharmacol.*, **13**, 492 (1961).
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by the paper chromatographic analysis which had been used to detect the metabolites of BPB in our previous report.[1)]

In Table I, the hepato-biliary transport characteristics of the major compounds used in this report are listed. About BPB and PAAH, more detailed investgation had been done in our previous report. Since with respect to BTB the plasma disappearance was so rapid that the plasma level at 30 min after the intravenous administration (10 μmole/300 g body

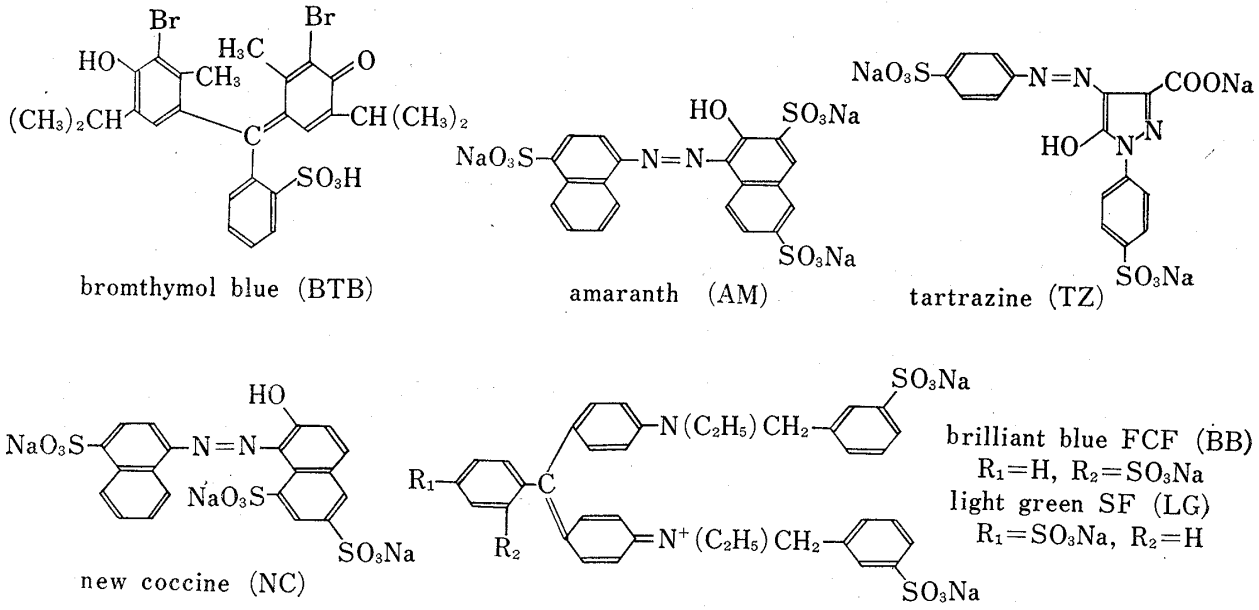

Fig. 1. Chemical Structures of the Compounds used

In addition to these compounds, bromphenol blue (BPB), sulfobromophthaleinsodium (BSP) and *p*-acetylaminohippuric acid (PAAH) which had been used in our previous study were used.

TABLE I. Hepato-biliary Transport Characteristics

Compounds[a)]	Dose[b)] (μmole)	Time[c)] (min)	Plasma level (μg/ml)	Liver[d)] level (μg/g)	Bile level (μg/ml)	B/P ratio[e)]	L/P ratio[f)]	B/L ratio[g)]	% recovery in bile
Bromthymol blue (BTB)[h)]	10	5	59±7	247±28	—	—	4.8±1.7	—	—
	10	30	—	93±32	11600±3500	∞	∞	125±24	—
Bromphenol blue (BPB)[i)]	5	30	3.6±0.6	80±10	3372±718	894±99	22±5	42±8	55.2±6.5 (30 min)
Amaranth (AM)	10	30	20.1±7.0	19±2	8282±725	456±128	1.09±0.34	424±26	72(2hr)[j)]
Tartrazine (TZ)	10	30	122±22	3.7±0.6	534±64	4.7±1.5	0.03±0.01	148±21	7.9±1.3 (30 min)
p-Acetyl-aminohippuric acid (PAAH)[i)]	6	30	11.4±3.5	5.4±1.6	570±74	43±15	0.47±0.27	126±30	21.4±3.0 (30 min)

a) These compounds being administered to rats, the liver and blood were removed at the definite time and bile samples were collected for three 10 min periods except BTB.
b) μmole/300 g rat body weight
c) At this time the liver and blood were removed.
d) μg/the liver gram wet weight
e) bile/plasma concentration ratio
f) liver/plasma concentration ratio
g) bile/liver concentration ratio
h) The uptake of BTB by the liver was so rapid that the plasma level of BTB at 30 min after the administration was negligibly low. At 5 min, the plasma level was measured though bile level could not be measured because of less sample volume.
i) These data are cited from our previous report.[1)]
j) Data from O'Reilly, *et al.*[14)]
Results are mean±S.E. for four to six rats for each compound.

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weight) could not be measured, the plasma level at 5 min after the administration was measured. But in this case the bile level of BTB could not be correctly measured because of less sample volume and of more contribution of the lag time. As the B/P, L/P and B/L ratios of BTB are by far greater than one, it is thought that BTB belongs to the "BPB type" compounds of which the characteristics are shown as follows:

bile level>liver level>plasma level

On the other hand, though AM and TZ are thought to be excreted into bile by an active transport system because of their large B/P ratios, their L/P ratios were less than one or equal to one. Then it is thought that both AM and TZ belong to the "PAAH type" compounds of which the characteristics are shown as follows:

bile level>plasma level≧liver level

Thinking that this difference between the two types is due to the states of compounds in the liver, the bindings of these compounds to the liver cytoplasmic fraction was measured. (Table II.) From this Table, it becomes clear that the percentages bound to the liver cytoplasmic fraction of "BPB type" compounds are greater than that of "PAAH type" compounds.

Table II. Binding to the Rat Liver Cytoplasmic Fraction

Compounds	% bound[a)]
Bromthymol blue	77.7±0.4
Bromphenol blue	64.1±1.0
Amaranth	52.9±1.8
Tartrazine	35.9±1.6
p-Acetylaminohippuric acid	15.8±4.9

a) Using cells for equilibrium dialysis, the binding of these compounds to the rat liver cytoplasmic fraction, the supernatant after the centri fugation of the 25% liver homogenate at 100000 g (av.) for 2 hr, was measured. The cell was shaken during two days at 4°. Each value is the mean S.E. for four to six rats.

However in "PAAH type" compounds, the fact that the percentage bound to the liver cytoplasmic fraction of AM was 52.9+1.8%, namely AM existed as a free form and a bound form at an equal ratio in the liver cytoplasmic fraction, consists with the fact that the L/P ratio of AM was nearly equal to one. For both TZ and PAAH, it is thought that their low percentages bound to the liver cytoplasm are related to their low L/P ratios. Then the affinity to the liver cytoplasmic fraction appears to be relative to L/P ratio which is an index for hepatic uptake. Next how much the liver cytoplasm level of BPB is changed by increasing the dose of BPB was examined. The whole liver levels of BPB at 30 min after the intravenously administration of BPB are cited from our previous report.[1)] In Table III, the whole liver levels and the liver cytoplasm levels of BPB are compared. From this table it is suggested

Table III. Comparison between the Whole Liver Level and the Liver Cytoplasm Level of BPB

Dose (μmole/300g)	Whole liver level[a)] ($\times 10^{-4}$ μmole/g w. wt.)	Liver cytoplasm level[b)] ($\times 10^{-4}$ μmole/ml)
5	1194±120	1064±80
10	2205±285	1476±396
20	2529±267	1936±644

a) These data are cited from our previous report.[1)]
b) At 15 min after the intravenous administration of BPB, 5, 10 and 20 μmole/300 g body weight, the liver was removed and was homogenized to a 25% homogenate. The homogenate was centrifuged at 100000 g (av.) for 2 hr and the BPB content of the resulting supernatant was measured. Results are mean±S.E. for three to five rats for each dose.

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that in these dose ranges the liver cytoplasm level of BPB have a tendency to become saturated as the whole liver level, and that the liver cytoplasm level reflects the major portion of the whole liver level of BPB.

The state of BPB in the liver cytoplasm, namely whether BPB exists in the liver cytoplasm as a bound form or a free form, was examined according to the method of Arias, *et al.*[5] After BPB (1 μmole) was added to 5 ml of the liver cytoplasmic fraction, the resulting mixture was applied to a Sephadex G-75 column by the use of flow adaptor after incubated for 2 hr at 4° and the effluent was collected per about 5 ml. Results are shown in Fig. 2 in which the first peak at 600 mμ appeared at the tube number 11. As this peak corresponds to the void volume, its protein concentration is very high and the optical density at 600 mμ which represents the existence of BPB was observed only a little. So, this peak of 600 mμ could be considered to be due to the turbidity of protein but not due to BPB. In the remaining tubes, the first and the second peaks are thought to be the Y and Z binding protein fractions respectively and the third peak is called T binder fraction in this report. However the last peak at the tube number 45—52 is thought to be due to the existence of the unbound BPB.

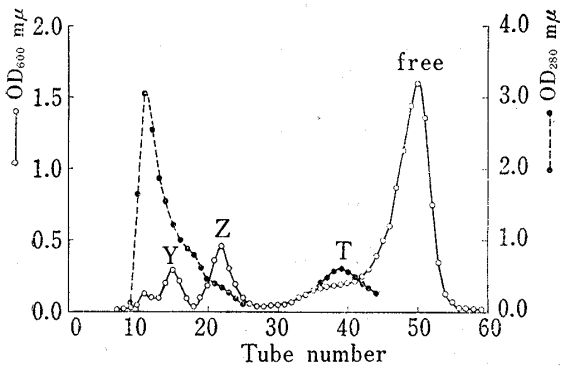

Fig. 2. Elution Pattern of BPB added to the Rat Liver Cytoplasmic Fraction (*in Vitro*)

BPB, 1 μmole, was added to the rat liver 100000 g (av.) supernatant and the mixture was incubated for 2 hr at 4° and was eluted from a column of Sephadex G-75. Collections were 5.4 ml/tube. Optical density at 280 mμ indicates protein and that at 600 mμ indicates BPB.

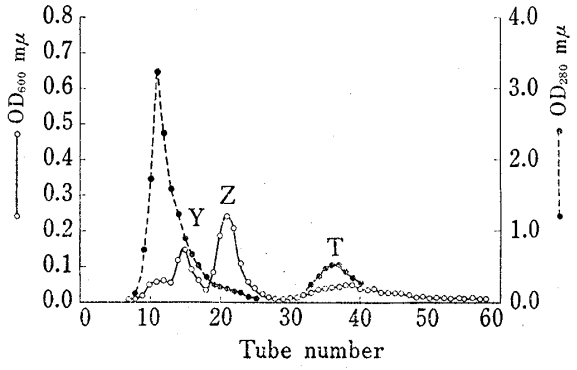

Fig. 3. Elution Pattern of BPB from the Rat Liver 100000 g (av.) Supernatant (*in Vivo*)

At 15 min after the *i.v.* administration of BPB, 5 μmole/300 g body weight, the liver was perfused with saline, removed, homogenized and the homogenate was centrifuged at 100000 g (av.) for 2 hr. 5 ml of the resulting supernatant was applied to a column of Sephadex G-75 and was eluted.

Also the binding of BPB to the liver cytoplasmic binding proteins was measured *in vivo*. At 15 min after BPB, 5 μmole/300 g body weight, was administered to rat from femoral vein, the liver was perfused with saline, was removed and was homogenized. And after the liver cytoplasmic fraction was obtained by ultra-centrifugation of the homogenate, gel filtration was performed using a column of Sephadex G-75 and the elution pattern is represented in Fig. 3. As shown in this figure, BPB was bound to Y and Z binding proteins and T binder respectively, but no unbound BPB was detected. As there is a great difference between *in vivo* and *in vitro* gel filtration experiments, the importance of *in vivo* experiment is suggested.

How BSP which was known to be bound to Y and Z binding proteins[6] influences the binding of BPB to these binding proteins was examined. BSP, 6 times molar ratio to BPB, being added to the liver cytoplasmic fraction, the bindings of BPB to the three binding proteins were perfectly inhibited by BSP all at once (Table IV).

However, different results were obtained *in vivo* experiment. When following BSP, 5 times molar ratio to BPB, BPB (5 μmole/300 g body weight) was administered to rats, the binding of BPB to Y binding protein was decreased about 65% compared to control but *vice versa* the binding of BPB to Z binding protein was increased 27% (Table V and Fig. 4).

Comparing the amounts of BSP and BPB bound to Y and Z binding proteins, it becomes clear that BSP is more bound to Y binding protein than BPB but BPB is more bound to

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Table IV. Effectof BSP on the Binding of BPB to the Liver Cytoplasmic Y and Z Binding Proteins and T Binder (*in Vitro*)

Condition	Amount of BPB bound to Y binding protein	Z binding protein	T binder
	($\times 10^{-4}$ μmole)		
100000 g sup. 5 ml +BPB 1 μmole	641.0	1121.3	882.4
100000 g sup. 5 ml +BPB 1 μmole +BSP 6 μmole[a)]	4.4	0.3	9.4

a) BSP 6 μmole and BPB, 1 μmole, were added to the rat liver 100000 g (av.) supernatant and the resulting mixture was incubated at 4° for 2 hr and was eluted from a column of Sephadex G-75. The amount of BPB bound to the liver cytoplasmic Y and Z binding proteins and T binder was measured spectrophotometrically.

Table V. Effect of BSP on the Hepatic Uptake of BPB (*in Vivo*)

Condition		Y binding protein	Z binding protein	T binder
		($\times 10^{-4}$ μmole/ml supernatant)		
BPB 5 μmole	BPB	364±30	689±78	—
BPB 5 μmole[a)]	BPB	127±19	870±172	—
BSP 25 μmole	BSP	951±110	261±4	624±96

a) At 15 min after BPB, 5 μmole/300 g body weight, was administered to rat from femoral vein following BSP, 25 μmole/300 g body weight, the liver was removed and was homogenized. The homogenate was centrifuged at 100000 g (av.) for 2 hr and 5 ml of the resulting supernatant was applied to a Sephadex G-75 column. After the elution, the amount of BPB bound to the rat liver cytoplasmic Y and Z binding proteins and T binder was measured. As the amount of BSP bound to them was measured spectrophotometrically, these values do not precisely represent the amount of BSP because BSP is metabolized in the liver. Results are mean±S.E. for three to five rats for each condition.

Z binding protein than BSP. The fact that the increased binding of BPB to Z binding protein by the pre-administration of BSP is thought to be able to explain our previous results that the whole liver level of BPB was increased by the pre-administration of BSP.[1)] In Fig. 5, the kinetics of the binding of BPB to Y and Z binding proteins is shown. When BPB, 5 μmole/300 g body weight, was administered to rats, at first BPB was bound to Y binding protein and this binding reached a peak at about 15 min after the intravenous administration of BPB. On the other hand, the binding of BPB to Z binding protein became superior to the binding to Y binding protein after 5 min and appeared to be increased step by step until 25 min.

The binding capacities of BPB to Y and Z binding proteins and T binder were also examined (Fig. 6). The amounts of BPB bound to Y and Z binding proteins and T binder at 15 min after the administration of BPB, 5, 10 and 20 μmole/300 g body weight, were measured. In this dose range, the binding of BPB to Y and Z binding proteins showed a tendency to become saturated, though the binding to T binder showed a linear relationship. The binding characteristics of the other compounds in the liver cytoplasm were studied. In Fig. 7, the elution patterns of BTB, TZ, AM, PAAH, NC and BB that were administered to rats from femoral veins from a Sephadex G-75 column are represented. In the liver cytoplasm, BPB was bound to Y and Z binding proteins, BTB bound to Y and Z binding proteins and T binder, AM bound to only Y binding protein, PAAH bound to Y binding protein and T binder, TZ and NC bound to only Y binding protein. In common all these compounds were bound to Y binding protein. To our surprise, the liver itself and the liver cytoplasmic fraction at 15 min after the intravenous administration of light green SF were colorless, though bile which was

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collected through a polyethylene tube was green. Therefore light green SF could not be detected spectrophotometrically in the liver cytoplasmic fraction. When one drop of light green SF solution was added to Y and Z binding proteins and T binder fraction, Y binding

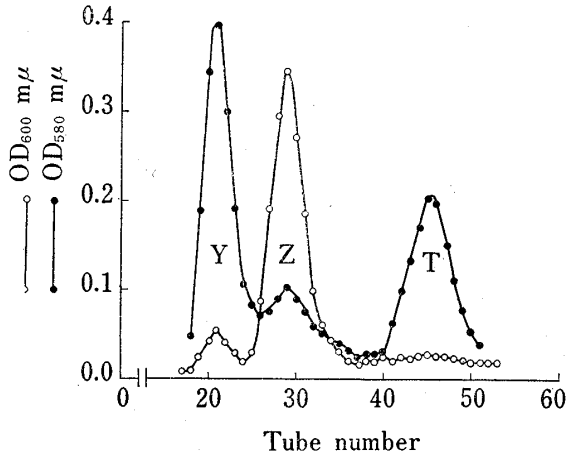
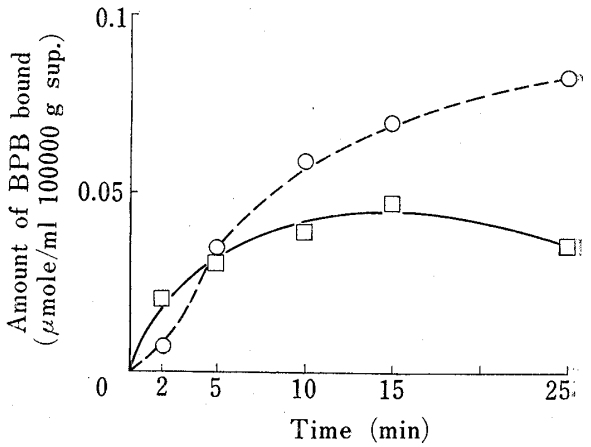


Fig. 4. Effect of BSP on the Bindings of BPB to the Liver Cytoplasmic Y and Z Binding Proteins and T Binder

At 15 min after BPB (5 μmole/ 300 g body weight) was administered to rat *i.v.* following BSP (25 μmole/300g body weight), the liver was removed. 5 ml of the supernatant which was obtained by the ultra-centrifugation of the liver 25% homogenate was appied to a Sephadex G-75 column and was eluted.



Eig. 5. Time-course of the Amount of BPB bound to the liver Cytoplasmic Binding Proteins Y and Z

At appointed times, 2, 5, 10, 15, and 25 min, after BPB was administered to rats, the livers were removed and the amounts of BPB bound to Y and Z binding proteins were measured by gel filtration method.
□——□: BPB bound to Y binding protein
○------○: BPB bound to Z binding protein

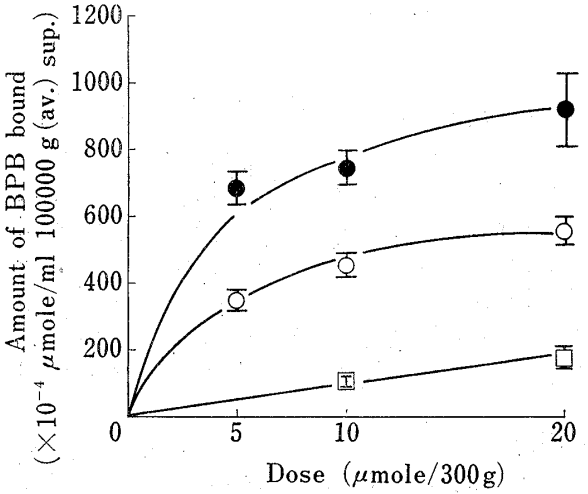


Fig. 6. Dose-dependency of the Binding of BPB to Y and Z Binding Proteins and T Binder

At 15 min after the administration of BPB, 5, 10 and 20 μmole/300 g body weight, the livers were removed and the amounts of BPB bound to the rat liver cytoplasmic Y and Z binding proteins and T binder were measured according to the usual method. Each point represents the mean of four or more animals±S.E.
○——○: BPB bound to Y binding protein
●——●: BPB bound to Z binding protein
□——□: BPB bound to T binder

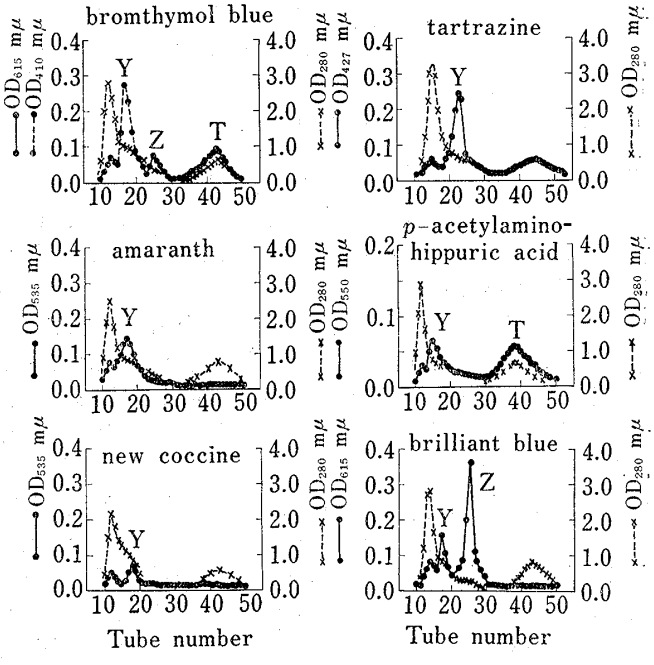


Fig. 7. Elution Patterns of BTB, TZ, AM, PAAH, NC and BB

At 15 min after these compounds (10 μmole/ 300 g) were administered *i.v.* to rats, the livers were removed, homogenized and were centrifuged. 5 ml of the resulting 100000 g (av.) supernatants was applied to a Sephadex G-75 column and was eluted. The compounds bound to the liver cytoplasmic binding protein fractions were detected spectrophotometrically: at 615 mμ for BTB, at 427 mμ for TZ, at 535 mμ for AM, at 550 mμ for PAAH, at 535 mμ for NC and at 615 mμ for BB. BTB bound to Y binding protein fraction was detected at 410 mμ because of its color shift.

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protein fraction was colorless though Z binding protein and T binder fractions became green. So, it was proved that light green SF was bound to only Y binding protein.

Discussion

As in our previous report the hepato-biliary transport of BPB and PAAH were studied from two steps, namely hepatic uptake from blood and secretion from the liver into bile, it was suggested that the hepatic uptake process probably depends on protein binding-like mechanism.[1] To clarify the uptake mechanism, non-metabolizing compounds were mainly used because intact drug and its metabolites are not always excreted into bile by the same transport system. For example, morphine ethereal sulfate and morphine glucuronide are typical case.[19] From the results of binding experiment (Table II), it becomes clear that there exists a relationship between the bindings to the liver cytoplasmic fraction and the *in vivo* L/P ratios. The compounds of which percentages bound to the liver cytoplasmic fraction are greater than 50%, BPB and BTB, considerably enter the liver and their L/P ratios become greater than one. However AM, TZ and PAAH of which the percentage bound to the liver cytoplasmic fraction were less than 50% do not so much enter the liver as BPB and BTB. When the liver cytoplasm levels of several compounds were studied from the viewpoint that the liver cytoplasm level is the major factor for the hepatic uptake of organic anionic compounds,[20,21] it was suggested that the liver cytoplasm level represents the major part of the whole liver level of BPB (Table III). As the metabolites of 3,4-benzpyrene and 3-methylcholanthrene were bound to the liver cytoplasm[11] and as unchanged and conjugated procaineamide ethobromide were highly concentrated in the liver cytoplasm,[12] BPB was also highly bound to the liver cytoplasmic fraction. So, it is thought that L/P ratio can be related to the binding ability to the liver cytoplasm.

In this report, the hepato-biliary transport characteristics of three organic anionic compounds, BTB, TZ and AM, that are actively excreted into bile were examined and it became clear that these compounds could be classified into two categories which were proposed by us previously.[1]

i) bile level$>$liver level$>$plasma level
ii) bile level$>$plasma level$\geqq$liver level

BTB and BPB belong to the upper class compounds, namely compounds pooled in the liver and rapidly appear into bile, but AM, TZ and PAAH belong to the lower class compounds, namely compounds not pooled in but rapidly pass through the liver. However, it is thought that this distinction can be explained with the binding ability to Y and Z binding proteins and T binder that are located in the liver cytoplasm. Though BTB was considerably bound to Z binding protein as BPB, AM and TZ were bound to only Y binding protein (Fig. 7). So, it is suggested that if a compound is bound to only Y binding protein in the liver cytoplasm its L/P ratio will become about one or less than one and if a compound is bound to Z binding protein as well as Y binding protein or T binder its L/P ratio will become greater than one. Accordingly it is probably due to the binding to Z binding protein whether an organic anionic compound that is actively excreted into bile belongs to type i) or type ii).

However in this time we need to pay attention to the fact that there was a quite difference in the elution patterns between *in vitro* (Fig. 2) and *in vivo* (Fig. 3) experiments. Although no free form of BPB was detected *in vivo*, it appeared *in vitro*. The same attention is necessary when the effect of BSP on the binding of BPB to the liver cytoplasmic Y and Z binding proteins and T binder was examined (Fig. 4 and Table IV). This phenomenon is supposed to be due to the existence of the lipid barrier, liver plasma membrane, and of the limited bind-

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ing capacities of Y and Z binding proteins and T binder. From these results, it is suggested that *in vivo* experiment is superior to *in vitro* one when the role of the liver cytoplasmic binding protein is examined.

Let us now consider the roles of the liver cytoplasmic Y and Z binding proteins and T binder on the hepato-biliarytransport of organic anionic compounds. In our previous report,[1] it was observed that the percentage excreted of BPB in bile for 30 min was decreased about 60% by the pre-administration of BSP, 5 times molar ratio to BPB. In this report, the binding of BPB to Y binding protein was decreased 65% but the binding to Z binding protein was increased reversely by the pre-administration of BSP (Fig. 4 and Table V). This result suggests that the role of Y binding protein is corresponding to the biliary secretion of BPB. On the other hand, BPB was bound faster to Y binding protein than to Z binding protein, and the binding of BPB to Y binding protein after the intravenous administration of BPB reaches maximum at 15 min when the extraction rate of BPB into bile reaches maximum too. Moreover all the compounds used in this report were bound to Y binding protein in common. Judging from these results, Y binding protein seems to have an important role for organic anionic compounds that are excreted into bile from the liver. But Z binding protein seems to act as a hepatic pool as mentioned by Arias, *et al.*,[6] because the bindings of compounds mainly used in this report significantly correlate to L/P ratios of them, the binding of BPB to Z binding protein was increased gradually after the administration of BPB, and became superior to the binding to Y binding protein (Fig. 6). Regarding T binder, it does not seem to so take part in the hepato-biliary transport of organic anionic compounds at low doses.

In this report, it was also tried to clarify the difference of the hepato-biliary transport characteristics between the two related compounds, AM and NC, BB and LG, with the binding characteristics to the liver cytoplasmic binding proteins. These four compounds were bound to the liver cytoplasmic fraction but it seems that there are some difference between the amounts of these compounds bound to the liver cytoplasmic binding proteins (Fig. 7). So, it is thought that the differences are due to the affinities to Y binding protein or to the affinities to the active transport system which takes part in the biliary secretory process.

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