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Biopharmaceutical Study of the Hepato-biliary Transport of Drugs. III.[1,2] Binding Characteristics of Bromphenol Blue and Amaranth to the Liver Cytoplasmic Y and Z Binding Proteins *in Vitro*

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Binding properties of the rat liver cytoplasmic organic anion binding proteins, Y and Z proteins, for two organic anionic model compounds, bromphenol blue (BPB) and amaranth (AM) which are transported from blood stream into the rat bile by an active transport system, were studied by means of chemical methods, modifying the proteins with sulfhydryl-blocking reagents and amino group modifiers and fluorescent probe. Moreover, the affinities to Y and Z proteins were compared with that to bovine serum albumin by means of the apparent association constants. AM had higher affinity to the liver proteins than to bovine serum albumin, but in the case of BPB reverse result was obtained. The hydrophobic binding is important for the bindings of the dyes to the both proteins and BPB had higher affinities to the both proteins than AM. However, the binding properties to the both proteins were different, namely the binding of AM to Y protein was thought to be mainly due to the hydrophobic forces but the contribution of ionic forces could not be neglected with respect to the binding of BPB to the both proteins.

In the previous reports,[2,4] we have proposed to classify the organic anionic compounds which are transported from blood into bile by an active transport system into two categories, namely, i) compounds pooled in the liver and rapidly appear into bile and ii) compounds not pooled in but rapidly pass through the liver. Among the model organic anionic compounds which are thought to have an advantage for the study of hepato-biliary transport because of their non-metabolizing properties, bromphenol blue (BPB) and bromthymol blue (BTB) belong to the former, and amaranth (AM), tartrazine and *p*-acetylaminohippuric acid belong to the latter. Using these compounds, the mechanisms which are involved in the hepato-biliary transport of drugs were supposed that, briefly speaking, the rat liver cytoplasmic Y and Z binding proteins play important roles in the hepato-biliary transport of these organic anionic compounds, particularly in the hepatic uptake from the blood stream and in the intracellular transport of the liver parenchymal cells. Probably these anions transport behavior would actually depend on the affinity to the liver cytoplasmic binding proteins and on the protein contents. About the binding properties, however, very little is known until recently, though the physico-chemical properties of these binding proteins are partly clarified by Arias, *et al.*[5,6] In this paper, the binding properties of Y and Z binding proteins for the organic anionic compounds were investigated by some chemical methods, namely modifying the proteins with sulfhydryl-blocking reagents and amino group modifiers and fluorescent probe, using BPB and AM as respective model compounds of the two categories as mentioned above.

1) Part I: K. Takada, Y. Mizobuchi, and S. Muranishi, *Chem. Pharm. Bull.* (Tokyo), **22**, 922 (1974).
2) Part II: K. Takada, M. Ohno, and S. Muranishi, *Chem. Pharm. Bull.* (Tokyo), **22**, 1477 (1974).
3) Location: *Yoshida-shimoadachi-cho, Sakyo-ku, Kyoto.*
4) K. Takada, S. Muranishi, and H. Sezaki, *J. Pharmacokin. Biopharm.*, **2**, 417 (1974).
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Experimental

Materials——*p*-Chloromercuribenzoic acid (PCMB), iodoacetamide (IAA), N-ethylmaleimide (NEM), succinic anhydride, maleic anhydride, acetic anhydride were obtained from Nakarai Chemicals Co., Ltd. *p*-Chloromercuribenzene sulfonic acid (PCMBS) was obtained from Sigma Chemical Co., Ltd. 8-Anilino-1-naphthalenesulfonic acid magnesium salt (ANS) was obtained from Tokyo Chemical Co., Ltd.

Ultrafiltration membrane, Abcor HFA-180 and Diafilter G-10T, was obtained from Bio-engineering Co., Ltd.

Preparation of Binding Proteins[2]——Male Wistar rats, 150—200 g, were anesthetized by an intraperitoneal injection of sodium pentobarbital. The liver was removed after perfusion with ice-cold saline through the portal vein to wash out the blood which was entrapped in the liver, immediately washed, weighed and homogenized to a 25% homogenate with 0.25 M sucrose–0.01 M phosphate buffer (pH 7.4). The homogenate was centrifuged at 100000 ***g*** (av) for two hours in a Hitachi 55P ultra-centrifuge at 4°. After the surface lipids were removed, the supernatant fraction was carefully collected and was placed on a Sephadex G-100 column (10×70 cm) equilibrated with 0.01 M phosphate buffer (pH 7.4) and elution was performed with the same buffer. After Y and Z protein fractions[2] were collected respectively, they were concentrated and was desalted by ultrafiltration using Abcor HFA-180 membrane for Y protein and Diafilter G-10T membrane for Z protein. These membranes retained the macromolecules under positive pressure of nitrogen. The partially purified Y and Z protein fractions were lyophilized and were stored at −20° respectively.

Binding Assays——The binding of BPB or AM by the binding proteins was studied by dialyzing 1 ml aliquots of protein solution, the concentration of which was 1 mg/ml for Y protein and 0.5 mg/ml for Z protein, in sacs of Visking dialysis tubing, inflated diameter 6.4 mm, against 4 ml of phosphate buffer, pH 7.4, containing BPB or AM of which concentration was 0.01 μmole/ml.

Used protein concentration and sample concentration were nearly correspond to the concentration of the eluted Y and Z protein fractions from Sephadex G-100 column on the condition described above. After shaken for two days at 4°, the concentration of the outer sample solution was measured spectrophotometrically and binding percentage was calculated by correcting the blank value which is due to the binding to the Visking tubing membrane itself.

Modification with Sulfhydryl-blocking Reagents——Each 1 ml aliquot of Y or Z protein solution of which concentration was 1 mg/ml for Y protein and 0.5 mg/ml for Z protein was incubated for 1 hour at 4° with 0.1 ml of 1×10^{-2} M solution of PCMBS, IAA, and NEM[7] and 0.1 ml of 1×10^{-3} M solution of PCMB because of its limited solubility. After the modification in which 0.02 M phosphate buffer, pH 7.4, was used, the binding of BPB or AM to these modified proteins was measured according to the method described above.

Modification with Amino Acid Group Modifiers[8]——Succinic Anhydride and Maleic Anhydride: To a 1 ml aliquot of Y or Z protein solution, 1 mg/ml for Y and 0.5 mg/ml for Z protein respectively, 0.1 ml of 1×10^{-1} M solution of succinic anhydride or maleic anhydride which was dissolved in 0.01 M carbonate buffer (pH 9.1) was added and the resulting mixture was incubated for 1 hour at 20°.

Acetic Anhydride: After the addition of 0.1 ml of 1×10^{-1} M solution of acetic anhydride which was dissolved in 0.02 M phosphate buffer, pH 7.4, to 1 ml of Y or Z protein solution, 1 mg/ml for Y and 0.5 mg/ml for Z protein respectively, the resulting mixture was incubated at 0° for 30 min. Using these modified proteins, the binding experiment was performed by an equilibrium dialysis method as mentioned above.

Fluorescent Probe[9,10]——To a 4 ml aliquot of Y and Z protein buffered solution (pH 7.4) of which concentration was 1 mg/ml for Y protein and 0.5 mg/ml for Z protein, 0.4 ml of 1×10^{-4} M solution of ANS was added and fluorometric measurement was made with a spectrophotofluorometer. The relative fluorescence intensities of bound probe was obtained from fluorometer readings. The emission spectrum was recorded from 400 nm to 600 nm in response to excitation at 375 nm.

Next, 0.1 ml of 10^{-3} M solution of BPB or AM was added to 3 ml of the resulting mixture and the fluorometric measurement was performed as mentioned above.

Results

Effect of Ionic Strength and pH on the Binding of BPB to Y and Z Proteins

The influence of the ionic strength of the medium at pH 7.4 on the binding percentage of BPB to both Y and Z proteins is shown in Fig. 1. The binding of BPB to Y and Z proteins appears to be independent on the ionic strength, but shows a little decrease at low ionic strength.

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On the other hand, the influence of pH on the binding of BPB to Y and Z proteins is obtained as shown in Fig. 2. Though the pH effect is much clearly detected in the case of the binding to Y protein, in this pH range, it is obvious that the more the pH increases the more the binding percentage decreases not only in the case of the binding to Y protein but also to Z protein.

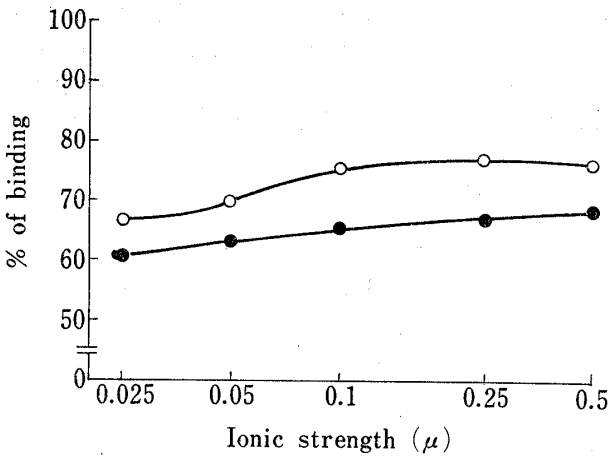

Fig. 1. Influence of Ionic Strength on BPB Binding to Y and Z Proteins

Using pH 7.4 phosphate buffer of which ionic strength was ranged from 0.025 to 0.5 μ, the binding percentage of BPB to Y and Z proteins was measured.
○—○ : Y protein
●—● : Z protein

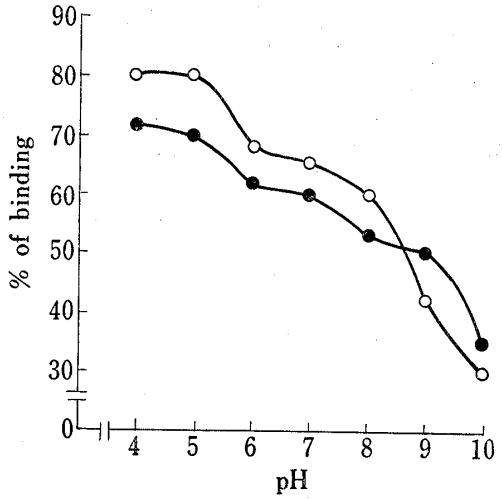

Fig. 2. Influence of pH on BPB Binding to Y and Z Proteins

At pH 3 and 4, acetate buffer, at pH 6, 7, and 8, phosphate buffer and at pH 9 and 10, bicarbonate buffer were respectively used.
○—○ : Y protein
●—● : Z protein

Properties of Binding Reactions

The apparent association constants of BPB and AM for the rat liver cytoplasmic Y and Z proteins were determined as compared with that for bovine serum albumin by an equilibrium dialysis method using Scatchard analysis,[11,12] and the obtained values are summarized in Table I. BPB has higher affinity to both liver cytoplasmic proteins, and bovine serum

Table I. Comparison of the Apparent Association Constants of Rat Liver Cytoplasmic Y and Z Proteins and Bovine Serum Albumin for BPB and AM

Compounds	Y protein (M^{-1})	Z protein (M^{-1})	Bovine serum albumin (M^{-1})
Bromphenol blue	1.29×10^5	1.39×10^5	6.58×10^5
Amaranth	5.67×10^4	1.43×10^4	1.33×10^4

By equilibrium dialysis method, apparent equilibrium association constants of Y and Z proteins and bovine serum albumin for BPB and AM were determined using 15 different concentrations of BPB and AM solutions, pH 7.4. The used concentrations of proteins were 1 mg/ml for Y protein and bovine serum albumin and 0.5 mg/ml for Z protein.

albumin than AM, and in the case of BPB, the apparent association constant for bovine serum albumin is about five times greater than that for liver cytoplasmic proteins. About AM, however, inverse result was obtained. Namely, the apparent association constants for Y as well as Z protein were greater, particularly about five times in the case of Y protein, than

11) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).
12) B.E. Pennock, *Anal. Biochem.*, **56**, 306 (1973).

that for bovine serum albumin. Though the used concentration of Z protein solution was half as much as that of bovine serum albumin because of its low concentration in the cytoplasmic fraction of the liver cells.

Effect of Sulfhydryl-blocking Reagents

To examine whether the sulfhydryl groups of Y and Z proteins are required or not for the binding to BPB and AM, four sulfhydryl-blocking reagents which are indicated in Table II were used and binding percentage was measured by an equilibrium dialysis method after the sulfhydryl groups of the proteins are masked. Results for BPB and AM are respectively represented in Table II and III in which the values are expressed as per cent of the control binding observed with native, unmodified, Y and Z proteins. From Table II, it is suggested

Table II. Influence of Sulfhydryl-blocking Reagents on the Binding of BPB to Y and Z Proteins

Reagents	Used concentration	Bromphenol blue bound to Y protein	Bromphenol blue bound to Z protein
	M	%	%
None	—	100	100
p-Chloromercuribenzoic acid	10^{-3}	83.3±2.4	78.6±1.5
p-Chloromercuribenzene sulfonic acid	10^{-2}	87.3±1.1	82.0±5.7
N-Ethylmaleimide	10^{-2}	95.8±1.0	76.9±0.6
Iodoacetamide	10^{-2}	95.4±0.7	86.6±0.4

The results are expressed as per cent of the control binding observed with native, unmodified, proteins. Each value is the mean ± S.E. for 3—4 experiments.

Table III. Influence of Sulfhydryl-blocking Reagents on the Binding of AM to Y and Z Proteins

Reagents	Used concentration	Amaranth Y protein	Amaranth Z protein
	M	%	%
None	—	100	100
p-Chloromercuribenzoic acid	10^{-3}	98.6±0.9	100.0±0.8
p-Chloromercuribenzene sulfonic acid	10^{-2}	97.3±1.0	97.5±2.0
N-Ethylmaleimide	10^{-2}	100.1±0.7	97.9±1.6
Iodoacetamide	10^{-2}	99.3±1.0	99.1±0.4

Each value is the mean ± S.E. for 3—4 experiments.

that sulfhydryl-blocking reagents do not so inhibit the binding of BPB to Y protein as compared with that to Z protein. But the inhibitory effect of these reagents on the binding of BPB to Z protein seems to be not so great. Moreover there detected no inhibitory phenomena in the binding of AM to both proteins as shown in Table III.

Amino Group Modifiers

To examine the effect of the modification of the amino groups of Y and Z proteins on the binding of BPB and AM to both proteins, three amino group modifiers, succinic anhydride, maleic anhydride and acetic anhydride were used at their proper reaction pH; at pH 9.1 for succinic anhydride and maleic anhydride, and at pH 7.4 for acetic anhydride, which have been used to modify the human serum albumin to examine the binding site for bilirubin.[8] Results are expressed as per cent of the control binding as in the case of sulfhydryl-blocking

reagents and are represented in Table IV and V. From Table IV, it is observed that the binding of BPB to Y protein as well as Z protein of which amino groups are modified is inhibited by this modification. On the other hand, the binding of AM to both modified proteins is not so decreased as compared with the binding to the native Y and Z proteins (Table V).

TABLE IV. Influence of Amino Group Modifiers on the Binding of BPB to Y and Z Proteins

Reagents	Used concentration	Bromphenol blue	
		Y protein	Z protein
	M	%	%
None	—	100	100
Succinic anhydride	10^{-2}	91.2± 0.5	91.2± 0.9
Maleic anhydride	10^{-2}	96.8± 0.6	84.5±10.5
Acetic anhydride	10^{-2}	50.2± 5.8	24.2± 5.2

The results are expressed as per cent of the control binding observed with native, unmodified, proteins.
Each value is the mean ± S.E. for 3—4 experiments.

TABLE V. Influence of Amino Group Modifiers on the Binding of AM to Y and Z Proteins

Reagents	Used concentration	Amaranth	
		Y protein	Z protein
	M	%	%
None	—	100	100
Succinic anhydride	10^{-2}	104.9±2.0	100.3±2.5
Maleic anhydride	10^{-2}	103.5±6.2	99.8±4.7
Acetic anhydride	10^{-2}	95.9±1.2	98.3±0.5

Each value is the mean ± S.E. for 3—4 experiments.

Fluorescent Probe

Using ANS of which fluorescence intensity is thought to be induced by binding to the hydrophobic region of proteins, the contribution of the hydrophobic force for the binding of BPB and AM to Y and Z proteins was examined. Fig. 3. shows the fluorescence emission spectra of the probe in the presence and absence of Y protein. The fluorescence intensity of the probe itself in pH 7.4 phosphate buffer was not significant (curve D), but when ANS was added to Y protein solution (1 mg/ml), the fluorescent intensity was greatly enhanced (curve A). Curve B and C show the fluorescence emission spectra of the probe in the presence of AM and BPB respectively and it is suggested by comparing these two curves that the diminution of fluorescence intensity by the addition of BPB was greater than that of AM. With respect to Z protein, results are represented in Fig. 4. When ANS was added to Z protein solution (0.5 mg/ml), the fluorescence intensity was increased (curve A) as added to Y protein. Moreover, by the addition of BPB to this resulting mixture the fluorescence intensity was extremely decreased (curve C). As shown in curve B, however, the effect of AM is slight.

Discussion

As the physico-chemical properties of rat liver cytoplasmic organic anion binding proteins themselves, Y and Z proteins, have been already studied,[5,6] in this report the binding properties of Y and Z proteins for the two organic anionic model compounds, BPB and AM, which

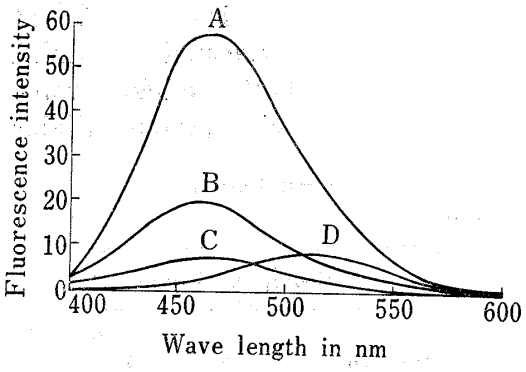

Fig. 3. Influence of BPB and AM on the Fluorescence Emission Spectra of Probe-Y Protein Complex

Fluorescence emission spectra of the ANS-Y binding protein complex was measured in the presence (curve A) and in the absence (curve D) of Y protein (9.1×10^{-6}M) in pH 7.4 phosphate buffer. Excitation was at 375 mμ and emission was at 400—600 mμ. Curves B and C are the emission spectra of the probe-protein complex in the presence of AM and BPB (3.2×10^{-5}M) respectively.

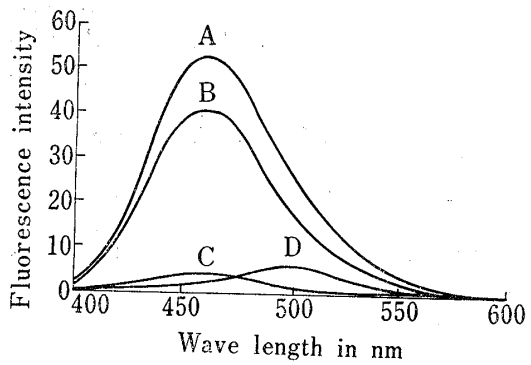

Fig. 4. Influence of BPB and AM on the Fluorescence Emission Spectra of Probe-Z Protein Complex

The method is the same as mentioned in Fig. 3.
curve A : ANS+Z protein
curve B : ANS+Z protein+AM
curve C : ANS+Z protein+BPB
curve D : ANS only

were confirmed to be actively transported from blood stream into bile,[1,2] were examined by chemical methods, chemical modification and fluorescent probe. Firstly, the effect of pH on the binding of BPB to Y and Z proteins was examined and it is suggested that the pH effect is considerably great with respect to the binding of BPB to both proteins (Fig. 2). It seems that the increase of binding percentage in lower pH region depends on an increase of hydrophobicity due to phenol group of BPB (pK_a=4.0). And the decrease of binding percentage from pH 8 to 10 is probably due to a change in ionization of proteins. However, the effect of ionic strength was not detected, (Fig. 1) and from this result, it is thought that the conformational changes of both proteins do not so influence the binding abilities of the proteins, because it is generally accepted that at low ionic strength most proteins form heavy complexes by aggregation and at high ionic strength dissociate to subunits.[13–15]

On the other hand, with respect to the information of the binding site, hydrophobic binding plays an important roles for the binding of not only AM but also BPB to both Y and Z proteins. In the case of AM, its binding to Y protein is thought to be due to mainly hydrophobic binding, because the fluorescence intensity of the probe complex was decreased by the addition of AM (Fig. 3.), namely AM competes the binding site of ANS which is thought to have a hydrophobic properties, and blocking the sulfhydryl groups and amino groups of Y protein did not have an influence on the binding of AM to Y protein (Table III and V).

About the binding of AM to Z protein, clear results were not obtained by fluorescent probe method, because of its low affinity to this protein (Table I).

However hydrophobic binding is thought to contribute for the binding of AM to Z protein as the fluorescence intensity was decreased, though slightly, by the addition of AM in spite of no influence of sulfhydryl-blocking reagents and amino group modifiers on the binding of AM to Z protein.

Though it is thought that the hydrophobic binding plays an important role on the binding of AM to Y and Z proteins, ionic forces are thought to be unable to be neglected with respect to the binding of BPB. From Table II and IV, it is suggested that amino groups seem to

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14) T.V. Gopalakrishnan and A. Sadgopal, *Biochem. Biophy. Acta.*, **287**, 164 (1972).
15) G. Giannopoulos, *J. Biol. Chem.*, **248**, 3876 (1973).

participate in the binding of BPB to Y protein and that the contribution of sulfhydryl groups seems to be unconsiderable.

Moreover, it is thought that both the sulfhydryl and the amino groups of Z protein do not so contribute to the binding of BPB to this protein, because the modification of these groups had a little effect.

Next the relationship between the association constants of BPB and AM to the rat liver cytoplasmic proteins and that to bovine serum albumin should be considered. Though both BPB and AM appear in rat bile rapidly after intravenous administration, the apparent association constants of BPB to the liver cytoplasmic Y and Z proteins are smaller than that to bovine serum albumin and the reverse result is obtained in the case of AM. With respect to BPB, its transport from blood into bile is not understandable but the transport of AM seems to be reasonable by comparing the association constants to bovine serum albumin and the liver cytoplasmic proteins. But in the former case, three theories have been proposed until lately as follows.

i) There exists a carrier-mediated process at the level of the liver plasma membrane which was proposed by Frezza[16] for the flux of BSP from plasma into the liver.

ii) The net flux of organic anionic compounds from blood stream into the liver is determined by the amount of and the binding affinity of albumin in plasma and the amount of and the binding affinities of Y and Z proteins in the liver cytoplasm. Influx is largely determined by dissociation of anionic compounds from protein in blood and diffusion across the plasma membrane of the liver cells. Efflux from the liver is largely determined by the amount of Y and Z proteins. Most organic anionic compounds are either metabolized or directly excreted by the liver cells and such transport continuously depletes the intracellular concentration of the organic anionic compounds.[5]

iii) As the albumin concentration is about one-tenth as compared with the plasma protein concentration in the extracellular fluid of the liver,[17] organic anionic compounds exist for the most part as free forms, even if their plasma protein binding is very great, and the plasma protein binding does not become a limiting step for the hepato-biliary transport of organic anionic compounds.

Though various possibilities have been presented up to date with respect to the transport of organic anionic compounds from blood into the liver cells against the gradient of affinity, the only clarified event is that the plasma protein binding does not determine the hepato-biliary transport of organic anionic compounds. But about this important point, more detailed investigation should be necessary as mentioned above.

Acknowledgement We thank Professor Hitoshi Sezaki for his helpfull discussions.

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