MULTIPLICITY OF SULFOBROMOPHTHALEIN-BINDING PROTEINS IN Y-FRACTION FROM RAT LIVER *

YUICHI SUGIYAMA, TATSUJI IGA, SHOJI AWAZU, ** AND MANABU HANANO

Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

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Several species of the sulfobromophthalein-binding proteins were isolated from the Y-fraction of rat liver cytosol. Each of these proteins, designated as C_v , C_1 , C_2 and C_3 according to the elution order from CM-Sephadex column, migrated to the same position as a single protein band in dodecylsulphate electrophoresis. They are basic proteins of approximately 46000 daltons, composed of two subunits of apparently equal size, and similar to the glutathione S-transferase AA, A, B (ligandin), C purified by Jakoby *et al.* Each of the C_v , C_1 and C_2 protein forms 1:1 primary complex with BSP, with the binding constant of about $5 \times 10^6 \text{ M}^{-1}$ or more. The C_1 protein has a somewhat higher binding constant than the others. The binding constant was little influenced by the purification. The binding constants of these proteins for other organic anions were determined by the competitive technique using 1-anilino-8-naphthalenesulfonate. These proteins show broad binding specificity for various organic anions which are all known to be rapidly transported into the liver. From these results, it is concluded that the binding of BSP by the Y-fraction is not due to a single protein like ligandin.

Keywords — sulfobromophthalein-binding protein; Y-fraction; ligandin; glutathione S-transferase; 1-anilino-8-naphthalenesulfonate; hepatic uptake; binding constant; scatchard plot

The plasma disappearance rate of sulfobromophthalein (BSP) is widely used to evaluate hepatic function.¹⁾ The mechanism responsible for the rapid and seemingly selective transfer of BSP from plasma into liver, however, remains unknown. In 1969, Levi *et al.*²⁾ discovered two hepatic cytoplasmic binding protein fractions (Y and Z) in rats by gel filtration, which were considered responsible for the selective transfer of organic anions such as BSP, bilirubin and indocyanine green. Our previous report also suggested that the Y-fraction is one of the determinants in BSP net transfer from plasma into liver.³⁾

A protein from the Y-fraction (45000 daltons) was purified by Fleischner *et al.*,⁴⁾ and shown to be identical with an azo-dye-binding protein by Ket-

terer *et al.*⁵⁾ and also a cortisol-binding protein by Morey and Litwack.⁶⁾ Because of the versatile binding of this protein, it was named ligandin.⁷⁾ Ligandin is known to be a basic protein (pI = 9.1) and represents 5% of the total cytosol protein.⁷⁾ Recently, Habig *et al.*⁸⁾ demonstrated that ligandin was identical with glutathione S-transferase B, one of the group of purified enzymes (glutathione S-transferase, AA, A, B, C, E). All these enzymes were found to be proteins of about 45000 daltons.⁹⁾ Ketley *et al.*¹⁰⁾ found that the rat liver glutathione S-transferases AA, A and C as well as B (ligandin), bind some organic anions such as bilirubin, indocyanine green and 3,6-dibromosulfophthalein, which are not their substrates.

However, no report can be seen concerning the

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** Present address: Tokyo College of Pharmacy, 1432-1 Horinouchi Hachioji, Tokyo 192-03, Japan.

binding affinity of BSP itself to these proteins except ligandin. Although Kaplowitz *et al.*²⁸⁾ indirectly found the binding of BSP to glutathione S-transferases (S-aryl, S-epoxide, S-alkyl and S-alkyl transferase) using rat liver cytosol, the lowest inhibitor constant ($1.6\ \mu\text{M}$) among them obtained in S-alkyl-transferase was far higher than the dissociation constant ($0.2\ \mu\text{M}$) obtained in the direct binding experiment of BSP to ligandin by Kamisaka *et al.*¹⁶⁾ and Ketterer *et al.*¹⁸⁾

On the other hand, Fleischner *et al.*⁴⁾ and Arias *et al.*²⁹⁾ showed that an antibody produced against ligandin precipitated the ligandin from liver supernatant and that the antibody specifically removed over 80% of the BSP-binding capacity from the Y-fraction. Based on this finding, many investigators determined the concentration of ligandin by measuring the binding capacity for BSP.²²⁻²⁴⁾ However, one must be more deliberate in using this method until the binding affinities for BSP of the other proteins in Y-fraction than ligandin are known. Isolation methods of rat ligandin from the Y-fraction based on the BSP binding activity was briefly reported by Fleischner *et al.*,⁴⁾ however, the detailed binding behavior for BSP during the isolation procedure is reported nowhere.

In this report the authors attempted to determine the binding of BSP to proteins in Y-fraction and estimated the contribution of ligandin to the binding of Y-fraction for BSP.

MATERIALS AND METHODS

Materials — 1-Anilino-8-naphthalenesulfonate (ANS), amaranth (AM) and bromphenol blue (BPB) were purchased from Tokyo Chemical Industries, Co., Ltd. Rose bengal (RB) and bilirubin (BLB) were purchased from Wako Pure Chemical Industries, Ltd. BSP, phenolsulfophthalein (PSP) and indocyanine green (ICG) were purchased as the Injection (Daiichi Chemical Industries, Ltd.). Standard proteins for the determination of molecular weight were from Böehringer Mannheim. Other reagents used were Sephadex G-75, DEAE-Sephadex A-50, and CM-Sephadex C-50 (Pharmacia); acrylamide and methylene *bis* acrylamide (Seikagaku Kogyo, Co, Ltd.). Rat albumin (Pentex,

Fraction V) was purchased from Research Products Division of Miles Lab., Inc. (Kankakee, IL, USA). All the chemicals were used without further purification. Cells for equilibrium dialysis were obtained from Kokugo Gomu, Co; Ltd. Visking tube (wall thickness 0.001 inch) was obtained from Visking company. **Animals** — Male Donryu rats (Nihon Rat Co. Ltd; Japan), weighing 270—330 g were used as liver source.

Purification of BSP-Binding Proteins in Y-fraction — All procedures were performed at 4°.

(Step 1.) Preparation of Supernatant Fraction: A 100000 g supernatant was prepared from a 50% homogenate of 77 g liver from 7 rats. Detail procedures were described previously.³⁾

(Step 2.) Sephadex G-75 Gel Filtration (First Time): An aliquot of 55 ml of the supernatant from step 1 was chromatographed on a Sephadex G-75 column (5.4cm×85cm), which had been equilibrated with 50 mM Tris-HCl buffer, pH 7.4 (buffer A). Elution was performed with buffer A using a pump-driven downward flow (45 ml/hr), and fractions of 10 ml each were collected. Protein concentration was determined spectrophotometrically at 280 nm. Y-fractions from each gel filtration were combined. The identification of Y-fraction was preliminarily determined as described later.

(Step 3.) Sephadex G-75 Gel Filtration (Second Time): The Y-fraction from the previous step (140 ml) was concentrated to 20 ml by lyophilization after dialysis against distilled water. The concentrated Y-fraction was chromatographed on the same Sephadex G-75 column under the same condition as described in step 2. The peak fractions which appeared at the same position as in step 2 were collected (70 ml).

(Step 4.) DEAE-Sephadex Chromatography: The sample from step 3 was concentrated to 20 ml by lyophilization, and dialysed against buffer A. The dialysed sample was applied to a DEAE-Sephadex A-50 column (1.5cm×17cm) equilibrated with buffer A. Elution was performed with 200 ml buffer A at a flow rate of 12 ml/hr, and aliquots of 3.5 ml were collected. Fifty five per cent of the applied protein was eluted and

designated as D_v-fraction. The adsorbed protein fraction was eluted with buffer A containing 0.5M NaCl, and designated as D_{NaCl}-fraction.

(Step 5.) CM-Sephadex Chromatography: The D_v-fraction (70 ml) was concentrated to 13 ml by ultrafiltration using Abcor HFA-180 membrane and dialysed for one day against 0.05M acetate buffer, pH 6.0 (buffer B). The dialysed sample was applied to a CM-Sephadex C-50 column (1.25 cm×11.5 cm) equilibrated with buffer B. After the column was rinsed with 150 ml of buffer B, a linear gradient composed of 200 ml of buffer B and 200 ml of buffer B containing 0.5M NaCl was used to elute the column. The flow rate was 12 ml/hr and aliquots of 4 ml were collected. Four protein fractions, which have binding activities for BSP, were obtained and designated as C_v, C₁, C₂ and C₃ according to the elution order.

(Step 6.) Sephadex G-75 Gel Filtration: The C_v, C₁, C₂ and C₃ fractions were concentrated to 3 ml by ultrafiltration and applied to a Sephadex G-75 column (2.8 cm×54 cm) equilibrated with buffer A, respectively. The flow rate was 12 ml/hr and aliquots of 3.0 ml were collected. Each of these fractions was eluted at the same position. The final protein samples thus obtained were named C_v, C₁, C₂ and C₃ protein, respectively. The molecular weights of these proteins were simultaneously estimated by using cytochrome C(12500), chymotrypsinogen (25000), hen egg albumin (45000) and bovine serum albumin (67000) as standard proteins. The void volume was determined by blue dextran.

Preliminary Test for BSP binding to Proteins during the Purification —(1) Preliminary Sephadex G-75 Gel Filtration: Ten ml of the supernatant from three rats as mixed with 10 μmol of BSP, and the mixture was chromatographed on the Sephadex G-75 column under the same conditions as described in the purification procedure. Protein and BSP concentrations in each aliquot were measured spectrophotometrically at 280 nm and 580 nm, respectively.

(2) Preliminary CM-Sephadex Chromatography: The D_v-fraction prepared from three rats was chromatographed on a CM-Sephadex column (1.3

cm×9.5 cm) under the same conditions as described in the purification procedure. Aliquots of 3 ml were collected, and dialysed against buffer A. After the protein concentrations of all the aliquots were measured, they were dialysed against 0.2 μM of BSP dissolved in 500 ml of buffer A. A Visking membrane of Type: 8/32 was used. After the equilibrium was achieved 80 hr later, the BSP concentration on both inside and outside of the membrane was measured. The concentration outside the membrane (*i. e.* unbound concentration) was 0.19 μM, and the bound concentration of BSP in each sample was calculated by subtracting the unbound concentration from the total concentration inside the membrane.

Protein Concentration —Protein concentrations were determined by the method of Lowry *et al.*,¹¹⁾ with bovine serum albumin as standard. Bovine serum albumin concentrations were determined from the extinction coefficient, $E_{279}^{1\%} = 6.7$.

Sodium Dodecyl Sulphate-polyacrylamide-gel Electrophoresis —Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis was performed as described by Weber and Osborn.¹²⁾ Prior to electrophoresis, proteins were incubated with 1% mercaptoethanol for 15–20 hr at room temperature. Solutions containing about 20 μg of protein were introduced to columns (10% gel), and a current of 4 mA/gel was applied for about 6 hr. The gels were fixed in 50% trichloroacetic acid for 1 hr and stained in 0.25% coomassie brilliant blue–20% trichloroacetic acid. Subunit molecular weights of these proteins were determined by using cytochrome C(12500), myoglobin (17000), chymotrypsinogen (25000), hen egg albumin (45000) and bovine serum albumin (67000) as standard proteins.

Equilibrium Dialysis —The protein samples were previously dialysed against buffer A for 20–30 hr. Equilibrium dialysis experiments were carried out using dialysis cells. The dialysis cell which has two chambers was divided with a Visking membrane (Type: 18/32). Three ml of protein sample was placed in one chamber and 3 ml of BSP solution was added in the other chamber. The concentration of BSP ranged from 0.6 to 15

μM . After the chambers were gently shaken for 80–95 hr at 4° , the concentration of unbound BSP was measured spectrophotometrically with Hitachi 356-type dual beam spectrophotometer ($\lambda_1 = 650 \text{ nm}$, $\lambda_2 = 580 \text{ nm}$). There was some evidence of adsorption of BSP to the membrane, but in no instance did this exceed 2.5% of the total BSP present and could be corrected for.

Analysis of Binding Results — When the pure proteins were used in the binding experiment, the binding parameters in eq.1 assuming two classes of site, were obtained with a non-linear iterative least square method by using Hitachi 8700/8800 digital computer.

$$r = n_1 K_1 C_f / (1 + K_1 C_f) + n_2 K_2 C_f / (1 + K_2 C_f) \quad \text{eq.1}$$

where C_f and r represent the concentration of unbound BSP, and the observed mol of bound BSP per mol of protein, respectively, and K_i and n_i represent the binding constant and the number of binding sites, respectively.

When the crude protein fractions were used in the binding experiment, the apparent binding parameters (n_1 , K_1) concerning the site which has the highest affinity for BSP, were obtained graphically from the straight part of the Scatchard plot. Such simplification has widely used by many authors.^{13,14)}

Determination of Binding Constant by Fluorescence Change of ANS — Competitive binding studies were performed with Hitachi MPF-4 spectrofluorometer at room temperature ($26\text{--}29^\circ$) by following the decrease in the fluorescence of ANS. To cuvettes containing 3 ml of mixture of about $0.8 \mu\text{M}$ protein, $10 \mu\text{M}$ ANS and buffer A, were added a maximum of $40 \mu\text{l}$ of the competitor in $2 \mu\text{l}$ or $5 \mu\text{l}$ aliquots. The solution was stirred with a glass rod after each addition and the fluorescence was measured at 480 nm during excitation at 400 nm . The calculation method of the binding constant from such competitive study was shown previously.¹⁵⁾

Determination of Binding by the Quenching of Intrinsic Fluorescence of Protein — The decrease in the intrinsic fluorescence of C_v -protein upon ad-

dition of RB was measured at 330 nm while exciting at 290 nm . To cuvettes containing 3 ml of C_v -protein solution, were added a maximum of $40 \mu\text{l}$ of RB in $2 \mu\text{l}$ or $5 \mu\text{l}$ aliquots. The analysis of these data were carried out according to the method of Halfman and Nishida.³⁰⁾ In this analysis, the fluorescence data obtained at two widely different concentrations of protein were used. These concentrations used at the present analysis were $0.22 \mu\text{M}$ and $1.54 \mu\text{M}$, and $0.22 \mu\text{M}$ and $3.08 \mu\text{M}$.

RESULTS

Purification of BSP Binding Proteins in Y-fraction

Figure 1 shows the elution profile after the preliminary Sephadex G-75 gel filtration of the supernatant containing BSP. Three peaks of BSP binding to proteins were obtained, and identified with X, Y and Z-fraction, respectively, following the nomenclature of Levi *et al.*²⁾ The Y-fraction which corresponds to the horizontal bars in the preliminary test (Fig. 1) were pooled in the gel filtration without BSP for the purification experiment (step 1), and used for further purification.

The D_v -fraction after DEAE-Sephadex chro-

FIG. 1. Preliminary Sephadex G-75 Gel Filtration of 10 ml of Rat Liver Supernatant after Addition of $10 \mu\text{mol}$ of BSP

A column of $5.4 \times 85 \text{ cm}$, and buffer A were used. Elution rate was 45 ml/hr and each tube contained 10 ml .

— elution pattern of protein (abs. 280 nm);

----- elution pattern of BSP (abs. 580 nm).

FIG. 2. Elution Pattern of Protein and BSP Binding Activities after Preliminary CM-Sephadex Chromatography of the D_v -fraction

The D_v -fraction (35 mg) was chromatographed on a CM-Sephadex column (1.3×9.5 cm) equilibrated with buffer B. Elution rate was 12 ml/hr and each tube contained 3 ml. BSP binding activities were measured with equilibrium dialysis. See details in the test. ----- protein (Abs. 280 nm); ——— BSP binding against the unbound BSP concentration of $0.19 \mu\text{M}$.

FIG. 3. CM-Sephadex Chromatography of the D_v -fraction

The D_v -fraction (83 mg) was chromatographed on a CM-Sephadex column (1.25×11.5 cm). Elution rate was 12 ml/hr and each tube contained 4 ml. Protein is designated by a dotted line (-----) and the NaCl concentration by a solid line.

The horizontal bars indicated the fractions used for further purification.

matography (step 4) bound BSP with much higher affinity than the D_{NaCl} -fraction, as described later. Therefore, the D_v -fraction was used for further purification.

Figure 2 shows the elution profile after the preliminary CM-Sephadex chromatography of the D_v -fraction. Four BSP binding fractions (C_v , C_1 ,

FIG. 4. Estimation of Molecular Weight of BSP Binding Proteins by Gel Filtration (Sephadex G-75) and SDS Gel Electrophoresis

See details in the text. Standard proteins are BSA (●), hen egg albumin (○), chymotrypsinogen (▲), myoglobin (△) and cytochrome C (■). ——— gel filtration; ----- SDS gel-electrophoresis.

FIG. 5. Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis of BSP Binding Proteins

Prior to electrophoresis, protein aliquots (about $20 \mu\text{g/ml}$) were incubated with 1% mercaptoethanol for 15–20 hr at room temperature. See details in the text. (a) C_v -protein, (b) C_1 -protein, (c) C_2 -protein, (d) C_3 -protein, (e) D_v -fraction.

TABLE I. *Binding Parameters of BSP for the High Affinity Site on Various Protein Fractions in the Purification*

Step ^{a)}	Fraction		Number of the binding sites (n_1), ($\pm$ S.E.)		Binding constant ($\pm$ S.E.)
1	Supernatant	4 ^{e)}	5.3 ^{b)} $\pm$ 0.8	c)	8.6 ^{d)} $\pm$ 0.8
2	Y-fraction	7	14.5 $\pm$ 1.4		7.3 $\pm$ 1.5
	D _V -fraction	4	17.0 $\pm$ 1.1		8.7 $\pm$ 0.9
4	D _{NaCl} -fraction	4	22.0 $\pm$ 1.7		1.1 $\pm$ 0.2
	C _V -protein	4	21.8 $\pm$ 2.0	0.98 $\pm$ 0.09	4.7 $\pm$ 0.3
	C ₁ -protein	4	16.0 $\pm$ 1.2	0.72 $\pm$ 0.05	7.1 $\pm$ 1.0
6	C ₂ -protein	3	19.0 $\pm$ 0.4	0.86 $\pm$ 0.02	4.5 $\pm$ 0.5
	C ₃ -protein	4			1.3 $\pm$ 0.3 ^{f)}

a) These correspond to the purification step described in "MATERIALS AND METHODS". b) $\mu\text{mol/g-protein}$. c) mol/mol-protein . d) $\times 10^6 \text{ M}^{-1}$. e) The number of experiments. f) Two of the four preparations show positive cooperativity. Apparent binding constant was obtained using a part of negative slope of the Scatchard plot.

C₂ and C₃) were obtained, and the aliquots except around each peak fraction had little binding activities for BSP. Therefore, the fractions from each peak, shown by the horizontal bars in Fig. 3, were used for the next gel filtration. The gel filtrations of the C_V, C₁, C₂ and C₃ fractions on a calibrated Sephadex G-75 column were carried out (step 6), and each protein was eluted at the same position. The molecular weight estimated by the gel filtration is 46000 (Fig. 4). Figure 5 shows the result of dodecylsulphate-gel electrophoresis of these proteins after final gel filtration. Each protein migrated to the same position as a single protein band. The molecular weight estimated by dodecylsulphate-gel electrophoresis was 25000 (Fig. 4). These results suggest that each protein is composed of two subunits of the same size. In order to confirm homogeneity in the molecular weight of subunits of these proteins, we carried out dodecylsulphate-gel electrophoresis of the D_V-fraction, which contained all these proteins. It also migrated as a single protein band (Fig. 5).

Binding of BSP to Crude Protein Fractions in the Purification

Table I summarizes the binding parameters determined by equilibrium dialysis of BSP to various protein fractions in the purification. As the purification goes further, the number of binding sites per gram of protein (n_1) becomes larger, while

the binding constant (K_1) shows little change. By comparing the K_1 values between the D_V and D_{NaCl} fractions, we know the D_V-fraction which is

FIG. 6. *Binding Curve of BSP to Binding Proteins in the Y-fraction*

Data were obtained by equilibrium dialysis experiments. See details in the text. r represents the number of moles of BSP bound per mole of protein (assuming its molecular weight to be 45000) and C_f is the concentration of unbound BSP.

(●) C_V-protein, (▲) C₁-protein, (○) C₂-protein, (△) C₃-protein.

FIG. 7. Scatchard Plot of BSP Binding to Binding Proteins in the Y-fraction

Binding data in Fig. 6 are replotted.

(a) BSP binding to C_V -protein (●) and C_2 -protein (○). The solid line (—) and dotted line (-----) are calculated curves using $n_1=1$, $n_2=5$, $K_1=4.1 \times 10^6 \text{ M}^{-1}$, $K_2=5.7 \times 10^4 \text{ M}^{-1}$ for C_V -protein, and $n_1=1$, $n_2=5$, $K_1=3.0 \times 10^6 \text{ M}^{-1}$, $K_2=6.8 \times 10^4 \text{ M}^{-1}$ for C_2 -protein, respectively.

(b) BSP binding to C_1 -protein (●) and C_3 -protein (○).

a group of basic proteins has higher affinity for BSP.
Binding of BSP to Purified Proteins

Typical stoichiometric binding curves of BSP to the C_V , C_1 , C_2 and C_3 proteins are shown in Fig. 6. The concentration of free BSP ranges from about $0.07 \mu\text{M}$ to $8 \mu\text{M}$. These four proteins exhibit similar binding curves except that the binding to the C_3 protein is lower than the others below $0.3 \mu\text{M}$ of the free concentration. Figure 7 shows the Scatchard plots for these binding studies. By the least square method, the converged parameters, $n_1=1$, $n_2=5$, $K_1=4.1 \times 10^6 \text{ M}^{-1}$, $K_2=5.7 \times 10^4 \text{ M}^{-1}$ for the C_V protein, and $n_1=1$, $n_2=5$, $K_1=3.0 \times 10^6 \text{ M}^{-1}$, $K_2=6.8 \times 10^4 \text{ M}^{-1}$ for the C_2 protein were obtained, however, in the C_1 protein the converged parameters could not be obtained when n_1 value was fixed to be integer. In this case, the binding parameters were obtained graphically. In the C_3 protein, the Scatchard plot shows an

anomalous pattern, that is, an initial positive sloping character. This character appeared in two of four preparations of the C_3 protein. The apparent parameters in C_3 protein are obtained using a part of negative slope of the Scatchard plot. The binding parameters thus obtained are summarized in Table I (average of three or four preparations). C_V , C_1 and C_2 proteins have a fairly high affinity for BSP, of which binding constants are about $5 \times 10^6 \text{ M}^{-1}$ or more. Among these proteins, C_1 protein has a somewhat higher binding constant than the others. The binding constant of C_1 protein ($7.1 \times 10^6 \text{ M}^{-1}$) is comparable to those of crude protein fractions such as the supernatant, Y-fraction, and D_V -fraction. The stoichiometry of high affinity binding of BSP to the three proteins (C_V , C_1 , C_2) can be considered as 1 within the experimental error.

Binding Specificity

In order to know the binding specificity of these

FIG. 8. Binding of ANS to C_v Protein by the Fluorescence Method in the Presence and Absence of BSP
 (a) Double reciprocal plots of the fluorescence titrations of C_v-protein with ANS. Excitation wave length, 400 nm; emission wavelength, 480 nm. The concentrations of C_v-protein were 0.79 μM (●), 1.58 μM (▲) and 3.16 μM (■).
 (b) Double reciprocal plot of inhibition by BSP of binding of ANS to C_v-protein. The concentration of C_v-protein was 0.79 μM. (■) without BSP, (●) with 0.83 μM of BSP.

proteins, we carried out the binding experiment for various organic anions by a competitive technique. For a comparison, the binding to rat albumin was also measured. The results in the C_v protein will be shown as an example. At first, the binding of ANS to the C_v protein was measured by fluorescence method. At any concentration of C_v protein (0.79–3.16 μM), the identical binding constant, $6 \times 10^4 \text{ M}^{-1}$ was obtained (Fig. 8). Addition of various organic anions to the ANS and C_v protein complex results in quenching of the fluorescence (Fig. 9). Figure 8 demonstrates that such quenching is due to the competition of anions for the ANS binding site on the protein. As shown in Fig. 9, the calibration curve can be calculated as a function of the binding constant for a competitor, assuming a single competition at a single site. The approximate binding constants for the competitors were estimated by comparing the calibration curves of fluorescence with the experimental values.

Binding constants of organic anions thus obtained are listed in Table II. The binding constants

of BSP to these proteins obtained by this method are in good agreement with those obtained from the equilibrium dialysis. In order to confirm the validity of such a competitive technique further, the binding of RB to C_v protein was measured by the method of Halfman and Nishida.³⁰⁾ This method, which was also adopted by Ketley *et al.*,¹⁰⁾ is based on the quenching of the intrinsic fluorescence of proteins by binding of substrates. Fig. 10 shows the Scatchard plot for this binding study. Binding parameters, $n = 1.1$ and $K = 9.1 \times 10^6 \text{ M}^{-1}$ were obtained and these values were in good agreement with those by the competitive methods. It is apparent from Table II that the binding affinity is dependent on the nature of the ligand rather than that of the protein. For example, RB and ICG bind strongly to all four proteins, whereas ANS and PSP have a low affinity. Rat albumin behaves somewhat differently. As compared to the proteins in the Y-fraction, rat albumin has shown a higher binding affinity for the anions, except BSP and ICG, by an order of magnitude.

FIG. 9. Competition of Various Organic Anions with ANS for Binding Site on C_v -protein

The decreases of fluorescence intensities were measured at constant concentration of C_v -protein ($0.79 \mu\text{M}$) and ANS ($10 \mu\text{M}$). See details in the text. Competitors used are PSP (●), AM (▲), BPB (○), BSP (△), ICG (■) and RB (□). The solid lines are theoretical curves calculated according to the method as previously reported. The binding parameters used in this calculation are, $K_a = 6 \times 10^4 \text{ M}^{-1}$ (binding constant for ANS), $n = 1$ (number of binding site for ANS), and K_b (binding constant for competitor) =, (a) $1 \times 10^5 \text{ M}^{-1}$; (b) $1 \times 10^6 \text{ M}^{-1}$; (c) $2 \times 10^6 \text{ M}^{-1}$; (d) $1 \times 10^7 \text{ M}^{-1}$.

FIG. 10. Scatchard Plot of RB Binding to C_v Protein measured from the Quenching of the Intrinsic Fluorescence of Protein

The decrease in the intrinsic fluorescence of C_v protein upon addition of RB was measured at 330 nm while exciting at 290 nm . See details in the text. The analysis of these data were carried out by the method of Halfman and Nishida.³⁰⁾ The concentrations of C_v protein used are $0.22 \mu\text{M}$ and $1.54 \mu\text{M}$ (●), and $0.22 \mu\text{M}$ and $3.08 \mu\text{M}$ (○).

TABLE II. Binding Constants of Various Organic Anions to Rat Albumin and Binding Proteins in Y-fraction^{a)}

	Binding constant ($\times 10^6 \text{ M}^{-1}$)							
	ANS	BSP	ICG	AM	BPB	BLB	RB	PSP
Rat albumin	11	5	15	20	50	100	100	1.5
C_v -protein	0.06	5	7	1	2	3	10	0.1
C_1 -protein	0.04	8	10	2	5	5	10	0
C_2 -protein	0.04	3	10	0.5	3	1	10	0.1
C_3 -protein	0.03	2	6	0.5	3	1	6	0.1

a) Binding constants were determined by the competitive techniques using ANS. See abbreviation of dyes in "MATERIALS AND METHODS".

DISCUSSION

A group of BSP binding proteins (C_v , C_1 , C_2 , C_3) was isolated from the Y-fraction of rat liver cytosol. These four proteins were found to be of ap-

proximately 46000 daltons, composed of two subunits of apparently equal size (Fig. 4). They seem to be basic proteins, because they passed through a DEAE-Sephadex column equilibrated

with 50 mM, Tris-HCl buffer (pH 7.4). The binding constants (K_1) of the C_v , C_1 and C_2 protein for BSP are $4.5 \times 10^6 \text{ M}^{-1}$, $7.1 \times 10^6 \text{ M}^{-1}$ and $4.5 \times 10^6 \text{ M}^{-1}$, respectively. These are fairly in good agreement with the values reported in the BSP binding to ligandin, $6 \times 10^6 \text{ M}^{-1}$ by Kamisaka *et al.*,¹⁶⁾ $3.5 \times 10^6 \text{ M}^{-1}$ by Arias¹⁷⁾ and $5.6 \times 10^6 \text{ M}^{-1}$ by Ketterer *et al.*¹⁸⁾ These proteins show broad binding capability (Table II), that is, they bind with high affinity not only BSP, but also other organic anions, which are known to be rapidly transported into the liver.^{19,20,21)} In not only the common physical properties such as molecular weight and basicity, but also the broad binding specificity for organic anions, are these proteins we isolated similar to the glutathione S-transferases (AA, A, B, C, E) purified by Jakoby *et al.*⁹⁾ In the recent experiment, the authors isolated proteins from Y-fraction in the similar procedure in order to study their glutathione S-transferase activities for BSP and 1-chloro-2,4-dinitrobenzene. In this experiment, we also obtained four major proteins all of which had glutathione S-transferase activities for 1-chloro-2,4-dinitrobenzene. The fraction eluted at the third position from CM-cellulose column which corresponded to C_2 protein in the elution order, had the highest enzymatic activities for BSP. This property is similar to glutathione S-transferase A which Habig *et al.*⁹⁾ reported. The details about the enzymatic activities of these proteins will be reported in the near future. The present result showed that the C_1 protein had the higher binding constant for most of organic anions than the others (Table I, Table II). This property of C_1 protein is similar to that of ligandin which was reported by Ketley *et al.*¹⁰⁾ Considering both the elution order in the present experiment and the binding and conjugating activities of the proteins for BSP, the most feasible correspondence of our proteins to the enzymes is as follows; C_3 to AA, C_2 to A, C_1 to B (ligandin) and C_v to C.

BSP binding by the Y-fraction has long been considered to be due to a single protein, *i.e.*, ligandin. For example, the quantity of ligandin in the Y-fraction has often been measured by BSP binding of liver supernatant fractions during gelfiltration.^{22,23,24)}

Our present results, however, show that the BSP binding by the Y-fraction is not due to a single protein. The best binders are C_v , C_1 and C_2 proteins in the low concentration of free BSP (below $0.3 \mu\text{M}$), and C_3 as well as the former three proteins, in the high concentration (above $1 \mu\text{M}$). The approximate ratios of the concentrations of glutathione S-transferases AA, A and C to that of transferase B (ligandin) are known to be 0.14, 0.22 and 0.52, respectively,³¹⁾ therefore, we can estimate the contribution of ligandin to the BSP binding of the Y-fraction, assuming that the C_v , C_1 , C_2 and C_3 proteins correspond to glutathione S-transferase C, B, A and AA, respectively. Since these four proteins exhibit similar binding curves for BSP as shown in Fig. 6, the contribution of ligandin to the BSP binding of the Y-fraction can be estimated only from the concentration ratio of ligandin to all of the binding proteins in the Y-fraction. This ratio is about 50%.³¹⁾ Our present observation thus supports the suggestion made by Ketley *et al.*¹⁰⁾ that the glutathione S-transferases other than ligandin also have a broad binding specificity for non-substrate organic anions. Recently, Meuwissen *et al.*²⁵⁾ observed that the binding activities for bilirubin did not show the same distribution as ligandin when the cytosol proteins were separated by zonal centrifugation and electrophoresis. This fact is also in accord with our result. Anyhow, it must be reconsidered to determine the quantity of ligandin in the Y-fraction by measuring the BSP binding.

The proteins we isolated form 1:1 primary complexes with BSP (Table I), though they are composed of two subunits of apparently equal size. Such a phenomenon is called "half of the site reactivity" by Levitzki *et al.*²⁶⁾ They presented four possible mechanism for such a reactivity. It is uncertain at present as to which mechanism will be applicable, but the most attractive mechanism is the conformational change induced by binding of one ligand, *i.e.*, negative cooperativity. Some preparations of the C_3 protein show the positive cooperativity in its binding for BSP (Fig. 7). In view of these phenomena, the studies about the change of conformation and the subunit-subunit in-

teraction of the protein will be needed to clarify the binding mechanism.

Ketley *et al.*¹⁰⁾ reported the enhancement by glutathione (GSH) of the affinity of ligandin for ICG, *i.e.*, the binding constant of $7.7 \times 10^4 \text{ M}^{-1}$ in the absence of GSH, and $3.6 \times 10^5 \text{ M}^{-1}$ in the presence of 1 mM GSH. On the contrary, Tipping *et al.*²⁷⁾ have shown that GSH and estrone sulphate compete for binding to ligandin. We also observed a result, which support the finding of Tipping *et al.* (unpublished data). The binding constant of ICG by the C_v , C_1 and C_2 proteins, which we obtained in the absence of GSH by the competitive technique, was about $1 \times 10^7 \text{ M}^{-1}$. This binding constant is larger by two orders than that reported by Ketley *et al.*¹⁰⁾ These discrepancies among the laboratories seem too large to be accounted for on the basis of the different methods and conditions employed, because we obtained the same binding constant of RB to C_v protein by the method based on the quenching of intrinsic fluorescence of a protein as that by the competitive technique (Table II, Fig. 10). On the other hand, we showed the existence of a secondary binding site in the BSP binding to the C_v , C_1 and C_2 proteins (Fig. 7). The binding constant to this site is lower by about two orders than that to the first site. Therefore, a working hypothesis, which accounts for these discrepancies, would be presented. There are two classes of binding sites for organic anions. The binding of organic anions by the first site, which has a high affinity, is competitively inhibited by GSH, and the binding by the secondary sites, which has a much lower affinity, is enhanced by GSH. This hypothesis is of much interest and under study in our laboratory, because these proteins also have glutathione S-transferase activity.

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