[Chem. Pharm. Bull. 26(1) 199—208 (1978)]

UDC 547.661.6.04 : 547.962.04

Studies on Ligand Binding Properties of Z-Fraction from Rat Liver Cytosol using 1-Anilino-8-Naphthalenesulfonate[1]

Yuichi Sugiyama, Tatsuji Iga, Shoji Awazu, and Manabu Hanano

Faculty of Pharmaceutical Sciences, University of Tokyo[2]

(Received June 4, 1977)

Spectrofluorometric studies using 1-anilino-8-naphthalenesulfonate (ANS) reveal that Z-fraction from rat liver has far the more hydrophobic binding sites than the other cytoplasmic binding fractions X and Y. Diagnostically important organic anions, such as sulfobromophthalein (BSP) and rose bengal (RB), are shown to displace ANS from Z-fraction by quenching the fluorescence of ANS and Z-fraction mixture without the change of quantum yield and emission maximum. The binding constants of these dyes to Z-fraction are determined by the competitive studies with ANS, and are coincident with those from other methods. Since the spectrophotometric technique reveals that the number of RB binding sites on Z-fraction is about twice as large as that of ANS binding sites, ANS and RB do not have necessarily the identical binding sites, though they must have partly common ones. The major binding force between organic anions and Z-fraction is supposed to be hydrophobic considering that the binding constant of Z-fraction for ANS is only about one-eight of that of bovine serum albumin (BSA) of which site is both hydrophobic and cationic. This property of sites accounts for the lower affinity for BSP of Z-fraction extracted from CCl_4 chronically intoxicated rats, which our former study has made clear.

Keywords——sulfobromophthalein; 1-anilino-8-naphthalenesulfonate; hepatic uptake; cytoplasmic binding protein; Y-protein; Z-protein; fluorescence spectrum; binding study; fluorescence quantum yield

It is well known that diagnostically important organic anions such as sulfobromophthalein (BSP), indocyanine green (ICG) and rose bengal (RB) are transported rapidly from blood to hepato-biliary system by a selective process.[3] This selective process seems to involve the protein binding-like mechanism, because it shows the following properties.[3,4] These are: (a) saturation with increasing dose; (b) mutually competitive inhibition. The clarification of this mechanism would be necessary in order to understand and predict the behavior of organic anions in the body.

Recently, Levi, *et al.*[5] discovered two hepatic cytoplasmic binding protein fractions (Y, Z), which were considered to be major determinants of the net flux of various organic anions (*i.e.* BSP, bilirubin, ICG) from plasma into the liver. Thereafter, it was reported by Takada, *et al.*[6] that these binding proteins might play an important role in the hepato-biliary transport of the non-metabolizing anions, bromphenol blue and amaranth. Also in our laboratory, the importance of these proteins in BSP transfer was shown by using CCl_4

1) This work was presented at the 95th Annual Meeting of Pharmaceutical Society of Japan, Tokyo, April 1973, and taken in part from a thesis to be submitted by Y. Sugiyama to the Graduate School, University of Tokyo, in partial fulfillment of the requirements for the Ph. D. degree.
2) Location: *Hongo, Bunkyo-ku, Tokyo.*
3) P. Jablonski and J.A. Owen, "Advances in Clinical Chemistry," Vol. 12 ed. by O. Bodansky and C.P. Stewart, Academic Press, New York and London, 1969, pp. 309—386.
4) C.A. Goresky, *Am. J. Physiol.*, **207**, 13 (1964); D.B. Hunton, J.L. Bollman, and H.N. Hoffman, *J. Clin. Invest.*, **40**, 1648 (1961); G. Paumgartner, P. Probst, R. Kraines, and C.M. Leevy, *Ann. N.Y. Acad. Sci.*, **170**, 134 (1970); C.D. Klaassen and G.L. Plaa, *Toxicol. Appl. Pharmacol.*, **15**, 374 (1969).
5) A.J. Levi, Z. Gatmaitan, and I.M. Arias, *J. Clin. Invest.*, **48**, 2156 (1969).
6) K. Takada, S. Muranishi, and H. Sezaki, *J. Pharmacokin. Biopharm.*, **2**, 495 (1974).

chronically intoxicated rats.[7]

Z-protein was known to be an acidic protein with a molecular weight of about 12000,[8] and was found in intestinal mucosa, kidney, myocardium and adipose in addition to liver.[8,9] However, about the binding properties and the nature of the binding sites, little has been known until recently, though they were partly clarified by circular dichroism analysis[10] and chemical modifications.[11]

The quantity of Z-protein in liver is not so sufficient that the binding studies using purified Z-protein by classical techniques was difficult. In order to overcome this difficulty, 1-anilino-8-naphthalenesulfonate (ANS) is used as a model anion whose binding can be measured very sensitively by spectrofluorometric techniques, and evidence is presented that BSP and RB competitively inhibit ANS binding to Z-fraction.

Bovine serum albumin (BSA) has been widely known to bind to organic anions very rigidly,[12] and its binding mechanism has become fairly clear.[13] We herein compare, therefore, the ANS binding behavior for Z-fraction with that for BSA in order to make the anion binding properties of Z-fraction clearer and clarify the reason why the binding constant of Z-fraction from intoxicated rats for BSP is smaller than that from intact rats.[7]

Experimental

Materials——ANS, amaranth (AM) and bromphenol blue (BPB) were purchased from Tokyo Chemical Industries, Co., Ltd. RB and BSA were purchased from Wako Pure Chemical Industries, Ltd. BSP, ICG and phenolsulfophthalein sodium (PSP) were purchased as the Injection (Daiichi Chemical Industries, Ltd.). All the chemicals were used without further purification. Sephadex G-50, G-75 and G-200 were obtained from Pharmacia Fine Chemicals, Inc.

Animals——Adult male Donryu (SD strain) rats weighing 280—340 g were used. CCl_4 chromically intoxicated rats were produced as described previously.[7]

Preparation of Cytoplasmic Binding Fractions——105000 *g* supernatant of a 50% homogenate of rat liver was prepared as described previously.[7] Twenty ml of the supernatant was placed on a Sephadex G-75 column (5.4×85 cm) equilibrated with 0.05 M Tris–HCl buffer (pH 7.4) and elution was performed at a flow rate of 45 ml/hr. X, Y and Z fractions was collected according to the designation by Levi, *et al.*[5] After dialysis against distilled water, each fraction was concentrated to about 3 ml by lyophilization and was applied to the following Sephadex column equilibrated with the same buffer. Columns used were Sephadex G-200 column (4.0×58 cm) in X-fraction, Sephadex G-75 column (2.0×65 cm) in Y-fraction and Sephadex G-50 column (2.2×65 cm) in Z-fraction, respectively. An elution rate of 12 ml/hr was maintained with the use of a Tokyo Rikakikai microtube pump. Column eluates were monitored photometrically at 280 nm. Protein was assayed by the Lowry method using crystalline BSA as a standard. According to this procedure, Z-fraction was prepared three times. Each preparative procedure started from five or six rats. These preparations were named 1, 2 and 3, respectively. In every experiment except for that shown in Fig. 2 (see the Result), preparation 3 was used. In the study to measure the infinite fluorescence (Fig. 2), every preparation of Z-fraction was used to be compared with one another.

Fluorometric Measurements——Fluorometric measurement was performed in a Hitachi MPF-4 fluorescence spectrophotometer equipped with a recorder in the air-conditioned room (26—29°). Experiments were performed in 0.05 M Tris–HCl buffer (pH 7.4). The fluorescence emission spectra were measured with right-angle optics using 1 cm light path quartz cuvette and 10 mm entrance and 2 mm exit slits. When necessary, the spectra were corrected by comparing the observed spectrum of quinine sulfate in 1 N H_2SO_4 with published spectrum for that compound.[14] The emission maximum of ANS bound to BSA obtained

7) T. Iga, Y. Sugiyama, M. Yokota, Y. Tomono, S. Awazu, and M. Hanano, *Biochemical. Pharmacol.*, **26**, 1867 (1977)
8) *a*) R.K. Ockner, J.A. Manning, R.B. Pappennausen, and W.K.L. Ho, *Science*, **177**, 56 (1972); *b*) G.M. Grodsky, H.J. Kolb, R.E. Franska, and C. Nemechek, *Metabolism.*, **19**, 246 (1970).
9) R.K. Ockner and J.A. Manning, *J. Clin. Invest.*, **54**, 326 (1974).
10) K. Kamisaka, I. Listowsky, Z. Gatmaitan, and I.M. Arias, *Biochim. Biophys. Acta.*, **393**, 24 (1975).
11) K. Takada, O. Naruyama, and S. Muranishi, *Chem. Pharm. Bull.* (Tokyo), **23**, 729 (1975).
12) J.F. Fostner, in The Plasma Proteins, Vol. 1, (F.W. Putnam, Ed.) New York, N.Y., Academic Press, 1960, p. 179; F.W. Putnam, *Proteins*, **3**, 187 (1965).
13) *a*) A. Jonas and G. Weber, *Biochemistry*, **10**, 1335 (1971); *b*) L.B. Swaney and I.M. Klotz, *Biochemistry*, **9**, 2570 (1970).
14) E. Lippert, W. Nagle, I. Seibold-Blankenstein, U. Staiger, and W. Voss, *Z. Anal. Chem.*, **170**, 1 (1959).

from thus corrected spectrum agreed well with that reported by Turner, *et al.*[15]

The fluorometric titration to measure the infinite intensities was carried out in the following manner. A 3.0 ml solution of ANS (5.0 μM) was titrated with a solution containing both ANS (5.0 μM) and proteins by a Termo micro-syringe so that the ANS concentration remained constant while the protein concentration increased. Each mixture solution was stirred with a micro-magnetic bar for 1 min, and the fluorescence was measured at 480 nm, with excitation at 380 nm. All other titrations were made in the similar manner. When necessary, the fluorescence intensities were corrected for both dilution and inner filter effect by the method as had been reported by Chignell.[16] In certain proteins, the titration was stopped on the way before the fluorescence intensities reached the plateau by the following reasons.

(1) The highly concentrated protein solution could not be prepared due to its aggregation (in the case of X-fraction).

(2) The protein solution had so high absorption either at 380 nm or at 480 nm that the correction for "inner filter" effect was difficult (in the case of Y-fraction).

(3) The affinity of the protein for ANS was not so high that higher concentration of the protein was needed for the titration to reach the plateau (in the case of X, Y and Preparation 1, 2 of Z-fraction).

The concentration of bound ANS was calculated by the following equation.

$$\mathrm{ANS_{bound}} = (5 \times F_{obs}/F_{bound})\mu M$$

where $\mathrm{ANS_{bound}}$ is the concentration of bound ANS, F_{obs} is the observed fluorescence, and F_{bound} is the fluorescence of a solution containing 5.0 μM ANS and large excess of protein, under which conditions the ANS is completely bound (details in "Results").

Spectrophotometric Measurements——Spectrophotometric measurements were performed in a Hitachi 356-type two wavelength spectrophotometer in an air-conditioned room (26—29°). Absorption spectra were measured by this spectrophotometer set as a split beam type. Spectrophotometric titration was made in the same manner as described in "Fluorometric measurements." The concentration of bound RB was calculated by the following equation.

$$\mathrm{RB_{bound}} = \mathrm{RB_{total}} \times (E_{obs} - E_f)/(E_b - E_f)$$

where $\mathrm{RB_{bound}}$ and $\mathrm{RB_{total}}$ are the concentrations of bound and total RB, respectively, and E_b and E_f are the molar extinction coefficients of bound and unbound RB at 562 nm. E_b was obtained from the absorbance of a solution containing 0.5—3.0 μM RB and 750 μg/ml Z-fraction, under which conditions RB is completely bound. E_{obs} is the observed molar extinction coefficient obtained by dividing the observed absorbance by $\mathrm{RB_{total}}$. The concentration of $\mathrm{RB_{total}}$ was from 0.5 μM to 4.0 μM.

Calculation of the Binding Parameters (n, K)——The concentration of unbound ligand was calculated by subtraction of the bound concentration from the total one. Results were plotted according to the method of Scatchard,[17] and the binding parameters were obtained by the non-linear iterative least square method using a Hitachi 8700/8800 digital computer.

Calculation of the Binding Constant from the Competitive Binding Data——The binding constants of various organic anions to Z-fraction were determined by the competitive techniques using equation 1.

$$K_b = \frac{K_a(A)}{(PA)} \times \frac{n(P_t)K_a(A) - K_a(A)(PA) - (PA)}{(B_t)K_a(A) - n(P_t)K_a(A) + K_a(A)(PA) + (PA)} \qquad \text{Eq. 1}$$

where K_a ($0.62 \times 10^6\ \mathrm{M^{-1}}$) and K_b are the binding constants for ANS and the competitor, respectively, (A) and (PA) are the concentrations of unbound and bound ANS, respectively, (P_t) and (B_t) are the total concentrations of Z-fraction and competitor, respectively, and n is the number of binding sites (18 μmol/g). Both n and K_a were determined in the other experiment (see Fig. 4 in Results"). This equation was derived assuming the simple competition by two ligands for equal and independent binding sites on protein.[18] The concentration of unbound ANS, (A) is expressed in equation 2.

$$(A) = (A_t) - (PA) \qquad \text{Eq. 2}$$

where (A_t) is the concentration of total ANS (2.0 μM). From Eq. 1 and Eq. 2, (PA) can be expressed in six

15) D.C. Turner and L. Brandt, *Biochemistry.*, **7**, 3381 (1968).
16) C.F. Chignell, in Methods in Pharmacology, Vol. 2, (C.F. Chignell, Ed.), Appleton-Century-Crofts, New York, N.Y., 1972, p. 33.
17) G. Scatchard, *Ann. N.Y. Acad. Sci.*, **51**, 660 (1949).
18) I.M. Klotz, H. Triwush, and F.M. Walker, *J. Am. Chem. Soc.*, **70**, 2935 (1948).

known parameters, *i.e.* K_a, K_b, n, (P_t), (B_t) and (A_t). Therefore, the fluorescence intensity which is proportional to (PA) can be calculated as a function of (B_t), when K_b is given.

Results

Fluorescence Spectra of ANS Bound to BSA and X, Y, Z-Fractions

Figure 1 shows the corrected fluorescence emission spectra of ANS in the presence of BSA and cytoplasmic binding fractions, X, Y, and Z. Since the fluorescence intensity of unbound ANS can be neglected compared with that of bound ANS under these conditions, these may well be considered as the spectra of ANS bound to the proteins. In buffer solution, ANS has very low fluorescence with a emission maximum at 550 nm, but when bound to these proteins the intensities are enhanced, and their emission maximums shift to 482 nm in BSA, 494 nm in X-fraction, 500 nm in Y-fraction and 480 nm in Z-fraction, respectively. When diagnostically important organic anions such as BSP and RB are added to the solution of ANS and Z-fraction, the fluorescence intensity decreases without any shift of the emission maximum (not shown in figure).

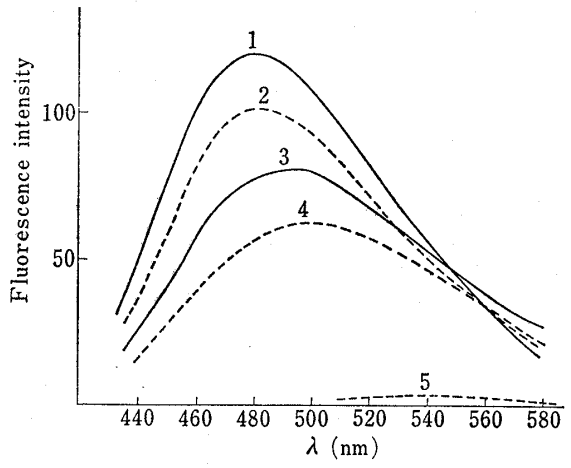

Fig. 1. Corrected Fluorescence Emission Spectra of ANS in the Presence of BSA and Cytoplasmic Fractions

Each solution contains 20 μM ANS, and the excitation wavelength is 380 nm. Curve 1, ANS+320 μg/ml Z-fraction; Curve 2, ANS+170 μg/ml BSA; Curve 3, ANS+250 μg/ml X-fraction; Curve 4, ANS+330 μg/ml Y-fraction; Curve 5, ANS only. Curve 3 and 4 are at a sensitivity of six times greater than 1,2 and 5.

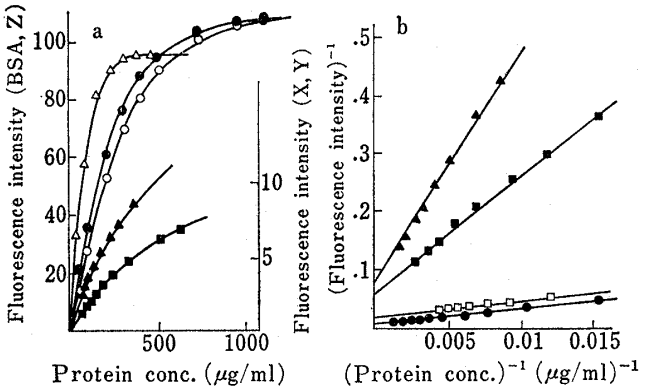

Fig. 2. Fluorometric Titration Curves of 5 μM ANS with BSA and Cytoplasmic Fractions to Obtain the Infinite Intensities

The excitation wavelength is 380 nm, and the emission wavelength is 480 nm. In the case of X and Y-fractions, the intensities are corrected for "inner filter" effect.

(a) The fluorescence intensity of ANS as a function of protein concentration. (△), BSA; (●), Z-fraction (preparation 3); (○), Z-fraction (preparation 3) with 5 μM BSP; (▲), X-fraction; (■), Y-fraction. In the experiment with BSP, both ANS solution and Z-fraction solution contain 5 μM BSP.

(b) Double reciprocal plots when the infinite intensities cannot be obtained directly. (▲), X-fraction; (■), Y-fraction; (●), Z-fraction (preparation 2); (□), Z-fraction from CCl_4 chronically intoxicated rats.

Fluorometric Titration to Obtain the Infinite Intensity

Figure 2 shows the fluorometric titration curves of a fixed concentration of ANS with BSA and the cytoplasmic fractions in order to obtain the infinite fluorescence intensities which correspond to the quantum yield of bound ANS. When 5 μM ANS is titrated with BSA or Z-fraction (preparation 3), the fluorescence intensities reach the plateau. In the titration with Z-fraction, the presence of equimolar BSP decreases the fluorescence intensities on the way of the titration curve, while the infinite intensity does not change. When the titration is stopped before the fluorescence intensities reach the plateau (see details in

"Experimental"), the infinite intensities are derived from the double reciprocal plots[19] as shown in Fig. 2(b). The infinite intensities thus obtained are 97 units in BSA, 14 units in X-fraction, 13 units in Y-fraction and 111 units (preparation 1), 118 units (preparation 2), 109 units (preparation 3) in Z-fraction, respectively. It is noteworthy that the infinite intensity of ANS bound to Z-fraction prepared from CCl_4 chronically intoxicated rats is 59 units which is about half of that obtained in intact rats.

Fluorescence Spectra of ANS and Z-Fraction Mixture Excited at 280 nm

Figure 3 shows the uncorrected fluorescence emission spectra of ANS and Z-fraction mixture excited at 280 nm. As the concentration of ANS increases, a decrease in the protein fluorescence at 310 nm and concurrent increase in the ANS fluorescence at 460 nm are shown, and there exists an isoemissive point at 406 nm as was observed in ANS and BSA mixture.[20]

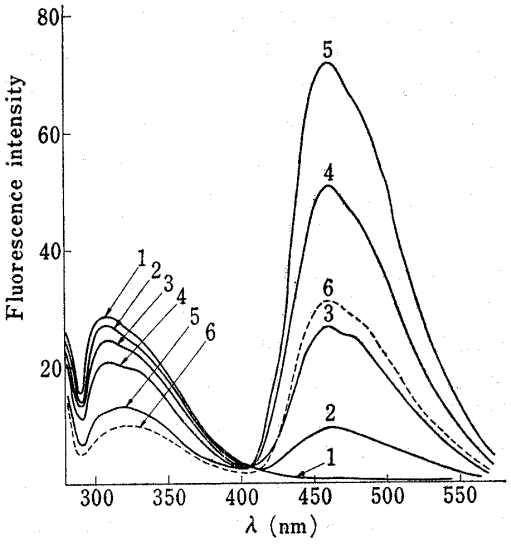

Fig. 3. Observed Fluorescence Emission Spectra of ANS and Z-Fraction Mixture as a Function of ANS Concentration

The excitation wavelength is 280 nm. The concentration of Z-fraction (preparation 3) is 388 μg/ml, and the ANS concentrations are as follows. (1), 0 μM; (2), 1.0 μM; (3), 3.32 μM; (4), 8.26 μM; (5), 26.0 μM; (6), 26.0 μM with 6.60 μM rose bengal(RB).

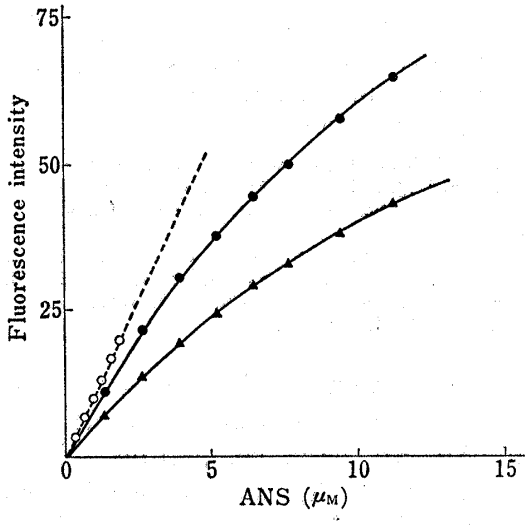

Fig. 4. Fluorometric Titration of Z-Fraction with ANS in the Presence and Absence of BSP

The excitation wavelength is 380 nm, and the emission wavelength is 480 nm. The fluorecence intensities are corrected for "inner filter" effect. The concentration of Z-fraction (preparation 3) is as follows. (○), 741 μg/ml; (●), 388 μg/ml; (▲), 388 μg/ml+5 μM BSP. The dashed line (—) is a theoretical curve calculated from Fig. 2(a) assuming that added ANS is completely bound to Z-fraction.

ANS Binding by Fluorescence Techniques

Fluorometric titration curves of Z-fraction (preparation 3) with ANS in the presence and absence of BSP are shown in Fig. 4. Data points in the Z-fraction concentration, 741 μg/ml coincides with a dashed line, which is a theoretical line calculated from Fig. 2(a). This fact suggests that added ANS below the concentration of 2 μM is completely bound to Z-fraction.

Figure 5(a) shows the Scatchard plot of ANS binding to Z-fraction in the presence and absence of BSP. In the absence of BSP, 18 μmol/g-protein for the number of binding sites (n), and 0.62×10^6 M^{-1} for the binding constant (K) are obtained. The presence of 5 μM BSP decreases K value to 0.17×10^6 M^{-1} without the change of n value.

ANS binding to BSA is measured in the same manner (Fig. 5(b)), and compared with that

19) *a*) B. Rubalcava, M.D. Martinez, and C. Giltler, *Biochemistry.*, **8**, 2742 (1969); *b*) L. Klungsoyr, *Biochemistry.*, 13, 1751 (1974).
20) E. Daniel and G. Weber, *Biochemistry.*, **5**, 1893 (1966).

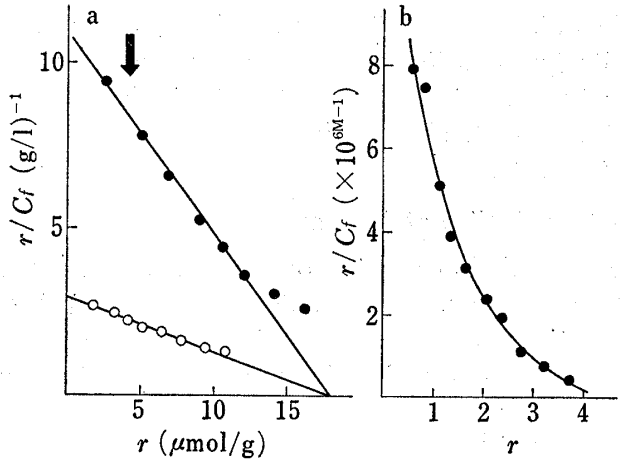

Fig. 5. Scatchard Plot of ANS Binding to Z-Fraction(preparation 3) in the Presence and Absence of BSP, and that to BSA

(a) ANS binding to Z-fraction. The data points are calculated from Fig. 4. (●), without BSP; (○), with 5 μM BSP. The arrow shows the condition used in the competitive study described in Fig. 6.

(b) ANS binding to BSA. r is expressed in moles ANS bound per mole BSA, regarding BSA molecular weight as 66000. The solid line is a theoretical curve computed using $n_1=2$, $n_2=3$, $K_1=4.9\times10^6$M^{-1} and $K_2=1.8\times10^5$M^{-1}.

to Z-fraction. The binding constant for the first site (K_1) is computed to be 4.9×10^6 M^{-1}, which is about eight times as large as that in Z-fraction.

Effects of Organic Anions on the Fluorescence of ANS and Z-Fraction Mixture

The effects of various anions as well as BSP on ANS binding to Z-fraction (preparation 3) are investigated by using fluorescence decrease (Fig. 6). Dyes used are BSP, RB, ICG, BPB, and AM, which have been known to have high affinities for hepato-biliary system,[6,21] and PSP widely known to be actively secreted by the tubules in the kidney.[22] Figure 6(a) shows the effects of these dyes on the fluorescence intensities of ANS and Z-fraction mixture. The concentrations of Z-fraction and ANS used in this experiment are 388 μg/ml and 2 μM, respectively, and under these conditions about 80% of added ANS is bound to Z-fraction and that to the first binding site, as shown by as arrow in Fig. 5(a). RB and BPB have large effects on the fluorescence intensities, BSP and ICG have intermediary effects, and PSP and AM are weak competitors. In order to carry out these competitive studies more quantitatively, the fluorescence intensities are measured as a function of the competitor concentration (Fig. 6(b)). PSP, BSP, and RB are selected as typical competitors. The solid lines in Fig. 6(b) are theoretical curves calculated according to Eq. 1 and Eq. 2 (see details in "Experimental"), taking $K_b=1\times10^5$ M^{-1}, 5×10^5 M^{-1}, 2×10^6 M^{-1}, 1×10^7 M^{-1} and 5×10^7 M^{-1} (top to bottom), respectively. By comparing the data with these theoretical curves the binding constants of these competitors are determined to be about 1×10^5 M^{-1} in PSP, 2×10^6 M^{-1} in BSP and 1×10^7 M^{-1} in RB, respectively.

RB Binding to Z-Fraction by Spectrophotometric Techniques

The binding of RB to Z-fraction (preparation 3) is investigated spectrophotometrically to confirm whether the binding constant determined by the competitive technique is reasonable or not. RB in aqueous solution has an absorption maximum at 548 nm (curve 1 in Fig. 7). When a high concentration of Z-fraction is added, RB is almost completely bound to Z-fraction and the absorption maximum shifts to 562 nm (curve 4 in Fig. 7). There exists an isosbestic point at 556 nm, which indicates that the molar extinction coefficient of bound RB is constant in spite of the multiplicity of RB binding sites, therefore, it is possible to carry out binding studies spectrophotometrically.

Figure 8(a) shows the binding curve obtained by the spectrophotometric techniques (see details in "Experimental"). RB binding to Z-fraction does not seem to be affected by the protein concentration, because it is expressed in the identical binding curve under any concentration of Z-fraction, namely 19.4 μg/ml, 38.8 μg/ml and 129 μg/ml. Fitting these

21) S.L. Schanker, in Handbook of Physiology, Vol. 5., (W. Heidel, Ed.) Waverly Press, Inc., Baltimore, Maryland, 1968, p. 2433.
22) I. Sperber, *Pharmacol. Rev.*, **11**, 109 (1959).

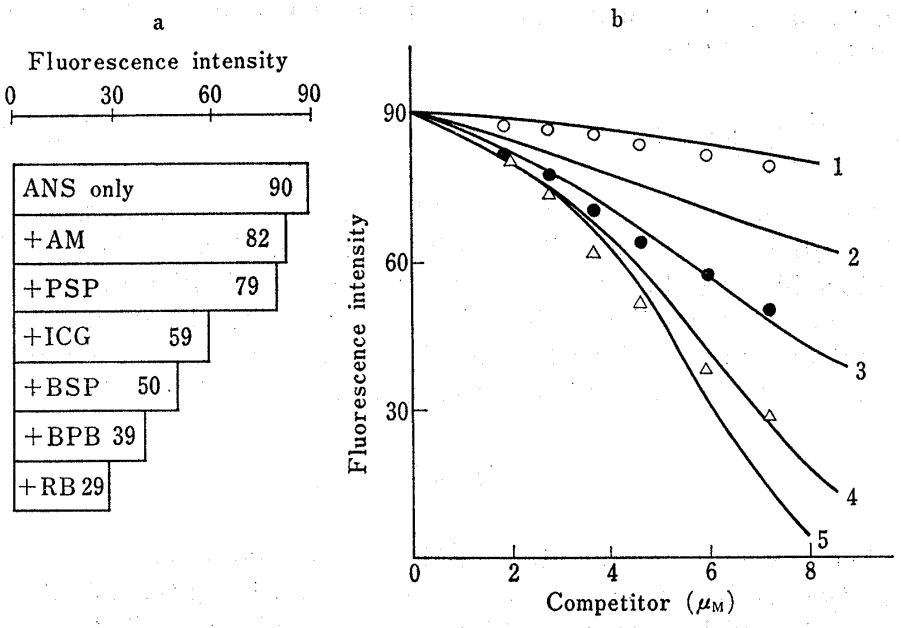

Fig. 6. The Competition of Various Organic Anions with ANS for the Binding Sites on Z-Fraction

The concentration of Z-fraction and ANS is 388 μg/ml and 2 μM, respectively. Under these conditions, about 80% of added ANS is bound and that to the first binding sites (see the arrow in Fig.5(a)).

(a) The decrease of fluorescence intensities by adding various competitors. Each competitor concentration is 7.2 μM.

(b) The titration of ANS and Z-fraction mixture with PSP (○), BSP (●) and RB (△). The solid lines are theoretical curves calculated according to Eq.1 and Eq.2 on the basis of $K_a=0.62\times10^6\text{M}^{-1}$, $n=18$ μmol/g, and $K_b=$, (1), $1\times10^5\text{M}^{-1}$; (2), $5\times10^5\text{M}^{-1}$; (3), $2\times10^6\text{M}^{-1}$; (4), $1\times10^7\text{M}^{-1}$; (5), $5\times10^7\text{M}^{-1}$.

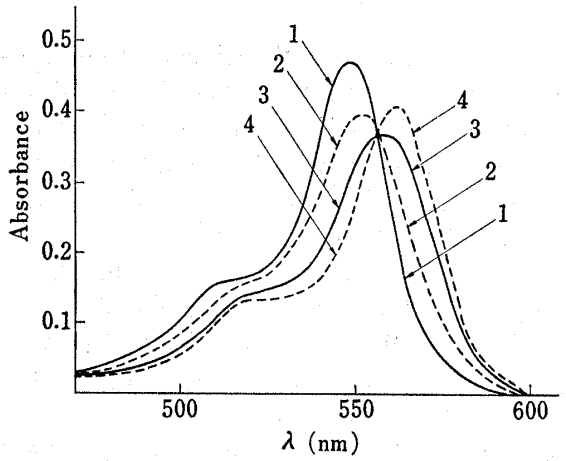

Fig. 7. Absorption Spectra of RB as a Function of Z-Fraction Concentration

Each solution contains 5 μM RB. The concentration of Z-fraction is as follows. (1), 0; (2), 35 μg/ml; (3), 82 μg/ml; (4), 370 μg/ml.

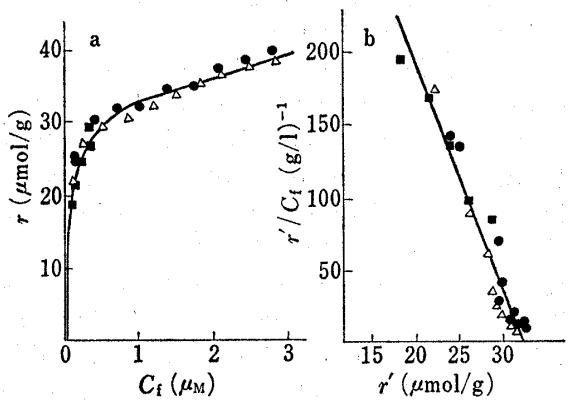

Fig. 8. The Binding of RB to Z-Fraction Determined by Spectrophotometric Titration

The concentration of Z-fraction (preparation 3) is, (●), 19.4 μg/ml; (△), 38.8 μg/ml; (■), 129 μg/ml.

(a) Binding curve. The solid line is a theoretical curve computed using $n=32$ μmol/g, $K=1.62\times10^7\text{M}^{-1}$, and $n'K'=2.57$ $(\text{g/l})^{-1}$ in Eq. 4.

(b) The Scatchard plot of RB binding to the tight binding sites. r' is obtained by dividing RB concentration bond to the tight binding sites by Z-fraction concentration. (See details in the text).

binding data with general Langmuir-type equation (Eq. 3) cannot give the converged parameter.

$$r=\sum_{i=1}^{2}\frac{n_iK_iC_f}{1+K_iC_f} \qquad \text{Eq. 3}$$

Therefore, Eq. 4 is used intead of Eq. 3 considering that the latter half of the binding curve exhibits almost the straight line.

$$r = \frac{nKC_f}{1+KC_f} + n'K'C_f \qquad \text{Eq. 4}$$

In this case, the converged parameters are obtained, *i.e.* $n=32$ μmol/g, $K=1.62\times10^7$ M^{-1} and $n'K'=2.57$ (g/l)$^{-1}$. The nonspecific binding which the term $n'K'C_f$ implies has often been seen in other binding studies.[23] Subtracting the contribution of nonspecific binding from the whole binding gives the straight Scatchard plot, which indicates the presence of homogenous tight binding sites for RB (Fig. 8(b)). The binding constant thus obtained is 1.62×10^7 M^{-1}, and in good agreement with that from the competitive study.

Discussion

The emission maximum and quantum yield of fluorescent probes have been known to reflect the polarity of active sites in several proteins. Namely, as the polarity of active sites decreases, the quantum yield increases and the emission maximum shifts toward the blue.[15,24] From the emission maximum shown in Fig. 1, it can be seen that Z-fraction has fairly hydrophobic binding sites similar to BSA, and that X and Y-fractions have polar binding sites considering that chymotrypsin having emission maximum at 490 nm is known to be a very polar protein.[15,25] The quantum yield gives the similar results about the polarity of binding sites on cytoplasmic fraction as the emission maximum does. Namely, the quantum yield of ANS bound to each fraction is determined to be 0.11 in X-fraction, 0.10 in Y-fraction and 0.87 in Z-fraction, respectively (Fig. 2), when the quantum yield of ANS bound to BSA is assumed to be 0.75.[26] Judging from both the emission maximum and quantum yield of bound ANS, Z-fraction as well as BSA has much more hydrophobic binding sites than the other cytoplasmic binding fractions X and Y.

Therefore, the binding of ANS to Z-fraction is investigated quantitatively using fluorescence enhancement in order to make its binding mechanism clearer. This binding study is based on the assumption that the fluorescent quantum yield is the same for ANS bound to any site. This assumption has been justified in the binding of ANS to BSA,[20] however, in the case of Z-fraction it is uncertain because of its impurity. The validity of this assumption is ascertained in Fig. 3. The fluorescence spectra in Fig. 3 will probably be considered as a result of energy transfer from the aromatic amino acids in Z-fraction to the bound ANS. There is an isoemissive point at 406 nm, which suggests that the quantum yield of bound ANS is the same over the values covered by this experiment. When RB is added to the ANS and Z-fraction mixture, the isomissive point disappears. This comes from the fact that a RB-Z-fraction complex is newly produced in addition to two molecular species, namely Z-fraction and ANS-Z-fraction complex. A little curvature of the Scatchard plot of ANS binding to Z-fraction (Fig. 5(a)) would probably indicate the presence of secondary binding sites, however, the contribution of these sites is so small in the region of this experiment that the binding parameters to the only first site are determined.

It becomes clear that Z-fraction has fairly hydrophobic binding sites similar to BSA, however, the binding constant of Z-fraction for ANS is only about one-eighth of that of BSA (Fig. 5). Serum albumin was known to bind small molecules, especially anions with increas-

23) A.C. Mclaughlan, *J. Biol. Chem.*, **249**, 1445 (1974).
24) L. Stryer, *Science*, **162**, 526 (1968).
25) R.P. Haugland, and L. Stryer, in Conformation of Biopolymers, Vol. 1, (G.N. Ramachandran, Ed.), Academic Press, New York, 1967, p. 321.
26) G. Weber and L.B. Young, *J. Biol. Chem.*, **239**, 1415 (1964).

ing avidity as their hydrophobic character increased.[12,27] It was generally thought, therefore, that the binding sites on serum albumin might have complementary hydrophobic as well as cationic side chains. Recently, in human serum albumin, Swaney and Klotz[13b] found the cluster of six hydrophobic residues bracketed by two cationic ones, which has been proved to be major anion binding site. Also in BSA, similar result about the binding sites was obtained by Jonas and Weber.[28] Therefore, the lower affinity for ANS of Z-fraction than BSA in spite of the similar hydrophobicity around their binding sites, is likely to be a consequence of either the lack or few number of cationic amino acid residues around the binding sites on Z-fraction. That is to say, hydrophobic binding force would probably play an important role in the binding of organic anions such as ANS to Z-fraction. This suggests that BSA has more favorable binding sites for organic anions than Z-fraction. Warmer, *et al.*[29] suggested that the designation of Z-protein specifically as an organic anion binding protein was inappropriate because of its high affinity for uncharged hexachlorophene. Most recently, Ketterer, *et al.*[30] indicated that the affinities to Z-protein of the neutral compounds such as testosterone and oestradiol were similar to those of charged steroid sulphates. Our speculation described above also supports these results. In the binding of BSP and RB to Z-fraction, each binding constant was determined to be $2.5\times10^{6}\,\mathrm{M}^{-1}$ [5] and $1.6\times10^{7}\,\mathrm{M}^{-1}$ (Fig. 8), respectively, while their binding constants to BSA were reported to be $1.7\times10^{7}\,\mathrm{M}^{-1}$ [31] and $1.2\times10^{8}\,\mathrm{M}^{-1}$.[32] In each case, the binding constant for BSA is considerably larger than that for Z-fraction, which supports the conception described above.

The binding study between ANS and BSA was carried out in the same manner by Par-Lin, *et al.*,[27] who gave a straight Scatchard plot and obtain the binding parameters, $n=2.9$ and $K=1.1\times10^{6}\,\mathrm{M}^{-1}$. This deviation from our results perhaps came from the differences in BSA, such as the difference in fatty acid content, as has been sometimes pointed out.[33]

In our laboratory, Z-fraction from CCl_4 chronically intoxicated rats was shown to have about one-sixth of binding constant for BSP in comparison with that from intact rats, and this decrease in the binding constant has been supposed to be one of the reason for slower uptake of BSP from plasma into the liver in intoxicated rats.[7] While, in the present study, it is observed that the quantum yield of ANS bound to Z-fraction from intoxicated rats is about one half of that from intact rats (Fig. 2), therefore, such a lower affinity for BSP in intoxicated rats probably has resulted from the decrease in hydrophobicity of the binding sites. This speculation is reasonable considering that the main binding force between organic anions and Z-fraction is hydrophobic one. No difference could be found only in the disk gel electrophoretic pattern (unpublished data) but also in their gel filtration pattern[7] between Z-fraction from intoxicated rats and that from intact rats. These results indicated that the decrease in hydrophobicity of the binding sites on Z-fraction from intoxicated rats does not arise from the bulk change of Z-fraction which is usually accompanied by the alteration in molecular weight and isoelectric point, but from the local change around its binding sites.

It is indicated that BSP diminishes the fluorescence intensity of ANS and Z-fraction mixture without any change of the emission maximum (Fig. 2(a)). Such a diminution as above has been attributed to the two causes. One is the conformational change of proteins induced by the ligand binding,[23,34] and the other is the competition between the probe and

27) Hsu.Par-Lin, K.H. Ma. Joseph, H.W. Jun, and L.A. Luzzi, *J. Pharm. Sci.*, **63**, 27 (1974).
28) A. Jonas and G. Weber, *Biochemistry.*, **9**, 5092 (1970).
29) M. Warner and A.H. Neim, *Can. J. Physiol. Pharmacol.*, **53**, 493 (1975).
30) B. Ketterer, E. Tripping, and J.F. Hackney, *Biochem. J.*, **155**, 511 (1976).
31) K.J. Baker and S.E. Bradely, *J. Clin. Invest.*, **45**, 281 (1966).
32) L. Ibuki, Y. Sugiyama, S. Awazu, and M. Hanano, *in preparation.*
33) M. Sogami, *Kagakuno-ryoiki.*, **23**, 602 (1969).
34) G. Sudlow, D.J. Birkett, and D.N. Wadw, *Molecular Pharmacol.*, **9**, 649 (1973).

ligand for the binding sites on proteins.[35] In the former case, the changes of emission maximum and quantum yield of the probe have been often observed.[23] In our study, neither the emission nor the quantum yield changes (Fig. 2(a)), therefore, the competition between BSP and ANS for the binding sites on Z-fraction is supposed to occur. This is ascertained also in Fig. 5(a), that is to say, the presence of BSP decreases the binding constant between ANS and Z-fraction without the change of number of the binding sites. As the competition between ANS and BSP for Z-fraction becomes evident, the binding constants of various organic anions to Z-fraction are determined by the competitive techniques. Such competitive studies are shown to be available for the estimation of BSP and RB binding constants to Z-fraction considering that the binding constants thus obtained are comparable with those from the other independent binding studies. The competitive study also gives the binding constants of BPB and AM to Z-fraction, $4\times10^6\,\text{M}^{-1}$ and $0.8\times10^5\,\text{M}^{-1}$, respectively (Fig. 6(a)). While, the difference in the hepatic uptake rate between BPB and AM was attributed by Takada, *et al.*[6] to the more binding to Z-fraction of the former than the latter. The binding constants of these anions given in the present study also support their results.

A little deviation is observed between the experimental data and the curves theoretically obtained, especially in the case of RB (Fig. 6(b)). A possible explanation is that the assumption of simple competition by two ligands for equal and independent binding sites is not valid. In fact, the ANS binding sites on Z-fraction are regarded as homogeneous, though strictly speaking this is not correct as shown in a little curvature of the Scatchard plot (Fig. 5(a)). However, in order to neglect this effect, the competitive study is carried out on the condition that the ANS binding is almost limited to the first binding sites on Z-fraction, so that this deviation cannot be attributed to only the heterogeneity of ANS binding sites. While, spectrophotometric techniques reveal that the number of RB binding sites on Z-fraction is 32 μmol/g, which is about twice as large as that of ANS binding sites. Consequently, the differences in the number of binding sites on Z-fraction between ANS and RB would probably make the assumption of simple competition invalid, and lead to the deviation from the theoretical curve.

The present studies show that the quantum yield of ANS bound to Z-fraction exceeds far those to X and Y-fractions. Such a characteristic of Z-fraction will perhaps make it possible to use the ANS fluorescence enhancement as an excellent criterion of active Z-protein in the course of its purification.

Acknowledgement This work was supported by the Grant-in-Aid for Scientific Research provided by the Ministry of Education Science and Culture to which the authors are grateful.

35) H.W. Jun, R.T. Mayer, C.M. Himel, and L.A. Luzzi, *J. Pharm. Sci.*, **60**, 1821 (1971); C.F. Chignell, *Molecular Pharmacol.*, **5**, 244 (1969).