Drug Interactions. V.[1] Binding of Basic Compounds to Bovine Serum Albumin by Fluorescent Probe Technique[2]

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The binding of basic compounds to bovine serum albumin (BSA) was studied by the fluorescence method, using N-phenyl-1-naphthylamine (NPN) and 1-anilinonaphthalene-8-sulfonate (ANS) as fluorescent probes. The spectral property of these probes was used for an indirect measurement of the binding of basic compounds to BSA. The compounds used in the present investigation were chlorpromazine, diazepam, chlordiazepoxide, nitrazepam, aniline, and acetanilide. Its results demonstrated that these compounds bind to the hydrophobic regions on the albumin molecule. It appeared that these compounds may be located at least near a tryptophan residue of albumin and two probes may bind at the same site. In the case of chlorpromazine, this drug competed with NPN but not with ANS in binding to BSA. Competitive binding between these compounds, except chlorpromazine, and the probes was observed. The significance of these results was discussed in relation to various types of binding forces. NPN is a useful probe in the evaluation of interactions of basic compounds to serum albumin, but the usefulness of the probe technique may be limited in that binding studies are related only to the site to which the probe binds.

Keywords——albumin binding; fluorescent probe technique; 1-anilinonaphthalene-8-sulfonate; N-phenyl-1-naphthylamine; chlorpromazine; diazepam; chlordiazepoxide; nitrazepam; aniline; acetanilide

Our preceding report[1] described a fluorescent probe technique which is a convenient method for estimating the nature of binding and binding sites from chemical structure and spectral properties of the probe used. Numerous studies on the binding of acidic compounds to plasma protein have been reported[4] but publications on basic compounds are relatively scanty. In this study, 1-anilinonaphthalene-8-sulfonic acid (ANS) and N-phenyl-1-naphthylamine (NPN) were used to study the binding of some basic compounds to bovine serum albumin (BSA). ANS is the most popular fluorescent probe and ionizes completely at pH 7.4. In contrast, NPN is practically nonionized at pH 7.4. NPN was chosen for two reasons. In connection with the importance of nonpolar interactions in the binding of drugs to serum albumin, it is known that hydrophobic interactions play an important role in protein binding.[5] Furthermore, it is likely that NPN is deeply submerged in the hydrophobic pockets of BSA, whereas ANS is localized in the surface region.[6] Recently, these probes were used to analyse the sorption of fatty acids on molecules of BSA.[7] The fluorescence intensity of the probes in protein dispersion decreases by the introduction of certain drug molecules. This is taken as an indication that competition between the probe and the drug occurs and that the drug falls into the same category of the probe.[1,8]

1) Part IV: S. Ozeki and K. Tejima, *Yakugaku Zasshi*, **98**, 320 (1978).
2) Presented at the 95th Annual Meeting of the Pharmaceutical Society of Japan, Osaka, April 1975.
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4) M.C. Meyer and D.E. Guttman, *J. Pharm. Sci.*, **57**, 895 (1968).
5) *a)* C. Hansch and G.L. Lawrence, *J. Am. Chem. Soc.*, **87**, 5770 (1965); *b)* R. Franke, *Biochim. Biophys. Acta*, **160**, 378 (1968); *c)* J. Krieglstein, W. Meiler, and J. Staab, *Biochem. Pharmacol.*, **21**, 985 (1972).
6) V.S. Danilov, A.S. Shevchenko, V.G. Rebrov, and S.N. Orlov, *Biofizika*, **20**, 822 (1975).
7) S.N. Orlov, V.G. Rebrov, Yu.A. Malkov, V.A. Fedin, and V.S. Danilov, *Biofizika*, **21**, 75 (1976).
8) L. Brand, J.R. Gohlke, and D.S. Rao, *Biochemistry*, **6**, 3510 (1967); P-L. Hsu, J.K.H. Ma, and L.A. Luzzi, *J. Pharm. Sci.*, **63**, 570 (1974).

The purpose of the present study is to define the usefulness of NPN as a fluorescent probe in studying the interaction of some basic compounds, and the binding parameters of these compounds. Further the difference of fluorescence changes in interaction of ANS or its non-sulfonated analog NPN with BSA due to competitor compounds may provide a useful information in understanding interactions between proteins and small molecules.

Experimental

Materials——Bovine serum albumin, Fraction V, was obtained from Wako Pure Chemical Industries, Ltd., and its molecular weight was assumed to be 69000. The fluorescent probe, ANS was purchased as sodium salt from Tokyo Kasei Co., Ltd.; NPN was obtained from Wako Pure Chemical Industries, Ltd. Chlorpromazine hydrochloride, diazepam, chlordiazepoxide, nitrazepam, aniline. acetanilide, and other chemicals of analytical grade were obtained from commercial sources, and used without further purification.

Instrument——Fluorescence measurements were made with a Hitachi MPF-3 fluorescence spectrophotometer equipped with a recorder. Fluorescence emission spectra in this study were not corrected. Absorption spectra were measured with a Hitachi 124 spectrophotometer.

Methods——Fluorescence intensity of the protein-probe complex as a function of probe concentration was measured at appropriate protein concentration (1.38×10^{-6} M) in pH 7.4 phosphate buffer. Two milliliters of protein solution was titrated with successive addition of 2 μl of 1×10^{-3} M NPN in MeOH or ANS in the phosphate buffer. Titration was performed manually at 27° with a microsyringe (MS-10, Jintan Terumo Co.). Methanol did not affect the binding of the probe in the concentration range used. Binding of the compounds was determined by titration of mixture of the compounds and BSA with the probe solution. The excitation and emission wavelengths for BSA, NPN, and ANS were 300, 350, and 400 nm, and 340, 410, and 465 nm, respectively. To minimize the photodecomposition of probes, samples were exposed to the light only for the short measurement period. Blank titration in the buffer was made to correct the fluorescence of free probe in the absence of protein. The inner filter effect was not observed by keeping absorbance low at the excitation wavelength (less than 0.04).[9]

Treatment of Data——Enhancement of the fluorescence of the probe upon addition to BSA and the subsequent decrease of fluorescence in the presence of binding competitors were used to calculate the binding parameters for the probe and competitors. The fraction of probe bound, X, was calculated using the following Eq. (1).

$$X = (I_0 - I_f)/(I_b - I_f) \tag{1}$$

where I_0 and I_f rafer to the fluorescence intensities of a given concentration of probe in solutions of lower protein concentration and in solutions without protein, respectively, and I_b refers to the fluorescence intensity of the same concentration of probe in solutions of high protein concentration, which gives fluorescence intensity of the probe in the presence of excess binding sites. In this study, the fluorescence titrations were carried out at several different protein concentrations, and the values of I_b were taken to be extrapolated values of the plots of $1/I$ versus $1/(P)$, to $1/(P) = 0$, where (P) represents the concentration of BSA. Competition experiments between probes and compounds were calculated with the assumption that bound fluorescence intensity was not changed. After the value X is found for each point along the titration curve, the Scatchard Eq. (2) may be applied to determine the association constant by methods used in preceding report.[1]

$$\bar{v}/D_f = nka - \bar{v}ka \tag{2}$$

where $\bar{v}$ is the number of mol of bound probe per mol of protein, D_f is the concentration of free probe, n is the number of binding sites on the protein molecule, and ka is the association constant of the probe.[10] Klotz et al.[11] derived the equation describing between two ligands for identical binding sites.

$$kb = \frac{nP_tkaD_f - kaD_fD_b - D_b}{B_tkaD_f - nP_tkaD_f + kaD_fD_b + D_b} \times \frac{kaD_f}{D_b} \tag{3}$$

where kb = association constant for competitor, ka = association constant for the probe, D_f = concentration of free probe, D_b = concentration of bound probe, n = number of binding sites, P_t = total concentration of protein, and B_t = total concentration of competitor. Hence, Eq. (3) can be written

$$kb = \frac{ka(n - \bar{v}) - \bar{v}/D_f}{B_t/P_t - n + \bar{v} + (\bar{v}/D_f)1/ka} \times \frac{1}{(\bar{v}/D_f)P_t} \tag{4}$$

where $\bar{v} = D_b/P_t$

The competitive binding of compounds to protein was determined by using Eq. (4).

9) C.F. Chignell, "Methods in Pharmacology," Vol. 2, Meredith, New York, 1972, pp. 33—61.
10) G. Scatchard, *Ann. N.Y. Acad. Sci.*, **51**, 660 (1949).
11) I.M. Klotz, H. Triwush, and F.M. Waker, *J. Am. Chem. Soc.*, **70**, 2935 (1948).

Partition Coefficient Measurements——Portions of octanol (saturated with water) containing NPN and water (saturated with octanol) were used. After shaking the mixture for 0.5 hr and centrifugation, the concentration of the probe in the two layers was determined by fluorometry. The partition coefficient was calculated from P.C.$=Co/Cw$, where Co is the concentration of the compound in the organic phase and Cw, the concentration of the compound in the aqueous phase.

The Study of Interaction between the Probes and Compounds——In the case of interactions between NPN and compounds, excess quantity of NPN was placed in a glass-stoppered bottle together with 50 ml of pH 7.4 phosphate buffer containing the compound ($1—2 \times 10^{-4}$ M). The bottles were placed in a mechanical shaker at room temperature ($20°—24°$) and equilibrated for some time. Each solution was centrifuged. One milliliter of the supernatant liquid was pippeted into a glass-stoppered centrifuge tube containing 5 ml of hexan and 6 ml of 0.1 N HCl. The tube was shaken and centrifuged at room temperature. The concentration of NPN in hexane was determined by fluorometry. The content of the compound in the aqueous extract was determined by spectrophotometry. In the case of interaction between ANS and compounds, excess amount of the compound was weighed into a series of glass-stoppered tubes containing 5 ml of pH 7.4 phosphate buffer containing ANS. The tubes were stoppered and shaken at room temperature ($20°—24°$). After equilibration was attained, each solution was centrifuged. One milliliter of the supernatant liquid was pippeted into a glass-stoppered centrifuge tube containing 8 ml of $CHCl_3$. The tube was shaken for 15 min and centrifuged. Concentration of the compound in the organic phase was determined by spectrophotometry.

Result and Discussion

Binding of NPN to BSA

The fluorescence emission spectra of NPN-BSA complexes, excited at 300 nm, are shown in Fig. 1. It is seen that as NPN concentration increases, NPN fluorescence (λ_{max} 410 nm) increases and concurrently the protein fluorescence (λ_{max} 340 nm) decreases. It is considered that the quenching of BSA by NPN, like that observed in other protein interactions,[12] is due to energy transfer from excited state tryptophan residues to the bound probe. A general prerequisite for energy transfer to occur by Föster mechanisms[13] is that the emission band of
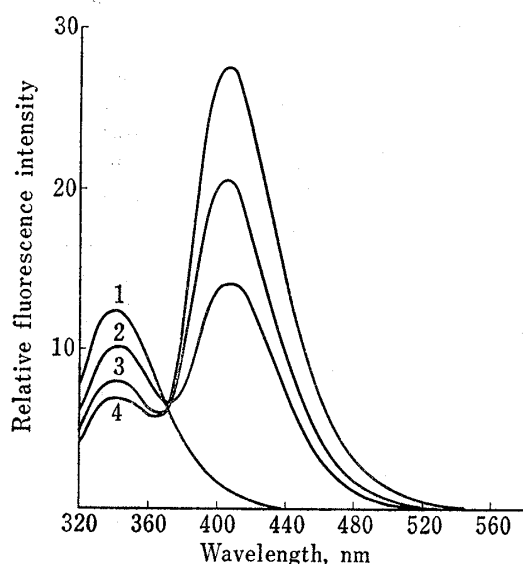

Fig. 1. Fluorescence Emission Spectra excited at 300 nm

1: 1.38×10^{-6} M BSA.
2: 1.38×10^{-6} M BSA in the presence of 1×10^{-6} M NPN.
3: 1.38×10^{-6} M BSA in the presence of 2×10^{-6} M NPN.
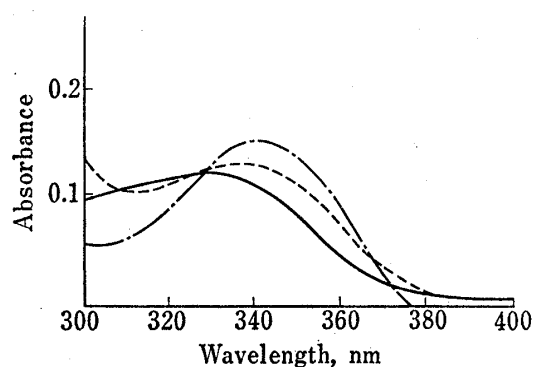4: 1.38×10^{-6} M BSA in the presence of 4×10^{-6} M NPN.

Fig. 2. Absorption Spectra of 2×10^{-5} M NPN

———: in phosphate buffer, pH 7.4.
———: in the presence of BSA in phosphate buffer, pH 7.4.
———: in ethanol.

12) C.F. Chignell, Mol. Pharmacol., 6, 1 (1970); G. Weber and L.B. Young, J. Biol. Chem., 239, 1415 (1964).
13) T. Föster, Discussions Faraday Soc., 27, 7 (1959).

the donor (*i.e.*, tryptophan residues of BSA) overlaps the absorption band of the acceptor. Fig. 1 and 2 show that this prerequisite is met in the system (NPN-BSA), since the protein fluorescence band (λ_{max} 340 nm) overlaps the NPN absorption band (λ_{max} 330 nm). The fact that NPN strongly quenches the fluorescence of albumin, therefore, indicates that the binding site for this probe is in the vicinity of tryptophan residues.

In phosphate buffer at pH 7.4, NPN has an absorption maximum at 330 nm (Fig. 2). The binding of NPN to BSA resulted in the shift of the absorption maximum to 340 nm, with a slight increase in the intensity of absorption. The red shift of the NPN absorption and increase of the intensity observed when NPN was bound to BSA were similar to the spectral changes provoked by changing the solvent from polar water to a more apolar ethanol (Fig. 2). It can be concluded from these observations that a part of the aromatic moieties of NPN molecule must be located in hydrophobic regions of the albumin molecule, when the probe is bound to the binding sites.

Competitive Binding between NPN and Compounds

Fig. 3 shows fluorescence emission spectra of the probe-BSA complex in the presence and absence of diazepam. A decrease in fluorescence of the probe-BSA complex in the presence of diazepam is an evidence of the competition between the probe and the drug for the same binding sites on BSA. Compounds used in this experiment did not alter the shape of the emission spectrum or the wavelength of maximum fluorescence of the probe-BSA complex. Fig. 4 shows Scatchard plots of NPN made from the binding data. The plots represent experimental data. The solid lines are theoretical curves generated by Eq. (4). Association constant (ka) was taken as 4.8×10^5 M^{-1}, kb as 5.5×10^3 M^{-1}, P_t as 1.38×10^{-6} M, and $n=2.0$. The data are consistent with simple competition between diazepam and NPN for the binding sites on BSA. In addition, to study the effect of different concentrations in binding experiments were carried out at three diazepam concentrations and results are shown in Fig. 4. Similar experiments were carried out with chlorpromazine, aniline, chlordiazepoxide, nitrazepam, and acetanilide. In the case of nitrazepam and chlordiazepoxide, titrations were monitored by fluorometry by excitation at 375 nm, a wavelength at which BSA and drug show no appreciable absorption. These results are shown in Fig. 4—6.
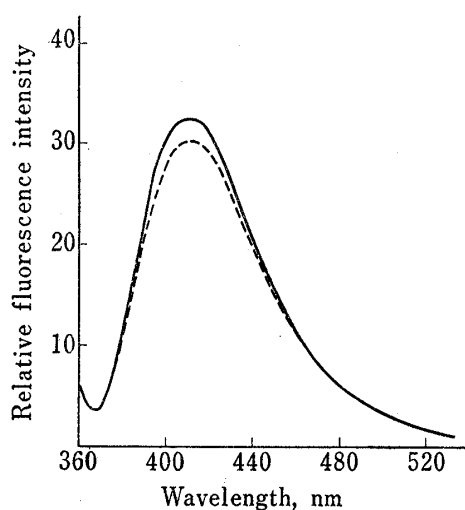

Fig. 3. Fluorescence Emission Spectra excited at 350 nm for Solutions containing 1.38×10^{-6} M BSA and 5×10^{-6} M NPN

——: in the absence of drug.
----: in the presence of 1×10^{-4} M diazepam.

Fig. 4. Scatchard Plots of NPN binding to BSA at 27° and pH 7.4

—○—: in the absence of drug.
—●—: in the presence of 5×10^{-5} M diazepam.
—□—: in the presence of 1×10^{-4} M diazepam.
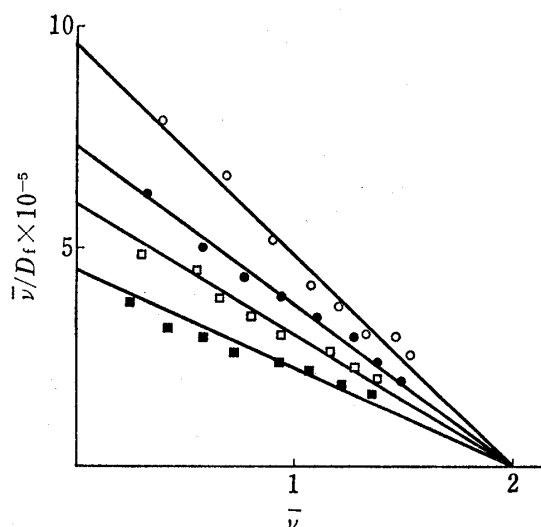—■—: in the presence of 2×10^{-4} M diazepam.

Fig. 5.   Scatchard Plots of NPN binding to BSA at 27° and pH 7.4

—○—: in the absence of drug.
—●—: in the presence of 5×10^{-5} M chlorpromazine.
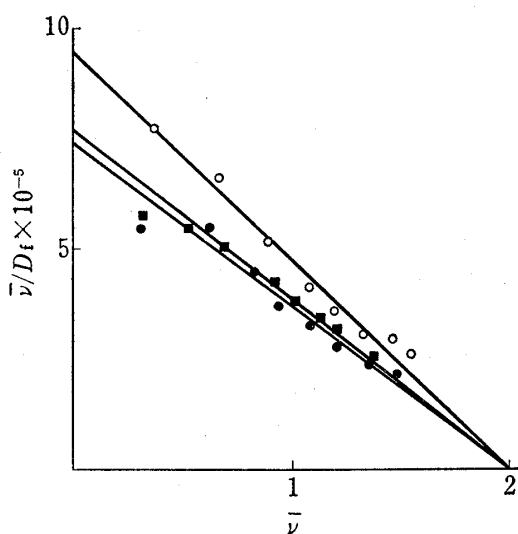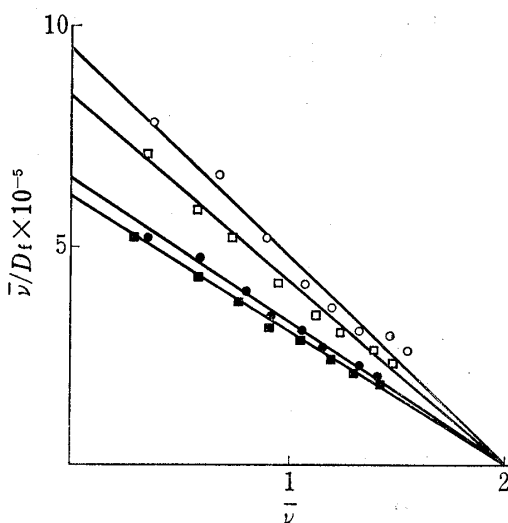—■—: in the presence of 1×10^{-4} M aniline.

Fig. 6.   Scatchard Plots of NPN binding to BSA at 27° and pH 7.4

—○—: in the absence of drug.
—●—: in the presence of 2.5×10^{-5} M chlordiazepoxide.
—■—: in the presence of 2.5×10^{-5} M nitrazepam.
—□—: in the presence of 1×10^{-4} M acetanilide.

TABLE I.   Summary of Binding Parameters and Some Physicochemical Properties for Compounds

Compound	This study			Literature		log $P^{b)}$	pKa
	n	$ka \times 10^{-3}$ M^{-1}	$nka \times 10^{-3}$ M^{-1}	$nka \times 10^{-3}$ M^{-1}	Method$^{a)}$		
Nitrazepam	2.0	19.4	38.8	30.4$^{c)}$	GF	2.1$^{h)}$	3.2$^{k)}$
Chlordiazepoxide	2.0	13.6	27.2	10.5$^{d)}$	UF	2.4$^{i)}$	4.6$^{k)}$
				13.3$^{e)}$	GF		
Chlorpromazine	2.0	5.6	11.2	12.3$^{e)}$	ED	5.4$^{i)}$	9.4$^{l)}$
				21.0$^{5a)}$	GF		
				25.7$^{f)}$	SP		
Diazepam	2.0	5.5	11.0	10.4$^{d)}$	UF	2.8$^{i)}$	3.3$^{k)}$
				45.9$^{c)}$	GF		
Aniline	2.0	2.0	4.0			1.0$^{i)}$	4.6$^{m)}$
Acetanilide	2.0	1.4	2.8	21.5$^{g)}$	DD	1.2$^{i)}$	0.3$^{m)}$
NPN	2.0	480.0	960.0			4.2$^{j)}$	

a) ED: equilibrium dialysis, GF: gel filtration, SP: spectral method, UF: ultrafiltration, DD: dynamic dialysis; pH 7.0—7.4.
b) The partition coefficient (n-octanol/water) was calculated for the undissociated molecule.
c) W.E. Muller and U. Wollert, Biochem. Pharmacol., 25, 147 (1976).
d) E. Van Der Kleijn, Arch. Int. Pharmacodyn., 179, 225 (1969).
e) N. Nambu and T. Nagai, Chem. Pharm. Bull. (Tokyo), 70, 2463 (1972).
f) H. Zia and J.C. Price, J. Pharm. Soc., 64, 1177 (1975).
g) J.C. Dearden and E. Tomlinson, J. Pharm. Pharmacol., 22, 53s (1970).
h) W.E. Muller and U. Wollert, Naunvn–Schmiedeberg's Arch. Pharmacol., 278, 301 (1973).
i) A. Leo, C. Hansch and D. Elkins, Chem. Rev., 71, 525 (1971).
j) This study.
k) J. Barrett, W. Franklin Smyth and I.E. Davidson, J. Pharm. Pharmacol., 25, 387 (1973).
l) A. Hulshoff and J.H. Perrin, Pharm. Acta Helv., 51, 65 (1976).
m) L.S. Schanker, P.A. Shore, B.B. Brodie and C.A.M. Hogeben, J. Pharmacol. Exp. Ther., 120, 528 (1957).

The binding parameters are summarized in Table I.   In the previous papers,[14] we have shown that the affinity of various organic compounds to albumin molecule was closely correlated with the degree of hydrophobicity of the compound as measured by the oil/water parti-

14) T. Tukamoto, S. Ozeki, F. Hattori, and T. Ishida, Chem. Pharm. Bull. (Tokyo), 22, 389 (1974); S. Ozeki and K. Tejima, ibid., 22, 1297 (1974); idem, ibid., 25, 1952 (1977).

tion coefficient. This suggests that the hydrophobic interaction plays an important role in protein binding of organic compounds. Furthermore the importance of hydrophobic inter-actions for the binding of organic compounds to serum albumin has been shown by several authors.[15] The binding affinity of the compounds used in the present study, therefore, ought to be related to the log partition coefficient (log P). However, results obtained for the binding affinity indicate that this relation is not quite as close as might be expected, e.g., comparison of chlordiazepoxide ($ka=13.6\times10^3$ M^{-1}, log $P=2.4$) with nitrazepam ($ka=19.4\times10^3$ M^{-1}, log $P=2.1$) shows that nitrazepam has a much greater affinity than its hydrophobicity would indicate. Also, one might have expected that the affinity of chlorpromazine ($ka=5.6\times10^3$ M^{-1}, log $P=5.4$) or diazepam ($ka=5.5\times10^3$ M^{-1}, log $P=2.8$) would be much greater than that of chlordiazepoxide or nitrazepam. Thus, it is considered that not only hydrophobic interac-tions can be present in protein binding of the compounds, but other binding mechanisms such as ionic, hydrogen, and charge-transfer binding, or steric effect must also be involved. Investigations[15] using a gel filtration method have shown that the binding of benzodiazepine derivatives to human serum albumin is highly dependent on their structure. This observa-tion is supported by the results of the present investigation, because we can show that there are marked differences in the binding affinity of benzodiazepine derivatives (nitrazepam, chlordiazepoxide, and diazepam). Comparison of published data with the data reported here (Table I) shows that the binding strength expressed as nka obtained for the compounds are generally in good agreement except in the case of acetanilide.

The solubility of NPN decreased by the presence of chlorpromazine and concurrently the concentration of chlorpromazine decreased, but this was not so with other compounds such as diazepam, aniline, acetanilide, chlordiazepoxide, and nitrazepam. From these results NPN may react with chlorpromazine to form a complex. Therefore, it appears that the value of the binding constant obtained for chlorpromazine in this study seems to be unreliable.
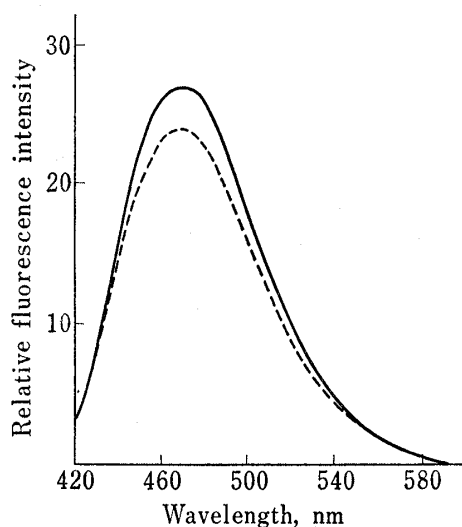
Fig. 7. Fluorescence Emission Spectra excited at 400 nm for Solutions con-taining 1.38×10^{-6} M BSA and 5×10^{-6} M ANS

——: in the absence of drug.
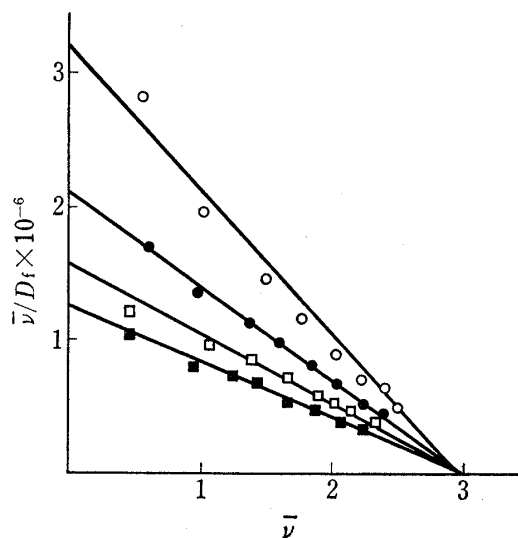----: in the presence of 1×10^{-4} M chlordiazepoxide.

Fig. 8. Scatchard Plots of ANS binding to BSA at 27° and pH 7.4

—○—: in the absence of drug.
—●—: in the presence of 5×10^{-5} M chlordiazepoxide.
—□—: in the presence of 1×10^{-4} M chlordiazepoxide.
—■—: in the presence of 2×10^{-4} M chlordiazepoxide.

15) W. Müller and U. Wollert, Naunyn-Schmiedebergs Arch. Pharmacol., 280, 229 (1973).

Displacement of ANS by Basic Compounds

Interaction of basic compounds used in this study to BSA was studied using ANS as another probe. Fig. 7 shows the effect of chlordiazepoxide on the fluorescence emission spectra of the ANS-BSA complex, and the addition of chlordiazepoxide resulted in quenching of the fluorescence intensity. Fig. 8 illustrates Scatchard plots of ANS for the effect of chlordiazepoxide. These results suggested that chlordiazepoxide competes with ANS for the same binding sites on the BSA molecule. The Scatchard plots (Fig. 8 and Table II) are reasonable quantitative agreement with the theoretical curves generated by Eq. (4). However, the situation here is not well suited for competition studies because of the difference in binding constants by different concentrations of this competitive drug. It was found, also, that nitrazepam competes with ANS for the same binding sites on BSA from similar experimetns. The results are shown in Table II. The binding constants for nitrazepam, similarly, varied from $13.7 \times 10^3 \, \text{M}^{-1}$ to $10.4 \times 10^3 \, \text{M}^{-1}$. The results obtained from Table II give some indication of a decrease in kb with increasing drug concentrations. In contrast, the difference in binding constants for diazepam by different concentrations was small (Table II). Other compounds such as aniline and acetanilide did quench the fluorescence of the binding of ANS to BSA. The binding constants of these compounds were less than $1 \times 10^3 \, \text{M}^{-1}$.

TABLE II. Association Constants of Drugs with BSA from Displacement Experiment at pH 7.4 and 27°

Probe	Drug	$B_t{}^{a)} \times 10^4$, M	$ka \times 10^{-3}$, M^{-1}
ANS	Chlordiazepoxide	0.5	10.0
		1.0	9.5
		2.0	7.5
ANS	Nitrazepam	0.5	13.7
		1.0	12.0
		2.0	10.4
ANS	Diazepam	2.5	1.0
		5.0	1.2

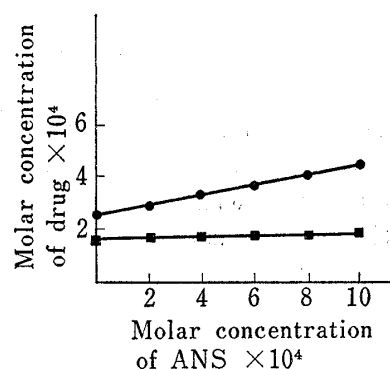a) Total concentration of competitor.

Fig. 9. The Solubility of Drugs in the Presence of ANS at 27° and pH 7.4

—●—: chlordiazepoxide.
—■—: nitrazepam.

Fig. 9 shows that the solubility of chlordiazepoxide or nitrazepam increased by the addition of ANS at concentrations up to 1×10^{-3} M, but this was not so with diazepam. These are presumably due to the formation of complexes, and the intermolecular forces between ANS and chlordiazepoxide or nitrazepam may involve electrostatic and localized charge-transfer interactions.[16] Therefore, variation of the binding constants by different concentrations may be due to complex formation of the probe and drug or the interaction of the ANS-BSA complex and drug. Furthermore, stability constants were calculated assuming the formation of 1:1 complex from the straight-line portion of the solubility diagram.[17] The stability constants of ANS-chlordiazepoxide and ANS-nitrazepam complexes were determined to be 4.4×10^2 M^{-1} and 2.8×10^2 M^{-1}, respectively. Therefore, the fact that the ANS-BSA complex gave a very large binding constant $(1.1 \times 10^6$ M$^{-1})$ compared with the ANS-chlordiazepoxide or ANS-nitrazepam complex suggests the effect of displacement of ANS by the ANS-drug complex in the binding sites of ANS are small. The experimental Scatchard plots agree well with theoretical curves generated by Eq. (4) on the assumption that both ligands are absorbed on the same site, and a mechanism of inhibition of ANS-BSA binding by chlordiazepoxide, nitraze-

16) R.S. Schnaare and A.N. Martin, *J. Pharm. Sci.*, **54**, 1707 (1965).
17) T. Higuchi and J.L. Lach, *J. Am. Pharm. Assoc., Sci. Ed.*, **43**, 349 (1954).

pam, or diazepam may be based on competition for the same binding sites.   In the ANS-BSA system, it was found that the fluorescence intensity of ANS was enhanced by chlorpromazine. Our studies showed that ANS reacts with chlorpromazine to forn an insoluble complex in pH 7.4 phosphate buffer.   Thus, the enhancement of fluorescence may be due to the formation of a complex.   More detailed description of the ANS-chlorpromazine complexing will be reported elsewhere.[18]

The association constants for ANS and NPN with BSA were found to be $1.1 \times 10^6 \, \text{M}^{-1}$ and $4.8 \times 10^5 \, \text{M}^{-1}$, respectively.   ANS has a stronger binding than NPN.   The difference in binding affinity between the two probes may be due to the contribution of sulfonic group of naphthalene ring.   Our experimental results indicate that the binding sites for the probes may be present in the immediate vicinity of at least one of the tryptophan residues of BSA from quenching of the fluorescence of tryptophan by the two probes.   Furthermore, the two probes were displaced from the sites of BSA by chlordiazepoxide, nitrazepam, and diazepam. Therefore, it seems highly probable that the probes and the compounds used in this experiment may bind to the same sites on BSA molecule.   Swaney and Klotz[19] have shown that one of the strong organic anion binding sites of human albumin contains the amino acid sequence of Lys-Ala-Try(214)-Ala-Val-Ala-Arg.   The five nonpolar side chains form the hydrophobic pocket for binding of organic compounds, and a cationic group is present at each end.   On the other hand, amino acid sequences in the neighborhood of two tryptophan residues, positions 134 and 212, of BSA have been established as sequence (1) of Lys-Phe-Try(134)-Gly-Lys-Tyr-Leu and (2) of Lys-Ala-Try(212)-Ser-Val-Ala-Arg by Brown.[20]   It is apparent from these sequence that sequence (2) corresponds to the binding site of human albumin mentioned above. These findings on the chemical structure and their affinity permit one to draw certain conclusions about the characteristics of the binding site on albumin molecule.   The attractive force that holds an organic compound in binding with its binding site from concerted operation of numerous bonds of several types.[21]   The surface of the binding site, by virtue of its polar groups, can be thought of as covered by a sheath of water molecules.   The binding of organic compounds to the site must then entail mutual squeezing out of the intervening water layers. The interaction as a whole is therefore sometimes described as the formation of hydrophobic bonds.   The bond energy of dipole-induced dipole bond is only approximately 0.5 kcal/mol and the force of attraction is inversely proportional to the 7th power of the distance.   It might be imagined, because of their weakness and because they decrease so rapidly with slight increase of interatomic distance, that the van der Waals force will fall off to a negligible value for the primary attractive force that comes into play as an organic molecule approaches its site. On the contrary, the bond strength of an ionic bond is about 5 kcal/mol, and the coulombic forces, which diminish only as the square of the distance, will continue to be felt farther from the center of the charge density.   Therefore, it is considered that the earliest attraction that draws an approaching organic molecule toward its site is often an electrostatic one.   For example, when hydrocarbons contain an anionic group, first, an electrostatic interaction probably occurs between the anions and the positively charged residues of the binding site, and then the primary interaction must be reinforced by accessory bonds.   Organic compounds may contain potential cationic and anionic groups of all kinds, capable of forming ionic bonds with oppositely charged groups.   Electrostatic attraction may operate between the positively charged residues and atoms of an organic molecule that are not frankly ionized but have a partial ionic character.   Here the concerted effect of ionic bonds, hydrogen bonds, dipole-induced dipole bonds, etc., may yield a stable binding.   It is noteworthy that these spefrom these specula-

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19) J.B. Swaney and I.M. Klotz, Biochemistry, 9, 2570 (1970).
20) J.R. Brown, Fed. Proc., 34, 591 (1975).
21) A. Goldstein, L. Aronow, and S.M. Kalman, "Principles of Drug Action," Hoeber Medical Division, Harper and Row, New York, 1968, p. 25.

tions are related to the characteristics of the binding site on albumin offer a consistent and reasonable explanation of the binding affinities of the compounds used in the present study.

As a consequence, it is suggested that NPN may indeed prove to be a useful probe in the evaluation of interaction of basic compounds to BSA and understanding of binding mechanisms. However, the usefulness of the probe technique may be limited in that the binding studies are related only to the sites to which the probe binds, and the probe is not competitively interacting with the drugs for the different protein binding sites. In addition, the fluorescent probe technique shows that one should examine the effect of interactions between probes and competitor compounds such as ANS-basic compound complex.