Drug Interactions. VI.¹⁾ Binding of 1-Anilinonaphthalene-8-sulfonate with ChlorpromazineSHOJI OZEKI^{2a)} and KIKUO TEJIMA^{2b)}Faculty of Pharmaceutical Sciences, Nagoya City University^{2a)} and
Seto Plant, Showa Shinyaku Co., Ltd.^{2b)}

(Received December 19, 1978)

The complex of 1-anilinonaphthalene-8-sulfonate (ANS) and chlorpromazine was studied by chemical and spectroscopic methods. Elemental analysis indicated that the complex is composed of equimolecular quantities of ANS and chlorpromazine. Spectral data suggested that there is a strong interaction of the anilino hydrogen with the sulfonate group, and that hydrophobic stacking between the phenothiazine ring of chlorpromazine and the aromatic ring of ANS may occur. The fluorescence intensity of ANS in the presence of chlorpromazine was more markedly enhanced in chloroform than in water. Thus, it is likely that molecular rigidity and coplanarity of the aromatic rings of ANS are the dominant factors influencing the quantum yield of fluorescence of ANS. Finally, the enhancement in fluorescence of ANS in the presence of serum albumin and chlorpromazine is interpretable in terms of complex formation on the binding sites of albumin.

Keywords—drug interactions; bovine serum albumin; complex of 1-anilinonaphthalene-8-sulfonate with chlorpromazine; stoichiometric ratio of complex; synthesis of complex; binding constant; partition experiment; mechanism of fluorescence enhancement

1-Anilinonaphthalene-8-sulfonate (ANS) is known to form complexes with proteins and has been widely used in various fields.³⁾ Generally, the fluorescence of the probe increases with a decrease in the polarity of the solvent, and the same effects occur upon addition of proteins in aqueous solution. Thus, it was thought that the change in the fluorescence characteristics of probes on binding to a protein could be related to the nonpolar character of the binding sites,⁴⁾ but recent studies have demonstrated that the fluorescence spectra of probes are too complex for this to be so.⁵⁾ Further, Ainsworth and Flanagan suggested that the spectroscopic properties of naphthalenesulfonic acids depend not only on the polarity of the solvent but also on its rigidity and its ability to solvate the probe.⁶⁾ Although the mechanism

of fluorescence enhancement in proteins is not clearly understood, it has been suggested that the relative orientation of the two ring systems in the probe may influence their fluorescence characteristics.^{5a,7)}

In the preceding paper, we reported complex formation by ANS with chlorpromazine, and observed enhancement of the fluorescence of the probe upon addition to bovine serum albumin (BSA) and subsequent further enhancement of

Fig. 1. Structures of ANS and Chlorpromazine

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the fluorescence in the presence of chlorpromazine.¹⁾ Recently, Jun and Ruenitz reported that ANS exhibited an increase in fluorescence intensity accompanied by a hypsochromic shift of the emission maximum in the presence of chlorpromazine and imipramine.⁸⁾ The object of this study was to apply chemical and spectroscopic analytical techniques to determine the nature and structure of the ANS-chlorpromazine complex, and to explore the mechanisms of fluorescence enhancement of ANS. The chemical structures of ANS and chlorpromazine base are shown in Fig. 1.

Experimental

Materials—1-Anilidonaphthalene-8-sulfonic acid was purchased from Tokyo Kasei Co., Ltd. It was purified by recrystallization from water. 1-Anilidonaphthalene-8-sulfonic acid sodium was from Tokyo Kasei Co., Ltd. Bovine serum albumin (BSA), Fraction V, (Wako Pure Chemical Industries, Ltd.) was used in this study, and its molecular weight was assumed to be 69000. Chlorpromazine hydrochloride, chlorpromazine base and other chemicals of analytical grade were obtained from commercial sources and were used without further purification.

Instruments—Fluorescence measurements were made with a Hitachi MPF-3 spectrophotofluorometer equipped with a recorder. Emission spectra in this study are not corrected. Ultraviolet absorption spectra were measured with a Hitachi 124 spectrophotometer. Infrared (IR) spectra were measured by the potassium bromide disk technique, using a Jasco IR-S spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded at 100 MHz with a JEOL JNH-MH-100 spectrometer. Tetramethylsilane was used as an internal standard in CDCl_3 . Thin-layer chromatography (TLC) on Kieselgel G (E. Merck, Darmstadt, Germany) activated at 110° was used. The plates were spotted with a methanol solution of each compound, and spots were visualized with iodine vapor. The following solvents were used: CHCl_3 -EtOH (9:1) and CHCl_3 -EtOH (19:1).

Synthesis of the Complex—Exactly 85 mg of chlorpromazine hydrochloride was added to 20 ml of water containing $1.2 \times 10^{-2} \text{ M}$ ANS. The resulting precipitate was filtered, then dried over P_2O_5 . The complex gave a melting range of 158 – 162° (dec.), as pale yellow prisms. *Anal.* Calcd. for 1:1 complex, $\text{C}_{33}\text{H}_{32}\text{ClN}_3\text{O}_3\text{S}_2$: C, 64.15; H, 5.18; N, 6.80. Found: C, 63.64; H, 5.13; N, 6.71.

Stoichiometric Ratio of the Complex—The complex (20 mg) was dissolved in 50 ml of methanol. Five milliliters of the solution was pipetted into a glass-stoppered centrifuge tube containing 40 ml of *n*-heptane and 10 ml of 0.5 N sodium hydroxide. The tube was shaken and centrifuged at room temperature. The concentration of chlorpromazine in *n*-heptane was determined from the absorbance at 312 nm. The concentration of ANS in methanol-water was determined from the absorbance at 370 nm. The results indicate that the complex is of 1:1 type, one chlorpromazine molecule reacting with one ANS molecule.

Evaluation of the Binding Constant—The intensity of the fluorescence was determined with solutions of chlorpromazine base and ANS under various conditions. The excitation and emission wavelengths were taken to be 400 and 480 nm, respectively. The fraction of bound ANS, X , was calculated using the following Eq. (1),

$$X = (I_o - I_f) / (I_b - I_f) \quad (1)$$

where I_o and I_f refer to the fluorescence intensities of a given concentration of ANS in solutions of low chlorpromazine concentration and in solutions without chlorpromazine, respectively. I_b refers to the fluorescence intensity of the same concentration of ANS in solutions of high chlorpromazine concentration, *i.e.*, the fluorescence intensity of ANS in the presence of excess chlorpromazine. In this study, determinations of the fluorescence intensity were carried out at several different chlorpromazine concentrations, and the ANS concentration was $2 \times 10^{-6} \text{ M}$ in all cases. The values of I_b were extrapolated to $1/C=0$ from plots of $1/I$ versus $1/C$, where C represents the concentration of chlorpromazine. Let $[A_o]$ denote the total concentration of ANS, $[C_o]$ that of chlorpromazine, and $[AC]$ the concentration of complex. Then, $[Af]$ =free ANS concentration $= (1-X)[A_o]$, $[AC] = X[A_o]$, $[Cf]$ =free chlorpromazine $= [C_o] - X[A_o]$. Applying the law of mass action, we have

$$K = [AC] / [Af][Cf] \quad (2)$$

$$K = X / (1-X)([C_o] - X[A_o]) \quad (3)$$

In this experiment, no inner filter effect was observed since the absorbance was kept low at the excitation wavelength (less than 0.04).⁹⁾

Partition Experiment—The partition coefficient was obtained by equilibrating 5.0 ml of $5 \times 10^{-5} \text{ M}$ ANS in phosphate buffer, pH 7.4 with 5.0 ml of chlorpromazine hydrochloride chloroform solution at room

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temperature for 4 hr with shaking. Chloroform and buffer were saturated with each other before use. After shaking, the concentration of the probe in the water phase was determined by spectrophotometry.

TABLE I. *R_f* Values for ANS, Chlorpromazine Base and the Complex

Solvent system	<i>R_f</i>		
	ANS	Chlorpromazine base	Complex
CHCl ₃ -C ₂ H ₅ OH (9 : 1)	0	0.74	0.07
CHCl ₃ -C ₂ H ₅ OH (19 : 1)	0	0.80	0.09

Fig. 2. IR Spectra of the Complex, ANS (1-Anilinonaphthalene-8-sulfonic acid sodium) and Chlorpromazine

Results and Discussion

Complex of ANS with Chlorpromazine

The results of TLC studies of the complex are summarized in Table I. A complex of ANS and chlorpromazine was isolated and demonstrated to have physical and chemical properties different from those of the individual compounds.

The IR spectrum of the complex between ANS and chlorpromazine differed greatly from the spectra of the individual compounds (Fig. 2). The NH⁺ stretching vibrations of chlorpromazine appeared between 2460 and 2640 cm⁻¹ in the spectrum of the complex; these bands might result from the protonation of chlorpromazine.¹⁰⁾ In the spectrum of ANS,

Fig. 3. NMR Spectra of the Complex, ANS (1-Anilinonaphthalene-8-sulfonic acid) and Chlorpromazine in CDCl₃

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the band at 3320 cm^{-1} is assigned as the bonded NH absorption because a nonbonded NH stretching vibration of N-phenyl-1-naphthylamine, which lacks the sulfonic acid group of ANS, was observed at 3400 cm^{-1} . The shift of the NH stretching vibration in the spectrum of ANS can be attributed to hydrogen bond formation between the sulfonate group and NH. The bonded NH absorption band was also observed at 3320 cm^{-1} in the spectrum of the complex, and it is suggested that the same hydrogen bond is formed within the complex. The aromatic 1,2,4-trisubstitution out-of-plane bending vibration of chlorpromazine is present at 926 cm^{-1} , and this bending vibration is shifted to higher frequency (940 cm^{-1}) upon complex formation. The shift might result from an electron transfer from the chlorpromazine ring to ANS within the complex, since chlorpromazine is a powerful electron donor.¹¹⁾

To gain further evidence for complex formation, the NMR spectra of ANS, chlorpromazine and the complex were examined. It is assumed that the reaction proceeds first by salt formation, followed by an alignment of aromatic groups on top of each other. The NMR spectra of the complex, ANS and chlorpromazine are shown in Fig. 3. The spectrum of the complex appears not to correspond to a simple superimposition of one spectrum on the other. The chemical shifts did not result from concentration differences. Assignments

TABLE II. NMR Data on Chlorpromazine

Compound	Chemical shifts, ppm		
	NCH_2CH_2-	$-\text{CH}_2\text{N}(\text{CH}_3)_2$	$-\text{N}(\text{CH}_3)_2$
Chlorpromazine base	3.88 t	2.38 t	2.20 s
Chlorpromazine HCl	4.08 t	3.16 t	2.72 s
Complex	3.76 t	2.80 t	2.40 s

s=singlet, t=triplet.

of chemical shifts to the protons were based on published data.^{3a,12)} Tertiary aliphatic amines containing one or more N-methyl groups show NMR absorption of the methyl protons as a single peak in the vicinity of 2.2 ppm downfield from tetramethylsilane. This single peak is characteristic of N-methyl tertiary amines as the free base.^{12a)} The increased chemical shift from 2.2 ppm for chlorpromazine base to 2.4 ppm for the complex makes it possible to establish the presence of the aliphatic amine salt structure in the complex.^{12a)} In the case of chlorpromazine hydrochloride, this peak was observed at 2.72 ppm. Now, an increased downfield shift of the N-methyl groups is attributable to a corresponding increase in the strength or degree of protonation of the nitrogen atom.¹³⁾ Thus, it appears that the partial positive charge in the vicinity of the nitrogen atom of chlorpromazine hydrochloride is greater than that in the complex. The methylene group $-\text{CH}_2\text{N}(\text{CH}_3)_2$ of chlorpromazine base showed a downfield shift from 2.28 to 3.08 ppm in chlorpromazine hydrochloride, while this doublet of the complex was found at 2.80 ppm. These differences may be due to a difference of the partial positive charge in the vicinity of the nitrogen atom. An upfield shift occurs in the $\text{NCH}_2\text{CH}_2\text{CH}_2\text{N}(\text{CH}_3)_2$ triplet of the complex from 3.88 to 3.76 ppm, whereas a downfield shift occurs in chlorpromazine hydrochloride to 4.08 ppm. In the case of the complex, the triplet which is proximate to the phenothiazine ring experiences a change in magnetic environment. This is compatible with the methylene group residing in a nonpolar environment and being influenced by the diamagnetic anisotropy of adjacent aromatic moieties of ANS.

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NMR spectrum of ANS is not clear except for the H_2 signal because of its low solubility in $CDCl_3$. In the case of the aromatic protons of the complex, the aromatic proton H_2 of ANS was shifted from 8.3 to 8.4 ppm. It seems that H_2 experiences abnormal deshielding because of the proximity of the phenyl ring.^{5a)} H_4' of the anilino ring of ANS was shifted to 6.86 ppm. Moreover, on comparing the spectra (Fig. 3), the most striking feature is the presence of a low field singlet at 9.7 ppm for the complex, which is absent for ANS. This resonance is assigned to the NH proton, which fails to exchange with deuterium in $CDCl_3$. Complex formation might cause a conformational change in ANS, which would account for the change in chemical shift of the aromatic protons and the appearance of the NH peak of ANS. Results of an NMR study on ANS have suggested that the aromatic rings of ANS are more nearly coplanar in alcohol than in water and that the anilino hydrogen participates in a strong interaction with the sulfonate group in alcohol. Furthermore, solvents which

favor intramolecular hydrogen bond formation will favor a conformation in which the rings are nearly coplanar.^{5a)} Therefore, it is assumed that the rings of ANS bound to chlorpromazine are more nearly coplanar, and the anilino hydrogen of ANS participates in a strong interaction with the sulfonate group.

Integration of the NMR signal as well as elemental analysis demonstrates that the complex is composed of equimolecular quantities of ANS and chlorpromazine, and though it is not possible to elucidate completely the structure of the complex, the spectral data suggest the protonation of chlorpromazine by ANS. Other types of bonds can be proposed to account for some of its unique properties. Face-to-face stacking of the aromatic rings may occur and allow interaction between the rings.¹⁴⁾

Fig. 4. The Emission Spectra of $5 \times 10^{-6} M$ ANS Excited at 400 nm in $CHCl_3$

—: in the absence of chlorpromazine,
 ---: in the presence of $5 \times 10^{-6} M$ chlorpromazine.

The Mechanism of Fluorescence Enhancement in ANS-Chlorpromazine Complex

Figure 4 shows fluorescence emission spectra of ANS in the presence and absence of chlorpromazine in chloroform. An increase in the fluorescence of ANS in the presence of chlorpromazine provides evidence of complex formation between ANS and chlorpromazine. The quantum yield of fluorescence of ANS in methanol is much larger than that in water. Solvents in which rapid exchange of the NH proton occurs are those in which the quantum yield of fluorescence of ANS is quenched, and the prime cause of the quenching of the fluorescence of ANS in water is loss of molecular rigidity.^{5a)} From the NMR and IR spectra in this study, too, it appears that the rings of ANS must align in a more nearly coplanar fashion on binding to chlorpromazine, and there may be a restriction of rotation between the phenyl and naphthyl rings in the complex. Further, it has already been suggested that increased viscosity, molecular rigidity^{7,15)} and coplanarity of the aromatic rings^{5b)} are necessary for efficient fluorescence. Thus, molecular rigidity and coplanarity of the aromatic rings may be the dominant factor influencing the quantum yield of fluorescence of the complex. The stoichiometry of the interaction of ANS with chlorpromazine was studied. The results are shown in Fig. 5, which indicates that one mole of chlorpromazine is bound per mole of ANS. The binding data of Fig. 5 can be used not only to determine the stoichiometry but also to obtain the binding constant, K , which was found to be $4 \times 10^7 M^{-1}$

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Fig. 5. Plots of Relative Fluorescence Intensity versus Chlorpromazine/ANS Ratio at a Fixed Concentration of ANS (2×10^{-6} M)

Fig. 6. The Emission Spectra of 1×10^{-6} M ANS Excited at 370 nm in Phosphate Buffer, pH 7.4

—: in the presence of 1×10^{-6} M BSA,
 ----: in the presence of 1×10^{-6} M BSA and 1×10^{-6} M chlorpromazine.

from Eq. (3). Figure. 6 illustrates the effect of chlorpromazine on the fluorescence of ANS bound to BSA. We have already reported that chlorpromazine enhanced ANS fluorescence in the presence of BSA, whereas nitrazepam and other compounds caused reductions.¹⁾ Chlorpromazine had no effect when only 1×10^{-6} M ANS was present in phosphate buffer, pH 7.4. To test whether the complex formed between chlorpromazine and ANS could bind within a nonpolar cleft of a protein, the partitioning of ANS between a polar solvent (pH 7.4 phosphate buffer) and a nonpolar solvent (chloroform) containing chlorpromazine was studied. The partition ratio of ANS was found to increase from almost zero in the absence of chlorpromazine to 0.3 at a chlorpromazine concentration of 2×10^{-4} M. Therefore, chlorpromazine is capable of solubilizing ANS in a nonpolar environment, and the binding sites of BSA for the complex are less polar than water. From these experimental results, the enhancement in fluorescence of ANS in the presence of serum albumin and chlorpromazine appears to be interpretable in terms of complex formation on the binding sites of albumin, which are nonpolar amino acid residues bracketed by cationic ones.¹⁾

Acknowledgement The authors are very grateful to Prof. J. Watanabe, Nagoya City University, for his kind guidance. We also thank Miss. T. Ito for the NMR spectral measurements and members of the Analytical Center of this Faculty for elemental analysis.