[Chem. Pharm. Bull. 21(2) 366—371 (1973)]

UDC 547.653.1.04 : 615.31.011.5

Binding of 1-Anilino-8-naphthalenesulfonate to Polyvinylpyrrolidone, Poly-N-vinyl-2-oxazolidone, Poly-N-vinyl-5-methyl-2-oxazolidone and Polyethylene Glycol-20M in Aqueous Solution[1-3]

KENJI KONO,[4a] TSUNEJI NAGAI,[4b] and HISASHI NOGAMI[4c]

Faculty of Pharmaceutical Sciences, University of Tokyo[4]

(Received August 11, 1972)

The binding mechanism of 1-anilino-8-naphthalenesulfonate (ANS), called "hydrophobic probe," to polyvinylpyrrolidone (PVP), poly-N-vinyl-2-oxazolidone (PVO), poly-N-vinyl-5-methyl-2-oxazolidone (PVMO) and polyethylene glycol-20M (PEG-20M) was investigated as an approach to understanding of the interaction between ANS and biological components.

A high fluorescence emission was observed when the above synthetic water soluble polymers were added to aqueous solution of ANS. The emission maximum appeared at 480 mμ by the excitation at 365 mμ in the cases of PVP K-15, PVP K-30, PVO and PVMO, and at 475 mμ in the case of PEG-20M. The fluorescence was polarized in ANS/PVP/water, as was described with Perrin's equation. The enhancement and the shift of fluorescence emission were not observed in ANS/polyethylene imine/water.

The shift of signals to higher field on the proton magnetic resonance spectra of the model systems ANS/N-methyl-2-pyrrolidone/D_2O and ANS/N-chloroethyl-2-oxazolidone/D_2O suggested that ANS may stack on pyrrolidone and oxazolidone rings, respectively.

PEG-20M gave a ultraviolet absorption spectrum and another infrared absorption around 1515 cm^{-1} than PEG 4000 and 6000 did and also gave two peaks on the gel permeation chromatogram. The fluorescence intensity at 310 mμ, excited at 265 mμ, decreased with the increase of concentration of ANS. Accordingly, it was considered possible that some aromatic residue was incorporated in the PEG-20M molecule as have been reported in some other cases and this residue played an important role in the binding of ANS to PEG-20M.

It has been reported that such polycyclic aromatic compounds as 1-anilino-8-naphthalenesulfonate (ANS), 2-*p*-toluidinylnaphthalene-6-sulfonate, and related compounds do not fluoresce in water but fluoresce strongly when bound to certain native proteins or other biological materials,[5,6] being called "hydrophobic probes" in biological field. Especially, ANS, has been used widely as a reagent for qualitative analysis of hydrophobic portion and microenvironment of proteins[7,8] or membranes.[9-11] However, the mechanism of the binding of ANS to biological components is not fully understood, as may be due to the complexity of the structures of these components.

1) This paper forms Part XXV of "Physico-chemical Approach to Biopharmaceutical Phenomena." Preceeding paper, Part XXIV: N. Nambu and T. Nagai, *Chem. Pharm. Bull.* (Tokyo), **20**, 2463 (1972).
2) A part of this work is outlined in a preliminary communication: K. Kono, T. Nagai, and H. Nogami, *Chem. Pharm. Bull.* (Tokyo), **18**, 1287 (1971), also being taken in part from the thesis of Kenji Kono for the degree of Doctor of Pharmaceutical Sciences, University of Tokyo, 1971.
3) "Polyethylene glycol 20M" is the product name of Union Carbide Chemicals Co.
4) Location: *Hongo 7-3-1, Bunkyo-ku, Tokyo*; Present address: *a) Kyorin Chemical Laboratory, Ukima 1-3-32, Kita-ku, Tokyo*; *b) Hoshi Institute of Pharmaceutical Sciences, Ebara 2-4-41, Shinagawa-ku, Tokyo*; *c) Igusa 2-16-3, Suginami-ku, Tokyo.*
5) Lubert Stryer, *Science*, **162**, 526 (1968).
6) G.M. Edelman and W.O. McClure, *Accounts Chem. Res.*, **1**, 65 (1968).
7) E. Daniel and G. Weber, *Biochemistry*, **5**, 1893 (1966).
8) Lubrt Stryer, *J. Mol. Biol.*, **13**, 482 (1965).
9) J. Vanderkooi and A. Martonosi, *Arch. Biochem. Biopys.*, **133**, 153 (1969).
10) D.F. Wallach, E. Ferber, D. Selin, E. Weidekamm, and H. Fischer, *Biochim. Biophys. Acta*, **203**, 67 (1970).
11) P.A.G. Fortes and J.F. Hoffman, *J. Membrane Biol.*, **5**, 154 (1971).

In this connection, it was reported in a preliminary communication,[2] that ANS fluoresces strongly also when bound to water soluble synthetic polymers, such as polyvinylpyrrolidone (PVP) and polyethylene glycol-20M (PEG-20M). The elucidation of the mechanism of this binding may give useful informations for an understanding of the interaction between ANS and biological components.

The present study was attempted to discuss the binding mechanism between ANS and these synthetic polymers on the basis of such data as fluorescence spectra, proton magnetic resonance spectra and polarization of fluorescence, including the related data on poly-N-vinyl-2-oxazolidone (PVO) and poly-N-vinyl-5-methyl-2-oxazolidone (PVMO) which are similar to PVP in structure and in complexing tendency with various pharmaceuticals.[12]

Experimental

Materials——Two kinds of polyvinylpyrrolidone (PVP) marketed as "PVP K-15" and PVP K-90" by Gokyo Sangyo Co., Ltd. were fractionated by gel filtration using Sephadex G-100 and then lyophilized. The average molecular weight was estimated from the viscosity according to the equation reported by Scholtan.[13] Poly-N-vinyl-2-oxazolidone (PVO), poly-N-vinyl-5-methyl-2-oxazolidone (PVMO) and N-chloroethyl-2-oxazolidone (NCO) were gratefully supplied by Dr. Takeshi Endo of Tokyo Institute of Technology.[14] Polyethylene glycol-20M (PEG-20M) marketed by Union Carbide Chemicals Co. was dissolved in benzene to remove the insoluble portion by filtering through No. 4 glass filter and then precipitated with ethyl ether. This procedure was repeated three times. The average molecular weight was calculated from the viscosity according to the equation reported by Bailey, *et al.*[15] Polyethylene imine marketed by Dow Chemicals Co. was used without purification. 1-Anilino-8-naphthalenesulfonate (ANS) and deutrium oxide used were of the reagent grade. N-methyl-2-pyrrolidone (NMP) was used after purifying by vacuum distillation. The water used as solvent was prepared by distillation after treated with ion-exchange column.

Procedures——Viscosity measurement was done by a Ubbelohde type viscometer at controlled temperature. Fluorescence intensity and spectra were measured with a Hitachi 203 fluorescence spectrophotometer with a QPD-53 recorder. Ultraviolet (UV) absorption spectra were measured with a Hitachi 124 double beam spectrophotometer. Proton magnetic resonance spectra were measured in various deutrium oxide solutions by a Nippon Densi 4H-100 spectrometer, using 3-(trimethylsilyl)propane-1-sulfonate as the internal reference. Fluorescence polarization studies were carried out by a Shimadzu PE-21 light scattering photometer equipped with temperature controlling bath,[16] excited by the natural light through 365 mμ filter, and the polarization of the light emitted was given by the following expression[17]: $P=(I_{//}-I_{\perp})/(I_{//}+I_{\perp})$, where $I_{//}$ and $I_{\perp}$ are the emission intensities given by positioning the analyzer paralell and vertical, respectively, to the direction of the cell. Molecular weight distribution was determined by a Shimadzu GPC-1A gel permeation chromatography, using tetrahydrofuran as the elution solvent.

Result and Discussion

Binding of ANS to PVP, PVO, and PVMO

A striking enhancement of fluorescence took place upon the addition of PVP to aqueous solution of ANS, and the emission shifted from green to blue. According to the differential absorbance spectrophotometry, any substantial change was not detected in the UV absorption spectrum of ANS upon the addition of PVP except for the shift of the maximum at 265 mμ to 268 mμ.

It was confirmed by equilibrium dialysis method separately that ANS binds to PVP. The enhancement of fluorescence upon the addition of PVP to ANS was considered due to the binding of ANS to PVP. In simple aqueous solution, ANS has a weak quantum yield and the emission maximum exists at 515 mμ. Binding to PVP, this maximum moved to 480 mμ

12) S.M. Blaug and A.G. Rich, *J. Pharm. Sci.*, **54**, 30 (1965).
13) W. Scholtan, *Makromol. Chem.*, **7**, 209 (1952).
14) T. Endo and M. Okawara, *Makromol. Chem.*, **112**, 49 (1968).
15) F.E. Bailey, Jr., J.L. Kucera, and L.G. Imhof, *J. Polymer Sci.*, **32**, 517 (1958).
16) Y. Nishijima, *Kobunshi*, **13**, 80 (1964).
17) G. Weber, *Adv. Protein Chem.*, **8**, 415 (1953).

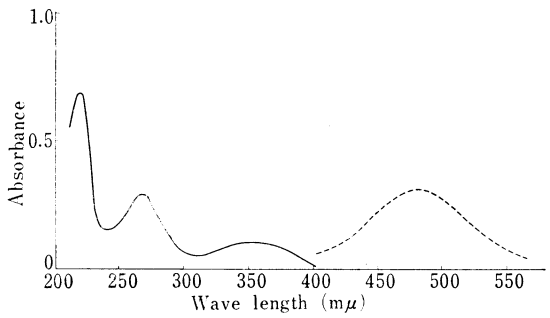

Fig. 1. Absorption Spectrum (——) of 5.5 μg/ml of ANS in Water and Fluorescence Emission Spectrum by the Excitation at 365 mμ (------) in Arbitrary Unit in the Binding of ANS to PVP

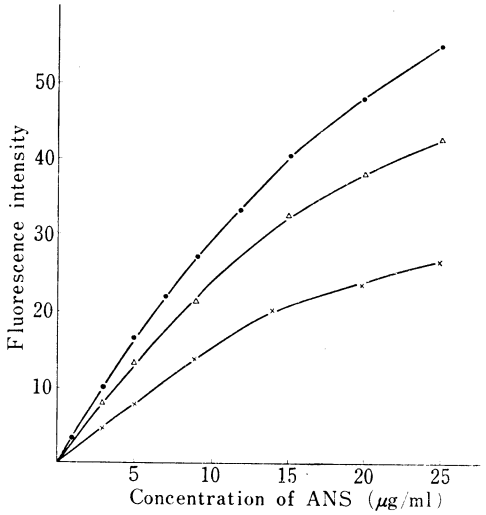

Fig. 2. Fluorescence Intensity in Arbitrary Unit at 480 mμ in the Binding of ANS to PVP K-15, Excited at 365 mμ at 25°

—●—: 3 mg/ml of PVP K-15
—△—: 2 mg/ml of PVP K-15
—×—: 1 mg/ml of PVP K-15

as shown in Fig. 1. Average molecular weights of PVP K-15 and K-90 were about 9×10^3 and 45×10^4, respectively, but the emission maximum appeared at an identical wave length. The effect of PVP K-15 on the fluorescence of ANS, excited at 365 mμ and analyzed at 480 mμ, is shown in Fig. 2.

If a fluorescent molecule is attached by a polymer or if it is in a high viscous solution, some degree of polarization may be retained in the fluorescence because of the restriction of rotational motion of the molecule.[17] In the present study, the fluorescence was polarized when ANS bound to PVP, and a linear relationship was obtained between $(1/P+1/3)$ and T/η, as shown for ANS/PVP K-15 system in Fig. 3, according to Perrin's equation for natural exciting light[17]: $(1/P+1/3)=(1/P_0+1/3)(1+RT\tau/\eta V)$, where τ is the lifetime of the exciting state, R the gas constant, T the absolute temperature, η the viscosity of the solution, V the volume of the fluorescent molecule, P the fluorescence polarization, and P_0 the value of polarization extraporated to $T/\eta=0$. This result also may confirm the binding of ANS to PVP.

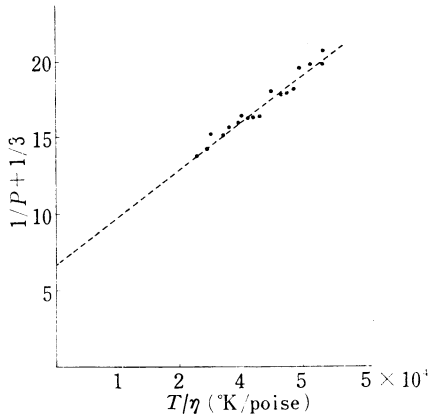

Fig. 3. Perrin's Plot for the Solution Containing 5 μg/ml of ANS and 3 mg/ml of PVP K-15, in which no Unbound ANS Molecule was expected to Exist

A high fluorescence emission was observed also when PVO or PVMO was added to aqueous solution of ANS. The wave length of emission maxima was the same as the case of PVP, *i.e.*, 480 mμ. The blue shift of emission maximum observed upon the binding of ANS to macromolecules was considered due in part to the polarity of the binding site.[5,6] From the above result, the binding site of PVP, PVO, and PVMO seemed to have the same polarity. However, there is given some contradiction. That is, chemically the molecules of PVO

and PVMO are considered more polar than PVP because they have three electronegative centers which may contribute to the polarization in the oxazolidone portion of the respective molecules.[12,18] An explanation for this contradiction may be given as follows: contrary to PVP, PVO, and PVMO in aqueous solution separate above 40° as the "cloud point" of nonionic surfactants consisting of ethylene oxide group, and thus the oxygen in the exazolidone monomer unit of PVO and PVMO is similar to that of ethylene oxide group of monionic surfactants in a high possibility of interacting with other molecules in aqueous solution.[19]

The enhancement of fluorescence emission and the shift of fluorescence were not observed when polyethylene imine was added to aqueous solution of ANS. The appearance of fluorescence of ANS is considered very sensitive to electrostatic interaction.[11] Therefore, if ANS bound to polyethylene imine with ion-bonding by sulfonate ion only, it should fluoresce strongly. On the other hand, though ANS fluoresced upon the binding to PVP, PVO, and PVMO as is described before, these polymers are considered to have no ionic portion in the respective monomer units because even the nitrogen atom of pyrrolidone or oxazolidone group is suggested to be of little charge.[20] Accordingly, a possibility of ionic interaction may be excludedregarding the mechanism of binding of ANS to PVP, PVO and PVMO.

The fluorescence of ANS is considered to be influenced also by the rigidity of the binding site.[21] Therefore, if an entanglement of polymer chain took place, ANS should be enclosed in the chain and a free rotation of ANS molecule should be blocked to result in a fluorescence emission. However, in the present study, the concentration of PVP, PVO or PVMO was very low and thus the effect of the entanglement of polymer chain on the fluorescence emission of ANS also might be negligible.

Moreover, though ANS has been used as a characterizing reagent of hydrophobic region of proteins or membranes, *i.e.*, "hydrophobic probe," it bound to PVP, PVO, and PVMO which seem less hydrophobic than the binding site of proteins or membranes because they have the hydrophobic portion in the respective hydrocarbon chains only. Therefore, an aditional mechanism of binding should be taken into consideration for an understanding of the present binding system. It has been known that a proton magnetic resonance spectroscopy is useful to an investigation of an interaction of polymer with small molecule.[22,23] Since it was impossible to assign the proton magnetic resonance spectra obtained for the present binding system because of a broadening of signals, NMP and NCO were used as the model compounds of PVP and PVO, respectively. The signals of NMP and NCO separated from that of ANS which appeared arround 6.5—8.5 ppm. In the presence of ANS, the spectrum of NMP made a marked change, and all methylene proton signals, except that of $N-CH_3$, moved to higher field,

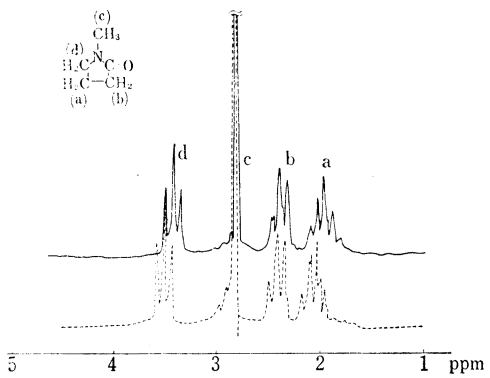

Fig. 4. Proton Magnetic Resonance Spectra of NMP/D_2O and ANS/NMP/D_2O at 100 MHz

- - - - - - : 3.7 mg/ml of NMP
——— : 3.7 mg/ml of NMP with the addition of 3.7 mg/ml of ANS

18) I.M. Klotz, *Federation Proceedings*, **24** (No. 2, Part III), S-24 (1965).
19) *a*) T. Higuchi and J.L. Lach, *J. Pharm. Sci.*, **43**, 465 (1954); *b*) D. Guttman and T. Higuchi, *ibid.*, **44**, 668 (1955); *c*) B.N. Kabadi and E.R. Hammerlund, *ibid.*, **55**, 1072 (1966).
20) S. Yoneda, I. Morishima, K. Fukui, and Z. Yoshida, *Kogyokagaku Zasshi*, **68**, 1074 (1965).
21) S. Ainworth and M.T. Flanagan, *Biochim. Biophys. Acta*, **194**, 213 (1969).
22) O. Jardetzky, N.G. Wade, and J.J. Fischer, *Nature*, **197**, 183 (1963).
23) O. Jardetzky, *Adv. Chem. Phys.*, **7**, 499 (1964).

as shown in Fig. 4. Similar changes were observed in various concentrations of ANS. In the case of NCO also, there was observed a shift of signals to higher field similar to the case of NMP. Although these model experiments carried out under the conditions different from those for the binding study of ANS to PVP and PVO described before, the shift of signals to higher field mentioned above suggested that ANS might stack on pyrrolidone and oxazolidone rings, respectively.[24] In this connection, Scholtan described the participation of hydrogen bonding in binding of some dye to PVP.[25] Conclusively, it seemed reasonable that a stacking phenomenon might participate together with hydrogen bonding in binding of ANS to PVP and PVO.

Binding of ANS to PEG-20M

It was reported in a preliminary communication[2] that the fluorescence emission maximum of ANS was observed at 475 mμ with addition of PEG-20M and at 500 mμ with addition of PEG 4000 and PEG 6000, and the fluorescent intensity of the former case was higher than the latter.

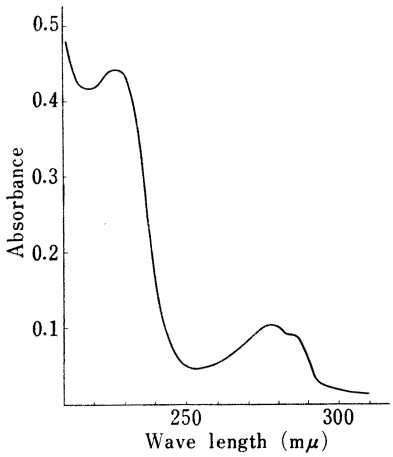

Fig. 5. Absorption Spectrum of 100 μg/ml of PEG-20M in Water

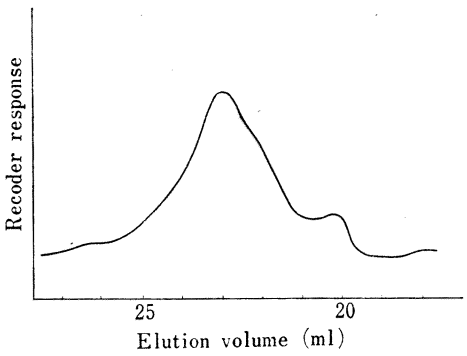

Fig. 6. Gel Permeation Chromatogram of PEG-20M (0.5% in Tetrahydrofuran) at 40°

The sample of PEG 20M of average molecular weight about 2×10^4 used was purified by reprecipitating with ethyl ether from dilute benzene solution several times, as described before, but its aqueous solution gave a UV absorption spectrum, as shown in Fig. 5. It is generally considered that PEG is a polymer of ethylene oxide monomer unit having no UV absorbing group, and thus the above result is very interesting. According to the gel permeation chromatography of PEG-20M, two peaks appeared in the elution pattern, as shown in Fig. 6, suggesting that a part of higher average molecular weight was included, and this tendency was not displayed in the case of PEG 4000 and PEG 6000. The infrared absorption spectrum of PEG-20M also showed an existence of another group than ordinary ethylene oxide one, *i.e.*, the absorption around 1515 cm^{-1}. From these results, it was considered possible that PEG-20M might have been made by polymerization from such a low molecular weight one as PEG 6000 with some aromatic diisocyanate compound, as is sometimes done in a synthesis of higher polymer accompanying a formation of urethane bonding.[26,27] Accord-

24) O. Jardetzky, *Biopolymers Symposia*, **1**, 501 (1964).
25) W. Scholtan, *Makromol. Chem.*, **11**, 131 (1953).
26) P.J. Flory and J.E. Mark, *J. Am. Chem. Soc.*, **87**, 1415 (1965).
27) Private communication form Dr. T. Kutsushita of the Faculty of Engineering, University of Tokyo.

ingly, the difference between ANS/PEG-20M/water and ANS/PEG 4000 or 6000/water in λ_{max} and intensity of the fluorescence was considered to be originated from the structure speciality of PEG-20M rather than the molecular weight dependency.

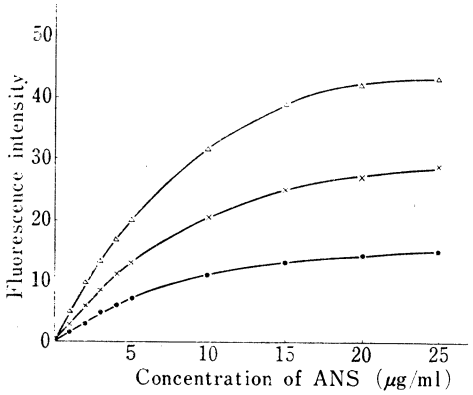

Fig. 7. Fluorescence Intensity in Arbitrary Unit at 475 mμ in the Binding of ANS to PEG-20M, excited at 365 mμ at 25°

—△—: 1.5 mg/ml of PEG-20M
—×—: 1.0 mg/ml of PEG-20M
—●—: 0.5 mg/ml of PEG-20M

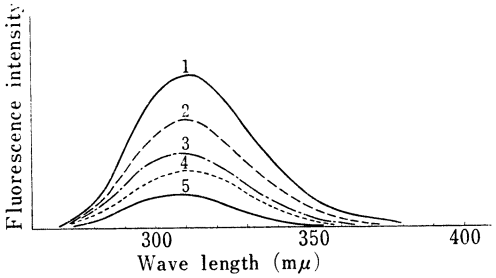

Fig. 8. Fluorescence Spectra in Arbitrary Unit of ANS/PEG-20M/Water in 2 mg/ml of PEG-20M and Various Concentrations of ANS, excited at 265 mμ

1: containing no ANS
2: 3 μg/ml of ANS
3: 7 μg/ml of ANS
4: 10 μg/ml of ANS
5: 20 μg/ml of ANS

In a similar way to the case of ANS/PVP/water, the fluorescence of ANS/PEG-20M/water, excited at 365 mμ, increased with the increases of concentration of ANS and PEG-20M, as shown in Fig. 7. The fluorescence spectrum of ANS/PEG-20M/water, excited at 265 mμ where both ANS and PEG-20M made absorption, gave two maxima at 310 mμ and 475 mμ, which were considered due to PEG-20M and ANS, respectively. The intensity at 310 mμ decreased with the increase of concentration of ANS, as shown in Fig. 8, while the one at 475 mμ increased in the same tendency as shown in Fig. 7. In this connection, it is known that an energy transfer or molecular association contributes to a quenching phenomenon of fluorescence,[28] and it has been reported that the quantum yield of aromatic amino acid quenches when ANS binds to proteins.[8] In the present case, therefore, the excitation energy level of the aromatic residue in PEG-20M mentioned already was considered to be influenced by the ANS molecule bound on it. In other words, this aromatic residue was considered to play an important role in the binding of ANS to PEG-20M.

As a result, it may be concluded that ANS binds to a very specific site of high polymers and not always to the hydrophobic portion of proteins of membranes.

Acknowledgement The authors gratefully acknowledge the award of Research Grants from Naito Foundation (to T.N.). Thanks are also given to Dr. Takeshi Endo of Tokyo Institute of Technology for generous supply of materials, to Prof. Yoshinobu Nakai of Chiba University for measurement of fluorescence polarization, to Dr. Etsuo Awada of Sankyo Co. for gel permeation chromatography, and to Dr. Mitsuji Sano of Daiichi Pharmaceutical Co. for proton magnetic resonance spectroscopy.

28) Y. Nishijima, *Kobunshi*, **13**, 80 (1964).