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Application of Quenching of Albumin-Induced Fluorescence of 8-Anilinonaphthalene-1-sulfonate to the Studies on Protein Bindings and Protein-Mucopolysaccharide Interaction[1)]

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A new fluorophotometric method for the determination of binding constant between chemical substances and protein utilizing quenching of albumin induced fluorescence of 8-anilinonaphthalene-1-sulfonate (ANS) is described. Binding constants were calculated employing Langmuir-type equation. The chemical substances and ANS were shown to compete for the same binding sites on BSA molecule. When this method was applied to the interaction of fatty acids with BSA, binding constant increased markedly with the chain length. ANS was thus demonstrated to reflect the hydrophobic interaction of substrates with protein. Binding constants for aromatic compounds were largely affected by the size of the aromatic nuclei. The influence of hydrophobic binding appeared to be more dominant in the bidings of polynuclear compounds. The present method was also proved to be effective for the investigation of protein-mucopolysaccharide interaction. Naturally occurring mucopolysaccharides showed smaller binding constants than synthetic sulfated polysaccharide. This fact may be due to the more ordered arrangement of sulfate groups in natural mucopolysaccharides. This method is simple and rapid, and may be applicable to wide range of substances.

Protein bindings[3)] has extensively been studied[4)] because it is one of the most important factors related to the transportation, absorption, metabolism, excretion, and especially to the biological activities of chemical substances which are administered to bioorganisms.[3)] Equilibrium dialysis[4a–c)] and metachromasy method[4d–i)] have been employed for the measurement of protein bindings. The former method is effective for measuring the number of molecules adsorbed on protein. However, it is considerably tedious and time-consuming and hence not well suited for comparative study in which a large number of substances are tested at one time. Moreover, this method is inapplicable to larger molecules which do not permeate the semipermeable membrane. On the contrary, the latter procedure is rapid and simple. It gives some information even on macromolecules. Nevertheless, the result of metachromasy measurement is liable to be influenced by the physicochemical properties of the dye employed in it. Anionic dyes, which have hitherto been used in this field, were assumed to reflect the electrostatic binding of the chemical substances.[3)] However, protein bindings may be governed by complex factors involving hydrogen bonding, electrostatic binding, and hydrophobic

1) *a*) This paper forms Part IV of "Microanalysis of Proteins and Peptides," Preceding paper, Part III: T. Kinoshita, F. Iinuma, and A. Tsuji, *Chem. Pharm. Bull.* (Tokyo), **22**, 2735 (1974); *b*) A part of this work is outlined in a preliminary communication: T. Kinoshita, F. Iinuma, and A. Tsuji, *Chem. Pharm. Bull.* (Tokyo), **19**, 861 (1971).

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3) I.M. Klots, "The Proteins," Vol. 1, ed. by H. Neurath and K. Bailey, Academic Press, New York, 1953, pp. 727—806.

4) *a*) M. Nakagaki, N. Koga, and M. Terada, *Yakugaku Zasshi*, **83**, 586 (1963); *b*) I.M. Klotz, F.M. Walker, and R.B. Pivan, *J. Am. Chem. Soc.*, **68**, 1486 (1946); *c*) I.M. Klotz and F.M. Walker, *ibid.*, **70**, 943 (1948); *d*) I.M. Klotz, *ibid.*, **68**, 2299 (1946); *e*) I.M. Klotz, H. Triwush, and F.M. Walker, *ibid.*, **70**, 2935 (1948); *f*) I. Moriguchi, S. Wada, and H. Sano, *Chem. Pharm. Bull.* (Tokyo), **16**, 592 (1968); *g*) *Idem*, *ibid.*, **16**, 597 (1968); *h*) I. Moriguchi, S. Wada, and T. Nishizawa, *ibid.*, **16**, 601 (1968); *i*) I. Moriguchi and S. Wada, *ibid.*, **16**, 1440 (1968).

interaction.[5] Recently, Jones and Weber[6] proved the participation of hydrophobic region of serum albumin in the protein binding.

In the course of our studies on the accumulation of lipophilic pharmaceuticals in tissues,[7] reference dyes, which reflect the hydrophobic binding of chemical substances, became necessary. In the present study, 8-anilinonaphthalene-1-sulfonate, one of the most effective hydrophobic probe,[8] was applied to the estimation of protein binding. This fluorochrome was also found to be effective for the study of protein-mucopolysaccharide interaction.

Materials and Methods

Bovine serum albumin (BSA, fraction V) was purchased from Armour Laboratories, Co. Sodium 8-anilinonaphthalene-1-sulfonate (ANS) and sodium salts of fatty acids were obtained from Tokyo Kasei Kogyo Co., Ltd. Chondroitin sulfate and heparin were purchased from Seikagaku Kogyo Co. Ltd. and Nakarai Chemicals, Ltd., respectively. Sulfated mono- and polysaccharides were the gift of Meito Sangyo Co. Fluorescence intensity was measured with a Hitachi Model 103 Fluorescence Spectrophotometer, thermostatted within an accuracy of 0.1°.

Procedure: To 1 ml of 9.52 μM of BSA in 0.4M phosphate buffer, pH 7.4, were added 1 ml of 64.0 μM ANS in water and 2 ml of aqueous solution containing chemical substances to be measured. Fluorescence intensity of the resultant mixture was measured at excitation and emission wavelengths of 365 and 469 nm,[7] respectively.

Calculation of Binding Constant: Assuming that the binding data of ANS and of chemical substances with BSA follow Langmuir-type equation[4b] and that ANS and chemical substance compete for the same binding site on albumin molecule, the binding constant K of chemical substance may be expressed as[4e]

$$K = K_A(a-x)y/(b-y)x \tag{1}$$

where K and K_A represent the intrinsic binding constant for the chemical substance and ANS to each site on albumin molecule, respectively, a and b the initial concentration of ANS and the substrate, respectively, and x and y the concentration of bound ANS and the bound substrate, respectively. The value y is obtained by equation (2)[4g]

$$y = np - x\{1+1/K_A(a-x)\} \tag{2}$$

where n is the number of binding site for ANS (that is, for chemical substances) on protein molecule, and p is the concentration of BSA present in the system. On the other hand, x is obtained by equation (3).

$$F = fx \tag{3}$$

where F is the fluorescence intensity of bound ANS. Since the fluorescence intensity of unbound ANS is negligible in aqueous media,[8] the observed fluorescence intensity of the sample can directly be regarded as F. f is the proportionality constant and determined according Moriguchi and co-workers[4f] by equation (4)

$$1/f = \lim_{1/p\to 0}(a/F) \tag{4}$$

Finally, n and K_A is obtained from Langmuir plot of x and y.

Results and Discussion

Competition between a few anionic dyes such as methyl orange and colorless compounds for binding sites on protein molecule has been demonstrated by spectral methods.[3,4d,e] Moriguchi and co-workers[4f-i] utilized 2-(4′-hydroxyphenylazo)benzoic acid (HABA) as a reference dye, which showed remarkable spectral change on interaction with protein and

5) K. Kakemi, H. Sezaki, and M. Nakano, *Yakuzaigaku*, **27**, 283 (1968).
6) A. Jones and G. Weber, *Biochemistry*, **10**, 1335 (1971).
7) A. Tsuji, T. Kinoshita, and F. Iinuma, *Chem. Pharm. Bull.* (Tokyo), **19**, 2209 (1971); K. Hiratsuka, T. Mayahara, S. Yamada, and T. Kinoshita, *Japan J. Pharmacol.*, **24**, 23 (1974).
8) *a*) R.F. Chen, H. Edelhoch, and R.F. Steiner, "Physical Principles and Techniques of Protein Chemistry," ed. by S.J. Leach, Academic Press, New York, 1969, pp. 214—216; *b*) L. Streyer, *J. Mol. Biol.*, **13**, 482 (1965); *c*) T. Kinoshita, "Keiko-Bunseki," ed. by Z. Tamura, T. Tabata, and K. Yasuda, Kodansha Scientific, Tokyo, 1974, p. 111.

established a rapid and accurate method for the determination of binding constant. These dyes were considered to be probes for electrostatic interaction[3,4f] between the colorless compounds and proteins. Recently, Moriguchi and co-workers[9] pointed out that HABA, when bound to BSA, was largely buried in hydrophobic region of BSA. This finding is well interpreted by the concept of Jones and Weber[6] that the basic groups of arginine residues of BSA are located in the vicinity of its hydrophobic region and binding of anionic compounds is largely dependent on this structure. They have also shown that this local structure is the binding site of ANS. Since HABA can be posturated to bind to such arginine residue, marked metachromasy observed for this compound[4f,g] may presumably be caused by the neighboring hydrophobic environment. However, this does not directly imply that the hydrophobic bonding also serves as a driving force in the binding of this dye with protein. ANS has widely been used as an effective hydrophobic probe[8a,b] for proteins. This fluorochrome fluoresces

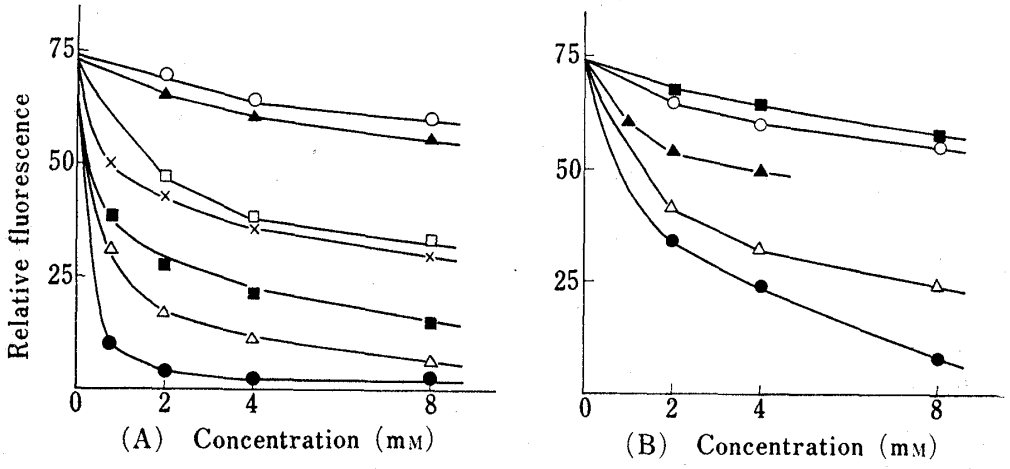

Fig. 1. Quenching of BSA–ANS System Caused by Various Substance at 25°

(A) —○—: sulfamic acid, —▲—: phenol, —□—: cyclohexylsulfamic acid and benzenesulfonic acid, —×—: benzoic acid, —■—: salicylic acid, —△—: α-naphthalenesulfonic acid, —●—: dodecylsulfuric acid

(B) —■—: *p*-aminophenol, —○—: phenol, —▲—: m-aminophenol, —△—: thymol, —●—: *o*-aminophenol

very little in aqueous solution but the fluorescence is greatly enhanced in the presence of proteins[8a,b] and other macromolecules having hydrophobic region such as cycloheptaamylose.[1a,8c] ANS was, therefore, expected to be useful probe for hydrophobic binding of chemical substances to proteins.

Figure 1 displays the plot of fluorescence intensity of ANS in the presence of BSA against varied concentration of substances added. This figure supports that the substances and ANS compete for the same binding sites on BSA molecule. It is the advantage of the present method that the fluorescence intensity of ANS in the absence of BSA is negligible whereas

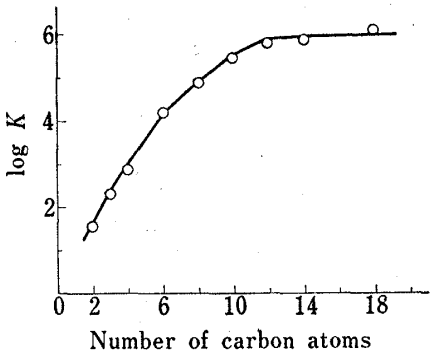

Fig. 2. Effect of Chain Length of Fatty Acids on their Binding Constants at 25°

concentration of fatty acids: C_2-C_4, 8.0 mM; C_6-C_{18}, 0.08 mM

9) I. Moriguchi, S. Fushimi, and N. Kaneniwa, *Chem. Pharm. Bull.* (Tokyo), **18**, 2447 (1970); *idem*, *ibid.*, **19**, 1272 (1971).

the dyes for the metachromasy methods show considerable absorbance in the absence of BSA.

Figure 2 illustrates the log K values for various saturated straight chain fatty acids plotted against their chain length. The binding constants increased markedly with the chain length, namely, with the hydrophobic character of the acids, and reached a plateau in the range from laurate (C_{12}) to stearate (C_{18}). This result indicates that the quenching of the fluorescence of ANS largely reflects the strength of hydrophobic binding of chemical substances to protein. Nagai and co-workers[10] have reported that ANS bound to a very specific site of synthetic polymers such as polyvinyl pyrrolidone, which seem less hydrophobic than the binding site of protein. The present data, however, indicates that ANS functions as a hydrophobic indicator in the competitive binding on protein molecule. In addition, the binding of free fatty acids to serum albumin is of clinical importance.[11] The free fatty acid in serum are known to have long carbon chain (C_{16} to C_{18}) which showed large log K values. Accordingly, the present method may also be utilized in this field.

Table I. log K Values for the Binding of Chemical Substances with Bovine Albumin

Substrate	Concentration of substrate in mM								Average
	8.0	4.0	2.0	0.8	0.4	0.08	0.04	0.02	
Phenol	2.75	2.66	2.69						2.70
Thymol	3.75	3.80	3.84						3.80
β-Naphthol		3.84	4.29	4.42					4.18
o-Aminophenol	4.32	4.06	4.09						4.16
m-Aminophenol		3.33	3.43	3.38					3.38
p-Aminophenol	2.44	2.50	2.52						2.49
Benzoic acid	3.57	3.69	3.78						3.68
Salicylic acid	4.08	4.18	4.28						4.18
Ammonium sulfamate	2.18	2.42	2.04						2.21
Cyclohexylsulfamic acid	3.49	3.59	3.68						3.59
Benzenesulfonic acid	3.49	3.56	3.74	3.79	3.85				3.69
β-Naphthalenesulfonic acid			3.92	4.08	4.18				4.06
α-Naphthalenesulfonic acid	4.49	4.57	4.64	4.58	4.65				4.59
Dodecylsulfuric acid						6.29	6.16	6.19	6.21
Myristic acid (C_{14})						5.86	5.84	5.91	5.87
Stearic acid ($C_{18:0}$)						6.12	6.09	5.83	6.01
Oleic acid ($C_{18:1}$)						5.54	5.48	4.95	5.32

a) in 0.1M phosphate buffer, pH 7.4, at 37°
Unit of K is liter/Avogadro number of binding.

Table I lists log K values for various chemical substances. Little change is observed in the values with concentration of the substrates added and this supports that the assumptions and procedure for calculation of binding constants (K) are valid. The following two series (acids and phenols) of decreasing log K values are observed: α-naphthalenesulfonic acid$>\beta$-naphthalenesulfonic acid$>$benzenesulfonic acid$\simeq$cyclohexylsulfamic acid$>$ammonium sulfamate; β-naphthol$>$thymol$>$phenol. There is fairly large differences between log K values for mononuclear phenols and mononuclear aromatic acids such as benzoic acid and benzenesulfonic acid, whereas no such difference is observed between β-naphthol and β-naphthalenesulfonic acid. These facts suggest that the influence of hydrophobic interaction is more dominant and that of electrostatic binding is comparatively small in the case of polynuclear compounds. In addition, dodecylsulfate shows particularly large binding constant and this value is greater than that calculated by Klotz.[3] Dodecylbenzeneslfonate and stearic

10) K. Kono, T. Nagai, H. Nogami, *Chem. Pharm. Bull.* (Tokyo), **21**, 366 (1973).
11) H. Kushiro and I. Fukui, *Jap. J. Clin. Chem.*, **1**, 42 (1971).

acid show the same order of binding constant with dodecylsulfate. These data are also explicable by considering the highly hydrophobic character of these acids. Oleic acid shows smaller binding constants than those of stearic acid whereas both fatty acids have the same number of carbon atoms. This difference may be caused by the more polar nature of the former acid owing to the presence of a double bond.

Aminophenols exhibits unique binding constants (Table I). *o*-Aminophenol has largest log K value which is of the same magnitude with that of naphthol. *m*-Aminophenol gives still larger binding constant than phenol. Log K for *p*-aminophenol is almost the same with that for phenol. Moriguchi and co-workers[12] reported that these three derivatives give essentially the same log K values by HABA method. Although these facts are not fully accounted for by the data herein described, it is suggested that *o*-aminophenol affects mainly the hydrophobic binding sites because the binding of HABA does not seem to be affected by aminophenols. Therefore, bifunctional nature of *o*-aminophenol may play a role in quenching of ANS by influencing its hydrophobic interaction with BSA. These phenomena indicate that the polarity of the probe affects the binding data. It is therefore recommended to use at least two dyes of different physicochemical properties in the study of protein binding.

Ammonium sulfamate shows log K of 2.21 and this may be primarily due to the electrostatic binding.

The present method was then applied to the investigation of protein-mucopolysaccharide interaction and the results are summarized in Table II. Since the substrate concentration used in the calculation of the binding constant was expressed in terms of the concentration of sulfate group in mmol/liter, the values listed here may be regarded as the relative strength of binding for a single sulfate group on the substrate. As shown in the table, Glucose-6-sulfate gives the smallest log K value among the sulfated carbohydrates tested. Binding of naturally occurring mucopolysaccharides (chondroitin sulfate A and heparin) are also not sizable. On the contrary, synthetic polysulfates of polysaccharides (dextran and cellulose) exhibits larger values, and much greater binding constants are observed for synthetic polysulfates of mono- and oligosaccharides (glucose, lactose, and sucrose). The mono- and oligosaccharide polysulfates carry 3 to 4 sulfate groups on one molecule and some sulfate groups may be arranged close together. High log K values for these substrates are again suggestive of some bifunctional effect. However, the binding constant of glucose polysulfate is fairly affected by its concentration. This suggests that some complex mechanism such as conformational change of BSA would be involved in the binding reaction.

Table II. log K Values[a] for Binding of Mucopolysaccharides with Bovine Serum Albumin

Substrate	Degree of sulfation in mol/monosaccharide unit	Concentration of sulfate group in mM				Average
		80	40	20	10	
Glucose-6-sulfate	1.0		0.43			0.43
Chondroitin sulfate A	0.5	0.88	0.88	0.72		0.83
Heparin	1.5	1.60	1.56	1.58		1.58
Dextran sulfate (1)	2.5	1.90	2.04	2.28		2.07
Dextran sulfate (2)	0.7	1.90	2.04	2.14		2.03
Cellulose sulfate	1.5	2.08	2.11	2.20		2.13
Sucrose polysulfate	3.3		2.18	2.36	2.54	2.36
Lactose polysulfate	2.9		2.45	2.67	2.82	2.65
Glucose polysulfate	3.9		2.40	2.80	3.08	2.76

a) refer to a) in Table I

Naturally occurring mucopolysaccharides have a ordered structure in which sulfate groups are located fairly apart.[13] Chondroitin sulfate A is consisted with galactosamine monosulfate and heparin with glucosamine-2,6-disulfate and glucuronic (or iduronic) acid, half of which is monosulfated. Accordingly, no vicinal sulfate groups are considered to be present in these mucopolysaccharides and it is appreciable that the binding constants for these polymers are similar to that for glucose-6-sulfate. On the other hand, sulfate groups on the synthetic sulfated polysaccharides may not be arranged in order and two or more sulfate groups may often be located in high proximity. This would cause the strong binding of these polysaccharides to protein. This result is of interest concerning to the toxicity of synthetically prepared sulfates of polysaccharides.[14]

Interaction of mucopolysaccharides with serum protein is of particular importance in the studies on their anticoagulant and fat clearing activities.[15] However, quantitative evaluation of the binding force has so far been difficult. The present results indicate that the binding constants obtained by ANS method do not appreciably change with the concentration of the mucopolysaccharides. Therefore, the data would be regarded to obey Langmuir-type of equation. Application of the present method is expected to be useful for the quantitative investigation of protein-mucopolysaccharide complexes of biological or clinical significance.

Acknowledgment The authors are greatly indebted to Mr. T. Kimizuka, Meito Sangyo Co. for his generous gift of sulfated carbohydrates.

13) J.S. Brimacombe and J.M. Webber, "Mucopolysaccharides," Elsevier Publishing, Amsterdam, 1964.
14) Ref. 13) p. 37.
15) P. Porter, M.C. Porter, and J.N. Shanberge, *Clinica Chimica Acta*, **17**, 189 (1967); E. Hasegawa, S. Suzuki, N. Seno, and S. Hirano, "Structure and Function of Mucopolysaccharides," (in Japanese), Nanko-do, Tokyo, 1968, pp. 1—21; Ref. 13), pp. 92—130.