of I-oxime, is 0.40 ppm, and it seems appropriate to consider the presence of an AcO group at C-5α in V. These observations prove the correctness of II structure.

The route of formation of II may be considered as follows. The *trans*(diaxial)-addition of anhydrous nitrous acid (N_2O_3) to the double bond in cholesteryl acetate would give 3β-acetoxy-5α-hydroxy-6-nitrosocholestane-5-nitrite (VIII), which tautomerized to a stable oxime (IX) and this oxime is acetylated to form II. Formation of I can be presumed as the result of hydrolysis of II or IX, or the Claisen degradation[6] of the oxime group in IX by nitrous acid, accompanied by hydrolysis of the nitrite function.

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Studies on Protein Bindings utilizing Quenching of Albumin-Induced Fluorescence of 8-Anilinonaphthalene-1-sulfonate

The binding of chemical substances with protein is one of the most important factors related to their biological activities. In the course of studies on the interaction of mucopolysaccharides with serum proteins through their quenching effect of albumin–induced fluorescence of 8-anilinonaphtalene-1-sulfonate(ANS),[1] we have found that this hydrophobic compound is also applicable to the investigation on binding of a wide range of chemical substances with proteins.

Determination of binding constants: To 1 ml of 9.52×10^{-6}M bovine serum albumin (fraction 5, Armour Laboratories Co.) in 0.4 M phosphate buffer, pH 7.4, were added 1 ml of 6.40×10^{-5}M ANS(sodium 8-anilinonaphtalene-1-sulfonate, Tokyo Kasei Co., Ltd.) in water and 2 ml of sample solution in water. The obtained solution was measured fluorometrically. Excitation and emission wavelength were 365 and 469 mμ, respectively. Assuming that the binding data of ANS and of chemical substances with bovine serum albumin are represented by a Langmuir–type equation[2] and that ANS and chemical substances compete for the same binding sites on albumin molecules, the binding constant of chemical substances may be expressed as[3]

$$K=K_A(a-x)y/(b-y)x$$

where K and K_A represent the intrinsic binding constant for the chemical substance and ANS to each site on albumin molecules, respectively a and b the initial concentration of and the ANS substrate, respectively, and x and y the concentration of bound ANS and

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the bound substrate, respectively. These values were obtained by calculation in a similar way as described by Klotz and co-workers[3] and Moriguchi and co-workers.[4]

TABLE I. Log K Values[a] for Binding of Chemical Substances with Bovine Serum Albumin

Substrate	Concentration of substrate in mM					Average
	8.0	4.0	2.0	0.8	0.4	
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(0.08)[b]		6.16(0.04)[b]		6.19(0.02)[b]	6.21

a) in 0.1 M phosphate buffer, pH 7.4, at 37° Unit of K is liter/Avogadro number of binding.
b) Binding constants for dodecylsulfate were measured at low concentration of the substrate. Numbers in parenthesis express the concentration of substrate in mM.

Table I shows the values of log K for various substrates. The following two series of decreasing log K values are observed: α-naphthalenesulfonic acid > β-naphthalenesulfonic acid > benzenesulfonic acid ≃ cyclohexylsulfamic acid > ammonium sulfamate; β-naphthol > thymol > phenol. Thus the values of log K appear to be largely dependent on hydrophobic character of the substrates rather than dissociation constant of the acids or phenols. This reasoning is supported by the fact that the binding constants of phenols and sulfonic acids are virtually the same when they have large hydrophobic nuclei such as naphthalene. Interestingly, there is another series of decreasing log K values: o-aminophenol > m-aminophenol > p-aminophenol. Moriguchi and co-workers[5] reported that these three derivatives give essentially the same log K values by the method utilizing metachromasy. Neither hydrophobicity nor dissociation constant simply account for this fact and some bifunctional effects may be involved.

The present method is expected to be used as an effective tool in estimating binding constants for hydrophobic interatcion between chemical substances and biopolymers. Studies are in progress in our laboratories on the determination of binding constants between a wide range of compound and various biopolymers.

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