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Drug–Biomolecule Interactions: Fluorescence Studies on Interaction of Aminonaphthalenesulfonic Acid Derivatives with Serum Albumins

H. W. JUN, L. A. LUZZI ˣ, and JOSEPH K. H. MA

Abstract □ The binding of the three aminonaphthalenesulfonic acid derivatives to human and bovine serum albumins was studied by measuring the fluorescence enhancement of the compounds. The number of binding sites of human and bovine serum albumins for these compounds appears to be one and two, respectively, under the experimental conditions. As the molar ratio of the fluorescent compounds to bovine serum albumin increased, the binding sites appeared to increase for the compounds. The quenching of the native fluorescence of albumin was examined by the successive addition of methanolic solutions of these compounds. 1-Anilinonaphthalene-8-sulfonate quenched the protein fluorescence to a greater extent than the other compounds studied, indicating that 1-anilinonaphthalene-8-sulfonate molecules are bound more closely to the tryptophan residues of albumin. The finding that the three compounds did not quench the fluorescence of tryptophan dissolved in water indicates no direct molecular interaction between tryptophan and the three fluorescent probes. The driving force for binding may be due to the structural characteristics of the amino acid sequence surrounding the tryptophan residues.

Keyphrases □ Aminonaphthalenesulfonic acid derivatives—binding to human and bovine serum albumins, fluorescence enhancement □ Albumin, human and bovine serum—binding of three aminonaphthalenesulfonic acid derivatives, fluorescence enhancement □ Probes—binding of three aminonaphthalenesulfonic acid derivatives to serum albumins, fluorescence enhancement □ Drug–biomolecule interactions—fluorescence studies on interaction of aminonaphthalenesulfonic acid derivatives with serum albumins □ Interactions—drugs with biomolecules, symposium

Interaction of organic molecules with body proteins has resulted in many facets of research interest in biomedical studies. When drugs are bound to plasma proteins, distribution, therapeutic efficacy, and elimination of the drugs may be altered. Biotransformation of organic compounds occurs through interactions of the compounds with metabolizing enzymes. The pharmacological effects of drugs are also thought to be mediated by interactions of the drug molecules with active site proteins.

Because of the clinical implications of such interactions, numerous studies have been carried out concerning these subjects (1, 2). Although aims and experimental techniques vary among investigators, most studies have been directed toward explaining the effects of the interactions.

In this study, fluorescence spectroscopy was employed to study the binding of three fluorescent probes to human and bovine serum albumins. A fluorescent probe is a compound with spectral properties such as optimal excitation and emission wavelengths, fluorescence intensity, and lifetime of the excited state that undergo changes reflecting its molecular environment. When probe molecules bind to protein binding sites, an enhancement of fluorescence emission and wavelength shift occur.

Table I—Excitation and Emission Maxima (Uncorrected) of the Fluorescent Probes in pH 7.5 Phosphate Buffer and Bovine and Human Serum Albumin Solutions

	pH 7.5 Phosphate Buffer		Bovine Serum Albumin		Human Serum Albumin	
	Excitation, nm	Emission, nm	Excitation, nm	Emission, nm	Excitation, nm	Emission, nm
1-Anilinonaphthalene-8-sulfonate	360	520	376	467	380	474
N-n-Butyl-(5-dimethylamino-naphthalene)-1-sulfonamide	330	540	343	494	340	498
5-Dimethylaminonaphthalene-1-sulfonamide	328	540	347	506	345	509

The enhancement of fluorescence emission of a probe can be monitored to obtain binding information for the probe (3). The quenching of the intrinsic fluorescence of protein can also be quantitated to calculate the binding parameters of the interactant (4). The purposes of this study were to compare and to characterize the binding of three structurally similar fluorescent probes to human and bovine serum albumins by measuring the fluorescence enhancement of the probes and the fluorescence quenching of the proteins in the hope that additional information would be obtained for the binding model.

EXPERIMENTAL

Materials—The following fluorescent probes were studied: 1-anilinonaphthalene-8-sulfonate[1], *N-n*-butyl-(5-dimethylamino-naphthalene)-1-sulfonamide[2], and 5-dimethylaminonaphthalene-1-sulfonamide[3]. Crystalline human[4] and bovine[5] serum albumins were purchased commercially. Spectroscopic grade methanol was employed as a solvent for the probes. The water used was double distilled from glass. All other chemicals used were of reagent grade.

Instruments—All fluorescence measurements were made on a spectrophotofluorometer[6] equipped with a 150-w xenon lamp and 1P21 photomultiplier tube. Spectra were recorded with an *X-Y* recorder[7]. The entrance slit for the excitation light and the exit slit for the fluorescence emission were 3 and 4 mm, respectively.

Methods—Two milliliters of human and bovine serum albumin solution (1.37×10^{-6} *M*) in pH 7.5 phosphate buffer (0.05 *M*) were titrated with successive additions of 2 μl of 1×10^{-3} *M* of probe dissolved in methanol. The excitation and emission wavelengths (uncorrected) for the probes are recorded in Table I.

Titrations were carried out in fluorescence quality cells with fitted Teflon stoppers, and titrants were added manually with microsyringes[8]. After each titration, the enhancement of fluorescence intensity was recorded as a function of probe concentration. Separate titrations with successive additions of 2 μl of a 1×10^{-3} *M* probe solution were also performed at higher protein concentrations (34.2×10^{-6} *M* for bovine serum albumin and 68.5×10^{-6} *M* for human serum albumin). It was then assumed that all of the probe was bound to protein at this concentration.

Temperature was controlled at 23 ± 0.5° with thermostated cell compartments through which water from a constant-temperature bath[9] was circulated. To minimize the photodecomposition of protein, samples were exposed to the light only for the short measurement period. Blank titrations in the buffer were performed to correct the fluorescence of free probe in the absence of protein.

Treatment of Data—Enhancement of the fluorescence intensity of the probe upon addition to human and bovine serum albumins at two protein concentrations was measured, and these data were used to calculate the fractions of free and bound probe and the molar ratio of probe bound to protein (5). The binding constants and the number of binding sites of the compounds were determined by the use of the Scatchard equation (6):

$$\bar{V}/D_f = nK_a - \bar{V}K_a \qquad \text{(Eq. 1)}$$

where $\bar{V}$ is the number of moles of bound probe per mole of protein, D_f is the concentration of free probe, n is the number of binding sites on the protein molecule, and K_a is the binding constant of probe to the protein.

Measurement of Fluorescence Quenching—The quenching of the native fluorescence of human and bovine serum albumins was measured at the emission wavelength of 358 nm with the excitation at 283 nm following successive additions of probe. In titration, 2 μl of a 1×10^{-4} *M* probe solution in methanol was successively added to 2 ml of protein solution (1.37×10^{-6} *M* in pH 7.5 phosphate buffer) and the degree of quenching was recorded. The concentration of probe added to the protein solution was chosen so that it did not absorb the exciting radiation at 283 nm.

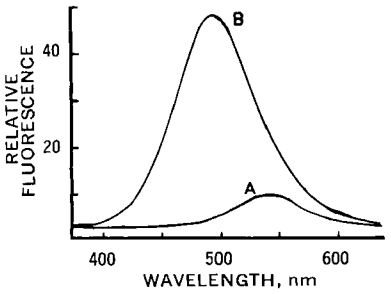

Figure 1—*Emission spectrum of* N-n-*butyl-(5-dimethylaminonaphthalene)-1-sulfonamide* (10×10^{-6} M) *in the absence (curve A) and presence (curve B) of bovine serum albumin* (1.37×10^{-6} M) *in pH 7.5 phosphate buffer. Excitation wavelengths were at 330 and 343 nm, respectively.*

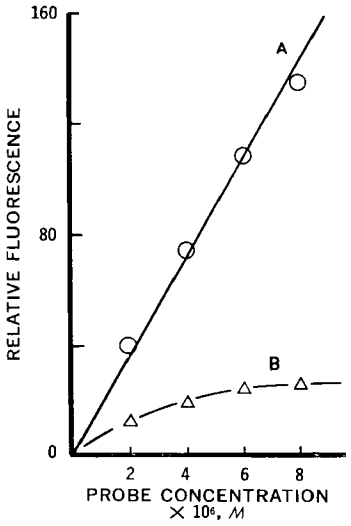

Figure 2—*Fluorescence titration curves of bovine serum albumin with* N-n-*butyl-(5-dimethylaminonaphthalene)-1-sulfonamide at higher protein concentration* (34.2×10^{-6} M) *(curve A) and lower protein concentration* (1.37×10^{-6} M) *(curve B).*

[1] Recrystallized in water, Aldrich Chemical Co., Milwaukee, Wis.
[2] Obtained from the Department of Entomology, University of Georgia, Athens, Ga.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Control No. 4619, Nutritional Biochemicals Corp.
[5] Control No. 1693, Nutritional Biochemicals Corp.
[6] Aminco-Bowman, American Instrument Co., Silver Spring, Md.
[7] Omnigraphic, Houston Instrument, Bellaire, Tex.
[8] Hamilton.
[9] Haake model FJ, Polyscience Corp., Evanston, Ill.

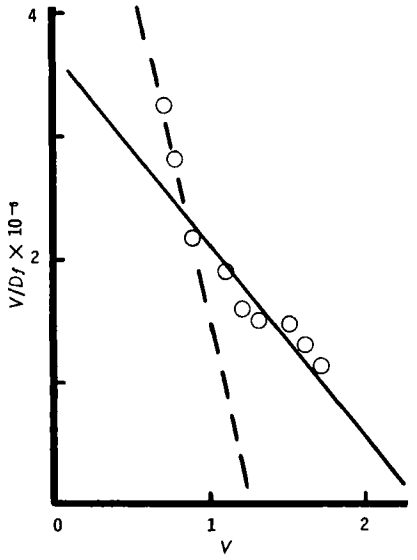

Figure 3—*Scatchard plot for 1-anilinonaphthalene-8-sulfonate–bovine serum albumin binding.*

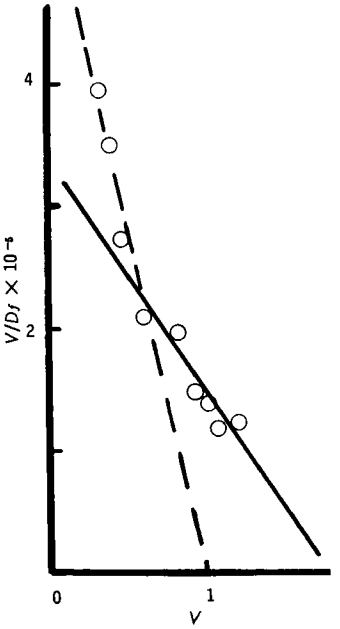

Figure 5—*Scatchard plot for 5-dimethylaminonaphthalene-1-sulfonamide–bovine serum albumin binding.*

RESULTS AND DISCUSSION

The use of fluorescence spectroscopy to study the binding of small molecules to proteins has been well established (7, 8). The aminonaphthalenesulfonic acid derivatives used in this study show an increase in fluorescence emission when bound to serum albumins. In this study the binding data were obtained by monitoring the increased fluorescence intensities of the probes (9, 10) and by measuring the quenching of the fluorescence of protein by the probes (11, 12).

Figure 1 is an emission spectrum of *N-n*-butyl-(5-dimethylaminonaphthalene)-1-sulfonamide in the absence and presence of bovine serum albumin in pH 7.5 phosphate buffer (0.05 *M*). The fluorescence emission of this compound in a buffer is minimal (curve A); but in the presence of albumin, the fluorescence intensity is greatly enhanced (curve B), with a 46-nm hypsochromic shift in the wavelength of the emission maximum. Jun *et al.* (13) reported the quantum yield of 0.059 for this compound in water and indicated that it was increased about 12 times to 0.7 when bound to bovine serum albumin.

In Fig. 2, the enhancement of fluorescence intensity is essentially dependent on the amount of the probe added at the high protein concentration (curve A). At lower protein concentration, fluorescence intensity reaches a maximum, indicating a saturation of binding sites (curve B). The large increase in fluorescence emission and hypsochromic shift in the presence of protein has been attributed to binding of the probe at hydrophobic sites (14, 15). These spectral modifications are responses to changes in the polarity of the probe environment as a result of binding.

McClure and Edelman (16) observed a significant increase in fluorescence emission of the fluorescent probe 2-*p*-toluidinylnaphthalene-6-sulfonate when placed in a solvent of lower polarity. Similar spectral shifts and fluorescence enhancement were observed for the other compounds studied. Table I summarizes the spectral shifts of the compounds in phosphate buffer and human and bovine serum albumin solutions.

Figures 3–5 are Scatchard plots obtained for probe–bovine serum albumin complexes. The plots were obtained by the method described under *Experimental* and were used to calculate the binding constant and the number of binding sites. Under the experimental conditions, the number of binding sites for the probes appears to increase from one to two as the molar ratio of probe to protein increases. The dotted line representing lower molar ratios of probe to protein indicates the number of binding sites to be close to one with possibly higher binding affinity and the solid line indicates two binding sites.

Since the quenching studies showed that the binding sites may

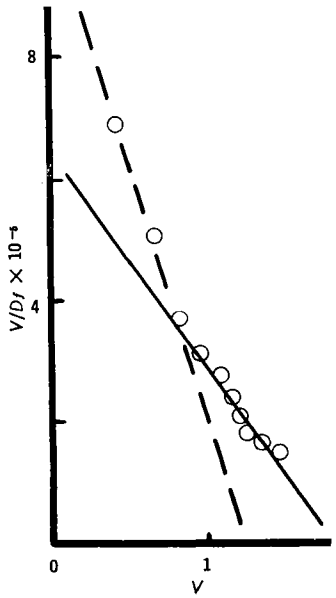

Figure 4—*Scatchard plot for* N-n-*butyl-(5-dimethylaminonaphthalene)-1-sulfonamide–bovine serum albumin binding.*

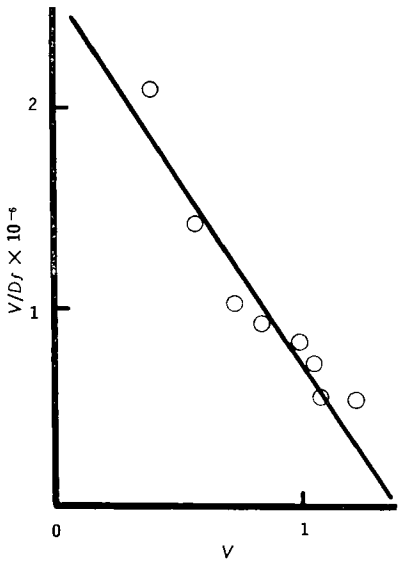

Figure 6—*Scatchard plot for 1-anilinonaphthalene-8-sulfonate–human serum albumin binding.*

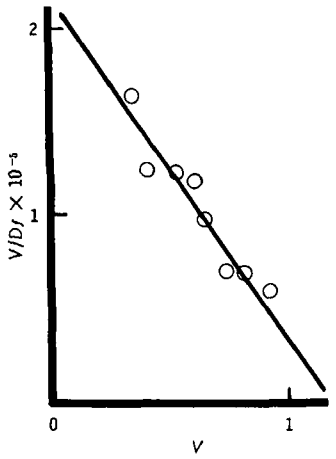

Figure 7—*Scatchard plot for* N-n-*butyl-(5-dimethylaminonaphthalene)-1-sulfonamide–human serum albumin binding.*

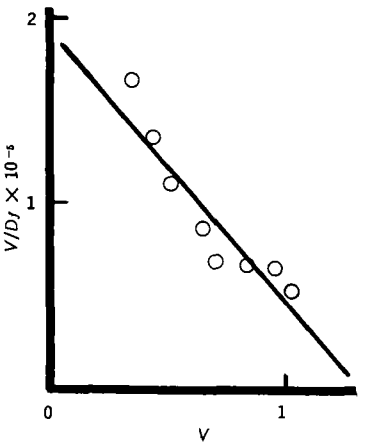

Figure 8—*Scatchard plot for 5-dimethylaminonaphthalene-1-sulfonamide–human serum albumin binding.*

be near the tryptophan residues and since bovine serum albumin is known to have more than one tryptophan binding site (17), the Scatchard plots suggest that the molecular environment of the binding sites may not be identical. At the low molar ratio of probe to protein, certain binding sites are initially available; as the probe concentration increases, more binding sites become available until all binding sites are saturated. This view may be supported by the report (18) that the total amount of salicylate bound to plasma protein was a function of drug concentration and that the capacity of binding sites increased at higher drug concentration.

The binding affinities of the probes to bovine serum albumin were in the following order: 1-anilinonaphthalene-8-sulfonate ($1.70 \times 10^6/M$), *N-n*-butyl-(5-dimethylaminonaphthalene)-1-sulfonamide ($3.66 \times 10^5/M$), and 5-dimethylaminonaphthalene-1-sulfonamide ($1.72 \times 10^5/M$). 1-Anilinonaphthalene-8-sulfonate had the highest binding constant. The difference in binding affinity between the two amide probes may be due to the involvement of the side chain in binding. Although the hydrophobic naphthalene moiety appears to be primarily responsible for the binding (19), the side chain of these compounds apparently affects binding.

Figures 6–8 are the Scatchard plots of the probes bound to human serum albumin using the same experimental conditions as with bovine serum albumin. The number of binding sites for the probes appears to be one. The orders of wavelength shift and binding affinity of the probes were similar to those for the bovine serum albumin–probe interactions. 1-Anilinonaphthalene-8-sulfonate ($1.60 \times 10^6/M$) showed the highest binding affinity followed by *N-n*-butyl-(5-dimethylaminonaphthalene)-1-sulfonamide ($1.82 \times 10^5/M$). The fluorescence enhancement was generally lower than that for the bovine serum albumin–probe complexes.

Figure 9 shows the quenching effects for the native fluorescence of albumin following the successive addition of probes. The probe, in the concentration used, did not contribute to the quenching. When 2 ml of bovine serum albumin solution (1.37×10^{-6} *M* in pH 7.5 phosphate buffer) was successively titrated with 2 μl of 1-anilinonaphthalene-8-sulfonate solution (1×10^{-4} *M* in methanol), the fluorescence emission of protein at 358 nm was diminished (curve A) when excited at 283 nm. The stoichiometry of the quenching curve shows the number of binding sites to be one and indicates that the binding sites are not saturated at this molar ratio of probe to protein.

Earlier workers (8, 11, 12) reported that the quenching of protein fluorescence in the presence of small molecules is an indication of binding. For fluorescence quenching to occur in a solution of two compounds, the quenching substance must be within a critical transfer distance (20) to the fluorophor so that the energy transfer between the two molecules takes place. Forster suggested (21) that for an energy transfer to occur the emission band of the fluorophor must overlap the absorption band of the quencher. Although the concentration of 1-anilinonaphthalene-8-sulfonate dissolved in buffer did not absorb the light at the emission wavelength of the protein (358 nm), the absorption band of the bound probe may overlap the emission band of the protein.

The finding that 1-anilinonaphthalene-8-sulfonate strongly quenches the fluorescence of albumin indicates that the binding site for this probe is in the vicinity of the tryptophan residues. Studies (22, 23) have shown that serum albumin has strong binding sites for small organic molecules located in the immediate vicinity of the tryptophan residue. Swaney and Klotz (23) reported that the environment of tryptophan residues of human serum albumin forms a cluster of apolar amino acids. This arrangement of hydrophobic clusters may be responsible for the interaction of the probes to serum albumins.

The quenching of the fluorescence of albumins by the other probes was less significant (Figs. 9B and 9C). This may be due to the molecules of 1-anilinonaphthalene-8-sulfonate being more closely bound to the tryptophan residues of the albumin. The finding that other organic compounds did not quench the fluorescence of serum albumin suggests the specificity of binding sites to the aminonaphthalenesulfonic acid derivatives. Chen and Kernohan (12) observed that 1-anilinonaphthalene-8-sulfonate did not quench the fluorescence of erythrocyte carbonic anhydrase. This observation also indicates the specificity of binding between serum

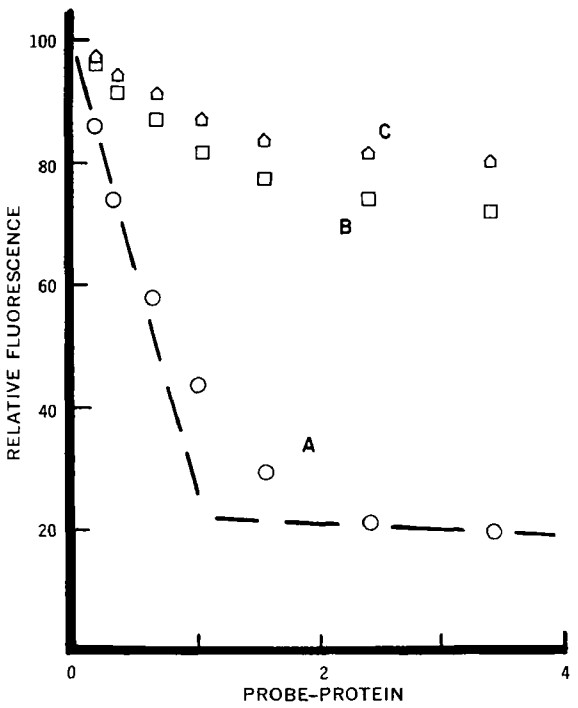

Figure 9—*Quenching curves of bovine serum albumin fluorescence by 1-anilinonaphthalene-8-sulfonate (curve A),* N-n-*butyl-(5-dimethylaminonaphthalene)-1-sulfonamide (curve B), and 5-dimethylaminonaphthalene-1-sulfonamide (curve C). The bovine serum albumin concentration in pH 7.5 phosphate buffer was 1.37×10^{-6} M. Fluorescence was measured at 358 nm (emission) and 253 nm (excitation).*

albumins and the probes used. Carbonic anhydrase may lack the structural requirement for the binding of 1-anilinonaphthalene-8-sulfonate.

In contrast to the quenching of the native fluorescence of protein by the probes, no quenching was observed upon addition of the probes to tryptophan dissolved in buffer. This indicates that the driving force for the probe–protein binding is not mediated by direct interaction between the probe and tryptophan molecules, although tryptophan has been reported to have a binding affinity to various other organic molecules (24, 25). This observation also shows that the quenching of protein fluorescence by the probes was not due to concentration or inner filter effects.

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Drug–Biomolecule Interactions: Spin-Probe Study of Effects of Anesthetics on Membrane Lipids

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Abstract □ The electron spin resonance spectra of probes were used to study the organization and motion of molecules in hydrated stacked bilayer or liposome model membrane systems. The same steroid structures required for reduction of membrane permeability were required to produce well-ordered films of brain lipid. Alcohols and anesthetic agents influence the structure of model membranes, with their order of efficacy paralleling their pharmacological effectiveness. Spin probes were also used to demonstrate effects of calcium and local anesthetics on the rate of penetration of ascorbate into lipid bilayers.

Keyphrases □ Anesthetics—effects on membrane lipids, electron spin resonance spectra □ Lipids, membrane—effects of anesthetics, electron spin resonance spectra □ Drug–biomolecule interactions—effects of anesthetics on membrane lipids, electron spin resonance spectra □ Interactions—drugs with biomolecules, symposium

Electron spin resonance spectroscopy (ESR) examines the spectra of free radicals in an applied magnetic field. In a filled electron orbital, there are two electrons whose magnetic moments cancel; but in a free radical, there is an unpaired electron with a net magnetic moment. This electron has two different energy states, which can be qualitatively described as having the magnetic moment parallel and antiparallel to an applied magnetic field. The energy difference between these states is equal to the energy of a quantum of microwave radiation (for the usual applied magnetic fields of 3–12 kilogauss). A single absorption line is observed in the case of an isolated electron. In a free radical, the magnetic environment of the electron can split this resonance into a number of lines.

In the spin-label method, a free radical with a suitably designed functional group is reacted with the system. In the spin-probe method, selected molecules with free radical moieties are intercalated into the