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Dimer Formation from 1-Anilino-8-naphthalenesulfonate Catalyzed by Bovine Serum Albumin. A New Fluorescent Molecule with Exceptional Binding Properties*

Carl-Gustaf Rosen† and Gregorio Weber

ABSTRACT: At pH values around 2, nitrite induces a series of chemical changes in 1-anilino-8-naphthalenesulfonate. From the resulting mixture of products a compound has been isolated, the spectroscopic properties of which are very similar to the parent compound, but with an affinity for bovine serum albumin nearly two orders of magnitude greater than that of the latter. The number of strong binding sites displaying fluorescence enhancement is two. If albumin is present during the formation of the compound, the yield, which otherwise amounts to only 1–2%, approaches 100%, *i.e.*, the protein appears to behave in an enzyme-like fashion. Three different preparative procedures are described. Experimental evidence is given to support the conclusion that the new molecule is a dimer of 1-anilino-8-naphthalenesulfonate.

Several aromatic dyes, which are virtually nonfluorescent in water solution, become strongly fluorescent in nonaqueous solvents, or when bound to apparently hydrophobic sites in proteins (Weber and Laurence, 1954). They have for this reason been applied to studies of protein–ligand interactions and binding sites in proteins. In the course of studies by Daniel and Weber (1966) on cooperative effects on binding by BSA ANS[1] was utilized, and it was observed that when a dilute solution of ANS and BSA (concentration $\sim 10^{-7}$ M) was left at pH 2, the ANS fluorescence increased slowly with time so that doubling of the initial fluorescence intensity took place in about 20 min. Since the quantum yield of fluorescence for the bound dye is initially as high as 0.7, the cause of this effect must obviously be an increase of the binding rather than an increase of the quantum yield, and accordingly there must be a slow process taking place, which affects the binding. This could be either a chemical change in the dye and/or the protein, or a conformation change of the protein modifying the binding sites.

The work presented in this paper was initiated as a search for factors that were responsible for the fluorescence increase. It was found that the process was inhibited by millimolar concentrations of ferrocyanide ions and could be observed only at concentrations of BSA and ANS $\lesssim 10^{-5}$ M. It was finally realized that the process required the presence of nitrous ions. These were always present, albeit in small concentrations, because the fluorescence cuvets were cleaned by soaking in

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[1] Abbreviations used are: ASN, 1-anilino-8-naphthalenesulfonate; BSA, bovine serum albumin; DNS, dimethylaminonaphthalenesulfonate.

TABLE I: Stoichiometry of Bis-ANS–BSA Formation.[a]

No. of ANS + $NaNO_2$ Additions	Protein Concn (M)	OD_{400} (max)	Bis-ANS Concn[b] (M)	Ratio Bis-ANS:BSA	Comment
1	1.2×10^{-5}	0.465	2.7×10^{-5}	2.3	
2	1.2×10^{-5}	0.93	5.4×10^{-5}	4.5	
3	1.3×10^{-5}	1.14	6.6×10^{5}	5.1	Slightly opalescent

[a] Sodium chloride (0.5 M)–phosphate buffer (0.1 M), pH 2. Initial concentration increases after each addition: ANS (10^{-4} M)–sodium nitrate (2×10^{-5} M). [b] Calculated from an extinction coefficient of 17×10^{3} cm^2 $mole^{-1}$.

commercial nitric acid and the subsequent exhaustive washing failed to remove the traces of nitrous ion adsorbed to the quartz walls. Once this requirement was recognized we were able to reproduce with great regularity the process of fluorescence increase by addition of $NaNO_2$ to a dilute solution of ANS–BSA at pH 2. As shown further in the paper, the new compound has an extremely high affinity for BSA. A number of experimental observations indicate that it is a dimeric product of ANS and in the following we shall refer to it as bis-ANS.

Materials and Methods

Bovine serum albumin was a crystalline preparation from Armour and Co.

ANS was the magnesium salt prepared from Eastman Kodak 1-anilino-8-naphthalenesulfonic acid as described by Weber and Young (1964).

The recording spectrofluorometer has been described by Weber and Young (1964), the fluorometer utilized for the titrations by Anderson and Weber (1965). References to other instruments used in this investigation are given in the text.

The theoretical background and practical procedures of the fluorescence titrations are given in Daniel and Weber (1966).

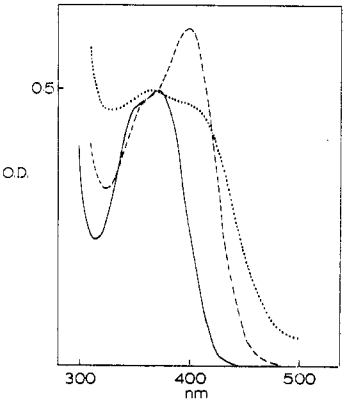

FIGURE 1: Absorption spectra of solutions recorded after additions in various orders into 0.5 M NaCl, pH 2. (——) ANS + BSA. (– – – –) ANS + BSA + $NaNO_2$. (. . . . . .) ANS + $NaNO_2$ + BSA. Final concentrations: ANS, 10^{-4} M; BSA, 10^{-5} M; and $NaNO_2$, 2×10^{-5} M.

Results

Preliminary experiments showed that a compound with high affinity and fluorescence enhancement for BSA could be obtained by action of $NaNO_2$ in acid upon ANS, but that the presence of BSA resulted in much higher yields. The high affinity of the product for the protein must be judged by the fact that (a) hardly any separation occurred when the neutralized reaction mixture was run through a G-25 Sephadex column and (b) 48 hr of dialysis against 0.5 M KCl buffered at pH 2 or 6 and frequently changed removed less than 25% of the fluorescent material. Covalent bonding to the protein was first suspected but this was excluded when association with an amino acid residue could not be detected after acid hydrolysis or extensive digestion of the protein with the *Streptomyces protease* (Nomoto *et al.*, 1960). Also, no free amino acid could be made to react with ANS in the presence of nitrite to yield a similar compound.

The three spectra of Figure 1 correspond to ANS–BSA neutral complex, to $NaNO_2$ added to a BSA–ANS mixture, and to BSA added to a mixture of ANS and $NaNO_2$. The spectrum of the third as compared with the second indicates the formation in the latter case of a smaller number of components. In fact, the second spectrum is not appreciably different from that of purified bis-ANS adsorbed to BSA so that the protein must have a favorable influence upon its formation as compared with alternative reaction products.

Preparation of Bis-ANS. As shown in Figure 1 the presence of BSA enhances the formation of bis-ANS. In a protein-free solution the yield is much lower and depends upon the relative ANS and nitrite concentrations. Nonfluorescent by-products are formed in yields which increase with the nitrite concentration, and the maximum yield obtained for bis-ANS under such conditions is of the order of 1–2%. Three distinct methods of obtaining bis-ANS were used.

METHOD A. A small volume of sodium nitrite solution giving a final concentration of 2×10^{-5} M and ANS giving a final concentration of 10^{-4} M are added to a solution of 10^{-5} M BSA in 0.5 M sodium chloride previously titrated to pH 2 with concentrated hydrochloric acid (pH not critical). At 20-min intervals, identical amounts of nitrite and ANS are added again twice. The resulting yellow to brownish-yellow solutions are dialyzed against several large volumes of 0.5 M NaCl and finally against distilled water for 2 days, after which the samples are freeze dried and the absorption spectra were recorded on weighed samples. Using an extinction coefficient of

TABLE II: Spectral Characteristics of ANS and Bis-ANS.

Approximate Maxima	ANS (nm)	Bis-ANS (nm)
Absorption maximum, water solution	350	387
Absorption maximum adsorbed to BSA	370	400
Fluorescence maximum of BSA complexes	478[a]	500[a]

[a] Corrected for photomultiplier response and grating transmission.

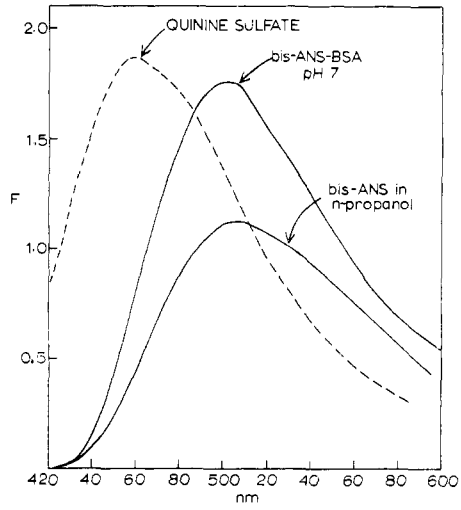

FIGURE 2: Fluorescence spectra of bis-ANS in 1-propanol and of bis-ANS–BSA complex in neutral buffer. The spectra are corrected for grating transmission and photomultiplier response. The spectral areas are proportional to the fluorescence yield. The absolute value of the fluorescence yield may be gauged by comparison with the included spectrum of quinine sulfate.

17×10^3 cm^2 mole^{-1} (determined from crystallized bis-ANS with the assumption of a molecular weight of 600), the ANS/BSA ratios were calculated as presented in Table I. Bis-ANS was isolated from the conjugate in two different ways: by butanol extraction of a protease digest, or by direct extraction from a conjugate solution with butanol in which case a large number of extractions were required.

METHOD B. Sodium nitrite is added dropwise during 5–10 min from a separatory funnel to a final concentration of 2×10^{-3} M to a 10^{-2} M ANS solution in 0.5 M NaCl containing 0.05 M potassium phosphate and previously titrated to pH 2.0 with concentrated HCl. The brownish mixture is left under constant stirring for 3 min after completion of the addition. The reaction is stopped by the addition of phosphate buffer to 0.1 M bringing the pH to 7. A bluish intermediate is observed in the reaction.

METHOD C. ANS is dissolved to saturation in concentrated sulfuric acid, and a few milligrams of sodium nitrite added. A dark blue color is developed and nitrous gases evolved. On dilution with a large volume of neutral phosphate buffer the color changes to brown.

In methods B and C the resulting dye mixtures are extracted from the water phase by shaking with one-third volume of butanol. The butanol extracts are dried by addition of anhydrous magnesium sulfate (suitable because of its low solubility in butanol) and taken to dryness in a flash evaporator. The dyes synthesized according to methods b and c were shown by thin-layer chromatography to consist of one major and a few minor fluorescent compounds in addition to unchanged ANS as a major fluorescent component. Although the dye mixtures separated on a silica gel column, some of them appeared not to be stable when adsorbed on that material, whereas neutral alumina (Bio-Rad) proved to be a satisfactory separation medium. ANS and two strongly colored by-products were eluted with methanol containing 30% water buffered to pH 7, and bis-ANS and a brownish, almost nonfluorescent component were then eluted halfway down the column with 0.1 M neutral phosphate buffer. The alumina was then taken out of the glass column and the two fractions were separated mechanically. Extraction was carried out with 50% methanol–buffer solution and was followed by evaporation of the solvent until the green product precipitated. The compound could be crystallized from water solution and dried in a vacuum desiccator over P_2O_5, mp $>300°$.

The compounds isolated after synthesis according to the three methods were studied with respect to R_F value on thin-layer chromatography, ultraviolet and infrared absorption spectra, fluorescence spectra, and elementary composition. The dye synthesized in the protein-free systems (methods B and C) was found to be identical with the bis-ANS formed in the presence of BSA.

Identification and Properties of Bis-ANS. A comparison was made between ANS and bis-ANS. The carbon:nitrogen:sulfur proportions of ANS and bis-ANS were determined by elementary analysis and found to be identical within the experimental errors. The determination of absolute compositions as well as of hydrogen contents was obscured by the hygroscopic properties of the molecules; if one to two molecules of water were added to the formula, both sets of data agreed with the composition calculated for ANS. However, these data were sufficient to prove: (a) that no splitting of the ANS molecule occurs, and (b) that no nitrogen is introduced.

The infrared absorption spectra of ANS and bis-ANS were found nearly identical. Some differences appeared only in the 1300–1420-cm^{-1} region, which may correspond to aromatic substitution.

The ultraviolet absorption and fluorescence spectra are very similar, the only difference being slight shifts toward longer wavelengths for bis-ANS as compared to ANS (Table II and Figure 2). Further, the fluorescence quantum yields of the two dyes were found to be similar and to vary identically (min 0.004, max 0.8) in different environments (water < propylene glycol < isopropyl alcohol < BSA complex < dimethylformamide).

A closer study of the long-wave-absorbing oscillators was made in the following way. ANS and bis-ANS were dissolved in propylene glycol, and the polarization excitation spectra at $-55°$ were run in the instrument described by Weber and Bablouzian (1966). These spectra exhibit in both substances two leveled regions in the spectral range covered by the absorption bands in the 300–400-nm region. These two leveled regions

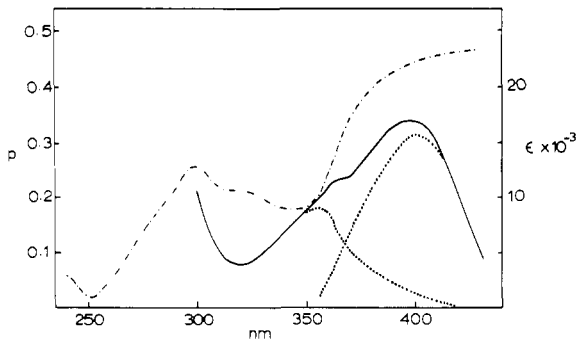

FIGURE 3: Polarization of fluorescence spectrum at −55° (-.-.-) and molar extinction coefficient at 25° (———) of bis-ANS in propylene glycol. (. . . . . .) Molar extinction coefficients of the separate oscillators in the longest wavelength absorption band.

correspond to different absorption oscillators, and the percentage contributions of each oscillator to the total absorption at any wavelength may be calculated if the polarization spectra are combined with the absorption spectra. The full details for bis-ANS are given in Figure 3. Similar resolution has been carried out for ANS by Anderson and Weber (1969). The wavelengths of the oscillator maxima and the intermediate points of equal absorption are for ANS 345 (max,) 378 (max), and 358 (intermediate) nm, respectively, and for bis-ANS 355 (max), 401 (max), and 368 (intermediate) nm, respectively.

Our conclusion that the compound that we call bis-ANS is actually a dimer of ANS needed support from an unambiguous observation of increased molecular size and for this purpose we made a determination of the rotational relaxation time of both compounds by observations of the fluorescence polarization of their solutions. According to the classical equation of Perrin (1926).

$$\rho_h = 3\tau \frac{1/P_0 - 1/3}{1/P - 1/P_0}$$

ρ_h is the mean relaxation time of the direction of the transition moment in emission, P is the observed polarization, P_0 the polarization in the absence of Brownian rotation, and τ the lifetime of the excited state of the fluorescence. If ρ_h is a linear

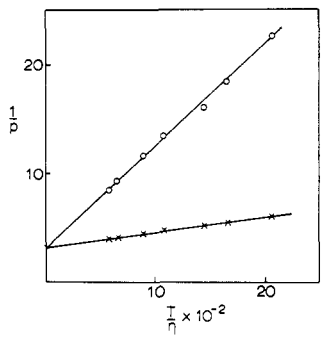

FIGURE 4: Reciprocal of polarization, $1/p$, as a function of T/η of ANS (O———O) and bis-ANS (×———×) in propylene glycol. Concentrations ~10^{-5} M. Excitation wavelength 366 nm. Emission observed through a 0.5-cm layer of 0.1 M $NaNO_2$ and Corning filter CS 3-72.

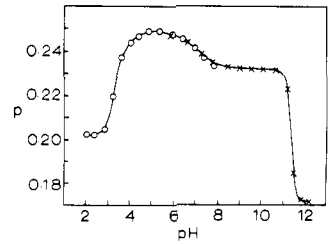

FIGURE 5: Fluorescence polarization of bis-ANS–BSA complex as a function of pH. (O—O) Titrated with HCl from neutrality. (×—×) Titrated with NaOH from pH 5.5. Excitation and emission as in Figure 4.

function of T/η (T, absolute temperature in °K; η, viscosity coefficient of the solvent) a plot of $1/p$ against T/η is a straight line with intercept $1/P_0$. Such plots (Figure 4) were obtained by variation of the temperature (18–40°) of propylene glycol solutions in the instrument described by Weber (1956). The temperature dependence of the relative viscosity of propylene glycol was determined with an Ostwald viscometer and the values were converted into absolute viscosity using the value of η = 44.79 cP at 20° given by Gartenmeister (1890). Lifetimes of the excited state of bis-ANS in dry propanol and in propylene glycol and of the bis-ANS–serum albumin complex at pH 7 were measured by phase delay in the cross-correlation fluorometer of Spencer and Weber (1969). The results are collected in Table III. From the values of that table and P and P_0 of Figure 4 the rotational relaxation times in *propylene glycol* at 25° are: ANS = 10.4 nsec; bis-ANS = 40 nsec. The corresponding times in water at 25° are ANS = 0.207 nsec; bis-ANS = 0.795. Using the Debye relation (Debye, 1929), $\rho_h = 3\eta V/RT$, the effective volumes V are 200 ml/mole for ANS and 737 ml/mole for bis-ANS. The difference is large enough to be accounted for by the doubling of the molecular size plus the elongation of the hydrodynamic ellipsoid in going from ANS to bis-ANS.

Further evidence for the dimer character of bis-ANS was obtained by osmometry. The vapor pressure of the potassium salt of bis-ANS was measured in a "Mecrolab" Model 301A osmometer using dibenzyl (mol wt 210) as reference. Assuming complete dissociation into three ions the molecular weight was calculated as 732. Thus, within the large error (±20%) of

TABLE III: Fluorescent Lifetimes of Bis-ANS.[a]

Solvent	nsec
Propanol	4.1 ± 0.2
Propylene glycol	4.6 ± 0.1
1% BSA, pH 7	9.4 ± 0.2
H_2O	0.1 ± 0.06
ANS in propylene glycol	8.5 ± 0.1

[a] Values were calculated from phase delay at a modulation frequency of 28.4 MHz; excitation wavelength = 366 nm. Emission was observed through a Corning CS-3-72 and 0.1 M $NaNO_2$ layer of 0.5 cm; concentration of bis-ANS = 10^{-5} M; temperature = 25°.

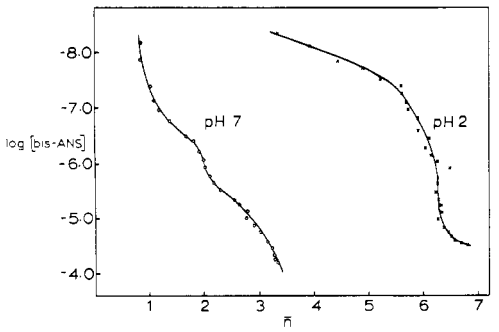

FIGURE 6: Binding curves of bis-ANS by BSA at 25° in 0.1 M phosphate. (O—O) pH 7; (×—×) pH 2. Excitation and emission as in Figure 4.

the method this value agrees with the expected 672 for the dimer.

Properties of Bis-ANS–Albumin Complexes. The complex formation does not appear to change the protein in any significant way. The sedimentation constants of the native albumin and of the albumin–bis-ANS complex (1:2) were calculated from sedimentation velocity runs of identical solutions of the two in the ultracentrifuge. They were found to be 4.22 and 4.16 S, respectively. Also, the polarization of fluorescence of the complex as a function of pH (Figure 5) demonstrated acid and alkaline expansions analogous to those recorded for the conjugate of BSA with DNS (Weber, 1952). In the polarization curve of the complex there is, however, a hump around pH 5 not found in BSA–DNS conjugates. The binding of bis-ANS to BSA was characterized by the titration curve as described by Daniel and Weber (1966). Such curves were obtained at pH 2 and 7 by additions of increasing volumes of a stock solution containing 7.6×10^{-4} M bis-ANS and 9×10^{-5} M BSA to a 0.1 M phosphate buffer solution and to excess BSA (10^{-4} M) in the same buffer, as described by Daniel and Weber (1966). In Figure 6 we have plotted the two curves, *i.e.*, log free bis-ANS *vs.* $\bar{n}$, at the two pH values. $\bar{n}$ is the average number of ligand molecules bound per protein molecule, and it is calculated as $(F_c/F_b)(X_0/P_0)$, where F_c is the bis-ANS fluorescence of a given solution of bis-ANS–BSA, and F_b the fluorescence, under the same experimental conditions, of an equal concentration of bis-ANS in excess BSA, *i.e.*, a concentration large enough to ensure complete binding of all the bis-ANS present. X_0 is the total bis-ANS concentration and P_0 the total BSA concentration.

The titration curve at pH 7 depicts the successive binding to two kinds of sites, separated by an inflexion at $\bar{n} = 2$, through the concentration range investigated. Of interest here is to note that the binding constant of the stronger sites is more than two orders of magnitude greater than the average binding constant of the five titrable sites in BSA which are accessible to ANS. As with the latter compound, the acid expansion of BSA makes an additional number of sites accessible to bis-ANS. As seen from Figure 6, six binding sites are titrated simultaneously at pH 2. The average binding constant for these sites is even greater than that of the strong sites at neutral pH. The situation is therefore quite different from that of ANS binding, where the affinity is considerably lower at pH 2 than at pH 7 (Daniel and Weber, 1966). The stoichiometry (at pH 7) is apparent from Figure 7 in which we have plotted $(\bar{q}/q_0)$·

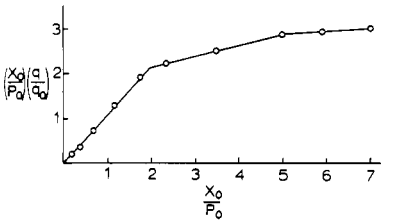

FIGURE 7: Stoichiometric plot of relative fluorescence efficiency of bis-ANS–BSA mixtures in 0.1 M phosphate (explanation in text). X_0, molarity of total bis-ANS. P_0, molarity of total protein. Excitation and emission as in Figure 4.

(X_0/P_0) against (X_0/P_0), where $\bar{q}$ is the average quantum yield at a particular concentration, and q_0 is the quantum yield at $\bar{n} = 1$ (*i.e.*, at a concentration yielding unsaturated binding). As elaborated by Daniel and Weber (1966) due to the fact that the quantum yield of the bound ligand is 180 times that of the free ligand, $\bar{q}/q_0$ will vary between 1 for complete binding and 0 for no binding, making the plot ideally reach a value N when all N possible binding sites are occupied. In Figure 7, the curve follows a straight line up to the point where X_0/P_0 equals 2, which reflects strong binding with equivalent fluorescence enhancement to two sites. At higher X_0/P_0 ratios the lesser slope of the curve indicates either decreasing strength of the third binding site, or less fluorescence enhancement upon binding to this site. An interesting factor is that the polarization of fluorescence is constant on binding to the first two binding sites, whereas it declines on binding to the third site (Figure 8), reflecting excitation transfer between the ligands to take place only after the introduction of a third ligand molecule.

Discussion

The binding with fluorescence enhancement of ANS and similar dyes to hydrophobic binding sites has in a number of investigations in recent years been used as a tool for studies of protein structures (Weber and Young (1964), Anderson and Weber (1966), Stryer (1965), McClure and Edelman (1966), Daniel and Weber (1966), Weber and Daniel (1966), and Anderson and Weber (1969)). As with other kinds of ligands, this binding is influenced not only by the size and shape but also by the charge of the ligand (Foster, 1960). Naturally, the

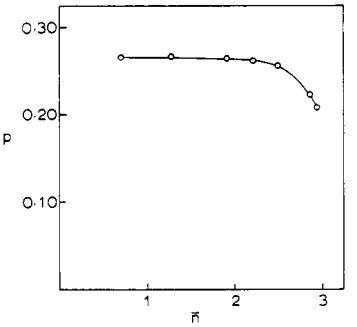

FIGURE 8: Polarization of fluorescence of bis-ANS–BSA mixtures at various degrees of binding (*cf.* Figure 6 and text). $\bar{n}$, average number of bis-ANS molecules bound per BSA molecule. Excitation and emission as in Figure 4.

larger the number of ligands per protein molecule the more complex is the interpretation of the data. A convenient complementary tool in this kind of studies would be a set of molecules with similar chemical and spectroscopic properties but differing in size. The compound studied in this work fills such a need. There are only two strong binding sites in BSA at neutral pH, and since there is no depolarization accompanying the introduction of the second ANS molecule (Figure 5), these sites are probably located far apart in the BSA molecule. Since binding to the third site is accompanied by depolarization, this site or sights might be located in between the two strong binding sites.

The catalysis by BSA in the formation of bis-ANS (Figure 1) is of particular interest. We have investigated a number of other proteins in this respect, and we found that only those proteins which bind ANS with fluorescence enhancement catalyze the reaction (BSA > ovalbumin > γ-globulin). With other proteins, as in the absence of protein, nitrite instead predominantly induces the formation of nonfluorescent products. It thus appears that the adsorption of ANS to BSA is the basic requirement for the enzymelike action of the protein.

The chemistry of the formation as well as alternative synthetic pathways are still under study. The results will be published elsewhere, but we will here give a summary of the facts that lead to the conclusion that the new compound studied here is a dimer of ANS. The elementary analysis of bis-ANS shows that no splitting or introduction of new groups takes place. This conclusion is also supported by the fact that nitrite acts in catalytical concentrations. The smaller number of molecules binding to BSA (as compared with ANS), the much longer rotational relaxation time, and the osmometry measurements make it clear that bis-ANS is a larger molecule than ANS. The formation of the compound through a blue intermediary under conditions identical with those of formation of a blue compound in the classical nitrate–nitrite test indicate that bis-ANS is formed according to a similar reaction scheme, which in the case of diphenylamine proceeds through quinoid structures and ends up with a dimeric product (Sidgwick, 1937). In the ANS case, the initial attack would be the formation of the *N*-nitrosamine. It is well known (*cf.* the Fischer–Hepp rearrangement, which is also catalyzed by chloride ions) that in this type of compound the *para* position is labilized. In ANS we have two such positions, the *para* position of the phenyl ring and the 4 position in the naphthalene ring system. The slight shifts in absorption and fluorescence maxima of bis-ANS as compared to ANS make any substitution in the naphthalene residue which is exclusively responsible for the lowest lying electronic state in the former highly unlikely. The resolution of the longest wavelength absorption band into two electronic transitions very similar to those of ANS and only slightly displaced toward longer wavelengths obtained by polarization spectral measurements (Figure 3) speaks in the same direction. The conclusion must therefore be that bis-ANS is the dimer of ANS formed by uniting the *para* positions of the phenyl rings of two molecules.

Apart from the suggested usefulness of bis-ANS in protein–ligand binding studies, the compound could also find some use in a more practical context. Because of the exceptionally high affinity for albumin paired with the strong fluorescence enhancement, it should be possible to use it with advantage in the detection and quantitative determination of albumin in minute concentrations.

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