A Fluorescence Quenching Technique for the Investigation of the Configurations of Binding Sites for Small Molecules*

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ABSTRACT: A method is described for the investigation of the exposure, to the solvent, of a small fluorescent molecule which is bound to a protein. The method is based upon determinations of fluorescence intensity emitted from aliquots of a solution containing the protein and a fluorochrome which reversibly binds to it. Measurements are made before and after equilibration with an external quencher. It is suggested that the intensity of the fluorescence is related to exposure of the fluorochrome to encounters with the quencher. Results indicate that: (1) 6-aminonaphthalene-2-sulfonate is extensively shielded when bound either by bovine serum albumin or by homologous antibody directed, in part, to the charged sulfonate. (2) The polarity of the binding sites of the particular antibody preparations used in this study does not differ greatly from that of water but the albumin binding site for the same fluorochrome is of a much lower dielectric constant.

In the present report the degree of exposure of a protein-bound small fluorescing molecule is characterized as a function of the intensity of a partially quenched emitted fluorescence. The method may be thought of as a variation of a solvent perturbation technique (Leherer, 1967), but the investigations to be discussed are several orders of magnitude more sensitive than classical solvent perturbation studies.

There are three requirements which must be satisfied by a system before it can be studied by what we have termed the fractional quenching method; *viz.*, (1) a distinctively fluorescent molecule, the fluorochrome, must be selectively bound to the protein at the site to be investigated; (2) the emission of the fluorochrome must be inversely related to the concentration of a third molecule; (3) the third molecule, the concentration of which can be independently varied, must be able to effect a substantial decrease in fluorescence intensity at concentrations which do not significantly alter the primary reaction between the protein and its substrate.

Aminonaphthalenesulfonic acids were chosen for these studies. All the isomers of the acids are commercially available, and derivatives formed by covalent coupling with other groupings can be readily prepared. Bromate ion effectively quenches the fluorescence of the aminonaphthalenesulfonic acids if the amino group is not blocked.

Fractional quenching has been applied to the study of the specific binding sites of both homologous antibody and bovine serum albumin. Both proteins appear to afford extensive shielding against the bromate ion quenching of 6-aminonaphthalene-2-sulfonate, but antibody binding fails to significantly alter the emission spectra of 6-aminonaphthalene-2-sulfonate, whereas a large blue shift accompanies the binding of 6-aminonaphthalene-2-sulfonate by bovine serum albumin. If this shift is related to the effective dielectric constant at the binding site (Stryer, 1965), these results indicate that antibody homologous to 6-aminonaphthalene-2-sulfonate possesses a specific binding site with dielectric constant similar to that of the aqueous milieu while bovine serum albumin binding sites have a lower dielectric constant.

Materials and Methods

6-Aminonaphthalene-2-sulfonic acid was obtained as the Technical Grade from Fisher Scientific Corp., Pittsburgh, Pa. Subsequent to the activated charcoal decolorization of a hot basic solution, it was crystalized, as the sodium salt.

Normalized emission spectra of the resulting silky pale violet crystals were invariant in energy distribution at 240-, 280-, 300-, and 320-mμ excitation. It was assumed, consequently, that within experimental limits only one fluorescent species was present.

Sodium bromate was Fisher Reagent grade. All alcohols or glycols were Spectral Grade (Fisher) or Chromatoquality (Matheson Coleman and Bell) and used without further purification.

The buffer (borate)[1] used to prepare all solutions, unless otherwise noted, was 0.2 M boric acid adjusted to pH 8 with 0.16 N NaOH and a final ionic strength of 0.16 with NaCl.

Antibody to 6-aminonaphthalene-2-sulfonate was incited in unselected New Zealand albino rabbits by subdermal and intramuscular injections of 10 mg of bovine γ-globulin-6-azonaphthalene-2-sulfonate (6-aminonaphthalene-2-sulfonate diazotized and coupled to a Pentex bovine γ-globulin) in complete Freunds adjuvant. A booster of 10 mg of the same antigen in 1 ml of borate was injected into an ear vein of each animal 1 month later. Animals, under ether anesthesia, were bled by cardiac puncture 6 days after the booster injection. Sera with more than 100 μg/ml of antihapten antibody were pooled and the globulin fraction was precipitated at 14% Na_2SO_4 concentration (Keckwick, 1940). Purification of the

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[1] Borate, 0.2 M boric acid adjusted to pH 8 with 0.16 N NaOH and adjusted to 0.16 ionic strength with NaCl.

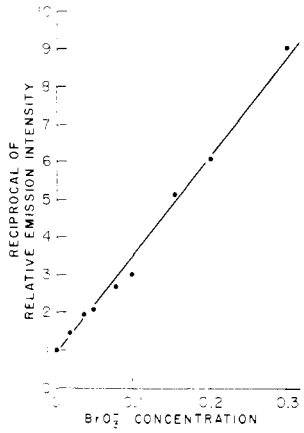

FIGURE 1: The reciprocal of the maximum relative emission intensity of 5×10^{-5} M 6-aminonaphthalene-2-sulfonic acid as a function of bromate ion concentration. Fluorescence excited at 300 mμ. Ionic strength of buffer, 0.16, pH 8.

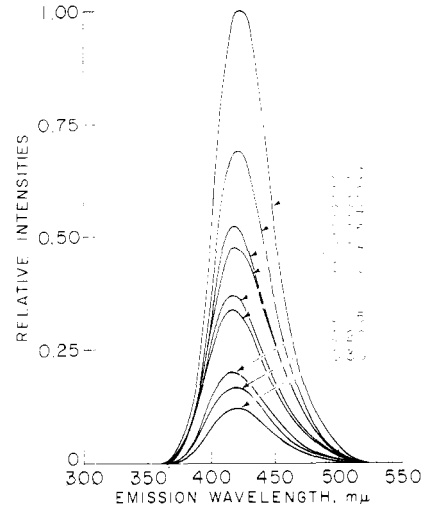

FIGURE 2: The emission spectra of the solutions of Figure 1 at 300-mμ excitation. The numbers refer to total molarity of BrO_3^-.

antibody was affected by a procedure previously described (Winkler, 1963). After antibovine γ-globulin was removed by specific adsorption antihapten antibody was adsorbed with homologous antigen rendered insoluble with antibovine γ-globulin. Antihapten antibody was eluted with 0.1 M 6-aminonaphthalene-2-sulfonate in borate and further purified by dialysis and passage through a column of Amberlite GC 400. The antibody was 85% precipitable with rabbit γ-globulin azo-6-aminonaphthalene-2-sulfonate.

Antibenzoate sera were purified in the same manner as anti-6-aminonaphthalene-2-sulfonate. The γ-globulin of unknown specificity was prepared by three Na_2SO_4 precipitations at 18, 14, and 9% (Keckwick, 1940).

Sera with low unrelated antihapten titers were used in experiments requiring antibovine γ-globulin. Absorption spectra were determined with a Cary 11 spectrophotometer.

The apparatus used for the determination of fluorescence emission spectra has been previously described (Winkler *et al.*, 1966).

Emission spectra were obtained from solutions maintained at 23° in square semimicrocuvets having a 3-mm light path. All solutions had an optical density in 1-cm.[2] cells of less than 0.9 at 280 mμ (Winkler, 1965).

Equilibrium dialyses were performed at 4° in 1-ml clear plastic cells obtained from Chemical Rubber Corp., Cleveland, Ohio.

In no case did excitation or emission band widths exceed 1.6 mμ.

Results

The emission intensity, I_m (at 300-mμ excitation), of the fluorochrome in bromate-containing solutions relative to I_m in bromate-free solutions, $I_{m\,rel}$, had been determined at a number of bromate ion concentrations. (It should be noted that although the OD_{300} for a 1-cm path length was 0.29 only a 3-mm light path was used. Therefore, since there is very little overlap between the absorption and emission of the fluorochrome, inner filter effects were negligible.) The reciprocals of $I_{m\,rel}$ *vs.* bromate ion concentrations are presented in graphical form in Figure 1. The straight-line relationship is consistent with a first-order reaction (Pringsheim, 1949). The relative energy distribution as a function of wavelength is almost constant in this range of bromate concentrations (0–0.3 M, Figure 2). All absorption spectra, taken over this same range, were found to be superimposable in shape, position, and unlike the emission spectra in magnitude. A representative curve is reproduced in Figure 3.

The mechanism of quenching must ultimately be dependent upon encounters between 6-aminonaphthalene-2-sulfonate and BrO_3^- since the latter has no appreciable absorption overlapping the emission spectra of the fluorochrome. An expression which relates the encounter frequency, Z, between molecules in solution to T/η was derived by Smoluchowski (1918). According to that derivation Z is directly proportional to T/η and for a 0.1 M aqueous solution at room temperature should be approximately $6.6 \times 10^{+8}$ encounters/sec. If one makes the assumption that Z is equal to the quenching frequency, this value can be substituted into the Stern–Volmer expression, $I/I_0 = 1/1 + Z\tau_0$, τ_0 (the mean lifetime of fluorescence) for 6-aminonaphthalene-2-sulfonate has been measured in borate and

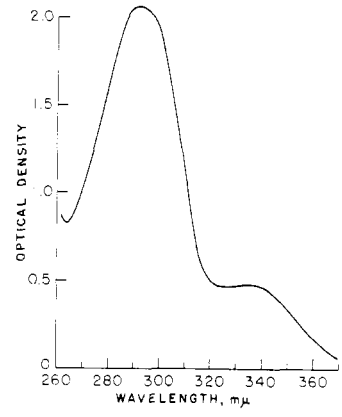

FIGURE 3: Absorption spectrum of 3.3×10^{-4} M 6-aminonaphthalene-2-sulfonate in pH 8 borate buffer. The spectral curves are superimposable between 0 and 0.3 M BrO_3^-.

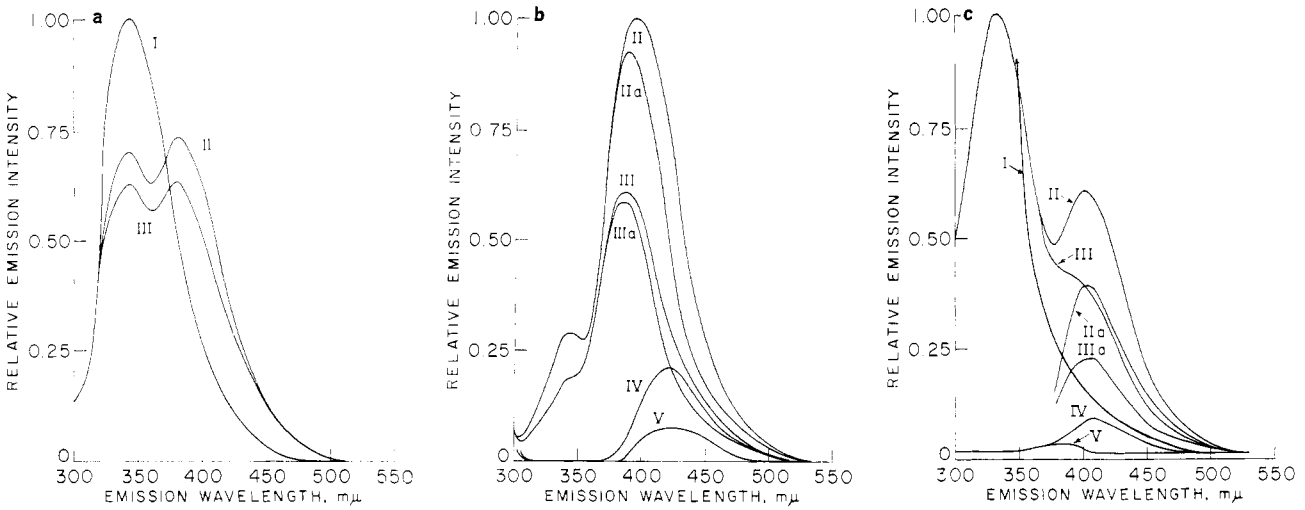

FIGURE 4: Emission spectra of identical volumes of solutions which had been dialyzed to equilibrium across Visking membranes. Initial concentrations are for protein, 1 mg/ml, and for 6-amino-naphthalene-2-sulfonic acid, 6×10^{-6} M. Results were obtained in the absence or presence of 0.1 M bromate ion. The over-all ionic strength is that of the pH 8 borate buffer (I 0.16) plus that contributed by the designated amount of bromate. The curves which appear in parts a–c are numbered as follows: curve I, protein alone; curve II, protein and fluorochrome; curve III, protein and fluorochrome in 0.1 M bromate; curve IV, free fluorochrome equilibrated with protein in the absence of bromate; curve V, free fluorochrome equilibrated with protein in the presence of bromate; curve IIa, bound dye in the absence of bromate, derived by subtracting curve I (where significant) and curve IV from II; curve IIIa bound dye in the presence of 0.1 M bromate. (Curves I and V subtracted from III.) In 4a (normalized to curve I) 1 mg/ml of bovine serum albumin. Excitation at 287 mμ: (b) (normalized to curve II) as (a) but excitation at 300 mμ: (c) (normalized to curve II) as (a) but 1 mg/ml of purified anti-6-aminonaphthalene-2-sulfonate substituted for albumin.

found to be 1.6×10^{-8} sec (M. H. Winkler, unpublished results). Therefore I/I_0 should be 1/11.6 approximately. The fact that in protein-free solution it is closer to one-third may indicate that an additional factor should be considered; however, the theoretical value of I/I_0 is close enough to the experimental value to very strongly suggest that translational diffusion of the quencher is the most important if not the only factor which need be considered.

We consider these results to support our initial assumption that the degree of quenching depends upon the accessibility of negatively charged bromate ions to the fluorochrome. Therefore, 6-aminonaphthalene-2-sulfonate has been employed as another "molecular probe" to characterize protein binding sites. It differs from a "molecular probe" proposed earlier (Winkler, 1962) in that it may provide a measure of the accessibility of a small negatively charged ion to a protein-bound ligand. To evaluate this use, 6-aminonaphthalene-2-sulfonate was allowed to equilibrate, across a Visking membrane, with bovine serum albumin either in the presence or in the absence of 0.1 M bromate. The emission spectra of both protein-containing and protein-free sides of the dialysis cells are given at 287 and 300 mμ, respectively, in Figure 4a,b. The emission spectra of the ligandfree protein is also included in 4a(curve I). Curves II are the emission spectra of the equilibrium mixture of protein and fluorochrome. The energy-transfer contribution to spectra excited at 287 mμ is clearly evident in the reduction of the protein fluorescence appearing at 340 mμ. This reduction cannot be attributed to absorption by the fluorochrome since its OD_{287} at these concentrations is negligible. Curves III represent the emission of the protein plus the fluorochrome in the presence of 0.1 M bromate. Bromate ion apparently has a significant effect on bovine serum albumin fluorescence. Therefore, the derived curves IIa and IIIa which represent the emission only of the bound fluorochrome were obtained from spectra excited at 300 mμ where protein excitation is small.

The change in the extent of binding affected by the addition of 0.1 M bromate to either of the protein fluorochrome systems used in this study cannot be directly evaluated with available instrumentation. The effect of bromate on the emission of bound 6-aminonaphthalene-2-sulfonate is not known and equilibrium concentrations of the free fluorochrome are too low for reliable optical density measurements to be made. The relative magnitudes of emission from the protein-free fluorochrome solutions equilibrated with bovine serum albumin in the absence or presence of 0.1 M bromate (curves IV and V of Figure 4b), however, are consistent only with similar degrees of binding both in the absence or presence of the quencher. A comparison of these curves with those obtained from fluorochrome solutions which are 0.0 and 0.1 M in bromate (Figure 2) reveal that similar fractional decreases in fluorescence intensity are induced by 0.1 M bromate. This result is only possible if the amount of unbound fluorochrome on the protein-free side of the dialysis membrane is unchanged by the addition of bromate ion.

The most immediately obvious features of the bound fluorochrome emission spectra are: (1) the uncorrected reduction in I_m of protein-bound fluorochrome by 0.1 M bromate is considerably less than that found for free fluorochrome (Figure 1); and (2) that the binding to bovine serum albumin is accompanied by a blue shift in the emission spectra. In order to most directly evaluate the quantitative aspects of the uncorrected results, 5×10^{-6} M solutions of fluorochrome in borate, in 1 mg/ml of bovine serum albumin, and in 1 mg/ml of bovine γ-globulin were studied. Emission spectra excited at 300 mμ are given in Figure 5. The absolute magnitude of all three curves is quite similar. It is possible to estimate the contribution of free fluorochrome

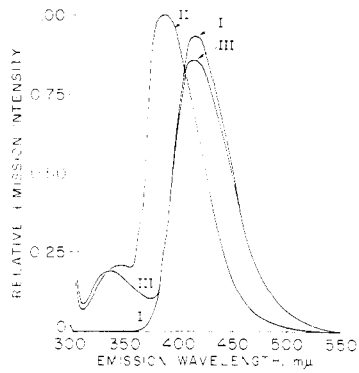

FIGURE 5: Emission spectra of 5×10^{-6} M 6-aminonaphthalene-2-sulfonate in: pH 8 borate buffer (curve I), 1 mg/ml of bovine serum albumin (curve II), and 1 mg/ml of bovine γ-globulin (curve III).

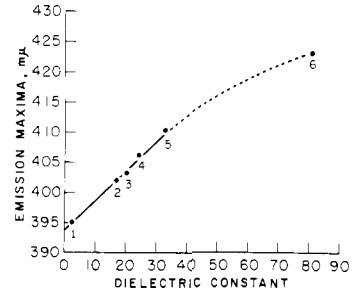

FIGURE 6: Wavelength of maximal emission of 6-aminonaphthalene-2-sulfonic acid as a function of the dielectric constant of the solvent. Solvents are: (1) dioxane, (2) 1-butanol, (3) 1-propanol, (4) ethanol, (5) methanol, and (6) aqueous pH 8 borate buffer.

emission at 418 mμ to the bound fluorochrome curve which has an emission maxima of 395 mμ from the data recorded in Figure 4b. The free hapten contributes about 7% to the intensity of the bound hapten emission. A reduction of 7% in the fluorochrome–bovine serum albumin emission recorded in Figure 5 produced an I_m which is very close to the I_m of the free fluorochrome, even though λ_{max} is different. Therefore, it seems that any alteration in instrumental characteristics over this short-wavelength span are compensated by either the higher energy content per quanta at the shorter wavelength or by a suitable alteration in quantum yield which may, for instance, result from an increased effective viscosity in the binding site (Winkler, 1967).

The area under the recorded uncorrected emission curves, obtained after subtraction of the contributions made by equilibrated free ligand and protein, was used as the basis for the conclusions presented below. The intensity of emission of the bound fluorochrome in 0.1 M bromate is found to be 66% of that of bound fluorochrome in the absence of bromate (Figure 4b, curves IIa and IIIa). Using Figure 1 it is seen that this corresponds to the quenching of free fluorochrome affected by 0.020 M bromate ion, but the actual solution concentration of bromate ion was 0.1 M. Therefore, based on the reasoning outlined above, the binding protein reduces the accessibility of bromate ion to the binding site by 80%.

It should be noted that all of the above results were obtained after equilibration with "protein-free" buffer. Hence, unbound bromate concentrations are equal in protein and protein-free solutions. The fact that fluorescence emission is greater in the presence of bovine serum albumin (Figure 4a,b) or antibody (Figure 4c) clearly indicates that binding has taken place. Binding of BrO_3^- ion by bovine serum albumin (Scatchard *et al.*, 1957) would not be expected to effect the results. The concentration of BrO_3^- is about 0.1 M, that of protein is of the order of 10^{-5} M. Hence an unreasonably large amount of small ions would have to be bound to the respective proteins to have any detectable effect.

It would seem that the blue shift in the emission spectra of 6-aminonaphthalene-2-sulfonate is occasioned by a reduced dielectric constant in the binding site. To help evaluate this possibility emission spectra were obtained at 300-mμ excitation for the fluorochrome dissolved in borate, methanol, propanol, butanol, and *p*-dioxane. These data, depicted in Figure 6, reveal a reasonably straight-line relation between λ_{max} and dielectric constant in the organic solvents. This result is not actually in conflict with that previously reported (McLure and Edelman, 1967). That study was made at a band pass of 10 mμ and would not be expected to show the small differences we are reporting. The results depicted in Figure 6 were obtained at a 0.8-mμ band pass. Judging by these results the microdielectric constant of the bovine serum albumin binding site is similar to the dielectric constant of dioxane. In contrast when the same fluorochrome is allowed to complex with homologous rabbit antibody (Figure 4c), the wavelength maxima of emission of bound fluorochrome is similar to that of fluorochrome in borate buffer. As in the previous case the intensity of emission is found to be unaffected by binding. The antibody binding site offers about 65% protection against the quenching of the fluorochrome by bromate ion.

Discussion

The fluorescence of 6-aminonaphthalene-2-sulfonate in solution is reduced to 32.6% of its value in borate by the presence of 0.1 M bromate ion. When complexed with bovine serum albumin, 0.1 M BrO_3^- reduces the fluorescence to 66% of its initial value, and when complexed with homologous antibody the 0.1 M BrO_3^--induced reduction is to 59%. These values for bovine serum albumin–fluorochrome and antibody–fluorochrome would correspond, respectively, to the quenching that would be realized by 0.020 and 0.035 M bromate. These results suggest a protection of 80% by bovine serum albumin and 65% by homologous antibody; however, if permissible energy levels involved in the fluorescence transition are altered by binding, as is indicated by the blue shift experienced when the ligand binds to the bovine serum albumin, then this simple interpretation may be in error. If the bound ligand is not susceptible to quenching by bromate ion due to alterations in permitted transitions, the accessibility may be high, even though quenching is minimal. If susceptibility is increased the values given are minimal ones. Neither of these situations is likely, however, because binding to antibody, which induces a minimal shift in emission spectra, yields similar results to those obtained with bovine serum albumin.

Binding of 6-aminonaphthalene-2-sulfonate by antibody results in minimal change in emission. Hence it is probable that the lowest vibrational level of the first excited electronic state and all levels of the ground state involved in either transition are

unperturbed. Therefore quenching should occur as in the unbound fluorochrome limited only by the "effective" concentration of bromate ion in the immediate vicinity of the bound fluorochrome. These results demonstrate the feasibility of using externally quenched small fluorescent molecules, which will bind to proteins, as indicators of the exposure of such molecules, when bound, to the solution environment, or at least, in this instance, to a small, negatively charged ion. This type of probe could be useful in the investigation of antihapten binding sites. For this purpose the progressive removal of the fluorescent moiety from the binding protein might be affected by the use of a series of specially synthesized compounds characterized by a haptenic moiety covalently bound to a fluorescent moiety by chains of known lengths. It should then be possible to determine the degree of influence of the bulky protein molecule at specific distances from the binding site. A suitable choice of bound molecules would enable one to map the topography of the binding macromolecule in the vicinity of the specific binding site.

The fact that λ_{max} of the emission spectra of antibody-bound 6-aminonaphthalene-2-sulfonate is almost identical with that obtained for a borate solution of 6-aminonapthalene-2-sulfonate is of interest in connection with an earlier report (Winkler, 1962). It was noted at that time that there was an increase in fluorescence when an *N*-phenyl derivative of naphthalenesulfonic acids was bound to antibody prepared against the 1–4 isomer of azonaphthalenesulfonic acid. We had suggested that a possible explanation for this observation was that the degree of intramolecular rotation experienced by the fluorochrome in solution was reduced when the fluorochrome was bound to antibody. Subsequently, an alternate suggestion which connected the increased emission to a lower dielectric constant in the binding site was offered (Stryer, 1965; McLure and Edelman, 1967). If the preceding interpretation of these results is correct, however, the dielectric constant of the binding site for the 6–2 isomer does not greatly differ from that of water. It does not seem probable that the binding site for the 4–1 isomer is considerably less polar than that for the 6–2 isomer. If the sites are similar, one could not attribute the observed increase in fluorescence to a lower dielectric constant in the binding site.

Parker *et al.* (1967) have found that the binding of the haptenic grouping 5-dimethylaminonaphthalene-1-sulfonamidolysine to homologous antibody results in a 56-mμ blue shift in the emission spectrum. This shift is similar to that induced by the solution of 5-dimethylaminonaphthalene-1-sulfonamidolysine in dioxane. Consequently they have concluded that the dielectric constant of the antibody binding site is similar to that of dioxane. The similarity of 5-dimethylaminonaphthalene-1-sulfonamidolysine to the hapten used in this study is only apparent, however. The antibody to 5-dimethylaminonaphthalene-1-sulfonamidolysine which they had prepared must be directed to the uncharged dimethylamino grouping rather than the negatively charged sulfonic acid moiety. It is quite reasonable, therefore, that a less polar binding site should be characteristic of antibody to 5-dimethylaminonaphthalene-1-sulfonamidolysine.

Binding of 1-anilino-8-naphthalenesulfonate by homologous antibody also induces a large blue shift in the emission spectra of the hapten (Yoo and Parker, 1968). In this case the antibody binding site includes the charged sulfonate grouping. It should be noted, however, that Yoo and Parker have suggested that their observed results were actually due to a composite of factors which might include interactions at the amino nitrogen. In view of the present results, we would like to suggest that the antibody binding sites to both 1-azonaphthalene-8-sulfonate and to 6-aminonaphthalene-2-sulfonate have dielectric constants similar to water and the blue shift noted by Yoo and Parker is, as they propose, due to interactions at the amino nitrogen. The fact that we obtain a similar shift when 6-aminonaphthalene-2-sulfonate is bound to bovine serum albumin along with a greater degree of protection may indicate that the bovine serum albumin site encompasses the amino nitrogen and, additionally that the amino nitrogen is at a quenching sensitive area of the hapten.

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