Fluorescent Probes for the Study of the Antibody–Hapten Reaction. I. Binding of the 5-Dimethylaminonaphthalene-1-sulfonamido Group by Homologous Rabbit Antibody*

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ABSTRACT: When rabbit antibody specific for the 5-dimethylaminonaphthalene-1-sulfonamido (DNS) group interacts reversibly with ε-DNS-lysine (the homologous hapten), the fluorescence quantum yield of the bound dye at 25° is increased 25–30-fold. The increase in the fluorescence of the bound dye is accompanied by a marked shift in the emission maximum from 556 to about 500 mμ. Control studies indicate that ε-DNS-lysine binding is limited to the Fab portion of anti-DNS antibody; "nonspecific" rabbit γ-globulins and rabbit antibodies specific for structurally unrelated nonpolar haptens do not bind the dye to any significant degree. An approximation of the dielectic constant of the antibody combining site has been obtained by correlating changes of antibody-bound dye fluorescence with changes in dye fluorescence in various solvents. When the dye is transferred from an aqueous to a nonpolar solvent like dioxane, alterations in dye fluorescence occur which mimic those of the antibody-bound dye. On the basis of this similarity it is concluded that the specific combining region of anti-DNS antibody is essentially a hydrocarbon environment.

In the study of the correlation between antibody structure and function one of the salient questions is the nature of the active site. By means of chemical-labeling techniques and amino acid analysis it has become evident that antibodies differ in regard to amino acid composition and active site reactivity. Antibodies to charged haptens have a small increase in the proportion of one or more oppositely charged amino acids (Koshland and Englberger, 1963). While experimental data are quite limited it seems reasonable to assume that at least one oppositely charged amino acid residue is actually in or near the antibody active site. In this location the charged amino acid would be capable of forming a salt-type linkage with the hapten contributing Coulombic energy to the antibody–hapten interaction. If the idea of active site complementarity is extended to nonpolar haptens one would postulate an active site composed of nonpolar amino acids. A collection of uncharged amino acid side chains in proper orientation could provide a hydrocarbon milieu for the ligand permitting hydrophobic bond formation. Current attempts to define the structure of the active site of antibodies to uncharged haptens do not permit definite conclusions. However, judging from the studies of Singer and his collaborators, labeled peptides in or near the active sites of antibodies specific for the 2,4-dinitrophenyl (DNP) group appear to have an unusually high proportion of uncharged amino acids (Singer and Doolittle, 1966).

A recent approach to the study of combining regions on proteins which provides a different means of attacking this question involves the use of environmentally sensitive chromophoric groups. Such molecules undergo marked changes in absorption or fluorescence depending on the polarity of their local environment. Inferences in regard to the nature of the combining region on the protein are possible by relating absorption or fluorescence changes in the protein-bound state to changes produced by perturbation of the molecule in solvents of varying polarity. The use of fluorescent probes in the study of reversible ligand–protein interactions was introduced by Weber and Laurence (1954). They defined a group of anilinonaphthalenes and anilinoacridines which undergoes marked increases in fluorescence on combination with various serum albumins and denatured proteins. One of the anilinonaphthalenesulfonates, 8-anilinonaphthalene-1-sulfonate (8-1-ANS),[1] has recently been employed by Weber and his colleagues in a series of extensive studies on albumin–ANS and lactic acid dehydrogenase–ANS interactions (Weber

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[1] Abbreviations used: 8-1-ANS, 8-1-anilinonaphthalene-1-sulfonate; DNS, the 5-dimethylaminonaphthalene-1-sulfonyl group; AcS, 2-methoxy-6-chloro-9-acridinyl-*N*-*p*-aminobenzenesulfonic acid; 4-1-ANS, 4-1-anilinonaphthalenesulfonate; DNS_{15}-BγG, a DNS–bovine γ-globulin conjugate with an average of 18 DNS groups/molecule of protein; phosphate–saline is 0.15 M NaCl–0.01 M phosphate (0.008 M dipotassium phosphate and 0.002 M monosodium phosphate) (pH 7.4).

and Young, 1964; Daniel and Weber, 1966; Anderson and Weber, 1965). Stryer (1965) has demonstrated that 8-1-ANS also interacts strongly with the heme binding regions of apohemoglobin and apomyoglobin. Gally and Edelman (1965) have described the use of ANS to study thermal transitions in the configuration of Bence–Jones proteins. Several other fluorescent probes have been used to characterize nonpolar binding sites in or near the catalytic binding site of chymotrypsin (Deranleau and Neurath, 1966; Bernhard *et al.*, 1966; McClure and Edelman, 1967).

Surprisingly little effort has been made to develop and characterize antibody–hapten systems involving environmentally sensitive haptens. Certain nonfluorescent chromophoric haptens like the ε-DNP-lysine group do undergo changes in absorption maxima in the presence of homologous antibody (Eisen and Siskind, 1964). However the magnitude of the effect is quite small relative to the changes observed when fluorescent molecules like 8-1-ANS are bound by serum albumins. In a preliminary report Winkler (1962) noted marked increases in the fluorescence of two different anilinonaphthalenesulfonates in the presence of a cross-reacting antibody. However no attempt was made to correlate changes in ligand fluorescence with antibody concentration or affinity. More recently, Berns and Singer (1964) studied alterations in the fluorescence of an acridine substituted with *p*-aminobenzenearsonate in the presence of rabbit antibenzenearsonate antibody. While significant increases in the fluorescence of the antibody-bound ligand were observed, a far greater change in fluorescence occurred when the same molecule was bound to bovine serum albumin. Moreover, solubility considerations required that the binding studies be performed at pH 9.5, a pH region which is not optimal for antibody stability.

In the present report we will characterize changes in the fluorescence of the ε-(5-dimethylaminonaphthalene-1-sulfonamido) derivative of lysine (ε-DNS-lysine) in the presence of homologous rabbit antibody, "normal" rabbit globulin, serum albumin, and various organic solvents. In the companion paper results obtained using ε-DNS-lysine as a probe to elucidate changes in the binding site of anti-DNS antibody with time after immunization will be described. The selection of the ε-DNS-lysyl group as a desirable antigenic determinant for the evaluation of antibody interactions with fluorescent haptens was based on results of systematic studies with a variety of substituted naphthalenes (Yoo and Parker, 1966) and several derivatives in the acridine, fluorescein, and rhodamine families (C. W. Parker, unpublished results). Some of the reasons for selecting the DNS group in preference to certain of the other determinants are outlined in the discussion. A brief resumé of a portion of this work was published earlier (Parker, 1966).

Materials and Methods

DNS-chloride, DNS-sulfonic acid, and DNS-amide were prepared as described by Weber (1952). ε-DNS-lysine was prepared by reaction of the copper complex of lysine with DNS-chloride, followed by decomposition of the copper complex with EDTA. A mixture of 2.424 g of L-lysine·HCl and 4.035 g of cupric carbonate in 150 ml of water was refluxed for 1 hr. The warm solution was filtered and the precipitate was washed with 30 ml of water. The solution was cooled to room temperature, and 3.9 g of solid sodium bicarbonate was added followed by the addition of 3.0 g of DNS-chloride in 108 ml of acetone. After stirring at room temperature overnight, 600 ml of water was added, and the precipitate was isolated by filtration and washed with water, ethanol, and ether. The isolated copper complex was treated with 11.9 g of Na_2EDTA, 62 ml of water, and enough 5 N NaOH to bring the pH to about 10–10.5. The mixture was stirred at room temperature until the precipitate dissolved, the pH of the solution was then adjusted to 6.0 with 2 N HCl, and the gummy yellow precipitate which formed was isolated by centrifugation and washed with a saturated solution of Na_2EDTA and water. The gum was suspended in water, and the pH of the suspension was adjusted to 4.0 with dilute HCl. The precipitate was isolated by centrifugation and crystallized by trituration with acetonitrile–acetone (1:1). The crystals were isolated by filtration and washed with cold acetonitrile–acetone. The yield was 45%, mp 200–202°. For analysis a sample was recrystallized from acetonitrile–acetone. *Anal.* Calcd for $C_{18}H_{25}N_3O_4S_1 \cdot 0.3HCl$: C, 55.3; H, 6.47; Cl, 3.2; N, 10.75; S, 8.19. Found: C, 55.32; H, 6.65; Cl, 3.49; N, 10.6; S, 8.09. To remove the chloride the product was dissolved in 1 N HCl and neutralized by the addition of 1 N NaOH. The precipitate was isolated by filtration and washed with water, mp 200–202. *Anal.* Calcd for $C_{18}H_{25}N_3O_4S_1$: N, 11.1. Found: N, 11.0.

The ε-DNS-lysine gave a single fluorescent, ninhydrin-positive spot by paper chromatography in four solvent systems and it exhibited the characteristic 330-mμ absorption peak of DNS-amides. The molar extinction coefficient at 330 mμ in 0.15 M NaCl–0.01 M phosphate (pH 7.4) was 4.57×10^3.

Tritium-labeled ε-DNS-lysine (sp act. 24 mc/mmole) was synthesized from tritium-labeled L-lysine (Nuclear-Chicago, Chicago, Ill.) by a semimicroadaptation of the above procedure. 8-Anilinonaphthalene-1-sulfonate (magnesium salt) was prepared according to Weber and Young (1964). Its extinction coefficient was 4950 cm^2/mmole at 350 mμ in water. Tritium-labeled 8-1-ANS was prepared as described by Parker and Osterland (1966).

2-Methoxy-6-chloro-9-acridinyl-*N*-*p*-aminobenzenesulfonic acid (AcS) was generously provided by Dr. G. Weber and Dr. C. Todd. Organic solvents were the highest purity available. Solutions of sucrose and Ficoll were made up according to McClure and Edelman (1966).

DNS–protein conjugates, rabbit anti-DNS antisera, and specifically purified anti-DNS antibody were prepared as described by Parker *et al.* (1967). The antisera utilized in the present paper were obtained

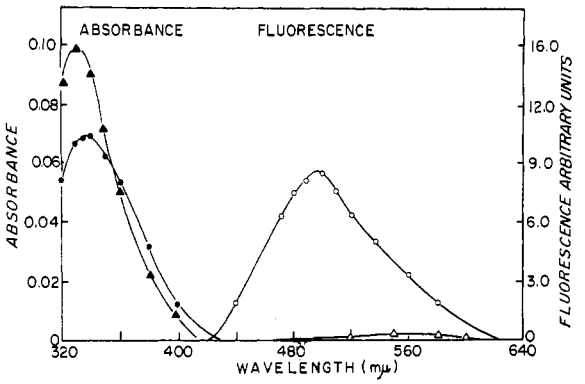

FIGURE 1: Absorption and emission spectra for free and antibody bound ϵ-DNS-lysine. (▲) Absorbance of free ϵ-DNS-lysine. (△) Emission of free ϵ-DNS-lysine. (●) Absorbance of bound ϵ-DNS-lysine. (○) Emission of bound ϵ-DNS-lysine. Free and bound hapten were at a concentration of 2.1×10^{-5} M. The absorption and emission spectra of bound ϵ-DNS-lysine were corrected for protein and free dye. The amount of dissociated dye was calculated from parallel equilibrium dialysis experiments at the same antibody concentration. Temperature, 25°; solvent, phosphate–saline.

1–1.5 months after immunization with DNS-hemocyanin. "Nonspecific" γ-globulin and anti-DNS γ-globulin fractions were prepared by ammonium sulfate precipitation at 40% of saturation followed by DEAE chromatography. The protein and the resin were equilibrated with 0.02 M phosphate (pH 7.0). The initial protein fraction eluted from the column was used. Sources for nonspecific γ-globulins included purified rabbit γG-globulin (the Pentex Corp., Kankakee, Ill.) and antisera from animals immunized with structurally unrelated haptens in complete Freund's adjuvant. Specifically purified rabbit anti-DNP antibody was prepared according to Eisen (1964b). Immunoelectrophoresis of the purified γ-globulins and antibodies against sheep antirabbit serum (Hyland Laboratories, Los Angeles, Calif.) failed to reveal any proteins other than γG-globulin. The technique employed for equilibrium dialysis is described in the succeeding paper (Parker *et al.*, 1967).

Crystalline rabbit Fc was obtained from a DEAE-purified γG-globulin fraction containing anti-DNS antibody according to the procedure of Nisonoff (1964). Fab fragments were separated from residual Fc by chromatography on CM-cellulose under the conditions given by Porter (1959) for isolation of "fraction I." The rabbit serum albumin and human serum albumin were obtained commercially (the Pentex Corp., Kankakee, Ill.).

Fluorometric titrations were performed in an Aminco Bowman spectrofluorophotometer. Temperature control was maintained by utilizing a cell compartment which was equipped with a hollow copper block connected to a constant-temperature water bath. Incident and emission spectra were corrected for instrument factors according to the procedure described by Melhuish (1962) using the Aminco solid sample accessory and a Rhodamine B quantum counter. Corrected emission spectra were obtained by replacing the quantum counter with a mirror, measuring light intensity at each wavelength, and applying the previously determined correction factor for variation in incident light intensity at each wavelength (Melhuish, 1962). Quantum yields were determined at 25° by the method of Parker and Rees (1960) using quinine sulfate as a standard

$$\phi_s = \phi_q \frac{(Abq)(\text{area } s)I\lambda_q}{(Abs)(\text{area } q)I\lambda_s}$$

where ϕ_s is the quantum yield of the sample; ϕ_q is the quantum yield of quinine in 1.0 N H_2SO_4 (taken to be 0.546 at a concentration of 2×10^{-6} M) (Melhuish, 1961); and area q and area s are the respective integrated areas under the fluorescence curves for quinine and sample as determined by plotting and weighing the paper under the peak. $I\lambda_q$ and $I\lambda_s$ are the relative lamp intensities at the wavelengths for excitation of quinine (350 mμ) and sample (ϵ-DNS-lysine at 340 mμ); Abs and Abq are the absorbances of the sample and quinine at their respective excitation wavelengths.

Results

The absorption and emission spectra of ϵ-DNS-lysine in phosphate–saline are given in Figure 1. The naphthalene derivative exhibited an activation maximum at 330 mμ and a fluorescence maximum at 556 mμ. The absorption spectrum corresponded closely to the activation spectrum, at least over the range from 300 to 400 mμ. The absorption and emission spectra did not overlap to any significant extent.

In aqueous solution the fluorescence quantum yield of ϵ-DNS-lysine at 25° was only 0.026–0.029 (Table I). With the addition of increasing proportions of dioxane there was a progressive increase in quantum yield until a value of 0.45 was reached in pure dioxane (dielectric constant 2.2). The increase was accompanied by a decrease in λ_{max}, the wavelength of the emission maximum. The change in λ_{max} was essentially monotonic with increases in the weight per cent of dioxane in the medium (and corresponding decreases in the dielectric constant) until an 80:20 dioxane–water mixture was reached. McClure and Edelman (1966) have observed similar anomalous effects on 2-*p*-toluidinylnaphthalene-6-sulfonate fluorescence at high dioxane concentrations. To explain this effect they suggested that the structure of dioxane was ordered by small amounts of water, resulting in marked alterations of the thermodynamic properties of dioxane–water mixtures in the range from 80–100% dioxane.

The relationship between ϵ-DNS-lysine fluorescence and the dielectric constant of the solvent was further examined in a homologous series of unbranched aliphatic alcohols. The fluorescence quantum yield increased with increases in the chain length of the

TABLE I: Variation in ε-DNS-lysine Fluorescence in Selected Solvents.[a]

Solvent	Quantum Yield	λ_{max}
Phosphate–saline	0.029	556
H_2O	0.026	557
H_2O–dioxane (90:10)	0.05	554
H_2O–dioxane (80:20)	0.09	552
H_2O–dioxane (70:30)	0.12	550
H_2O–dioxane (60:40)	0.18	547
H_2O–dioxane (50:50)	0.23	544
H_2O–dioxane (40:60)	0.27	543
H_2O–dioxane (30:70)	0.32	540
H_2O–dioxane (20:80)	0.34	535
H_2O–dioxane (10:90)	0.40	528
Dioxane	0.45	496
Methanol	0.28	536
Ethanol	0.33	535
1-Butanol	0.36	526
1-Hexanol	0.40	524
1-Heptanol	0.41	522
Antibody bound	0.4–0.95[b]	495–512[b]

[a] λ_{max} is given in millimicrons. Excitation was at 340 mμ. The values given for quantum yield and λ_{max} were corrected for instrument factors (see text). Temperature was 25°. The concentration units for the dioxane–H_2O mixtures were in volume to volume. [b] These values vary with the individual antibody preparation (Parker *et al.*, 1967).

TABLE II: Effect of Solvent Viscosity on the Fluorescence Quantum Yield of ε-DNS-lysine in Aqueous Solution.[a]

Expt	Composition of Mixture: Wt % 20% Sucrose	Composition of Mixture: Wt % 20% Ficoll	Sp Viscosity[b] (Cp)	Fluorescence Quantum Yield
1	100	0	1.6	0.040
2	80	20	3.0	0.044
3	60	40	4.5	0.048
4	40	60	9.0	0.056
5	20	80	12.0	0.059
6	0	100	19.5	0.062

[a] The fluorescence quantum yield of ε-DNS-lysine in the indicated amounts of 20% Ficoll and 20% sucrose was determined at 25° as described in the text. [b] Based on data given by McClure and Edelman (1966).

alcohols, corresponding to a more nonpolar environment for the hapten.

The fluorescence quantum yield of ε-DNS-lysine in water and other solvents was only slightly affected by temperature over the range from 273 to 313°K. There was actually a 5% increase in fluorescence quantum yield in water at 303°K as compared with 273°K.

In accord with the relative lack of temperature effect on the fluorescence of ε-DNS-lysine its emission was not markedly affected by changes in solvent viscosity (Table II). A series of six solutions containing varying proportions of 20% sucrose and 20% Ficoll were prepared as described by McClure and Edelman (1966). Despite a marked increase in solvent viscosity in going from pure 20% sucrose to pure 20% Ficoll the increase in quantum yield was only 50%.

The activation and emission spectra of antibody-bound ε-DNS-lysine are illustrated in Figure 1. The 330-mμ absorption band of the unbound dye was shifted to 340 mμ for the antibody-bound dye. The red shift in the absorption band in this region was accompanied by a diminution in oscillator strength. The emission maximum of the bound dye was shifted to the 495–510-mμ region depending on the antibody preparation (Parker *et al.*, 1967). With most antibody preparations there was a 25–30-fold increase in the fluorescence quantum yield as compared with that of the free dye. Bound ε-DNS-lysine generally underwent a 150–200-fold increase in fluorescence intensity at 480 mμ.

The activation spectrum of the antibody-bound dye also displayed a well-defined maximum at 280 mμ where the dye itself has a relatively low absorbance. The 280-mμ peak is due to excitation energy transfer from excited tyrosine and tryptophan residues to the dye, producing sensitized fluorescence (Weber and Teale, 1965).

A representative fluorometric titration of specifically purified rabbit anti-DNS antibody is shown in Figure 2. Similar increases in fluorescence occurred when globulin fractions containing both nonspecific γ-globulin and anti-DNS antibody were titrated. Nonspecific rabbit γG-globulin alone and rabbit anti-DNP antibody did not alter the fluorescence of ε-DNS-lysine significantly.

A structurally unrelated molecule (AcS), which has been used to probe hydrophobic binding sites on serum albumin (Berns and Singer, 1964), was not bound to any significant extent by the anti-DNS antibody.

The extent to which rabbit anti-DNP antibody and nonspecific rabbit γG-globulin bind ε-DNS-lysine also was examined by means of fluorescence quenching (Velick *et al.*, 1960; Parker, 1963; Eisen, 1964a). As shown in Figure 3 the dye did not quench the fluorescence of the two proteins significantly. For comparison, quenching curves obtained by titrating anti-DNP antibody with ε-DNP-lysine and anti-DNS antibody with ε-DNS-lysine are shown.

A comparison of the fluorescence enhancement of 8-1-ANS and of ε-DNS-lysine in the presence of rabbit serum albumin at a concentration of 1.2 mg/ml is shown in Figure 4. The activation and emission maxima

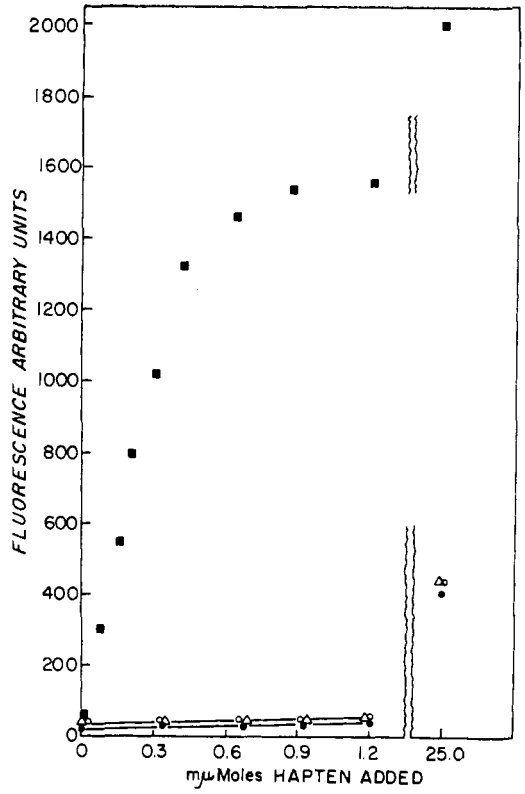

FIGURE 2: Fluorescence enhancement. Changes in fluorescence on addition of ϵ-DNS-lysine to purified rabbit anti-DNS antibody (■–■–■), purified rabbit anti-DNP antibody (○–○–○), purified nonspecific rabbit γG-globulin (Δ–Δ–Δ), and phosphate–saline (●–●–●). The anti-DNP and anti-DNS antibodies were at 40 μg/ml. The γG-globulin was at a concentration of 1000 μg/ml. Activation 340 mμ, fluorescence 480 mμ. The fluorescence was corrected for solvent blank and for changes in solution volume during the titration. Temperature, 30°; solvent, phosphate–saline.

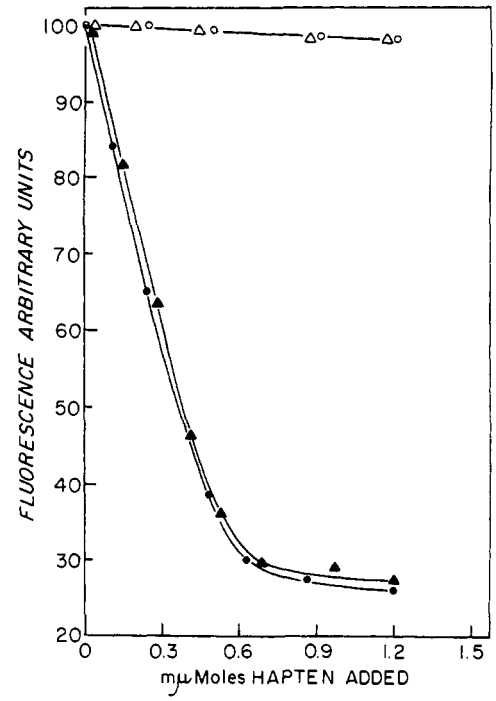

FIGURE 3: Fluorescence quenching. ○–○–○ is the curve obtained when purified rabbit anti-DNP antibody was titrated with ϵ-DNS-lysine; Δ–Δ–Δ is purified rabbit γG-globulin titrated with ϵ-DNS-lysine; ●–●–● is anti-DNP antibody titrated with ϵ-DNP-lysine; ▲–▲–▲ is rabbit anti-DNS antibody titrated with ϵ-DNS-lysine. The antibody solutions were at a concentration of 40 μg/ml. Fluorescence is given in arbitrary units. Excitation 290 mμ, fluorescence 350 mμ. The fluorescence was corrected for solvent blank and changes in volume during the titration. Temperature, 30°; solvent, phosphate–saline.

are the ones which give the maximal increments in fluorescence in the presence of the respective specific antibodies. The number of DNS binding sites on the albumin was difficult to calculate because of the weakness of the binding. If a value of 5 was chosen (the number of 8-1-ANS sites on bovine serum albumin (Weber and Young, 1964), the association constant for the rabbit albumin–DNS interaction was only about 5×10^3 M^{-1}. A similar value was estimated by equilibrium dialysis against ^{3}H-labeled ϵ-DNS-lysine. The degree of enhancement of ϵ-DNS-lysine fluorescence (excitation 340 mμ, emission 480 mμ) on binding to albumin was estimated by correlating the fluorescence titration curves with equilibrium dialysis data. The increase of fluorescence of the albumin-bound dye was only about 6% of that of the antibody-bound dye.

The number of binding sites on purified rabbit anti-DNS antibody was determined by fluorometric titration and equilibrium dialysis. Figure 5 is a plot of R/C *vs.* R, where R is the number of moles of hapten bound per mole of antibody and C is the free hapten concentration. An extrapolated value of 1.95 ± 0.05 (based on three points) was obtained by both methods in accord with the expected value of 2.0.

In order to further demonstrate the specificity of ϵ-DNS-lysine binding by rabbit antibody the papain proteolytic fragments of rabbit anti-DNS antibody were examined (Figure 6). Binding activity was recovered quantitatively in the Fab fragments. The crystalline Fc fragments of nonspecific rabbit γ-globulin and of rabbit anti-DNS antibody did not exhibit significant binding, nor did Fab fragments from nonspecific rabbit γG-globulin bind the dye.

The pK_a of the dimethylamino group of DNS can be estimated fluorometrically because the $(CH_3)_2NH^+$ form of the dye is virtually nonfluorescent (Weber, 1952; Klotz and Fiess, 1960). The pK_a of the dimethylamino group of free ϵ-DNS-lysine was found to be about 3.8, in accord with the earlier data of Weber for DNS-amide. By correlating results of equilibrium dialysis fluorescence polarization and fluorescence enhancement in various glycine·HCl buffers the pK_a

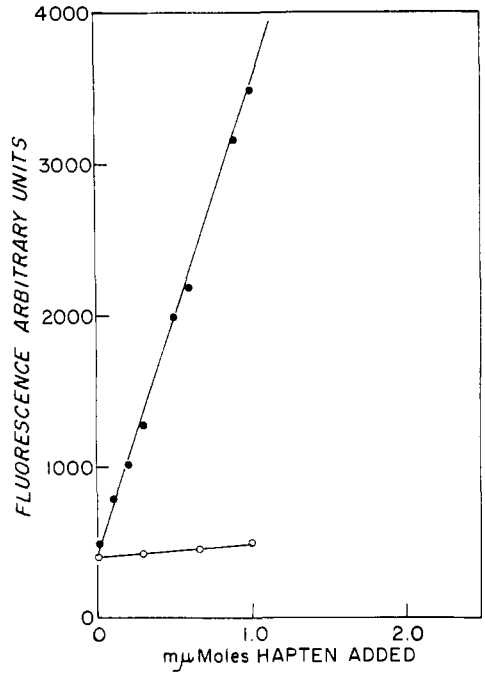

FIGURE 4: Titration of rabbit serum albumin at 1.2 mg/ml with ε-DNS-lysine (O–O–O) and 8-1-ANS (●–●–●). For the DNS titration excitation and emission were at 340 and 480 mμ, respectively. The corresponding wavelengths for the ANS titration were 365 and 470. The fluorescence was corrected for solvent blank and changes in solution volume during the titration. Temperature, 30°; solvent, phosphate-saline.

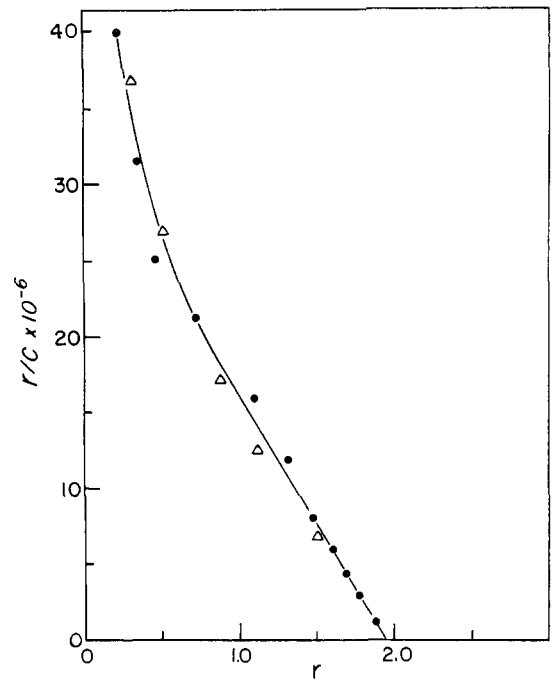

FIGURE 5: Curves for the binding of ε-DNS-lysine by anti-DNS antibody as measured by fluorescence enhancement (●–●–●) and equilibrium dialysis (△–△–△). The rabbit anti-DNS purified antibody was at a concentration of 40 μg/ml. *c* is free hapten concentration (millimicro moles per milliliter) and *r* is moles of hapten bound per mole of antibody (assuming an antibody molecular weight of 145,000 (Merler *et al.*, 1963). Temperature, 30°. Calculation of the fluorometric data was based on changes in ε-DNS-lysine fluorescence (excitation 340 mμ, fluorescence 480 mμ) (Parker *et al.*, 1967).

of the dimethylamino group of antibody-bound dye was estimated to be about 1.5.

The fluorescence of antibody-bound ε-DNS-lysine was compared with that of a variety of covalent DNS–protein conjugates. The conjugates included DNS–BγG, DNS–hemocyanin, DNS–β-lactoglobulin, and DNS–human serum albumin. Corrected emission maxima of the conjugates ranged between 520 and 545 mμ. The fluorescence quantum yield of a DNS_{18}–BγG protein conjugate was about 0.28 with a maximum at 540 mμ. When the DNS–BγG conjugate was complexed with Fab fragments of rabbit anti-DNS antibody there was a blue shift in the DNS emission and an increase in its fluorescence quantum yield. Judging from the increase observed in the region of far antibody excess, the increase in quantum yield of protein DNS-lysyl residues after binding to antibody was at least 1.5-fold. This value is minimal because it was not established that all the DNS-lysyl residues were sterically accessible to antibody.

Discussion

The binding of ε-DNS-lysine by rabbit anti-DNS antibody in aqueous solution resulted in marked alteration of the spectral properties of the dye. (1) A diminution in the oscillator strength of the 330-mμ absorption band as well as a red shift. (2) A blue shift of approximately 60 mμ in the emission maximum. (3) An increase of as much as 30-fold in the fluorescence quantum yield. (4) A decrease in the quenching of DNS fluorescence in dilute acid solution. (5) Evidence of excitation energy transfer from protein tyrosine and tryptophan residues to the dye.

A number of lines of evidence clearly established that the binding of DNS-lysine by anti-DNS antibody was specific and localized to the antibody combining site. (1) Nonspecific rabbit γ-globulins and rabbit antibodies specific for unrelated antigens exhibited virtually no binding of ε-DNS-lysine. (2) Both by equilibrium dialysis and fluorometric analysis purified rabbit antibody specific for DNS had 1.95 (±0.05) combining sites/molecule. (3) In papain proteolytic fragments of anti-DNS antibody binding activity was quantitatively recovered in the Fab fragments. No binding was exhibited by the Fc fragment. (4) The magnitude of the binding constant increased progressively with time after immunization in a manner con-

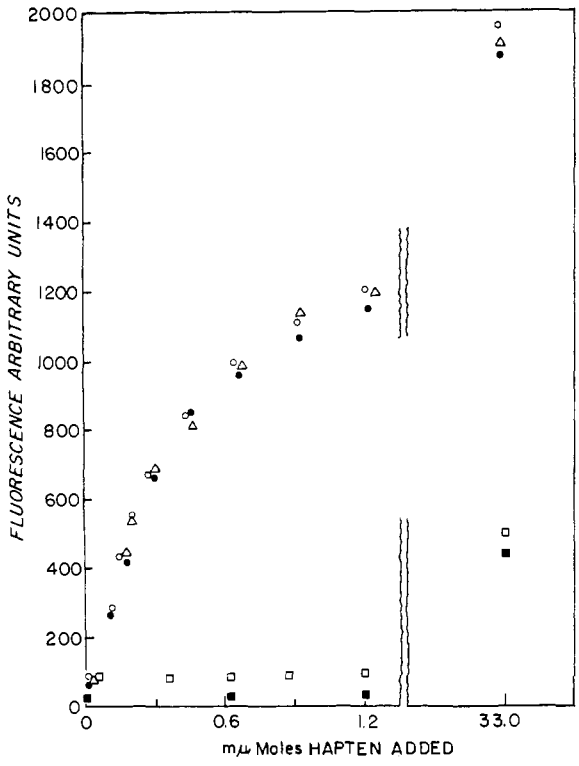

FIGURE 6: Fluorescence enhancement. Titration of rabbit anti-DNS antibody and its papain proteolytic fragments. (O–O–O) Intact antibody; (Δ–Δ–Δ) digested but unfractionated antibody; (●–●–●) isolated Fab (Porter's piece I); (□–□–□) Fc; (■–■–■) phosphate–saline. The intact antibody was a γ-globulin fraction purified by DEAE chromatography (see text) containing 0.38 mg of nonspecific globulin and 0.5 mμmole/ml of anti-DNS body sites per ml; the digested unfractionated antibody was at the same concentration. Porter's piece I was at a concentration of 0.26 mg/ml (assuming E_{280}, 1 mg/ml, 1-cm light path = 1.4). Using 145,000 and 50,000 as the molecular weights of intact γ-globulin and Fab, respectively, this theoretically would have given about the same antibody concentration as above (0.5 mμequiv/ml). Fc was at a concentration of 0.3 mg/ml (assuming E_{280}, 1 mg/ml, 1-cm light path = 1.3). A curve obtained with the Fab fragment of nonspecific rabbit globulin (0.4 mg/ml) was very similar to that of Fc. Excitation 340 mμ, fluorescence 480 mμ. The fluorescence was corrected for changes in volume during the titration. Solvent, phosphate–saline; temperature, 30°.

sistent with evolution of an antibody response (see Discussion of Parker *et al.*, 1967). (5) Antibodies to DNS did not bind heterologous, hydrophobic molecules such as ε-DNP-lysine or AcR to a significant extent.

An approximation of the local dielectric constant of the antibody site was obtained by relating the above changes to variation in dye fluorescence in different organic solvents. Of the various organic solvents examined dioxane solutions of the dye had emission characteristics which corresponded most closely to those of the antibody-bound dye. Higher members of a homologous series of unbranched aliphatic alcohols (hexanol and heptanol) also exerted effects on ε-DNS-lysine fluorescence which approximated those of the antibody. Judging from the low dielectric constant of these solvents (for example, dioxane, 2.2), the combining site of rabbit anti-DNS antibody is essentially a hydrocarbon environment. While we are inclined to attribute the shift in the fluorescence of antibody-bound dye solely to nonspecific factors related to the polarity of the environment additional specific contributions by individual amino acid residues in the site are difficult to exclude.

Justification for the premise that a hydrophobic binding region on a protein is capable of bringing about the type of fluorescence change exhibited by ε-DNS-lysine in the presence of antibody can be obtained from the studies of Stryer (1965). He demonstrated that 8-1-ANS underwent similar changes in fluorescence when it was bound at the hydrophobic haem binding site of apomyoglobulin (Kendrew, 1962).

As yet chemical-labeling techniques which might permit a direct analysis of the amino acid composition of the DNS binding site of rabbit antibody have not been applied. A study of this kind has been undertaken with rabbit antibodies specific for another nonpolar hapten the 2,4-dinitrophenyl (DNP) group (Singer and Doolittle, 1966). Tyrosine residues in the active site of rabbit anti-DNP antibody were affinity labeled with [^{3}H]*m*-nitrobenzenediazonium fluoroborate. On the basis of the solubility properties of the labeled peptides it appeared that the binding region of anti-DNP antibody was composed of unusually hydrophobic peptides.

Despite the evidence that the binding environments of anti-DNP and anti-DNS antibodies are essentially hydrophobic in character the nature of the forces which stabilizes the antibody–hapten complex is not entirely clear. It might seem logical to assume that a purely hydrophobic interaction is involved but the careful thermodynamic studies of Eisen and Siskind (1964) with rabbit anti-DNP antibody and ε-DNP-lysine indicate that the explanation is not that simple. If the sole source of binding energy were hydrophobic bond formation the reaction should be entropy driven (a positive value for $\Delta S°$) and relatively athermal. Instead, $\Delta S°$ was negative and $\Delta H°$ had a very large negative value. The large enthalpy effect can be explained if it is assumed that hydrogen-bond formation also contributes to the interaction (Eisen and Siskind, 1964; Green, 1966). Hydrogen-bond formation is suggested from the nature of the shifts exhibited by ε-DNP-lysine in the presence of homologous antibody. The oscillator strength of the 360-mμ absorption band of the ligand was diminished and there was also a red shift in the absorption maximum in this region (Eisen and Siskind, 1964). Absorption changes exhibited by ε-DNS-lysine in the presence of homologous antibody (Figure 1) were very similar to those occurring in the DNP–anti-DNP interaction and to carry the analogy

further, the thermodynamic constants, $\Delta S°$ and $\Delta H°$ appeared to be rather similar for the two haptenic systems.

The basis for the strong solvent effects on the fluorescence quantum yield of ε-DNS-lysine is not entirely clear. The relative lack of variation in quantum yield with temperature and solvent viscosity tends to exclude diffusional or collisional quenching of the singlet state as the major factor in the low quantum yield in water. Bowen and Seaman (1962) have made similar observations on the synthetic precursor of ε-DNS-lysine, 1-naphthylamine-5-sulfonate. To account for the absence of a temperature effect on fluorescence in the presence of marked solvent influence on quantum yield, they suggested the possibility of a charge-transfer reaction which facilitates singlet–triplet conversion and subsequent solvent quenching. Another possibility would be a reversible photochemical reaction in which there is transfer of a hydrogen ion between an excited dye molecule and a ground-state solvent molecule (Seliger and McElroy, 1965).

The DNS group has been used extensively as a marker in fluorescent protein studies (Steiner and Edelhoch, 1962) and in protein end-group analysis (Hartley, 1961). Considering the relatively low fluorescence quantum yield of ε-DNS-lysine in aqueous solution the usefulness of the dye in studies which utilize DNS fluorescence may seem surprising. Actually, DNS groups covalently bound to proteins have a relatively high fluorescence efficiency, even in aqueous solution. The increase in fluorescence is accompanied by a slight to moderate blue shift (10–30 mμ) in the emission maximum. These effects are less striking than the ones observed when DNS-lysine interacts reversibly with homologous antibody but they are in the same general direction. The increase in fluorescence after conjugation may be due at least in part to reduced molecular motion. In addition, several lines of evidence suggest that the DNS-lysyl residues can undergo noncovalent interactions with internal regions of proteins to which they are attached (Weber, 1952; Klotz and Fiess, 1960; Young, 1963).

Because of the versatility of the antibody response numerous other antigen–antibody systems which utilize environmentally sensitive haptens are possible. Yoo has confirmed the earlier qualitative results of Winkler in which antibodies specific for azonaphthalenesulfonates were shown to markedly increase the fluorescence of the appropriate anilinonaphthalenesulfonates (Yoo and Parker, 1966). Conjugation in this case was carried out by diazotization of the aminonaphthalenesulfonate in acid solution followed by reaction of the diazonium salt with protein at neutral or slightly alkaline pH. The general nature of the change in fluorescence exhibited by the anilinonaphthalenesulfonates in the presence of antibody was similar to that for the DNS–anti-DNS system and the increase in fluorescence quantum yield was even more striking. One interesting observation by Yoo was that the augmentation in fluorescence of antibody-bound 4-1-ANS was substantially greater than that of bound 8-1-ANS even where antibodies involved were specific for 1-azonaphthalene-8-sulfonate.

Despite the greater magnitude of the fluorescence changes obtained in specific antibody–hapten interactions which involve anilinonaphthalenesulfonates, the DNS–anti-DNS system has several advantages as a binding system. (1) DNS–protein conjugates are quite stable over a wide temperature and pH range and DNS substitution is largely limited to protein lysyl residues. By contrast a significant degree of antigen instability is usual with the azoproteins and possible reaction products on the protein include mono- and bisazotyrosine, mono- and bisazohistidine, and a triazine derivative of lysine (Tabachnick and Sobotka, 1959). Moreover, the anilinonaphthalenesulfonates used for titrating antiazonaphthalenesulfonate antibodies are cross-reacting haptens and the contribution of amino acid side chains of the protein to antigenic specificity cannot be evaluated. (2) Another advantage of the DNS–anti-DNS system has to do with nonspecific interactions of the dye with other serum proteins. ε-DNS-lysine, which has both a positive and negative charge at neutral pH, is bound very weakly by serum albumins and other nonantibody proteins in serum. Assuming approximately five binding sites per rabbit serum albumin molecule the nonspecific binding constant is only about 5×10^3 M^{-1} at 25°. This is very similar to the value found by Carsten and Eisen (1953) for the ε-DNP-lysine–bovine serum albumin interaction. Most fluorescent molecules including the anilinonaphthalenesulfonates interact strongly with albumin. For example, the association constant for the 8-1-ANS–albumin interaction is about 3×10^5 M^{-1} (Weber and Young, 1964). Minor degrees of contamination of anti-DNS–γ-globulin fractions by other serum proteins do not seriously influence the titration results; rigorous purification is required when ANS titrations are contemplated. (3) There are weak nonspecific binding sites on the γ-globulins themselves primarily in the Fab portion of the molecule (Parker and Osterland, 1966). The DNS interaction with these sites is extremely limited and not detectable at all under the usual titration conditions. The nonspecific interactions of ANS with γ-globulin, while weak, is of sufficient magnitude that it requires consideration when computations involving ANS–antibody interactions are made.

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