Hydrophobic Binding Sites on Immunoglobulins*

C. W. Parker† and C. K. Osterland

ABSTRACT: 8-Anilinonaphthalene-1-sulfonate (ANS) fluoresces strongly when bound to hydrophobic regions of proteins whereas the unbound dye is virtually nonfluorescent in aqueous solution. In the present study ANS has been employed as a probe for hydrophobic binding sites on immunoglobulins. Rabbit, guinea pig, and human immunoglobulins including "nonspecific" γ-globulins, purified antibodies of five different specificities and human myeloma proteins and macroglobulins have been evaluated. In the presence of immunoglobulin changes in ANS fluorescence consistent with a hydrophobic interaction were regularly demonstrable. The increase in ANS fluorescence could be correlated in a general way with antibody specificity and affinity suggesting that binding was predominantly in the Fab region. By equilibrium dialysis intact γ-G antibodies and normal γ-G globulins were demonstrated to have at least two binding sites for ANS. Average intrinsic association constants for the ANS–immunoglobulin interaction ranged from $\sim 8 \times 10^2$ to 2×10^4 l. mole^{-1} at 23°. Studies of Fab and Fc fragments of rabbit immunoglobulins (obtained by papain proteolysis) further indicated that the Fab region was the major site of ANS binding. Fc binding of ANS was weak and could not be correlated with antibody specificity. Homologous hapten interfered with ANS binding, presumably due either to competition of ANS and hapten for the same binding site or to a hapten-induced configuration change in an independent ANS binding region. Two other hydrophobic probes, 2-*p*-toluidinyl-6-sulfonate (TNS) and 2-methoxy-6-chloro-9-acridinyl-*N*-*p*-aminobenzenesulfonate (ACS), interacted with immunoglobulins in much the same way as ANS. The possible relevance of the demonstration of nonspecific hydrophobic interactions of fluorescent molecules with immunoglobulin to reports of "hapten specific" binding sites on myeloma proteins is discussed.

With the use of fluorescent probes like 8-anilinonaphthalene-1-sulfonate (ANS)[1] proteins can be screened for hydrophobic binding regions (Weber and Laurence, 1954; Stryer, 1965; McClure and Edelman, 1966; Deranleau and Neurath, 1966). Apomyoglobin, apohemoglobin, and bovine serum albumin interact strongly with ANS producing marked changes in the emission spectrum and fluorescence quantum yield of the dye (Weber and Young, 1964; Stryer, 1965). Somewhat weaker ANS binding is exhibited by α-chymotrypsin, β lactoglobulin, ovalbumin, and bovine γ-globulin (Stryer, 1965; see also McClure and Edelman, 1966). The nature and location of ANS binding regions on bovine γ-globulin and other immunoglobulins is of special interest because of their possible relationship to the antibody combining site and specialized areas on the Fc portion of the molecule. In this paper we present fluorometric and equilibrium dialysis studies of ANS binding by rabbit, guinea pig, and human immunoglobulins including nonspecific γ-globulins, purified antibodies of various specificities, and human myeloma proteins and macroglobulins.

* From the Departments of Internal Medicine and Preventive Medicine, Washington University School of Medicine, St. Louis, Missouri. *Received October 3, 1969.* Supported by U. S. Public Health Service Grants AI04646, AI00219, AM08490, and TIAM05548. Presented in part at the meeting of the American Federation for Clinical Research, Chicago, Nov 1, 1966 (Parker and Ostertand, 1966).

† Recipient of a Research Career Development Award No. AI09881.

[1] The following abbreviations were used: ANS, 8-anilinonaphthalene-1-sulfonate; TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate; ACS, 2-methoxy-6-chloro-9-acridinyl-*N*-*p*-aminosulfonic acid; BA, benzenearsonate; phosphate–saline, 0.15 M NaCl–0.01 M phosphate, pH 7.4; PNO, benzylpenicilloyl; CHO, carbohydrate; R, moles of hapten bound per mole of antibody; C, free hapten concentration; K_0, average intrinsic association constant in l. mole^{-1} (at 23° unless otherwise specified).

Materials and Methods

Ligands and Other Chemicals. Ligands and chemicals not otherwise described were obtained commercially and were the highest grade of purity available. 2-*p*-Toluidinylnaphthalene-6-sulfonate (TNS) was prepared and characterized according to McClure and Edelman (1966). The 2-methoxy-6-chloro-9-acridinyl-*N*-*p*-aminobenzenesulfonic acid (ACS) preparation was described previously (Parker *et al.*, 1967a). 8-Anilinonaphthalene-1-sulfonate (ANS) (Eastman Kodak, Rochester, N. Y.) was purified by preparing the magnesium salt and repeated recrystallization (Weber and Young, 1964). The molar extinction coefficient of the purified product was 4.97×10^3 cm^{-1} at 350 mμ. ϵ-Benzyl penicilloylaminocaproate was prepared as the benzylammonium salt (Levine, 1964).

α-*N*-[^{3}H-*acetyl*]-ϵ-DNP-lysine was the generous gift of Dr. J. R. Little, Washington University School of Medicine. α-[^{3}H]DNP-valine was prepared by the reaction of [^{3}H]dinitrofluorobenzene with valine (Bailey, 1962). The product was radiochemically pure by paper and thin-layer chromatography in six solvent systems and had the expected melting point (132°) and absorption maximum at 350 mμ in ethanol (ϵ 1.55×10^4 cm^{-1}).

^{3}H-Labeled ANS was prepared as follows: 153 mg of recrystallized ANS, 89 mg of aniline·HCl, and 230 mg of aniline (including 6.88 mg of [^{3}H]aniline at 135 mCi/mmole) were mixed and heated in a sealed vial for 16 hr at 150°. After cooling to room temperature the product was dissolved and transferred by the addition of about 1.0 ml of 30% NH_4OH and 4.0 ml of water. The aqueous solution was extracted four times with 3.0 ml of benzene and adjusted to a volume of 6.0 ml. After the addition of 200 mg of animal charcoal and heating to 60° the solution was filtered and 600 mg of $MgSO_4$

was added to the filtrate. The greenish crystals were isolated by filtration and purified by repeated recrystallization from a dilute solution of magnesium sulfate. The product was radiochemically pure by paper and thin-layer silica gel chromatography in six solvent systems and it had the expected absorption and emission properties of authentic ANS (Weber and Young, 1964). The molar extinction coefficient was 4.99×10^3 cm^{-1} at 350 mμ.

Antibodies and γ-Globulins. Unless otherwise specified rabbit and guinea pig antibodies were obtained by immunization with 1–2 mg of 2,4-dinitrophenyl (DNP)-bovine γ-globulin in complete Freund's adjuvant in the footpads as described previously (Parker *et al.*, 1967a). Antisera were obtained by cardiac puncture 2 weeks to 2 months later. Specifically purified rabbit and guinea pig anti-DNP antibodies[2] were prepared by method I of Eisen (1964b). Anti-DNP antibody preparations were at least 90% precipitable with DNP-human serum albumin at an antibody concentration of 2 mg/ml. Rabbit antibenzenearsonate antibodies were prepared by the method of Koshland *et al.* (1962).[2] The immunizing antigen in this case was BA–bovine γ-globulin. Rabbit anti-ovalbumin antibody was purified as described by Singer (1964). The two rabbit antipenicilloyl antibodies (immunizing antigen D α-benzylpenicilloyl$_{37}$-bovine γ-globulin) (Parker, 1967a) were purified by the absorption of pooled antisera to a penicilloylaminoethylcellulose column followed by elution with 0.1 M sodium benzyl penicilloate. Purified antistreptococcal carbohydrate antibody from New Zealand red rabbits was prepared by a previously described method (Osterland *et al.*, 1966). Purified anticarbohydrate, penicilloyl, ovalbumin, and BA antibodies were at least 86% precipitable with specific antigen and were free of nonimmunoglobulins by immunoelectrophoresis. All specifically purified rabbit antibody preparations were very largely or entirely composed of Ig immunoglobulin molecules as judged by immunoelectrophoresis. The rabbit γ-globulin fraction containing anti-8-azonaphthalene-1-sulfonate antibody was described previously (Yoo and Parker, 1968).

Purified human myeloma proteins were obtained by a combination of salt fractionation, starch gel electrophoresis, Sephadex, and ion-exchange chromatography and were homogeneous by immunoelectrophoresis. Gm typing and determination of L-chain classes was carried out according to standard methods. Human guinea pig and rabbit γ-G globulins were isolated by precipitation at 33% ammonium sulfate followed by DEAE chromatography (Parker *et al.*, 1967b).

Fc fragments of rabbit anti-DNP antibody, rabbit anti-BA antibody and rabbit γ-G globulin were obtained in crystalline form following papain digestion as described by Nisonoff (1964). The respective Fab fragments were obtained by papain digestion and fractionation on CM-cellulose (Porter, 1959; Nisonoff, 1964). Immunochemical purity was demonstrated by immunoelectrophoresis with goat antisera specific for rabbit Fc, Fab, IgG, and whole serum. Free H and L chains were produced by reduction and alkylation (Edelman, 1959; Fleischman *et al.*, 1963) followed by chromatography on Sephadex G-100 in 1 M propionic acid–4 M urea. Antibody fragment concentrations were based on dry weight determinations.

Fluorescence Titrations. Fluorescence enhancement and quenching titrations were carried out by previously described methods (Eisen, 1964a; Parker, 1967a; Parker, 1967b) using an Aminco Bowman spectrofluorophotometer. Unless otherwise specified titrations were performed in 0.15 M NaCl–0.01 M phosphate (pH 7.4) (phosphate–saline) at 25°. Fluorescence quantum yield was calculated as described previously (Parker *et al.*, 1967a,b). Activation wavelengths were 365 mμ for ANS, 366 mμ for TNS, and 400 mμ for ACS. For rabbit and guinea pig antibodies and γ-globulins a 1-mg/ml solution in phosphate–saline was assumed to have an optical density of 1.5 cm^{-1} at 280 mμ (Little and Donahue, 1968). The concentrations of myeloma proteins and macroglobulins were based on dry weight determinations. Ligand concentrations were standardized on the basis of known molar extinction coefficients (Little and Donahue, 1968; McClure and Edelman, 1966; Weber and Young, 1964).

Equilibrium dialysis was carried out as described by Eisen (1964a), using Lucite cells with an 0.8-ml chamber capacity. Dialysis was carried out in duplicate or triplicate over a period of 17–20 hr at ambient temperature (at or near 23°) on a slowly rotating wheel. The buffer was phosphate–saline. Radioactivity was measured in 0.25-ml duplicate aliquots from each compartment, utilizing Bray's (1960) solution as the phosphor and a Packard liquid scintillation spectrometer. Recovery of radioactivity was routinely well in excess of 90% of the theoretical maximum based on the size of the aliquots used. Variation in replicate values was usually less than 2%. Effects of proteins and ligands on counting efficiency were evaluated utilizing internal standards and the addition of known amounts of radioactive ligand to control solutions. Small corrections for variations in counting efficiency in the presence of high protein concentrations were applied which did not markedly affect the binding calculations. Analysis of data was essentially as described by Eisen and Siskind (1964), using the Sips equation to evaluate the average intrinsic association constant and relative homogeneity: $\log (R/(N - R)) = a \log c + a \log K_0$. K_0 is the average association constant, R is the number of moles of hapten bound per mole of antibody, c is the free hapten concentration, and a is the heterogeneity index. In the calculations of ANS binding the molecular weights of human, guinea pig, and rabbit γ-G globulins and antibodies were assumed to be 145,000 (Cohen and Porter, 1964).

Competitive equilibrium dialysis was carried out using α-DNP-valine or DNP-toluene as an inhibitor for [^{3}H]ANS binding and using unlabeled ANS as an inhibitor of α-[^{3}H]-DNP-valine binding.

Results

Fluorescence Studies. Human, rabbit, and guinea pig γ-globulins and purified antibody preparations all produced enhancement of ANS fluorescence. The fluorescence maximum of the bound dye was routinely at or near the 475-mμ region (Figure 1). This represents a blue shift of about 45 mμ from the fluorescence maximum exhibited by the unbound dye. The quantum yield, calculated with the help of equilibrium dialysis binding determinations, varied from 0.03 to 0.15. The quantum yield for the unbound dye is about 0.004 (Weber

[2] We wish to thank Dr. H. N. Eisen and Dr. J. R. Little of the Washington University School of Medicine, St. Louis, and Dr. J. McGuigan of the University of Florida School of Medicine, Gainesville, Fla., for samples of purified antibody.

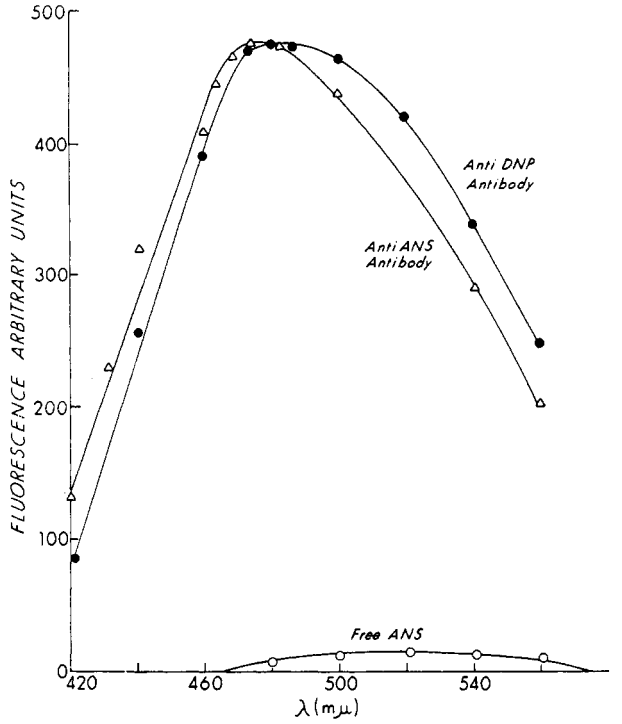

FIGURE 1: Emission spectra of free (○—○—○) and immunoglobulin-bound (●—●—●) ANS. The later curve was obtained in the presence of rabbit anti-DNP antibody (see Figure 2, third dot from top). Bound dye (estimated by parallel equilibrium dialysis measurements) and free dye are each at 1×10^{-6} M concentrations. Curve △—△—△ is the spectrum for ANS specifically bound to rabbit anti-8-azonaphthalene-1-sulfonate antibody. The concentration of specifically bound ANS is $\sim 1 \times 10^{-7}$ M.

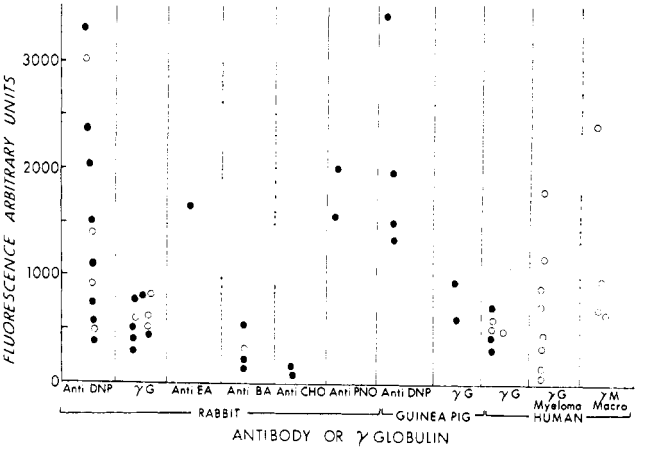

FIGURE 2: A comparison of the relative increases in bound ANS fluorescence in the presence of various immunoglobulins. The immunoglobulin concentrations were 0.8 mg/ml and the total ANS concentration was 40 mμM/ml. Closed circles are antibodies or γ-globulin prepared from pooled sera. Open circles are preparations from individual sera. Fluorescence is measured at 470 mμ.

and Young, 1964). The various immunoglobulins also bound TNS and ACS, again producing a blue shift and a marked increase in fluorescence quantum yield.

The fluorescence spectrum of ANS bound nonspecifically to immunoglobulin is qualitatively similar to the spectrum obtained for ANS bound to homologous antibody (rabbit antibody specific for 8-azonaphthalene-1-sulfonate; Yoo and Parker, 1968). The emission maximum in both instances is near 475 mμ. However, the band width (peak area divided by peak height; (McClure and Edelman, 1966) is less in the case of specifically bound ANS (Figure 1) and the quantum yield is much higher, usually 0.5 or more (Yoo and Parker, 1968).

Figure 2 compares the degree of enhancement of ANS fluorescence by various γ-globulin preparations evaluated at the same protein concentration. The increase in ligand fluorescence varied markedly, the differences being especially striking between individual myeloma proteins, macroglobulins, and purified antibodies. Variations in fluorescence quantum yield, binding affinity, or both may contribute to these differences. In examining the basis for differences in the behavior of specifically purified antibodies a major contribution by components of serum complement could be readily excluded. Purification of antibody in the presence of 0.01 M EDTA or using sera decomplemented by the formation of heterologous antigen–antibody precipitates (ovalbumin and rabbit anti-ovalbumin at equivalence) did not reduce ANS binding. Allotypic variations did not appear to exert a major influence on ANS binding since preimmunization γ-globulin fractions from individual outbred and 1,1,4,4 inbred animals enhanced ANS fluorescence to about the same extent. Contamination of purified antibody preparations by albumin and other serum proteins which enhance ANS fluorescence seemed an unlikely explanation for the following reasons: (1) Non-γ-globulin serum components were not detectible by immunoelectrophoresis using goat antisera to rabbit and guinea pig serum at high rabbit and guinea pig antibody concentrations. (2) Further purification of antibody by reprecipitation with antigen or DEAE chromatography did not markedly affect ANS binding. (3) The binding affinity and stoichiometry in equilibrium dialysis studies was not consistent with effects of low levels of serum components which bind ANS strongly.

It thus appeared that the most important factor in the differences in ANS binding was antibody specificity *per se*. To examine this question further two anti-DNP antibody preparations purified from early and late bleedings of the same homozygous 1,1,4,4 rabbit were compared (Figure 3). The late-antibody preparation enhanced ANS fluorescence to a much greater extent than the early-antibody preparation. Since allotypic differences in the two antibody molecules could be excluded the results strongly suggested that the differences in ANS binding resided in the variable regions of the antibody molecule. In examining results of titrations of anti-DNP antibodies as a whole there was a general correlation between the average affinity of anti-DNP antibody for homologous ligand and ANS binding. Preparations which bound ε-DNP-lysine especially strongly also bound ANS relatively well. Thus in Figure 2 the top three dots of the rabbit anti-DNP column represent antibodies with an average affinity for ε-DNP-lysine 6×10^7 l. mole^{-1} at 30°, the bottom three dots represent antibodies with an average affinity of 1×10^6 l. mole^{-1} for ε-DNP-lysine at 30°.

To examine further the localization of ANS binding on the γ-globulin molecule, two preparations of "normal" rabbit γ-globulin, two preparations of rabbit anti-DNP antibody, and one preparation of rabbit anti-BA antibody were digested with papain and the antibody fragments were compared with

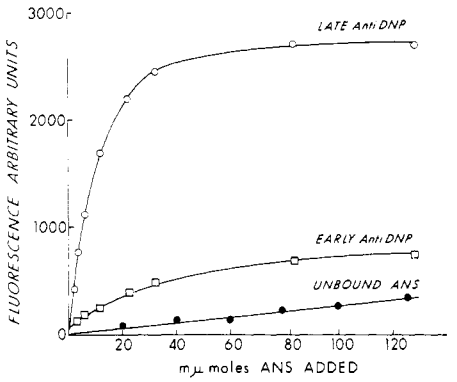

FIGURE 3: ANS titration of two anti-DNP antibody preparations obtained by serial bleedings of a single homozygous 1,1,4,4 rabbit. Curve □—□—□ (early anti-DNP) is that of an anti-DNP antibody preparation purified from serum obtained 2 weeks after immunization (K_a for DNP-lysine 5×10^5 l. mole^{-1} at 30°). Curve ○—○—○ (late anti-DNP) is that of an anti-DNP antibody preparation purified from serum obtained 6 months after immunization (K_a for DNP-lysine about 1×10^8 l. mole^{-1} at 30°). Both antibodies were at 200 μg/ml. ●—●—● is the curve for unbound ANS. Fluorescence was measured at 470 mμ.

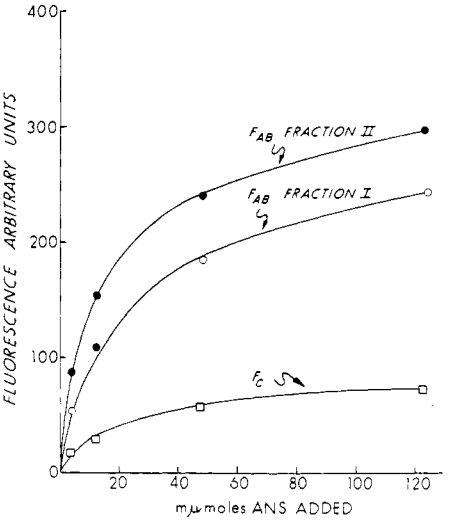

FIGURE 4: Bound ANS fluorescence in the presence of Porter's fractions I (○—○—○), II (●—●—●), and III (□—□—□). The fragments were derived from rabbit anti-DNP antibody. The initial protein concentrations were 0.58 mg/ml. Fluorescence values are corrected for protein fluorescence and free ANS fluorescence. Fluorescence was measured at 470 mμ.

respect to ANS enhancement. A representative titration, utilizing fragments of anti-DNP antibody (this preparation is represented by the third dot from the bottom in the anti-DNP column, Figure 2), is shown in Figure 4. Under the titration conditions indicated in the legend (and assuming that intact rabbit IgG is composed of two Fab fragments and one Fc fragment, each with a molecular weight of approximately 50,000) nearly 90% of the total enhancing activity of the fragments was localized to the Fab fragments. The overall recovery of ANS enhancement was about 75% of that exhibited by the intact γ-G molecule. Fc preparations from anti-DNP antibody, normal rabbit γ-G globulin, and anti-BA antibody increased ANS fluorescence to about the same extent. Enhancement of ANS fluorescence by anti-BA Fab fragments was actually a little less than that obtained with anti-BA Fc whereas with normal rabbit γ-G globulin Fab enhancement was about fourfold higher than Fc enhancement. It was thus established that the variable and (usually) large contribution to enhancement of ANS fluorescence was exerted by the Fab region and that the contribution by the Fc moiety was relatively constant.

H and L chains from rabbit anti-DNP, rabbit anti-egg albumin, and rabbit γ-G globulins were compared with respect to ANS binding. All three H-chain preparations enhanced ANS fluorescence markedly and to essentially the same extent. The increase in ANS fluorescence produced by free H chains was much greater than that produced by the parent γ-globulin fractions. Binding of ANS by isolated L chains differed in that the increase in ANS fluorescence was less marked and L chains from anti-DNP antibody enhanced ANS fluorescence to a considerably greater degree than L chains from anti-BA and rabbit γ-globulin (Figure 5). The demonstration of ANS binding by free L chains is consistent with the results of Gally and Edelman (1965) who demonstrated binding of 4-anilinonaphthalene-1-sulfonate to dimerized L chains.

Since ANS binding was largely localized to the Fab fragment, the relationship between the ANS binding site and the antibody active site was examined. The addition of homologous ligands to antibody–ANS mixtures markedly diminished bound ANS fluorescence. For example, D-α,ε-*N*-benzylpenicilloylaminocaproate, added at the beginning of an ANS-rabbit anti-benzylpenicilloyl antibody titration, largely prevented the development of bound ANS fluorescence (Figure

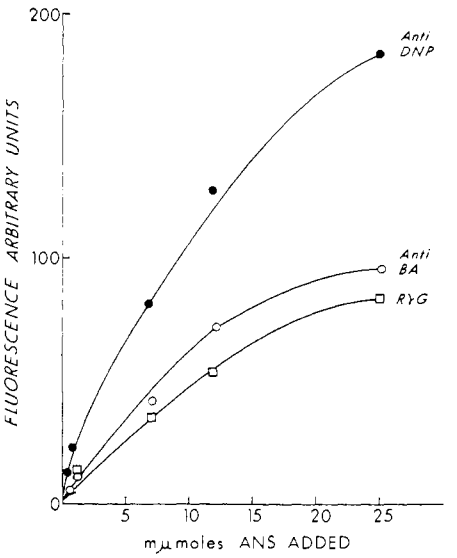

FIGURE 5: Bound ANS fluorescence in the presence of isolated L chains from rabbit anti-DNP antibody (○—○—○), rabbit anti-BA antibody (●—●—●), and normal rabbit γ-G globulin (□—□—□). The fluorescence values are corrected for protein and free ANS contributions to total fluorescence. The titrations were carried out in 0.01 M acetate (pH 5.2) at initial protein concentrations of 0.100 mg/ml. Fluorescence was measured at 475 mμ.

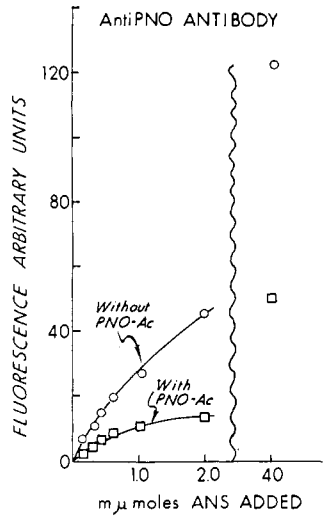

FIGURE 6: Changes in ANS fluorescence produced by rabbit anti-D-α-benzylpenicilloyllysyl antibody in the presence (□—□—□) and absence (○—○—○) of 0.1 μM D-α-ε-benzylpenicilloylaminocaproate. Bound ANS fluorescence is corrected for free hapten and protein fluorescence. The initial antibody concentration was 90 μg/ml. Fluorescence is measured at 470 mμ.

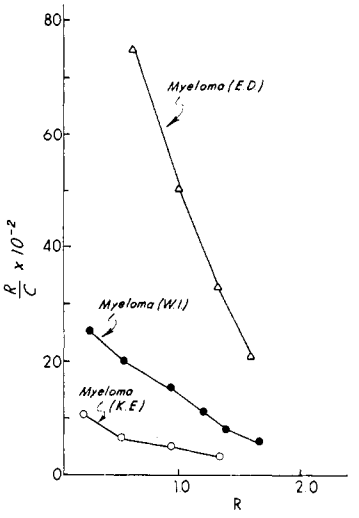

FIGURE 7: Binding of [^{3}H]ANS by three human γ-G myeloma proteins: W. I. (●—●—●), E. D. (△—△—△), and K. E. (○—○—○). Each protein was at a concentration of 11.4 mg/ml. C is the free ANS concentration in millimoles per milliliter, R is moles of ANS bound per mole of antibody. The calculated average K_{os} (see footnote 2 and text) are 1.2×10^3 l. mole^{-1} (W. I.), $<8 \times 10^2$ l. mole^{-1} (K. E.), and 5×10^3 l. mole^{-1} (E. D.).

6). The effect of benzylpenicilloylaminocaproate on the ANS-antipenicilloyl–antibody interaction appeared to be entirely specific in that the penicilloyl ligand did not alter bound ANS fluorescence with heterologous (anti-DNP and anti-ovalbumin) antibodies. Similarly, DNP ligands interfered with the increase in ANS, TNS, and ACS fluorescence normally observed in the presence of anti-DNP antibody. The degree of inhibition exerted by DNP ligands varied depending on the particular DNP antibody preparation but was usually marked (>40%). Inhibitory effects of DNP ligands on the fluorescence of immunoglobulin bound ANS were largely but not entirely limited to anti-DNP antibodies. With 4 out of 23 heterologous antibodies and myeloma proteins DNP ligands produced a 15–30% diminution of bound ANS fluorescence. The nonspecific effect of DNP ligands was most marked with a preparation of rabbit anti-ovalbumin antibody. Taken as a whole, however, nonspecific effects of DNP ligands with heterologous antibodies were small compared with what was observed with anti-DNP antibodies, where marked effects were the rule rather than the exception. In contrast to what was observed with anti-DNP and anti-penicilloyl antibodies and their homologous haptens, sodium arsanilate did not inhibit the increase of ANS fluorescence produced by anti-BA antibody. The relatively slight increase in ANS fluorescence produced by anti-BA antibody may be largely due to binding of ANS in the Fc region. The fluorescence increase produced by intact anti-BA antibody is nearly comparable with that obtained with isolated rabbit Fc alone.

Equilibrium Dialysis. By means of equilibrium dialysis γ-G antibodies and "normal" γ-G globulins were demonstrated to have at least two binding sites for ANS. Maximal experimental values for R, the number of moles of ANS bound per mole, varied from 1.3 to 2.2 (±0.4) depending on protein concentration and affinity. Extrapolated values for R (obtained by a plot of R/C *vs.* R, see Figure 7) were consistently 2.0 or higher. To localize the ANS binding sites isolated Fab and Fc fragments prepared from rabbit anti-DNP antibody and normal rabbit γ-G globulin were compared with respect to ANS binding. At protein concentrations of 1 mg/ml of ANS binding to Fab fragments was readily demonstrable whereas binding to Fc fragments was not observed.

Average intrinsic association constants, K_0's based on results of equilibrium dialysis at 25° (using an assumed valence of 2.0 and ignoring possible contributions by weaker binding sites) ranged from $\sim 8 \times 10^2$/(a human γ-G myeloma protein) to 2.0×10^4 l. mole^{-1} (a preparation of rabbit anti-DNP antibody with a K_0 of 6×10^7 l. mole^{-1} for ε-DNP-lysine at 25°). Pooled rabbit and human γ-G globulins had values of ~ 2–4×10^3 l. mole^{-1}. Association constants for five individual IgG myeloma proteins ranged from 8×10^2 to 8×10^3 l. mole^{-1}. This relatively broad range may have been due in part to the selection of myeloma proteins to include strong and weak enhancers of ANS fluorescence. There was a consistent relationship between fluorescence enhancement in fluorometric titrations and direct binding constants as determined by equilibrium dialysis. Those immunoglobulins which produced the most marked enhancement of ANS fluorescence (and ANS fluorescence quantum yield) bound ANS relatively strongly. Figure 8 illustrates the greater degree of ANS binding in a preparation of purified rabbit anti-DNP antibody obtained 10 weeks after immunization as compared with rabbit anti-DNP antibody obtained 4 weeks after immunization.

With two myeloma proteins, W. I. and E. D., which bound ANS relatively strongly (K_0's of $\sim 1.2 \times 10^3$ l. mole^{-1} at 25°, respectively), binding was essentially homogeneous over a range in values of R from 0.2 to 1.7 (W. I.) and 0.6–1.6 (E. D.) Figure 7. In the Sips plot of these data (Figure 9) a slope of 1.0 (±0.1) was obtained in both instances confirming the relatively high degree of binding homogeneity over the range of values of R evaluated. Heterogeneity of ANS binding was demonstrated in rabbit and guinea pig anti-DNP preparations

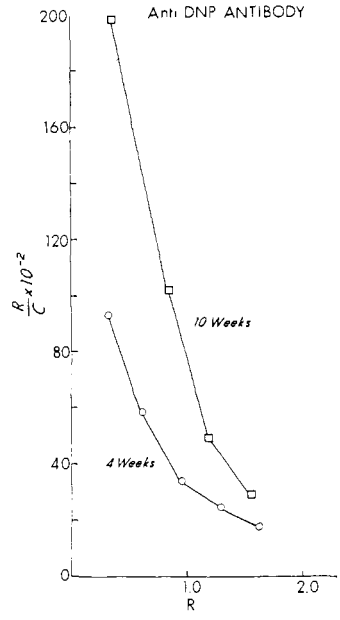

FIGURE 8: Binding of [^{3}H]ANS by two rabbit anti-DNP antibody preparations; □—□—□ obtained 10 weeks after immunization and ○—○—○, obtained 4 weeks after immunization. For an explanation of R and C, see legend to Figure 1; C is in millimoles per milliliter. The two antibodies were at a concentration of 7 mg/ml. The calculated average K_0's (see Figure 2) are 6×10^3 l. mole^{-1} (10 weeks) and 3.5×10^3 l. mole^{-1} (4 weeks).

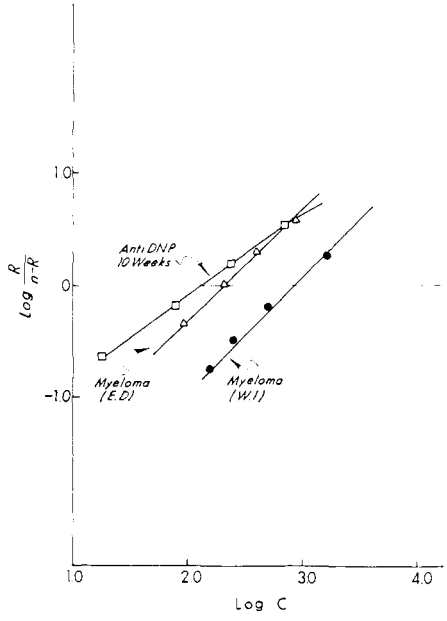

FIGURE 9: Sips plot of the ANS binding data of two of the myeloma proteins shown in Figure 7 (W. I. and E. D.) and one of the anti-DNP proteins shown in Figure 8 (10 weeks). C is in millimicromoles per milliliter. Over the ranges in C which are shown in the figure, a, the regression coefficient, is very close to 1.0 for the two myeloma proteins, indicating that binding is essentially homogenous. For the anti-DNP antibody $a = 0.7$.

(Figures 8 and 9). For the four preparations of anti-DNP antibody which were evaluated in this regard (each with an average K_0 for ϵ-DNP-lysine in excess of 1×10^7 l. mole^{-1}) values of a, the heterogeneity index ranged from 0.6 to 0.8.

As noted in Figure 6 bound ANS fluorescence was reduced in the presence of homologous hapten. The mechanism for hapten inhibition of ANS fluorescence was investigated by competitive equilibrium dialysis. With each of three rabbit anti-DNP preparations evaluated in detail binding of [^{3}H]-ANS by anti-DNP antibody was inhibited by DNP-toluene and α-DNP-valine. In each case the degree of inhibition was relatively small considering that the average direct binding constant for the interaction of the DNP ligand with anti-DNP antibody (measured by fluorescence quenching) was in excess of 1×10^6 l. mole^{-1} whereas the average direct binding constant for ANS was much lower ($<2 \times 10^4$ l. mole^{-1}). A representative experiment is shown in Figure 10. Various myeloma proteins and normal γ-globulins were evaluated in the same manner. With each of four normal γ-globulin preparations studied DNP-toluene and α-DNP-valine failed to significantly inhibit [^{3}H]ANS binding. A representative experiment is shown in Figure 10. With five human myeloma proteins, inhibition of ANS binding was observed in only one instance and in this case was considerably less striking than the degree of inhibition obtained with anti DNP antibodies. (DNP inhibition of bound ANS fluorescence was also observed with this protein.) Thus DNP ligand inhibition of ANS binding was largely but not entirely restricted to antibodies specific for DNP.

A similar result was obtained when inhibition was carried out in the reverse direction. Using unlabeled ANS in combination with [^{3}H]α-DNP-valine or α-*N*-[^{3}H-*acetyl*]-ϵ-DNP-lysine, ANS inhibition of DNP binding was observed with each of six anti-DNP preparations evaluated (representative data are shown in Figure 11).

To evaluate the possibility that immunologic cross-reactivity might be involved in ANS inhibition of the α-DNP-valine anti-DNP antibody interaction the effect of another hydrophobic molecule, the sodium salt of octanoic acid, on DNP binding was studied. With a preparation of rabbit anti-DNP antibody of relatively high affinity for α-DNP-valine (2×10^6 l. mole^{-1}), octanoate, and ANS were essentially equivalent as inhibitors (on a molar basis).

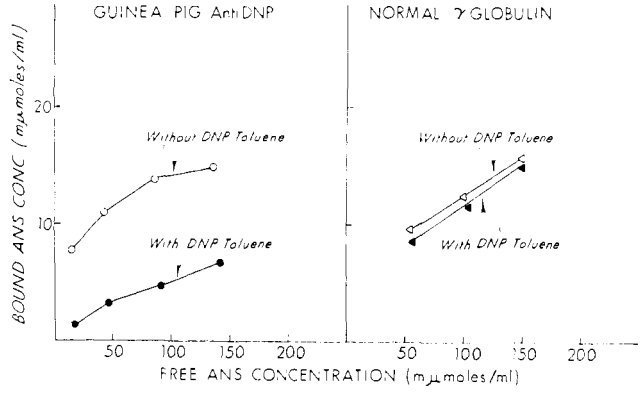

FIGURE 10: ANS binding studies. (a) [^{3}H]ANS binding to guinea pig anti-DNP antibody in the presence (●—●—●) and absence (○—○—○) of 112 mμM DNP-toluene. The antibody concentration was 2.5 mg/ml. (b) ANS binding to normal rabbit γ-G globulin in the presence (▼—▼—▼) and absence (▽—▽—▽) of 112 mμM DNP-toluene. The γ-G globulin concentration was 5.0 mg/ml.

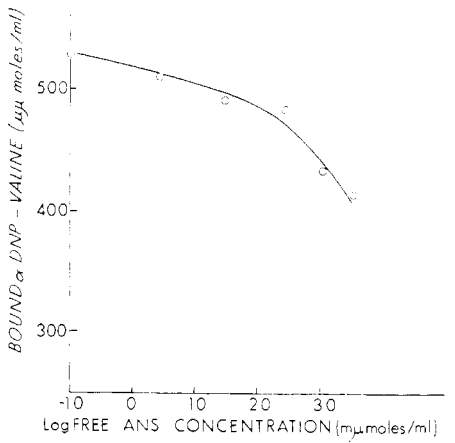

FIGURE 11: Binding of [^{3}H]α-DNP-valine by guinea pig anti-DNP antibody at various ANS concentrations. The antibody concentration (100 μg/ml) and the total quantity of α-DNP-valine in the dialysis chambers were kept constant. The K_0 of the antibody for α-DNP-valine was 3×10^6 l. mole^{-1}. The direct ANS binding constant (as determined in independent binding studies) was $\sim 5 \times 10^3$ l. mole^{-1}.

Discussion

Using several hydrophobic probes (ANS, TNS, and ACS) immunoglobulins and purified antibodies from rabbits, guinea pigs, and human beings have been examined for nonspecific binding sites. Each of the three dyes was demonstrated to be bound by immunoglobulin as evidenced by changes in the fluorescence spectrum of the dye in the presence of the protein. ANS binding to immunoglobulin was confirmed by equilibrium dialysis in which it was shown that there are at least two ANS binding sites per molecule of protein. Association constants for the interaction ranged from $\sim 8 \times 10^2$ to 2×10^4 l. mole^{-1}. Immunoglobulin-bound ANS was found to have a fluorescence quantum yield of 0.03–0.15 and an emission maximum in the region of 475 mμ. Unbound ANS has a quantum yield of 0.004 (Weber and Young, 1964) and an emission maximum at about 520 mμ in aqueous solution. The changes in fluorescence of immunoglobulin-bound ANS are similar to those exhibited by the dye in nonpolar solvents such as dioxane (Stryer, 1965), indicating that the binding site is hydrophobic in character.

Binding of ANS by immunoglobulins could be correlated in a general way with antibody specificity and affinity, suggesting that the major contribution to binding is in the Fab region. Thus, the average anti-DNP antibody preparation bound ANS to a greater extent than normal γ-globulin and anti-BA antibody. This behavior is assumed to reflect the fact that the DNP group is an uncharged aromatic determinant which would be expected to select for an antibody combining site which is essentially hydrophobic in character. In studies of proteolytic fragments of rabbit γ-globulins the presence of binding sites in the Fab region was confirmed both by equilibrium dialysis and fluorometric titration. The degree of fluorescence enhancement by Fab fragments was found to correlate with the enhancement properties of the parent γ-globulin molecule. In addition fluorometric studies indicated relatively weak ANS binding in the Fc region. Fc fragments from normal rabbit γ-globulin, rabbit anti-DNP antibody, and rabbit anti-BA antibody, each largely or entirely of IgG immunoglobulin molecules, enhanced ANS fluorescence to approximately the same extent. This finding is in accord with the relative constancy of Fc structure within a given species and immunoglobulin class. ANS binding to Fc was not demonstrable by equilibrium dialysis at the concentrations of Fc obtainable in neutral aqueous solution. On the basis of these observations the two relatively strong ANS binding sites demonstrated by equilibrium dialysis on intact IgG immunoglobulins can be assigned to the Fab regions.

If ANS binding sites in Fab regions of immunoglobulins differ markedly depending on antibody specificity clearly defined differences in the emission spectra of the bound dye might be expected. However, the emission maxima of dye bound to isolated Fab fragments and intact antibodies uniformly fell near 475 mμ (± 3 mμ). By contrast, on other proteins the emission maxima of bound ANS have a relatively broad range extending as low as 454 mμ (apomyoglobulins) (Stryer, 1965). Considering the substantial variation in the ANS binding sites of various proteins, the relatively narrow range in emission maxima observed with antibodies of differing specificities is almost certainly not fortuitous and would suggest a basic similarity in their ANS binding sites. Since myeloma and macroglobulins influence ANS fluorescence spectra in the same way as normal γ-globulins and purified antibodies it is evident that they possess similar hydrophobic regions. Specific ANS binding sites on homologous antibody, *e.g.*, rabbit anti-8-azonaphthalene-1-sulfonate antibody, produce a bound ANS emission maximum which is close to the maximum for dye bound nonspecifically to heterologous antibody. However, with specific ANS–antibody interactions there is a slightly greater blue shift, the band width is narrower and the quantum yield is much higher, often in excess of 0.5 (Yoo and Parker, 1968).

The nature of the area(s) on Fc which interacts with ANS is obscure. The Fc region is known to contain sites with the ability to bind reversibly to the surface membranes of homologous and heterologous tissues. The Fc region also interacts with various bacterial and plant products, with rheumatoid factors and with the first component of complement. While it is quite possible that the observed ANS binding is occurring in one or more of these areas, it may involve Fc regions which do not have clearly defined biological functions.

The relationship of the ANS binding site to the antibody combining site was investigated by measurements of ANS binding in the presence and absence of homologous hapten. With purified anti-DNP antibodies, ANS and DNP ligands exhibited mutual inhibition. With both combinations (ANS inhibition of [^{3}H]-α-DNP-valine binding and α-DNP-valine or DNP-toluene inhibition of [^{3}H]ANS binding) the level of inhibition was significantly below that expected on the basis of average direct binding constants, K_0's, suggesting that the ANS and DNP binding sites may not be identical. While ANS binding sites on Fc could contribute to poor DNP inhibition of ANS binding the results of direct measurements of ANS binding to Fc did not support this possibility. Antibody heterogeneity also might contribute to the lack of correspondence between direct binding constants and effective inhibitory concentrations of ligand. If binding sites with an especially high affinity for ANS were of lower than average affinity for α-DNP-valine, α-DNP-valine inhibition of ANS binding would be significantly less than that expected on the

basis of the direct binding constant. This possibility is lessened by the general observation that antibodies which bind DNP strongly also tend to bind ANS strongly. Nonetheless, a contributory role of antibody heterogeneity cannot be completely excluded. It therefore cannot be stated with certainty whether the ANS and DNP binding regions are the same or independent sites. If independent sites are involved inhibition could occur by means of ligand induced alterations in protein structure analogous to allosteric modifications of enzyme active sites.

It could be argued that ANS binding to anti DNP antibody reflects a limited degree of immunologic cross-reactivity since ANS and DNP molecules contain aromatic rings. However, octanoic acid, an aliphatic hydrophobic molecule, inhibited the α-DNP-valine–anti-DNP interaction just as effectively as ANS. If inhibition were based solely on structural similarity between α-DNP-valine and the inhibitor, ANS should have been the better inhibitor. Indeed, if immunological cross-reactivity were the only factor in "nonspecific" binding, octanoic acid should not have inhibited at all since α-DNP-valine does not contain a long unbranched alkyl chain with which the octanoic acid molecule might compete.

The demonstration that molecules with little structural similarity to specific hapten can bind to antibodies with association constants in excess of 1×10^4 l. mole^{-1} raises interesting theoretical possibilities in regard to human immunologic disease. Association constants of this magnitude might be sufficient to lead to manifestations of hypersensitivity *in vivo*. Conceivably, autoimmune phenomena, or *in vitro* reactions of antibodies with purified tissue fractions may in some instances be caused by nonspecific or cross-reactive hydrophobic interactions rather than the reaction of an autoantigen with homologous antibody (Parker and Osterland, 1966).[3]

The demonstration that immunoglobulins regularly contain hydrophobic binding sites in Fab regions requires consideration in light of recent observations that certain mouse and human myeloma proteins have antibody-like specificities (Stone and Metzger, 1968; Eisen *et al.*, 1967; Potter and Leon, 1968; Schubert *et al.*, 1968; Hannestad, 1969). If one were to summarize what is known about ANS binding sites the following general properties could be listed: (1) They are localized primarily in Fab regions. (2) There is one ANS site for each Fab fragment. (3) With myeloma proteins, the binding is essentially homogeneous. (4) There is considerable variation in ANS binding from one myeloma protein to another. If it were not clear that most (rather than a few) myeloma proteins bind ANS with association constants in excess of 1×10^3 l. mole^{-1} at 25° one could argue that myeloma proteins with K_0's of this magnitude contain specific ANS binding sites (*e.g.*, sites equivalent to those on specifically induced anti ANS antibodies). The frequency of DNP-specific myeloma proteins is also far in excess of what might be expected on the basis of random immunogenetic selection. Using a spectrophotometric assay, Eisen and his colleagues (1968) have demonstrated DNP binding with 7 of 116 mouse myeloma proteins. Schubert and his colleagues (1968) screened 240 myeloma proteins and observed precipitation of polynitrophenylated proteins with 16. In view of the high incidence of DNP binding in the mouse myeloma protein population, the possibility that DNP binding is also nonspecific must be seriously considered. In connection a human macroglobulin recently studied by Hannestad (1969) was found to precipitate both with polynitrophenylated proteins and aggregated human γ-G globulin, two seemingly unrelated antigens. Screening procedures for DNP binding are considerably less sensitive than those for ANS binding and may fail for proteins with association constants below 10^3 l. mole^{-1} making it appear that DNP binding is far more restricted in the myeloma protein population than ANS binding is. Only with myeloma proteins which bind DNP ligands with K_0's well in excess of 10^4 l. mole^{-1} and which bind other hydrophobic molecules such as ANS comparatively poorly can a convincing argument for a DNP-specific binding site be made. These criteria have been completely fulfilled by only one of the more than 25 DNP binding myeloma proteins thus far studied. This unusual protein, from mice with plasma cell tumor MOPC 315, has been characterized in detail by Eisen and his colleagues (1968). It displays an association constant for ϵ-DNP-lysine in excess of 1×10^7 l. mole^{-1} at 4° and binds ANS and other hydrophobic molecules relatively poorly. On the basis of these properties, the 315 protein would appear to be suitable for detailed sequence studies with the goal of elucidating the chemical nature of specific binding regions of anti-DNP antibodies. The significance of low-affinity interactions of hydrophobic molecules with myeloma proteins is less clear and the assumption that the binding sites involved are analogous to hapten-specific sites on antibody may be incorrect.

[3] Nonspecific hydrophobic interactions also might contribute to the elicitation of passive cutaneous anaphylaxis by "toxic" univalent haptens (Amkraut *et al.*, 1963). For example, in immediate skin responses produced by α-carbobenzoxy-ϵ-DNP-lysine and anti-DNP antibody in guinea pigs the carbobenzoxy group might be combining nonspecifically with anti-DNP antibody so that a bridge is formed between two antibody molecules.

Acknowledgments

We wish to thank Mrs. Shirley Godt and Miss Mary C. Johnson for their skillful technical assistance.

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