Fluorescein. Hapten and Antibody Active-Site Probe*

Dennis E. Lopatin and Edward W. Voss, Jr.†

ABSTRACT: Fluorescein groups conjugated to a γ-globulin protein carrier were shown to elicit a strong antibody response. Antibodies specific for the fluorescein group were purified by immunoadsorption and the IgG antibodies resolved. A fluorometric binding assay was developed based on the observation that the ligand, fluorescein disodium, is quenched when bound to the antibody's active sites. Results of the fluorescence ligand binding assay were compared with results obtained from equilibrium dialysis. This comparison indicated that the fluorometric assay accurately measured the average intrinsic association constant and heterogeneity index. Because the fluorescence ligand quenching assay depends on a reduction in the fluorescence of the ligand rather than a measurement of the fluorescent chromophores within the antibody protein the assay was applicable for use with immune sera to indicate the presence of antibody.

Fluorescent dyes provide special advantages as molecular probes to study protein–small molecule interactions. Generally, extrinsic dyes (Stryer, 1968) or chromophores are useful because they are sensitive to the structure of, or changes in, the environment. These probes reflect the environment by measureable changes in the absorption properties or fluorescence emission characteristics. Extrinsic dyes are placed in different classes dependent on what changes occur in response to the environment. Some extrinsic dyes are nonfluorescent in aqueous (polar) solution but become fluorescent in a hydrophobic (nonpolar) environment. An example of this type dye is (ANS)[1] whose use was described by Weber and Laurence (1954) in binding studies with serum albumin. Parker *et al.* (1967) utilized a structural analog of ANS, namely DNS, as a hapten to study the environment of the active site of specifically purified antibody. Recently ANS was used as a general probe (Parker and Osterland, 1970) to study the binding sites of various immunoglobulins. Thus, a fluorescence assay based on fluorescence enhancement of the bound dye has become useful in measuring hapten–antibody interactions. This assay contrasts in principle to the fluorescence quenching assay developed with the nitrophenol ligands (Velick *et al.*, 1960; Eisen, 1964) which measures the fluorescence quenching of the antibody's intrinsic chromophore, tryptophan, by bound ligand.

In contrast to both of the above dyes this paper reports the use of the fluorescent dye fluorescein as a hapten, and the development of an assay based on the specific quenching of the dye's fluorescence when bound to specifically purified rabbit antifluorescein antibody.

Materials and Methods

Preparation of Antigen. Porcine γ-globulin (Pγ-G) (Pentex) was dissolved (30 mg/ml) in distilled water, adjusted to a pH of 10.0–10.5 with an equal amount (wt/wt) of potassium carbonate. Fluorescein isothiocyanate (FITC), isomer I (Sigma Chemical Co.), was added (1 mg of FITC/10 mg of protein) to the sclution, incubated 24–48 hr at 37° with agitation, and shielded from light. Unreacted FITC and other by-products were removed by passage through Dowex 1-X8, 200–400 mesh, Cl^- form (J. T. Baker Chemical Co.) equilibrated in 0.05 M PO_4, pH 8.0, followed by dialysis against 0.05 M PO_4, pH 8.0, overnight at room temperature. Determination of the FITC:protein molar ratio was based on dry weight analysis and absorbance of substituted fluorescyl at 493 nm (McKinney *et al.*, 1964; Chen, 1969) measured with a Beckman spectrophotometer. Substitution of conjugated proteins were expressed as $Fl_xP\gamma$-G, where the subscript x indicated the number of fluorescyl groups per mole of protein.

Immunization and Bleeding. Adult albino rabbits were immunized in the foot pads and intrascapularly with a total of 5.0 mg of $Fl_{24}P\gamma$-G in complete Freund's adjuvant. Prior to injection, a test for emulsion was made by placing a drop of the emulsion in contact with distilled water. All rabbits were boosted with equivalent amounts of immunogen by the same routes of immunization. Rabbits were bled through the marginal ear vein.

Amino Acid Analysis. Hydrolysis of $Fl_{24}P\gamma$-G was performed in constant boiling HCl in sealed evacuated tubes at 110° for 24 hr. A Beckman Model 120 amino acid analyzer was used and 200 μg of protein was placed on both the long and short columns. A sample of unsubstituted Pγ-G was treated in a similar manner. A Beckman standard mixture of amino acids was analyzed for calibration.

Preparation of Immunoadsorbent. Bromoacetyl-cellulose was prepared by the method of Robbins *et al.* (1967). To 10 g of derivatized cellulose was added 3.0 g of bovine serum albumin (Armour) conjugated with twelve fluorescyl residues (Fl_{12}-bovine serum albumin), in 0.5 M acetate buffer (pH 5.6). The cellulose protein conjugate was centrifuged and resuspended in 0.10 M bicarbonate (pH 9.0–9.5) allowing formation of covalent bonds. Extensive washing of the adsorbent in bicarbonate, 8.0 M urea, and 0.05 M PO_4, pH 8.0, preceded use in antibody purification studies.

Purification of Antibody. To 50 ml of antisera, in which EDTA was added to a final concentration of 0.001 M to inhibit the complexing of complement components, was added a 3-ml packed volume of immunoadsorbent. Reaction mixtures were incubated 2 hr at room temperature under mild stirring conditions. Following 4–5 washes with 0.05 M PO_4, pH 8.0, the

* From the Department of Microbiology, University of Illinois, Urbana, Illinois 61801. *Received July 13, 1970.* Supported by U. S. Public Health Service Research Grant AI-08288, National Institute of Allergy and Infectious Diseases.

† To whom correspondence should be addressed.

[1] Abbreviations used are: ANS, 1-anilino-8-naphthalenesulfonate; DNS, 5-dimethylaminonaphthalene-1-sulfonamido; Pγ-G, porcine γ-globulin; FITC, fluorescein isothiocyanate; Fl, fluorescyl; DNP, dinitrophenyl; TNP, trinitrophenyl.

immunoadsorbent was subjected to two incubations at 37° for 1 hr in 0.05 M PO_4, pH 8.0, and 1–2 incubations at 37° for 1 hr, with 0.10 M *N*-CBZ-glycine (carbobenzoxyglycine, Sigma Chemical Co.) in 0.05 M PO_4, pH 8.0. Incubations or mock elutions were controls for temperature, buffer, time, and ionic conditions. All supernatants were monitored at 278 and 493 nm. Anti-Fl was eluted with 0.10 M fluorescein (used throughout as the disodium salt) (Aldrich Chemical Co.) in 0.05 M PO_4, pH 8.0, at 37° for 1 hr.

Free fluorescein was resolved from antibody by molecular sieve chromatography over Bio-Gel P-10 (Bio-Rad Laboratories) equilibrated in 0.05 M PO_4, pH 8.0 (1.5 × 50 cm), followed by passage through Dowex 1-X8, 200–400 mesh, Cl^- form, in 0.05 M PO_4, pH 8.0 (2 × 10 cm). Further resolution into IgG and IgM fraction was effected by chromatography on DEAE-Sephadex A-50 anion-exchange resin (Pharmacia Fine Chemicals, Inc.) (2 × 10 cm) equilibrated in 0.05 M PO_4, pH 8.0. Pure IgG was eluted in the first fraction and IgM was eluted at 0.03 M PO_4, pH 8.0 (Voss and Eisen, 1968).

Preparation of [^{3}H]Ligand. Fluorescein disodium salt was tritiated by the method of Wilzbach (1957) and purified by thin-layer silica gel using 95% methanol as solvent (R_F 0.74). The product was diluted with the appropriate amount of 0.05 M PO_4, pH 8.0, to the desired concentration for use as ligands in equilibrium dialysis and appropriate elutions from the immunoadsorbant. The [^{3}H]fluorescein ligand was quenched equally to unlabeled fluorescein based on equal moles added in the fluorescence ligand quenching assay (see Results).

Nitrogen Analysis. Micro-Kjeldahl nitrogen analysis (Kabat and Mayer, 1961) of purified antibody was utilized to verify the extinction coefficient ($E^{1\%}_{1cm,278nm}$) of 15 (based on a N content of 16%; Brand, 1946) and to see if it varied with ligand in the site. Samples were dialyzed in 0.01 M PO_4, pH 8.0, to reduce salt concentration and adjusted to equal absorbance at 278 nm. Equal aliquots were submitted to nitrogen analysis. Low-affinity without bound fluorescein and high-affinity antibody with bound fluorescein both gave an extinction coefficient of 15.

Pepsin Digestions. Pepsin digestions of purified antibody were performed according to the method of Nisonoff (1964).

Fluorescent Ligand Quenching. Binding of fluorescein by the fluorescence assay was measured in an Aminco-Bowman spectrophotofluorometer at room temperature.

Equilibrium Dialysis Studies. Equilibrium dialysis experiments were performed as previously described (Voss *et al.*, 1969). Samples (70 μl) of purified antibody were dialyzed against 70 μl of increasing concentrations of tritiated [^{3}H]-ligand using Plexiglass chambers (0.1 ml total capacity/side). Antibody and ligand were dissolved in 0.05 M PO_4, pH 8.0. After 72-hr equilibration (fluorescein ligand appeared to require longer equilibration period) 50-μl samples were removed with Drummond microliter pipets from both sides of the chambers and dispersed in 8.0 ml of Bray's (1960) scintillation fluid. Samples were counted in a Nuclear-Chicago liquid scintillation spectrometer. Binding data were expressed in terms of r and c, where r is moles of ligand per mole of purified antibody and c is the concentration of free ligand. The ratio r/c was plotted as a function of r (Scatchard, 1949). Valence (n) was derived by extrapolation of the curve to infinite c. Average intrinsic association constant, K_0, was calculated as the reciprocal of c at 0.5 n. Binding data were analyzed according to the Sips distribution function (Sips, 1948; Karush, 1962) to obtain the heterogeneity index log $(r/n - r) = a \log c + a \log K_0$.

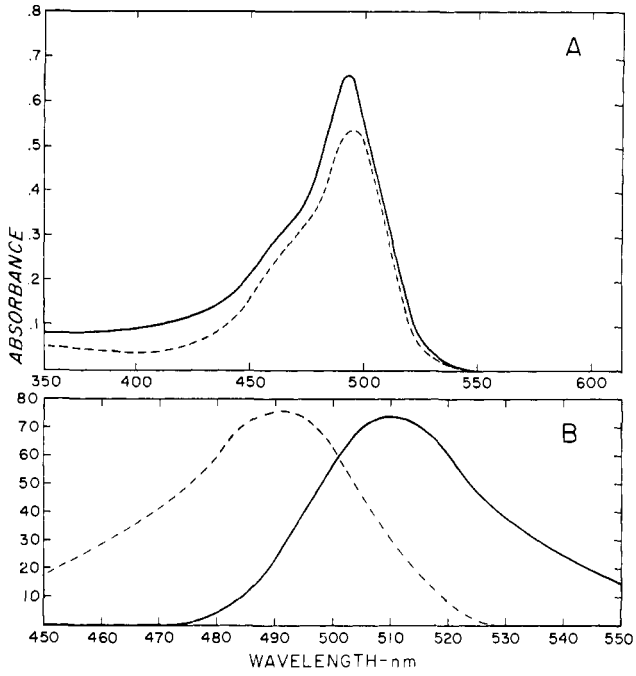

FIGURE 1: (A) Comparison of spectra of (——) fluorescein (8.8 nmoles/ml, 0.05 M PO_4, pH 8.0) and (----) Fl_{12} bovine serum albumin (43 μg/ml, 0.05 M PO_4, pH 8.0). (B) (——) Emission and (---) excitation maxima of fluorescein (0.05 M PO_4, pH 8.0).

Results

Fluorescein–Protein Conjugates and Ligands. Two carrier proteins were utilized throughout these studies for the fluorescein hapten, (a) porcine γ-globulin as the immunogen, and (b) bovine serum albumin as the test antigen. Under the conditions described in Materials and Methods an average of 25–30 fluorescein groups could be substituted per mole of porcine γ-globulin (mol wt 150,000) and 12–13 for bovine serum albumin (mol wt 67,000). Figure 1A shows the absorption spectrum of $FITC_{12}$ bovine serum albumin and an absorption maximum at 493 nm. $FITC_{27}P\gamma$-G showed a similar spectrum and fluorescein also showed a maximum at 493 nm. Thus, the absorption properties of fluorescein were not altered by high levels of substitution to the protein carrier. Fluorescyl–protein conjugates were subjected to acid hydrolysis (12 N HCl, 110° for 24 hr) to determine which amino acids were involved in the substitution reaction. It was found that all amino acids were recovered quantitatively, based on analysis of equal amounts of unsubstituted protein, suggesting that the fluorescyl bond was acid labile unlike the DNP or TNP haptens (Little and Eisen, 1966). Thus, it is unclear which amino acids are involved in the formation of the fluorescein thiocarbamide linkage.

Figure 1B shows the excitation and emission spectra of fluorescein disodium. The excitation maximum was at 493 nm corresponding to the absorption maximum. The fluorescence emission maximum was at 510 nm. All spectra (Figure 1A,B) were based on a pH of 8.0. It should be noted that the excitation and emission spectra appeared to be mirror images. At 493 nm the molar extinction coefficient of fluorescein was determined to be 72,000. McKinney *et al.* (1964) reported an extinction coefficient of 72,000 for the attached fluorescein thiocarbamide group at 493 nm.

Purification of Antifluorescein Antibody. Bleedings were obtained from rabbits at various time intervals, immunized

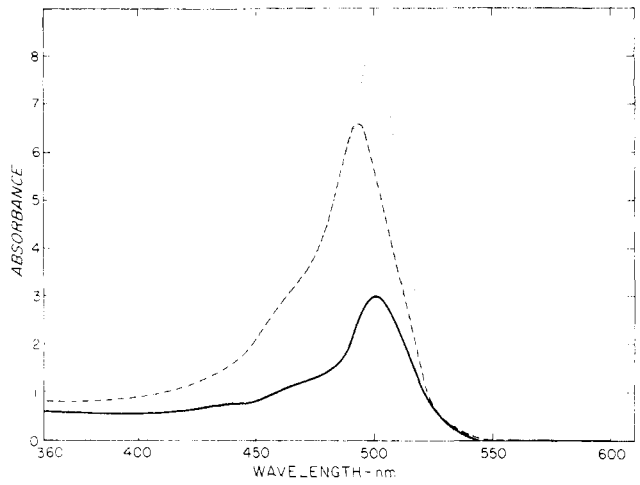

FIGURE 2: Difference spectra of fluorescein in spectral shift studies: (----) unbound fluorescein in 0.05 M PO_4, pH 8.0; (· · · ·) bound fluorescein with high-affinity antibody anti-Fl (1 mg/ml) after Dowex 1-X8; (——) bound fluorescein with high-affinity anti-Fl antibody (0.4 mg/ml) from equilibrium dialysis chambers; starting ligand concentration, 0.69×10^{-5} M. Equilibrated for 48 hr at 4°.

with $Fl_{25-30}P\gamma$-G. Purified antifluorescein antibody was obtained by immunoadsorption as described in Materials and Methods. After chromatography on a Dowex 1-X8 column (10 cm × 2 cm) the purified antibody was resolved into IgG and IgM fractions by anion-exchange chromatography (DEAE). The DEAE column (10 cm × 2 cm) was equilibrated in 0.05 M PO_4, pH 8.0, and rabbit IgG is eluted in the void volume under these ionic conditions. IgM binds to DEAE at 0.05 M PO_4, pH 8.0, and is eluted when the molarity of the buffer is raised to 0.30 M. IgM was found to represent 10–15% of the total purified antibody. Protein purity was verified by immunoelectrophoresis against goat antirabbit serum and acrylamide disc gel electrophoresis. Purified antibodies were 95–100% readsorbable to the Fl-bovine serum albumin immunoadsorbent. Thus, the conditions employed resulted in purified IgG and IgM which were totally active.

Repeated purifications indicated that after Dowex chromatography considerable fluorescein ligand was still bound to the IgG fractions. In order to determine what percentage of sites were filled it was necessary to measure the extinction coefficient of the bound ligand. Therefore, antifluorescein antibody was purified by immunoadsorption but the antibody was eluted with [^{3}H]fluorescein (0.05 M, 1500 cpm/nmole). The unbound ligand was removed by P-10 and Dowex 1-X8 chromatography as described above. Percentage of sites filled could be verified by counting the purified antibody at high concentrations to obtain significant levels of radioactivity. Radioactive measurements were then compared with spectrophotometric determinations.

Purified antibody after Dowex chromatography with bound [^{3}H]fluorescein was analyzed spectrophotometrically (Cary 15). Figure 2 shows that the bound ligand peaks at 500 nm. Thus, there is a red shift of 7 nm relative to the unbound ligand. This spectral shift was verified by dialyzing 1.5 ml of fluorescein (A_{493} 0.500) against 1.5 ml of purified antifluorescein IgG antibody (A_{278} 0.650) in large Lucite equilibrium dialysis chambers. After 48-hr equilibration 1.0 ml was removed from each side of the chamber and read as a difference spectrum in the Cary 15. Figure 2 shows the results of this experiment. The bound fluorescein shows identical spectral properties with those of the ligand measured in the dialysis experiment. In both instances the bound ligand shows an absorption maximum at 500 nm. The purified IgG antibody was concentrated to about 3.5 mg/ml, the optical density measured at 278, 493, and 500 nm, and the tritium content measured. Multiple measurements indicated that the extinction coefficient was 72,000 ± 10,000 at 500 nm. The scatter noted in the data is probably due to the low specific activity of the [^{3}H]fluorescein (1500 cpm/nmole). Thus, the bound and free fluorescein showed similar molar extinction coefficients despite a red shift of 7 nm.

The determination of the molar extinction coefficient of the bound ligand allowed the measurement of the per cent sites filled from spectral analyses.

Fluorescence Ligand Quenching Assay. In the above analyses of the bound ligand (fluorescein) it was noted that bound ligand did not fluoresce. This suggested the potential of a quantitative assay based on the quenching of the fluorescence of fluorescein when bound to the antibody active site.

To test the feasibility of a quantitative assay purified antifluorescein antibody was obtained at various time periods after immunization. These purified antibodies (IgG) were titrated with 8.8×10^{-6} mole/l. of fluorescein in 0.05 M PO_4, pH 8.0. Antibodies were titrated at various concentrations and it was found that the amount of quenching was proportional to the amount of antibody present: However, a protein concentration of 2.2 nmoles/ml (A_{278} 0.5) was considered optimum for various reasons. First, the assay gave most accurate results when 0.5 ml of fluorescein (8.8×10^{-6} mole/l.) was titrated into 1.0 ml of antibody. Thus, 4.4 nmoles of ligand was then equal to the number of sites present on bivalent IgG (A_{278} 0.5). This would allow for accurate determinations of moles of ligand bound per mole of antibody based on percentages of the amount added. Second, if too much antibody was present the bound ligand was shown to quench the fluorescence of the unbound ligand. Therefore, the concentration of antibody had to be minimized to prevent autoquenching. Third, if the antibody concentration is too low then problems would arise in titrating low affinity antifluorescein antibody ($K_0 = 10^4$–10^5 l./mole).

Figure 3A shows the results of the fluorescence ligand quenching assay. It can be seen that two comparative titrations of 4.4 nmoles of fluorescein against 1.0 ml of 0.05 M PO_4, pH 8.0, and 2.2 nmoles of normal rabbit IgG gave identical results. Since these curves are superimposable, it indicates that there is no quenching by either normal rabbit IgG or buffer. The curve represents a simple addition of fluorometric units in proportion to the volume added. The ligand is added in aliquots of 0.05 ml up to 0.5 ml (*i.e.*, 10 readings). The apparent nonlinearity of the curve at the higher points is due to the autoquenching by the fluorescein ligand at higher concentrations. Figure 3A also shows distinct differences between primary, secondary, and hyperimmune antibody and reflects differences in average intrinsic association constants (K_0). Primary antibody is antifluorescein antibody obtained 10 days after a primary immunization. Secondary antibody was obtained 81 days after the initial injection (*i.e.*, 67 days after primary immunization and 14 days after a booster injection). Approximately 25% of the antibody sites (secondary antibody) were filled with fluorescein ligand after purification. Purified antifluorescein IgM also gave significant quenching. The titration of hyperimmune antibodies and its $F(ab)_2$ fragment represent a special case and potential weakness in the assay. Figure 3A shows the titration of purified rabbit IgG antifluorescein about 4 months after a primary immunization.

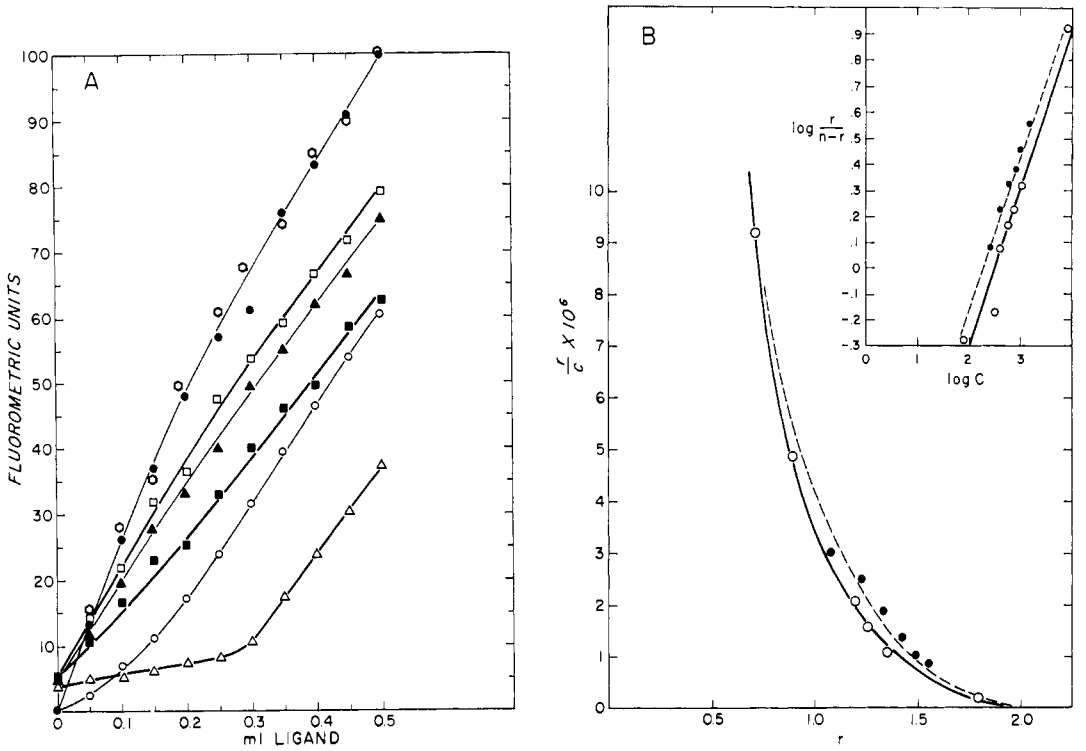

FIGURE 3: (A) Comparison of relative fluorescence ligand quenching of various purified immunoglobulins. All protein concentrations were adjusted to 2.2 nmoles/ml and were titrated with a total of 4.4 nmoles of fluorescein ligand in 0.05 M PO_4, pH 8.0: (●-●) normal rabbit immunoglobulin (IgG), (○-○) 0.05 M PO_4, pH 8.0, (○-○) primary purified rabbit anti-Fl (IgG), (△-△) secondary purified rabbit anti-Fl (IgG), (□-□) purified hyperimmune rabbit anti-Fl (IgG), (▲-▲) pepsin digest of purified hyperimmune rabbit anti-Fl (IgG), (■-■) purified rabbit anti-Fl (IgM). (B) Correlation of (○-○) equilibrium dialysis binding studies of [^{3}H]fluorescein with (●---●) fluorescence ligand quenching in Scatchard plots. A protein concentration of 2.2 mmoles/ml was used in equilibrium dialysis and fluorescence ligand quenching. Insert: Sip's plot of the data derived from equilibrium dialysis and fluorescence ligand quenching.

The antibody is of such high affinity that 50% of the sites ($r = 1$) were filled with ligand. Therefore, this curve represents the titration of only the unfilled sites. However, since the percentage of sites filled can be measured, then the proper correction factors can be applied. Also shown is the pepsin fragment obtained from the hyperimmune antibody used above. This titration shows that the antibody activity resides in the Fab active site fragments, since the Fc fragment is hydrolyzed to dialyzable peptides by pepsin. The shapes of the various curves are important since they probably reflect the heterogeneity of the purified antibody population in respect to the average intrinsic association constant. Heterogeneity is clearly shown in the titrations of the early and late antifluorescein antibodies.

The principle of the assay therefore would be that r (moles of ligand bound per mole of antibody) and c (free ligand) can be determined from the results of a titration. Appropriate plots of the calculated data would then yield K_0 (average intrinsic association constant) of the purified antibody. These calculations are explained below.

Determination of r, c, and K_0. The basic concept is that fluorescein is quenched when bound to the active site. This is probably due to the hydrophobic nature of the antibody active site. Dissolving fluorescein into various organic solvents suggested that fluorescein fluoresced in solvents with a dielectric constant above 10, and did not fluoresce in solvents below 10. For example, titration of fluorescein in phenol totally quenched fluorescence. The question remained as to whether the fluorescence of bound fluorescein was totally or partially quenched. This was difficult to prove and it seemed theoretically some fluorescence should be measured due to the equilibrium between free and bound ligand. However, to arrive at an approximation of the degree of quenching and if it was a factor in the assay the following experiment was performed. High affinity antibody with 50% (2.2 nmoles of ligand) of the sites filled was placed in the fluorometer. An equivalent amount of free ligand would have registered 55–60 fluorescence units (meter multiplier = 1, sensitivity = 34). However, the bound ligand did not register any fluorescence units. This indicates that the Dowex column removes unbound ligand and that bound ligand is essentially totally quenched by the antibody active site. Thus, the determination of c and r are related to either the amount of fluorescence by the free ligand or the absence of fluorescence by the bound ligand. Calculation of c is therefore based on the final amount of fluorescence at 0.5 ml of fluorescein added. In a typical titration against buffer or rabbit normal IgG (reference curve) the results represent total fluorescence (*i.e.*, 100 corrected for volume) obtained from the addition of 0.5 ml of 4.4 nmoles of fluorescein. By comparison secondary antibody in Figure 3A shows only 37 fluorescence units upon titration with purified antifluorescein antibody. Thus c can be calculated as 37% of 4.4 nmoles or read off directly from a comparable point on the reference curve. Since the moles of antibody are known (2.2 nmoles) and the amount of ligand bound taken as 63% of 4.4 nmoles then r is 1.25.

In those cases where ligand is bound to the antibody site prior to a titration (*i.e.*, high affinity) the appropriate correction can be made. The percentage of sites filled (r prior to titration) is measured by readings at 278 and 500 nm (the

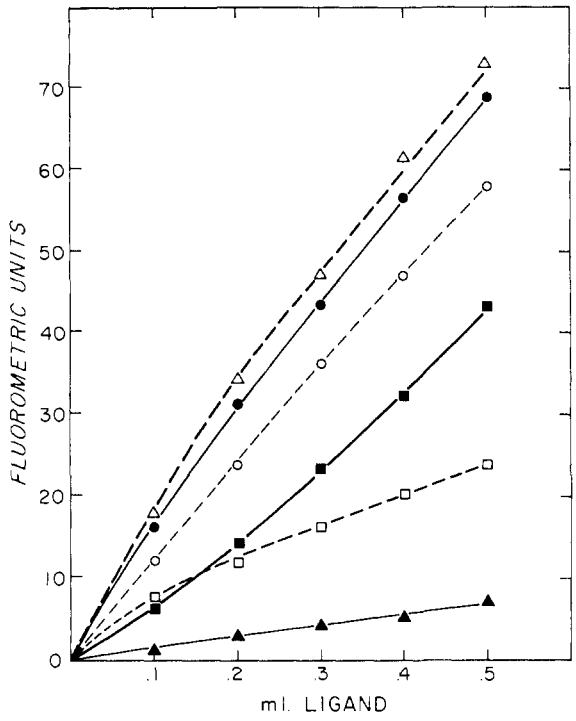

FIGURE 4: Comparative fluorescence ligand quenching titrations of various anti-Fl antisera diluted twofold with 0.05 M PO_4, pH 8.0. Diluted antiserum (1 ml) is titrated with 0.5 ml in 0.05-ml aliquots of 1×10^{-5} M fluorescein in 0.05 M PO_4, pH 8.0: (●-●) normal rabbit serum; (△-△) 6-day post primary immunization; (○-○) 10-day post primary immunization; (■-■) 18-day post primary immunization; (□-□) 81-day post primary immunization; (▲-▲) hyperimmune anti-Fl antiserum.

appropriate extinction coefficients of 1.5 and 72,000 can be applied). To verify that the fluorescence ligand quenching assay measures the correct average intrinsic association constant, secondary antibody used in Figure 3A was measured by equilibrium dialysis. The antibody was eluted with [^{3}H]-fluorescein of the same specific activity as that used in subsequent equilibrium dialysis measurements. Thus, the radioactive ligand was present and reflected the true r. Figure 3B shows the results of this experiment. A final extrapolated value of 2.1 was calculated for n (valence) and a K_0 of 4.0×10^6 l./mole was measured for the 81-day (late) purified antibody by the fluorescence ligand quenching assay. An n of 2.1 and a K_0 of 4.0×10^6 l./mole were measured by equilibrium dialysis. The Sips plots of both measurements (insert, Figure 3B) gave a heterogeneity index (a) of 0.6. Thus, it appears that the fluorescence ligand quenching assay accurately measures the ligand binding parameters.

Titration of Antiserum. Because the fluorescence ligand quenching assay is dependent on the reduction of fluorescence of the ligand rather than the quenching of the tryptophan such as the fluorescence quenching assay with the DNP system (Velick *et al.*, 1960) certain advantages are evident. Primary among these is the potential of titrating antiserum. Figure 4 shows the results of these titrations. It was found that undiluted antisera substantially quenched the fluorescence of the fluorescein ligand. Serum diluted twofold with 0.05 M PO_4, pH 8.0, proved adequate. This dilution also served to reduce any affect excess hemoglobin may exert on the system. It can be seen in Figure 4 that relative to the normal serum control there was an increase in quenching directly related to the increase in time after immunization. The 6-day bleeding did not show detectable antibody; however significant levels were detected at 10 days. Very late antibody showed substantial amounts of quenching. It is believed that the quenching reflects both the amount of antibody and the increasing average intrinsic association constant (Eisen and Siskind, 1964).

Discussion

Results of studies reported above suggest that fluorescein is a potent haptenic immunogen when conjugated to a carrier protein. Large quantities of antibodies have been elicited and purified specific for the fluorescyl hapten. Antibody levels of 0.5 mg/ml of sera adsorbed have been obtained within 2 weeks of a primary immunization, while hyperimmune sera have yielded 1.0–2.0 mg/ml. This antibody is generally characterized as being 10% IgM and 90% IgG. Antifluorescein antibody was originally elicited by Dandliker and Feigen (1961) and then utilized in studies related to the application of fluorescence polarization to antigen–antibody reactions (Dandliker *et al.*, 1964). Dandliker, using fluorescein-labeled immunogen, observed a decrease in fluorescence intensity with the carrier–anticarrier interaction. This decrease was not apparent in similar studies by Haber and Bennett (1962), and they noted that the difference was probably related to the use of fluorescyl-conjugated proteins as immunogens. Thus, Dandliker and Feigen (1961) observed both an antifluorescein reaction as well as an anticarrier reaction. Haber and Bennett (1962) used nonfluorescein labeled proteins as immunogens and therefore only measured the interaction of the nonconjugated protein with its specific antibody. Studies reported here also utilize purified antifluorescyl antibody as a specific reagent while previous studies utilized only the IgG serum fraction (Dandliker and Feigen, 1961, and Dandliker *et al.*, 1964).

Thus, these studies are an extension and quantification of these original observations. The assay is based on the quenching of the fluorescence of the fluorescein ligand when bound to the antibody active site. Since the effect is not measured with normal γ-globulin and is restricted to the Fab fragments, we conclude this is a true antigen–antibody interaction. The decrease in fluorescence is probably related to the low dielectric constant of the active site (Parker *et al.*, 1967; Little and Eisen, 1967) or the close positioning of the ligand with amino acids (Little and Eisen, 1967) within the site establishing conditions for loss of the electronic excitation energy before it can be dissipated as fluorescence. The excitation and emission maxima of fluorescein are sufficiently different from the excitation (300 nm) and emission (350 nm) spectra of proteins that no background corrections need be applied to the data. Experiments were attempted to show if the binding of fluorescein to the active site resulted in the quenching of the antibody's tryptophan fluorescence (350 nm). No quenching was observed and it is believed that this is due to a lack of overlap between the emission maxima of tryptophan (350 nm) and the absorption maxima of fluorescein (493 nm). Considerable overlap is necessary for efficient energy transfer as is the case with the nitrophenol ligands (Eisen, 1964).

The fluorescence assay as shown in Figures 3A and 3B results in reliable determinations of binding parameters as confirmed by equilibrium dialysis measurements. Since the assay is based on the fluorescence properties of the ligand it allows for accurate measurements of c. This becomes a critical parameter since antifluorescein IgG antibody have been of relatively high affinity. If c is accurately measured then it

follows that the accuracy of calculating r is increased. The comparison of a K_0 of 4.0×10^6 l./mole by the fluorescence ligand quenching assay to 4.0×10^6 l./mole by equilibrium dialysis shows the reliability of the assay. Both assays gave a heterogeneity index (a) of 0.6 by the Sips plot.

For most antifluorescein IgG antibodies the correlation between the fluorescence ligand quenching assay and equilibrium dialysis is very close. Some scatter has been noted in data obtained from the first few points in the titration. Because most of the ligand is bound by the antibody at these points there is little measurable fluorescence. Amounts measured fall in the nonlinear portion of the fluorometer scale (*i.e.*, 0–10). This can be rectified by properly adjusting the sensitivity of the meter, thereby magnifying the measurable fluorescence into a linear portion of the scale (>10). Hyperimmune purified antibody that after purification results in a significant number of sites filled presents a special problem. Titration of this antibody with fluorescein is in reality a titration only of the remaining unoccupied sites. Thus, if the number of sites filled (r) are determined spectrophotometrically prior to the fluorescence ligand titration a correction factor can be employed.

The correction is made by determining the percentage of unfilled sites at the onset of the fluorometric titration as previously described and dividing all subsequent experimental r values by this percentage to yield a corrected value which is equivalent to all sites being available. The direct addition of the predetermined r to each point gives values of K_0 that are too high. Thus, this correction factor implies that since the ligand is being monitored to obtain the appropriate measurements the results are interpreted in terms of the ligand. Therefore, the results are more consistent with the idea that a specified additional percentage of ligand would have been bound upon each addition if all sites had been unoccupied.

Data reported above further indicate the hydrophobicity of the antibody active site and describe the use of fluorescein in a fluorescence ligand quenching assay which could serve as a valuable probe to further elucidate the structure of the antibody active site. The use of fluorescein as a hapten differs from the more classical use of fluorescein as a fluorescent tag (Coons, 1958) and an aid in identifying antibody producing cells. However, the use of fluorescein as a hapten may in fact enhance the use of this dye in these studies. Spleen or lymph node cells obtained from rabbits producing antifluorescein antibody can be incubated directly with fluorescein-conjugated proteins (*e.g.*, Fl_{15} bovine serum albumin) to identify antifluorescein producing cells. Only a certain percentage of the fluorescein groups in the carrier protein will be bound by cells and their fluorescence quenched; however, the unbound groups on the same carrier will fluoresce, identifying the appropriate cells. Thus, fluorescein appears to provide a very flexible probe for immunological studies.

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