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Preparation and Characterization of Antibodies Specific for the 2,4,6-Trinitrophenyl Group*

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ABSTRACT: Rabbit antibodies specific for the 2,4,6-trinitrophenyl (TNP) group can be isolated from antisera in high yield by employing various combinations of reagents for specific precipitation and elution, the choice of reagents depending on the time of bleeding after immunization and the anticipated average affinity of the antibody molecules. In particular the use of a cross-reacting hapten–protein conjugate (2,4-dinitrophenyl (DNP) protein) to precipitate antibody from serum, and a homologous hapten to elute specifically from the immune precipitates, has provided the means of obtaining very high affinity molecules in excellent yield. The binding of univalent ligands by anti-TNP antibodies has been examined by the method of fluorescence quenching and the intrinsic association constants obtained have been validated by equilibrium dialysis. Anti-TNP molecules are conspicuously less quenchable than antibodies specific for the 2,4-DNP group. The Q_{max} obtained when antibody sites are saturated by a TNP- or DNP-hapten was found to vary between 52 and 62% for anti-TNP molecules, compared to 70–80% for anti-DNP molecules. The greater quenchability of rabbit anti-DNP antibodies is a property of the antibody molecules, not the ligands. Anti-TNP antibodies have binding constants in the same range as those previously reported for anti-DNP antibodies. Binding of univalent TNP-haptens is relatively unaffected by changes in pH or ionic strength and there is a rise in average intrinsic association constants for homologous and cross-reacting haptens with time after immunization. Proteolytic cleavage with papain has no detectable influence on the binding strength or heterogeneity of anti-TNP antibody–ligand reactions.

Considerable information has been accumulated in the past decade on the specific binding and structural properties of antibodies to the 2,4-dinitrophenyl (DNP)[1] group and on their heterogeneity. Because the 2,4,6-trinitrophenyl (TNP) group differs only by a third NO_2 group on the benzene ring, and is also potent immunogenically, it seemed possible that detailed comparison of the antibodies to the TNP group with the antibodies to the DNP group would shed some light on the structural features of antibodies that are relevant for their specificity.

In the present paper we describe procedures for obtaining substantial amounts of purified antibodies to the TNP group. In addition the reactions of several ligands with these antibodies and their proteolytic

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[1] Abbreviations used in this work: TNP or TNB, the 2,4,6-trinitrobenzene group; DNP or DNB, the 2,4-dinitrobenzene group; BγG, bovine γ-globulin; HSA, human serum albumin; TNT, 2,4,6-trinitrotoluene; DNT, 2,4-dinitrotoluene. The immunoglobulin nomenclature and abbreviations used are those proposed by the World Health Organization Committee on Nomenclature (1964), *Bull. World Health Organ. 30*, 447.

fragments, obtained by papain and pepsin digestion, have been examined by fluorescence quenching and equilibrium dialysis. The fluorescence of rabbit anti-TNP antibodies is much less quenchable by TNP or DNP ligands than the fluorescence of rabbit anti-DNP antibodies, though the affinities of both types of antibody for their homologous ligands fall within the same range. The affinity of anti-TNP antibodies for several TNP ligands, like that of anti-DNP antibodies for DNP ligands, increases with time after immunization. In later publications we shall present a comparison of the structural properties of various populations of anti-DNP and anti-TNP antibodies, as well as an analysis of their cross-reactions.

Materials and Methods

2,4,6-Trinitrobenzenesulfonic acid was prepared as described by Golumbic *et al.* (1946), or was obtained from Nutritional Biochemical Corp., Cleveland, Ohio. Recrystallized from 1 N HCl, it had an uncorrected melting point of 168–169° (Fisher-Johns melting point apparatus). Descending paper chromatography on Whatman No. 1 in water-saturated methyl ethyl ketone revealed a single spot, R_F 0.62; a spot corresponding to 2,4-dinitrobenzenesulfonic acid, R_F 0.34, was not observed. By this criterion the trinitrobenzenesulfonic acid contained less than 0.2 mole % 2,4-dinitrobenzenesulfonic acid as a possible contaminant.

TNP-Glycine was synthesized from glycine and TNB-sulfonic acid according to Okuyama and Satake (1960) and was recrystallized from aqueous methanol; mp 161–162°; λ_{max} 345 mμ (ε 14,990) in 0.02 M potassium phosphate, pH 7.4. *Anal.* Calcd for $C_8H_6N_4O_8$: C, 33.55; H, 2.12; N, 19.60. Found[2]: C, 33.82; H, 2.12; N, 19.39. Aqueous solutions of TNP-glycine are rapidly decomposed by light and hence were prepared immediately before use and scrupulously protected from light throughout.

ε-*TNP-L-Lysine* was prepared according to Okuyama and Satake (1960) and was recrystallized from 1 N HCl. The melting point was 198–199°; λ_{max} 348 mμ (ε 15,400) in 0.1 M potassium phosphate, pH 7.4. *Anal.* Calcd for $C_{12}H_{15}N_5O_8 \cdot HCl$: C, 36.70; H, 4.12; N, 17.83. Found: C, 36.87; H, 4.33; N, 17.95.

ε-TNP-L-[^{3}H]lysine was prepared by tritiation of 25 mg of ε-TNP-L-lysine by New England Nuclear Corp. in [^{3}H]glacial acetic acid using a platinum catalyst. The crude product was dried, taken up in 1 N HCl, extracted with peroxide-free ether, and purified by thin layer chromatography on silica gel G with water-saturated methyl ethyl ketone as solvent. The major yellow spot, with the same R_F as the starting material, was eluted with water. Its absorption spectrum between 260 and 400 mμ was identical with that of the authentic compound. The specific activity was 456,000 cpm/mμmole in Bray's (1960) solution.

ε-*TNP-Aminocaproic acid* was prepared according to Benacerraf and Levine (1962), and was recrystallized four times from ethanol–water; mp 144°; λ_{max} 348 mμ (ε 15,700) in 0.01 M potassium phosphate, pH 7.4. *Anal.* Calcd for $C_{12}H_{14}N_4O_8$: C, 42.20; H, 4.13; N, 16.20. Found: C, 41.81; H, 4.13; N, 16.20.

[1-^{14}C]-2,4-Dinitroaniline was a gift from Dr. James McGuigan and was prepared essentially as described (Eisen and Siskind, 1964) from [1-^{14}C]-2,4-dinitrochlorobenzene (Nuclear Equipment Chemical Corp., Farmingdale, N. Y., 8.5 mc/mmole). The [1-^{14}C]-2,4-dinitroaniline had a specific activity of 14,000 cpm/mμmole in a toluene solution of 2,5-diphenyloxazole and 1,4-di[2-(5-phenyloxazolyl)]benzene (Davidson and Feigelson, 1957).

DNP proteins were prepared with sodium dinitrobenzenesulfonate as described elsewhere (Eisen *et al.*, 1953; Eisen, 1964). Preparations of DNP-BγG and DNP-HSA had 50–57 moles of DNP/160,000 g of BγG and 34–37 moles of DNP/70,000 g of HSA, respectively.

Picric acid was obtained from Fisher Scientific Co. (St. Louis, Mo.) and was recrystallized three times from hot water. 2,4,6-Trinitrotoluene and 2,4-dinitrotoluene were obtained from Distillation Products Industries (Rochester, N. Y.) and were recrystallized from ethanol–water.

Additional compounds were used without further purification. 2,4-Dinitrophenylated amino acids were obtained from Mann Chemical Corp. (New York, N. Y.). Sodium dodecyl sulfate was the product of Matheson Coleman and Bell (Chicago, Ill.). The mineral oil (Bayol-F) and Arlacel A (Mannide Monooleate) used in emulsions for immunization were obtained from Humble Oil and Refining Co. (Linden, N. J.) and from the Atlas Powder Co. (Wilmington, Del.), respectively. Papain and pepsin were the products of the Sigma Chemical Co. (St. Louis, Mo.). Dowex 1-X8 (200–400 mesh) was obtained from J. T. Baker Chemical Co. (Phillipsburg, N. J.); DEAE-cellulose, 0.51 mequiv/g, and CM-cellulose, 0.68 mequiv/g, were Serva resins from Gallard-Schlesinger Chemical Manufacturing Co. (Garden City, N. Y.). The BγG and HSA used for the preparation of antigens were fraction II of bovine plasma, from Armour and Co. (Chicago Ill), and crystallized human serum albumin, from Pentex Inc. (Kankakee, Ill.).

Preparation of Antigens. BγG and HSA were trinitrophenylated by reaction with 2,4,6-trinitrobenzenesulfonic acid in 0.2 M sodium borate, pH 9.2 (Little and Eisen, 1966). Preparations of TNP-BγG had 50–61 moles of TNP/160,000 g of BγG and 34–37 moles of TNP/70,000 g of HSA.

Immunization. TNP-BγG in 0.15 M NaCl–0.02 M potassium phosphate, pH 7.4, was emulsified with an equal volume of mineral oil–Arlacel A (4:1, v/v). The mineral oil contained dried, heat-killed *Mycobacterium butyricum* (10 mg/100 ml) and immunization was performed by injecting 1–6 mg of TNP-BγG in 1.6-ml emulsion into the four foot pads of randomly bred albino rabbits.

[2] Elemental analyses were performed by the Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

Animals were bled by cardiac puncture at intervals after immunization. Serum pools were obtained by combining the bleedings from 3 consecutive days from groups of 25–50 rabbits. Sera from individual rabbits represented the combined bleedings of 2 or 3 consecutive days.

Precipitin Analyses. The concentration of anti-TNP antibodies in serum was measured by precipitin analyses using TNP-BγG or TNP-HSA as antigens, and often DNP-HSA or DNP-BγG as well. Antigen–antibody mixtures were incubated for 1 hr at 37° and then 18–24 hr at 4°. The amount of antibody in each washed precipitate, dissolved in 0.5% sodium dodecyl sulfate, was calculated from the total absorbance at 278 mμ minus the absorbance attributable to antigen; the latter was calculated from the ratio of the extinction coefficients at 278 and 360 mμ of the particular antigen preparation used. The E_{278}^{1cm} of rabbit antibodies was taken to be 15.5 for 1% solution at neutral pH.

Purification of Antibody. The procedures used for the isolation of anti-TNP antibodies were analogous to those described for anti-DNP antibodies (Farah *et al.*, 1960; Eisen, 1964). Briefly stated the following sequential steps were employed. (1) Antibodies were precipitated from serum with a TNP or DNP protein preparation in which the protein component did not cross-react with the protein of the immunogen. (2) Anti-TNP antibodies were eluted specifically from the washed specific precipitate with an appropriate univalent hapten (*e.g.*, picrate ion). (3) Antigen and hapten were removed from the dissolved specific precipitate by ion exchange on DEAE-cellulose and Dowex 1, respectively. (4) The antibody recovered from the ion-exchange columns was dialyzed against buffered saline and then against 0.001 M phosphate, pH 7.6–8.0, to remove "euglobulins." The final product was soluble in 0.001 M phosphate. Various antigens and haptens can be used in the purification procedure, depending on the anticipated average affinity of the antibody population in a given antiserum. The considerations that govern the choice of specific reagents are discussed below.

Fluorescence Quenching. The binding of haptens by purified anti-TNP antibodies was measured by the method of fluorescence quenching in an Aminco-Bowman spectrophotofluorometer as described elsewhere (Velick *et al.*, 1960; Eisen and Siskind, 1964). Samples (1 ml) with antibody at a concentration of 40–50 μg/ml were titrated with increments of a hapten solution at a concentration of 4.0–6.0 mμmoles/ml. Antibody fluorescence was activated with incident light at 295 mμ and emission intensity was measured at 345 mμ. All titrations were performed at 30° unless otherwise indicated. Bound and free hapten were determined on the basis of the fluorescence quenching observed, relative to the quenching when all antibody combining sites are occupied by ligand (Q_{max}). The magnitude of the value of Q_{max} was determined on each purified antibody preparation by the method of Day *et al.* (1963), using TNP-aminocaproate as ligand. The Q_{max} value with other ligands was not determined but, in general, we have found that the differences between homologous ligands are small and do not significantly affect the association constants. With selected samples the results were verified by comparing the association constants determined by fluorescence quenching with those measured by equilibrium dialysis.

Antibody concentrations were determined by absorbance at 278 mμ in neutral aqueous solution assuming the molecular weight of the rabbit antibodies to be 180,000. It has been pointed out previously that when lower values for molecular weight are used (*e.g.*, 150,000), the method of calculation employed underestimates the association constants of very high affinity antibodies ($>1 \times 10^8$ M^{-1}) but the values reported for moderate and low affinity antibodies (1×10^5–1×10^7 M^{-1}) are not significantly altered (Eisen and Siskind, 1964).

The average intrinsic association constant, K_0, was calculated from fluorescence quenching and equilibrium dialysis data according to the logarithmic form of the Sips equation (Nisonoff and Pressman, 1958; Karush, 1962)

$$\log r/(n - r) = a \log c + a \log K_0$$

where r is moles of hapten bound per mole of antibody at free hapten concentration c; n is the maximum number of moles of ligand that can be bound per mole of antibody; and a is the heterogeneity index which describes the dispersion of association constants about the average intrinsic association constant, K_0. When $a = 1$, the binding sites are homogeneous, *i.e.*, they all have the same intrinsic association constant; as a becomes smaller the dispersion of association constants increases.

Antibody samples were titrated in duplicate or triplicate with each hapten, and for each titration log c *vs.* log $r/(n - r)$ was plotted by the method of least squares; a was obtained from the slope and K_0 from the intercept. Calculations were performed with the aid of an IBM computer, Model 7072.[3] Conformity of the data to the linear regression was computed as the coefficient of correlation, which varied between 0.90 and 0.99 for all the data cited. In replicate titrations the average deviation of K_0 values from their mean was ±30%.

Equilibrium Dialysis. Measurements of antibody affinity by equilibrium dialysis were performed as described elsewhere (Eisen, 1964). Plastic chambers of the type available from Technilab Instruments, Los Angeles, Calif., were employed with 1 ml in "outside" and "inside" compartments separated by a flat sheet of washed Visking dialysis casing. Purified antibody concentrations in buffered saline were 80–100 μg/ml in each experiment. The experimental chambers were rotated 3–5 rpm for 6.5 hr at 30° and then equilibrium

[3] Automatic computation of values from fluorescence quenching titrations was generously provided by Mr. Richard A. Dammkoehler and Mr. Tom L. Gallagher of the Washington University Computer Facility.

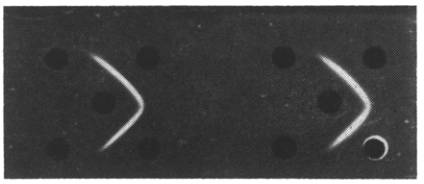

FIGURE 1: Double-diffusion precipitin reactions in agar gel. Center well on left contains undiluted antiserum TN-9 evoked by immunization with TNP-BγG; center well on right, purified anti-TNP antibodies from antiserum TN-9. The peripheral wells contain 500 μg of antigens/ml distributed as follows: upper left, BγG; upper right, TNP-HSA: lower right, DNP-HSA; lower left, HSA.

across the membranes was verified by counting aliquots of control chambers containing ligand but no protein. The extent of "bag binding" was less than 5% of the free ligand concentration with ε-TNP-L-[^{3}H]lysine and about 5% of the free ligand concentration with [1-^{14}C]-2,4-dinitroaniline. At equilibrium aliquots were taken from "inside" as well as "outside" compartments for liquid scintillation counting. When the aliquots contained ε-TNP-L-[^{3}H]lysine they were added directly to Bray's solution (1960). When the aliquots contained [1-^{14}C]-2,4-dinitroaniline they were first mixed in the counting vial with 0.2 ml of 1 M NaOH and then extracted into a toluene–phosphor counting solution (Davidson and Feigelson, 1957). Neither ligand was bound significantly by normal rabbit γ-globulin at a concentration of 100 μg/ml (*e.g.*, less than 1% of the free ligand concentration with 3×10^{-6} M ε-TNP-L-[^{3}H]lysine and 1×10^{-6} M [1-^{14}C]-2,4-dinitroaniline).

Proteolytic Cleavage of Rabbit Antibodies. Papain and pepsin digestion of anti-TNP antibodies were carried out at pH 7.5 and 4.5, respectively, under the conditions described by Nisonoff (1964). Papain fragments were resolved on CM-cellulose at pH 5.4, after removal of the crystalline fraction that separates from the reaction mixture during dialysis against 0.01 M potassium phosphate, pH 7.4 (Porter, 1959). The bivalent 5S antibody fragment obtained by pepsin digestion was separated from smaller peptides on Sephadex G-75 under the condition described by Utsumi and Karush (1965).

Results

The Immunogen. A preliminary characterization of the immunogen, TNP-BγG, was undertaken to establish the specificity of trinitrophenylation for the ε-NH_2 groups of lysine. Acid hydrolysates of TNP-BγG revealed that only the lysine content was reduced when compared with control samples of unsubstituted BγG (Table I). The formation of ε-TNP-lysine was confirmed by thin layer chromatography on silica gel G using water-saturated methyl ethyl ketone as solvent.

TABLE I: Amino Acid Composition of BγG and TNP-BγG.[a]

Amino Acid	Residues/Molecule			
	BγG	σ	TNP-BγG	σ
Lysine	*72.9*	2.8	*14.3*	1.0
Histidine	21.3	1.4	20.6	1.5
Arginine	54.7	0.1	57.1	2.1
Cysteic acid	23.7		23.0	
Aspartic acid	120.4	2.7	124.4	3.0
Threonine	144.0	3.6	142.4	4.0
Serine	184.7	4.2	185.5	3.8
Glutamic acid	122.2	2.5	123.7	1.8
Proline	102.6	2.1	101.7	0.8
Alanine	87.7	1.1	87.7	0.6
Valine	158.0	0.9	154.2	1.2
Methionine	14.2	1.0	13.3	1.1
Isoleucine	42.2	0.7	41.5	1.0
Leucine	103.7	1.1	104.9	0.4
Tyrosine	51.9	2.1	50.0	2.5
Phenylalanine	42.8		42.8	

[a] The values are averages for five analyses of BγG and four analyses of TNP-BγG. Two samples each of BγG and TNP-BγG were performic acid oxidized before acid hydrolysis (Hirs, 1956) and the cysteic acid values were obtained from the averaged duplicates. All samples were hydrolyzed in 6 N HCl at 110 ± 1° under reflux conditions for 24 hr. The calculation of residues per molecules was made assuming the molecular weight of BγG to be 160,000 and by normalizing the recovery of each amino acid to be a value of 42.8 residues of phenylalanine/mole of protein. The significant difference in lysine content is shown in italic type.

Chromatograms of acid hydrolysates (6 N HCl, 110°, 24 hr) of TNP proteins and of authentic ε-TNP-lysine were identical. In both chromatograms there were three faint spots in addition to the major yellow band, which was undegraded ε-TNP-lysine.

Precipitin Analyses. Extensive cross-reactivity with DNP proteins was observed in precipitin reactions with all antisera prepared against TNP-BγG (J. R. Little and H. N. Eisen, in preparation). The cross-reactions appeared as lines of identity between DNP and TNP proteins in double diffusion reactions carried out in agar gel either at pH 7.4 or 8.6 (Figure 1). The purified anti-TNP molecules also gave reactions of apparent identity with DNP and TNP proteins (Figure 1). The purified antibodies failed to precipitate in gel with the underivatized carrier proteins, BγG or HSA, and even unfractionated antisera made against TNP-BγG precipitated little if at all with the unsubstituted carrier protein.

The maximal amounts of antibody precipitated by

TABLE II: Effect of Precipitating Antigen on Antibody Yield in Purification.[a]

Serum Pool	Precipitating Antigen	Max Amt of Antibody Precipitated from 1 ml of Serum (mg)	Recov of Antibody in Purifcn (%)	
TN-8	TNP-HSA	0.321	14.1	14.4
TN-8	DNP-HSA	0.202	12.0	13.6
TN-8	TNP-BγG	0.325		
TN-8	DNP-BγG	0.202		
TN-9	TNP-HSA	1.97	7.0	
TN-9	DNP-HSA	1.87	44.5	

[a] Antiserum TN-8 was an early bleeding and antiserum TN-9 was a late bleeding after immunization with highly substituted TNP-BγG. TNP-HSA and DNP-HSA contained 36 moles of TNP and 37 moles of DNP/70,000 g of HSA, respectively. The antibody purification steps which followed precipitation by each of the antigens were identical (see text). Recovery of antibody was calculated from the amount of purified antibody soluble in 0.001 M phosphate, pH 7.4 (pseudoglobulin), relative to the amount of antibody precipitated from serum at the equivalence point in the precipitin curve with TNP-HSA.

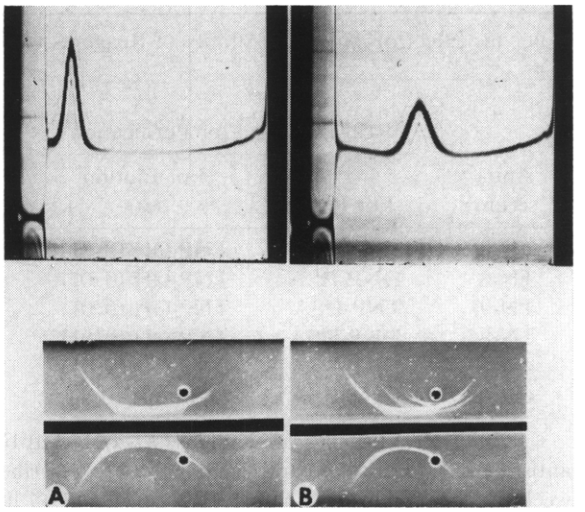

FIGURE 2: Upper panel: photographs taken with schlieren optics during ultracentrifugation of purified antibody TN-9, 3 mg/ml in 0.1 M KCl, 0.1 M Tris–Cl, pH 7.44. Rotor speed 52,640 rpm. Exposures were taken at 8 (left) and 64 min (right). Calculated $s_{20,w}$ = 6.30. Lower panels: immunoelectrophoreses performed in 1.5% agar, Barbital buffer, pH 8.6. Samples placed in wells and troughs are as follows. (A) Upper well, normal rabbit serum; lower well, purified antibody TN-9; trough, guinea pig antiserum against rabbit anti-TNP antibodies (TN-9) obtained as described in text. (B) Upper well, normal rabbit serum; lower well, purified antibody TN-0; trough, goat antiserum to crude rabbit serum globulin fraction.

DNP and TNP proteins are shown in Table II. One antiserum pool (TN-8) was obtained 16 days after immunization of each animal with 2.0 mg of TNP-BγG in complete Freund's adjuvant. This represents "early" antibody, since precipitating anti-TNP antibody is not usually detectable in antisera until approximately 14 days after the initial injection of immunogen. The second pool, TN-9, was "late" antibody; it was obtained 21 days after injecting 0.5 mg of TNP-BγG in complete adjuvant into animals that had received 5.9 mg of TNP-BγG as one injection 1 year previously. The binding studies described below establish that the anti-TNP molecules isolated from the early and later sera had low and high affinity, respectively, for ε-TNP-lysine.

The extent of cross-reactions of these sera with DNP proteins also differed and similar differences were found in many other comparable sera. In general, the early, low affinity anti-TNP antibodies cross-react less extensively with DNP proteins than the late, high affinity antibody populations; *i.e.*, the antibodies obtained from early bleedings appear more specific. With the low and high affinity pools the antibodies were precipitated as well by TNP groups on an unrelated carrier protein (TNP-HSA) as they were by the immunogen, TNP-BγG. This suggests that the most important portion of the antigenic determinant resides in the TNP-lysyl group and that neighboring amino acid residues probably make only a small energetic contribution, if any, to the binding of antigen by the antibody.

Antibody Purification. In the purification of anti-TNP molecules the yield and the average affinity of the isolated molecules reflect the selective reactions of the various reagents used for precipitation and elution (Eisen *et al.*, 1966). To illustrate these principles, the serum pools TN-8 and TN-9 were purified by precipitation with both the homologous and heterologous antigens, TNP-HSA and DNP-HSA, using the picrate ion for elution in both instances. The relative yields and the average affinities of the antibodies obtained from the several purification procedures are shown in Tables II and III. The yield of purified antibody from both low and high affinity populations was low when the initial specific precipitate was made with TNP protein. With the high affinity antibody population, however, the yield was improved about sixfold utilizing DNP-HSA, the cross-reacting antigen, for precipitation. Similarly, anti-DNP antibodies can be recovered from late anti-DNP sera in higher yields when initially precipitated with the cross-reacting protein, TNP-HSA (Eisen *et al.*, 1966). It can also be seen in Table III that antibodies have essentially the same affinity for a homologous ligand (either TNP-aminocaproate or TNT) whether purified by precipita-

TABLE III: Effect of Antibody Affinity of Reagents Used in Purification.[a]

Anti-serum	Reagents Used for Purification: For Pptn	Reagents Used for Purification: For Elution (M)	Affinity of Anti-TNP Antibodies for Haptens (M^{-1}): 2,4,6-TNP-Amino-caproate	2,4-DNP-Amino-caproate	TNT	DNT
TN-8	TNP-HSA	TNP-OH (0.01)	6.9×10^7	2.7×10^6		
TN-8	DNP-HSA	TNP-OH (0.01)	4.7×10^7	6.3×10^6		
TN-9	TNP-HSA	TNP-OH (0.01)	$\geq 1 \times 10^9$		1.8×10^7	6.0×10^6
TN-9	DNP-HSA	TNP-OH (0.01)	$\geq 1 \times 10^9$		1.4×10^7	9.0×10^6
423	DNP-HSA	TNP-OH (0.01)	4.1×10^7			
423	DNP-HSA	DNP-OH (0.1)	3.2×10^7			

[a] Affinity of antibodies for haptens, K_0, obtained by fluorescence quenching titrations as described in text. Early antiserum TN-8 and late antiserum TN-9 are described in text. Antiserum 423 was obtained from a single rabbit 5 weeks after immunization with 1.0 mg of TNP-BγG in complete Freund's adjuvant. TNP-OH and DNP-OH refer to 2,4,6-trinitrophenolate (picrate) and 2,4-dinitrophenylate ions, respectively.

tion with TNP-HSA or DNP-HSA. As a result of this and similar experience with other antisera it was generally preferable to isolate anti-TNP antibodies by using DNP-HSA, rather than TNP-HSA, for initial precipitation, unless the antiserum was obtained very soon after immunization, *i.e.*, unless the antibodies were of very low affinity.

Since picric acid is a well-known denaturing agent for proteins it was of interest to compare the antibodies recovered by elution with picrate (0.01 M) and 2,4-dinitrophenylate (0.1 M). Both antibodies were the same in binding properties (Table III), and those isolated with picrate were not aggregated (Figure 2).

Antibody Purity. The purity of each lot of anti-TNP antibodies was evaluated by quantitative precipitin analysis using TNP-HSA as antigen and by immunoelectrophoresis. The extent of precipitability varied somewhat with the concentration of antibody in the test solution, especially with antibodies of low affinity. At a concentration of 1 mg/ml, high affinity antibodies were 90–95% precipitated, and at 2 mg/ml they were >95% precipitated. The low affinity preparations were 90% precipitated when tested at 1 mg/ml.

Immunoelectrophoresis was performed in agar gel at pH 8.6 on purified antibody samples with a goat antiserum to whole rabbit serum or to a crude rabbit globulin fraction to develop the precipitin lines. All rabbit antibody samples were found to produce a single line of γG mobility (Figure 2b).

Selected antibody samples were also examined in a Spinco Model E ultracentrifuge equipped with schlieren optics (Figure 2). The pseudoglobulin antibody preparations had uncorrected sedimentation coefficients of 6.3–6.7 $\times$ 10^{-13} sec with a symmetrical boundary and without detectable faster or slower sedimenting material.

An additional test for purity was carried out with one antibody preparation (TN-9). This purified protein was used to immunize intensively ten randomly bred Hartley-strain guinea pigs. The antisera from individual animals were then used to develop an immunoelectrophoresis performed with normal rabbit serum. Only one line of γG mobility was observed in each sample (Figure 2A). Had the purified rabbit antibody preparation used as an immunogen contained any non-γG immunogenic impurities the antisera would probably have revealed the formation of additional precipitation lines when treated with normal rabbit serum, in which all possible contaminants presumably reside.

Dowex 1 did not succeed completely in removing picrate during the purification of those anti-TNP antibodies of particularly high affinity for the picryl group. A maximum estimate of the proportion of antibody sites occupied by picrate was made from the absorbance at 360 mμ. With antibodies of low or moderately high affinity the amount of picrate retained corresponded to 2–6 mole % of the antibody; with high affinity antibody the picrate contamination amounted to 10 mole %.

Q_{max} Values. For the calculation of an antibody–ligand association constant from fluorescence quenching it is necessary to establish the antibody's Q_{max} value, *i.e.*, the extent to which the antibody fluorescence is quenched when its active sites are saturated by ligand. The Q_{max} values were the same for different samples of purified anti-TNP molecules isolated from a given antiserum, but these values varied from Q_{max} = 52% to Q_{max} = 62% for antibodies isolated from different antisera. In each case the ligand used to saturate the antibody active sites was TNP-aminocaproate (Figure 3). If a Q_{max} value is accurate then association constants determined by fluorescence quenching, based on this Q_{max}, should agree well with association constants determined by an independent method, *e.g.*, equilibrium dialysis.

With two different antibody populations the average

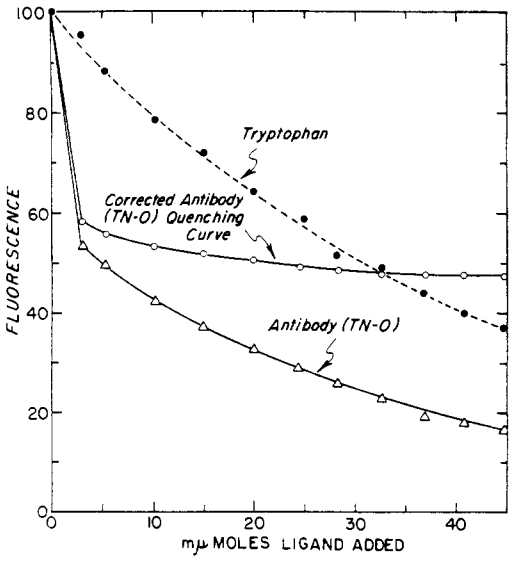

FIGURE 3: The Q_{max} value for antibody, TN-0. One milliliter of purified antibody, 60 μg/ml, was titrated with 5.0 × 10^{-4} M TNP-aminocaproate in buffered saline. The changes in fluorescence intensity after additions of hapten represent the combined effects of quenching by hapten bound specifically by the antibodies and the attenuation of fluorescence intensity due to the high concentration of unbound ligand. Δ——Δ, change in fluorescence of antibody. ●----●, change in fluorescence of tryptophan solution, both titrations with 5 × 10^{-4} M TNP-aminocaproate. The corrected quenching curve for antibody shown by O——O was obtained according to the method of Day *et al.* (1963). The Q_{max} value calculated from these data, 52%, was validated by equilibrium dialysis (see text).

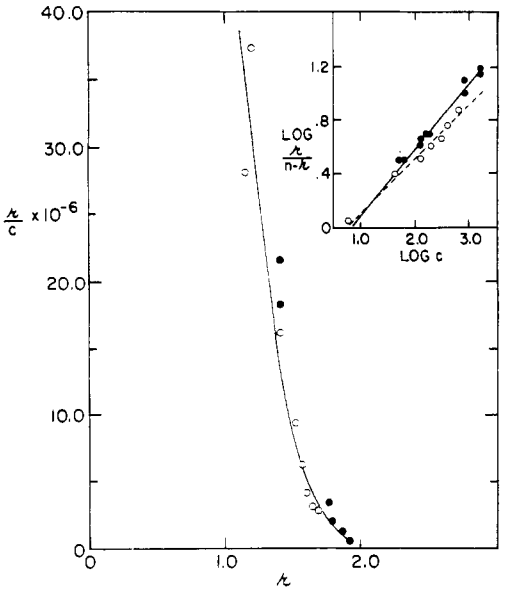

FIGURE 4: Binding of ε-TNP-L-lysine at 30° by purified antibody preparation TN-0. Fluorescence quenching (open circles) and equilibrium dialysis (closed circles) were performed in 0.1 M Tris–Cl at pH 7.4. Equilibrium dialysis with ε-TNP-L-[^{3}H]lysine yielded K_0 = 1.59 × 10^8 M^{-1}. Using the Q_{max} for this antibody population (52%) derived from the data in Figure 3, the K_0 determined by fluorescence quenching was 1.42 × 10^8 M^{-1}. The antibody preparation TN-0 was purified from an antiserum pool obtained from 51 rabbits 5 weeks after immunization with TNP-BγG. The concentration of free hapten in the insert is given in micromicromoles per millliter.

association constant determined by fluorescence quenching was compared with that obtained by equilibrium dialysis (Figures 4 and 5). The association constants measured by the two methods are in agreement. However, the slightly lower slope in the Sips plots of the fluorescence quenching data indicates that this method tends to overestimate the heterogeneity with respect to affinity.

The affinity of sample TN-9 for TNP-aminocaproate was too high to be measured accurately, and essentially all of the combining sites were saturated in the presence of relatively low concentrations of this ligand, as shown in Figure 6. At saturation the antibody fluorescence was 44% of its initial value and further addition of ligand resulted only in absorbance but no further quenching (Figures 6 and 7). The Q_{max} was thereby empirically determined to be 56% with TNP-aminocaproate. With this value (Q_{max} = 56%) in the calculation of fluorescence quenching titrations of antibody TN-9 with 2,4-dinitroaniline, the K_0 was found to be 3.33 × 10^7 M^{-1}, in good agreement with the equilibrium dialysis data which gave a K_0 with [1-^{14}C]-2,4-dinitroaniline of 3.63 × 10^7 M^{-1} (Figure 5). These results imply that the Q_{max} value for a given anti-TNP antibody is the same when the active sites are saturated with a TNP or a DNP ligand. This implication was shown to be valid by fluorescence quenching titrations of antibody TN-9 with high concentrations of TNP-aminocaproate and dinitroaniline. The same Q_{max} value was found for the two haptens (Figure 7).

Proteolytic Fragments. Figure 6 compares the quenching behavior of intact antibody molecules with *unseparated* papain proteolytic fragments (Fab and Fc). The unresolved papain digest and the intact antibody molecules behaved indistinguishably in fluorescence quenching titrations with TNP-aminocaproate, whether or not the fragments had been alkylated with iodoacetic acid. These results support earlier findings that univalent fragments of a given antibody have the same affinity for the homologous hapten and the same heterogeneity with respect to affinity as the bivalent antibody molecules from which the fragments were derived (Nisonoff *et al.*, 1960). Thus the binding of ligand at one active site of a bivalent antibody molecule does not appear to influence the affinity of the other site, at least when the ligands are small molecules. These results also indicate that the fluorescence arising in

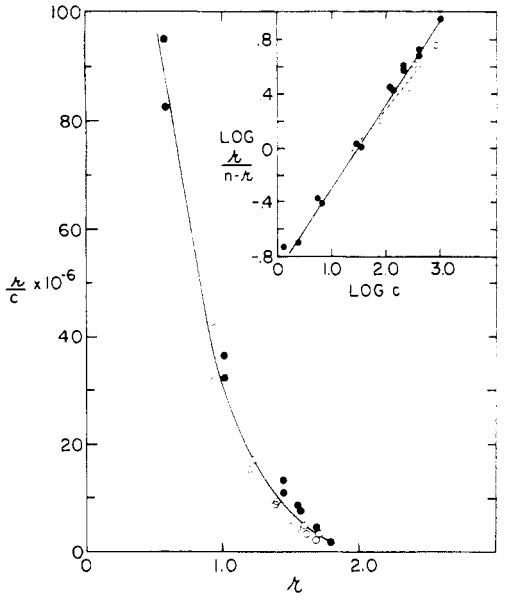

FIGURE 5: Binding of [1-^{14}C]-2,4-dinitroaniline at 30° by purified antibody preparation TN-9. Fluorescence quenching (open circles) and equilibrium dialysis (closed circles) were performed in buffered saline. Association constants and Q_{max} value are discussed in text.

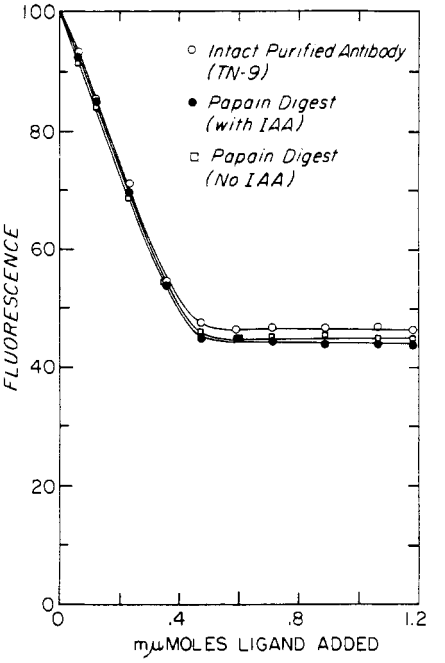

FIGURE 6: Essentially identical fluorescence quenching curves obtained with purified antibody TN-9 and the papain digest of TN-9, with and without alkylation with iodoacetic acid. Antibody (0.22 mμmole) was titrated with TNP-aminocaproate at 30° in buffered saline. The values shown are the average of duplicates. K_0 was $\geq 1 \times 10^9$ M^{-1} and fluorescence levels off after addition of the equivalent amount of ligand, which is 0.43 mμmole, since the antibody is bivalent (see Figures 4 and 5).

the Fc domain is not quenched when ligand is bound in the antibody combining sites. In support of this conclusion Figure 8 illustrates that the fluorescence of the active fragments, Fab and F(ab')2, was quenched 82% at saturation. This is in contrast with the Q_{max} of 56% for the intact antibody molecules from which these fragments were derived.

Dependence of Binding Constants on pH and Ionic Strength. Various lots of anti-TNP antibodies showed only minor changes in ligand binding constants over a broad pH range. Values for K_0 were essentially identical with a given hapten in Tris–acetate buffer between pH 4 and 8. At pH 1.5 and 10.5 the average association constant was usually one decimal order of magnitude lower than that observed in the neutral pH range. Similar results have been reported for anti-DNP

TABLE IV: Binding Constants and Thermodynamic Properties for a Preparation of Anti-TNP Antibody (TN-0).[a]

Hapten	K_0 (M^{-1} × 10^{-7})	Hetero-geneity Index (a)	$-\Delta F°$ (kcal mole^{-1})	$-\Delta H°$ (kcal mole^{-1})	$-\Delta S°$ (eu mole^{-1})
TNP-Aminocaproate	15.1	0.5	11.30	24.35	42.8
ε-TNP-L-Lysine	14.2	0.6	11.28		
TNP-Glycine	0.427	0.8	9.15		
Trinitrotoluene	0.393	0.3	9.13	17.41	27.3
Trinitrophenol	0.0165	0.4	7.15		
DNP-Aminocaproate	0.0930	0.5	8.27	9.43	3.8
ε-DNP-L-Lysine	0.0305	0.2	7.61		

[a] The antiserum pool is the same one described in the legend of Figure 4 and Table V. The $\Delta H°$ values were calculated from the Arrhenius plots in Figure 9. The K_0 and a values were obtained by fluorescence quenching titrations in 0.1 M Tris–Cl, pH 7.4, at 30°.

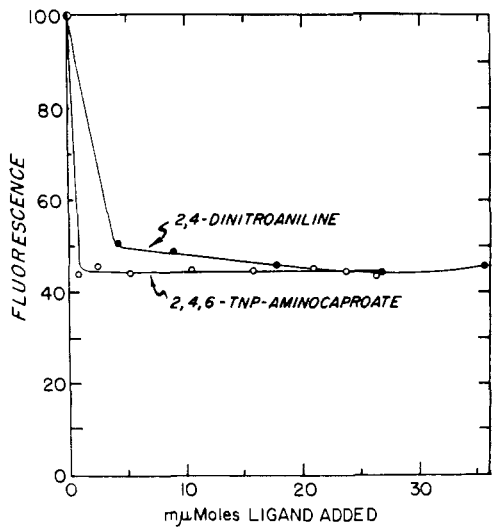

FIGURE 7: Identical results are shown for the estimation of maximal fluorescence quenching, Q_{max}, for antibody TN-9 with highly concentrated solutions of TNP-aminocaproate and 2,4-dinitroaniline. Both quenching curves have been corrected for nonspecific attenuation of fluorescence by the method shown in Figure 3 (Day *et al.* 1963), and described in the text. All titrations were performed at 30° in buffered saline.

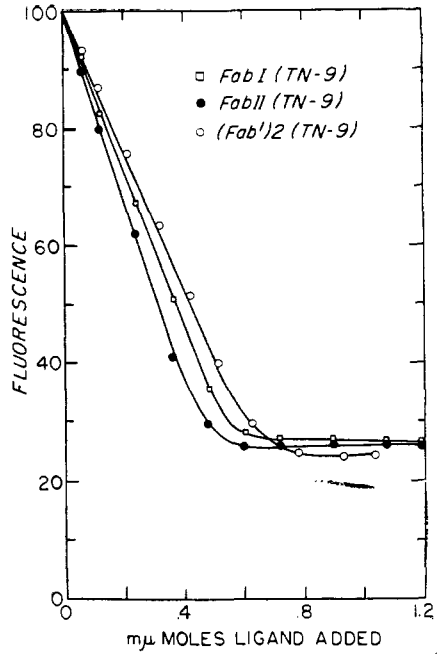

FIGURE 8: Fluorescence quenching curves obtained with the active fragments of purified antibody TN-9. (See Figure 6 for quenching curves of the intact molecules and unseparated fragments.) The ligand used was TNP-aminocaproate and K_0 was $\geq 1 \times 10^9$ M^{-1}, assuming $Q_{max} = 82\%$ and the molecular weight of the Fab and (Fab')2 fragments to be 45,000 and 92,000, respectively. Assuming the Fab and F(ab')2 fragments to have one and two sites per fragment, respectively, the equivalent amount of ligand was 0.55 mμmole.

antibodies (Velick *et al.*, 1960). No significant difference was observed in hapten binding at pH 7.4 at NaCl concentrations varying from 0.005 to 5.0 M.

Affinity and Specificity for Simple Ligands. In Table IV binding strengths are shown for one antibody population and various ligands. TNP-aminocaproate was bound slightly better than ε-TNP-L-lysine. This difference, which was also observed with several other antibody preparations, probably reflects the greater solubility in nonpolar solvents of the aminocaproate derivative compared to ε-TNP-L-lysine. Other ligands were bound less strongly, and the antibody's affinity for them decreased in accord with their structural differences from the homologous haptenic group, the ε-TNP-L-lysyl residue.

The rise in average association constant of antibodies purified from rabbit antisera obtained at different times in the immune response to DNP-BγG has been extensively documented (Eisen and Siskind, 1964). A similar increase in affinity with time has also been observed with rabbit antibodies to a large anionic group in which *p*-(*p*-*N*-acetylphenylazo)hippurate is linked *via* *S*-acetylsuccinyl to lysine residues (the SU_p group) (Fujio and Karush, 1966). Table V indicates an increase in average intrinsic association constants of purified anti-TNP antibodies for several haptens. The increase was especially pronounced with trinitrophenol. The antibodies were isolated from the same group of rabbits 5 and 10 weeks after immunization.

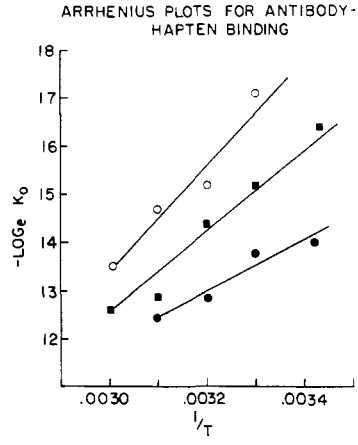

FIGURE 9: Arrhenius plots for the determination of $\Delta H°$, the standard enthalpy change in the formation of hapten–antibody complexes, for a single purified antibody preparation (TN-0, 5 weeks) and three univalent ligands, ε-TNP-L-lysine (open circles), TNT (closed squares), and ε-DNP-aminocaproate (closed circles).

Discussion

There is considerable similarity in the reactions of amino acid side chains with 2.4.6-trinitrochlorobenzene

TABLE V: Association Constants and Binding Energy for Two Antibody Preparations Obtained at Different Times after Immunization.[a]

	Antibody Purified from Antiserum Pool (TN-0) Obtained			
	5 Weeks after Immunization		10 Weeks after Immunization	
Hapten	K_0 ($M^{-1} \times 10^{-7}$)	$-\Delta F^\circ$ (kcal $mole^{-1}$)	K_0 ($M^{-1} \times 10^{-7}$)	$-\Delta F^\circ$ (kcal $mole^{-1}$)
TNP-Amino-caproate	15.1	11.30	34.7	11.84
TNT	0.393	9.13	0.988	9.65
TNP-OH	0.0165	7.15	5.31	10.70
DNP-Amino-caproate	0.0930	8.27	2.63	10.28

[a] Rabbits (51) were immunized by foot-pad injection of 2 mg of TNP-BγG in complete Freund's adjuvant. Each animal was bled by cardiac puncture 5 weeks after immunization and the 48 surviving animals were bled 10 weeks after immunization. Antibodies were purified from the separate 5- and 10-week serum pools and the K_0 values were obtained by fluorescence quenching titrations in 0.1 M Tris–Cl, pH 7.4, 30°.

(picryl chloride) or trinitrobenzenesulfonic acid and the comparable reactions with 2,4-dinitrochlorobenzene or -dinitrobenzenesulfonic acid. The presence of the third nitro group on the aromatic ring of TNB-sulfonic acid renders C_1 more reactive than with 2,4-dinitrobenzenesulfonic acid. Consequently desulfonative trinitrophenylation reactions proceed with moderate speed at room temperature in water solution at pH 8 (Okuyama and Satake, 1960); these conditions are somewhat milder than those usually employed for dinitrophenylation with DNB-sulfonic acid (Eisen *et al.*, 1953; Eisen, 1964). No special effort was made in the present work to minimize denaturation during the trinitrophenylation reaction. Nonetheless completely soluble and easily characterized preparations of highly substituted proteins were obtained routinely. Trinitrophenylation of BγG with picryl chloride, on the other hand, always yielded a large proportion of insoluble protein.

The amino acid analyses confirm the high degree of specificity of TNB-sulfonate for amino groups of proteins at pH 8–9; of the residues analyzed only the lysine content was reduced (Table I). There was no significant substitution of the OH group of tyrosine, as would have occurred if picryl chloride had been used for trinitrophenylation (Okuyama and Satake, 1960).

The purification scheme given above (see Methods) is in accord with recent modifications proposed for the isolation of anti-DNP antibodies (Eisen *et al.*, 1966). One of the advantages in undertaking a comparative study of anti-TNP and anti-DNP antibodies arises from the feasibility of using the same methods and reagents for purifying each of them, thereby minimizing possible artifacts due to selection from the respective antisera of antibody populations with differences that are unrelated to specificity, *e.g.*, differences in net charge.

When antibody is solubilized from a specific precipitate by the introduction of high concentration of a weakly bound univalent ligand, such as picrate or 2,4-dinitrophenolate, the precipitated antigen (TNP or DNP protein) is also solubilized. Though streptomycin precipitates these highly anionic substituted proteins almost quantitatively, traces remain soluble, leading to substantial loss of antibody by reprecipitation when the univalent hapten is removed by dialysis. In the present scheme passage of the solubilized specific precipitate, consisting of antibody, hapten, and antigen, through a double-layered column was effective in first removing the antigen, on the upper DEAE-cellulose layer, and then removing the hapten, on the lower Dowex 1 layer.

The solubilization of the specific precipitate, a critical step in the purification procedure, depends on competition between the univalent hapten and the antigen for the active sites of antibody. A cross-reacting but precipitating antigen competes less well with a homologous hapten and leads to a higher yield of purified antibody. Since cross-reacting antigens are bound less strongly than homologous antigens, they would also be expected to yield greater recovery of purified antibody even when nonspecific means (*e.g.*, dilute acid) are used to solubilize antigen–antibody precipitates.

The 30 samples of purified rabbit anti-TNP antibodies examined by immunoelectrophoresis and by ultracentrifugation were all 6.3- to 6.7S γG-immunoglobulins. Despite particular attention to antibodies isolated from early bleedings, no γM-immunoglobulin was detected.

By the method of fluorescence quenching rabbit anti-TNP molecules have Q_{max} values of 52–62% and are therefore less quenchable at saturation than rabbit anti-DNP molecules (Q_{max} = 70–80%). Both antibodies appear to display their respective Q_{max} values whether they are saturated with DNP or TNP ligands. This implies that these two antibodies, despite their extensive cross-reaction, differ substantially in the efficiency with which excitation energy is transferred to bound ligands. However, these differences are not strictly related to specificity since the corresponding antibodies isolated from guinea pig sera had the same Q_{max} value: 85–90% for both anti-TNP and anti-DNP antibodies, when either TNP or DNP ligands were bound (unpublished data). Since the active fragments (Fab or (Fab')2) of anti-TNP molecules are not completely quenched (Figure 8), some of the tryptophans in the active fragments do not transfer their excitation energy to the bound ligand in the active site. Alternatively it is possible that some denaturation occurred

during separation of the fragments. The difference in quenching between Fab I and Fab II (Figure 8) could be due either to the presence of a small amount of undigested antibody in Fab I or to a real but slight difference in their structures. The indistinguishable fluorescence quenching titrations with the intact and papain-digested antibodies (Figure 6) indicate that the binding of ligand at one antibody-combining site does not result in excitation energy transfer from the Fc region or from the other Fab region of the same molecule.

The lack of wide differences in association constants with changes in ionic strength and pH is expected since trinitrobenzene, the central element of the haptenic group, is not ionizable. The decreased binding at the extremes of pH (1.5 and 10.5) is probably due to changes in conformation of the antibody, though we cannot rule out the possibility that the formation of molecular adducts of trinitrobenzene with OH^- ions (charge-transfer complexes) is responsible for the decreased binding at high pH.

As is shown in the Arrhenius plots of Figure 9 and the ΔH° values in Table IV the large negative free energy change accompanying the binding of ligands by anti-TNP antibodies arises from the change in enthalpy. The magnitude of the negative enthalpy change is surprisingly large, even exceeding that observed when DNP ligands are bound by high affinity anti-DNP antibodies. The sources of the large enthalpy changes are obscure.

An additional point of interest is that the decrease in strength of hapten binding observed at high temperatures was completely reversible. Dilute antibody samples heated at 60° for 30 min and then cooled to 30° were indistinguishable in their association constants from unheated samples of the same antibody preparation.

Analysis of the ligand binding reactions of anti-TNP antibodies reveals striking similarities with the results previously reported for anti-DNP antibodies. Intrinsic association constants are in the same range; they are relatively unaffected by changes in pH or ionic strength, and they increase with time after immunization. Yet rabbit antibody molecules specific for the TNP group are different in their fluorescence quenching properties when compared with anti-DNP antibody molecules. These observations suggest that significant structural differences exist between the two antibody populations. A detailed analysis of these structural differences will be the subject of a forthcoming publication.

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