# Recognition of Carrier Residues Adjacent to Hapten by Anti-Trinitrophenyl Antibodies*

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ABSTRACT: 2,4,6-Trinitrophenyl (TNP) groups were conjugated to lysine residues in a protein carrier and guanosine residues in a deoxyribonucleic acid carrier. Antibodies specific for the TNP group were purified by immunoadsorption and their relative binding properties studied in relation to various ligands. Equilibrium dialysis binding and fluorescence quenching studies indicated small but apparently significant differences between the two antibody sites. However, anti-TNP-guanosyl antibodies bound guanosyl ligands directly in fluorescence quenching inhibition studies and in equilibrium dialysis. The anti-TNP-lysyl antibodies did not bind these ligands to a significant extent. Binding data indicated that the average intrinsic association constant for the TNP site in the anti-TNP-guanosyl antibody was 200× greater than the quanosyl site. The TNP site was more heterogeneous than the weaker binding site. Data are presented showing that the two antibodies produced distinguishable difference spectra in spectral shift studies.

Reports in recent years have strongly supported the concept that antibody specificity is dictated by a unique amino acid sequence (Haber, 1964; Buckley *et al.*, 1963; Whitney and Tanford, 1965; Freedman and Sela, 1966; Koshland *et al.*, 1966). Amino acids conveying specificity can be classified as "contact" amino acids with close spatial orientation to the bound antigenic determinant group (ligand) or may be "noncontact" but influence the conformational (tertiary) structure of the site. Studies utilizing haptens and/or various chemical reagents have served as valuable probes to determine which amino acids are important in antibody specificity (Wofsy *et al.*, 1962; Little and Eisen, 1967; Pressman and Grossberg, 1968). These studies have indicated amino acids such as tyrosine and tryptophan are important, but have not delineated their precise function or location within the site. Similarly, results comparing the amino acid content of purified anti-hapten antibodies (Koshland *et al.*, 1966) have suggested that various amino acids are important. Generally, amino acid studies have compared data as they relate to the haptenic group but have not studied the site in relation to the effect moieties adjacent to the hapten may have on the formation or selection of the antibody site.

The problem becomes more complex if the antibody combining site is visualized as a series of cavities (cleft) similar to the active site of lysozyme as shown by X-ray crystallography studies (Blake *et al.*, 1965). Each cavity within the antibody cleft may contain a unique amino acid sequence which conveys specificity to the appropriate ligand. Support for this concept is found in studies which have attempted to estimate the size of the antibody active site (Kabat, 1960, 1966, 1968). Inhibitory studies with derivatives of the isomaltose series on the dextran–human antidextran precipitating system showed the antibody site combined efficiently with a hexasaccharide or heptasaccharide. Analysis of antigenic fragments of silk fibroin (Cebra, 1961) indicated that the antibody combined with an antigenic peptide fragment consisting of 8–12 amino acids. Thus, the antibody site can bind complex polymers and it is important to know how specificity is encoded into the active site to provide binding ability to each component or subunit of the complex antigen.

To begin to study this problem one must compare the same hapten combined to two different subgroups. In each case a stringent requirement of a monospecific linkage is mandatory to the success of the experiments. We recently reported (Voss *et al.*, 1969) the specific covalent linkage of 2,4,6-trinitrophenyl groups to deoxyguanosine residues in deoxyribonucleic acid (carrier). Induction of antibody by this complex yields an antibody protein whose site (*i.e.*, anti-TNP-guanosyl) can be studied and compared with the more conventional anti-TNP-lysyl site induced by the TNP–protein carrier system (Little and Eisen, 1966).

This report presents the comparative binding studies between these two antibodies and provides a basis for future studies.

## Materials and Methods

*Preparation of Trinitrophenylated Deoxyribonucleic Acid (TNP-DNA).* Soft roe DNA (150 mg, Sigma Chemical Co.) was dissolved, with mild stirring, in 100 ml of 2.5% sodium dodecyl sulfate–0.15 M NaCl–0.0.15 M sodium citrate buffer (pH 8.0) for 48 hr under an ultraviolet light at room temperature. The detergent and ultraviolet light were used to prevent bacterial contamination. Dissolved DNA was heated to 90° ($T_m$) and an equal weight of anhydrous potassium carbonate was added followed by 2,4,6-trinitrobenzenesulfonate (ten times by weight, sodium dihydrate, Pierce Chemical). Temperature was maintained at 90° for 20 min and then 37° for 24 hr. Low molecular weight impurities and by-products were removed from the reaction mixture by extensive dialysis or

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precipitation of the substituted DNA with two volumes of 95% ethanol. Determination of the degree of substitution was based on dry weight analysis and absorbance at 420 mμ. A molar extinction coefficient of 15,000 was used for the *N*-TNP-deoxyguanosine conjugate (pH 8.0).

TNP-DNA preparation were treated with 0.04% pronase (Calbiochem, B grade) by weight in 0.001 M $CaCl_2$ at 37° for 4 hr to remove protein contaminants. The preparation were further dialyzed against five changes of the sodium citrate buffer and 0.001 M EDTA.

*Preparation of TNP-DNA and Methylated Albumin Complexes.* MBSA[1] was prepared according to the method of Mandell and Hershey (1960). BSA (1 g; Pentex, Inc.) was dissolved in 100 ml of absolute methanol containing 1 ml of 12 N HCl. After sitting in the dark for 72 hr with occasional shaking, the precipitate was collected and washed twice with absolute methanol and then with anhydrous ether. Ether was partially evaporated in air, and final drying was done *in vacuo* over NaOH pellets.

To 100 ml of TNP-DNA (1–2 mg/ml) was added a 1% solution of MBSA with mild stirring until the final weight ratio of MBSA:TNP-DNA was 1.

The orange precipitate was pelleted at 10,000 rpm for 30 min, rinsed twice with buffer, and suspended in 0.04 volume (4 ml) of buffer with an 18 gauge needle and syringe.

*Preparation of (TNP-BGG).* $TNP_{47}BGG$ was prepared as previously described (Little and Eisen, 1966).

*Preparation of Antisera.* TNP-DNA-MBSA was emulsified was an equal volume of complete Freund's adjuvant (Difco). Albino rabbits (2–3 kg) were given primary immunizations of 0.5 ml of the TNP-DNA-MBSA emulsion (24 mg/ml) in each hind footpad. One week later each rabbit received a 1.5-ml intramuscular injection of the emulsion. Two weeks after the initial injection each rabbit received 0.75 ml/hind footpad. Rabbits were bled at 21 and 22 days and the antisera were pooled. This pool (pool 1) served as the source of antibodies to be referred to as anti-TNP-guanosyl in the next.

Rabbits immunized with $TNP_{47}BGG$ in complete Freund's adjuvant (10 mg/rabbit) were boosted (5.0 mg/rabbit) 4 months after the primary immunization. Bleedings were obtained 14, 17, and 20 days after the booster injection and antibodies specifically purified from this pool (pool 2) were referred to as anti-TNP-lysyl antibodies in the text. Both antibody pools were greater than 95% active when assayed by readsorption to the adsorbent or by immune precipitation.

*Preparation of Immunoadsorbent.* DNP and TNP immunoadsorbents were prepared as previously described (Voss *et al.*, 1969) according to the method of Robbins *et al.* (1967).

*Antibody Purification.* Antisera were adsorbed with a tenfold excess of immunoadsorbent based on the capacity of the adsorbent (200 mg of antibody protein/g of derivatized cellulose) and an estimated amount of antibody present per milliliter of sera. Adsorptions were carried out for 2 hr at room temperature under mild stirring conditions. EDTA was added to a final concentration of 0.001 M to inhibit complement complexing. The antibody–immunoadsorbent complex was sedimented by centrifugation at 10,000 rpm for 10 min and washed four to five times in 0.05 M $PO_4$ pH 8 buffer. Supernates were monitored at 278 mμ for release of nonspecific protein. When the absorbancy at 278 mμ was less than 0.05, three mock elutions were performed based on the specific elution with hapten. Two 1-hr 37° incubations in 0.5 M $PO_4$ (pH 8) were performed in addition to two 1-hr 37° incubations with 10 ml of 0.1 M Cbz-Gly in $PO_4$ buffer. These mock elutions served as controls for the release of nonspecific proteins due to temperature, buffer, pH, and ionic environment. Antibodies were then eluted with two 1-hr 37° incubations with 5 ml each of 0.1 M TNP-Gly in 0.05 M $PO_4$ (pH 8).

Supernatants of the two elutions were pooled and passed over Dowex 1-X8 (in a column 10 × 2 cm equilibrated in 0.05 M $PO_4$, pH 8) to remove the hapten.

Both anti-TNP-guanosyl- and anti-TNP-lysyl-purified antibodies were judged pure by radioimmunoelectrophoresis, acrylamide disc gel electrophoresis, and were 95–100% active by readsorption to the adsorbent as previously described (Voss *et al.*, 1969).

*Preparation of ε-TNP-L-Lys.* ε-TNP-L-Lys was obtained by the trinitrophenylation of the copper derivative of lysine in which the α-amino group was blocked in a copper complex with the carboxyl group (Porter and Sanger, 1948; Okuyama and Satake, 1960).

A solution containing 1.7 g of L-Lys (Nutritional Biochemical Corp.), 800 mg of NaOH, and 800 mg of $CuSO_4$ in 17.5 ml of water was heated to 60° for 20 min to form the copper complex. To the reaction mixture was added 1 g of $NaHCO_3$ and 2.5 g of 2,4,6-trinitrobenzenesulfonate and the reaction was stirred at 37° for 60 min. The precipitate was collected, washed in 0.05 M $PO_4$ (pH 8.0), and resuspended in 6 N HCl. The product was further resolved and harvested from silica gel in water-saturated methyl ethyl ketone ($R_F$ 0.72). It was possible to distinguish between ε-TNP-L-Lys and α,ε-di-TNP-L-Lys on silica gel. The disubstituted compound was made as a control and was found to migrate close to the solvent front.

[$^3$H]ε-TNP-L-Lys was prepared from 0.03 mg of L-[$^3$H]Lys (New England Nuclear, lot 443-139) which after drying at 50° to remove the alcohol was suspended in water. To the labeled lysine was added 50 μl of $CuSO_4$ (10 mg/ml) and 50 μl of 0.1 M $NaHCO_3$. The reaction mixture was heated to 60° for 15 min, cooled, and then 16 μl of 2,4,6-trinitrobenzenesulfonate (12.5 mg/ml) was added. The precipitate and compound were then prepared as described above.

*Preparation of TNP-guanosine Derivatives.* *N*-TNP-guanosine compounds were prepared by adding an equal weight of potassium carbonate to guanosine or deoxyguanosine dissolved in water. A ten times by weight excess of 2,4,6-trinitrobenzenesulfonate was added and the reaction was incubated at 37° for 1 hr. *N*-TNP-guanosine was purified from the reaction mixture by chromatography on silica gel in water-saturated methyl ethyl ketone. [$^3$H]*N*-TNP-deoxyguanosine was prepared by substitution of 8-[$^3$H]deoxyguanosine (Schwarz BioResearch, Inc.; lot 6902, 13.1 Ci/mmole). Chromatographic purification was achieved on silica gel with 1 N HCl saturated methyl ethyl ketone as the solvent ($R_F$ 0.14) at 37°.

*Equilibrium Dialysis Studies.* Equilibrium dialysis experiments were performed as previously described (Voss *et al.*, 1969). Samples (50 μl) of purified antibody were dialyzed against 50 μl of increasing concentration of tritiated ligands

[1] Abbreviations used are: MBSA, methylated bovine serum albumin; BGG, bovine γ-globulin; Cbz, carbobenzoxy.

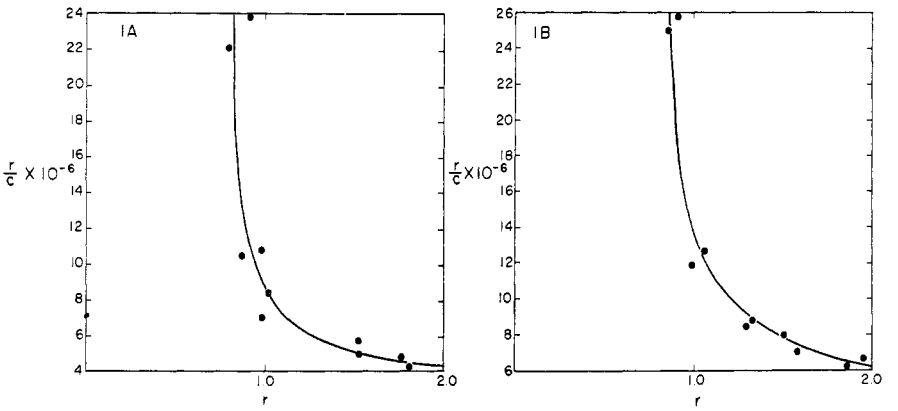


FIGURE 1: Comparison of binding of the ligand [$^3$H]$\epsilon$-TNP-L-Lys with anti-TNP-guanosyl (pool 1) and anti-TNP-lysyl (pool 2) antibodies in equilibrium dialysis. (A) Anti-TNP-guanosyl antibody at a concentration of 650 $\mu$g/ml. (B) Anti-TNP-lysyl antibody at a concentration of 610 $\mu$g/ml. Equilibration was achieved in 22 hr at 4°. The molar extinction coefficient for the ligand was $\epsilon^{348}_{1\,cm}$ 15,400. All antibodies and ligands were dissolved in 0.05 M phosphate (pH 8.0).

using Plexiglass chambers (0.1-ml total capacity/side). Antibody and ligands were dissolved in 0.05 M $PO_4$ (pH 8.0). After 24–48-hr equilibrium 25-$\mu$l 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 the moles of labeled ligand per mole of purified antibody. $c$ is the concentration of free labeled ligand. The ratio $r/c$ was plotted as a function of $r$ (Scatchard, 1949). Valence ($n$) was derived by an extrapolation of the curve to infinite $c$. Average intrinsic association constant, $K_0$, were obtained as the reciprocal of $c$ at $0.5n$. Binding data were analyzed according to the Sips distribution function (Sips, 1948; Karush, 1962) to obtain $a$, the heterogeneity index: $\log [r/(n-r)] = a \log c + a \log K_0$.

*Spectral Shift Studies.* Difference spectra of free and bound ligands with both purified anti-TNP-guanosyl and anti-TNP-lysyl antibodies were obtained using a Cary Model 15 recording spectrophotometer with thermostatic control (20°) as described previously (Little and Eisen, 1967).

*Fluorescence Quenching.* Binding of various ligands by purified antibodies was measured in an Aminco Bowman spectrophotofluorometer at 0–4° by the method of Eisen (1964).

*Pepsin digestions* of purified antibodies were performed according to the method of Nisonoff (1964).

## Results

Anti-TNP-lysyl and anti-TNP-guanosyl antibodies were purified and analyzed independently as described in Materials and Methods. Antibodies purified by means of a TNP-protein adsorbent were deliberately selected for TNP-lysyl specificity. This experimental approach was based on the premise that if differences are shown between sites when purification favors similarity, then the demonstrated divergent properties are significant. Antibodies were shown to be pure by acrylamide disc gel electrophoresis and radioimmunoelectrophoresis as previously described (Voss *et al.*, 1969). Anti-TNP-lysyl and anti-TNP-guanosyl antibodies were compared in equilibrium dialysis binding studies with the ligand [$^3$H]$\epsilon$-TNP-L-Lys. Figure 1A shows a Scatchard binding curve of the ligand with the anti-TNP-guanosyl antibody. An average intrinsic association constant, $K_0$, of 9.0 $\times$ $10^6$ l./mole was measured for the antibody purified from pool 1. Figure 1B shows the binding curve between [$^3$H]$\epsilon$-TNP-L-Lys and the homologous anti-TNP-lysyl antibody purified from pool 2. An average intrinsic association constant of 13 $\times$ $10^6$ was measured. Thus, the homologous ligand was bound somewhat better than the same ligand in the anti-TNP-guanosyl binding site.

Both the anti-TNP-guanosyl site and the anti-TNP-lysyl sites gave a heterogeneity index, $a$, of 0.57 as calculated from a Sips plot shown in Figure 2.

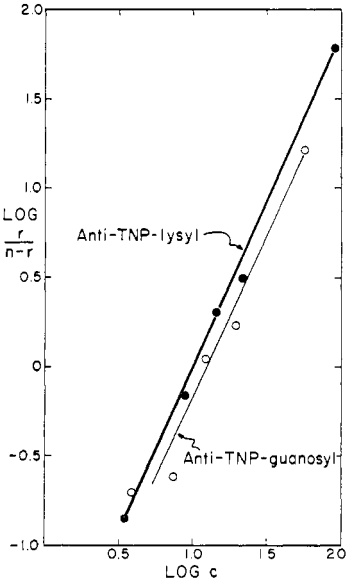


FIGURE 2: Comparative Sips plots of equilibrium dialysis binding data in Figure 1A,B. Both anti-TNP-guanosyl (○—○) and anti-TNP-lysyl (●—●) antibodies gave a heterogeneity index, $a$, of 0.57 with the ligand [$^3$H]$\epsilon$-TNP-L-Lys.

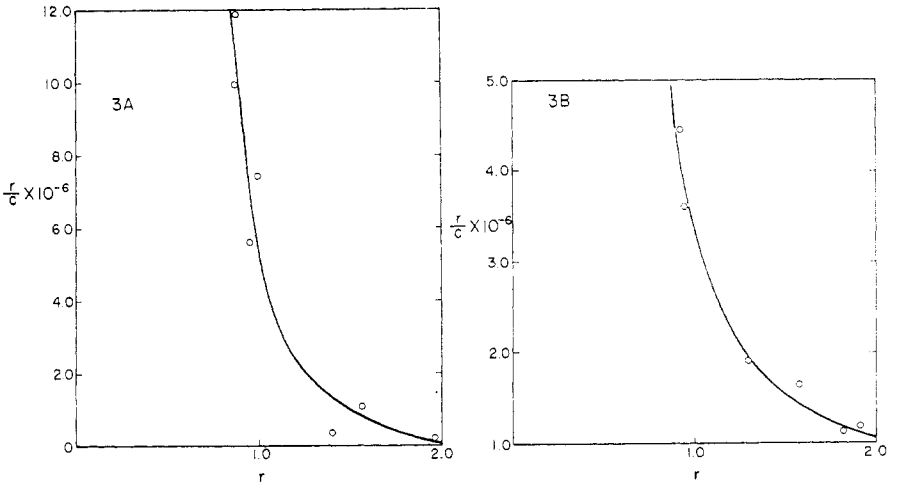


FIGURE 3: Comparison of binding of the ligand [$^3$H]*N*-TNP-guanosine with anti-TNP-guanosyl (pool 1) and anti-TNP-lysyl (pool 2) antibodies in equilibrium dialysis. (A) Anti-TNP-guanosyl antibody at a concentration of 1050 $\mu$g/ml. (B) Anti-TNP-lysyl antibody at a concentration of 720 $\mu$g/ml. Equilibration was achieved in 22 hr at 4°. The molar extinction coefficient for the ligand was $\epsilon^{422}_{1\,cm}$ 15,000. All antibodies and ligands were dissolved in 0.05 M phosphate (pH 8.0). Heterogeneity indices of $a$ = 0.65 and 0.70, for 3A and 3B, respectively, were derived from Sips plots of these data.

Thus, both antibodies are similar in their binding properties to the $\epsilon$-TNP-L-Lys ligand.

Figure 3A,B shows the binding curves of the anti-TNP-guanosyl (pool 1) and anti-TNP-lysyl (pool 2) with [$^3$H]-*N*-TNP-guanosine, respectively. The average intrinsic association constant for the homologous fit in Figure 3A was $5 \times 10^6$ and $3 \times 10^6$ l. per mole for the anti-TNP-lysyl site (3B). Thus, again the association constant for the homologous fit was slightly better than the comparative heterologous site. Therefore, these binding experiments measured the valence and binding constants but did not reveal gross distinguishable differences in the two sites.

Both antibodies were compared in fluorescence quenching studies with various 2,4-DNP and 2,4,6-TNP ligands as shown in Figure 4A,B. Figure 4A shows that the fluorescence emitted from the tryptophan residues in the anti-TNP-guanosyl site was quenched to a greater degree by the *N*-TNP-guanosyl ligands than the TNP- or DNP-amino acid derivatives. The quenching efficiency of the TNP-guanosyl compounds was unexpected since these ligands show peak absorbancy at 258–260 and 420–425 m$\mu$ (Voss *et al.*, 1969). There is no significant overlap with the fluorescence emission from tryptophan at 350 m$\mu$. Thus, one would have predicted a very inefficient energy transfer between the fluorescence energy of the tryptophan residues and the *N*-TNP-guanosyl derivatives. Figure 4B shows that $\epsilon$-TNP-L-Lys and $\epsilon$-TNP-aminocaproate derivatives, in general, quenched more efficiently than the TNP-*N*-guanosyl derivatives. Thus, fluorescence quenching results revealed some differences in the binding and quenching efficiencies between the two antibody populations. In both cases the homologous or related ligands showed significantly higher quenchability.

The maximum quenching or $Q_{max}$ (*i.e.*, when all antibody sites are saturated) was determined for both the anti-TNP-guanosyl and anti-TNP-lysyl sites. Maximum quenching of the antibody's tryptophan fluorescence was studied using $\epsilon$-TNP-aminocaproate and $\epsilon$-TNP-Lys. The anti-TNP-guanosyl molecule gave a $Q_{max}$ of 63.4% and the anti-TNP-lysyl site a $Q_{max}$ of 55.6%. Anti-DNP-lysyl antibody was quenched 65.4% when saturated with $\epsilon$-TNP-aminocaproate ($A_{348}$ 5.5). $Q_{max}$ values of 69 and 40% were obtained, respectively, when anti-TNP-guanosyl and anti-TNP-lysyl were saturated with *N*-TNP-deoxyguanosine.

*Inhibition.* Because of the differences noted in fluorescence quenching and $Q_{max}$ determinations it was necessary to distinguish more critically differences between the two antibody populations. Experiments were designed based on inhibition by deoxyguanosine of the fluorescence quenching with TNP-ligands. These experiments were based on the hypothesis that if every anti-TNP-guanosyl antibody site contained a site for deoxyguanosine, then if this site were saturated, the TNP group may be inhibited from binding to its site. Controls for cross-reactions were provided by reactions with anti-TNP-lysyl- and anti-DNP-lysyl-purified antibody sites. Figure 5 shows the inhibition of TNP quenching by guanosine 5′-monophosphate tested between $1 \times 10^{-6}$ and $5 \times 10^{-3}$ mole per l. It is noteworthy that 100% inhibition was measured. Anti-TNP-lysyl controls showed some binding starting at $7.5 \times 10^{-5}$ mole/l. However, there is a 25× difference in the concentration effects between the two antibody populations. Concentrations of guanylic acid up to $10^{-4}$ mole/l. showed no direct quenching in control studies. No inhibition of quenching was noted when guanylic acid was tested at high concentrations ($1 \times 10^{-4}$ M) in competition with $\epsilon$-DNP-L-Lys and anti-DNP-lysyl antibody. Thus, despite a low level of apparent nonspecific binding with anti-TNP-lysyl antibodies there is a significant difference in the ability of the TNP-guanosyl site to bind guanylic acid. Adenosine, thymine, and cytosine did not inhibit the binding of the TNP ligands in this assay with any of the antibodies studied.

*Spectral Shift Studies.* Differences between the two types of binding sites were studied by spectral shift analyses performed by difference spectroscopy. Figure 6 shows spectral shift studies reacting $\epsilon$-TNP-aminocaproate with anti-TNP-lysyl and anti-TNP-guanosyl antibodies. The free ligand

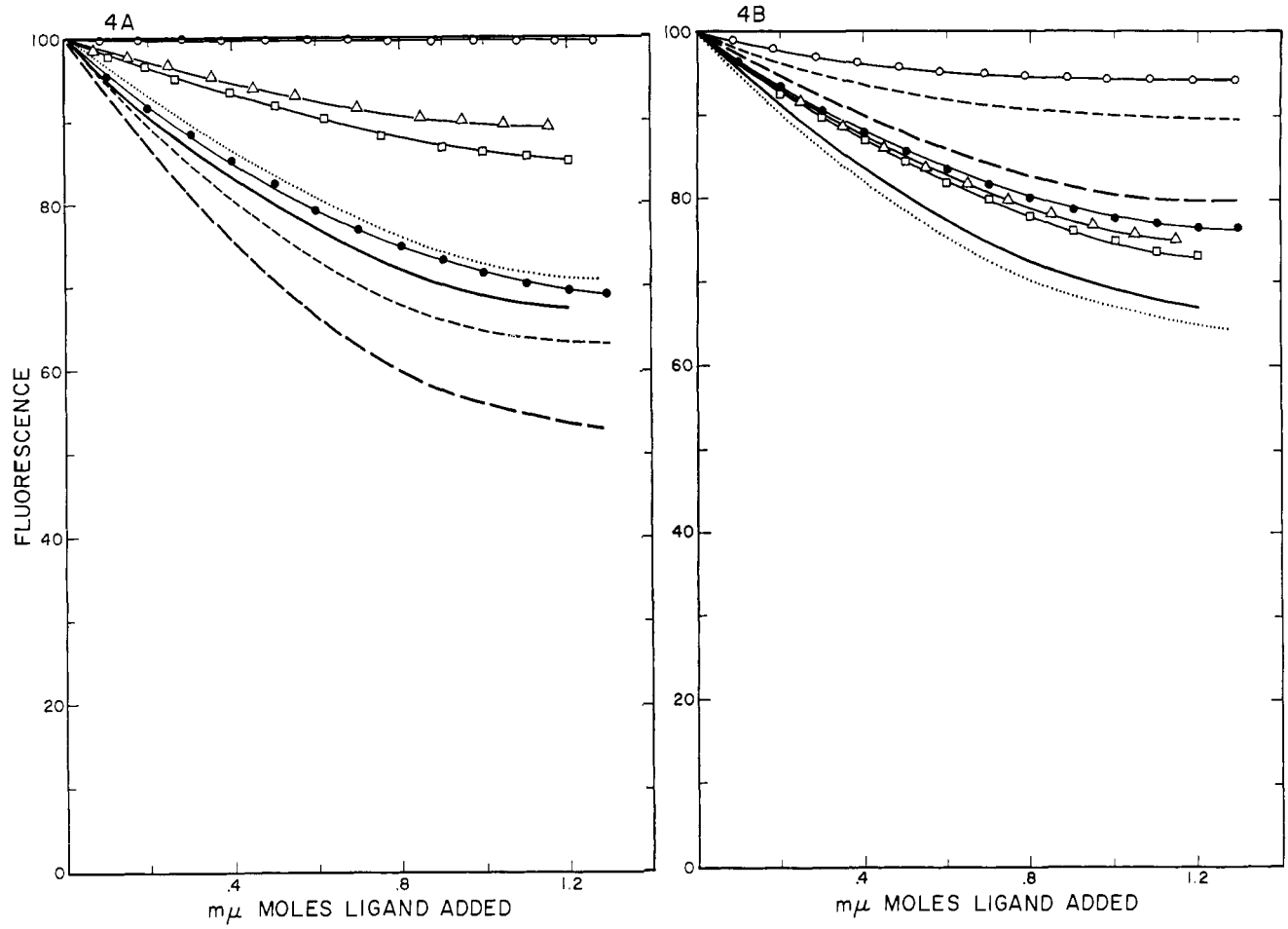


FIGURE 4: Comparative fluorescence quenching titrations of anti-TNP-guanosyl (pool 1) and anti-TNP-lysyl (pool 2) antibodies with DNP- and TNP-ligands. (A) Anti-TNP-guanosyl antibody concentration was 65–67 μg/ml. (B). Anti-TNP-lysyl antibody concentration was 65–68 μg/ml. All titrations were done at 4° by adding aliquots over a range of 0.02–0.2 ml to 1.0 ml of antibody. Antibodies and ligands were dissolved in 0.05 M phosphate (pH 8.0). Ligands: (○—○) DNPOH ($\epsilon^{360}_{1\,cm}$ 14,600, 6.8 mμmoles/ml); (△—△) ε-DNP-L-Lys ($\epsilon^{360}_{1\,cm}$ 17,530, 5.7) mμmoles/ml); (□—□) DNP-glycine ($\epsilon^{360}_{1\,cm}$ 15,890, 6.3 mμmoles/ml); (· · · ·) ε-TNP-aminocaproate ($\epsilon^{348}_{1\,cm}$ 15,700, 6.4 mμmoles/ml); (●—● ε-TNP-L-Lys ($\epsilon^{348}_{1\,cm}$ 15,400, 6.5 mμmoles/ml); (——) *N*-TNP-deoxyguanosine ($\epsilon^{420}_{1\,cm}$ 15,000, 6.7 mμmoles/ml); (---) *N*-TNP-guanosine ($\epsilon^{426}_{1\,cm}$ 15,000, 6.7 mμmoles/ml); (— —) *N*-TNP-guanosine 5′-monophosphate ($\epsilon^{420}_{1\,cm}$ 15,000, 6.7 mμmoles/ml).

(ε-TNP-aminocaproate) shows absorbance maxima, $\lambda_{max}$, at 348 and 430 mμ. Two absorbance peaks, measured by difference spectra, resulted from the binding of this ligand to anti-TNP-guanosyl antibody. These peaks appeared at 370–375 and 470 mμ. When the ligand was bound to anti-TNP-lysyl antibody absorbance maxima were noted at 375 and 450–455 mμ.

Preliminary studies had shown that when *N*-TNP-guanosine was reacted with the TNP-guanosyl antibody site maxima resulted at 345 and 420 mμ. However, when the TNP-nucleotide ligand was reacted with the TNP-lysyl antibody site maxima at 360 and 460 mμ were evident. The free ligand showed a peak at 422–425 mμ. To balance the free and bound ligand these experiments were performed as equilibrium dialysis binding experiments in large dialysis chambers (3.0 ml). After equilibration 0.1-ml aliquots were removed to measure the number of moles of ligand bound per mole of antibody, *r*. Concomitantly, 1.0-ml aliquots were removed from each side and placed in respective cells for difference spectroscopy studies. This permitted accurate reading of the unbound ligand side, *c*, *vs.* the bound ligand, *r*. These experiments resulted in a perfect balance of the free ligand on each side, so that in difference spectroscopy only the spectrum of the bound ligand was measured. The molar extinction coefficient of absorbance peaks can be measured by this method. Figure 7a shows the results using a high-affinity anti-TNP-lysyl antibody purified from later bleedings than used in pool 2. Figure 7a gives the spectra with $r = 1.18$ and 1.65 for the anti-TNP-guanosyl and with the high-affinity anti-TNP-lysyl sites, respectively. Figure 7b shows the spectrum with *r* values of 2.02 and 2.12 for anti-TNP-guanosyl and anti-TNP-lysyl, respectively. In the Discussion reference will be made to difficulties encountered in measuring a precise molar extinction coefficient.

Spectra from the spectral shift studies showed that the two sites produced significantly different results with a given ligand. Nonspecific rabbit IgG caused no spectral shift with either ε-TNP-aminocaproate or *N*-TNP-guanosine.

Indications to this point were that some differences did exist between the two sites. However, except for the ability

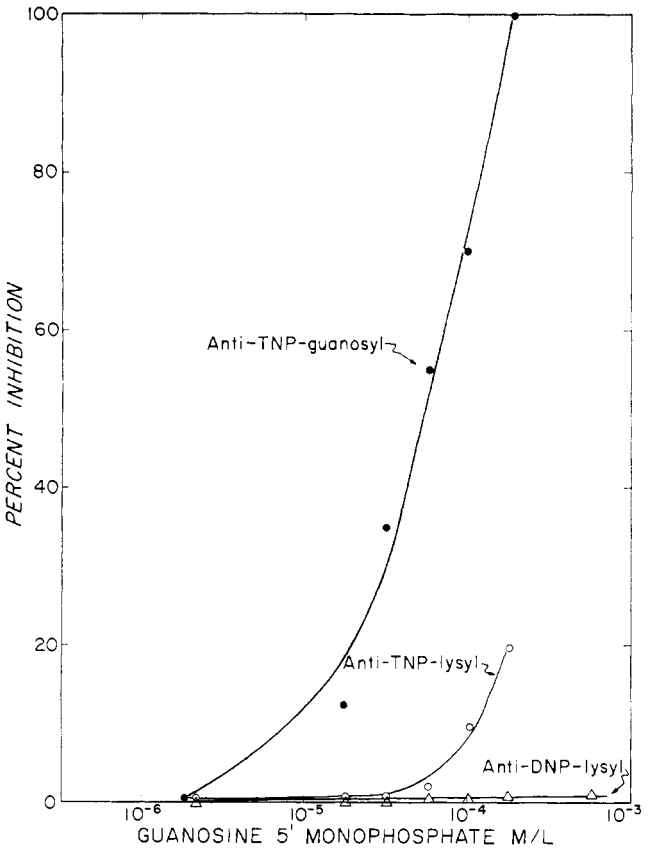


FIGURE 5: Inhibition of fluorescence quenching by guanosine 5′-monophosphate. Per cent fluorescence quenching was standardized with ϵ-TNP-aminocaproate binding to anti-TNP-guanosyl (pool 1) or anti-TNP-lysyl (pool 2) antibodies. Prior to titration with ϵ-TNP-aminocaproate ($\epsilon^{348}_{1\,cm}$ 15,700, 6.4 mμmoles/ml) 0.1 ml of guanosine 5′-monophosphate ($\epsilon^{260}_{1\,cm}$ 12,000) was added to 0.9 ml of antibody (65–67 μg/ml). Reactions were carried out at 4° in 0.05 M phosphate (pH 8.0). After addition of the inhibitor ϵ-TNP-aminocaproate was added in 0.02-ml aliquots to a final of 0.2 ml.

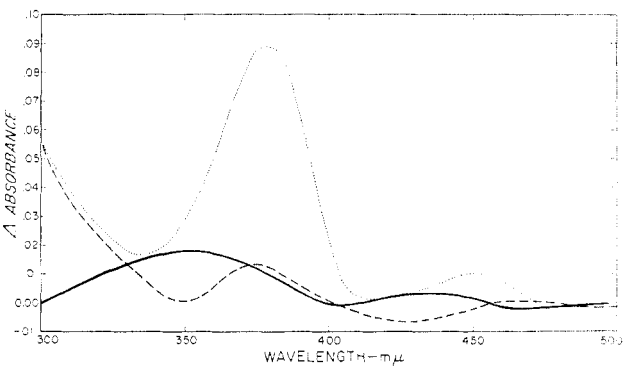


FIGURE 6: Difference spectra of ϵ-TNP-aminocaproate in spectral shift studies with anti-TNP-guanosyl (pool 1) and anti-TNP-lysyl (pool 2) antibodies. (——) Unbound ϵ-TNP-aminocaproate in 0.05 M phosphate (pH 8.0). (---) Bound ligand with anti-TNP-guanosyl antibody (1.05 mg/ml) and (· · ·) anti-TNP-lysyl antibody (0.95 mg/ml).

of guanosine to inhibit the binding of TNP ligands in fluorescence quenching experiments, direct binding data had not been obtained. Therefore, equilibrium dialysis binding studies were attempted by reacting [$^3$H]guanosine with both anti-TNP-guanosyl (pool 1) and anti-TNP-lysyl antibodies (pool 2).

Figure 8 clearly shows the binding of the guanosyl ligand to the anti-TNP-guanosyl site. It is important to note that the concentrations of ligand necessary for binding were of the same magnitude as those used for inhibition of fluorescence quenching. An average intrinsic association constant of $2.3 \times 10^{-4}$ l./mole was measured. Anti-TNP-lysyl antibodies and normal rabbit IgG showed minimal binding of [$^3$H]guanosine. To clarify the apparent nonspecific binding observed with these proteins as opposed to the anti-TNP-guanosyl antibody, all antibodies were subjected to pepsin digestion. Table I shows the comparison of the binding of the F(ab)$_2$ fragments with the IgG (7 S) molecules. When the Fc portion of the molecule was removed the anti-TNP-guanosyl antibodies still bound the guanosyl ligand. However, the removal of the Fc portion of the anti-TNP-lysyl molecule and normal IgG eliminated all binding of the guanosyl ligand. Thus, the guanosyl ligand was binding to the Fab fragments of the anti-TNP-guanosyl antibody but was apparently interacting with the Fc portion of the anti-TNP-lysyl and normal IgG molecules. None of the Fab fragments showed significant binding with $10^{-4}$ M [$^3$H]adenosine in equilibrium dialysis studies.

## Discussion

These data indicate that the moiety adjacent (carrier) to the primary haptenic group has a significant effect on the nature of the antibody active site. Previous studies indicated a similar effect (Eisen and Siskind, 1964; Parker *et al.*, 1966; Haber *et al.*, 1967; Little and Counts, 1969; Richards *et al.*, 1969). Studies presented here involved the comparison of two antibody sites induced by immunogens in which the primary haptenic group (TNP) was the same, but the moieties directly conjugated to the hapten were different. These sites can be visualized as shown in Figure 9. Data obtained in comparative studies of the two sites suggested that in equilibrium dialysis binding studies the sites were similar. Small differences noted in the average intrinsic association constants indicated that the homologous ligands demonstrated a slightly better fit (Figures 1 and 3). Fluorescence quenching studies confirmed trends noted in equilibrium dialysis studies. Figure 4A shows that side group effects were directly correlated to quenching efficiency. TNP-guanosyl ligands quenched more efficiently than TNP-amino acid derivatives (*i.e.*, ϵ-TNP-aminocaproate and ϵ-TNP-L-Lys). The greater binding efficiency of the TNP-amino acid derivatives compared with the DNP derivatives showed that the TNP group was immunodominant to the related DNP derivatives. It was previously noted that *N*-TNP-guanosine was very inefficient in quenching when bound to an anti-DNP-lysyl site (Gallagher and Voss, 1969). Relative quenching data with the anti-TNP-lysyl antibody site were likewise consistent. In general, the TNP-nucleotide derivatives quenched less well than the TNP and DNP derivatives. It would appear that fluorescence from tryptophan residues in the anti-TNP-lysyl site were quenched more efficiently with the amino acid derivatives. In both cases

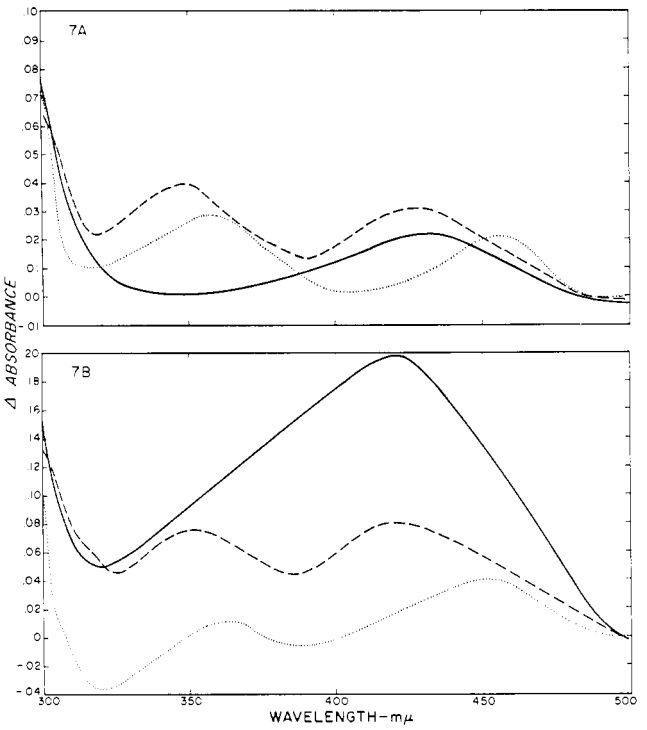


FIGURE 7: Difference spectra of *N*-TNP-guanosine in spectral shift studies utilizing equilibrium dialysis chambers. (A) (—) Unbound *N*-TNP-guanosine in 0.05 M phosphate (pH 8.0); (— —) bound ligand with anti-TNP-guanosyl antibodies (pool 1, 870 μg/ml); (· · ·) bound ligand with high-affinity anti-TNP-lysyl (820 μg/ml). Starting ligand concentration was 18.5 mμmoles/ml. Chambers were equilibrated for 20–22 hr at 4° before samples were removed for determination of *r* and spectral shift studies. (B) Difference spectra of *N*-TNP-guanosine as in part A with a starting ligand concentration of 50 mμmoles/ml.

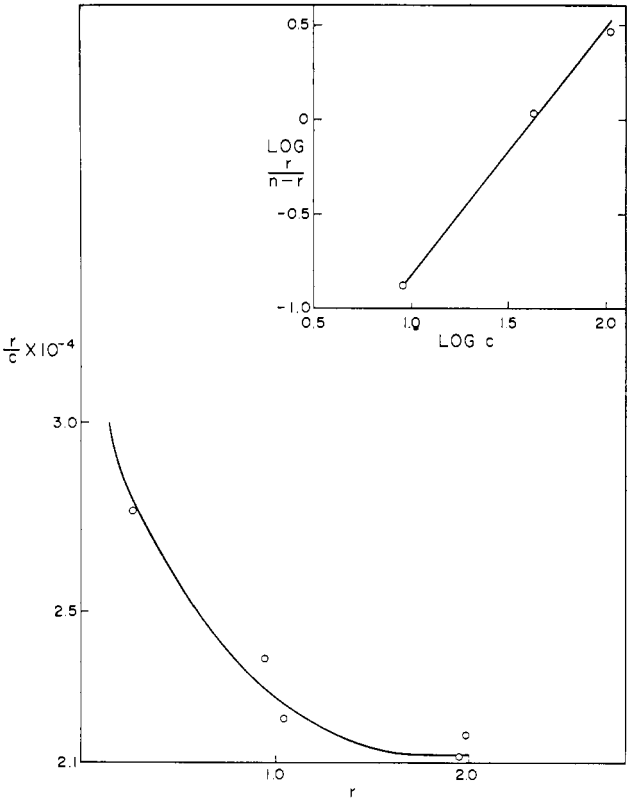


FIGURE 8: Equilibrium dialysis binding studies of [$^3$H]guanosine with anti-TNP-guanosyl antibody (pool 1, 1.0 mg/ml). Chambers were equilibrated for 20–22 hr at 4°. The insert shows a Sips plot of the data derived from the equilibrium dialysis experiment.

amino acid or nucleotide derivatives were more efficient than the phenolic (DNPOH) ligand.

Comparison of the two sites by direct binding of the guanosine ligand (minus the TNP group) proved the best assay for distinguishing these sites. Inhibition of fluorescence quenching indicated that the anti-TNP-guanosyl site bound the ligand as predicted from the model in Figure 9. The anti-TNP-lysyl site showed minimal binding of guanosine. In experiments with pepsin-derived $F(ab)_2$ fragments (Table I) this binding was eliminated, indicating it was nonspecific and probably involved an interaction with the Fc fragment. Since 100% inhibition was achieved with guanylic acid it would appear that every antibody molecule in pool 1 contained a site for the guanosyl moiety. This would support the idea that the TNP conjugation in the immunogen is to the guanosine base only and there is little or no protein contamination in the DNA immunogen (Voss *et al.*, 1969). Similarly, adenosine, thymine, and cytosine showed no inhibitory effects in competition with the TNP-ligand. If 100% inhibition is equivalent to two sites filled per mole of antibody then 50% should approximate an *r* of 1. The 50% inhibition was achieved with a ligand concentration of $8 \times 10^{-5}$ mole/l. This value would approximate *c* since the amount of antibody present in this assay is negligible. Thus, the reciprocal of the free-ligand concentration is $1.2 \times 10^{-4}$ l./mole. This value should approximate the average intrinsic association constant, $K_0$. The shape of the curve in Figure 5 also indicates a relative homogeneous site for the guanosyl residue. Comparison of these data derived from the fluorescence–inhibition studies with those of equilibrium dialysis binding studies (Figure 8) show some interesting correlations. The average intrinsic association constant of $2.4 \times 10^4$ l./mole measured in equilibrium dialysis is comparable with the value of $1.2 \times 10^4$ cited above. The binding of the guanosyl residue with anti-TNP-guanosyl antibodies was achieved over a short range of ligand concentrations and is the reason for the modified Scatchard plot in Figure 8. The Sips plot (insert Figure 8) shows an *a* of 0.8. This would coincide to a limited heterogeneity implied from the fluorescence quenching–inhibition experiment. Thus, there is a 200-fold difference between the association constant of the immunodominant TNP group ($5 \times 10^6$ l./mole) and the site for the guanosyl moiety ($2 \times 10^4$ l./mole). The 100–1000 molar excess of guanylic acid over ε-TNP-aminocaproate required for 100% inhibition of fluorescence quenching (Figure 5) is consistent with these association constants of the two parts of the determinant. However, the guanosyl site is relatively more homogeneous than the TNP site.

The valence of 2 for the guanosyl site correlates with the 100% inhibition obtained in fluorescence quenching studies. Since guanosyl ligands would not bind significantly to the

TABLE I: Comparative Valences, Association Constants, and Heterogeneity Indices of Anti-TNP-guanosyl and Anti-TNP-lysyl Antibodies and Their F(ab)$_2$ Fragments.

| Antibody | Ligand: TNP-L-Lys $n$[c] | TNP-L-Lys $K_0$[d] | TNP-L-Lys $a$[e] | *N*-TNP-guanosine $n$ | *N*-TNP-guanosine $K_0$ | *N*-TNP-guanosine $a$ | 5′-Monophosphate Guanosine $n$ | 5′-Monophosphate Guanosine $K_0$ | 5′-Monophosphate Guanosine $a$ |
|---|---|---|---|---|---|---|---|---|---|
| Anti-TNP-guanosyl | | | | | | | | | |
| IgG[a] | 2 | $9 \times 10^6$ | 0.57 | 2 | $5 \times 10^6$ | 0.65 | 2 | $2.3 \times 10^4$ | 0.81 |
| F(ab)[b] | 2 | $9 \times 10^6$ | 0.60 | | *f* | | 2 | $2.4 \times 10^4$ | 0.81 |
| Anti-TNP-lysyl | | | | | | | | | |
| IgG | 2 | $13 \times 10^6$ | 0.57 | 2 | $3 \times 10^6$ | 0.70 | | | |
| F(ab) | 2 | $14 \times 10^6$ | 0.59 | | *f* | | | | |

[a] Assumed molecular weight of 155,000. [b] Assumed molecular weight of 100,000. [c] Valence or the limiting value for $r$ at infinite $c$. [d] Average intrinsic association constant expressed as liters per mole. [e] Heterogeneity index obtained from a Sips plot of the binding data. [f] Not measured.

anti-TNP-lysyl site this was considered as evidence against serological cross-reaction. Binding of guanosyl ligands to the anti-TNP-guanosyl site must represent a separate distinct cavity within a site.

Spectral shift studies indicated that the sites provided a different environment for the ligands. Unbound $\epsilon$-TNP-aminocaproate (Figure 6) showed absorption maxima at 348 and about 430 m$\mu$. Bound $\epsilon$-TNP-aminocaproate showed maxima at 375 and about 450–455 m$\mu$ with the anti-TNP-lysyl site. Previous studies (Little and Eisen, 1967) showed absorption maxima at 380 and 470 m$\mu$ in spectral shift studies. Bound $\epsilon$-TNP-aminocaproate with anti-TNP-guanosyl showed maxima at 375 and 470 m$\mu$. Thus, there was no difference at the shorter wavelength and a 15–20-m$\mu$ difference at the longer wavelengths. It should be noted that the magnitude of the 370-m$\mu$ peak was much greater with the homologous anti-TNP-lysyl site. This may be explained by a stronger binding constant for the homologous site although the binding constants were similar and the protein concentration of each antibody was essentially identical. Thus, the spectral shifts are both qualitatively and quantitatively different. The magnitude of the spectral shift correlates with the fluorescence quenching ability of the $\epsilon$-TNP-aminocaproate noted in Figure 4A.

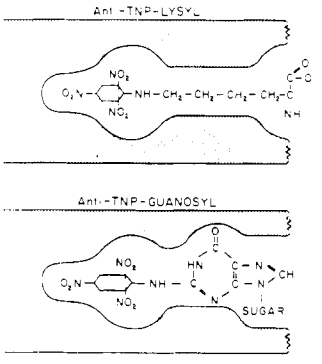


FIGURE 9: Schematic representation of the proposed anti-TNP-lysyl (top) and anti-TNP-guanosyl (bottom) antibody sites reacting with homologous ligands.

Similarly, the spectral shift studies with the ligand *N*-TNP-guanosine differed qualitatively and quantitatively in respect to the sites with which it reacted. *N*-TNP-guanosine peaked at 345 and 420 m$\mu$ with the homologous antibody anti-TNP-guanosyl but gave maxima at 360 and 460 m$\mu$ with anti-TNP-lysyl. The homologous ligand–antibody pair also gave the strongest effect in terms of absorbance. This appears significant because the protein concentrations for both antibodies was the same but the anti-TNP-lysyl antibody was of very high affinity ($r$ = 1.63) being specifically prepared for this experiment.

The ratio of the absorbance peaks obtained in spectral shift studies varied in relation to $r$. The ratio of $\lambda_{345}/\lambda_{422}$ at $r$ = 1.18 (*N*-TNP-guanosine reacted with anti-TNP-guanosyl) was 1.3. When $r$ = 2.02 the ratio was 0.9. Binding of *N*-TNP-guanosine with the anti-TNP-lysyl site gave similar results ($\lambda_{360}/\lambda_{460}$). Molar extinction coefficients for the 460-m$\mu$ peak varied from 2510 at $r$ = 1.6 to 3540 at $r$ = 2.1. These values are close to previously reported coefficients (Little and Eisen, 1967).

Thus, these two populations of antibodies are significantly different in respect to their active sites. The guanosyl residue in place of the lysyl residue in the immunogen has induced a different binding site. Fluorescence inhibition studies indicate that the cavities with the anti-TNP-guanosyl site may be arranged in a linear "tube" fashion and that when the guanosyl residue resides in the site it blocks the TNP ligand from reaching its cavity or site. However, these results are also compatible with the nonindependence of the two sites. Bound guanosyl ligands may produce a conformational change in the TNP site and result in reduced quenching by TNP ligands. Data presented here do not allow projection as to how the sites differ in amino acid composition or conformation. Studies are currently in progress to investigate these properties. These results are important since the biosynthesis of these sites are in question. Do the anti-TNP-lysyl and anti-TNP-guanosyl sites differ because of a selection of molecules for the best fit? If so, these results suggest the

selection was not based entirely on the TNP group. Conversely, if the site is constructed *de novo* from the cooperation of many genes then the cavity for TNP is dictated by one gene and the guanosyl site by another locus.

It is not clear how the antibody described above relates to the "multispecific" myeloma proteins described previously (Schubert *et al.*, 1968). These mouse myelomas precipitated with DNP-, TNP-, adenylic acid, 5-acetyluracil-, and purine-derivatized proteins. It is possible these myelomas have sites corresponding to the anti-TNP-guanosyl antibody.

The apparent or effective "double specificity" exhibited by the anti-TNP-guanosyl antibody may have importance in autoimmune diseases, such as systemic lupus erythematosis. Anti-DNA antibodies have been demonstrated in sera obtained from patients afflicted with the disease (Samter, 1965; Morse *et al.*, 1962). The anti-TNP-guanosyl antibody described above is "double specific" because it can react with the TNP group and separately with the guanosyl residue. Thus, one can extrapolate that similar antibody may be involved in system lupus erythematosis. Perhaps during the disease process certain bases become derivatized and this substitution results in a "foreign hapten–DNA" immunogenic group. Antibodies are then induced by this complex. However, the autoimmune condition would not necessarily result from one set of antibodies against the substituent group and a separate set against the DNA molecules. The antibodies induced could be double specific by virtue of one site for the foreign group as well as one for the base. This type of antibody would bind to host DNA molecules creating the pathology described in systemic lupus erythematosis patients. Stimulation of the "double-specific" antibody would also obviate the need to terminate tolerance to the host's DNA. It has been noted that the rabbits producing anti-TNP-guanosyl antibodies do not exhibit an autoimmune syndrome. Therefore, single-stranded DNA (antigen) in the diseased host may be an additional requirement for antibody reactivity in systemic lupus erythematosis patients.

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