Evidence for Tryptophan in the Active Sites of Antibodies to Polynitrobenzenes*

J. Russell Little† and Herman N. Eisen

ABSTRACT: 2,4-Dinitrophenyl (DNP) and 2,4,6-trinitrophenyl (TNP) haptens undergo characteristic spectral shifts when bound specifically by anti-DNP and anti-TNP antibodies. The shifts are reproduced qualitatively and quantitatively when the same haptens form complexes with tryptophan, as the free amino acid, but they are not reproduced by other amino acids, by inorganic solvents (methanol, dimethylformamide, and dimethyl sulfoxide), or in concentrated solutions of sodium chloride. It seems possible, therefore, that the spectral shifts that arise from specific binding to antibodies reflect the presence of tryptophan in the protein's active site. Since the spectral shifts are observed with antibodies obtained from the rabbit, guinea pig, goat, and horse, and with rabbit antibodies that differ over 1000-fold in affinity, it is suggested that tryptophan in the active site may be a general feature of all anti-DNP and anti-TNP antibodies.

It has been suggested that the shifts in the absorbance spectra of 2,4-dinitrophenyl (DNP) ligands bound to rabbit anti-DNP antibodies could be due to a charge-transfer complex between the bound ligand and a tryptophan residue in the antibody's active site (Eisen and Siskind, 1964). This suggestion was consistent with the following observations. (1) Polynitrobenzenes have a high affinity for electrons and readily form charge-transfer complexes with a variety of electron donors (Andrews and Keefer, 1964). (2) Because of its ionization potential tryptophan has, of all the naturally occurring amino acid residues, the greatest likelihood of serving as an electron donor (Isenberg and Szent-Gyorgyi, 1958; Szent-Gyorgyi, 1960). (3) Tryptophan fluorescence of anti-DNP antibodies and their active proteolytic fragments is strongly quenched by bound DNP ligands (Velick *et al.*, 1960).

Antibodies specific for the 2,4,6-trinitrophenyl (TNP) group have recently been found to resemble anti-DNP antibodies in many respects, and to have similar, though not identical, fluorescence quenching properties (Little and Eisen, 1966). The present work was initiated, therefore, to examine the spectral changes of TNP ligands bound to anti-TNP antibodies. Because anti-TNP molecules also bind DNP ligands and anti-DNP molecules bind TNP ligands, we have compared the spectral shifts that accompany the binding of both homologous and cross-reacting ligands.[1] In all instances hypochromicity and shifts of absorbance maxima to longer wavelengths have been observed. In addition, difference spectra between free and bound ligands were characterized by two absorbance peaks at 380–390 and 470 mμ. Because the spectral changes caused by specific binding to antibodies were reproduced in the complexes formed by DNP and by TNP ligands with tryptophan (as the free amino acid),[2] but not with other amino acids or in other solvents, it is suggested that a tryptophan residue in the active sites of anti-TNP and anti-DNP antibodies could account for the observed spectral shifts. Since these antibodies have been obtained from a variety of species (rabbit, guinea pig, goat, and horse) and cover a wide range in binding affinities (1.3×10^5 to $>1.0 \times 10^8$ M^{-1}), it appears that tryptophan may be present as a contact amino acid in the active sites of all these immunoglobulins.

Materials and Methods

Antibody Isolation and Characterization. Methods described previously were used for the preparation of DNP and TNP antigens (Little and Eisen, 1967) and for the isolation of the respective antibodies (Eisen *et al.*, 1967). Rabbit antibodies specific for the *p*-azophenylarsonate group were isolated as described (Lark *et al.*, 1965), following in outline the

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[1] Antibodies formed against 2,4-dinitrophenylated proteins and those formed against 2,4,6-trinitrophenylated proteins often cross-react extensively. They are referred to as anti-DNP and anti-TNP, respectively, because each forms more stable complexes with homologous ligands than with heterologous ones (J. R. Little and H. N. Eisen, manuscript in preparation).

[2] The free amino acids used in this work were of the L-series optical activity

method of Koshland *et al.* (1962). Each preparation of purified rabbit antibody gave a single precipitin arc of γG mobility on immunoelectrophoresis developed with a goat antiserum to a crude rabbit globulin fraction. The purified guinea pig antibody preparation consisted of a major γ2 and a minor γ1 fraction (Benacerraf *et al.*, 1963), as indicated by immunoelectrophoresis with a rabbit antiserum to guinea pig serum. Anti-DNP antibodies were also isolated from antisera obtained from a horse and a goat, but were not characterized by immunoelectrophoresis. Binding constants of the anti-DNP and anti-TNP antibody preparations were determined by the method of fluorescence quenching (Velick *et al.*, 1960; Eisen and Siskind, 1964; Little and Eisen, 1966).

Other Reagents and Ligands. The synthesis, purification, and characterization of DNP and TNP ligands have been described previously (Carsten and Eisen, 1953; Eisen and Siskind, 1964; Little and Eisen, 1966). 1,3,5-Trinitrobenzene[3] and 2,4,6-trinitrotoluene were obtained from Distillation Products Industries (Rochester, N. Y.) and were recrystallized twice from ethanol–water. Spectral grade dimethyl sulfoxide was obtained from Matheson Coleman and Bell (Chicago, Ill.) and used without further purification. Crystallized amino acids, tyrosine methyl ester, and dimethylformamide were obtained from the Sigma Chemical Co., St. Louis. Solutions of ligands, amino acids, and purified antibodies were prepared in 0.15 M NaCl–0.02 M potassium phosphate (pH 7.4, buffered saline), unless otherwise noted.

Spectral Measurements. Absorbance spectra were obtained without thermostatic control in a Cary Model 14 recording spectrophotometer using quartz cells with a 10-mm light path. Difference spectra were usually obtained with a slide wire that gave full-scale pen deflection for an absorbancy of 0.100.

Results

TNP Ligands with Rabbit Antibodies. The absorbance spectra of TNP-aminocaproate and of 2,4,6-trinitrotoluene (TNT) in the absence and in the presence of rabbit anti-TNP antibodies are shown in Figure 1. Because immunoglobulins and TNT absorb strongly at 230–290 mμ the spectrum of bound TNT was obtained as the difference between a solution of antibody (in the reference cell) and an aliquot of the same antibody solution to which TNT had been added (in the experimental cell). Under conditions where approximately 90% of the TNT was bound, its absorbance maximum (λ_{max}) was shifted 10 mμ higher than free TNT and the hypochromic effect (at λ_{max}) amounted to 26%. The red shift and the hypochromic effect were less pronounced with bound TNP-aminocaproate.

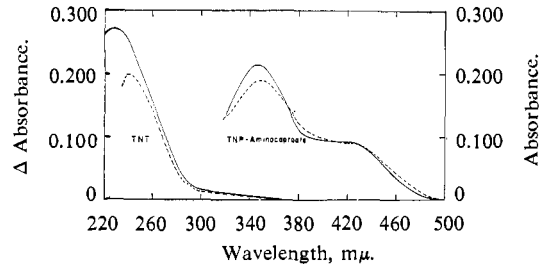

FIGURE 1: Absorbance spectra of free and antibody-bound TNT and TNP-aminocaproate. Left: Absorbance spectra of free and antibody-bound TNT. The solid line (right ordinate), with λ_{max} 230 mμ, is 1.57×10^{-5} M TNT in buffered saline. The dotted line (left ordinate), with λ_{max} 240 mμ, is the spectrum of a mixture of 1.57×10^{-5} M TNT plus 1.10×10^{-5} M anti-TNP antibody. Since the binding constant for this antibody–ligand pair was 1.8×10^{7} M^{-1}, more than 90% of the ligand was bound. Because immunoglobulins and TNT absorb strongly between 220 and 240 mμ the dotted line was obtained as a difference spectrum (left ordinate) with antibody and TNT in the experimental cell and antibody alone at the same concentration in the reference cell. Right: The absorbance spectra of free and antibody-bound TNP-aminocaproate. The solid line, with λ_{max} 348 mμ, represents 1.36×10^{-5} M TNP-aminocaproate in buffered saline. The dotted line, λ_{max} 350 mμ, is the spectrum of 1.36×10^{-5} M TNP-aminocaproate plus 1.23×10^{-5} M anti-TNP antibody; more than 95% of the hapten was bound since the average intrinsic association constant for this antibody–ligand pair was $\geqslant 1 \times 10^{8}$ M^{-1}. (———) Free; (- - - -) bound.

Nonspecific rabbit γG-immunoglobulins caused no spectral shift with either of these ligands.

The difference spectrum obtained with TNP-aminocaproate in the reference cell and the same ligand plus rabbit anti-DNP antibody in the experimental cell is shown in Figure 2 at wavelengths (360–500 mμ) where the antibody is transparent. For comparison, control difference spectra were obtained with (a) purified rabbit antibodies specific for the *p*-azophenylarsonate group and (b) nonspecific γG-immunoglobulin. The interaction with anti-DNP antibody resulted in two positive peaks for bound TNP-aminocaproate. The control immunoglobulins caused no spectral shifts. In additional difference spectra, some of which are shown in the succeeding figures, the binding of TNP-aminocaproate or TNP-lysine by several other rabbit anti-DNP and anti-TNP antibodies always resulted in the appearance of two difference spectral peaks like those in Figure 2, *i.e.*, one at 380 ± 3 mμ and the other at 470 ± 3 mμ. The magnitude of each peak was related to the concentration of bound ligands; *i.e.*, both to the concentration of reactants and to the average affinity of the antibody population for the ligand.

DNP Ligands with Rabbit Antibodies. The binding

[3] Abbreviations used: TNB, 1,3,5-trinitrobenzene; TNT, 2,4,6-trinitrotoluene; DMSO, dimethyl sulfoxide. In DNP-lysine, TNP-lysine, DNP-aminocaproic acid, and TNP-aminocaproic acid the DNP or TNP substituents are on the ϵ-amino group of L-lysine or the amino group of ϵ-aminocaproic acid.

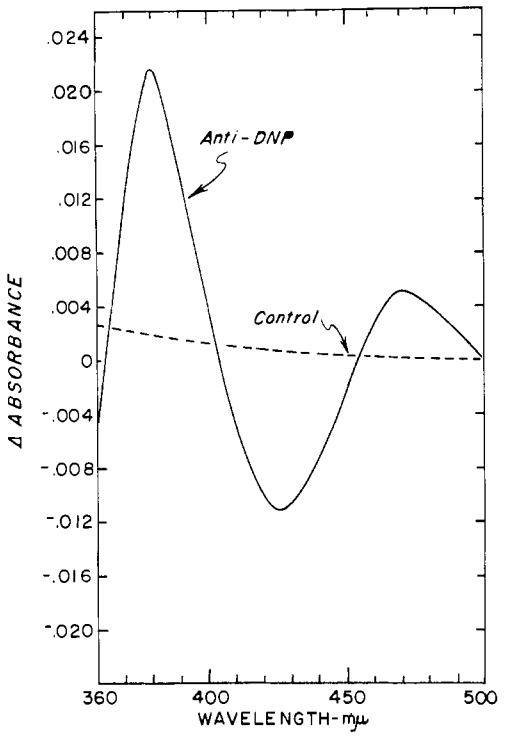

FIGURE 2: Difference spectrum between free and antibody-bound TNP-aminocaproate. The experimental cell contained rabbit anti-DNP antibody (5.27×10^{-6} M) and TNP-aminocaproate (1.43×10^{-5} M). The reference cell contained 1.43×10^{-5} M TNP-aminocaproate. The maxima are at 380 and 470 mμ. The control was obtained with purified anti-*p*-azophenylarsonate antibody (6.89×10^{-6} M) and TNP-aminocaproate (1.43×10^{-5} M) in the experimental cell and the same concentration of TNP-aminocaproate in the reference cell. Similar controls obtained with the same ligand and nonspecific γG-immunoglobulin also showed no spectral shifts.

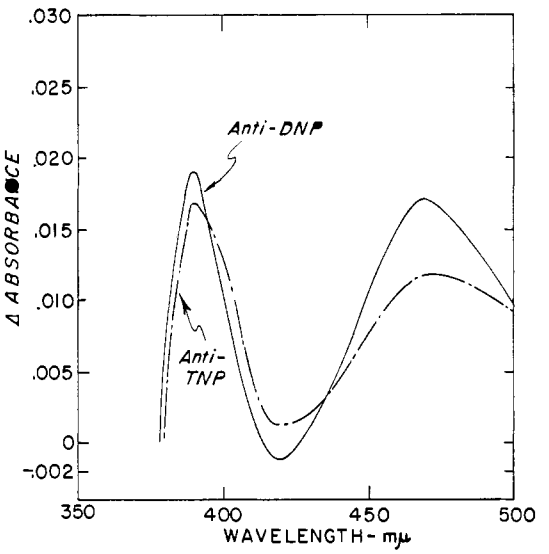

FIGURE 3: Difference spectra of free and antibody-bound DNP-lysine. Anti-DNP and anti-TNP antibodies were isolated from individual rabbits homozygous at the known light- and heavy-chain loci (Aa 3,3 and Ab 4,4) 1 month after immunization of one animal with DNP-protein and a second animal with TNP-protein. The sample cells contained 1.0 ml of antibody (4.6×10^{-6} M), and the reference cell 1.0 ml of buffered saline. To each cell was added 0.1 ml of ε-DNP-lysine (9.7×10^{-4} M). The spectra are differences between ligand plus antibody *vs.* ligand alone. Ordinate values (Δ absorbance) are uncertain because of the potential error involved in introducing the small volume of ligand at high concentration. Nevertheless, the positions of maxima and minima and the coincidence for the two antibodies are clear. The greater magnitude of the maxima with anti-DNP is consistent with its greater affinity for the ligand (DNP-lysine).

of DNP-lysine and of DNP-aminocaproate by rabbit anti-DNP and anti-TNP antibodies also resulted in difference spectra with two maxima, one at 470 ± 3 mμ (indistinguishable from that of the corresponding TNP ligands bound to antibody) and the other at 390 ± 2 mμ, 10 mμ higher than the corresponding peak of antibody-bound TNP ligands (Figure 3).

DNP and TNP Ligands with Tryptophan. DNP and TNP ligands undergo spectral shifts in concentrated solutions of tryptophan (as the free amino acid), indicating the formation of ligand–tryptophan complexes. Displayed as difference spectra (by using ligand alone in the reference cell), the spectra of tryptophan complexes with DNP-aminocaproate and with TNP-aminocaproate are shown in Figure 4 at wavelengths where tryptophan is transparent. As with the antibody-bound ligands the difference spectra of tryptophan-bound ligands showed two maxima, one at 470 ± 3 mμ, and the second at either 380 ± 3 (with TNP-aminocaproate) or 390 ± 2 mμ (with DNP-lysine and DNP-aminocaproate). The amplitude of each deflection varied with the concentrations of ligand and tryptophan (see below) but the position of each peak was constant for a given ligand–tryptophan pair.

Extinction Coefficients of Antibody–Ligand and Tryptophan–Ligand Complexes. The extinction coefficient of ligand bound to antibody was determined by dialyzing a sample of antibody (inside the dialysis sac) against a solution of ligand until concentration equilibrium was achieved (overnight at 25°). The absorbance of the bound ligand at 470 mμ was then determined directly as the difference between the inside and outside solutions; and the concentration of bound ligand was calculated from the difference between free ligand concentration at equilibrium and the total amount of ligand added initially, corrected for bag binding (1–2%) in the usual way (*e.g.*, Eisen, 1964). The values obtained are given in Table I.

By means of the analysis proposed by Isenberg and Szent-Gyorgyi (1958) the extinction coefficient of ligand bound to tryptophan was determined from

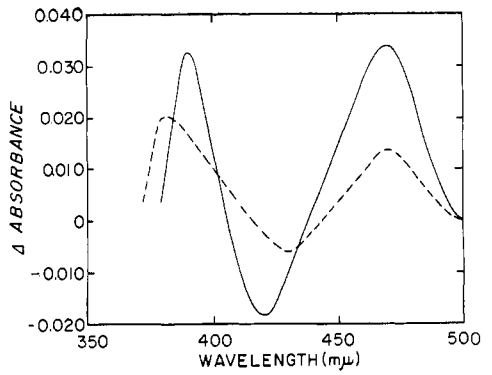

FIGURE 4: Difference spectra of DNP-aminocaproate and TNP-aminocaproate with tryptophan as the free amino acid. The solid curve was obtained with 0.024 M tryptophan plus 8.23×10^{-4} M DNP-aminocaproate in the experimental cell and an equal concentration of DNP-aminocaproate in the reference cell. The dotted curve is the difference spectrum obtained with 0.024 M tryptophan plus 9.37×10^{-5} M TNP-aminocaproate in the experimental cell and an equal concentration of TNP-aminocaproate in the reference cell. The solvent was buffered saline.

TABLE I: Molar Extinction Coefficients of Antibody–Ligand[a] and Tryptophan–Ligand[b] Complexes.

Ligand Complex with	Molar Extinction Coefficient of Complex at 470 mμ	
	DNP-aminocaproate	TNP-aminocaproate
Tryptophan	2980	2520
Anti-DNP antibodies	2920	
Anti-TNP antibodies		2510

[a] Based on equilibrium dialysis in which 4.68 mg of anti-DNP antibody or 5.34 mg of anti-TNP antibody was dialyzed against 0.183 μmole of DNP-aminocaproate and 0.211 μmole of TNP-aminocaproate, respectively, with the initial volume being 3.0 ml inside and 3.0 ml outside the bag. At equilibrium the free concentration of ligand (outside) was 1.94×10^{-5} M DNP-aminocaproate or 2.39×10^{-5} M TNP-aminocaproate. [b] Based on eq 3 and Figure 5. The molar extinction coefficients at 470 mμ for the free ligands are 2370 for DNP-aminocaproate and 1760 for TNP-aminocaproate.

the difference in absorbance (at 470 mμ) between a reference solution of ligand and several solutions with ligand at the same concentration but with varying concentrations of tryptophan in excess. On the assumptions that (1) the complexes form reversibly and consist of one molecule of tryptophan plus one molecule of ligand, and (2) the absorbancy of the complex obeys Beer's law, it follows that

$$A = \Delta\epsilon[\overline{TL}]l \tag{1}$$

and

$$K = \frac{[\overline{TL}]}{[T][L]} \tag{2}$$

where A is the net absorbance (difference between ligand alone in the reference cell and ligand plus tryptophan in the experimental cell); $\Delta\epsilon$ is the change in the ligand's molar extinction coefficient in the complex; $[\overline{TL}]$, [L], and [T] are the molar concentrations of the complex, unbound ligand, and unbound tryptophan, respectively; K is the association constant for the formation of the complex; and l is the path length (1.0 cm). With tryptophan in large excess the free and total tryptophan concentrations are essentially equivalent; and if $[L_t]$ is the total ligand concentration

$$\frac{1}{[T]} = K[L_t]\Delta\epsilon\frac{1}{A} - K \tag{3}$$

A plot of $1/[T]$ *vs.* $1/A$ is linear; the abscissa intercept is $1/[L_t]\Delta\epsilon$, while the ordinate intercept is $-K$. As is shown in Figure 5, K was 3 M^{-1} for DNP-aminocaproate and 10 M^{-1} for TNP-aminocaproate (at 25°). The molar extinction coefficients at 470 mμ for each ligand were the same when bound by antibody or tryptophan (Table I).

Effects of Other Amino Acids, Salt, and Other Solvents. While TNP-lysine underwent a variety of spectral shifts in concentrated solutions of various amino acids, NaCl, and some organic solvents, in no instance did these changes duplicate those observed with binding to antibody or to tryptophan. For example, at 0.1 M (in buffered saline) alanine, aspartate, glutamate, glycine, histidine, lysine, proline, threonine, serine, valine, methionine, phenylalanine, isoleucine, leucine, and arginine produced only minor positive or negative deflections at 420 mμ in difference spectra when each was mixed independently with TNP-lysine at 4×10^{-5} M. Cysteine was not tested as there is at most one anomalous SH group per molecule of γG-immunoglobulin. The different effects of all these amino acids and tryptophan are illustrated in Figure 6 using the phenylalanine effect as representative. At saturation (2×10^{-3} M) tyrosine caused no spectral shift, whereas in 2×10^{-3} M tryptophan a spectral shift in TNP-lysine was evident at 380 mμ.

A special effort was made to demonstrate spectral changes with tyrosine and cystine, and because of their limited aqueous solubility some spectra were also obtained in dimethyl sulfoxide. In this solvent tryptophan formed an orange-red complex with TNP-aminocaproate and an orange complex with 1,3,5-

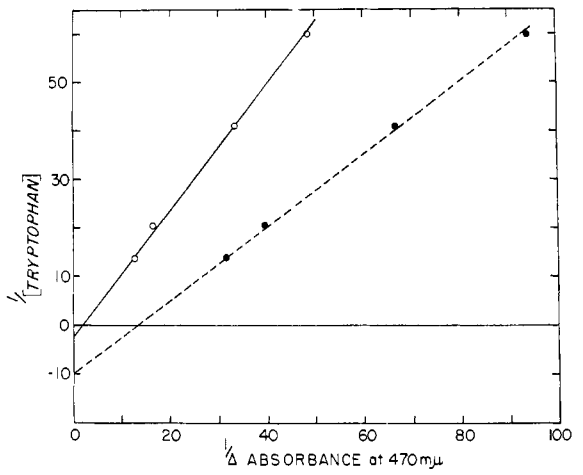

FIGURE 5: Determination of molar extinction coefficients of tryptophan-bound ligands and of equilibrium constants for complex formation. Δ absorbance at 470 mμ refers to the difference between reference cell with ligand and experimental cell with ligand plus tryptophan. The concentrations of DNP-aminocaproate and TNP-aminocaproate were 8.23×10^{-4} and 9.37×10^{-5} M, respectively. Tryptophan concentrations ranged from 0.05 to 0.0099 M. The abscissa intercept ($1/[L_t]\Delta\epsilon$) was 14 for TNP-aminocaproate and 2 for DNP-aminocaproate, corresponding to $\Delta\epsilon$ values of 760 and 610, respectively (see Table I). DNP-aminocaproate (O——O); TNP-aminocaproate (●– –●).

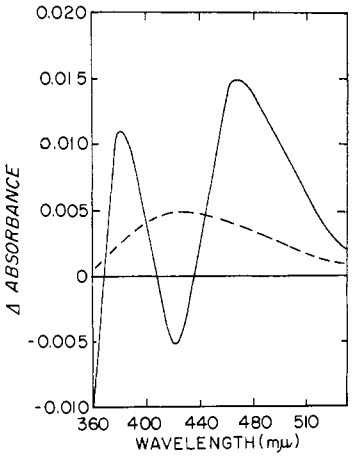

FIGURE 6: Difference spectra of TNP-lysine with tryptophan or phenylalanine as the free amino acids. Solid curve: TNP-lysine (5.52×10^{-5} M) plus tryptophan (0.05 M) in the experimental cell and the same concentration of TNP lysine in the reference cell. Dotted curve: TNP lysine (5.52×10^{-5} M) plus phenylalanine (0.05 M) in the experimental cell and the same concentration of TNP lysine in the reference cell.

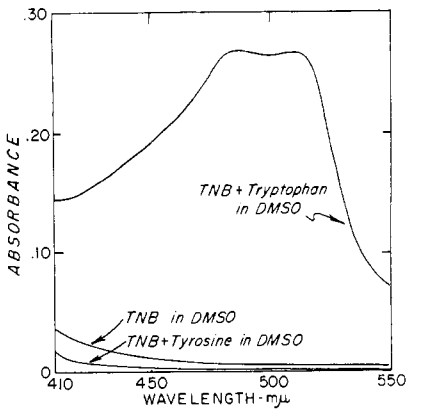

FIGURE 7: Absorbance spectra of 1,3,5-trinitrobenzene (TNB) alone and mixed with tyrosine methyl ester or tryptophan in dimethyl sulfoxide (DMSO). The upper curve shows the spectrum of the orange complex formed by 3.8×10^{-5} M TNB and 3.3×10^{-5} M tryptophan. The lower curves illustrate the absence of significant visible absorbance of TNB alone or a mixture of 3.8×10^{-5} M TNB plus 3.3×10^{-5} M tyrosine methyl ester.

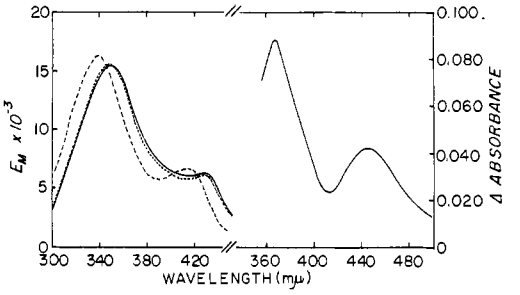

FIGURE 8: Absorbance and difference spectra of TNP-lysine. Left-hand panel: Absorbance spectra of TNP-lysine in 95% methanol (– – – –), buffered saline (. . . .), and 2 M NaCl (————). Right-hand panel: Difference spectrum of TNP-lysine at 6.73×10^{-5} M in 2 M NaCl (pH 7.4) in the reference cell and TNP-lysine at the same concentration in buffered saline in the reference cell. The λ_{max} values are 368 and 445 mμ and λ_{min} is 414 mμ.

trinitrobenzene (TNB), whereas at the same amino acid concentrations, tyrosine methyl ester, cysteine, and cystine failed to form colored complexes with TNB.[4] The absorption spectra of TNB with and without tryptophan and tyrosine methyl ester are shown in Figure 7.

Because disulfides serve as electron donors in charge-transfer complexes (Moreau and Weiss, 1966), the effect of oxidized glutathione was also examined. In the presence of 0.1 M glutathione at pH 7.4 the

[4] In dimethyl sulfoxide but not in buffered saline a saturated solution of phenylalanine formed a faint orange complex with TNP-aminocaproate but not with DNP-lysine or DNP-aminocaproate.

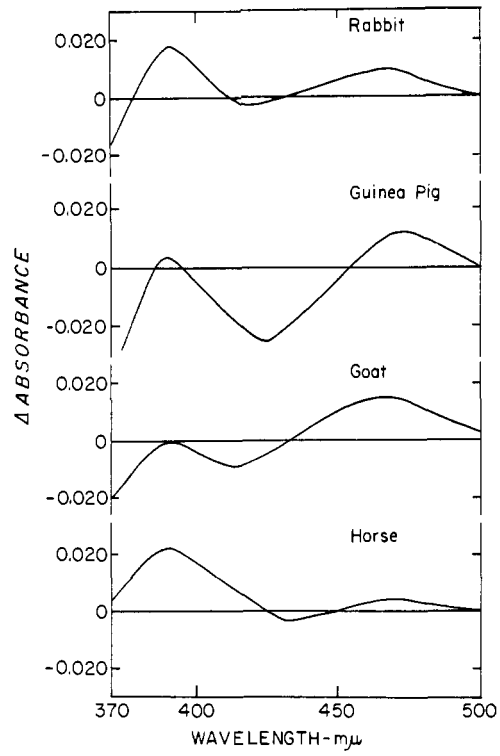

FIGURE 9: Difference spectra with purified anti-DNP antibodies from rabbit, guinea pig, goat, and horse. Each spectrum was obtained as the difference between antibody plus ligand in the experimental cell and the same total ligand concentration in the reference cell. The following concentrations were employed: rabbit antibody (0.82 mg/ml) and DNP-aminocaproate (1.03×10^{-4} M), guinea pig antibody (1.28 mg/ml) and DNP-aminocaproate (1.43×10^{-5} M), goat antibody (0.97 mg/ml) and DNP-lysine (8.73×10^{-6} M), and horse antibody (1.44 mg/ml) and DNP-lysine (8.73×10^{-6} M).

absorbance maxima characteristic of TNP-lysine in buffered saline were replaced by a single broad band with λ_{max} at 404 mμ. This spectrum resembled that of TNP-lysine in 0.1 M KOH, but bore no resemblance to that of the antibody-bound or tryptophan-bound ligand.

TNP-lysine underwent a blue shift in dioxane and in methanol (Figure 8), and TNB has also been reported to undergo a blue shift in ethanol (Abe, 1959). In 2.0 M NaCl, TNP-lysine underwent a red shift as shown in Figure 8. However, the shift did not duplicate that observed with binding to antibodies or to tryptophan.

DNP and TNP Ligands with Antibodies from Various Mammalian Species. When DNP-aminocaproate or DNP-lysine was bound by anti-DNP antibodies from guinea pig, goat, or horse (Figure 9), difference spectral peaks were also observed at 390 and 470 mμ. Similar maxima (at 380 and 470 mμ) also appeared when guinea pig anti-TNP antibodies bound TNP-aminocaproate (see figure in Little and Donahue, 1967).

Discussion

The present study shows that DNP and TNP ligands exhibit similar spectral shifts when bound by anti-DNP antibodies, anti-TNP antibodies, and by tryptophan, as the free amino acid. The wavelength maxima of the difference spectra coincide, and the molar extinction coefficients of the antibody-bound and tryptophan-bound ligands are also the same (at 470 mμ). Despite this coincidence the possibility must be considered that the spectral changes in the antibody-bound ligands result from relatively nonspecific solvent effects attributable to a difference between the dielectric environments of the antibody combining site and the bulk solvent. However, in solvents of lower dielectric constant than water, such as methanol, DNP and TNP ligands undergo a blue shift (Figure 8). In solvents of higher dielectric constant (0.7–5.0 M NaCl in water) TNP-lysine, taken as a representative ligand, underwent a red shift, but no hypochromicity was observed and maxima in the difference spectra were distinctly different than those of the antibody-bound ligand (Figures 2, 3, and 8). It seems more likely, therefore, that the spectral change observed with specific binding by antibody is due to interaction with a tryptophan residue in the protein's active site.

Since the specificity of an antibody must arise from the amino acid residues that constitute its active site, antibodies from different species should have similar active sites if specific for the same antigenic determinant. Similarly, residues that are essential for specificity should be present in all antibody molecules of the same specificity, regardless of the antibody's affinity for the ligand. It is of interest, therefore, that DNP ligands undergo similar spectral shifts when bound by anti-DNP antibodies synthesized in the rabbit, guinea pig, goat, and horse, and when bound by rabbit anti-DNP molecules that differ at least 1000-fold in affinity. Similar findings apply to TNP ligands and anti TNP molecules from rabbit and guinea pig. Accordingly, it seems reasonable to propose that tryptophan may be present in the active sites of all anti-DNP and anti-TNP antibodies. Since these two kinds of antibodies cross-react extensively they should have some common structural elements in their active sites.

The complexes formed by polynitrobenzenes with tryptophan or with tryptophan residues of protein are expected to be of the charge-transfer type (Szent-Gyorgyi *et al.*, 1961). A recent report, in fact, provides evidence for the formation of a charge-transfer complex between 2,4-dinitroacetophenone and a tryptophan in or near the active site of chymotrypsin (Sigman and Blout, 1967). In the present study, however, the spectral change of bound ligand (to tryptophan or to antibody) produces a red shift without the appearance of distinct charge-transfer band. Moreover, the red shift is roughly the same for complexes with DNP and with TNP ligands, whereas complexes involving the more extensively nitrated group should exhibit a shift to a longer wavelength (Jurinski and de Maine, 1964). It seems possible, nevertheless, that DNP

and TNP ligands do form charge-transfer complexes with tryptophan and with a tryptophan residue in the active site of antibody. A new band does appear at long wavelengths in mixtures of TNB and tryptophan in DMSO (Figure 7), and in aqueous solutions of other ligand mixtures a charge-transfer band could be masked in the region 420–490 mμ.

For electron-acceptor molecules increasing nitration of the benzene ring increases electron affinity and stability of charge-transfer complexes. In accord with this generalization ΔF° (at 25°) for the formation of the tryptophan complex with TNP-aminocaproate was −1.5 kcal as compared with −0.65 kcal for DNP-aminocaproate (Figure 5). These values are in the range found for charge-transfer complexes of diverse nitrobenzenes with a variety of electron donors (Jurinski and de Maine, 1964).

The ΔF° values for the formation of complexes with tryptophan, as the free amino acid, are far smaller than the standard free-energy changes observed in the specific binding of DNP and TNP ligands by the homologous or cross-reacting antibodies (−6 to −11 kcal; Eisen and Siskind, 1964; Little and Eisen, 1966). Thus, the proposed interaction between bound ligand and a tryptophan residue could only make a relatively small energetic contribution to the stability of the antibody–ligand complex.

The interesting studies of Metzger *et al.* (1963) with the method of "affinity labeling" have indicated that with rabbit anti-DNP antibodies tyrosine residues are in or near the active site, but a tryptophan residue was not detected. Possibly the diazonium salts used for affinity labeling do not form stable azo derivatives of tryptophan (Wofsy *et al.*, 1967), or, by making contact with a susceptible tryptophan residue, shield it from reaction with the diazonium substituent. In any event, we believe that the spectral changes associated with hapten binding to anti-DNP or anti-TNP molecules are due to contact between bound ligand and tryptophan, not tyrosine, residues.

There is no reason to suppose that if tryptophan is present in the active sites of antibodies that bind DNP and TNP groups selectively it would be limited to them. Tryptophan could well be similarly involved in active sites of antibodies for other haptenic groups with high electron affinity, such as the *p*-iodophenylsulfonyl (pipsyl) group.

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