is not catalyzed by the addition of octanol is consistent with this hypothesis. In COHb, the heme would not be expected to be displaced by the alcohol because of the strong covalent bond between the heme iron and proximal imidazole, which remains intact even on denaturation (Geddes and Steinhardt, 1968; Allis and Steinhardt, 1970).

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Alterations of Antibody Binding Properties and Active-Site Dimensions in the Primary and Secondary Immune Response*

J. C. Hsia† and J. Russell Little‡

ABSTRACT: Rabbit antibodies specific for the 2,4,6-trinitrophenyl (TNP) determinant group were obtained from the primary and the secondary response and their active sites were studied with two spin-labeled 2,4,6-trinitrophenyl ligands using electron spin resonance spectroscopy and fluorescence quenching. The substitution of the homologous TNP group with a 3-amino- or 3-methylamino-2,2,5,5-tetramethylpyrrolidine nitroxide introduced steric perturbations to the antibody–hapten complex formation. Differences in structure of the combining sites of antibodies produced during the primary or secondary immune response were inferred from the extent and the nature of these perturbations and the binding energy of antibody–hapten complex formation. These results indicate that antibodies produced during primary and secondary immune responses contain distinctive and relatively nonoverlapping subsets with different active-site structures. Antibodies from the primary response: (1) are less tolerant to steric perturbations; (2) form less rigid complexes with haptens; and (3) are more susceptible to organic solvent denaturation. The dimension of the secondary anti-TNP antibody active site parallel to the long axis of the spin-labeled ligand is at least 10 Å.

Many previous studies have documented differences in the ligand binding properties of antibodies produced in primary and secondary immune responses. After the initial exposure to immunogen, a second exposure several weeks or even years later often evokes a more vigorous immune response, and the antibodies produced usually form more stable complexes with antigen than those formed at a comparable time after the first injection (Eisen and Siskind, 1964; Little and Eisen, 1966; Fujio and Karush, 1966; Parker *et al.*, 1967). Antibodies from the secondary response are also more cross-reactive, *i.e.*, less discriminating (Little and Eisen, 1969). In addition, there is evidence of differences in structure between IgG antibody molecules obtained early or later in the immune

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† Supported in part by Research Grant MA-4129 MRC-DA-6 from the Medical Research Council of Canada and National Research Council Post Doctoral Fellowship 1969–1970.

‡ Supported in part by Research Grant AI 09723 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, by Research Grant T-560 from the American Cancer Society, Inc., and by a Public Health Service Research Career Program award (1-K3-AM-38,620) from the National Institute of Arthritis and Metabolic Diseases.

response, including differences in amino acid composition (McGuigan *et al.*, 1968) and in idiotypic determinants that may reflect changes in the structure of the active sites (MacDonald and Nisonoff, 1970; Brient and Nisonoff, 1970).

It was also shown that the increase in average affinity for 2,4-dinitrophenyl (DNP)[1] antigens resulted from a sequential change in the nature of antibodies synthesized by rabbit lymph node cells (Steiner and Eisen, 1967). It seems possible that these sequential changes in antibody affinity and cross-reactivity can be directly related to the changes at the antibody combining site by using the spin-labeled hapten technique (Stryer and Griffith, 1965). Recent experiments with spin-labeled haptens and rabbit anti-DNP molecules have yielded information concerning the dimensions (Hsia and Piette, 1969a), flexibility (Hsia, 1968; Hsia and Piette, 1968), and cross-reactivity (Hsia and Piette, 1969b) of the active sites.

The present study was undertaken in an attempt to correlate the changes in the affinity and cross-reactivity of antibodies specific for the 2,4,6-trinitrophenyl (TNP) determinant group (Little and Eisen, 1969) to structural alterations and physical properties of the hapten combining sites.

TABLE I: Binding Constants Calculated from Fluorescence Quenching Titrations of Primary and Secondary Anti-TNP Antibodies.

	Hapten	K_0 ($M^{-1} \times 10^{-8}$)	Heterogeneity Index (a)
Primary[a] anti-TNP antibodies	ε-TNP-L-lysine	0.051[b] (0.12[c])	0.36[b] (0.43[c])
	TNP-SL(5)	0.010[b]	1.2[b]
	TNP-methylene-SL(5)	0.020[b]	0.69[b]
Secondary[a] anti-TNP antibodies	ε-TNP-L-lysine	24[b] (13[c])	0.45[b] (0.34[c])
	TNP-SL(5)	0.17[b]	0.82[b]
	TNP-methylene-SL(5)	6.4[b]	0.48[b]

[a] Anti-TNP antibody concentrations were approximately 5×10^{-7} M. [b] Stock solution of TNP ligands (6×10^{-6} M) contain 10% v/v acetonitrile in 0.02 M phosphate buffer (pH 7.4). [c] Stock solution of ε-TNP-L-lysine containing no acetonitrile.

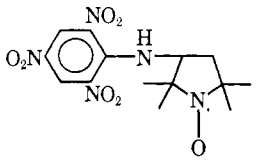
Materials and Methods

Antibody Isolation. The methods employed for production, quantitation, and isolation of anti-TNP antibodies have been described previously (Little and Eisen, 1966). All antisera were obtained from rabbits immunized with heavily substituted TNP-BγG, prepared by methods described elsewhere (Little and Eisen, 1967). Each lot of purified antibodies showed a single arc of IgG mobility on immunoelectrophoresis developed with goat antiserum to a crude rabbit globulin fraction and each was more than 90% precipitable with a different hapten–protein conjugate (HSA bearing 36–37 moles of TNP/mole of albumin). All antisera were pools derived from 25 to 50 randomly bred albino rabbits. Bleedings were generally obtained on 3 consecutive days from each animal, and the interval from immunization was counted on day 1 of bleeding. Anti-TNP antibodies from the primary immune response were obtained from bleedings 13 days after initial immunization. Antibodies from the secondary response were obtained 21 days after a second injection of the same immunogen (TNP-BγG) used for the initial immunization one year earlier.

Fluorescence Quenching. The binding of haptens by purified antibodies was measured in an Aminco-Bowman spectrophotofluorometer by the method of fluorescence quenching (Velick *et al.*, 1960; Little and Eisen, 1966). All titrations were performed at 26°. Bound and free hapten were determined on the basis of the fluorescence quenching observed, relative to the quenching when all antibody combining sites were occupied by ligand (Q_{max}). As has been pointed out previously (Little and Eisen, 1968; McGuigan and Eisen, 1968) Q_{max} valued obtained as these were by the method of Day *et al.* (1963) are only accurate for antibody–ligand pairs that interact strongly, since the method requires saturation of the antibody binding sites. Since the range of values for Q_{max} is narrow (52–62%) for a large number of preparations of rabbit anti-TNP antibodies, we have employed the previously determined average value, 55%, for our preparation of primary antibodies, and the previously validated value of 56% for our lot of secondary antibodies (Little and Eisen, 1966). Since Q_{max} for a given antibody population has not been found to vary due to the particular nitrophenyl ligand employed, we have confidence in the validity of the quenching method in providing reasonably accurate comparative values for binding constants and heterogeneity indices (Table I).

Antibody concentrations in 0.15 M NaCl–0.02 M phosphate (pH 7.4) (buffered saline) were determined by absorbance at 278 nm ($\epsilon^{1\%}_{1cm}$ 16.0 and 15.8 for the primary and secondary antibodies, respectively) assuming a molecular weight of 150,000 (Little and Eisen, 1968; Little and Donahue, 1968). The average intrinsic association constant, K_0, was calculated from the fluorescence quenching data according to the logarithmic form of the Sips equation (Nisonoff and Pressman, 1958; Karush, 1962).

Preparation of Spin-Labeled Haptens. TNP-SL(5) (I). *N*-(1-Oxyl-2,2,5,5-tetramethyl-3-aminopyrrolidinyl)-2,4,6-trinitrobenzene was prepared by the following reaction.



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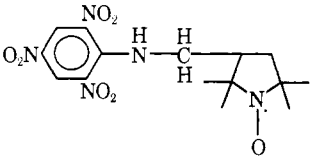

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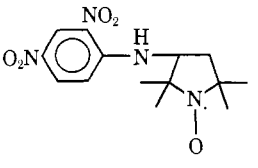

III

[1] Abbreviations used are: TNP, the 2,4,6-trinitrophenyl group; DNP, the 2,4-dinitrophenyl group; BγG, bovine γ-globulin; HSA, human serum albumin; RγG, rabbit γ-globulin. TNP-SL(5), TNP-methylene-SL(5) and DNP-SL(5) are five-membered pyrrolidine nitroxide spin-label (SL(5)) derivatives of the indicated hapten. Antibodies formed against 2,4-dinitrophenylated proteins or 2,4,6-trinitrophenylated proteins are referred to as anti-DNP antibodies and anti-TNP antibodies, respectively. Primary anti-TNP antibody or primary antibody refer to anti-TNP molecules isolated from the pooled bleeding of 25 rabbits, 13 days after antigen injection. Secondary anti-TNP antibody or secondary antibody refer to antibodies obtained 21 days after a second antigenic stimulation of animals that had received their first injection with the same antigen 1 year previously.

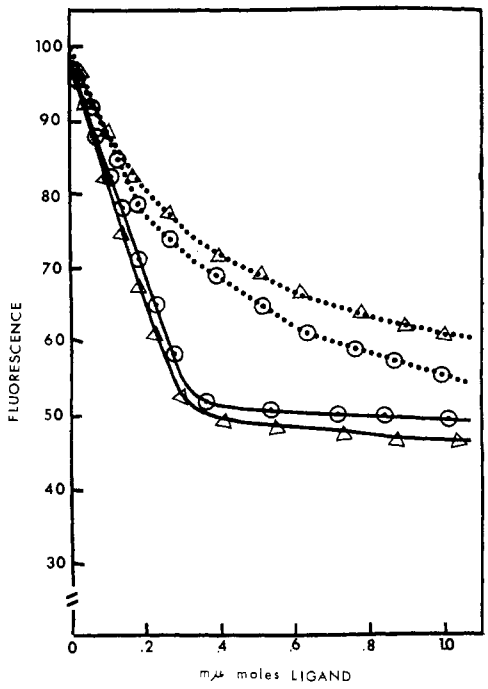

FIGURE 1: Fluorescence quenching of primary (· · · · · ·) and secondary (——) anti-TNP antibodies with ε-TNP-L-lysine stock solution in 0.02 M potassium phosphate buffer (pH 7.4) with (△) and without (○) 10% acetonitrile, *i.e.*, since the last addition of ligand was 0.02 ml of the stock solution, this corresponds to a maximum concentration of 1.96% acetonitrile by volume in each titration. Each titration was obtained with 1 ml aliquots of antibody in 50 μg/ml in buffered saline at 26°. Fluorescence values have been corrected for solvent fluorescence and for volume change due to ligand addition.

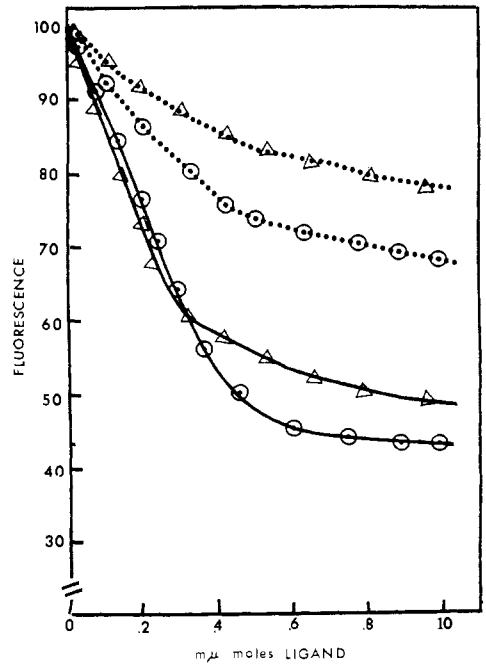

FIGURE 2: Fluorescence quenching titrations of primary (· · · · ·) and secondary (——) anti-TNP antibodies with spin-labeled TNP ligands; TNP-SL(5) (△) and TNP-methylene-SL(5) (○). Conditions are the same as those given in the legend to Figure 1.

1-Oxyl-2,2,5,5-tetramethyl-3-aminopyrrolidine prepared by the procedure of Rozantzev and Krinitzkaya (1965) was reacted with an equal molar amount of picryl chloride in chloroform with magnetic stirring for 12 hr. The reaction mixture was filtered and chromatographed on a silicic acid column equilibrated with chloroform. The second yellow band was collected and dark purple microcrystals were obtained after the solvent was reduced under vacuum. After recrystallization from chloroform and ether, metallic purple crystals were obtained: mp 141–142°, λ_{max} 337 nm (ε 15,400) in acetonitrile. The spin concentration determined from known standards was 6.0 (±0.2) × 10^{23} spins $mole^{-1}$. *Anal.* Calcd for $C_{14}H_{18}N_6O_4$: C, 45.7; H, 5.06; N, 19. Found: C, 45.6; H, 5.05; N, 19.

TNP-METHYLENE-SL(5) (II). *N*-(1-Oxyl-2,2,5,5-tetramethyl-3-methylaminopyrrolidinyl)-2,4,6-tritrobenzene was prepared by the following reactions. 1-Oxyl-2,2,5,5-tetramethyl-3-methylaminopyrrolidine prepared by the procedure of Hsia and Piette (1969a) was reacted with picryl chloride analogous to that of TNP-SL(5). After recrystallization, the purified crystalline material had a melting point of 194–195°. The molecular weight determined by mass spectroscopy was 368. The absorption spectrum of II in acetonitrile had a λ_{max} at 337 nm (ε 15,600). The spin concentration determined from known standards was 5.2 × 10^{23} spins $mole^{-1}$.

DNP-SL(5) (III). *N*-(1-Oxyl-2,2,5,5-tetramethyl-3-aminopyrrolidinyl)-2,4-dinitrobenzene was prepared by the procedure of Hsia and Piette (1969b): mp 205–206°, λ_{max} at 350 nm (ε 17,400).

Electron Spin Resonance Titrations. The titration apparatus was built around a standard quartz flat cell for the electron spin resonance spectroscopy study of aqueous solutions. It was fitted at the bottom end with a 2-ml syringe *via* a Luer-Lok connector and a Teflon plug. The antibody solution (1.0 ml) was introduced *via* the wide-top end of the cell and pulled into the narrow flat compartment using the syringe. The esr instrument was then tuned and locked onto the resonance frequency of the cavity-sample configuration. Thenceforth, the flat cell was retained in position by clamps, and its vertical and horizontal orientations were not varied. The sample was raised into the wide neck of the cell by depressing the syringe plunger. Using a microliter pipet, aliquots of a spin-labeled hapten were introduced *via* the wide top end of the cell. After gentle manipulation of the plunger to facilitate mixing, the sample was introduced into the observation compartment. An esr spectrum was taken, and the procedure was repeated with further aliquots of hapten. Small corrections in signal intensity were made to correct for the volume change due to the addition of ligand. A preliminary account of this technique has been reported (Hsia, 1968). Esr spectra were recorded on a Varian E-6 or E-3 X-band spectrometer at 22 ± 2°.

Results

Fluorescence Quenching Measurements. The binding of spin-labeled TNP ligands was compared to the binding of ε-TNP-L-lysine by the method of fluorescence quenching (Velick *et al.*, 1960). ε-TNP-L-lysine quenches approximately 45 and 52% of the total fluorescence of primary and secondary antibodies, respectively (Figure 1), at a total ligand concentration of 8.33 × 10^{-7} M. However, the same concentration of TNP-SL(5) was found to be much less effective in quenching the fluorescence of primary antibodies (20% quenching), and TNP-methylene-SL(5) gives 30% quenching at the end of titration (Figure 2). The average association constants, K_0, calculated according to Sips distribution function, are sum-

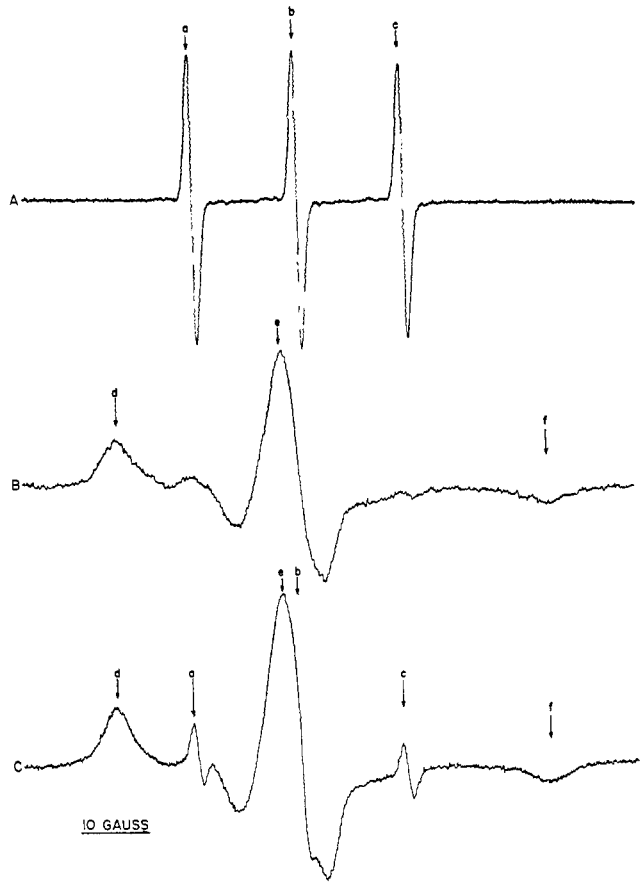

FIGURE 3: Electron spin resonance spectra of *N*-(1-oxyl-2,2,5,5-tetramethyl-3-aminopyrrolidinyl)-2,4,6-trinitrobenzene, *i.e.*, TNP-SL(5). (A) Free TNP-SL(5) (1×10^{-5} M) spectrum in the presence of 1×10^{-5} M normal RγG. (B) Pure immobilized spectrum on TNP-SL(5), 1.5×10^{-5} M, at secondary anti-TNP-antibody (5×10^{-5} M) active sites. (C) Mixture of free and immobilized spectrum. TNP-SL(5) and secondary antibody concentrations are 4×10^{-5} and 5×10^{-5} M, respectively. Peaks a,b,c and d,e,f are explained in the text. All spectra were recorded at 22 ± 2° in 0.15 M NaCl–0.02 M phosphate buffer at pH 7.4.

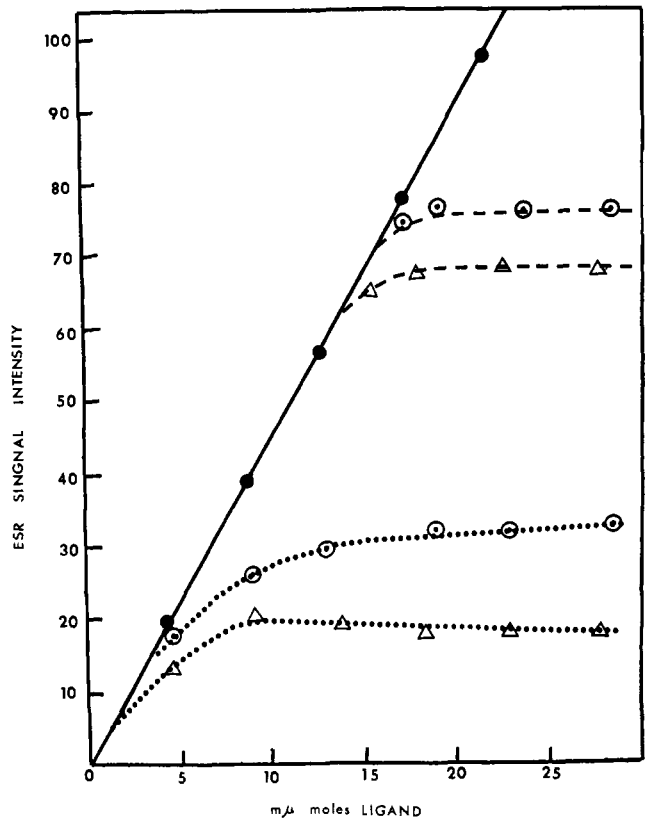

FIGURE 4: Spin-labeled TNP ligand titration of anti-TNP antibody from primary (· · · · ·) and secondary (- - - - -) immune response. Each titration was performed with 1 ml of 1×10^{-5} M of antibody and stock solutions of spin-labeled TNP ligands in acetonitrile. Standard free spin-labeled hapten concentrations were obtained in the presence of 1×10^{-5} M, of nonimmune RγG (●). Concentrations of antibody-bound TNP-SL(5) (△) and TNP-methylene-SL(5) (⊙) were obtained from the difference in concentratio n of the free TNP spin label in the presence of anti-TNP antibody or nonimmune RγG. The percentage of available sites was obtained from the total bound TNP ligand and the total antibody concentration assuming two sites per molecule.

marized in Table I. The low values obtained for the binding of spin-labeled ligands by primary antibodies may be due to a reduced affinity for these ligands compared to ε-TNP-L-lysine or to the inability of a fraction of the active sites to accommodate the bulky pyrrolidine nitroxide groups. The latter alternative is supported by esr titration experiments (see below). On the contrary, TNP-SL(5), TNP-methylene-SL(5), and ε-TNP-L-lysine gave similar fluorescence quenching titrations with the secondary antibodies (compare Figures 1 and 2) indicating a generally higher affinity for these ligands and a greater ability to accommodate the pyrrolidine nitroxide group than was observed with the primary antibodies.

Esr Titration of Available Antibody Active-Site Concentration. The principle of distinguishing the free and antibody-bound TNP spin labels relies on the fact that the resonance spectrum of the nitroxide spin label is dependent on its motional freedom in solution. A sharp triplet with hyperfine splitting constant of 16 ± 1 gauss (Figure 3A) is typical of a freely tumbling nitroxide spin label in aqueous medium having a rotational correlation time (τ_c) of approximately 10^{-11} sec (Stone *et al.*, 1965). A characteristic antibody-bound spectrum is shown in Figure 3B, which is the result of immobilization of the spin-labeled hapten at the antibody active site; therefore, motional freedom of the label is determined by the correlation time of the antibody as a whole ($\tau_c = 1.68 \times 10^{-7}$ sec, Yguerabide *et al.*, 1970). The distance between the spin label and the haptenic group must be short (3–4 Å) in order to obtain distinctive free and bound spectra.

The peaks a and c in Figure 3A are completely separated from peaks d and f in Figure 3B, whereas peaks b and e are almost superimposable. This is much more evident when additional spin-labeled hapten was added in Figure 3C. When antibody-bound and free spin-labeled hapten are at equilibrium, peaks a and c represent free hapten and peaks d and f represent the bound hapten. These resonance peaks appear at a different magnetic field, making it convenient to quantitate the two species independently. Furthermore, it is apparent from the nearly identical height of the free-ligand peaks a and c (Figure 3C) that *only* two ligand states are detected, *i.e.*, free and bound. There is no evidence that an intermediate state (*e.g.*, inequality of peaks a and c) results from the rapid exchange between the antibody-bound and free states of the ligands used in this work. In addition, peaks d, e, and f were converted into a, b, and c when ε-TNP-L-lysine was added to displace the spin-labeled ligand at the antibody active site. Therefore, the bound spin-labeled hapten concentration (*i.e.*, occupied active site concentration) can be obtained by mea-

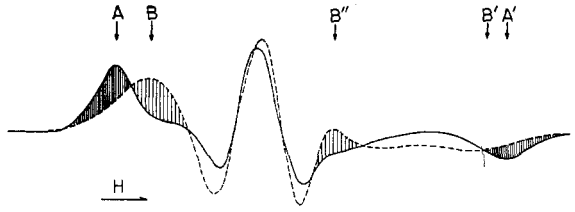

FIGURE 5: Electron spin resonance spectra of 1-oxyl-2,2,6,6-tetramethyl-4-piperidinol, 1×10^{-4} M in glycerol containing buffered saline. The correlation time (τ_c) of the piperidine nitroxide calculated from the viscosity of the medium is 20 nsec at 1.58 P and 283°K (- - - -) and 70.6 nsec at 5.77 P and 273°K (——) using the Stokes and Einstein equation, $\tau_c = 4\pi\eta a_0^3/3kT$, where η is the viscosity of the medium in poise and a_0 is assumed to be 5 Å, the effective radius of the nitroxide, k is the Boltzman constant, and T, the absolute temperature. A_{max} is measured as the separation between A and A′ or B and B′, respectively. Due to the ill-defined high-field peak (B′) of a weaker immobilized label, differences in mobility of the spin label are generally indicated as the separation between the low-field peaks (A and B). The shaded area under B′′ is also characteristic of increased motional freedom of the label.

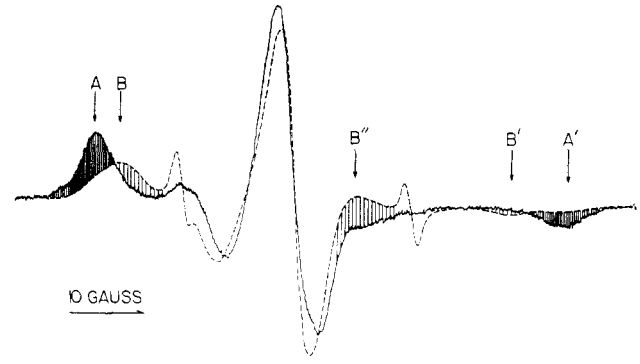

FIGURE 6: Electron spin resonance of 1-oxyl-2,2,5,5-tetramethyl-3-aminopyrrolidine derivatives of TNP and DNP ligands at secondary (——) and primary (- - - -) anti-TNP antibody active sites. Antibody concentrations were 5×10^{-5} M and concentrations for TNP-SL(5) and DNP-SL(5) were 2×10^{-5} and 1×10^{-5} M, respectively. The separation of low-field peaks, A and B, is 2.7 G. The sharp peaks appearing on both sides of the center peak are due to presence of unbound DNP-SL(5). Shaded area under the arrow at B′′ also indicates the less immobilized DNP-SL(5).

suring the difference in the intensity of peak c at a particular concentration of antibody and RγG, respectively. Using this method, the fraction of the active sites that bind TNP-methylene-SL(5) is 88 and 42% for secondary and primary antibodies, respectively (Figure 4), assuming two sites per IgG molecule. Less than complete availability of the active sites of the secondary antibodies could be accounted for by the high affinity of this antibody preparation and a resultant small contamination with one of the TNP ligands used in the isolation of these antibodies from serum. However, the active sites available to TNP-SL(5) were slightly lower in both primary (25–30%) and secondary (75–80%) antibodies (Figure 4). Therefore, we conclude that because of steric perturbations not all sites available to ε-TNP-L-lysine were available to the spin-labeled TNP ligands. The primary antibodies were more susceptible to this perturbation and the mobility and homogeneity of the motional characteristics were different in the primary and secondary antibody active sites (see Figure 7 below).

Stability of Primary and Secondary Antibody Sites. Due to the low solubility (approximately 5×10^{-5} M) of the spin-labeled TNP ligands in aqueous solvents, stock solutions of these ligands were dissolved in acetonitrile. Minute amounts of acetonitrile were found to have different effects on both fluorescence quenching and esr titrations of the primary and the secondary antibodies. Two per cent by volume of acetonitrile slightly decreased the fluorescence quenching of the primary antibodies and increased the quenching of the secondary antibodies with ε-TNP-L-lysine (Figure 1) suggesting that the primary antibodies were more sensitive to denaturation by acetonitrile. However, an alternative possibility is that acetonitrile may form complexes with the polynitrobenzene ligands and these complexes could have distinctive binding properties with primary and secondary antibodies. Using the esr titration method, we have found a decrease of 25% in the availability of primary antibody sites to TNP-SL(5) when the acetonitrile final concentration was 10%. However, no effect on the available site concentration was observed when 10% acetonitrile was present in the secondary antibody preparation. (A loss of 25% of the available sites was observed at 20% acetonitrile by volume.) All esr measurements were made within 10 min after each ligand addition.

Justification for Using Changes in A_{max} for Estimating Variation in Immobilization of Antibody-Bound Ligands. Qualitative differences in immobilization of the spin label were measured in a simple empirical way. Generally, the maximum splitting between the high- and low-field peaks, A_{max}[2] (separations between A and A′ or B and B′) (Figure 5), is a sensitive measure of the magnitude of motional freedom of the label. The shift of the high- and low-field peaks toward the center of the spectrum (*i.e.*, decrease in A_{max}) results from the decrease in spin-label relative correlation time (Hsia and Piette, 1969a). In addition, the shaded area (Figure 5) under B′′ is also characteristic of higher mobility of the spin label. An example of this behavior was clearly seen in the superposition of the resonance spectra of two structurally identical spin labels on different haptens, *i.e.*, DNP-SL(5)[3] and TNP-SL(5) complexed, respectively, to the active sites of primary and secondary anti-TNP antibodies (Figure 6). The separation between the low-field peaks marked A and B is 2.7 G. The A_{max} values are approximately 52 G (separation between B and B′) and 62.5 G (separation between A and A′), respectively, for DNP-SL(5) and TNP-SL(5). The spectra in Figure 6 were chosen to illustrate an unusually large variation in A_{max} which results from additive contributions made by the greater immobility of the homologous interaction (TNP ligand bound by anti-TNP antibodies) than the cross-reaction and by the greater immobility of ligands bound in high-affinity combining sites. Therefore, we are confident that the variation in A_{max} can be used to distinguish small but subtle differences in the motional freedom of spin-labeled ligands in antibody active sites.

Difference in Motional Freedom of TNP-SL(5) in Primary and Secondary Antibody Active Site. The resonance spectrum of TNP-SL(5) in the active sites of the secondary anti-TNP molecules is a typical strongly immobilized spectrum (Figures 6 and 7). The motional freedom of TNP-SL(5) appears to be determined by the rotational and translational motion of the antibody. Fluorescence measurements of the rotational relax-

[2] The term A_{max} was first introduced by Hsia and Piette (1969a,b) to interpret variation of maximum peak separation of the immobilized spectrum of antibody bound spin-labeled hapten.

[3] Results on cross-reactions of anti-TNP antibodies will be reported in a subsequent publication.

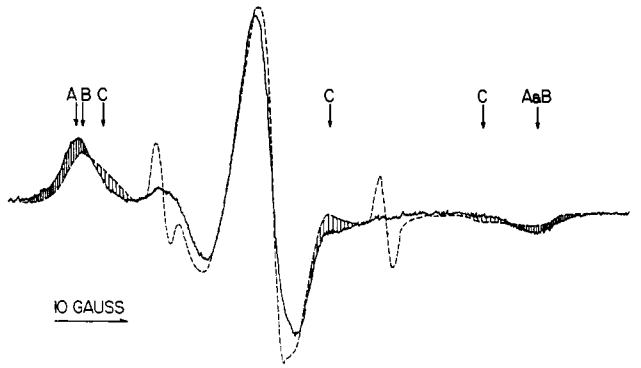

FIGURE 7: Electron spin resonance spectra of *N*-(1-oxyl-2,2,5,-5-tetramethyl-3-aminopyrrolidine-2,4,6-trinitrobenzene TNP-SL(5) at primary (- - - - -) and secondary (——) antibody active sites. Concentrations of various reagents in buffered saline are: antibody, 5×10^{-5} M; TNP-SL(5), 1×10^{-5} M at primary and 2×10^{-5} M at secondary antibody active site. The separation of low-field peaks under the arrows at A and B, is 0.8 G.

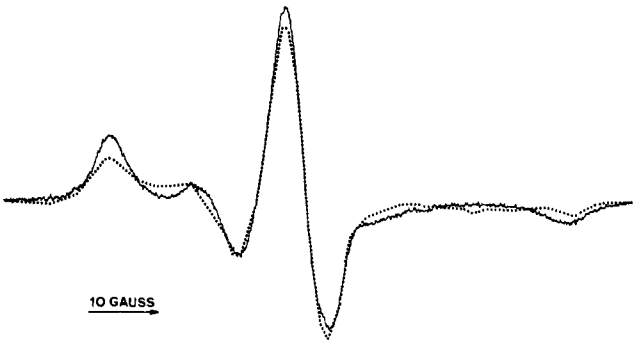

FIGURE 8: Electron spin resonance spectra of TNP derivatives of 1-oxyl-2,2,5,5-tetramethyl-3-aminopyrrolidine TNP-SL(5) and 1-oxyl-2,2,5,5-tetramethyl-3-aminomethylpyrrolidine TNP-methylene-SL(5) at secondary anti-TNP antibody active site. Antibody and TNP-SL(5) concentration for the solid line spectrum (——) is 5×10^{-5} and 2×10^{-5} M, respectively. Antibody and TNP-methylene-SL(5) for the dotted line (· · · · ·) spectrum were 1×10^{-5} and 0.88×10^{-5} M, respectively, in buffered saline.

ation time of rabbit antibody to the dimethylaminonaphthylsulfonamide group has been reported by Yguerabide *et al.* (1970) to be 168 nsec which is much slower in frequency than the anisotropic hyperfine energy, *i.e.*, 13 nsec, of the nitroxide spin label. Therefore, one would expect a broad assymetric spectrum as observed. Based on thermodynamic calculations, Eisen and Siskind (1964) postulated that the norleucine site of anti-DNP antibodies produced during the early primary response is not as well developed as in antibodies produced later. Therefore, one might expect the TNP-SL(5) would be less immobilized at the primary antibody active site. This was found to be the case. The differences in extent of immobilization of the same spin-labeled TNP ligand, TNP-SL(5), at primary and secondary anti-TNP antibody active sites are illustrated in Figure 7. There was a definite decrease in A_{max} of the TNP-SL(5) at active sites of primary antibodies compared to secondary antibody active sites; the difference in separation between high- and low-field peaks under arrows at A and B in Figure 7 is 0.8 G. In addition, the spectrum of a more weakly immobilized spin label (in the active sites of the primary antibodies) had the characteristic peaks indicated in the shaded areas under arrows at C. The immobilized resonance spectra compared in Figure 7 represent 70 and 27% of the secondary and primary antibody active sites, respectively, as calculated from the esr titrations. Furthermore, in the reaction between TNP-SL(5) and the primary antibodies, the broad asymmetry of the low-field peak (Figure 7) indicates the presence of at least two subsets among those active sites that can accommodate the spin label in addition to the major subset that fails to bind ligand under these experimental conditions. In contrast to this, the same ligand (TNP-SL(5) gives a symmetrical low-field peak (A in Figure 7) with a uniform A_{max} regardless of the percentage of the active sites occupied (up to 70%, the maximum value obtained).

Dimension of Active Sites of Anti-TNP Antibodies from Secondary Response. Using spin-labeled DNP ligands, the longitudinal dimensions of the active sites of rabbit anti-DNP antibodies obtained after a second antigenic stimulation were found to be 10 ± 1 Å (Hsia and Piette, 1969a). The current experiments demonstrate that the lower limit of one dimension of secondary anti-TNP antibody active sites is at least 10 Å. The characteristic spectra of TNP-SL(5) and TNP-methylene-SL(5) in secondary antibody active sites is shown in Figure 8. Both TNP-SL(5) and TNP-methylene-SL(5) gave strongly immobilized spectra indicating that C-3 of the pyrrolidine ring was bound by the protein. The distance between C-3 of the pyrrolidine ring and the 4-nitro group of TNP is 8.8 and 10 Å, respectively, for these two spin-labeled ligands. It would seem, therefore, that the secondary antibody active site is greater than 10 Å in a dimension measured along the long axis of the spin-labeled haptens. The TNP-methylene-SL(5) has a lower intensity of its low- and high-field peaks due to the introduction of the methylene group between the spin label and the haptenic group. A possible explanation for this spectral characteristic is that even when the TNP group is firmly held in the active-site, high-frequency, small-angle vibrations in the plane of the pyrrolidine ring are permissible due to the configuration of the region of the active site that accommodates the spin label. This type of anisotropic vibration in the plane of the nitroxide ring would reduce the g and hyperfine anisotropy perpendicular to the largest hyperfine anisotropy along the $2p\pi$ orbital of the N–O bond. Also, this type of vibration can alter the ratio of outer peak to center peak intensity without affecting A_{max}. Nevertheless, from the exent of immobilization of TNP-methylene-SL(5), we believe part of the pyrrolidine ring of the nitroxide is physically within the antibody site with limited anisotropic motional freedom.

Discussion

Steric perturbation of the nitroxide spin label is a serious limitation for the use of this sensitive method as a conformation probe. For example, placing a nitroxide at the α-amino group of the phenylalanine methyl ester makes it an inhibitor of α-chymotrypsin (Hamilton and McConnell, 1968). However, since antibody active sites are heterogeneous and no catalysis is involved, the steric perturbations induced by the spin label can be used to detect differences in the active sites of different antibody populations of the same specificity or from subsets of the same antibody population.

Our failure to detect the stoichiometric number of binding sites of primary antibodies with TNP-SL(5) and TNP-methylene-SL(5) suggests that the majority (70–75%) of the primary antibody active sites were not flexible enough to accommodate the bulky spin-label substituent. By this it is meant

that the affinity was so low (*i.e.*, $K \leqslant 1 \times 10^4$ M^{-1}) that a large subset of the active sites was not detected under the experimental conditions employed. The perturbation of the spin label on TNP-SL(5) was especially serious where only 25–30% of the sites were able to accommodate the spin-labeled ligand. According to the dimensions or depending on the physical and chemical structure of the antibody active sites, the presence of the pyrrolidine nitroxide group could impose either a positive or a negative contribution to the free energy of complex formation. Therefore, not all the active sites were available to the spin-labeled ligands for steric reasons. However, this provides additional information concerning the dimensions of the portion of the active site that is adjacent to the nitrobenzene site, *i.e.*, the norleucine site. The bulky nitroxide group itself provides a steric probe for the dimensions and flexibility of active sites. By this criterion, primary and secondary antibodies can each be divided into two subsets—those whose sites can accommodate the spin-labeled probe, and those whose sites cannot ($K \leqslant 1 \times 10^4$ M^{-1}). Most of the sites of secondary antibodies (70%) appear to be indifferent to the close proximity of the pyrrolidine nitroxide to the TNP group, perhaps because strong affinity for the TNP group overcomes the steric hindrance of the nitroxide. Those sites which can accommodate the spin-labeled hapten seem to have a relatively flexible structure of larger volume (Figure 7).

The large percentage (70–75%) of primary antibody active sites that cannot be titrated with TNP spin-labeled ligands indicates that less than 30% of the primary antibody persist in the secondary immume response. Similarly, less than 30% of the total primary antibodies resembled high-affinity secondary antibodies. This evidence is in accord with other data suggesting distinctive structural features of the active sites of early and late antihapten antibodies (Brient and Nisonoff, 1970). The heterogeneity of the secondary antibodies is very difficult to estimate with spin-labeled TNP ligands due to the exceedingly high affinity. However, it should be possible to study this property with a series of spin-labeled di- and mononitrobenzene ligands for which the affinity is much lower.

Acknowledgments

It is indeed a pleasure for author, J. C. H., to acknowledge the advice and support of Professor L. H. Piette, Dr. I. C. P. Smith, and Dr. H. Schneider in this investigation. We thank Dr. H. N. Eisen for the initial suggestion which prompted undertaking this project.

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