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Variations in Affinities of Antibodies during the Immune Response*

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The affinities of anti-2,4-dinitrophenyl (DNP) antibodies for a variety of 2,4-dinitrobenzenes have been measured by fluorescence quenching and the values obtained have been verified in representative instances by equilibrium dialysis. All populations of anti-DNP molecules examined, whether isolated from pooled sera or from single bleedings of individual rabbits, were heterogeneous in respect to affinity for dinitrobenzenes. The heterogeneity in virtually every instance could be described by the Sips distribution function over a wide range of binding data, and each titration was thus characterized by an average intrinsic association constant (K_o), and by a heterogeneity index (a). Fractional precipitation of antibodies from serum, by addition of limiting amounts of antigen, yielded from the serum of individual rabbits anti-DNP populations that differed as much as 10,000-fold in K_o. Changes in affinity for ε-DNP-lysine were followed by isolating antibodies from sera at various times after a single injection of DNP-bovine-γ-globulin. The K_o increased progressively with time after immunization and the rate of increase was most conspicuous when small quantities of DNP-bovine-γ-globulin were injected initially. The rise in K_o was markedly delayed when large doses of antigen were injected. Antibody populations isolated early after immunization had nearly the same low affinity for both ε-DNP-L-lysine and for 2,4-dinitroaniline, and it is inferred that their specific binding sites are poorly adapted to the dinitroanilino group and insensitive to the norleucine moiety of ε-DNP-lysine. In contrast, antibodies isolated late after immunization bind ε-DNP-lysine strongly, and from their interactions with a variety of dinitrobenzenes it is inferred that their binding sites, on the average, are just about large enough to accommodate ε-DNP-L-lysine. Complex formation was enthalpy driven: ΔH° values ranged from -8 to -20 kcal mole^{-1} for different antibody-ligand pairs, and ΔS° values ranged from -5 to -30 entropy units mole^{-1}. When representative 2,4-dinitrobenzenes were bound by anti-DNP molecules, bathychromic and hypochromic spectral shifts were observed; in the case of antibody-bound 2,4-dinitrotoluene a new absorption peak appeared at 300 mμ. The possibility is raised that charge-transfer contributes to the stability of antibody-dinitrophenyl complexes.

A fundamental difference between antibodies and enzymes is manifested in the specific binding of their respective ligands. Enzyme preparations of a given specificity generally behave as though composed of homogeneous molecules with uniform capacity to bind their substrate. Antisera of a given specificity, on the other hand, invariably seem to contain antibodies with diverse affinities for any appropriate ligand (e.g., Landsteiner and van der Scheer, 1936; Heidelberger and Kendall, 1935; Pauling *et al.*, 1944). The heterogeneity of antibodies in respect to affinity, which is implied by the properties of antisera, has been confirmed in studies of the reversible binding of haptens by purified antibodies (e.g., Eisen and Karush, 1949; Karush, 1956; Nisonoff and Pressman, 1958). In view of this heterogeneity, average association constants have been determined on the assumption that the specific binding sites in any population of antibody molecules are characterized by a normal distribution function in respect to affinities (Pauling *et al.*, 1944; Karush, 1956). The average values thus found for a variety of antibody-hapten systems have, until recently, been remarkably similar, ranging from about 10^5 to 10^6 liters mole^{-1} (Karush, 1962; Eisen and Pearce, 1962), and the uniformity of these values has been interpreted (e.g., by Nisonoff *et al.*, 1959a) as evidence in support of Pauling's theory of antibody formation. According to this theory (Pauling, 1940), antibodies of exceptionally high affinity for antigen would be infrequent since, during biosynthesis, their dissociation from hypothetical antigen-templates would be improbable, whereas antibodies of unusually low affinity would escape detection and isolation.

Within the past few years, however, two studies of the interaction between ε-DNP-lysine[1] and antibodies specific for the 2,4-dinitrophenyl group have given

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[1] Abbreviations used in this work: DNP, 2,4-dinitrophenyl; BγG, bovine γ-globulin; Tris, tris(hydroxymethyl)aminomethane. Where ε-DNP-L-lysine appears unqualified by L or D it represents ε-DNP-L-lysine.

widely divergent average association constants, 10^5 liters mole^{-1} in one case, and about 10^8 in the second (Carsten and Eisen, 1955; Velick *et al.*, 1960). Preliminary experiments suggested that this difference could have arisen from differences in the methods of immunization used in the two studies (Parker *et al.*, 1962). In the present study we have attempted, therefore, to determine systematically just how the average affinities of antibody populations vary as a function of the conditions used to initiate and follow antibody formation.

The specific binding of 2,4-dinitrobenzenes by purified anti-DNP antibodies quenches the protein's intrinsic fluorescence (Velick *et al.*, 1960), and this effect has been used empirically to measure antibody-hapten association constants. The validity of the values obtained by this procedure has been established in the present study by independent determination of association constants by means of equilibrium dialysis. Fluorescence-quenching analyses can be carried out on samples as small as 40 μg purified antibody, and do not require direct measurement of unbound hapten; accordingly, it has been possible to determine a wide range of association constants of antibody fractions and subfractions isolated from individual rabbits. The results reported here demonstrate that the affinities of antibodies vary systematically during the immune response and are dependent on the time elapsed after initial injection of antigen and, to some extent also, on the amount of antigen given. The results provide an example, moreover, of how widely the anti-DNP molecules that populate the serum of a rabbit at any one time vary in affinity for the 2,4-dinitrophenyl group.

Reagents

Antigen.—Dinitrophenyl bovine γ-globulin was prepared and characterized as described previously (Eisen *et al.*, 1953, 1959). The modified proteins contained, on the average, 40–50 moles 2,4-dinitrophenyl per mole BγG, substituted in lysine residues.

[1-^{14}C]-2,4-Dinitroaniline.—Twenty mg [1-^{14}C]-2,4-dinitrochlorobenzene (Isotope Specialties, Inc., Glendale, Calif.; 0.4 mc/mmole) was heated with 40 mg dry ammonium acetate in a sealed tube at 120° for 2 days. The mixture was extracted first with peroxide-free ether and then with 0.01 M potassium phosphate, *p*H 7. The dry residue was then crystallized three times from dilute ethanol; mp 178° (sintered at 155°). The absorption spectrum in 0.1 M Tris-Cl, *p*H 7.5, was identical with that of authentic 2,4-dinitroaniline (mp 177°, sintered at 155°). Molar absorbancy was 14,000 at 345 mμ (λ_{max}). Free concentrations were determined in dialysis-equilibrium experiments by adding 1 ml of the "outside" solution (free of protein) to 0.5 ml Hyamine[2] and 10 ml of the solvent described by Bray (1960). Counting efficiency for ^{14}C was 55%, and background was 80 cpm. The specific activity was 520 cpm/mμmole.

[^{3}H]-2,4-Dinitrotoluene.—2,4-Dinitrotoluene (75 mg) was tritiated by the New England Nuclear Corp. (Boston, Mass.) in ^{3}H-glacial acetic acid with a platinum catalyst. The crude product was dried from 5 ml benzene and taken up in warm ethanol, and the insoluble residue was discarded. Recrystallization five times from ethanol-water yielded white crystals, mp 70°, with an absorption spectrum identical to that of authentic 2,4-dinitrotoluene (in 95% ethanol the molar absorbancy at λ_{max} [242 mμ] is 14,200). Titration of an antibody solution with the authentic compound and with the ^{3}H-labeled compound gave identical fluorescence-quenching curves. In equilibrium dialysis experiments nonspecific binding to inert components of the system ranged from 40 to 80% of the free dinitrotoluene concentration and it was necessary, therefore, to measure hapten concentrations in both compartments of the dialysis tubes. For this purpose, to 1.0-ml aliquots were added 0.2 ml 1 N NaOH and 10 ml toluene containing 4 g/liter 2,5-diphenyloxazole and 1 mg/liter 1,4-bis-2′-(5′-phenyloxazolyl)benzene. The alkali was necessary to inactivate the antibody, when this was present, and to allow quantitative transfer of the hapten into the toluene phase. Under these conditions, the specific activity was 3830 cpm/mμmole. Purified rabbit antibody specific for *p*-aminobenzenearsonate did not bind [^{3}H]dinitrotoluene to a detectable extent in control dialysis-equilibrium experiments.

ϵ-Dinitrophenyl-L-[^{3}H]-lysine.—ϵ-DNP-L-lysine (25 mg) was tritiated by the New England Nuclear Corp. in ^{3}H-glacial acetic acid, using a platinum catalyst. The crude product was dried, taken up in 20% HCl, extracted repeatedly with peroxide-free ether, and then rapidly taken to dryness. After thin-layer chromatography in Silica Gel-G, with water-saturated methylethylketone as solvent, the major yellow band, with the same R_F as the authentic compound, was eluted with water. In equilibrium dialysis experiments with ϵ-DNP-L-[^{3}H]lysine, 1.0-ml aliquots were added to 0.5 ml Hyamine and 10 ml of the scintillation solvent described by Bray (1960). Counting efficiency was 4%, and the specific activity was 22,500 cpm/mμmole. Inside and outside solutions were counted, although nonspecific binding was negligible, as previously noted with authentic ϵ-DNP-L-lysine (Carsten and Eisen, 1955).

ϵ-DNP-D-lysine.—This was prepared from D-lysine by the method of Porter and Sanger (1948). It was crystallized from water and recrystallized from 20% HCl; mp 196°. The absorption spectrum in 0.1 M Tris-Cl, *p*H 7.5, was identical with that of ϵ-DNP-L-lysine (λ_{max}, 363 mμ). In glacial acetic acid (10 mg/ml), $[\alpha]_D$ was +16° and −17° for the optical enantiomorphs, respectively.[3] ϵ-DNP-D-lysine was an active substrate for D-amino acid oxidase, 80% of the theoretical amount of oxygen being taken up in one experiment in about 2 hours, whereas with 4-fold more ϵ-DNP-L-lysine as substrate no oxygen consumption was detectable under the same conditions.[3]

Other Reagents.—Unless otherwise specified, amino acids and 2,4-dinitrophenylamino acids were obtained from the Sigma Chemical Co. (St. Louis), the Mann Chemical Corp. (New York City), or the Cyclo Chemical Corp. (Los Angeles). Other dinitrophenyl and mononitrophenyl derivatives were obtained from Distillation Products Industries (Rochester, N.Y.) and recrystallized until correct melting points were obtained. Rabbit pseudo γ-globulin was prepared from normal rabbit serum by sodium sulfate precipitation (Kekwick, 1940) followed by chromatography on DEAE-cellulose (Sober *et al.*, 1956). Bovine γ-globulin (fraction II of bovine plasma) was obtained from Armour and Co. (Chicago).

Methods

Immunization.—DNP-BγG in 0.15 M NaCl–0.01 M phosphate, *p*H 7.4, was emulsified with an equal volume

[2] 1 M *p*-(diisobutyl-cresoxyethoxyethyl)dimethylbenzylammonium hydroxide (in methanol), supplied by Packard Instrument Co., Inc., La Grange, Ill. Hyamine is the registered trademark of Rohm and Haas.

[3] We wish to thank Prof. Elijah Adams, University of Maryland, for optical rotation measurements and D-amino acid oxidase assays of ϵ-DNP-L-lysine and ϵ-DNP-D-lysine.

of 4:1 mineral oil–Aralcel A (v/v); unless otherwise indicated, the mineral oil contained *Mycobacteria butyricum* (10 mg/100 ml), and immunization was carried out by injecting a total of 5 mg DNP-BγG in 1.6 ml emulsion into the four foot pads (Eisen *et al.*, 1959). In some experiments immunization was carried out with various quantities of DNP-BγG in similar emulsions but lacking mycobacteria ("incomplete adjuvant"; Freund, 1947). These special immunization conditions are specified in legends to the appropriate tables and figures.

Blood was obtained by cardiac puncture. Purified antibodies were isolated from either (a) individual serum samples derived from single bleedings of individual rabbits; or (b) serum pools from individual rabbits, obtained by combining the bleedings from 3 consecutive days; or (c) pools of serum from twenty to fifty rabbits, each of the rabbits contributing to the pool having been bled on 3 successive days.

Precipitin Analyses.—Concentrations of anti-DNP antibodies in antisera were measured by precipitin reactions, using DNP-BγG as antigen. Specific precipitates were collected, washed at 4°, dissolved in 0.5% sodium lauryl sulfate (recrystallized from ethanol or 1-butanol), and absorbances were determined at 278 and 360 mμ (Eisen *et al.*, 1954); the detergent solutions were warmed for 30 minutes at 37° to obtain optically clear solutions.

Purification of Antibody.—Unless otherwise indicated, purified antibodies specific for the DNP group were prepared as previously described (Farah *et al.*, 1960). Several other methods were used for special purposes (see Fig. 3 and Tables I and II). When large samples of purified antibody were prepared (0.1–1.0 g), the protein was lyophilized and stored at 4°. Smaller quantities were maintained in solution at 4°, or frozen and kept at −12°.

The purity of antibody preparations was evaluated by determining their precipitability by DNP-BγG. The dependency of precipitability on the initial concentration of antibody was shown in an experiment in which 5, 1, 0.5, 0.25, and 0.1 mg antibody in a final volume of 1.1 ml were precipitated 95, 93, 90, 86, and 76%, respectively, upon addition of the equivalent amount of DNP-BγG (assumed to be one-fifth the weight of antibody; Farah *et al.*, 1960). The apparent solubility of specifically precipitated antibody is thus about 20 μg/ml, with approximately 4% nonprecipitable protein in the particular sample here described. In nearly all samples the purified antibody was about 90% specifically precipitable by DNP-BγG when tested at an antibody concentration of 1 mg/ml. The purified anti-DNP antibodies did not precipitate BγG. Uncorrected sedimentation coefficients for several antibody preparations (e.g., 2A and 5; *vide infra*) have been in the range 6.5–7 $\times 10^{-13}$ seconds. In addition, immunoelectrophoresis of representative preparations obtained at various times after immunization have revealed in each instance only a single precipitin band which corresponded to γ_2-globulin. While these preliminary observations will have to be extended, it is likely that all the antibody samples described herein are 7S-γ_2 immunoglobulins.

Fluorescence Quenching.—Fluorometric titrations to measure binding of haptens by purified antibodies were carried out in an Aminco-Bowman spectrophotofluorometer, modified to incorporate a thermostatically controlled cell housing.[4] One-ml samples of antibody solution were titrated with 0.2 ml hapten solution, added in increments of 0.01–0.03 ml. Protein fluorescence was activated with incident light at about 290 mμ, and emission was measured at 345 mμ. Before the initial addition of hapten 5–10 minutes was allowed for temperature equilibration of the antibody solution. This was necessary in order to obtain a stable initial fluorescence value. Samples were shielded from incident light except when readings were taken. Fluorescence values are expressed as a fraction of the initial fluorescence (i.e., before addition of hapten), after correcting for solvent blank and for dilution owing to the volume of the hapten solution added. Bound and free hapten were distinguished on the basis of the degree of quenching observed (Q_i) relative to the quenching when all binding sites are occupied (Q_{max}). Bound hapten (in moles) was thus taken as $Q_i/Q_{max} \cdot 2 \cdot Ab$, where Ab is the total amount of antibody titrated (moles) and 2 is the number of binding sites per antibody molecule (see equilibrium dialysis experiments below). The concentration of free hapten was determined from the difference between added and bound hapten. Antibody concentrations were determined by absorbance at 278 mμ in neutral solution, taking $E_{1\,cm}^{1\%}$ to be 15.5 and the molecular weight of rabbit antibody to be 180,000.[5] The maximal amount of specific quenching expected when all antibody sites are occupied by hapten, Q_{max}, was taken as 0.72; the validity of this value is discussed below.

Titrations were generally performed at an antibody concentration of 40 μg/ml, with addition of a total of 1.2–3.0 mμmoles hapten. In a few experiments an antibody concentration of 100 μg/ml and a correspondingly higher concentration of hapten were used. The average intrinsic association constant, K_o, was calculated from the generalized adsorption isotherm as defined by Sips (1948) (see Klotz, 1953):

$$\frac{r}{n} = \frac{(K_o c)^a}{1 + (K_o c)^a} \qquad (1)$$

where r is moles hapten bound per mole antibody at free hapten concentration c, and n is the maximum number of moles hapten that can be bound per mole antibody (i.e., 2); a is a constant (heterogeneity index) which describes the dispersion of association constants about the average constant, K_o. When $a = 1$, all sites have the same association constant and equation (1) reduces to the standard Langmuir adsorption isotherm; decreasing values of a correspond to increasing heterogeneity in respect to association constants. Nisonoff and Pressman (1958) first used the Sips function to describe the heterogeneity of affinities of antibody-binding sites, and estimated a as that value which afforded the closest approach to linearity for a plot of n/r vs. $1/c^a$. Equation (1) may, however, be recast as:

$$\log \frac{r}{n - r} = a \log c + a \log K_o \qquad (2)$$

and a is then readily obtained (Karush, 1962).

[4] The modified cuvet holder was constructed by Mr. Oliver Treter, Department of Chemistry, Washington University, St. Louis.

[5] Molecular weights reported for rabbit 7S antibodies have ranged from 180,000 to 140,000. The former value was selected arbitrarily when this work was initiated, but recent analyses suggest that 150,000, or even 140,000, may be the more accurate figure. The results of recalculating four representative titrations, using various molecular weights (180,000–140,000), may be summarized by the ratio (R) of K_o based on 150,000/K_o based on 180,000, this ratio is given in parentheses after each of the following K_o values (calculated on 180,000 mw): 1.29×10^5 (1.0); 2.56×10^6 (1.2); 1.17×10^7 (1.9); 1.63×10^8 (7.1). If the "true" mw is 150,000, the values reported in this paper significantly underestimate the affinities of very high-affinity antibodies, but the values reported for moderate- and low-affinity antibodies remain valid.

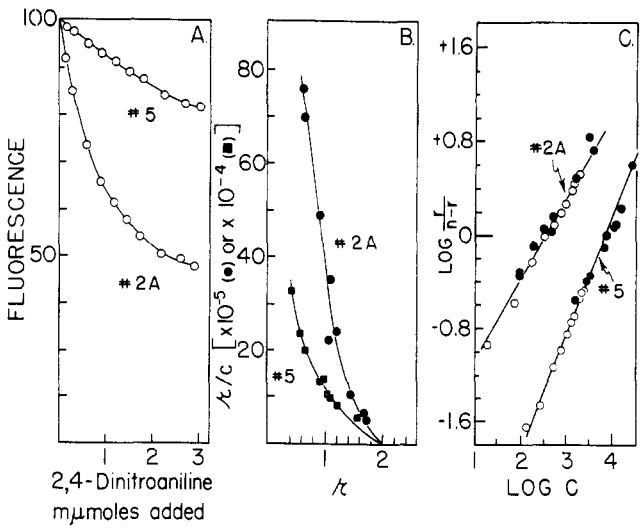

FIG. 1.—Binding of 2,4-dinitroaniline by antibody preparations 2A and 5 at 31°. (A) Fluorescence quenching. Antibody concentrations were 95 μg/ml for 2A and 90 μg/ml for 5. The solvent was 0.1 M Tris-Cl, *p*H 7.6. Titrations were the same in this solvent and in 0.1 M KCl–0.1 M Tris-Cl, *p*H 7.6. (B) Equilibrium dialysis with [1-^{14}C]-2,4-dinitroaniline. Antibody concentrations were 225 μg/ml for 2A and 825–865 μg/ml for 5. The solvent was 0.1 M KCl–0.1 M Tris-Cl, *p*H 7.6. (C) Combined data of (A) and (B) plotted according to equation (2). Fluorescence-quenching data are calculated from Q_{max} = 0.72. The K_o values for 2A and 5 were 3.5 ± 0.7 × 10^6 and 1.3 ± 0.3 × 10^5 by equilibrium dialysis (●) and 2.3 ± 0.7 × 10^6 and 1.4 ± 0.4 × 10^5 by fluorescence quenching (○), respectively. In the case of 2A, 10–85% of the binding sites were occupied, and in the case of 5, 2–80% of the binding sites were occupied.

Calculations were performed with the aid of an IBM computor, Model 7072.[6] For each titration log $r/n - r$ was plotted vs. log c by the method of least squares, and a was obtained as the slope and K_o from the intercept. Here K_o is the reciprocal of c, the free hapten concentration, when one-half the binding sites are occupied. Conformity of the data to the linear regression was computed as the correlation coefficient which ranged from 0.90 to 0.99. In replicate titrations, the average deviation of K_o values from their mean was ±30%.

Equilibrium Dialysis.—One- or 2-ml aliquots of antibody solution were dialyzed against equal volumes of solvent containing various initial concentrations of haptens. All tubes were set up in duplicate and concentration equilibrium was established by rotating the tubes at 3 rpm for 5 hours at 30° (±1°). In the case of *o*- and *p*-mononitroaniline and [1-^{14}C]-2,4-dinitroaniline, association constants were based on measurements of free hapten concentrations in the compartments which lacked antibody ("outside"). With [^{3}H]-2,4-dinitrotoluene and with ε-DNP-L-[^{3}H]lysine, however, hapten concentrations were determined on aliquots of the solutions inside and outside the dialyzing sacs; in these cases volume changes were determined by measuring the volume outside the sac at the end of the dialysis period.

Hapten concentrations were measured by absorbance or by counting in a Packard Tri-Carb liquid scintillation spectrometer. The λ_{max} and molar absorbancies were 370 mμ and 12,530 for *p*-mononitroaniline, and 410 mμ and 4510 for *o*-mononitroaniline. Counting

[6] We wish to thank Mr. R. A. Dammkoehler and Mr. T L. Gallagher of the Washington University Computing Facilities for developing the computor program.

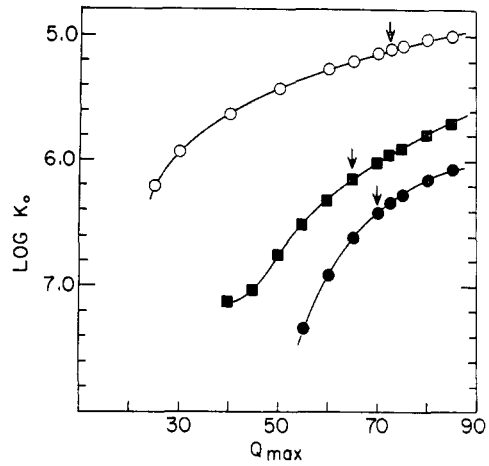

FIG. 2.—Effect of assuming different quenching maxima (Q_{max}) on the calculation of average association constants (K_o) from fluorometric titrations. Arrows (↓) indicate K_o values obtained by equilibrium dialysis with the following antibody-ligand pairs: antibody preparation 5, [1-^{14}C]-2,4-dinitroaniline (○); antibody preparation 2A, [1-^{14}C]-2,4-dinitroaniline (■); antibody preparation 12, [^{3}H]-2,4-dinitrotoluene (●).

conditions and specific activities for the radioactive haptens were given above (see under Reagents). Hapten binding was calculated in the usual way, after correcting for nonspecific binding of haptens to dialysis membranes when measurements of hapten concentration were limited to the "outside" compartment.

VALIDATION OF METHODS

Comparison of Fluorescence-Quenching and Dialysis Equilibrium.—In the calculation of average intrinsic association constants (K_o) from fluorescence-quenching data we have assumed that when all binding sites in any sample of anti-DNP antibody are occupied by hapten, the protein's residual fluorescence is 0.28 of the value observed in the absence of hapten, whatever 2,4-dinitrophenyl ligand is used, and whatever the antibody's affinity for it may be (i.e., Q_{max} = 0.72, see under Methods). The validity of this assumption was tested with two antibody preparations of widely different affinities for ε-DNP-L-lysine: both were titrated fluorometrically with 2,4-dinitroaniline, and their binding of [1-^{14}C]-2,4-dinitroaniline was measured by means of equilibrium dialysis. The values obtained for K_o were the same by both methods, using Q_{max} = 0.72 for the fluorometric titrations (Fig. 1). The assumption of two specific binding sites per antibody molecule of mw 180,000 (see under Methods) is supported by the equilibrium dialysis experiments (Fig. 1B), in agreement with older observations (e.g., Eisen and Karush, 1949; Karush, 1956). Further validation of the fluorescence-quenching method was provided by experiments in which the binding of [^{3}H]-2,4-dinitrotoluene was also measured fluorometrically and by equilibrium dialysis. The K_o values by both methods were in agreement when Q_{max} for fluorescence-quenching was taken as 0.70 (Fig. 2). Finally, with another antibody preparation, fluorometric titration with ε-DNP-L-lysine gave a K_o value of 2.0 × 10^7 liters mole^{-1} (taking Q_{max} = 0.72), in agreement with the value found for the same antibody preparation by equilibrium dialysis with ε-DNP-L-[^{3}H]lysine (2.2 × 10^7). In a few preparations of unusually high affinity the initial additions of strongly bound ligands (ε-DNP-L-lysine and ε-DNP-aminocaproate) resulted in excessive damping of fluorescence,

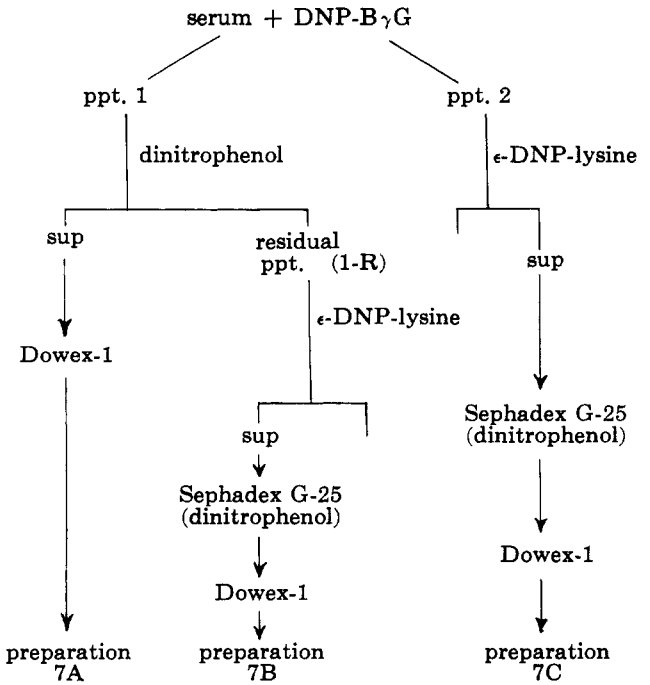

FIG. 3.—Effect of purification procedure on affinities of isolated antibodies for the reference ligand ε-DNP-L-lysine. From pooled serum (DN-28) of rabbits that had been immunized with 2 mg DNP-BγG and then bled 8 weeks later, specific precipitates (1 and 2) were formed from 20-ml aliquots by addition of 4 mg DNP-BγG to each. The precipitates were washed with 0.15 M NaCl–0.01 M phosphate, *p*H 7.4. Anti-DNP antibodies (preparation 7A) were isolated from precipitate 1 in the standard manner by elution with 0.1 M 2,4-dinitrophenol (Farah *et al.*, 1960). The residue of precipitate 1 (ppt. 1-R) was suspended for 1 hour at 37° in 2 ml ε-DNP-L-lysine (1×10^{-3} M), streptomycin (35 mg/ml), NaCl (0.15 M), phosphate (0.01 M), *p*H 7.4, and the supernatant was passed through Sephadex G-25 (2.5 × 7 cm) which was prepared in, and developed with, 0.1 M 2,4-dinitrophenol, 0.1 M Tris-Cl, *p*H 7.8; flow rate 0.3 ml/min. The emerging protein (in 6 ml) was put through Dowex-1 × 8, 1 × 17 cm, which was prepared in, and developed with, 0.15 M NaCl–0.01 M phosphate, *p*H 7.4. Protein from the latter column was precipitated with ammonium sulfate and then dialyzed (as had been preparation 7A) to yield preparation 7B. Preparation 7C was obtained directly from precipitate 2 by the same treatment which yielded 7B from the residue of precipitate 1, except that the Sephadex G-25 column was 2.5 × 14 cm. Purified antibody recovered in 7A, 7B, and 7C was 6.4, 6.9, and 16.3 mg, amounting to 25%, 26%, and 68%, respectively, of the antibodies initially precipitated from serum (24 mg antibody in ppt. 1 and in ppt. 2).

indicating that in some of the anti-DNP molecules in these preparations occupancy of one binding site by these ligands quenched perhaps as much as 50% of the protein's fluorescence. In titrations of this type, calculations of K_o and heterogeneity indices (a) were based on subsequent ligand additions in which unbound ligand was detectable on the basis of the above assumptions (i.e., that $Q_{max} = 0.72$).

Evidence that Purified Antibodies are Representative Samples.—In the method used here to isolate antibodies from sera about 50% of the serum antibodies that form specific precipitates with the immunizing antigen (DNP-BγG) are extracted from the precipitates with 0.1 M 2,4-dinitrophenol, and of this fraction only about one-half is recovered in the purified product (Farah *et al.*, 1960). The following experiments were carried out to determine if the affinities of antibodies thus isolated are representative of the total antibody mass initially precipitated from serum.

TABLE I

EFFECT OF PURIFICATION PROCEDURE ON SPECIFIC BINDING OF ε-DNP-L-LYSINE AND OF 2,4-DINITROPHENOL[a]

	Ligand			
	ε-DNP-L-lysine		2,4-Dinitrophenol	
Antibody Preparation	K_o (liters/mole × 10^{-7})	a	K_o (liters/mole × 10^{-7})	a
7A	85	0.4	0.2	0.4
7B	75	0.2	0.004	0.4
7C	85	0.3	0.03	0.5

[a] The procedure used to isolate the three antibody preparations are given in Figure 3. K_o and a are defined under Methods, and were calculated from duplicate fluorometric titrations at 30° in 0.1 M Tris-Cl, *p*H 7.6. Duplicates agreed to within ±20% of their average value.

Duplicate specific precipitates (nos. 1 and 2) were formed from aliquots of a serum pool, and three samples of antibodies were isolated from them as outlined in Figure 3. Preparation 7A was obtained from precipitate 1 by the standard method used throughout this study; i.e., by extracting the precipitate with 0.1 M 2,4-dinitrophenol. Since ε-DNP-L-lysine at 1×10^{-3} M specifically solubilizes at least 95% of the anti-DNP antibodies from these precipitates (Farah *et al.*, 1960), this ligand was used to extract the remaining antibodies from the residue of precipitate 1 (preparation 7B), and was also used directly to extract the anti-DNP molecules from precipitate 2 (preparation 7C). Procedural details and yields are given in the legend to Figure 3, and K_o values for the binding of ε-DNP-lysine and of 2,4-dinitrophenol are shown in Table I. The antibody recovered in 7C amounted to 68% of what was present in precipitate 2 (Fig. 3), and we ascribe the failure to recover about 30% of the solubilized antibody to the fact that it was necessary

TABLE II

EFFECT OF PURIFICATION PROCEDURE ON AFFINITIES OF ANTIBODIES[a]

		Ligand			
		2,4-Dinitrophenol		ε-DNP-L-lysine	
Antibody Fraction	2,4-Dinitrophenol in Extracting Solvent (mM)	K_o (liters/mole × 10^{-7})	a	K_o (liters/mole × 10^{-7})	a
a	1	3.5	0.6	18	0.9
b	10	1.3	0.6	25	1.0
c	100	0.29	0.6	18	0.7

[a] About 124 mg antibody was precipitated from 100 ml of a serum pool by 12.5 mg DNP-BγG. The precipitate was washed five times with 0.15 M NaCl–0.01 M phosphate, *p*H 7.5, and then extracted three times in succession with 2,4-dinitrophenol in increasing concentration. Conditions for the first extraction were as follows: The precipitate was suspended at 37° for 45 minutes in 3 ml of 2,4-dinitrophenol (1 mM), streptomycin sulfate (35 mg/ml), NaCl (150 mM), and phosphate (10 mM), *p*H 7.5. After centrifugation at room temperature, anti-DNP antibody (fraction a) was isolated from the supernatant as described (Farah *et al.*, 1960). Conditions for the second and third extractions, which yielded fractions b and c, were identical, save that the dinitrophenol concentrations were 10 and 100 mM. Yields of purified antibody were 6.5, 8.3, and 5.7 mg for fractions a, b, and c, respectively. Repetition of the third extraction (100 mM dinitrophenol) yielded only about 10% as much antibody as in fraction c. Fluorometric titrations were performed in 0.1 M Tris-Cl, *p*H 7.6 at 30–32°.

TABLE III

BINDING OF ε-DNP-L-LYSINE BY ANTIBODIES ISOLATED AT VARYING TIMES AFTER INJECTION OF 2,4-DINITROPHENYL-BOVINE-γ-GLOBULIN[a]

Amount of DNP-BγG Injected[b] (mg)	Rabbit No.	Time after Initial Injection of DNP-BγG: 2 weeks K_o (liters/mole × 10^{-6})	2 weeks a	5 weeks K_o (liters/mole × 10^{-6})	5 weeks a	8 weeks K_o (liters/mole × 10^{-6})	8 weeks a
5	1	0.60	0.7	32.0	0.5		
	2	1.6	0.7	27.0	0.7		
	3	0.32	0.7	1.6	0.6	20.0	0.3
	4	1.0	0.8	5.9	0.6	250.0	0.3
	5	0.78	0.9	1.5	0.5	80.0	0.5
50	6			0.21	0.6	0.55	0.7
	7	0.21	0.7	0.36	0.7	0.97	0.7
	8	0.26	0.6	0.20	0.6	1.4	0.5
	9	0.78	0.9	2.7	0.9	32.0	0.8
	10	0.28	0.8	0.20	0.7		
100	11	0.21	0.7	0.29	0.6		
	12	0.37	0.7	0.59	0.6	0.89	0.5
	13	0.17	0.6	0.13	0.6	0.23	0.9
	14	0.87	0.8	1.0	0.8	0.55	0.7
	15	0.26	0.7	0.19	0.8	0.37	0.9
250	16	0.14	0.9	0.13	0.8	0.10	0.7
	17	0.36	0.9	0.23	0.7	0.38	0.9
	18	0.13	0.8	0.12	0.8	0.16	0.7
	19	0.26	0.6	0.19	0.7	0.11	0.7

[a] a and K_o are defined in equation (2); see Methods. [b] The quantities of DNP-BγG indicated were injected at zero time in oil-in-water emulsion without mycobacteria; i.e., in "incomplete adjuvant" (Freund, 1947). Rabbits 1–15 received 2.75 ml emulsion distributed evenly among the foot pads and a deep subcutaneous site. Rabbits 16–19 received 7 ml emulsion distributed among the foot pads and three separate deep subcutaneous sites. Three weeks after the initial immunizing injection all animals were given 1 mg DNP-BγG intravenously. The same changes in affinity with time have been repeatedly observed when the latter intravenous injection is omitted.

to use exceedingly large Sephadex columns in order to completely displace antibody-bound ε-DNP-L-lysine (Fig. 3). We believe it is reasonable, therefore, to regard preparation 7C as being representative of the antibody mass initially precipitated from serum. The three preparations (7A, 7B, and 7C) had indistinguishable affinities for ε-DNP-lysine. Thus the antibody preparations examined in this study appear to be representative of the total serum populations of DNP-BγG-precipitable antibodies in respect to affinity for the reference hapten ε-DNP-L-lysine. As expected, however (Farah *et al.*, 1960), antibodies which were extracted with 0.1 M 2,4-dinitrophenol (7A) had greater affinity for 2,4-dinitrophenol than those which resisted extraction with this solvent (7B); preparation 7C had the intermediate affinity for this hapten expected if, as appears to be the case, it is a mixture in approximately equal amounts of the antibodies found in 7A and in 7B.

Further evidence that the isolation from serum of anti-DNP antibodies on the basis of affinity for 2,4-dinitrophenol does *not* select populations with anomalous affinities for ε-DNP-lysine was provided by an experiment in which a specific precipitate was subjected to three successive extractions with 2,4-dinitrophenol at increasing concentrations: 1, 10, and 100 mM. Procedural details, yields of purified antibodies, and the results of fluorometric titrations are given in Table II. The three preparations differed in affinity for dinitrophenol, their K_o values for this hapten varying inversely with the concentration of dinitrophenol used for their isolation. However, K_o values for ε-DNP-lysine were the same, within experimental error, for all three preparations. These results reinforce the evidence given above that antibody populations with the same average affinity for one hapten (in this case ε-DNP-lysine) can differ considerably from each other in respect to their affinities for a cross-reacting hapten (in this case 2,4-dinitrophenol).

RESULTS

Variations in K_o during the Course of the Immune Response.—Average association constants for fifty-two preparations of purified antibody, isolated from various bleedings of nineteen rabbits, are summarized in Table III. When large amounts of antigen were injected (250 mg) average affinities for ε-DNP-lysine were low, and did not change over a 2–8 week interval after immunization. When relatively small amounts of antigen were used to induce antibody formation (5 mg) the antibodies isolated had higher average affinities for ε-DNP-L-lysine, and their affinities increased as the interval between the immunizing injection and bleeding was prolonged from 2 to 8 weeks. At intermediate doses of antigen, the values obtained fell between these extremes (compare 50- and 100-mg doses in Table III).

Heterogeneity of Antibodies in Respect to Affinity.—The antibody samples examined in the foregoing experiments were inhomogeneous in respect to affinity for ε-DNP-lysine and for 2,4-dinitrophenol ($a < 1.0$ in Tables I–III). The possibility that the inhomogeneity was an artifact of purification seemed unlikely since many studies have shown other manifestations of binding heterogeneity in unfractionated antisera (e.g., Landsteiner and van der Scheer, 1936; Pauling *et al.*, 1944). We have, nevertheless, sought independent evidence concerning the heterogeneity of anti-DNP antibodies in serum. For this purpose, the individual sera from three rabbits were subjected to successive additions of small amounts of DNP-BγG, each addition amounting to about 10% of what was required for maximal precipitation of antibody. From each precipitate antibodies were isolated and

TABLE IV
HETEROGENEITY OF ANTI-DNP ANTIBODIES IN SERA OF INDIVIDUAL RABBITS[a]

Fraction No.	Rabbit A K_o (liters/mole $\times 10^{-6}$)	Rabbit A a	Rabbit B K_o (liters/mole $\times 10^{-6}$)	Rabbit B a	Rabbit C K_o (liters/mole $\times 10^{-6}$)	Rabbit C a
1	16	0.2	>1000	0.2	1.4	0.4
2	8.8	0.2	330	0.4	1 7	0.3
3	4 2	0.4	89	0.5	3.4	0.5
4	1.9	0.4	19	0.6	1.1	0.5
5	1.8	0.4	8.1	0 4	0 36	0.5
6	0.85	0 4	1.0	0.5	0.23	0.7
7	0.16	0.4	0.53	0.5	0.08	0.7
8			0.23	0.7	0.05	0.7
9	0.07	0.6	0.17	0.5	0.04	0.6
10	0.09	1.0	0.11	0.6		

[a] Each fraction was prepared by adding to the serum, or to the supernatant remaining after prior addition of antigen and removal of precipitate, 25 μg DNP-BγG per ml (6–8% of the amount of DNP-BγG required for maximal precipitation when antigen was added all at once). The serum-antigen mixtures were held at 37° for 1 hour and then kept at 4°. In fractions 1 through 7 the precipitates were collected after 24 hours at 4°; in fraction 8 the precipitates were collected after 48 hours at 4°; and in fractions 9 and 10 the precipitates were allowed to stand 96 hours at 4°. All precipitates were washed and antibody was extracted from them with 0.1 M 2,4-dinitrophenol as described (Farah *et al.*, 1960). The values are based on duplicate titrations of fractions from rabbit A and on single titrations of the fractions from rabbits B and C. Titrations were performed with ϵ-DNP-L-lysine in 0.1 M Tris-Cl, *p*H 7.6, without temperature control (30–32°). Accuracy of K_o values in excess of 1×10^9 is low. The total volumes of serum used for the sequential precipitations were: rabbit A, 80 ml; rabbit B, 88 ml; rabbit C, 86 ml. Each rabbit's serum was obtained by combining the bleedings of 3 successive days. The quantities of DNP-BγG required as a single addition, per ml of serum, for maximum precipitation of antibody were: rabbit A, 300 μg; rabbit B, 400 μg; rabbit C, 300 μg; and the total amounts of antibody precipitated, per ml serum, with these quantities of DNP-BγG were 1.36, 1.29, and 1.39 mg, respectively.

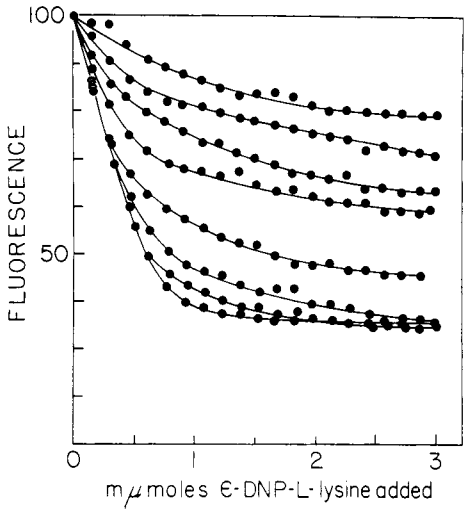

FIG. 4.—Fluorometric titrations of purified fractions isolated from the serum of a single rabbit by sequential precipitation with limiting amounts of DNP-BγG. Procedural details are given in Table IV. The above curves were obtained with fractions 1–10 of rabbit B in Table IV. Lowermost curve is fraction 1. Proceeding upward, the fractions are 3, 4, 5, 6, 7, 9, and 10. Fractions 2 and 8 are omitted to avoid overcrowding of data.

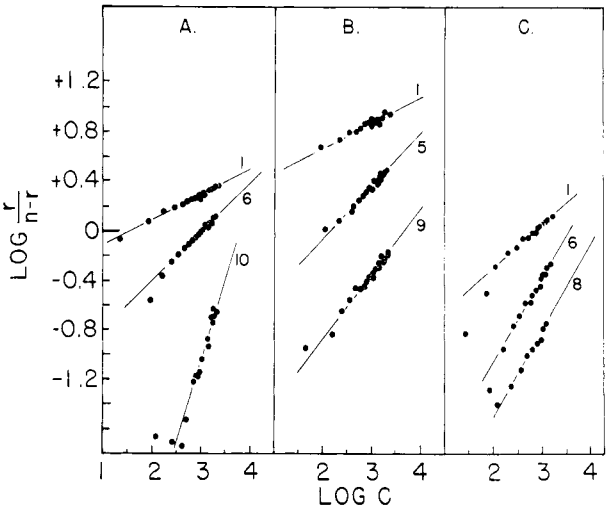

FIG. 5.—Sips plots of fluorometric titrations of representative antibody fractions from individual sera of three rabbits, A, B, and C. Fraction numbers on the curves and the letters correspond to the designations of Table IV.

titrated fluorometrically. Procedural details, K_o values, and heterogeneity indices (a) are given in Table IV. In Figure 4 the fluorometric titrations are shown for the antibodies obtained from the successive precipitates of one serum, and in Figure 5, binding data are shown for representative samples from each of the three sera. In general, the antibodies isolated from the successively formed precipitates exhibited progressively decreasing affinity for ϵ-DNP-lysine. The results are in accord with the expectation that when a limiting amount of antigen is added to a heterogeneous population of antibodies, specific complexes are formed preferentially by the antibodies of highest affinity. The fractions of higher average affinity were, in general, more heterogeneous (see also Table III).

Variations in Affinity with Hapten Structure.—Association constants for the binding of a variety of nitrobenzenes by one lot of antibody (2A) are listed in Table V. With the exceptions noted, K_o values were obtained by fluorometric titrations, using $Q_{max} = 0.72$. The values given for *o*- and *p*-mononitroaniline were obtained by equilibrium dialysis since their binding was too weak to be determined fluorometrically with the equipment available. In the dialysis experiments only the "outside" solutions (which lacked antibody) could be analyzed. In the case of *p*-mononitroaniline, K_o has a precision of about $\pm 20\%$, but in the case of *o*-mononitroaniline there is considerably greater uncertainty because this substance was bound nonspecifically to an exceptionally high degree by inert

TABLE V

BINDING OF SOME 2,4-DINITROPHENYL DERIVATIVES BY ONE LOT OF ANTI-DNP ANTIBODIES[a]

Hapten	K_o (liters mole^{-1} $\times 10^{-7}$)	Heterogeneity Index (a)	$-\Delta F°$ (kcal mole^{-1})
ε-DNP-L-lysine	2.30	0.6	10.30
ε-DNP-D-lysine	1.20	0.8	9.82
δ-DNP-L-ornithine	0.86	0.8	9.66
S-DNP-L-cysteine	0.72	0.6	9.44
O-DNP-L-tyrosine	0.19	0.6	8.70
α-DNP-L-alanine	0.88	0.5	9.63
α-DNP-L-norleucine	0.54	0.4	9.33
α-DNP-L-phenylalanine	0.31	0.5	8.99
ε-DNP-aminocaproate[b]	2.85	0.7	10.37
γ-DNP-aminobutyrate	2.00	0.6	10.12
β-DNP-alanine	1.86	0.6	10.08
DNP-glycine[c]	0.46	0.6	9.27
2,4-Dinitrophenol (*p*H 2.96)[d]	0.044	0.6	7.81
2,4-Dinitrophenol (*p*H 7.6)	0.027	0.7	7.52
2,4-Dinitrotoluene	0.13	0.6	8.50
m-Dinitrobenzene	0.06	0.6	8.02
2,4-Dinitroaniline	0.22	0.7	8.86
p-Mononitroaniline[e]	0.00075		5.47
o-Mononitroaniline[e]	(0.00060)		(5.2)

[a] Preparation 2A, isolated from a serum pool obtained 4–8 weeks after immunization with 5 mg DNP–BγG. Values are averages of two or more fluorometric titrations in 0.1 M Tris-Cl, *p*H 7.6 (except as noted in footnotes), all calculated on the basis of Q_{max} = 0.72. Titrations were performed without temperature control and at the end of each titration temperatures ranged from 30 to 32°. [b] At 5.4°, $K_o = 1.6 \times 10^8$ liters mole^{-1}; $\Delta F° = -10.45$ kcal mole^{-1}. [c] At 5.4°, $K_o = 1.3 \times 10^7$ liters mole^{-1}; $\Delta F° = -9.07$ kcal mole^{-1}. [d] In 0.1 M glycine, *p*H 2.96. In this solvent K_o for the binding of ε-DNP-L-lysine was 2.7×10^6 (*cf.* top line this table for binding of ε-DNP-lysine at *p*H 7.6). [e] Measured by equilibrium dialysis in 0.1 M KCl–0.1 M Tris-Cl, *p*H 7.6, using 5.0 mg antibody per ml and 1.0-ml volumes inside and outside the dialysis bags. Data in parentheses are subject to uncertainty because of large "bag-binding" correction.

components of the system (dialysis membrane, etc.). Within an order of magnitude, however, K_o appeared to be the same for *o*- and *p*-mononitroaniline.

Thermodynamic Properties.—Association constants for the binding of ε-DNP-lysine and of 2,4-dinitroaniline by two antibody preparations that differed widely in their affinities for these ligands were determined at several temperatures. Fluorometric titrations of one of these preparations with ε-DNP-L-lysine at 12–40° are shown in Figure 6, and plots of log K_o vs. $1/T$ are given in Figure 7 for both antibody preparations and both haptens. Thermodynamic values, calculated from these data in the usual way, are listed in Table VI.

Absorption Spectra of Bound Haptens.—Tryptophan residues excited by the incident light (290 mμ) donate their excitation energy to bound hapten molecules, and the absorption spectrum of the bound hapten is therefore more relevant to the transfer process than is the spectrum of unbound hapten. In Figure 8 absorption spectra are shown for ε-DNP-L-lysine when free in an aqueous medium and when localized in the antibody's specific binding site. Binding leads to a hypochromic change and a bathochromic shift of the major absorption band. In *p*-dioxane, with which the NO_2 groups cannot form hydrogen bonds, the major

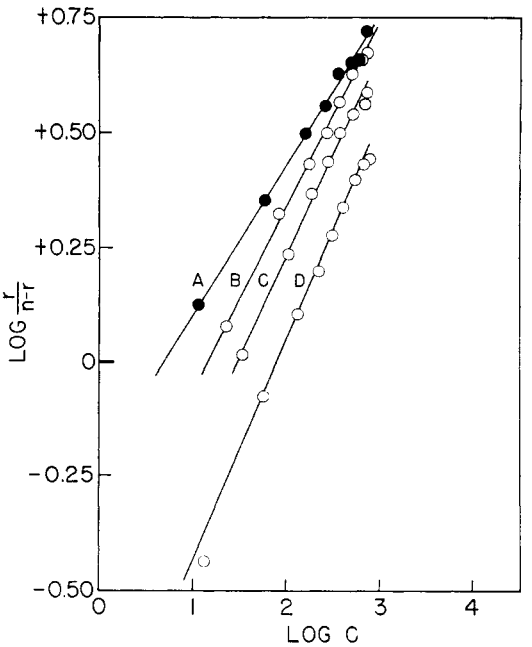

FIG. 6.—Fluorometric titrations of antibody 2A with ε-DNP-L-lysine at 12.8, 22.2, 30.5, and 39.5°. Solvent: 0.1 M Tris-Cl, *p*H 7.6.

absorption peak of ε-DNP-L-lysine is at a still lower wavelength than in water (see legend of Fig. 8).

Under the conditions used to determine the absorption spectrum of bound 2,4-dinitrotoluene, about 90% of this ligand was bound to antibody (Fig. 9). Without applying a correction for unbound ligand it is apparent that the major absorption peak of this ligand also exhibits a hypochromic effect with a bathochromic shift (λ_{max} shifts from 252 mμ to 257 mμ) when this substance is transferred from water to the antibody's binding site. Of particular interest is a new absorption peak of low intensity at 300 mμ in the spectrum of bound dinitrotoluene. The significance of this peak is discussed below in respect to the possibility that charge-transfer complexes are formed.

TABLE VI

THERMODYNAMIC RESULTS FOR THE BINDING OF ε-DNP-L-LYSINE AND 2,4-DINITROANILINE BY HIGH- AND LOW-AFFINITY ANTIBODY PREPARATIONS[a]

Antibody Preparation	Hapten	Temperature (°C)	$-\Delta F°$ (kcal mole^{-1})	$-\Delta H°$ (kcal mole^{-1})	$-\Delta S°$ (eu mole^{-1})
2A	ε-DNP-L-lysine	12.8	10.89	19.6	30.4
		22.2	10.62		
		30.5	10.43		
		39.5	10.12		
2A	2,4-Dinitroaniline	5.4	9.42	16.8	26.1
		17.5	9.40		
		32.0	8.87		
5	ε-DNP-L-lysine	5.4	7.96	13.1	18.6
		17.5	7.62		
		29.4	7.54		
5	2,4-Dinitroaniline	5.4	7.20	8.7	5.2
		12.5	7.32		
		20.2	7.25		
		30.6	7.26		

[a] Preparation 2A was isolated from a serum pool taken 4–8 weeks after immunization with 5 mg DNP-BγG. Preparation 5 was isolated from a serum pool taken 2 weeks after immunization with 250 mg DNP-BγG. $\Delta S°$ values are averages for the values found at the several temperatures for each antibody-ligand pair.

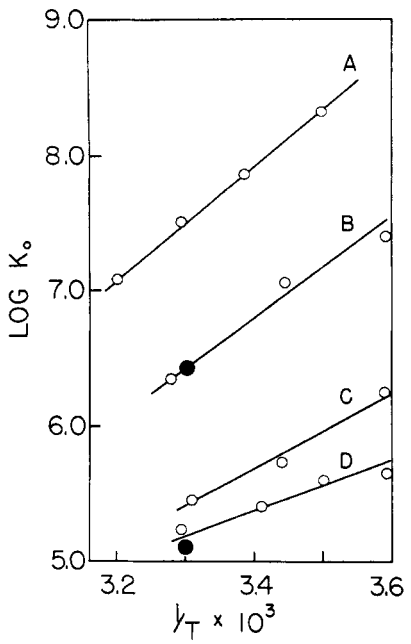

FIG. 7.—Temperature dependence of binding determined from fluorometric titrations (O). Solvent: 0.1 M Tris-Cl, *p*H 7.6. (A) Antibody 2A and ε-DNP-L-lysine (from Fig. 6). (B) Antibody 2A and 2,4-dinitroaniline. (C) Antibody 5 and ε-DNP-L-lysine. (D) Antibody 5 and 2,4-dinitroaniline. Closed circles (●) are data obtained by equilibrium dialysis in 0.1 M KCl–0.1 M Tris-Cl, *p*H 7.6.

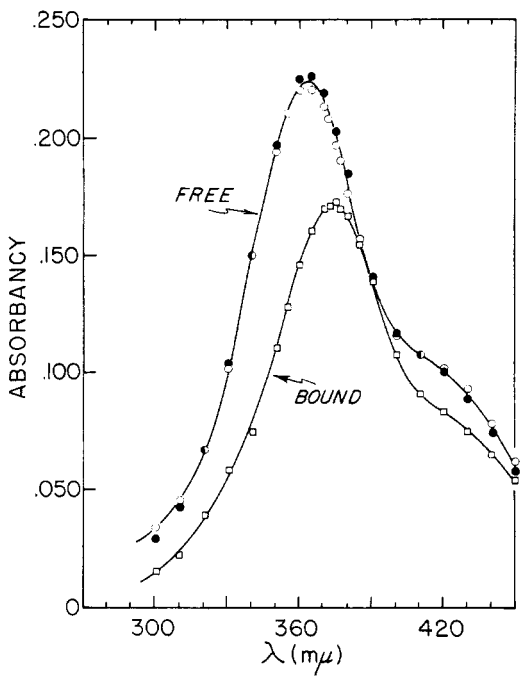

FIG. 8.—Absorption spectrum of ε-DNP-L-lysine (1.25×10^{-5} M) in 0.1 M KCl–0.1 M Tris-Cl, *p*H 7.6 (O), in the same solvent containing 1.6 mg/ml rabbit pseudo γ-globulin, not anti-DNP (●), and in the same solvent containing 1.6 mg/ml purified rabbit anti-DNP antibody (□). The K_o for the binding of the hapten by this antibody preparation (6) was 8×10^7 liters mole^{-1} at 30°. Absorbance of free and bound ε-DNP-lysine decreased steadily beyond 450 mμ and was undetectable at 510–600 mμ; from 450 to 510 mμ the absorbance of bound ε-DNP-lysine was 1.5–2.0 times greater than that of the free hapten, but a new peak was not observed (see Fig. 9). For free ε-DNP-L-lysine, $\epsilon = 17{,}530$ at λ_{max} (363 mμ). For antibody-bound ε-DNP-L-lysine, $\epsilon = 13{,}800$ at λ_{max} (375 mμ). In 1,4-dioxane, for ε-DNP-L-lysine, $\epsilon = 15{,}940$ at λ_{max} (348 mμ).

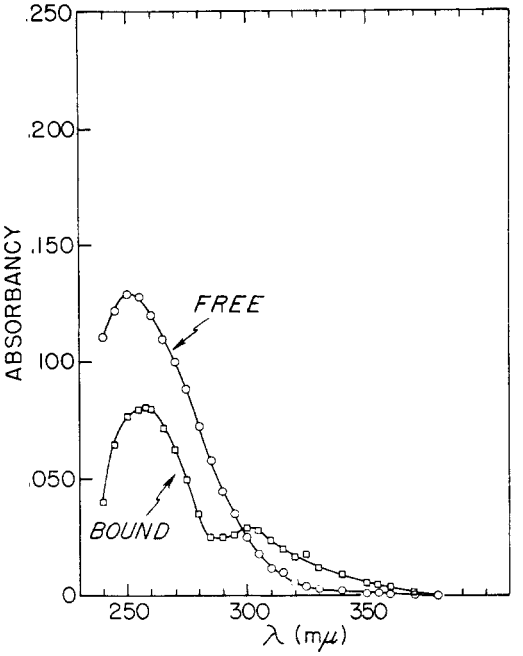

FIG. 9.—Absorption spectrum of 2,4-dinitrotoluene (8.6×10^{-6} M) in 0.1 M Tris-Cl, *p*H 7.6 (O), and in the same solvent containing 950 μg/ml anti-DNP preparation 7A (□). For free dinitrotoluene, $\epsilon = 15{,}100$ at λ_{max} (252 mμ). For antibody-bound dinitrotoluene, $\epsilon < 9400$ at λ_{max} (257 mμ).

Antibody fluorescence was recorded at 345 mμ in the fluorometric titrations. At this wavelength the molar absorbancy of antibody-bound dinitrotoluene is about 500, whereas the absorbancy of unbound dinitrotoluene was not measurable. Although excitation energy is transferred from antibody to ε-DNP-lysine and to 2,4-dinitrotoluene with approximately equal effectiveness (Q_{max} for both was about 0.7; see Fig. 2), the molar absorbancies of these haptens, when bound to antibody, differ about 20-fold at 345 mμ. However, the areas under the absorption bands of these haptens differ only about 5-fold in the region of the principal emission band of tryptophan residues (300–360 mμ). The transfer of excitation energy depends not only on spectral overlap between donor emission and acceptor absorbance but also on the acceptor's geometric orientation in respect to, and distance from, the excited tryptophan donors (Stryer, 1960). It becomes qualitatively understandable, therefore, that haptens which differ spectrally as much as ε-DNP-lysine and 2,4-dinitrotoluene could be so remarkably alike in the effectiveness with which they quench antibody fluorescence (*cf.* Figs. 2, 8, 9).

DISCUSSION

Variations of Average Association Constants.—Despite long recognition of the hapten-binding heterogeneity of antihapten antibodies it has been widely assumed that the interaction of antibodies of a given specificity with a homologous hapten is characterized by a unique average association constant and by unique thermodynamic values. The present study demonstrates that this viewpoint is incorrect. The average association constants observed for the binding of ε-DNP-lysine by different anti-DNP preparations varied over a 10,000-fold range. Even greater variation exists since the purification methods employed select against antibodies of low affinity ($<10^5$ liters mole^{-1}) and the method used for measuring association constants does not discriminate among values greater than 10^9

liters mole^{-1}. It seems likely that, in general, the range of affinities will be greatest for antibodies to those haptenic groups which possess an intrinsic energetic capacity to form the most stable specific complexes with antibody. Thus the 2,4-dinitrophenyl system, which can display exceptionally strong binding, has a wide range in affinities, whereas the benzenearsonate and other ionic systems, which are probably incapable of interacting so strongly, will probably turn out to have a more restricted range of affinities (e.g., Eisen and Karush, 1949; Doty and Epstein, 1954; Baker *et al.*, 1956; Velick *et al.*, 1960).

The anti-DNP molecules synthesized at increasing time intervals after the injection of DNP-BγG exhibit progressively increasing average association constants for the binding of the reference hapten ϵ-DNP-L-lysine. The time-dependent changes have been followed from a low of about 10^5 liters mole^{-1} 1–2 weeks after immunization to about 10^8 liters mole^{-1} 1–2 months later. The maximum affinity attainable with the present system is not yet evident because of limitations in the fluorescence-quenching method as used herein (*vide supra*).

It is very likely that the progressive increase in average association constant with increasing time after immunization, here described for anti-DNP antibodies, is characteristic of the immune response in general. It has, for example, often been observed that, with progressive immunization to diphtheria toxin, bovine serum albumin, and other proteins, antisera exhibit increasing avidity, i.e., increasing tendency to react rapidly with antigen, or to form less dissociable complexes with antigen (e.g., Jerne, 1951; Talmage and Maurer, 1953; Farr, 1958). Nonspecific interactions, however, doubtless play a significant role in determining the stability of the specific complexes formed by antibodies and high-molecular-weight antigens. Moreover, most antigens have a diversity of antigenic determinants per protein molecule; hence increasing avidity could be a consequence of the progressive appearance and accumulation of classes of antibodies, each specific for a distinctly different antigenic determinant. It has never been certain, therefore, just which properties of antisera and of antibodies are measured by avidity. It seems clear, nevertheless, that the frequent observation of time-dependent increases in avidity of antisera are explicable, at least in part, by the present observation of increasing association constants, with time, of the antibodies specific for one kind of determinant.

Increasing affinity with time could also account for the frequent observation (e.g., Hooker and Boyd, 1941) that specificity of antisera decrease with progressive immunization since antibodies of high affinity should be capable of displaying a wider range of cross-reactions than antibodies of low affinity.

Two considerations provide the basis for an extracellular mechanism which might account for the dependence of antibody affinity on the time elapsed after immunization, and on the dose of antigen administered. These considerations are: (a) the expectation that circulating free antigen would form complexes preferentially with antibodies of highest affinity in the serum, and (b) the fact that antigen-antibody complexes are rapidly eliminated from the blood stream (Glenny and Hopkins, 1923). Hence, the more free antigen available, the lower would be the average affinity of the residual free-antibody molecules. The free antigen remaining from the initial immunizing inoculum is expected to decrease with time, and this mechanism could therefore account for the increasing average affinity of antibodies with time. Support for this mechanism is provided by the preferential precipitation of high-affinity antibodies *in vitro* by the addition to serum of limiting amounts of antigen (Table IV and Figures 4, 5). Alternatively, or perhaps in addition, the lymph node cell populations that form antibodies may synthesize anti-DNP molecules of progressively increasing average affinity for the DNP group with increasing time after initiation of antibody synthesis. Experiments concerned with the latter possibility will be described elsewhere (L. A. Steiner and H. N. Eisen, to be published).

Heterogeneity of Association Constants.—Binding-heterogeneity has been evident in virtually every one of several hundred preparations of purified anti-DNP antibodies that have been examined in this laboratory (see also Day *et al.*, 1963), and in each preparation the dispersion of association constants about the mean (K_o) can be described by the Sips distribution function which closely approximates the Gaussian distribution function (Figs. 1, 5, 6). Thus there is very likely a large number of classes of anti-DNP molecules (e.g., 100 or 1000 or more), each distinguished by a single association constant for a specified ligand, with varying mixtures of the classes providing the basis for the wide range of average affinities observed. We cannot, however, rule out as yet the alternative but extreme possibilities that (a) no two anti-DNP sites are identical in affinity, or (b) there are only a small number of classes (e.g., <10) in respect to affinity.

The capacity of antibodies to recover their native configurations after denaturation (e.g., Karush, 1958; Buckley *et al.*, 1963) suggests that hapten-binding heterogeneity is not an artifact of the purification procedure. Moreover, the isolation of antibody populations of progressively decreasing association constants after stepwise precipitation of serum with limiting amounts of antigen (Figs. 4 and 5 and Table IV) establishes that the anti-DNP population in serum is, prior to purification, a heterogeneous assembly of molecules of widely different affinities for ϵ-DNP-lysine.

The haptenic groups of the DNP-BγG used herein to induce antibody formation are more uniform chemically than are the corresponding groups of the azoproteins ordinarily used to stimulate the formation of antihapten antibodies. Nevertheless, the microenvironments about individual DNP-lysyl residues in DNP-BγG are doubtless quite diverse. However, the anti-DNP antibodies isolated from animals immunized with bovine pancreatic ribonuclease substituted with one DNP group per protein molecule (on the lysine residue in position 41; see Hirs *et al.*, 1962) are just as heterogeneous in their binding of ϵ-DNP-L-lysine as most of the samples described in the present study (Eisen *et al.*, 1964). Likewise, we have found that antibodies isolated from a guinea pig immunized with DNP-poly-L-lysine (Kantor *et al.*, 1963) were heterogeneous in affinity for ϵ-DNP-L-lysine. It does not appear, therefore, that variations in the residues neighboring on the DNP-lysyl residues of the DNP-BγG used for immunization can account for the heterogeneity of binding affinities.

It seems likely that the explanation for hapten-binding heterogeneity will be found in the possibilities, which are not mutually exclusive, that (a) antibody-producing cells are functionally heterogeneous, each cell, or group of cells, producing antibody molecules of a uniform and distinctive affinity; or (b) fluctuations in the biosynthetic mechanisms are of such magnitude that even a single cell secretes antibody molecules with different affinities for any given ligand. In fact, even the two sites on individual anti-DNP molecules

may have different affinities for the DNP-determinant. This possibility, which is consistent with variations of heterogeneity indices shown in Table IV and in Figure 5, requires consideration even though in earlier studies it has been found that the two binding sites per antibody molecule (including anti-DNP molecules) are specific for the same ligand (Haurowitz and Schwerin, 1943; Eisen *et al.*, 1954; Nisonoff *et al.*, 1959b).

Variations in Affinity with Hapten Structure.—The anti-DNP antibodies isolated at different times after immunization must vary in the conformation of their specific binding sites since their affinities vary for ε-DNP-lysine. Accordingly, it is anticipated that different anti-DNP populations discriminate differently among various dinitrophenyl derivatives. This expectation is evaluated here with two preparations: 2A, isolated 1–2 months after immunization with 5 mg DNP-Bγ-G; and 5, isolated 2 weeks after immunization with 250 mg DNP-Bγ-G (see Fig. 1, Table V, and Table VI).

In the case of preparation 2A, the unitary free-energy changes (ΔF_u; Kauzmann, 1959; Karush, 1962) for the binding of ε-DNP-L-lysine, δ-DNP-L-ornithine, and 2,4-dinitroaniline were (at 30–32°) −12.58, −12.09, and −11.29 kcal mole^{-1}, respectively (Table V). 2,4-Dinitroaniline and δ-DNP-L-ornithine are thus bound 90 and 96% as strongly as ε-DNP-L-lysine. The small energetic advantage of ε-DNP-L-lysine over δ-DNP-L-ornithine is probably due to selective conformational features of the antibodies' binding sites since ε-DNP-L-lysine was bound slightly more strongly than ε-DNP-D-lysine. α-DNP-L-alanine was bound more tightly than α-DNP-L-norleucine or α-DNP-L-phenylalanine, and we infer, therefore, that the butyl and benzyl substituents of the latter derivatives sterically interfere with binding. For β-DNP-alanine, ΔF_u is −12.51, or 0.45 kcal mole^{-1} more negative than for α-DNP-alanine (Table V), indicating the close fit of the average binding site in this antibody preparation (2A) to the 1-carbon substituent of the 2,4-dinitrophenyl group. However, α-DNP-L-alanine is bound slightly better than DNP-glycine, so that the α-methyl group can make a small energetic contribution (−0.36 kcal mole^{-1}) despite its unfavorable position; possibly this signifies a certain amount of flexibility in the site.

From these results, the average binding site of antibody 2A, or at least that part of the site which can be explored with simple dinitrophenyl derivatives, appears to conform closely to the van der Waals contour of ε-DNP-L-lysine, and to be not much larger, if at all larger, than is necessary to accommodate this ligand. This view is consistent with the K_o values found for the DNP-derivatives of the homologous series: ε-aminocaproate, γ-aminobutyrate, β-alanine, and glycine (Table V).

The haptenic groups which are most prevalent in the immunizing antigen (perhaps even exclusively present) are ε-DNP-L-lysine residues. On the basis of general experience it is expected, therefore, that ε-DNP-L-lysine should, of all the DNP amino acids, be bound most strongly. This proved to be the case. Of all the haptens examined, only ε-DNP-aminocaproate was bound more strongly than ε-DNP-L-lysine (Table V); the difference was of borderline significance but consistent in replicate titrations. This difference is probably related to the observation that the *n*-amyl alcohol–water partition coefficient of ε-DNP-aminocaproate is greater than that of ε-DNP-L-lysine (1.56 and 1.10, respectively; Eisen *et al.*, 1959), since among homologous series of haptens the more apolar substances are, in general, bound more strongly than the more water-soluble substances. Thus protonated 2,4-dinitrophenol has relatively greater affinity for the anti-DNP binding site than 2,4-dinitrophenylate (Table V), and Metzger *et al.* (1963) observed that the protonated form of a 2,4-dinitrophenylazonaphthol is bound more strongly by anti-DNP antibodies than is the ionized form of this dye.

In contrast to preparation 2A, with antibodies isolated early after immunization (preparation 5) the binding of ε-DNP-L-lysine is hardly any stronger than that of 2,4-dinitroaniline: at 30°, ΔF_u for the binding of dinitroaniline was 98% that of ε-DNP-L-lysine, an insignificant difference. The difference in affinity for ε-DNP-lysine between antibody 2A and antibody 5 is 2.8 kcal mole^{-1} (Table VI), and this difference can be accounted for by the insensitivity of antibody 5 for the norleucine moiety of this hapten and by its having lower affinity for the dinitroanilino group. For preparation 5, therefore, it seems likely that the average binding site does not accommodate all of ε-DNP-L-lysine, or, if its volume is the same as that of the average site in preparation 2A, the shape precludes effective interaction with the norleucine moiety of this hapten. In addition, the average binding site of antibody 5 is less well adapted to the 2,4-dinitroanilino group than is the case for the average anti-DNP molecule synthesized later in the course of the immune response. Differences in the conformation of antibody-binding sites in different antisera to dextran and to the capsular polysaccharide of pneumococcus type III have also been inferred by Kabat (1960) and by Mage and Kabat (1963), but these differences have not previously been related to sequential changes in the course of the immune response.

In the case of antibody 2A the large difference in binding between the mononitroanilines (*o*- and *p*-) and 2,4-dinitroaniline indicates that each nitro group in the homologous hapten contributes (at 30°) about −3.5 kcal mole^{-1} to the stability of the hapten-antibody complex. From the binding of *m*-dinitrobenzene (Table V) it is estimated, therefore, that the benzene ring contributes about −3.5 kcal mole^{-1} to ΔF_u. These values are in agreement with the estimates of Karush (1962) and of Nisonoff *et al.* (1959a), who studied specific interactions of antibodies for phenyl-(*p*-azobenzoylamino)-acetate and for azonitrobenzenes, respectively. The accord is, however, likely to be fortuitous as these values are expected to vary at different stages of the immune response, at least for anti-DNP molecules.

Thermodynamic Results.—The 2,4-dinitrobenzenes utilized in this study are only sparingly soluble in water, except for 2,4-dinitrophenylate which is bound exceptionally weakly. Thus, despite the competitive effects of water the specific binding of these substances could be stabilized by hydrogen bonds and perhaps also by dipole-dipole and dipole-ion interactions. The last two interactions probably do not make much of a contribution, however, since equilibrium constants are relatively insensitive to changes in ionic strength (see footnotes to Figures 1 and 7) and in *p*H (Velick *et al.*, 1960). But the energetic contribution of hydrogen bonds would be amplified if, in the binding of these relatively hydrophobic ligands, water were extruded from the interstitial space between ligand and the side chains lining the protein's binding site. The 4–8 hydrogen bonds that might be formed per molecule of bound ligand could, in a relatively anhydrous environment, account for the enthalpy changes observed. The different absorption maxima for ε-DNP-L-lysine in 1,4-dioxane, in water, and when bound to antibody (Figure 8) are consistent with the possibility that

hydrogen bonds are involved in specific binding of this hapten.[7]

In view of the limited water-solubility of the dinitrobenzenes, apolar bonds (Karush, 1962) would be expected to play a conspicuous role in their binding, but it is not clear that the thermodynamic results (Table VI) support this expectation. According to a widely accepted view of the nature of apolar bonds (Kauzmann, 1959), their formation should be entropy driven and essentially athermal. With the binding of ϵ-DNP-lysine by antibody 2A, however, $\Delta S°$ is negative and $\Delta H°$ is the largest negative enthalpy value recorded for any hapten-antibody system. Even with an antibody preparation of low affinity (i.e., preparation 5) the negative $\Delta H°$ value is sufficient to account for $\Delta F°$. The binding of the norleucine moiety of ϵ-DNP-L-lysine to antibody 2A is also estimated to be exothermal (Table VI), and by this criterion we have no evidence that even this apolar structure is bound by apolar bonds. Conformational changes in the protein, accompanying complex formation, could account for the energetic results, but such changes are improbable in view of the kinetics of the association reaction which are so rapid as to appear to be diffusion-limited and which have an extremely low activation energy (Day *et al.*, 1963; Froese *et al.*, 1962). Tentatively it may be concluded, therefore, that apolar interaction between ligand and binding site, with extrusion of interstitial water, facilitates the formation of hydrogen bonds which are unusually energetic for an aqueous system.

The well-known capacity of polynitrobenzenes to form charge-transfer complexes raises the possibility that electron transfer of this type might contribute to the unusual stability of some antibody-dinitrobenzene complexes. The peak which appears at 300 mμ in the absorption spectrum of antibody-bound 2,4-dinitrotoluene supports this notion. Of all the amino acids, tryptophan is the most effective electron donor for charge-transfer (Isenberg and Szent-Gyorgi, 1958), and the high efficiency ($Q_{max} \cong 70$) with which anti-DNP antibody fluorescence is quenched is consistent with the possibility that a tryptophan residue is part of, or very close to, the hapten-binding site. The magnitude of the negative enthalpy changes observed are, however, considerably greater than have been measured with well-studied charge-transfer complexes (Briegleb, 1961), and, in addition, association constants for model complexes of this type are only of the order of 1–100, far less than here observed. Finally, electron-donating substituents on dinitrobenzenes would be expected to decrease their capacity to form charge-transfer complexes, but 2,4-dinitroaniline and 2,4-dinitrotoluene were bound more strongly than *m*-dinitrobenzene (Table V). It seems reasonable to conclude, therefore, that if charge-transfer occurs, and this might well be the case, it makes only a small energetic contribution to the stability of antibody dinitrophenyl complexes.

Concluding Remarks.—From a teleonomic viewpoint, binding heterogeneity would increase the range of cross-reactions which antibody populations can display, and thus broaden their protective value. In addition, the decline in serum antibody concentrations commonly observed long after immunization would be compensated for, in respect to antigen-binding capacity, by the higher affinities of the antibodies synthesized late in the immune response. Accordingly, both binding-heterogeneity and increasing affinity with progressive immunization may be visualized as contributing to the fulfillment of the biologic obligations of the antibody-forming apparatus. Both of these features might, therefore, have offered selective advantages in the evolutionary development of the immune response.

[7] In the nonspecific-serum-albumin binding of some 2,4-dinitrobenzenes, on the other hand, hypochromic effects were also observed, but shifts in absorption maxima to longer wavelengths were not (Carsten and Eisen, 1953).

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