Affinity and Heterogeneity of Antibodies Induced by ε-2,4-Dinitrophenylinsulin*

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ABSTRACT: Bovine insulin, substituted exclusively on its sole lysine residue with the 2,4-dinitrophenyl group, acts as a potent immunogen in guinea pigs. Anti-2,4-dinitrophenyl antibodies have been isolated from sera at various times after immunization with ε-2,4-dinitrophenylinsulin and these have been compared with guinea pig antibodies made against heavily dinitrophenylated bovine γ-globulin. Despite the relative homogeneity of ε-2,4-dinitrophenylinsulin, the antibodies induced by this immunogen display heterogeneity of ligand binding constants and electrophoretic migration comparable with antibodies evoked by heavily dinitrophenylated bovine γ-globulin. By analysis of the average association constants for binding a homologous series of 2,4-dinitrophenyl ligands including the immunogen, it appears that portions of the immunogen adjacent of the 2-4-dinitrophenyl group vary in their contributions to the total free energy of the ligand–antibody reaction depending upon the time of antibody isolation during the immune response. Although the affinity for each ligand increases with time after immunization, the rate of increase seems to be greater for smaller 2,4-dinitrophenyl ligands. As a result, antibody molecules formed late in the immune response form nearly equally stable complexes with various members of a structurally homologous series of 2,4-dinitrophenyl compounds. This property of high-affinity molecules seems to correspond qualitatively to their broader range of cross-reactions with structurally similar ligands.

It has been suggested (Singer, 1964) that the heterogeneity of association constants that is characteristic of antigen–antibody reactions arises in part from the structural heterogeneity of immunogens. Though the degree of structural heterogeneity of an immunogenic macromolecule cannot be known after injection into an animal, the heterogeneity of a specified haptenic determinant can be minimized. This has been achieved in the present study by the use of an immunogen of known primary structure bearing a chemically defined substituent (the 2,4-dinitrophenyl group) at a single known site on the carrier protein. Many previous studies have provided evidence for the heterogeneity of antibody populations formed against single antigenic determinants. Work in Kabat's laboratory on the antidextran system (summarized in Kabat, 1966) and anti-isomaltotrionic acid (Arakatsu *et al.*, 1966) has documented a variation in the binding of oligosaccharides of increasing chain length by subsets of antibodies from individual antisera. Similar observations have been made by others using polypeptide (Cebra, 1961; Maurer, 1964; Sage *et al.*, 1964; Arnon *et al.*, 1965; Van Vunakis *et al.*, 1966; Goodman and Nitecki, 1966), polynucleotide (Stollar *et al.*, 1962; Sela *et al.*, 1964), or hapten–protein conjugate (Eisen and Siskind, 1964; Kitagawa *et al.*, 1965, 1967; Schlossman *et al.*, 1965; Cheng and Talmage, 1966) antigens. It has also been reported that guinea pigs and rabbits can be stimulated to produce antihapten antibodies by immunization with a preparation of protein molecules each bearing only one haptenic group (Pepe and Singer, 1959; Eisen *et al.*, 1964; Brenneman and Singer, 1968).

The present study was therefore addressed to the following questions: (1) Do antibodies elicited by an immunogen bearing a single haptenic group show heterogeneity of association constants? (2) Do antibodies made later in the immune response to such an immunogen show higher average affinity than antibodies made at earlier times? (3) Do amino acid residues adjacent to the haptenic group in the immunogen contribute to the total free-energy change of the antibody–ligand reaction? ε-DNP-insulin (Figure 1) was chosen as the immunogen because its primary structure is known and because the molecule contains only one lysyl residue, the penultimate amino acid in the phenylalanine chain.

Materials and Methods

The Immunogen, ε-DNP-Insulin. The monosubstituted immunogen was prepared by a method described by Li (1956). In a typical preparation 500 mg of crystalline bovine insulin (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 80 ml of aqueous 0.1 M sodium carbonate solution and to this was added 1.0 g of recrystallized sodium 2,4-dinitrobenzenesulfonate. The reaction was allowed to proceed at 4° for 9 days. With some preparations an additional 0.5 g of 2,4-dinitrobenzenesulfonate was added to the reaction after 4 days and the mixture returned to the cold for 3 days. The DNP-insulin (ε-DNPI[1]) was then precipitated at pH 5.5 by the addition of 6 N HCl to the reaction mixture. The yellow precipitate was

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[1] Abbreviations used in this work: BγG, bovine γ-globulin; HSA, human serum albumin; ε-DNPI, bovin insulin bearing an ε-*N*-2,4-dinitrophenyl group on the sole lysine residue; DNP-HSA and DNP-

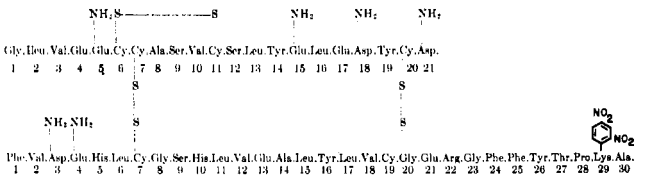

FIGURE 1: The primary structure of ε-DNPI. The sequence of bovine insulin as determined by Sanger and collaborators (Ryle *et al.*, 1955) is depicted with a 2,4-dinitrophenyl group conjugating the ε-NH_2 group of the sole lysine, residue 29 of the phenylalanine chain.

dissolved in 1 M $NaHCO_3$ and applied to a Sephadex G-25 column equilibrated with water. The yellow fraction appearing with the void volume was adjusted to pH 1.8 with 1 N HCl and the protein was precipitated by the addition of 5 M NaCl. Precipitation with NaCl was repeated and the final precipitate was dissolved in a small volume of 1 M $NaHCO_3$. Samples were removed from this stock solution and dialyzed against water or buffered saline (0.15 M NaCl–0.02 M phosphate, pH 7.4) as required. All preparations of ε-DNPI were contaminated with unreacted bovine insulin and attempts to remove this contaminant were unsuccessful (see Results).

Immunization and Isolation of Antibodies. Randomly bred male Hartley strain guinea pigs were immunized by footpad injection of 0.5 mg of ε-DNPI or of DNP-BγG in complete Freund's adjuvant. Blood was collected by cardiac puncture at various intervals from 2 to 6 weeks after immunization. Antibodies specific for the 2,4-DNP group were isolated from single bleedings of individual animals of from serum pools of several animals obtained on 4 consecutive days. Anti-DNP antibodies were purified by the method described by Eisen *et al.* (1967) by precipitation with highly substituted DNP-HSA and hapten elution with 0.1 M 2,4-dinitrophenol.

Preliminary attempts to stimulate anti-DNP antibody formation in rabbits following a single injection of ε-DNPI in complete Freund's adjuvant were unsuccessful.

Proteolytic Cleavage of ε-DNPI. Tryptic hydrolysis of ε-DNPI was performed at pH 8.0 in 0.1 M sodium phosphate. The incubation was carried out in a dialysis casing at 25° with a weight ratio of trypsin (Worthington Biochemical Corp., Freehold, N. J.) to ε-DNPI of 1:50. The appearance of 360-mμ-absorbing material in the diffusate (100 ml) was linear up to 8 hr and at that time the total diffusate was concentrated by flash evaporator (Laboratory Glass Supply Co., New York, N. Y.) and applied to an 0.9 × 150 cm Sephadex G-25 column equilibrated with 0.01 M NaCl–0.01 M $NaHCO_3$. The DNP-peptide material was retarded on Sephadex G-25 as was found by Hirs (1962) in the purification of a DNP-peptide derived from mono-DNP-ribonuclease. The effluent fractions comprising the single symmetrical yellow peak were pooled, concentrated by flash evaporation, and freed of salt on a mixed-bed ion-exchange resin (AG11A8, Bio-Rad Laboratories, Richmond, Calif.).

α-Chymotryptic hydrolysis of ε-DNPI was performed with a chymotrypsin to ε-DNPI weight ratio of 1:50. A reaction mixture in 0.1 M Tris-Cl (pH 8.0) was dialyzed against 100 ml of the same buffer for 6 hr at 25°. The yellow diffusate was concentrated by flash evaporation, and the DNP-peptide-containing material was applied to an 0.9 × 150 cm Sephadex G-25 column. The minor yellow fraction which eluted in the void volume was discarded and the major yellow peak was pooled, concentrated, and freed of salt by a mixed-bed resin (AG11A8).

ε-DNPI was digested with diisopropylfluorophosphate-treated carboxypeptidase A (Worthington Biochemical Corp., Freehold, N. J.) in 0.1 M Tris-acetate (pH 8.0) for 30 min at 37°. The reaction was terminated by acidification to pH 4.0 with 1 M acetic acid. For 0.0432 μmole of digested ε-DNPI, 0.0420 μmole of free alanine was detected by automatic amino acid analysis. Trace amounts of aspartic acid were also detected but no other amino acids. Another small aliquot of the reaction mixture was applied to a 0.9 × 50 cm Sephadex G-25 column equilibrated with buffered saline. All of the 360-mμ-absorbing material was eluted with the void volume indicating insignificant cleavage of the ε-DNP-lysyl residue of ε-DNPI. Additional evidence against the occurrence of significant cleavage of the peptide-bound ε-DNP-lysine was obtained by thin-layer chromatography on silica gel G in water saturated in methyl ehtyl ketone. Ascending chromatography of the carboxypeptidase digest failed to yield a spot with the R_F (0.2) of authentic ε-DNP-L-lysine.

α-Acetyl-ε-DNP-L-lysine was synthesized from ε-DNP-L-lysine (Sigma Chemical Co., St. Louis, Mo.) by the method of Fraenkel-Conrat (1957). The product was crystallized from aqueous methanol, mp 168°. *Anal.* Calcd for $C_{14}H_{18}N_4O_7$: C, 47.45; H, 5.12; N, 15.81. Found[2]: C, 47.36; H, 5.31; N, 14.35. The absorbance spectrum of crystallized α-acetyl-ε-DNP-L-lysine dissolved in buffered saline was identical with authentic ε-DNP-L-lysine between 270 and 400 mμ and the ε 360 mμ of α-acetyl-ε-DNP-L-lysine was therefore taken to be that of ε-DNP-L-lysine, *i.e.*, 17,400 (Carsten and Eisen, 1953). Tritiated α-acetyl-ε-DNP-L-lysine was synthesized from acetic [^{3}H]anhydride (400 mCi/mmole; New England Nuclear Corp., Boston, Mass.) by the same procedure. At the termination of the synthesis, the reaction mixture was extracted with freshly opened ether, acidified with HCl, and reextracted with ether several times until no further yellow color could be extracted from the aqueous phase. The ether extracts were pooled and evaporated to dryness. The yellow product was dissolved in 2.0 ml of 95% ethanol and applied to a 0.9 × 25 cm DEAE-cellulose column equilibrated with 0.01 M acetic acid. Radioactive colorless impurities were eluted with 300 ml of 0.01 M acetic acid and then the yellow band was eluted in 3–4 ml with 0.01 M HCl. An aliquot of the product was analyzed by thin-layer chromatography on silica gel G developed with water-saturated methyl ethyl ketone. The single yellow spot with an R_F of 0.9 was ninhydrin negative and was coincident in migration with the radioactivity detected by autoradiogram of the chromatogram. The product specific activity in Bray's (1960) solution was 26,000 cpm/mμmole. The yield of the radioactive synthesis was 71% compared with ε-DHP-L-lysine used in the reaction mixture. The absorbance spectrum and thin-layer chromatography of the radioactive and nonradioactive products were identical.

BγG were heavily substituted preparations bearing 37 moles of DNP/70,000 g of human serum albumin and 50–57 moles of DNP/160,000 g of bovine γ-globulin, respectively.

[2] Elemental analysis was performed by the Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

Amino Acid Analyses. Hydrolysis of proteins and peptides was performed in 6 N HCl at 110° under reflux conditions for 22 hr. Amino acid analyses were performed on a Beckman Model 120B analyzer equipped with long light-path photometer cells and an electronic scale expander.

Kjeldahl Nitrogen. The nitrogen content of ε-DNPI was determined by the micro-Kjeldahl technique described by Mayer (1961). The extinction coefficient at 360 mμ for ε-DNPI was calculated from Kjeldahl nitrogen values assuming bovine insulin to be 15.88% nitrogen (Tristram and Smith, 1963). Since this coefficient did not differ significantly from ε-DNP-L-lysine, 17,400 was the ε 360 mμ employed for the calculation of the concentration of ε-DNP groups in various preparations of ε-DNPI.

Immunoelectrophoresis. Antibodies were examined by immunoelectrophoresis in 1.5% Noble agar, barbituric acid–acetate buffer (pH 8.6). Precipitation arcs were developed with a rabbit antiserum to guinea pig serum. Unstained moist gels were photographed after 24 hr.

Ligand Binding Measurements. Average intrinsic association constants for various ligand–antibody combinations were obtained by equilibrium dialysis and fluorescence quenching. Measurements of antibody affinity by equilibrium dialysis were performed as described elsewhere (Eisen, 1964). The chambers were 10 × 75 mm disposable test tubes capped with rubber vial closures. The inside solution was contained in a sac of washed 11/32 Visking dialysis casing. The volume of outside and inside solutions was 1 ml. The inside compartment of the experimental chambers contained 40–70 μg/ml of antibody in buffered saline. The experimental chambers were rotated 3–5 rpm for 5.5–6.0 hr at 30° and then equilibrium across the membranes was verified by counting aliquots of control chambers containing ligand (with nonspecific γG-immunoglobulin) but no antibody. Bag binding was less than 3% of the free-ligand concentration with α-[^{3}H]acetyl-ε-DNP-L-lysine. At equilibrium, aliquots were taken from inside and outside compartments for counting in Bray's (1960) solution in a Packard liquid scintillation spectrometer and uniform quenching of all samples was confirmed with the Packard automatic external standard. None of the ligands were bound significantly by nonspecific rabbit γG-immunoglobulin at the concentrations of free ligand employed. r values in various equilibrium dialysis experiments ranged from 0.2 to 1.70. None were adequate to permit accurate estimate of antibody purity but by precipitation with DNP-HSA various lots of anti-ε-DNPI proved to be more than 85% precipitable at antibody concentrations of approximately 1 mg/ml.

Fluorescence quenching measurements of ligand binding by purified antibody preparations were performed in buffered saline at 30° in an Aminco-Bowman spectrophotofluorometer (Velick *et al.*, 1960; Little and Eisen, 1966). Bound and free hapten were determined on the basis of the fluorescence quenching observed, relative to the quenching when all of the antibody combining sites are occupied by ligand (Q_{max}). The value of Q_{max} was determined by comparison of equilibrium dialysis and fluorescence quenching data for a single ligand–antibody pair as described by McGuigan and Eisen (1968). The same Q_{max} value was then employed for calculation of all binding data for other aliquots of that particular antibody preparation. Various preparations of guinea pig antibodies had apparent Q_{max} values of 90–100. When antibody preparations were titrated with ε-DNPI as the ligand, an additional correction factor was employed due to the minimal but significant fluorescence of this protein ligand. A correction curve was obtained for each preparation of ε-DNPI by "titration" of 1-ml aliquots of buffered saline using the same ligand concentration and volumes of addition as in the antibody titrations. The fluorescence attributable to the ligand was then subtracted from the total fluorescence at each point in the titration of antibody. This resulted in corrections of the observed fluorescence of 0–5%. All antibody samples were titrated in duplicate or triplicate with each ligand and binding constants and heterogeneity indices were calculated with the aid of an IBM computer, Model 7072.[3] Average intrinsic association constants, K_0, were calculated from fluorescence quenching and equilibrium dialysis data according to the logarithmic form of the Sips equation (Nisonoff and Pressman, 1958; Karush, 1962). Conformity of the fluorescence quenching data to the linear regression was computed as the coefficient of correlation which varied between 0.99 and 0.90 for all the data cited. For all guinea pig antibody populations, the value of $\epsilon_{278}^{1\%}$ 14.0 was taken.

Additional compounds were used without further purification. 2,4-Dinitrophenylated amino acids were obtained from Mann Chemical Corp. (New York, N. Y.). DEAE-cellulose (0.51 mequiv/g) was a Serva resin from Gallard-Schlesinger Chemical Manufacturing Co. (Garden City, N. Y.). The BγG and HSA used for preparation of antigens were fraction II of bovine plasma, from Armour and Co. (Chicago, Ill.), and crystallized human serum albumin, from Pentex Inc. (Kankakee, Ill.). The ε-DNP-D-lysine was a gift from Dr. H. N. Eisen.

Results

Amino Acid Analysis. The amino acid composition of ε-DNPI (Table I) corresponded closely to bovine insulin. There was no evidence of DNP substitution of the N-terminal glycine or phenylalanine residues as these were present in the same amounts as in bovine insulin. Unsubstituted lysine remained in preparations of ε-DNPI to the extent of 0.5–0.6 residue/mole of protein (0.59 residue/mole of protein compared with 1.07 residue/mole in bovine insulin). The low values for isoleucine (0.42 residue/mole in ε-DNPI and 0.46 residue/mole in bovine insulin) were probably the result of incomplete hydrolysis.

Table II gives the composition of the DNP octapeptide derived from ε-DNPI by trypsin digestion. This corresponds to the known composition of this C-terminal octapeptide of bovine insulin.

Thin-Layer Chromatography. Aliquots of all ε-DNPI preparations were hydrolyzed in sealed tubes at 110° in HCl for 22 hr. The hydrolysates were reduced to dryness under vacuum, dissolved in 1 N HCl, and applied to thin-layer chromatography plates of silica gel in paralled with ε-DNP-L-lysine, DNP-glycine, DNP-L-phenylalanine, and *O*-DNP-L-tyrosine. The chromatograms were developed with methyl ethyl ketone saturated with water. For each ε-DNPI preparation only one yellow spot was present, which had the R_F of authentic ε-DNP-lysine (0.2). Under these conditions, the presence of DNP-glycine, DNP-phenylalanine, or *O*-DNP-tyrosine would have

[3] Automatic computations were carried out at the Washington University Computing Facilities, which is supported in part by a National Science Foundation grant (G222-96).

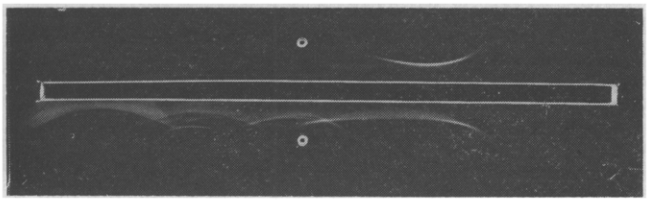

FIGURE 2: Immunoelectrophoresis of guinea pig antibody preparation. Upper well, anti-ε-DNPI isolated from antiserum of a representative single guinea pig 4 weeks after injection with 0.5 mg of ε-DNPI. Lower well, normal guinea pig serum. The trough contained rabbit antiserum to whole guinea pig serum. The analysis was performed in 1.5% agar, barbital–acetate buffer (pH 8.6), and the anode was at the left.

been detected at a level greater than 1% of the total DNP-amino acids. *O*-DNP-tyrosine was detected on chromatograms by quenching of a fluorescent indicator in the silica gel (Eastman chromagram sheets).

Characterization of Isolated Guinea Pig Antibodies. A representative immunoelectrophoresis pattern obtained with anti-ε-DNPI antibodies isolated from a single guinea pig bled 4 weeks after immunization is shown in Figure 2. Each lot of purified anti-DNP-BγG and anti-ε-DNPI antibodies proved to be predominantly γ_2 with smaller amounts of γ_1 when developed with rabbit antiserum to guinea pig serum. The immunoelectrophoresis patterns did not reveal significant differences between antibody populations isolated from 2 to 6 weeks after immunization, nor between antibodies specific for ε-DNPI or DNP-BγG.

TABLE I: Amino Acid Composition of Bovine Insulin and ε-DNPI.[a]

Amino Acid	Residues/Mole of Protein		
	ε-DNPI (Experimental)	Bovine Insulin (Experimental)	Bovine Insulin (Predicted)
Lysine	0.59	1.073	1
Histidine	2	2	2
Arginine	1.15	1.01	1
Aspartic acid	3.17	2.99	3
Threonine	1.11	0.95	1
Serine	2.97	2.92	3
Glutamic acid	7.28	7.28	7
Proline	1.36	1.09	1
Glycine	4.12	4.47	4
Alanine	3.19	3.04	3
Half-cysteine	5.31	5.47	6
Valine	4.42	4.28	5
Isoleucine	0.42	0.46	1
Leucine	6	6	6
Tyrosine	3.86	3.97	4
Phenylalanine	2.89	2.94	3

[a] The experimental values are averages for three analyses each of ε-DNPI and bovine insulin. The theoretical values for bovine insulin were obtained from Ryle *et al.* (1955). All samples were hydrolyzed in 6 N HCl at 110 ± 1° under reflux conditions for 22 hr. The calculation of residues per mole was made by assuming 2 and 6 moles of histidine and leucine, respectively, per mole of insulin and by normalizing the recovery of arginine and lysine to the histidine recovery and all other amino acids to the leucine recovery.

TABLE II: Amino Acid Composition of Tryptic DNP-peptide from ε-DNPI.[a]

Amino Acid Residue	Residues/Mole of Peptide	
	Experimental	Predicted
Threonine	0.98	1
Proline	1.08	1
Glycine	1.10	1
Alanine	1	1
Tyrosine	0.87	1
Phenylalanine	1.99	2
Lysine	0.05	1

[a] The experimental values are averages for duplicate analyses of the tryptic octapeptide isolated from ε-DNPI as outlined in Materials and Methods. The predicted values for bovine insulin are the same as those from Table I assuming the sequence shown in Figure 1 and the failure of trypsin of hydrolyze the peptide at the DNP-lysyl residue. Samples were hydrolyzed in 6 N HCl at 110 ± 1° under reflux conditions for 22 hr. The calculation of residues per mole was made by assuming 1 mole of alanine/mole of peptide and by normalizing the recovery of the other amino acids to the alanine recovery.

Antibody Intrinsic Association Constants and Heterogeneity Indices. The binding data obtained with a preparation of antibodies isolated from a single animal 2 weeks after immunization with ε-DNPI are shown in Figure 3. The data obtained by equilibrium dialysis and fluorescence quenching for the binding of α-Ac-ε-DNP-L-lysine are coincident. The index of heterogeneity of association constants is 0.59, comparable with values observed for the binding of ε-DNP-L-lysine by rabbit antibodies obtained after DNP-BγG immunization (Eisen and Siskind, 1964). The average intrinsic association constant calculated from these data is 2.9×10^6 M^{-1} (Figure 3), based on $Q_{max} = 95$. The average association constant for the binding of the immunogen, ε-DNPI, is 2.5×10^7 M^{-1} and the heterogeneity index is 0.30. Anti-ε-DNPI antibodies isolated from relatively early antisera consistently showed stronger binding to ε-DNPI than to ε-DNP-L-lysine in contrast to anti-DNP-BγG antibodies, whose binding constants for ε-DNPI and ε-DNP-L-lysine were generally indistinguishable.

Fluorescence Quenching. Figure 4 shows typical fluorescence quenching curves for anti-ε-DNP-insulin antibodies titrated with 2,4-dinitroaniline, ε-DNP-L-lysine, and ε-DNPI. ε-DNPI quenched antibody fluorescence better than dinitroaniline whereas the quenching by ε-DNP-lysine was intermediate; 6 weeks after immunization with ε-DNPI, the extent of antibody fluorescence quenching by each ligand was greater but the

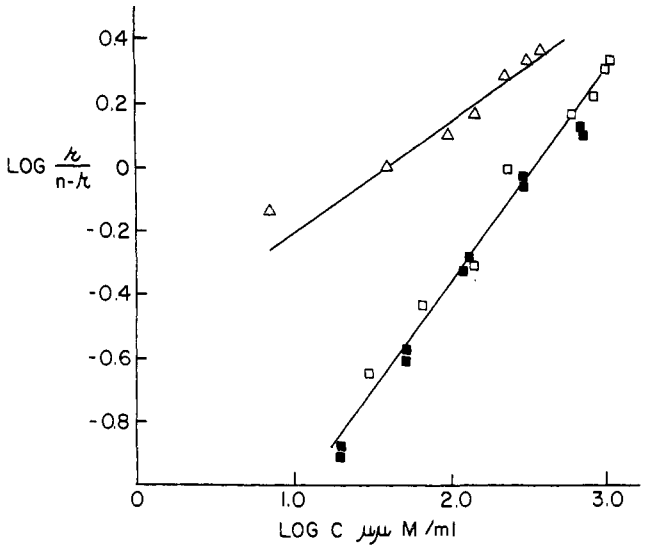

FIGURE 3: Sips plot of equilibrium dialysis and fluorescence quenching at 30° obtained with a single lot of anti-ε-DNPI antibodies obtained 2 weeks after immunization. Equilibrium dialysis (filled squares) and fluorescence quenching (open squares) were performed in buffered saline (0.15 M NaCl–0.02 M phosphate, pH 7.4) with the same ligand, α-[^{3}H]Ac-ε-DNP-L-lysine. K_0 calculated from the equilibrium dialysis data was 2.9 × 10^6 M^{-1} and the same value was obtained by fluorescence quenching assuming Q_{max} = 95%. The heterogeneity index, a, for these data was 0.59. Shown in open triangles is the fluorescence quenching titration of the same antibody preparation with ε-DNPI. The K_0 for ε-DNPI calculated from these data, assuming Q_{max} = 95, is 2.5 × 10^7 and the heterogeneity index is 0.30. All determinations were made at 30°.

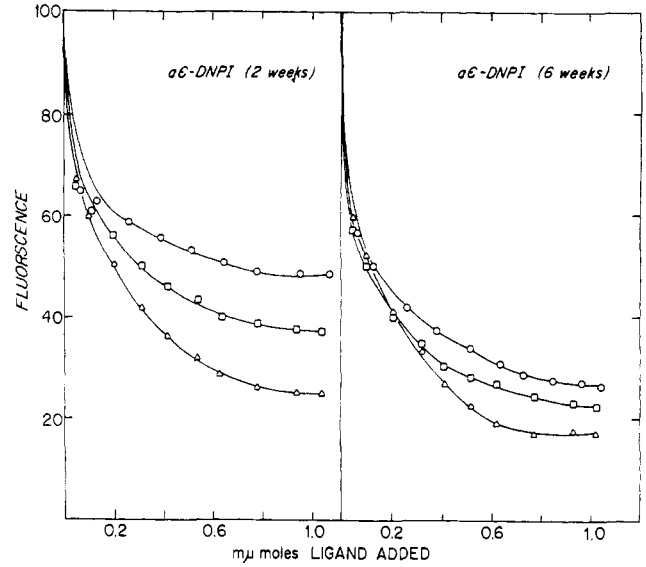

FIGURE 4: Fluorescence quenching of anti-ε-DNPI antibodies from a single guinea pig bled at 2 and at 6 weeks after immunization. The ligands employed were 2,4-dinitroaniline (circles), ε-2,4-DNP-L-lysine (squares), and ε-DNPI (triangles). Each titration was obtained with 1-ml aliquots of antibody at 50–55 μg/ml in buffered saline at 30°. Fluorescence values have been corrected for solvent fluorescence and for volume change due to ligand addition for all three ligands. An additional small correction has been applied to the ε-DNPI titration curves because this ligand (but not the others) has a small but significant fluorescence emission at 350 mμ. Each curve is the average obtained from duplicate or triplicate titrations. Assuming Q_{max} = 100, the K_0 values calculated from these data are given in Table IV.

differences in the quenching curves were smaller than was observed at 2 weeks. The binding of the purified C-terminal octapeptide bearing the ε-DNP-lysyl residue, prepared by tryptic digestion of ε-DNPI was indistinguishable from the binding of ε-DNPI by anti-ε-DNPI antibodies of low- or high-average affinity (Table III). ε-DNPI from which the residue adjacent to the DNP-lysyl group, the C-terminal alanine, had been cleaved with carboxypeptidase showed poorer quenching than ε-DNPI (Table III).

In Table III are the association constants, K_0, and the free-energy changes of binding, $-\Delta F°$, calculated from fluorescence quenching titrations of a preparation of purified anti-ε-DNPI antibodies obtained 2 weeks after immunization. The $-\Delta F$ for the reactions increased as larger ligands were used until a maximum value was reached with the DNP-tetrapeptide obtained by chymotrypsin digestion of ε-DNPI. There was no difference between the $-\Delta F°$ for the reaction of anti-ε-DNPI antibodies with the DNP-tetrapeptide, the tryptic octapeptide, or with ε-DNPI itself.

Fluorescence quenching curves for guinea pig antibodies isolated 3 and 6 weeks after immunization with DNP-BγG are shown in Figure 5. In contrast to the preparations of early anti-ε-DNPI antibodies, there was little difference in the binding of ε-DNP-L-lysine and ε-DNPI by the anti-DNP-BγG antibodies. There was increased quenching by all ligands at 6 weeks with the greatest increment shown by dinitroaniline.

A comparison of the association constants and $\Delta F°$ values for antibodies obtained from a representative animal 2, 4, and 6 weeks after immunization with ε-DNPI is given in Table IV. The average association constants systematically increased for each ligand with time after immunization, and the differences in the free energy of binding showed a systematic decrease such that dinitroaniline and ε-DNP-L-lysine were bound almost as well as ε-DNPI at 6 weeks.

TABLE III: Affinity of Early Antibodies Induced by ε-DNPI for Homologous Series of DNP Ligands.[a]

Ligand	$K_0 \times 10^{-6}$ (M^{-1})	$-\Delta F°$ (kcal M^{-1})
2,4-Dinitroaniline	0.19	7.31
ε-2,4-DNP-L-lysine	1.5	8.56
ε-2,4-DNP-D-lysine	1.3	8.47
ε-DNPI	8.1	9.57
ε-DNPI-octapeptide	7.9	9.56
ε-DNPI-tetrapeptide	7.8	9.55
Carboxypeptidase digest of ε-DNPI	2.0	8.73

[a] The values for average intrinsic association constants, K_0, were obtained from fluorescence quenching titrations with a single lot of guinea pig antibodies isolated 2 weeks after immunization with ε-DNPI. Each value is an average of duplicate or triplicate titrations performed at 30°, assuming Q_{max} = 100.

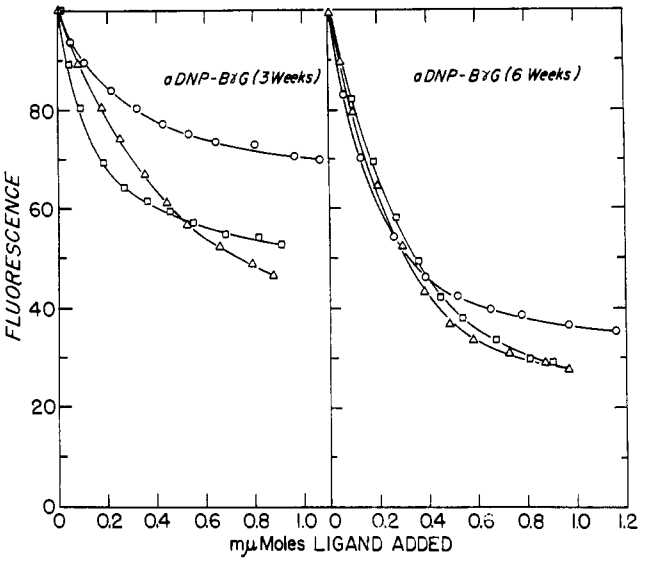

FIGURE 5: Fluorescence quenching of anti-DNP-BγG antibodies from a single guinea pig bled at 3 and at 6 weeks after immunization. The ligands employed were 2,4-dinitroaniline (circles), ε-2,4-DNP-L-lysine (squares), and ε-DNPI (triangles). Each titration was obtained with 1-ml aliquots of antibody at 50–55 μg/ml in buffered saline at 30°. Each curve is the average of duplicate or triplicate titrations and corrections for volume change and fluorescence blanks were made as outlined in Materials and Methods and in the legend to Figure 4. Assuming $Q_{max} = 100$, the K_0 values calculated from these data are: at 3 weeks 6.1×10^4 M^{-1} for dinitroaniline, 3.4×10^5 M^{-1} for ε-DNP-L-lysine, and 6.3×10^5 M^{-1} for ε-DNPI; at 6 weeks 3.7×10^7 M^{-1} for dinitroaniline, 5.1×10^7 M^{-1} for ε-DNP-L-lysine, and 5.9×10^1 M^{-7} for ε-DNPI.

TABLE IV: Increasing Affinity of Antibodies Obtained at Various Intervals after Immunization with ε-DNPI.[a]

Ligand	$K_0 \times 10^{-6}$ M^{-1}	$-\Delta F°$ (kcal M^{-1})	$\Delta\Delta F°$ (kcal M^{-1})
2 Weeks after Immunization			
ε-DNPI	7.8	9.50	
			0.98
ε-2,4-DNP-L-lysine	1.6	8.52	
			1.02
2,4-Dinitroaniline	0.26	7.50	
4 Weeks after Immunization			
ε-DNPI	12	9.74	
			1.04
ε-2,4-DNP-L-lysine	2.0	8.70	
			0.70
2,4-Dinitroaniline	0.64	8.00	
6 Weeks after Immunization			
ε-DNPI	21	10.2	
			0.30
ε-2,4-DNP-L-lysine	16	9.90	
			0.78
2,4-Dinitroaniline	8.2	9.12	

[a] The values for K_0 were obtained from fluorescence quenching titrations with preparations of antibodies from a single guinea pig obtained 2, 4, and 6 weeks after immunization with ε-DNPI. Each value is an average of duplicate or triplicate titrations at 30°, assuming $Q_{max} = 100$. Titrations with the same three ligands were compared with antibodies isolated from individual animals at 2 and 6 or 2 and 4 weeks after immunization with ε-DNPI and proved these data to be representative under the conditions employed for immunization. When the same data were calculated with several different Q_{max} values (from 80 to 100), different association constants were obtained but the relative differences in K_0 were not appreciably altered.

Discussion

ε-DNPI was found to be immunogenic in guinea pigs, consistently inducing antisera containing approximately 1–2 mg/ml of anti-DNP antibodies. The ease of preparation of this singly substituted immunogen and the facility with which it can be cleaved enzymatically to yield a homologous set of ligands of varying size, make ε-DNPI a useful material for the study of ligand–antibody reactions. The amino acid analyses (Table I) and thin-layer chromatography indicate that only lysine was substituted but that unsubstituted bovine insulin was found to contaminate preparations of ε-DNPI, comprising about 60% of each lot. It is possible that even in the recipient animal, ε-DNPI may be aggregated (Fredericq, 1953) and effectively multivalent with respect to the number of DNP groups per particle. The ability of monomeric ε-41-mono-DNP-ribonuclease (Eisen *et al.*, 1964) and of papain-*S*-DNP-lysine (Brenneman and Singer, 1968) to stimulate the formation of anti-DNP antibodies indicate, however, that multiple haptenic substituents are not essential for immunogenicity.

Guinea pig antibodies induced by ε-DNPI were first detectable by precipitation with DNP-HSA 10 days after immunization. Although anti-DNP antibody concentrations were low at this time, by 2 weeks the titers had risen to 1–2 mg/ml. Since antibody isolation was achieved by specific precipitation with DNP-HSA and elution with 2,4-dinitrophenol, selection was achieved for those antibody molecules whose binding sites were capable of forming stable complexes with the DNP-lysyl group and the dinitrophenylate ion. The same reagents were used for the isolation of anti-DNP-BγG and anti-ε-DNPI antibody populations from early and from late sera of each specificity. The antibodies formed early after immunization with ε-DNPI discriminated between ε-DNPI and ε-DNP-L-lysine by more stable complex formation with the immunogen, whereas the antibodies formed against DNP-BγG, whether from early or late antisera, did not distinguish between ε-DNPI and ε-DNP-L-lysine.

The possibility was considered that cooperative binding effects, attributable to the self-aggregation ε-DNPI (Fredericq, 1953, and unpublished data), were the cause of the stronger interaction of anti-ε-DNPI antibodies with the immunogen than with monovalent ε-DNP-L-lysine (Paul *et al.*, 1966). This seems unlikely in view of the generally indistinguishable binding of ε-DNPI and the ε-DNP-octapeptide. The latter is included by Sephadex G-25, is freely dialyzable, and inhibits specific precipitation of anti-DNP antibodies with DNP-HSA (unpublished data). Thus, the DNP-octapeptide has the prop-

erties of a functionally monovalent ligand in contrast to the properties of ε-DNPI. Efforts were also made to detect cooperative binding effects by comparison of fluorescence quenching titrations with ε-DNPI and ε-DNP-L-lysine by native and papain-digested antibody preparations. It was found, however, that even in titrations with ε-DNP-lysine the fluorescence quenching properties of the cleaved antibody molecules were significantly altered and direct comparison of the binding data with intact molecules could not be made.

The presence of unsubstituted bovine insulin in each lot of ε-DNPI would be expected to have little effect on the results because the method of antibody purification employed (Farah *et al.*, 1960) has been shown to select only those molecules whose active sites bind the DNP group and any antibodies specific for unsubstituted insulin would have been discarded. Unsubstituted bovine insulin failed to inhibit specific precipitation of DNP-HSA by anti-ε-DNPI sera (Table V), demonstrating that the only antibody molecules that precipitate with DNP-HSA are those for which the DNP group acts as the dominant portion of the antigenic determinant.

The binding studies using purified anti-ε-DNPI antibodies demonstrate heterogeneity of association constants with each ligand tested. This was confirmed by equilibrium dialysis experiments with α-acetyl-[^{3}H]ε-DNP-L-lysine. Despite the relative homogeneity of the immunogen, there exists the possibility of variations in the conformation of the DNP-antigenic determinant since the flexibility of the lysyl side chain may permit several alternative stable interactions of the DNP group with neighboring residues. In general, antibody–ligand reactions with higher association constants had greater heterogeneity. Though it seems likely that this reflects greater absolute heterogeneity in the immune response itself, it is possible that this direct relationship of the heterogeneity index to K_0 results in part from the purification procedure. It might be expected that for heterogeneous antibody populations of low average affinity ($K_0 < 1 \times 10^5$ M^{-1}) the antibodies with K on the lower part of the distribution curve might escape precipitation with DNP-HSA. This would yield purified antibody preparations with somewhat higher K_0 and would also reduce the *range* of affinities. For high-affinity antibodies, molecules with a wide range of association constants would form complexes of sufficient stability to be precipitated with antigen (although antibodies with very high K might be poorly eluted and lost). One would then expect to see the greatest heterogeneity in antibody populations of intermediate average association constant and this was not observed.

In the period from 2 to 6 weeks after immunization, there was an increase in the average affinity with time for all the ligand–antibody combinations tested. This is in accord with the findings of Eisen and Siskind (1964) who studied rabbit antibodies induced by the multiply substituted immunogen, DNP-Bγ G. In addition, the foregoing data (Figure 4) show that the increase in association constants with time varied with different ligands. Within a homologous series of DNP ligands there was less discrimination by late high-affinity antibodies than by early, lower affinity molecules. This result is in accord with the coordinate increase in affinity and cross-reactivity of rabbit antibodies produced during the primary response to immunization with DNP-Bγ G and TNP-Bγ G (Little and Eisen, 1969).

Also shown in Table III is the significant reduction in K_0 that accompanies removal of the alanine residue at the carboxy terminus of the phenylalanine chain of ε-DNPI. Since the carboxyl group of this alanine residue in the immunogen is probably charged at physiological pH, the translocation of the negative charge to the DNP-lysyl carboxyl group (following removal of the alanine) may explain the loss in binding energy with the desalanyl-ε-DNPI. Cleavage of the carboxy-terminal alanine of ε-DNPI may also constitute removal of one arm of a branched antigenic determinant of which the DNP-lysyl group comprises the second arm. The indistinguishable binding of ε-DNP-L-lysine and ε-DNP-D-lysine suggests that proximity to the DNP group *per se* is probably not a sufficient explanation for the differences in binding of ε-DNPI and carboxypeptidase-treated ε-DNPI. These data are in accord with those of Parker *et al.* (1966) who clearly showed the importance of residues neighboring the DNP-lysyl group in binding studies performed with antibodies made following immunization with a DNP-tetrapeptide determinant group.

The energy contributions of various portions of the ligand molecule can be estimated from comparisons of their binding constants with antibodies from early (2-week) and late (6-week) antisera. Tables III and IV and Figure 4 show that for antibodies obtained 2 weeks after immunization with ε-DNPI, amino acid residues adjacent to the DNP-lysyl group of the immunogen participate in the antibody–ligand reaction though their total energy contribution is small (approximately 1 kcal M^{-1}). For this same antibody preparation approximately 1 kcal M^{-1} of interaction energy seems to result from the presence of the norleucine side chain of ε-DNP-L-lysine. On the other hand, since the binding of the tryptic octapeptide and ε-DNPI are indistinguishable for both early and late antibodies (Table III), it seems that portions of the ε-DNPI molecule greater than six amino acid residues from the DNP-lysyl group make no detectable contribution to the total $-\Delta F^\circ$. Comparison of the binding constants with dinitroaniline, ε-

TABLE V: Failure of Insulin to Inhibit Specific Precipitation of Antiserum to ε-DNPI.[a]

Bovine Insulin Concn (M)	mg of Antibody Protein in Precipitate
0	0.364
2.7×10^{-6}	0.365
1.4×10^{-5}	0.350
2.7×10^{-5}	0.356

[a] The guinea pig antiserum was a pool of five sera obtained 3 weeks after immunization with ε-DNPI. A quantitative precipitin analysis performed with DNP-HSA and 0.5 ml of antiserum per tube established that equivalence precipitation required the addition of 35 μg of DNP-HSA. This amount of antigen was then added to 0.5-ml aliquots of antiserum containing increasing amounts of a stock solution of bovine insulin. The insulin concentrations refer to the mixture containing antigen and antiserum. Specific precipitates were formed during incubation for 1 hr at 37° followed by 18 hr. at 0°. After washing with iced-buffered saline, the specific precipitates were each dissolved in 1.0 ml of 0.5% sodium dodecyl sulfate for absorbance measurements.

DNP-L-lysine and ε-DNPI for the high-affinity antibody molecules (6-week) suggests that the relative contributions of binding energy made by the norleucine side chain and residues adjacent to the DNP-lysyl group of the immunogen are less than for low-affinity molecules.

These results do not accord with the observations of Eisen and Siskind (1964) whose binding data indicated that the norleucine side chain made a greater contribution to the binding of ε-DNP-L-lysine to high-affinity than to low-affinity antibodies. The reason for this discrepancy is not clear, but it may be related to the use by these authors of antibodies isolated from two (and only two) pools of rabbit antiserum rather than from individual animals or to their use of a different DNP-protein immunogen. Two possible explanations for these results are a progressive reduction with time in the size of that portion of the immunogen which serves as an antigenic determinant, or a relatively smaller size of the combing sites of high-affinity antibody molecules. A third possibility cannot be excluded, namely the selection during antibody purification of anti-DNP molecules which bind smaller ligand molecules preferentially. If hapten elution is more selective with specific precipitates made from high-affinity antibodies than with precipitates made from low-affinity molecules, one would expect to isolate antibodies with affinities showing a disproportionate increase with time for smaller ligands. However, parallel elution of antibodies with ε-DNP-L-lysine and with 2,4-dinitrophenol (Eisen and Siskind, 1964) suggest that antibody preparations obtained from specific precipitates by 2,4-dinitrophenol elution are representative of the total precipitable serum populations. Particularly important is the observation that antibodies isolated by 2,4-dinitrophenol or by ε-DNP-L-lysine elution did not differ in their affinity for ε-DNP-L-lysine. Therefore, it seems possible that anti-DNP molecules of high affinity may possess combing sites that differ from the active sites of low-affinity molecules in that they permit especially energetic reactions with small as well as larger ligands of a homologous series. This property of high-affinity molecules is in accord also with their broader range of cross-reactions (Little and Eisen, 1969).

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