The Antibody Response to a 2,4-Dinitrophenyl Peptide*

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ABSTRACT: Antibodies to a synthetic 2,4-dinitrophenyl (DNP) tetrapeptide, Val-ε-DNP-Lys-Leu-Phe-OEt, have been evaluated in regard to their immunologic specificity and heterogeneity. Immunization was performed in rabbits and guinea pigs with a conjugate of the DNP peptide with human serum albumin. Antibodies with DNP specificity were obtained in highly purified form by specific precipitation and elution with 2,4-dinitrophenol. In the precipitating antigen the DNP group was substituted directly on protein lysyl residues and the remainder of the peptide was absent. The purified anti-DNP antibodies were evaluated by fluorescence quenching, hapten inhibition of precipitation, equilibrium dialysis, and electrophoresis. About 50% of the purified rabbit antibodies bound the homologous DNP peptide to a greater degree than ε-DNP-lysine. By the use of DNP peptides lacking in various side chains or amino acids of the immunizing peptide, contributions by various portions of the peptide to binding were estimated. Antibodies with both peptide and DNP specificity bound DNP-lysine relatively ineffectively so that the over-all binding affinity for the entire peptide was not exceptionally high. Controls established that antibodies to DNP antigens in which the peptide moiety was absent bound ε-DNP-lysine as well as or better than the DNP tetrapeptide. Antibodies to the DNP tetrapeptide exhibited substantial binding and electrophoretic heterogeneity regardless of whether the amino acids of the immunizing tetrapeptide were in the L or D configuration. The results indicate that the specificity of antihapten antibodies can extend beyond the hapten and the amino acid to which it is directly attached to neighboring amino acids. The degree of heterogeneity exhibited by antibodies to the DNP tetrapeptide, an unusually homogeneous antigenic determinant, suggests that heterogeneity of antigen is probably a relatively minor factor in antibody heterogeneity.

Hapten-specific antibodies usually are prepared by injection of the hapten in covalent combination with a purified protein. The antibodies which are formed have a high degree of specificity for the haptenic group and the amino acid residue to which it is directly attached. A contribution by the protein carrier to hapten-specific immunologic phenomena is indicated in the elicitation of delayed hypersensitivity (Gell and Benacerraf, 1961), in the study of immune tolerance (Boyden and Sorkin, 1962), and in the stimulation of a secondary antibody response by antigen *in vitro* (Dutton and Bulman, 1964). Further delineation of the precise role of the protein moiety is difficult because of the complexity of protein structure and the unavailability of pure peptide fragments substituted with hapten. What is not entirely clear is whether or not the specificity of antihapten antibody extends to amino acids adjacent to points of attachment or whether the chemically modified protein and the hapten act as independent antigenic determinants. If contiguous amino acids do influence the type of antibody formed to a hapten, a hapten protein conjugate has many different haptenic antigenic sites. Heterogeneity of this kind could account for much of electrophoretic and binding heterogeneity of purified hapten-specific antibodies from single animals.

We have approached this problem by investigating the antibody response in rabbits to a synthetic tetrapeptide containing the 2,4-dinitrophenyllysyl residue at the 2 position, valyl(ε-*N*-DNP)lysylleucylphenylalanyl ethyl ester[1] (I, Figure 1).[2] Antibodies specific for the 2,4-dinitrophenyl (DNP) moiety have been purified using a DNP protein in which DNP groups are substituted directly on protein lysyl residues and the remainder of the peptide is absent. The purified anti-

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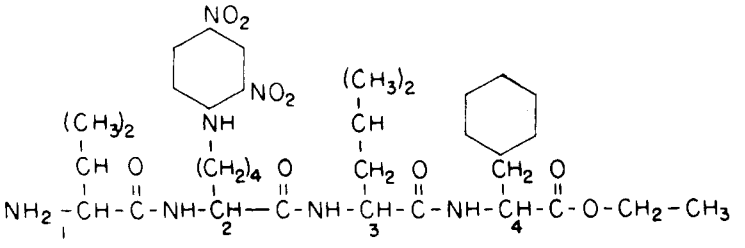
[1] The following abbreviations have been used in the text: OEt, ethyl ester; OMe, methyl ester; HSA, human serum albumin; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl; B-γ-G, bovine γ-globulin; Q_{max}, maximal per cent quench of antibody fluorescence; LT-A, LT-B, and LB-C are antibody preparations specific for the L-L-L-L DNP tetrapeptide; DT-A is an antibody preparation specific for the D-D-D-D tetrapeptide; PO_4–saline, pH 5.75, is 0.15 M NaCl–0.01 M phosphate, pH 5.75; BAW, butyl alcohol–acetic acid–H_2O; *sec*-BAM, *sec*-butyl alcohol–ammonia–H_2O; MEC, molar extinction coefficient; DMF, dimethylformamide; DCC, dicyclohexylcarbodiimide; CBZ, carbobenzyloxy.

[2] Amino acid residues are in the L configuration except where specified. Where a DNP-amino acid or peptide is coupled *via* its α-amino group to protein, the protein is listed first, *e.g.*, HSA-Val-ε-*N*-(DNP)-Lys-Leu-Phe-OEt. Amino acids and peptides coupled through the C-terminal amino acid are listed before the protein. Abbreviations of amino acids and blocking groups are according to standard nomenclature (Greenstein and Winitz, 1961). Peptide structural formulas and the Roman numerals by which peptides are designated in the text are given in Figure 1.



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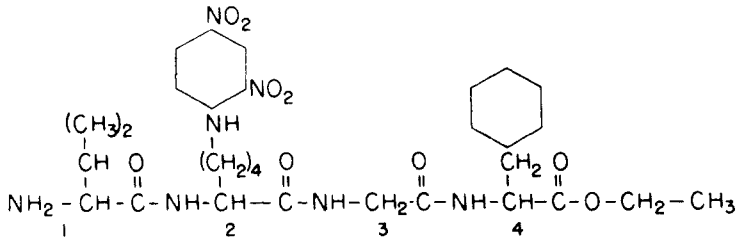

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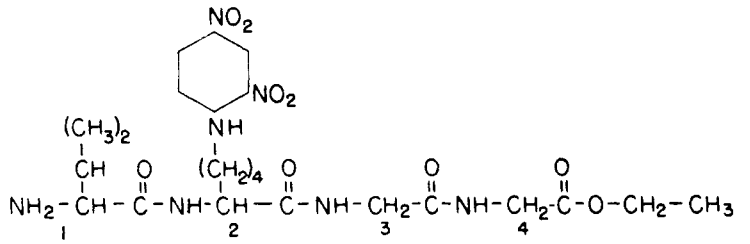

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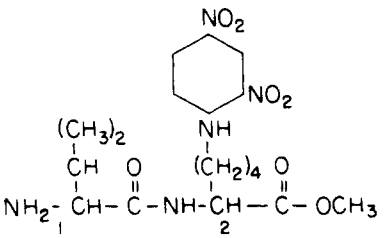

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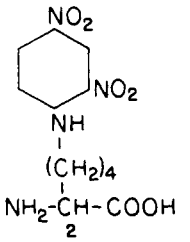

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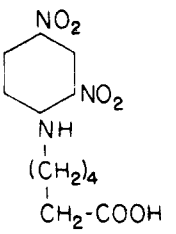

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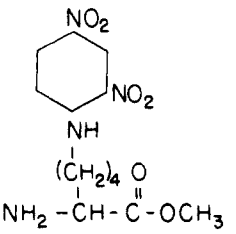

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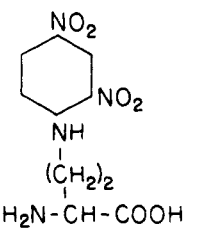

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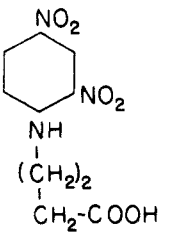

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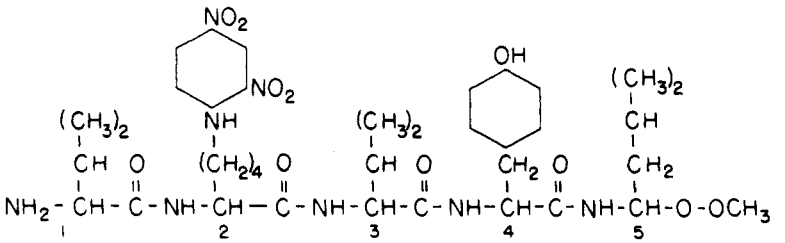

X

FIGURE 1: Structural formulas of various DNP derivatives. Derivatives I–X contain L-amino acids or lack an asymmetrical carbon. Derivatives XI and XII contain D-amino acids and have a methyl ester group at the C-terminal end rather than an ethyl ester.

bodies have been evaluated by fluorescence quenching and hapten inhibition of precipitation in regard to whether specificity was for the entire peptide or only the lysyl portion. It has been possible to demonstrate that about 50% of random bred rabbits makes antibodies with specificity for the entire peptide. Estimates of the relative contributions of various portions of the peptide to binding have been made.

Material and Methods

The syntheses of the peptides used in this study are described in the Experimental Section. Procedures for the preparation of derivatives V, VI, and XII are described in the literature (Porter and Sanger, 1948; Carsten and Eisen, 1953; Eisen and Siskind, 1964). Derivative IX was obtained commercially (Nutritional Biochemicals, Cleveland, Ohio). Derivative X will be described in a subsequent publication.

The DNP-lysyl tetrapeptide, Val-(DNP)-Lys-Leu-Phe-OET (I), was coupled in peptide linkage to carboxylate groups on human serum albumin (HSA) by means of a water-soluble carbodiimide (Goodfriend *et al.*, 1964; Permutt *et al.*, 1966). To a stirred solution of 100 mg of HSA (Pentex Inc., Kankakee, Ill.) in 50 ml of H_2O at pH 5.5 was added 60 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (EDC) (Ott Chemical Co., Muskegan, Mich.) followed immediately by the dropwise addition with stirring of 25 mg of the tetrapeptide I in 4 ml of dimethylformamide. After 10 min another 10 mg of EDC was added. Following incubation for 12 hr at room temperature the product was purified by prolonged dialysis *vs.* 0.001 M phosphate, pH 6.0, and concentrated by lyophilization. Several different preparations contained three to five DNP peptide groups per molecule of protein. Use of higher concentrations of protein or a greater percentage of organic solvent resulted in a partially insoluble product. The employment of a dilute protein during coupling is necessary in order to minimize formation of intermolecular peptide bonds between protein molecules. Even under the conditions described above small amounts of aggregated protein were formed. Other DNP-amino acids or peptide protein conjugates (see below) were prepared similarly.

Random bred white rabbits were immunized with 2 mg of the DNP tetrapeptide–HSA conjugate (HSA–Val-ε-DNP-Lys-Leu-Phe-OEt) or another DNP-antigen. The protein solution was emulsified with an equal volume of Bayol F–Arlacel (4:1) containing 2 mg/ml of *Mycobacteria butyricum*. Animals were given 0.4 ml of the emulsion in each footpad. Animals were reinjected with 1 mg of antigen in adjuvant at monthly intervals. Sera were obtained at 3 weeks and at 7–10 days after each boost. Hartley strain guinea pigs were immunized with 1.0 mg of the DNP tetrapeptide–HSA conjugate in Freund's adjuvant, 0.1 ml/footpad, and antisera obtained at various intervals after immunization. Rabbit and guinea pig sera containing significant amounts of precipitating antibody specific for the DNP group as judged by ring testing with DNP bovine γ-globulin (DNP-B-γ-G) were purified by the technique of Eisen (1964b). DNP-B-γ-G was used as the precipitating antigen in the purification. The DNP-B-γ-G was obtained by reaction of DNP sulfonate with B-γ-G and contained 42 DNP groups per molecule of protein. Yields of purified antibody ranged between 20 and 35%.

Fluorometric titrations were performed with an Aminco Bowman spectrofluorophotometer as described by Velick *et al.* (1960), Parker (1963), and Eisen (1964a). Fluorescent intensities in the figures are given in arbitrary units. Titrations routinely were carried out with 40 μg of purified antibody at 30°, usually at pH 5.75 because of relative peptide insolubility at pH 7.4. Previous studies have shown that binding of DNP ligands by antibody is not reduced at this pH (Velick *et al.*, 1960). Protein concentrations were determined by absorbance at 280 mμ using 1.5 as the value for a 1 mg/ml of solution of rabbit γ-globulin and 160,000 as the molecular weight of rabbit 7S γ-globulin. Antibodies to DNP-B-γ-G and DNP-HSA were taken to have a Q_{max} of 0.72 (Eisen and Siskind, 1964). The Q_{max} was determined for each preparation of rabbit antitetrapeptide antibody and ranged from 0.64 to 0.72. These values were based on titrations with high concentrations of ε-*N*-DNP-lysine and gave association constants which correlated well with the results of equilibrium dialysis with the DNP-pentapeptide X. Calculations were made with the aid of an IBM computer, Model 7072, as described by Eisen (1964a). The heterogeneity index, a, was obtained from the formula

$$\log \frac{r}{n - r} = a \log c + a \log K_0$$

where r is moles of hapten bound per mole of antibody, c is free hapten concentration, n is antibody valence (two), and K_0, the association constant, is the reciprocal of free hapten concentration where one-half of the antibody sites is occupied (Nisonoff and Pressman, 1958; Eisen, 1964a). Association constants were calculated using the last five points in the titration curve because of the difficulty in interpreting the early part of the quenching curve (see Discussion). Hapten inhibition of precipitation was carried out as described in Kabat and Mayer (1961), and the legend to Figure 4.

Equilibrium dialyses were carried out in phosphate–saline, pH 5.75, with 1 ml of antibody solution (20 μg/ml of antibody, 80 μg/ml of rabbit γ-globulin) inside the dialysis bag and 2 ml of outside solution containing the hapten. After equilibration (see legend to Table III) inside and outside solutions were counted in a well-type γ-counter to an average of 5000 counts over background. The average deviation of replicate tubes from their mean was ±4%. Two preparations of Val-ε-*N*-(DNP)-Lys-Val-Try-Leu-OET (X) were employed for dialysis studies. The pentapeptide was labeled with ^{125}I on the tyrosine residue by the method of Hunter and Greenwood (1962) and purified by chromatography on IRA 400. A trace-labeled product

(preparation A) had a specific activity of 1.3 mc/μmole (assuming 70% counting efficiency). Pentapeptide also was labeled with ^{125}I in the presence of an excess of cold iodide. This preparation (preparation B) had a specific activity of 32 mc/mmole and contained 1.92 I atoms per molecule of pentapeptide. The labeled peptides were radiochemically pure by paper chromatography.

Electrophoresis on cellulose acetate strips was carried out in barbital buffer, pH 8.6, as described by Kohn (1960). Immunoelectrophoresis was according to the procedure of Scheidegger (1955).

Results

Purified antibodies from 16 individual animals immunized with the DNP-lysyl tetrapeptide I have been examined by the method of fluorescence quenching. The DNP-lysyl tetrapeptide I quenched protein fluorescence to a greater degree than ε-*N*-DNP-lysine (V) in 9, indicating more effective binding of the tetrapeptide. An example of a fluorescence titration curve in which there was specificity for the peptide as well as DNP is shown in Figure 2A. Binding data of this antibody preparation with various haptens are given in Table I (antibody LT-A). The more effective binding of the tetrapeptide I in comparison with ε-DNP-L-lysine (V) may be due in small part to a negative effect of the charged α-amino group of ε-DNP-lysine on binding. The effect of charge here cannot be evaluated directly because the tetrapeptide I is insoluble in aqueous solution when its α-NH_2 group is uncharged. However, ε-DNP-aminocaproate (VI) which is lacking the α-amino group is bound more effectively than ε-DNP-lysine (V); the difference is even more striking between the corresponding γ-DNP-butyrate derivatives VIII and IX where the positive charge is closer to the DNP group. By way of contrast there was no apparent effect of the carboxylate charge on binding: the binding constants of ε-DNP-L-lysine and γ-DNP-diaminobutyrate (V and VIII) were indistinguishable from those of their methyl esters VII and the corresponding butyric derivative.

By comparing the binding of Val-ε-DNP-Lys-Leu-Phe-OET (I) and Val-ε-DNP-Lys-Gly-Gly-OET (III) it would appear that the phenylalanyl side chain increases the binding constant from about 1.2×10^7 to 1.0×10^8 at 30° (a contribution of about −1.3 kcal $mole^{-1}$ to the standard free energy of binding, $\Delta F°$). Contributions by valyl and leucyl side chains are less striking. The near equivalence of Val-ε-DNP-Lys-OMe (IV) and Val-ε-DNP-Lys-Gly-Gly-OET (III) indicates that the peptide backbone at positions 3 and 4 (numbered from the N-terminal end) has relatively little role in binding. The relatively small difference between the L and D forms of the tetrapeptides I and XI is in accord with the conclusion that the peptide backbone is relatively unimportant in binding (see also Eisen and Siskind, 1964).

The over-all titration pattern shown in Table I for LT-A is exhibited by other DNP tetrapeptide antibodies which bind I more effectively than V (Figure 2A). Given more effective quenching by I than V the other DNP peptides II–IV are bound to a greater extent than V and γ-DNP-aminobutyrate (IX) is bound to a greater extent than γ-DNP-diaminobutyrate (VIII). For DNP tetrapeptide antibodies which have a 2B titration pattern (7 of 16) the binding pattern is similar to that obtained with antibodies to DNP-B-γ-G (Table I, antibody LT-B as compared with anti-DNP-B-γ-G) Here in contrast to results obtained with antibody LT-A, V is bound to an equal or greater degree than the DNP peptides I–III and VIII is bound as well as IX.

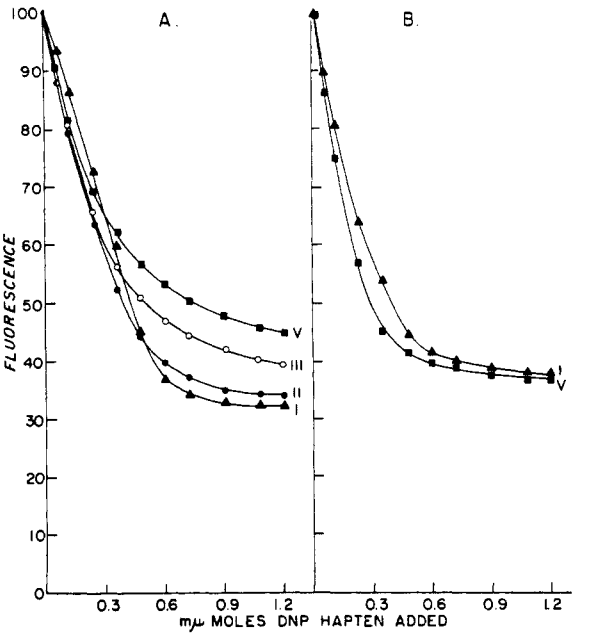

FIGURE 2: Fluorometric titrations of purified rabbit anti-DNP antibodies with ε-*N*-DNP-lysine and various DNP peptides (see Figure 1). (A) Antibody to HSA–Val-DNP-Lys-Leu-Phe-OEt obtained 8 weeks after immunization (preparation LT-A, see also Table I). (B) Antibody to DNP-B-γ-G obtained 8 weeks after immunization (preparation DNP-B-γ-G, Table I).

Purified antibodies from three animals immunized with HSA–Val-ε-DNP-Lys-Leu-Phe-OMe where the amino acids are in the D configuration were examined at 5 weeks after immunization. Two displayed little if any peptide specificity (type 2B pattern). The third had some degree of peptide specificity (Table I, DT-A). All three exhibited slightly more effective binding of the homologous D-D-D-D tetrapeptide (XI) than of the L-L-L-L tetrapeptide (for example, Table I, antibody DT-A, comparing I with XI). After immunization with the L-L-L-L tetrapeptide the converse was true, although again the difference was small (Table I, LT-A and LT-B).

Both the D- and the L-DNP tetrapeptide antibodies displayed charge heterogeneity similar in magnitude to other DNP antibodies on cellulose acetate electrophoresis and immunoelectrophoresis. Values for *a*,

TABLE I: Binding of Various DNP Derivatives by Purified Rabbit Antibodies.[a]

No.	Hapten	K_0 (l. mole^{-1} × 10^{-6})[e] LT-A[b]	LT-B[b]	Anti-DNP-B-γ-G[c]	DT-A[d]
I	Val-ε-DNP-Lys-Leu-Phe-OEt	110.0	50.0	80.0	5.7
II	Val-ε-DNP-Lys-Gly-Phe-OEt	100.0	150.0	200.0	
III	Val-ε-DNP-Lys-Gly-Gly-OEt	12.0	180.0	300.0	
IV	Val-ε-DNP-Lys-OMe	10.0	230.0	300.0	
V	ε-DNP-L-lysine	5.0	160.0	320.0	5.5
VI	ε-*N*-DNP-aminocaproate	7.0	180.0	350.0	
VII	ε-DNP-lysine-OMe	5.0	160.0	300.0	
VIII	γ-DNP-α-γ-diaminobutyrate	0.6	19.0	110.0	
IX	γ-DNP-γ-aminobutyrate	6.0	20.0	100.0	
X	Val-ε-DNP-Lys-Val-Tyr-Leu-OMe	20.0	50.0	60.0	
XI	D-Val-ε-DNP-D-Lys-D-Leu-D-Phe-OMe	80.0		70.0	9.2
XII	ε-DNP-D-lysine	4.5	150.0	300.0	6.0

[a] Fluorometric titrations were carried out in 0.15 M NaCl–0.01 M phosphate, pH 5.75, at 30°. [b] LT-A and LT-B are purified rabbit antibodies from individual animals immunized with HSA–Val-DNP-Lys-Leu-Phe-OEt (L-L-L-L). The fluorometric titration curve of LT-A is shown in Figure 2A. [c] The anti-DNP-B-γ-G antibody was purified from a pool of rabbit antisera to DNP-B-γ-G. The LT-A, LT-B, and anti-DNP B-γ-G antisera were obtained 8–9 weeks after immunization, after a total of 4 mg of antigen in Freund's adjuvant. [d] DT-A is purified antibody from an antiserum to HSA–Val-DNP-Lys-Leu-Phe-OMe (D-D-D-D) obtained at 5 weeks after a total of 3 mg of antigen, exhibiting a small degree of peptide specificity. [e] Values for the average association constant, K_0, have been calculated using points on the titration curve at 0.454, 0.64, 0.78, 0.915, and 1.0 mμM total hapten concentration.

the binding heterogeneity index in the Sips equation, appeared to cover as broad a range as with antibodies to DNP-B-γ-G, indicating substantial binding heterogeneity.

DNP-specific purified antibodies (38) (seven to DNP-B-γ-G, three to DNP-HSA, nine to HSA–*S*-DNP-glutathione dimethyl ester, five to α-DNP-valine–HSA, six to HSA–Gly-ε-DNP-Lys-Gly-Gly ethyl ester, five to *N*-acetyl-*S*-DNP-cysteine–HSA, and 3 to HSA–ε-DNP-lysine methyl ester (C. W. Parker, manuscript in preparation) have been used as controls for the specificity of the fluorescence quenching pattern seen in Figure 2A. With none of these antibodies has the DNP tetrapeptide I produced better quenching than ε-DNP-lysine (V) and other DNP peptides. In Figure 2B a representative titration with antibody to DNP-B-γ-G is shown. Control fluorometric titrations with purified rabbit pseudoglobulin (DEAE fractionation) indicated that the haptens I–V (Figure 1) were bound only slightly and to an equivalent extent by "normal" globulin.

The possibility had to be considered that small amounts of antibodies specific for non-DNP portions of tetrapeptide (but not specific for DNP) might be contaminating the purified antibodies. An impurity of this type might conceivably convert a titration pattern from 2B to 2A. This possibility has been excluded by three control experiments. First, an antibody with a type 2A titration curve (preparation LT-A) was purified a second time by precipitation with DNP-B-γ-G and elution with hapten. The titration curve of the product was almost identical with that of the once purified material (Figure 3A). A second control was to mix antisera to DNP-B-γ-G and to HSA–Val-Gly-Leu-Phe-OEt (1 mg of peptide-specific antibody/ml after mixing the sera) (C. W. Parker, manuscript in preparation) and purify for DNP-specific antibody. A type 2B titration pattern was observed indicating that antibodies with specificity for the peptide were not carried through the purification or were not quenched by the DNP-lysyl tetrapeptide I (Figure 3B). As further evidence that non-DNP antibodies are essentially eliminated during the purification, purification of anti-DNP antibody was carried out in the presence of rabbit antibodies to the *p*-toluenesulfonyl (tosyl) group. After the two sera were mixed, the antitosyl antibody had a concentration of 0.6 mg/ml. The purified anti-DNP antibody obtained failed to precipitate with tosyl HSA at an antibody concentration of 2.5 mg/ml. Moreover, similar titration curves were obtained with ε-*N*-DNP-lysine (V), the DNP-lysyl tetrapeptide I, and Val-ε-DNP-lysyl-Val-tosyllysine methyl ester (C. W. Parker, manuscript in preparation).

Changes in the titration pattern over the time range from 3 weeks to 3 months in the rabbit have not been striking. There was a rise in average antibody affinity with time after primary immunization. Antibodies with

TABLE II: Fluorometric Titration of Antibodies to the DNP Tetrapeptide I Changes in Antibody Affinity with Time.[a]

Antibody Prepn	Time after Immunizn (weeks)	ε-DNP-lysine (V)		DNP Tetrapeptide I		Ratio $\Delta F°$ Peptide I/$\Delta F°$ DNP-lysine
		$K_0 \times 10^{-6}$ (mole^{-1})	$-\Delta F°$ (kcal mole^{-1})	$K_0 \times 10^{-6}$ (l. mole^{-1})	$-\Delta F°$ (kcal mole^{-1})	
LT-A	4.5	3.6	9.1	16.0	10.0	1.10
	9	5.0	9.3	110.0	11.1	1.19
LT-D	4.5	1.2	8.4	2.6	8.9	1.06
	9	10.0	9.7	40.0	10.5	1.08
LT-E	4	4.6	9.2	15.0	9.9	1.08
	12	34.0	10.4	110.0	11.1	1.07
LT-F	4	4.2	9.2	3.6	9.1	0.99
	12	20.0	10.1	15.0	9.9	0.97

[a] Antibodies to HSA–Val-DNP-Lys-Leu-Phe-OEt were obtained at the indicated times and purified as described in the text. Fluorometric titrations were carried out at 30° in phosphate–saline, pH 5.75.

TABLE III: Equilibrium Dialysis of Antibody to the DNP-Lysyl Tetrapeptide with the DNP Pentapeptide X.[a]

Antibody Sites (mμmoles/ml × 10^6)	Bound Hapten (mμmoles/ml × 10^6)	Free Hapten (mμmoles/ml × 10^6)	K_0 (l. mole^{-1} × 10^{-6})
0.32	0.012	0.012	3.2
0.33	0.041	0.025	5.7
0.34	0.09	0.025	14.0
0.32	0.21	0.10	19.0
0.34	0.24	0.22	11.0
0.36	0.31	0.47	13.0

[a] The binding of Val-(ε-DNP)-Lys-Val-Tyr-Leu-OMe (X) (trace labeled with ^{125}I) (see text) by antibody to the DNP-lysyl tetrapeptide (I) (see fluorometric titration in Figure 2A). Dialysis was for 16 hr in 0.15 M NaCl–0.01 M PO_4, pH 5.75, at 31° and 1 rpm. Controls established that equilibrium had been reached and that there was no nonspecific binding to rabbit γ-globulin. Final antibody concentrations are corrected for volume changes of the inside solution during dialysis as determined by the weight of the bag contents.

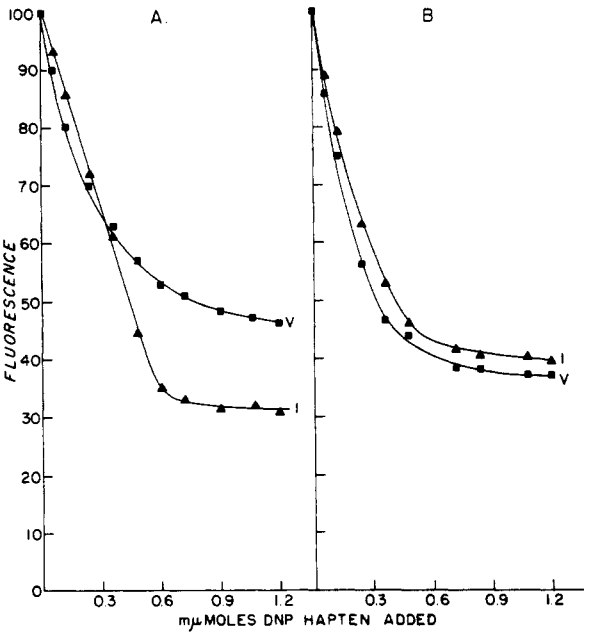

FIGURE 3: Fluorometric titrations. (A) Fluorometric titration of repurified rabbit anti-DNP tetrapeptide antibody. Compare with the titration pattern of the IX purified antibody (Figure 2A). (B) Fluorometric titration of rabbit anti-DNP B-γ-G antibody, purified in the presence of antibody to Val-Gly-Leu-Phe-OEt. Compare with the titration pattern in Figure 2B (the same antibody purified in the absence of the antitetrapeptide antibody).

no peptide specificity at 3–4 weeks did not display peptide specificity at a later time. Antibodies with peptide specificity showed a variable change with time in the relative contribution of the peptide, as compared with the DNP-aminoalkyl moiety. Four examples are shown in Table II.

The results of hapten inhibition of precipitation are shown in Figure 4. Higher peptide concentrations were not used because of limited solubility. An antibody with a tetrapeptide specificity pattern (see Figure 2A and Table I, LT-A) is shown in Figure 4A. At a 1×10^{-5} M hapten concentration, the order of inhibition is I > II > III > IV > V. This result was entirely consistent with the results of the fluorometric titration. To carry the analogy with the fluorometric titration still further, at low concentrations of the DNP-lysyl

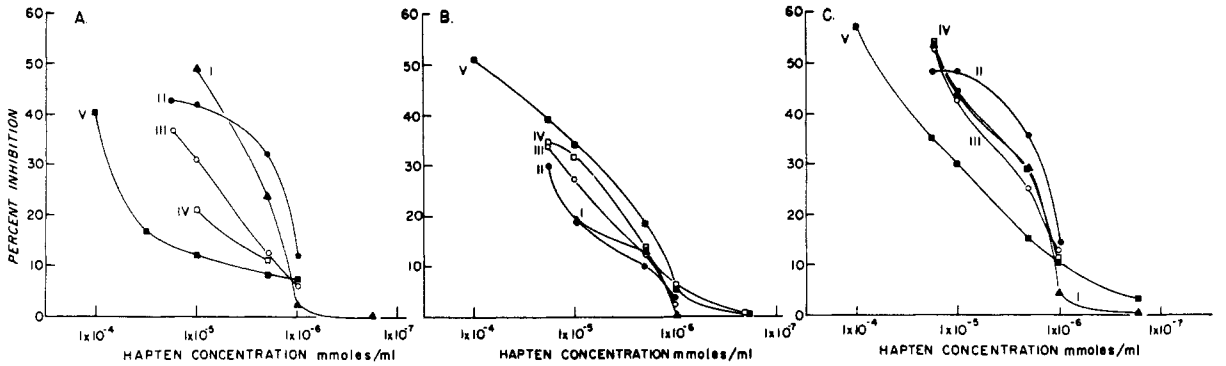

FIGURE 4: Hapten inhibition of precipitation. Precipitation was carried out at equivalence in duplicate with 90 μg of purified antibody/tube and 9 μg of DNP_{42}-B-γ-G, each in phosphate–saline, pH 5.75. After incubation at 37° for 1 hr and at 4° for 18 hr, precipitates were washed two times with cold saline and read on the spectrophotometer at 280 and 360 mμ in 0.4 ml of 0.5% sodium lauryl sulfate in water (the average deviation of the 280 and 340 readings of duplicates from their mean was 1.5%). Curve A, a pool of two purified rabbit antibodies to the DNP-lysyl tetrapeptide exhibiting considerable peptide specificity (fluorometric titration pattern 2A). Curve B, from a purified rabbit antibody to DNP-B-γ-G (fluorometric titration pattern 2B). Curve C, from a purified rabbit antibody to the DNP-lysyl tetrapeptide with "partial peptide specificity."

tetrapeptide I (1×10^{-6} M), it inhibited less effectively than the other haptens including ϵ-DNP-lysine (compare with early part of curve in Figure 2A). The order of effectiveness with antibodies to DNP-B-γ-G (peptide absent) was reversed (Figure 4B). Similar hapten inhibition patterns were obtained with antibodies to two other DNP proteins (peptide absent). Antibody LT-C which had a fluorometric titration and hapten inhibition of precipitation pattern intermediate between 2A and 2B ("partial peptide specificity") is shown in Figure 4C.

The results of equilibrium dialysis with ^{125}I-labeled pentapeptide X (preparation A) and tetrapeptide specific antibody (antibody LT-A) (Table I) are shown in Table III. The association constant at relatively high hapten concentrations was about 1.6×10^7 l. mole^{-1}, very similar to values obtained at corresponding points in the fluorometric titrations (about 2×10^7 l. mole^{-1}).

In two separate equilibrium dialyses, the association constants increased significantly (six- to eightfold) as the ratio of hapten to antibody increased. The effect of iodine substitution on the tyrosine of the DNP pentapeptide on pentapeptide binding by antibody was evaluated with a pentapeptide which had been labeled in the presence of excess cold iodine (preparation B). Fluorometric titration of preparation B with the above antibody gave binding constants similar to those obtained with the uniodinated peptide. The results of equilibrium dialysis with preparations A and B correlated well at low hapten concentrations (values of K_0 in the range of $2–8 \times 10^6$ l. mole^{-1}). Equilibrium dialysis at higher concentrations of preparation B could not be interpreted because of very slow equilibration. Thus binding of the iodinated pentapeptide appeared to be comparable to that of the unlabeled peptide, at least with the antibody prepar a tion studied in greatest detail (LT-A, Table I).

The results of immunization with the DNP-lysyl tetrapeptide in mixed Hartley strain guinea pigs were qualitatively similar to those in the rabbits. Out of 10 guinea pig antibodies studied, three showed evidence of peptide specificity. In the three the average contribution of the phenylalanine side chain to binding was considerably less striking than in the rabbits ($\Delta F°$ an average of -0.15 kcal mole^{-1} at 30° for antibodies obtained at 11 days). This contribution was no longer demonstrable with antibody obtained at 5 weeks in one but remained at about the same level in the other two. The binding of the butyric haptens displayed a similar pattern to that in the rabbits. γ-DNP-diaminobutyrate (VIII) was bound less well than γ-DNP-butyrate (IX) where the DNP tetrapeptide I was bound better than ϵ-*N*-DNP-lysine (V).

Discussion

Studies using hapten containing peptides of known sequence as antigens were carried out by Landsteiner and Van der Scheer many years ago (1932, 1934, 1939). Two- to five-membered peptides containing leucine and glycine in various combinations and an N-terminal *p*-aminobenzoyl group were diazotized and coupled to proteins. The results of these studies clearly indicated that short peptides could produce antibodies with a high degree of specificity for the homologous peptide. While antisera undoubtedly contained both antihapten and antipeptide specificities, effects due to an antibody with a combined specificity could not be distinguished from effects due to independent contributions by two different antibodies. In the present study antibodies with DNP specificity have been obtained in highly

purified form and examined for the ability to bind non-DNP portions of the immunizing peptide. The results clearly indicate that antibodies with a combined specificity have been formed in a substantial proportion of the immunized animals. Similar results have been obtained by Eisen *et al.* (1964) with antibodies to ε-DNP-lysine$_{41}$ ribonuclease and by Counts and Little (1966) with mono-DNP-lysyl-insulin.

Published estimates as to the size of an antigenic site have been largely obtained with a series of linear antigens including the dextrans (Kabat, 1960) and polyalanyl and polylysyl proteins (Sage *et al.*, 1964); Schechter and Sela, 1965). Present indications are that an antibody site can encompass at least as many as six to seven isomaltose residues or four to five amino acid residues. Estimates from studies with proteins such as ribonuclease (Brown, 1962) and silk fibroin (Cebra, 1961) raise the possibility that an antigenic site may be even larger. The DNP-peptide differs from oligosaccharide and polyamino acid antigens in that it contains an especially potent antigenic group (the DNP moiety) which might completely dominate antibody specificity. While the present experiments indicate that antibody specificity can extend beyond the DNP group they do not establish limits as to the size of a DNP peptide antigenic site. Although the present approach could be extended to larger units, unless charged groups were introduced into the peptide, solubility would become a serious problem. On the other hand, charged groups might participate in side reactions during coupling.

The particular DNP tetrapeptide used in these studies was selected in part because it contains several nonpolar side chains. There is now considerable evidence to support the view that hydrophobic interactions are very important in the stabilization of protein structure. (See, for example, Scheraga *et al.*, 1962.) Recently it has been suggested that antigen–antibody interactions of high affinity might involve hydrophobic bond formation between nonpolar groups on the antibody and the antigen (Karush and Eisen, 1962). On the basis of comparative results of fluorometric titration with the homologous hapten I and the tetrapeptides Val-ε-(DNP)-Lys-Gly-Gly-OEt (III) and Val-ε-DNP-Lys-Gly-Phe-OEt (II) (Table I) it would appear that the nonpolar side chain of phenylalanine can make a contribution of approximately 1.3 kcal/mole to the standard free energy for antibody binding at 30°. While this value should be regarded as provisional, the results of hapten inhibition of precipitation (Figure 4A) also indicate that the phenylmethylene side chain makes a substantial contribution to binding. Using statistical thermodynamic theory, Némethy and Scheraga (1962) have estimated the standard free energy change for the formation of hydrophobic bonds between various combinations of nonpolar amino acid side chains on proteins. According to these calculations, if the phenylalanyl side chain of the DNP peptide were optimally fitted to a nonpolar amino acid in the antibody site, a contribution to binding of as much as 1.4 kcal/mole at 25° might be anticipated. While it is not established that the contribution of the phenylalanyl side chain to antibody binding is due to hydrophobic bond formation, present estimates of the magnitude of the contribution are consistent with this possibility.

Despite the contribution of remote side chains to DNP binding there is no clear evidence that antibodies with a combined specificity have a uniquely high affinity of interaction with the peptide. Such antibodies seem to have a relatively low affinity for ε-DNP-lysine itself whereas antibodies with no peptide specificity have a relatively high affinity for ε-DNP-lysine. Thus the over-all affinity for the DNP tetrapeptide is about the same regardless of whether the adaptation is to ε-DNP-lysine alone or to the entire peptide.

It might be argued that the highly effective binding of the DNP tetrapeptide I by homologous antibody is in part nonspecific due to nonpolar amino acid residues in the antibody site. With this possibility in mind 38 purified antibodies with specificity for various other DNP antigens have been examined and in no instance has the tetrapeptide I been bound more effectively than ε-*N*-DNP-lysine (V). Moreover, the extent of nonspecific interaction of the various DNP peptides with "normal" rabbit γ-globulin is comparable to that of ε-*N*-DNP-lysine, as judged by fluorometric titration.

It has been established by Eisen and Siskind (1964) that the affinity of ε-*N*-DNP-lysine for antibody specific for the DNP moiety of DNP-B-γ-G rises progressively after immunization. In the present study the antibodies from animals immunized with the DNP-lysyl tetrapeptide I also have undergone increases in affinity with time, both for DNP-lysine V and the tetrapeptide I. Over the time range studied (3 weeks to 3 months in the rabbit, 11 days to 6 weeks in the guinea pig), the average contribution of the DNP moiety relative to the remainder of the peptide has not undergone any major change. In no instance has a pattern of no peptide specificity (2B) in the first bleeding evolved to a 2A type pattern. Thus the observed pattern has been more characteristic of the animal than of the duration of immunization. Presumably these differences among animals reflect genetic variations and efforts are in progress to substantiate this possibility.

An interesting aspect of the fluorescence quenching curve in titrations of anti-DNP antibody with the DNP tetrapeptide I is the relatively poor quenching at low hapten concentrations (Figure 2A). This effect was especially evident with two of the nine preparations of antibody which have peptide as well as DNP specificity. It was seen to a lesser extent or not at all with other DNP tetrapeptides, to some extent with the DNP pentapeptide, but was absent with ε-*N*-DNP-lysine. One possible explanation would be heterogeneity of the DNP antibodies with respect to their binding affinity and quenching maximum (Q_{max}). One population of antibody molecules with a relatively high Q_{max} would be as well or better adapted to ε-DNP-

lysine than to the tetrapeptide; the other population with a low Q_{max} would bind ε-DNP-lysine relatively ineffectively but would bind the tetrapeptide well. The second population would be titrated late by ε-DNP-lysine and early by the tetrapeptide. If heterogeneity of this kind were to produce the quenching anomaly one might be able to separate the antibody into populations with high and low Q_{max} values. Attempts have been made to fractionate the antibody by absorption with soluble and insoluble antigens and examining the antibody remaining in the supernatant. While experiments to date have been negative the possibility that heterogeneity of this type exists and accounts for the quenching curve is not excluded.

An alternative explanation (and the one that we favor) is that this phenomenon is not a quenching artifact but is an indication of poor binding of the tetrapeptide in the early part of the curve.[3] In support of this possibility hapten inhibition of precipitation and equilibrium dialysis also indicates weak binding of the tetrapeptide (or pentapeptide) at low ratios of hapten to antibody (Figure 4, Table III). Assuming the second explanation is correct the calculated association constants undergo an absolute rise both in equilibrium dialysis and fluorometric titrations as the hapten concentration increases (in the latter case from 8×10^6 to 1×10^8 l. mole^{-1}). Antibody heterogeneity as such does not provide an explanation for this increase. Antibody sites with relatively high affinity should be titrated first causing a fall in the average K_0 at high hapten concentrations and values of a, the heterogeneity index, which are <1. However, with antibody preparation LT-A the value for a is ≥ 1.5. To explain the variation in K_0 and the high values for a, it is possible to invoke cooperative binding effects, a subject recently discussed in detail by Weber (1965) and Weber and Anderson (1965). Let us suppose that the interaction of the DNP tetrapeptide with DNP tetrapeptide antibody were to produce a configurational change in the antibody molecule which favored binding of the peptide. At low hapten concentrations the majority of the antibody molecules would be in their usual configuration. At relatively high hapten concentrations the majority of the antibody molecules would be in the tautomeric form that favors binding. Following dissociation of a hapten molecule from the antibody site the altered antibody configuration would persist for a short time favoring binding of a second hapten molecule. The over-all result would be an increase in the association constant as the ratio of DNP peptide to antibody is increased.

While we have no direct evidence that the configuration of DNP antibody is altered by its interaction with DNP peptides, recent work from other laboratories with antibodies of other specificities supports this possibility. For example, Grossberg *et al.* (1965) have found that bivalent and univalent antibodies are less susceptible to proteolysis by chymotrypsin in the presence of specific hapten than in its absence. Feinstein and Rowe (1965) have been able to observe physical changes in antibody structure in the presence of antigen by electron microscopy but it is not clear that univalent hapten would produce the same effect. The possibility that the DNP-tetrapeptide is bound weakly by antibodies in their native configuration could be evaluated further by kinetic analysis. Day *et al.* (1963) have found that the association between ε-*N*-DNP-lysine and anti-DNP antibody is extremely rapid and the energy of activation very low. If the above interpretation is correct the kinetic curves for the combination of the DNP tetrapeptide with antibody should differ substantially from results obtained with ε-*N*-DNP-lysine.

In the L-DNP-lysyl tetrapeptide, the amino acid sequence immediately around the DNP group is specified. Despite this the purified antibodies examined to date have been heterogeneous in regard to electrophoretic mobility and hapten binding. The demonstration of heterogeneity by antibodies from a single animal in the extent to which antibodies are adapted to ε-DNP-lysine and the DNP tetrapeptide is in accord with the results of Eisen and Little and their colleagues with various DNP antigens including monosubstituted proteins and polylysines (Eisen *et al.*, 1964; Counts and Little, 1966). The observations of Schlossman and Kabat (1962) with human antibodies specific for dextran and of Nisonoff and Pressman (1958) with antibodies to azobenzenes further attest to the fact that antibody heterogeneity is a characteristic feature of the immune response. The former authors found that the antidextran antibodies from single subjects fell into two populations with differing relative affinities for smaller *vs.* larger oligosaccharides of the isomaltose series.

The basis for the heterogeneity of the antibody response to the DNP tetrapeptide is not clear. One possibility would be a degradation of the peptide within lymphoid cells leading to an antibody response to DNP-lysine alone, and to various DNP di- and tripeptides as well as the tetrapeptide. Under such circumstances the

[3] Still another possibility would be that the small degree of quenching observed at low hapten concentrations is due to aggregation of hydrophobic tetra- and pentapeptide molecules. Suppose, for example, that dimers formed at low hapten concentrations and trimers at higher concentrations. Only one of two DNP groups might be available for binding in the dimer and two of three in the trimer. As a result quenching efficiency would increase with increasing hapten concentration. However, we seriously doubt that aggregation of hapten has occurred under the titration conditions employed. The coefficient for the partition of ^{125}I-labeled DNP pentapeptide X between ethyl acetate and phosphate–saline, pH 5.75, is constant over a range of hapten concentrations from 6×10^{-6} to 2×10^{-3} M, the region covered in the antibody studies. Moreover, the degree of quenching at low hapten concentrations varies with the antibody preparation. The same hapten solution will quench the fluorescence of one antibody preparation as effectively as ε-DNP-lysine whereas inefficient quenching is observed with another antibody. If aggregation were involved there would be inefficient quenching early in the titration with every antibody preparation. Since the DNP peptides can and often do quench very effectively (*e.g.*, to an extent that one must assume that >90% of the ligand is bound) it seems unlikely that a significant proportion of hapten molecules are sterically inaccessible to antibody.

TABLE IV: Elemental Analyses.

	Formula	Calcd (%) C	H	Br	N	Found (%) C	H	Br	N	Mp (°C)	% Yield
I	$C_{34}H_{50}N_7O_9Br_1$	52.31	6.41	10.26	12.56	52.32	6.40	10.09	12.36	255–256	80
a	$C_{26}H_{34}N_5O_9$	55.8	5.9		12.5	56.13	6.00		12.31	113	76
b	$C_{25}H_{34}N_7O_8$	53.7	5.9		17.5	54.1	5.96		17.81	157–160	81
c	$C_{42}H_{56}N_7O_{11}$	60.5	6.72		11.73	60.83	7.08		12.06	185–187	54
II	$C_{30}H_{42}N_7O_9Br_1$			11.05	13.54			11.11	13.46	228–230	90
a	$C_{38}H_{47}N_7O_{11}$	58.69	6.05		12.61	58.79	6.03		12.80	130–133	54
III	$C_{23}H_{36}N_7O_9Br_1$			12.62	15.46			12.68	15.43	188–191	85
a	$C_{31}H_{41}N_7O_{11}$	54.15	5.97		14.26	54.12	5.94		13.98	210–211	71
IV	$C_{18}H_{28}N_5O_7Br_1$	42.69	5.53	15.81	13.83	42.49	5.70	15.62	13.74	217	80
VIII	$C_{10}H_{12}N_4O_6 \cdot HCl$	37.5	4.22		17.5	37.44	4.41		17.55	251–253	70
XI	$C_{33}H_{48}N_7O_9Br_1$	51.7	6.26	10.44	12.8	51.2	6.25	10.75	12.45	233–235	90
a	$C_{26}H_{34}N_5O_9$	55.8	5.9		12.5	56.01	6.13		12.66	105–108	50
b	$C_{25}H_{34}N_7O_8$				17.5				17.17	158–160	80
c	$C_{41}H_{54}N_7O_{11}$	60.0	6.59		11.95	60.07	6.48		12.10	175–178	67
d	$C_{24}H_{30}N_2O_5$	67.6	7.03		6.8	67.55	7.17		6.77	86–87	72

variation in antibody response between animals (Table I, LT-A compared with LT-B) could reflect a varying ability to break down the antigen. The DNP D-D-D-D tetrapeptide was used in this study because it would be expected to be far less susceptible to enzymatic cleavage than the corresponding L-L-L-L tetrapeptide. Studies with other peptides composed of D-amino acids indicate that they can be broken down to smaller units in the rabbit but the rate of degradation is very slow by comparison with corresponding peptides in the L configuration (Gill *et al.*, 1965). While our studies with the D tetrapeptide have been limited, it is evident that it induces DNP-specific antibodies with a variable degree of peptide specificity. Moreover, these antibodies appear to exhibit the same degree of binding and electrophoretic heterogeneity shown by those to the L tetrapeptide. These results suggest that antigenic breakdown is not a necessary condition for the production of a heterogeneous antibody response. Following a suggestion by Singer (1964), antibody heterogeneity may be due at least in part to the sequestration of portions of nonpolar tetrapeptide molecules in hydrophobic regions of the proteins. Whatever the explanation it is apparent that antibody response to DNP-lysyl residues can be influenced by contiguous amino acids. It follows that one aspect of heterogeneity of antihapten antibodies is heterogeneity of antigen, even where the hapten is substituted on a single kind of amino acid on a protein. Further studies with hapten-substituted peptides or with proteins monosubstituted in a specified area should help to clarify further the antigenic interrelationships between the hapten and the polypeptide carrier. There is no clear indication at present, however, that antigens of this type will provide a means of preparing unusually homogeneous antibody fractions for studies on antibody structure.

Experimental Section

Reagent grade chemicals were used throughout. Amino acids and peptides used for synthesis were obtained commercially or prepared according to procedures in the literature and were checked for optical rotation, melting point, and chromatographic purity. Melting points were obtained with a Fisher-Johns hot stage. Elemental analyses were carried out by Microtech Laboratories, Skokie, Ill., and Schwarzkopf Microanalytical Laboratory, New York, N. Y. R_F values were obtained by ascending chromatography. Chromatographic solvents were butyl alcohol–acetic acid–H_2O (60:15:25) (BAW) and *cis*-butyl alcohol–ammonia–H_2O (100:4:40) (*sec*-BAm). Analytical values are given in Tables IV and V.

N-CBZ-L-valyl-ε-DNP-L-lysine Methyl Ester (Ia). L-DNP-lysine methyl ester·HCl (4 mM) (1 g) was stirred with 50 ml of acetonitrile and 0.56 ml of triethylamine for 10 min at room temperature; 1.01 g of *N*-CBZ-L-valine (4 mM) was added and the resulting suspension was cooled to −3° in an ice–salt bath. DCC (1.04 g) dissolved in 4 ml of acetonitrile was added with stirring. The mixture was stirred for 6 hr at −5°, 16 hr at 4°, and 2 hr at room temperature. Glacial acetic acid (2 drops) was added and the mixture was filtered after 15 min. The precipitate was washed with three 8-ml portions of hot acetone. The combined filtrate and washings were taken to dryness. The residue was taken up in ethyl acetate and extracted with 1 N HCl, 1 M $NaHCO_3$, and water. The ethyl acetate solution was dried over Na_2SO_4 and taken to dryness *in vacuo*. The residue was crystallized from hot ethyl acetate. For analysis, a sample was recrystallized from ethyl acetate.

N-CBZ-L-valyl-DNP-L-lysine Hydrazide (Ib). N-

TABLE V: Chromatographic, Polarimetric, and Spectrophotometric Analyses.

	α_D (deg)				R_F		MEC	
	Temp	Solvent	Sp Rotation	Concn (g%)	BAW	*sec*-But–NH_3	DMF–EtOH (1:12) (350 mμ)	PO_4–saline (pH 5.75) (360 mμ)
I	24	DMF	−0.6	1.1	0.98	0.94	17,900	15,900
a	23.5	DMF	+0.5	1.0	0.98	0.98	17,200	
b	23.5	DMF	−11.8	2.4	0.98	0.96	17,900	
c	25	DMF	−15.3	0.8	0.98	0.96	18,700	
II	24.5	DMF	+14.6	0.7	0.89	0.96	18,300	17,200
a	24	DMF	−2.7	1.5	0.97	0.95	18,300	
III	25	DMF	+13.5	0.7	0.90	0.84	18,000	17,500
a	24	DMF	−4.57	1.3	0.98	0.92	18,150	
IV	24	DMF	+21.2	0.4	0.92	0.85	17,600	17,800
XIa	24	DMF	+5.5	0.7	0.95	0.97	17,500	
XI	24.0	DMF	+6.3	0.7	0.91	0.97	18,300	15,900
b	24	DMF	+14.7	0.7	0.95	0.96	17,400	
c	24	DMF	+21.6	0.7	0.96	0.96	18,200	
d	24.5	DMF	+17.0	0.7				

CBZ-L-valyl-DNP-lysyl methyl ester (Ia, 9.6 g) was dissolved in 375 ml of methanol with heating. The solution was filtered and 8 ml of anhydrous hydrazine was added. The solution was refluxed for 1 hr and then incubated at room temperature for 24 hr. The crystals which formed were stirred with additional methanol and water overnight in the cold, filtered, and washed with water, methanol, and ether.

*N-CBZ-*L*-Val-DNP-*L*-Lys-*L*-Leu-*L*-Phe-OEt* (*Ic*). DNP dipeptide hydrazide (1b, 1 mM, 559 mg) was dissolved in 12.5 ml of 1 *N* HCl, 11 ml of water, 22.5 ml of acetic acid, and a few drops of ethyl acetate. The solution was cooled to −10° and 1.5 ml of a 56 mg/ml of solution of sodium nitrite in water was added. After an additional 10 min, 150 ml of ice water was added. The precipitate which formed was extracted into 75 ml of ice-cold ethyl acetate. The organic layer was extracted two times with 10 ml of ice water, two times with 10 ml of 1 M $NaHCO_3$, and two times with 10 ml of ice water. The material was dried quickly over magnesium sulfate and taken to dryness at 0° *in vacuo*. The residue was dissolved in 8 ml of ice-cold dimethylformamide and added to 320 mg of L-Leu-L-Phe-OEt (1.05 mM). The latter had been freshly prepared from the hydrochloride by neutralization of a concentrated aqueous solution with ammonium hydroxide followed by extraction into ethyl acetate and evaporation to dryness. The reaction mixture was allowed to incubate for 48 hr at 4° and at room temperature for 6 hr. Cold water (225 ml) was added. After 10 min, the suspension was filtered and the precipitate was washed with 1 N HCl, water, 1 M $NaHCO_3$, and water. The precipitate was dissolved in 5 ml of hot dioxane and crystallized by the addition of 10 ml of 95% ethanol and 5 ml of water. The crystals were washed once with water, two times with 10 ml of cold 95% ethanol and three times with 20 ml of cold ether.

*HBr·*L*-Val-DNP-*L*-Lys-*L*-Leu-*L*-Phe-OEt* (*I*). Finely ground α-CBZ-DNP-lysyl tetrapeptide ethyl ester (Ic, 300 mg) was treated with 1.5 ml of 33% HBr in glacial acetic acid with stirring at room temperature. After 20 min, the product was precipitated by the addition of ether and washed by centrifugation four times with ether. For analysis a sample was recrystallized from hot 95% ethanol.

*N-CBZ-*L*-Val-DNP-*L*-Lys-glycyl-*L*-phenylalanine Ethyl Ester* (*IIa*). The free base of glycylphenylalanine ethyl ester was treated with DNP-lysyl dipeptide azide, prepared from 0.320 mM of the hydrazide Ib in a reaction volume of 2.7 ml of dimethylformamide. The reaction mixture was incubated for 48 hr at 4° and 6 hr at room temperature. The reaction mixture was taken to dryness. The residue was taken up in 95% ethanol and precipitated by the addition of 0.4 volume of water. For analysis a sample was recrystallized from 60% ethanol–water.

HBr·L-Val-DNP-L-Lys-Gly-L-Phe-OEt (II) was prepared from IIa by the action of hydrogen bromide in anhydrous acetic acid as described for derivative I. The product was crystallized from ethanol–ether.

N-CBZ-Val-DNP-Lys-Gly-Gly-OEt (*IIIa*). Glycylglycine ethyl ester free base (130 mg, 0.81 mM freshly prepared from the hydrochloride) was treated with *N*-CBZ-Val-DNP-Lys azide, freshly prepared from 410 mg of the hydrazide Ib in a volume of 6.2 ml of dimethylformamide. After 2 days at 4° and 12 hr at room temperature, the product was crystallized by the addition of equal volumes of 95% ethanol and ether. The product was filtered and washed with dimethylformamide–ethanol–ether (1:1:1) and with

ether. For analysis a sample was recrystallized from dimethylformamide–ethanol–ether.

HBr·L-Val-DNP-L-Lys-Gly-Gly-OEt (III) was prepared from IIIa by the action of hydrogen bromide in anhydrous acetic acid as described for derivative I. The product was recrystallized from ethanol ether.

HBr·L-Val-DNP-L-Lys-OMe (IV) was prepared from Ia by treatment with hydrogen bromide in anhydrous acetic acid as described for derivative I. The product was recrystallized from methanol ether.

γ-DNP-α-γ-L-diaminobutyric Acid·HCl (VIII). This was prepared by dinitrophenylation of the copper complex of diaminobutyric acid by the method of Porter and Sanger (1948). After liberation from the copper complex with H_2S, the product was crystallized two times from 20% HCl. For analysis a sample was recrystallized from hot 25% HCl.

CBZ-D-Val-D-DNP-lysine-OMe (XIa). To 252 mg of CBZ-D-valine and 362 mg of D-DNP-lysine-OMe·HCl were added 0.8 ml of ethyl acetate and 4.5 ml of dimethylformamide. After cooling to 0°, 0.14 ml of triethylamine was added. After 15 min the mixture was cooled to −5° and 220 mg of DCC was added. Further incubation and purification was as described for Ia with the exception that the insoluble dicyclohexylurea was washed with ethyl acetate. The product was crystallized from ethyl acetate–petroleum ether (bp 40–60°).

CBZ-D-Val-D-DNP-Lys hydazide (XIb) was prepared as described for the corresponding L-L derivative IIb with the substitution of the equivalent amount of hydrazide hydrate for anhydrous hydrazine. CBZ-D-Val-D-DNP-Lys-D-Leu-D-Phe OMe (XIc) was prepared from XIb and the free base of XIc (see below) as described under Ic. After incubation cold water was added and the precipitate was crystallized from dioxane–ethanol–water (1:2:2). D-Val-D-DNP-Lys-D-Leu-D-Phe-HBr·OMe (XI) was prepared as described for I.

CBZ-D-Leu-D-Phe-OMe (XId). CBZ-D-Leu (265 mg) in 5.0 ml of acetonitrile was stirred with 260 mg of *N*-ethyl-5-phenylisoxazolium 3′-sulfonate and 0.14 ml of triethylamine for 5 min. D-Phenylalanine methyl ester·HCl (215 mg) and 0.14 ml of triethylamine were added and the reaction mixture was stirred at room temperature overnight. The reaction mixture was taken to dryness, and the product was crystallized by trituration with water and recrystallized from ethanol water. D-Leu-D-Phe-OMe·HBr (XIe) was prepared from XId by the action of hydrogen bromide in glacial acetic acid.

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Energetics of Potassium Transport in Mitochondria Induced by Valinomycin*

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ABSTRACT: The valinomycin-induced transport of K^+ into mitochondria has been followed on a continuous basis, by means of the ion-specific electrode, as a function of [K^+], pH, temperature, energy source, and anionic environment. Simultaneous measurement of O_2 consumption during transport energized by substrate oxidation, or liberation of inorganic phosphate (P_i) during ATP-energized transport, yields values for the stoichiometry of K^+ transport per equivalent of adenosine triphosphate (ATP) expended. A maximum value over seven was obtained at pH 6.7, 25°, 2.5 mM K^+, and 20 mM acetate for ATP-energized transport, corresponding to a thermodynamic efficiency estimated to be about 80%.

The energy of activation of K^+ transport obtained from the temperature studies was 9.8 and 10.8 kcal for oxidizable substrate and ATP-energized transport, respectively. Evidence is offered that the mechanism of action of valinomycin involves not only increased mitochondrial permeability to K^+ but also stimulation of the transport-energizing process itself. The data obtained have been used for a critical assessment of proposed mechanisms of ion transport, particularly the chemiosmotic hypothesis of Mitchell.

The accumulation of K^+ by mitochondria, induced by valinomycin (Moore and Pressman, 1964), shares in common with the spontaneous, energy-dependent accumulation of the divalent ions, Ca^{2+},[1] Mn^{2+} (Maynard and Cotzias, 1955; Bartley and Amoore, 1958; Saris, 1963; Chappell *et al.*, 1963), Sr^{2+} (Saris, 1963; Chappell *et al.*, 1963; Carafoli, 1965), and Mg^{2+} (Brierley, 1963; Pressman and Park, 1963; Judah *et al.*, 1965a): (1) a requirement for energy from either oxidizable substrate or ATP;[2] (2) sensitivity to specific inhibitors, such as oligomycin (energy source, ATP), and to amytal or rotenone (energy source, oxidizable substrate); (3) stimulated respiration or ATPase activity; (4) movement of H^+ counter to movement of ion accumulated; and (5) facilitation by permeant anions, such as phosphate, arsenate, and acetate (Moore and Pressman, 1964; Rasmussen *et al.*, 1964; Pressman, 1965a; Chance and Yoshioka, 1965; Rasmussen *et al.*, 1965). This suggests that the underlying mechanisms of mono- and divalent ion accumulations are similar if not identical. Indeed, a mutual competition between monovalent and divalent cation accumulation (guanidinium and Mg^{2+}) has already been reported (Pressman and Park, 1963). Accordingly, information obtained for any one cation might be expected to apply generally to the phenomenon of energy-dependent cation accumulation by mitochondria.

The induced accumulation of K^+ has definite techni-

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[1] The rather extensive bibliography pertaining to the interaction of Ca^{2+} with mitochondria is reviewed exhaustively in the recent paper by Chance (1965).

[2] Abbreviations used: ADP and ATP, adenosine di- and triphosphate; DPN, diphosphopyridine nucleotide.