The Synthesis and Antigenic Properties of a Macromolecular Peptide of Defined Sequence Bearing the Dinitrophenol Hapten*

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ABSTRACT: A macromolecular polypeptide of defined sequence having an approximate molecular weight of 10,000 has been synthesized by polymerization of undecapeptide subunits having the formula [D-Ala-L-Ala]$_5$-ϵ-DNP-L-Lys. This polymer bears the dinitrophenol (DNP) hapten at regular intervals of 30 A in the extended form. It is designed to overcome some of the antigenic heterogeneity found in randomly hapten-substituted proteins and polypeptides. On injection, this polymer gives rise to a high titer of precipitating antibody in rabbits. The detailed methods of synthesis of the undecapeptide subunit by the solid-state method and its subsequent polymerization *via* a pentachlorophenol active ester intermediate are described. The degree of racemization in each residue has been estimated.

Antibodies directed against a small organic hapten may be produced by coupling the hapten to a macromolecular carrier, and injecting the complex into an animal (Landsteiner, 1945). It is known, however, that whole families of antibodies are produced against such a system. Each member of the family is able to combine with the hapten but may differ from another member in binding affinity by as much as four orders of magnitude (Eisen and Siskind, 1964). A number of workers have questioned whether the production of many antibodies to one determinant is not, in fact, due to the injection of multiple antigens. These could be produced, for instance, by coupling the hapten to a number of residues in the carrier each having a different chemical microenvironment. The DNP group attached to the ϵ carbon of lysine may for instance behave differently if the neighboring groups are largely polar than if they are apolar (Singer, 1964). Thus each hapten located in a specific environment might act as a separate antigen.

Among others, two principles have emerged in recent years from studies on antibody structure. First, the antibody combining site is considerably larger than most aromatic ring systems usually employed as haptenic determinants (Cebra, 1961; Maurer, 1964; Sage *et al.*, 1964; Kabat, 1966). Since the combining site extends beyond the determinant, the amino acid residues near the determinant may play a significant role in binding antibody (Parker *et al.*, 1966). Thus, from the hapten plus permutations of neighboring residues, many antigenic groupings can be envisioned each of which in turn might evoke separate antibodies.

A second principle stems from the work of Kabat (1960) who demonstrated that antidextran antibodies contained different fractions directed against the various multiples of the dextrose subunits constituting dextran. Thus, it may well be that if more than one of the specific determinants to be studied is found within that area of the antigen corresponding to the antibody combining site, a different antibody may be formed than if such an area contains one specific determinant only. Juxtaposition of two or more determinants on a carrier may therefore be another cause for antigenic heterogeneity.

A simple system which might avoid these complexities would be one in which a hapten is attached to a nonantigenic or weakly antigenic carrier. Since, however, such an attachment now renders the carrier antigenic (Sela *et al.*, 1964), the immediate environment of the hapten should be as simple as possible. If the carrier is a polypeptide it should be composed of as few types of amino acid residues as possible. For reasons described below, it is impractical to use most amino acid homopolymers. Where two or more amino acid residues constitute the carrier, these should be in defined sequence in order to avoid heterogeneity due to nonregular distribution of residues. To prevent juxtaposition of the haptenic groups, it is preferable to space them at intervals larger than the size of the antibody combining site. It is advantageous to have a carrier backbone which is not an α helix or in β conformation, since these arrangements increase the difficulty in separating the haptenic groups.

Described below are the synthesis and some of the antigenic properties of a macromolecular defined sequence polypeptide of molecular weight 10,000 carrying the DNP determinant at regular intervals of 30 A along the extended chain. This polymer approximates the requirements set out above. We wish to know if immunization with such a molecule leads to the production of more homogeneous antibody frac-

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tions within single antibody subgroups from single animals, or whether a high degree of antibody heterogeneity is retained even against highly ordered macromolecular antigens.

Experimental Section

The antigen described here has the formula [[L-Ala-D-Ala]$_5$-ε-DNP-L-Lys]$_n$, where n = 10.2. It is polymerized from undecapeptide subunits of molecular weight 1005, each one of which is composed of one DNP-L-lysine residue at the C-terminal end and five D-Ala and five L-Ala residues with D and L residues alternating. The subunits were synthesized by the solid-state method (Merrifield, 1964) and polymerized employing a pentachlorophenol active ester intermediate (Kovacs and Kapoor, 1965).

Synthesis of the Undecapeptide Subunit

The method employed follows in general that of Merrifield (1964). The outstanding advantage of the solid-state technique over classical methods is great speed and a high product yield. The main disadvantage is that products occur which are closely related to the final peptide but lack single amino acid residues. These "short-chain" peptides probably arise from incomplete substitution during the addition steps. The solid-state method is also well suited to the production of copolymers of relatively simple composition for which separation methods of high resolution exist. It is possible to separate small peptides of polylysine, polyglutamic acid, and polyalanine differing from each other only by a single amino acid residue (Miller, 1960; Schechter *et al.*, 1965; Yaron and Sober, 1964).

Solid-State Resin. Chloromethylated polysytrene resin, 200–400 mesh, containing 2% divinylbenzene and having a capacity of 1.5 mequiv/g, was purchased from Bio-Rad Laboratories, Richmond, Calif. (control no. 2904), and used without further treatment.

Amino Acids and Amino Acid Derivatives. The *t*-butyloxycarbonyl group (*t*-Boc)[1] (Carpino, 1957a,b) was used as the amino-protecting group throughout this synthesis, except that the ε-amino group of lysine was already substituted with the DNP group before assembly of the undecapeptide.

α-t-Boc-ε-DNP-L-Lysine. This compound was synthesized from ε-DNP-L-lysine hydrochloride prepared by the method of Sanger (1945). ε-DNP-L-lysine hydrochloride (30 mmoles, 10.5 g) was finely ground in a mortar with 90 mmoles (3.60 g) of magnesium oxide. The mixture was suspended in 200 ml of 50% aqueous dioxane and stirred at 47° for 1 hr. *t*-Butyl azidoformate (35 mmoles, 45.0 g) was added slowly to the mixture with constant stirring over 3 hr, and the mixture was stirred at 47° for a further 17 hr. It was then cooled to 4° and 300 ml of water was added. This alkaline solution was extracted three times with 300 ml of ethyl acetate. The ethyl acetate extract was back-extracted with 1 N sodium carbonate solution and the aqueous phases were pooled.

The aqueous phase was brought to pH 4.0 with a 20% solution of citric acid and reextracted three times with 200 ml of ethyl acetate. The organic phase was pooled and washed three times with 15 ml of saturated sodium chloride and three times with distilled water. The whole extract was dried over anhydrous magnesium sulfate. Subsequently, the volume of the solvent was reduced *in vacuo* until yellow crystals of *α-t*-Boc-ε-DNP-lysine began to separate out. The material was recrystallized from a dry ether–petroleum ether (bp 30–60°) mixture (1:10, v/v). The yield was 58% of theory, mp 51–53°. *Anal.* Calcd for N, 14.4. Found: N, 14.8. Electrophoresis at pH 2.0 confirmed that the α-amino group was protected. Chromatography in butanol–acetic acid–water (60:15:25, v/v) gave an R_F of 0.29 as compared with 0.80 for ε-DNP-L-lysine in the same system. The infrared spectrum revealed major peaks at 1158, 1162, 1758, 3000, and 3530 cm^{-1} in addition to the peaks present in the ε-DNP-lysine spectrum.

α-t-Boc-D-Alanine and α-t-Boc-L-[^{14}C]Alanine. These

TABLE I: Running Analysis of the Growing Polypeptide Chain.

Residue[a]	% Attachment	Method of Estn
1	100	A[b]
2	62	B[c]
3	63	C[d]
4	65	B
5	61	C
6	59	B
7	64	C
8	60	B
9	60	C
10	61	B
11	62	C

[a] Residues are numbered in order of attachment to the resin so that residue 1 is ε-DNP-lysine and the residues thereafter are alternately D- and L-alanine. The second column expresses the per cent attachment of residues to the resin on a molar basis. The quantity of residue 1 attached to the resin is taken as an arbitrary 100%. The subsequent residue attachment is expressed as percentages of the first residue attached. [b] A = optical density at 360 mμ in 1.0 N NaOH solution after hydrolysis with 20% NaOH in ethanol at 40° for 24 hr. This procedure also removes the peptide from the resin. [c] B, by automatic amino acid analysis of the supernatant after alkaline hydrolysis of the resin–peptide adduct. [d] C, by total radioactivity of the supernatant after alkaline hydrolysis of the resin–peptide adduct.

[1] Abbreviations used: *t*-Boc, *t*-butyl oxycarbonyl group; DMF, dimethylformamide.

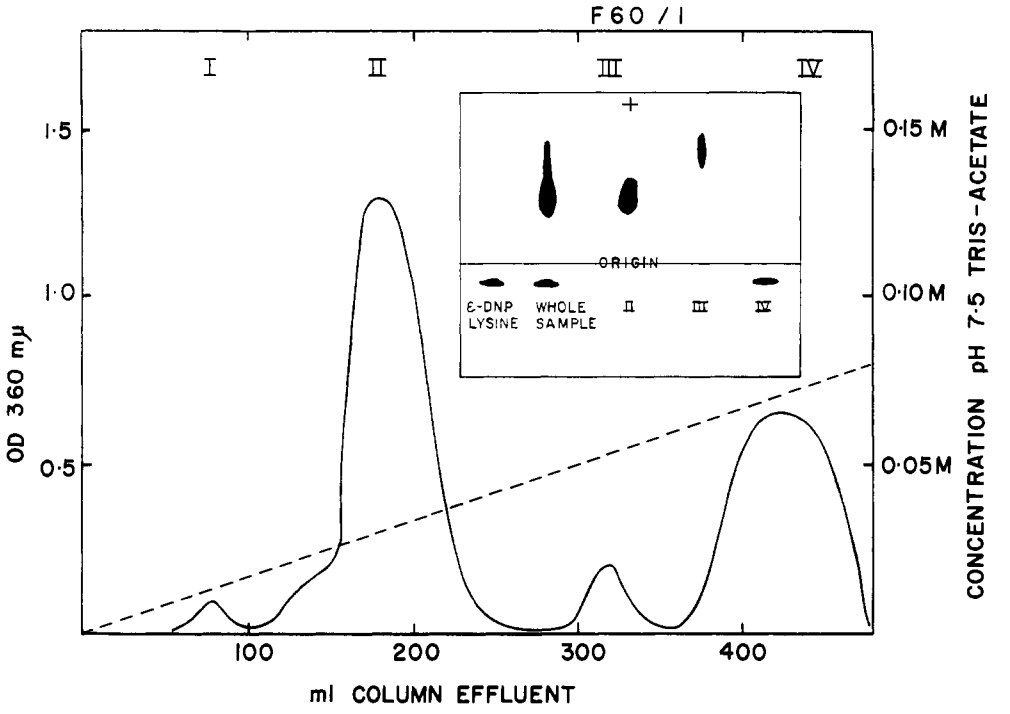

FIGURE 1: Separation of reaction products from the synthesis of the undecapeptide by eluting a column 60 × 1.9 cm packed with CM-cellulose (Whatman CM 23) with a Tris-acetate gradient. Peak II is the undecapeptide [L-Ala-D-Ala]$_5$-ε-DNP-L-Lys. The electrophoresis pattern at pH 7.5 of the chromatographic peaks was obtained on separation of the reaction products of the undecapeptide synthesis. Electrophoresis was carried out on No. 3MM Whatman paper for 1–2 hr at 30 v/cm, using a pH 7.5 pyridine–acetic acid buffer (Smith and Smith, 1960).

TABLE II: Analysis of Reaction Products in the Undecapeptide Synthesis.

Peak	Moles of Alanine per Mole of ε-DNP-Lysine: Acid Hydrolysis[a]	Moles of Alanine per Mole of ε-DNP-Lysine: Spectrogravimetric Anal.	% N Found
I	Insufficient for anal.	Insufficient for anal.	Insufficient for anal.
II	9.6	9.9	
II (rechromatographed)	9.9		20.2 (expected 19.8)
II (anterior shoulder)	9.9		
III	5.1		
IV	0.005	0	17.9 (expected for ε-DNP-lysine, 19.9)

[a] Acid hydrolysis was carried out in 5.7 N HCl at 110° for 24 hr *in vacuo*. For the spectrogravimetric analysis, the undecapeptide was dried at 60° *in vacuo* over P_2O_5 for 24 hr and weighed. The dried product was then dissolved in water and the optical density (at 360 mμ) was measured. A molar extinction coefficient of 17.7×10^3 was taken for the ε-DNP-lysine residue (Fraenkel-Conrat *et al.*, 1955).

were synthesized according to the method of Schwyzer *et al.* (1959). Into the L-alanine used in this synthesis, small quantities of uniformly labeled [^{14}C]alanine (New England Nuclear Corp.) were introduced to give a final specific radioactivity of approximately 4×10^4 dpm/mg of L-alanine residue. The D-alanine residue was not labeled.

Esterification Step (Resin–α-t-Boc-ε-DNP-L-Lysine Adduct). α-*t*-Boc-ε-DNP-L-Lys (10 mmoles) was esterified onto 25.2 g of chloromethylated resin using the conditions of Merrifield (1964). After alkaline hydrolysis of a resin aliquot (20% NaOH in ethanol at 45° for 24 hr), it could be shown that 5.7 mmoles of α-*t*-Boc-ε-DNP-L-Lys had attached to the resin.

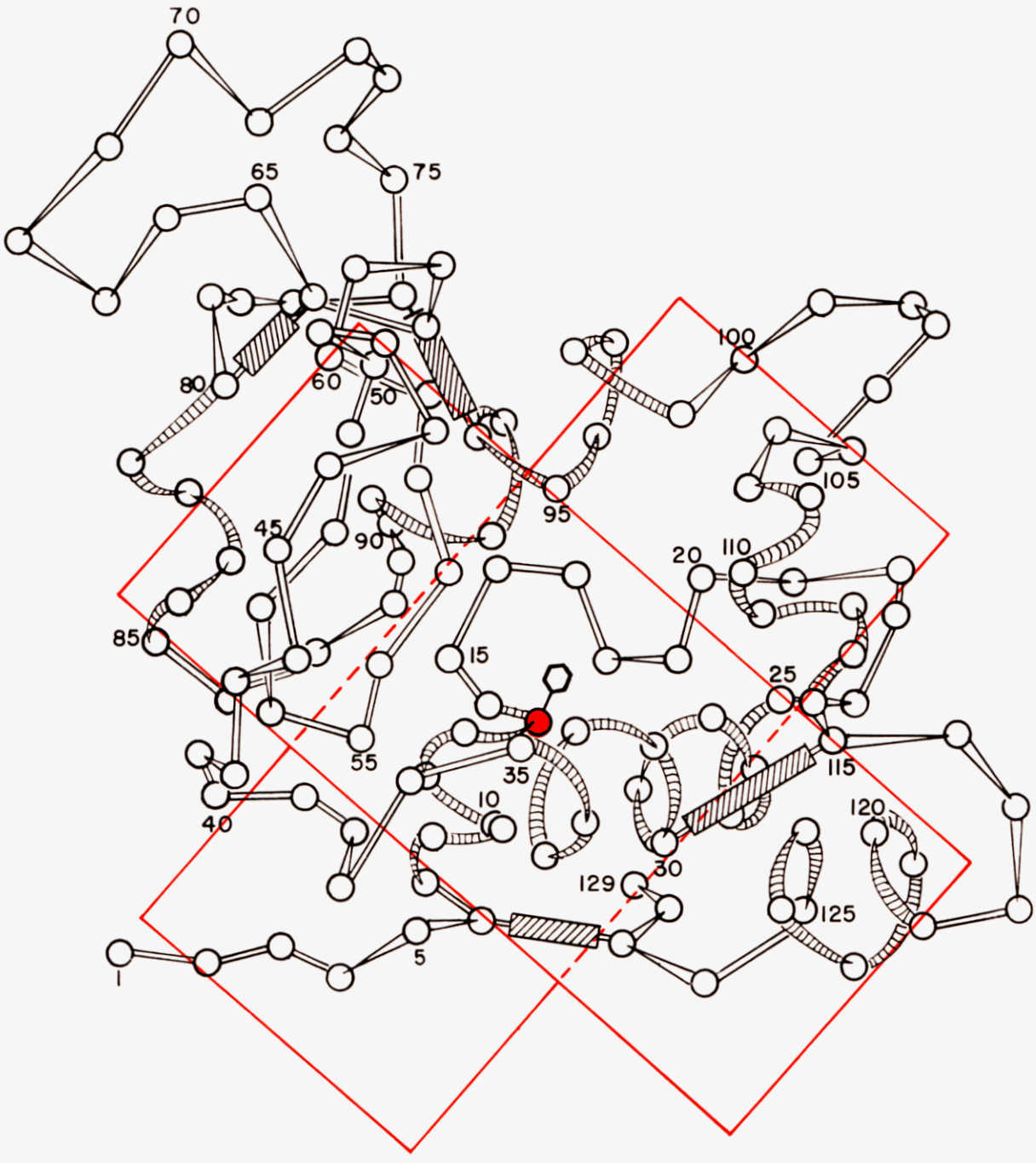

FIGURE 2: Schematic drawing of the lysozyme molecule. The ϵ-amino group of lysine residue 13 (red circle) is shown as substituted with the DNP group. Two rectangles (red) measuring 25 × 11 A centering on residue 13 have been drawn to represent antigenic sites. The drawing of the lysozyme molecule is based on Bragg's drawing in Blake *et al.* (1965). The numerals in the figure refer to the residue number of the amino acid sequence.

Addition of Peptide Residues. The order of residue addition is shown in Table I. Alanine (3.5 molar excess) was used at each step. The attachment of the residues was measured quantitatively at each step both by automatic amino acid analysis and by determining the attached radioactivity of the L-[^{14}C]alanine residues (specific radioactivity, 40,800 dpm/mg). The figures are given in Table I. The attachment of the second residue was only about 60% as great as that of the first residue; that is, for every 10 moles of ϵ-DNP-L-lysine attached to the resin, only six D-alanine residues had been attached. The next addition step and every subsequent addition step give essentially the same result. Between 58 and 62% of the expected maximum

attachment occurred (calculating the attachment of the first residue to the resin as an arbitrary 100%). From this result two situations seemed possible. Either the alanine addition steps were only 60% complete and there was present a high percentage of short chains, or only 60% of the ε-DNP-lysine groups esterified onto the resin was able to initiate chain formation and, once chain formation had started, each step went to completion. Chromatography and analysis of the reaction products (Table II) showed that the second situation obtained. The presence of ε-DNP-$(Ala)_5$ in peak III is difficult to explain. This short-chain peptide could have stopped growing at this point owing perhaps to accidental acetylation of the amino group by incompletely removed acetic acid, but the running analysis figures (Table I) gave no evidence of this. Another possibility is that this molecule could have arisen from a split between residues 6 and 7 in the isolation procedure. However, search in the reaction mixture for the other product of such a split, an alanine tetrapeptide, was negative. For the cleavage of the peptide from the resin with hydrogen bromide the conditions of Merrifield (1964) were followed exactly.

Separation and Characteristics of Products Obtained by Hydrogen Bromide Cleavage

The isolation methods employed involved two fractionations on CM-cellulose, a further fractionation on acrylamide gel, and characterization of the fractionation products by paper electrophoresis.

The material (100 mg) obtained by hydrogen bromide cleavage was dissolved in 25 ml of 0.001 M Tris-acetate buffer, pH 7.5, and eluted from a 6 × 80 cm column packed with a high-resolution carboxymethylcellulose (Whatman Column Chromedia CM 23) by means of a continuous gradient 0.001–0.02 M, pH 7.5, Tris-acetate buffer. Aliquots of the first peak were rerun on a 1.9 × 60 cm column packed with the same preparation and employing the same gradient. Later, however, identical results were obtained using distilled water as eluent. The products were eluted from the 1.9 × 60 cm CM-cellulose columns as three major peaks, one minor peak, and one shoulder (see Figure 1).

Paper electrophoresis (Whatman 3MM paper) at pH 2.0 gave single spots with peaks II–IV (see Figure 1). Single spots were also obtained by electrophoresis at pH 7.5 and by electrophoresis at pH 2.0 on cellulose acetate strips.

The major peak II containing the ε-DNP-L-Lys-$(Ala)_{10}$ product was subjected to electrophoresis at pH 1.5 (A. Berger and I. Schechter, private communication, 1966). Under these conditions a second product, present as a 3% contamination, separated out and could be shown to consist of chiefly ε-DNP-L-Lys-$(Ala)_9$. The shoulder preceding peak II has the same analysis as peak II itself. Peak III consisted of ε-DNP-L-Lys-$(Ala)_{5.1}$, while peak IV was ε-DNP-L-lysine. It could be shown that this contaminant was present almost exclusively in the tail of peak II. The middle section of one peak was collected and rechromatographed on acrylamide gel column (60 × 1.9 cm, Bio-Gel P2 50–100 mesh) (Bio-Rad Laboratories, Richmond, Calif.) in distilled water. This yielded a single symmetrical peak.

Identification and Analysis of the Undecapeptide

The major reaction products were identified by acid hydrolysis followed by automatic amino acid analysis and nitrogen content. The molecular weight of peak II was obtained by a spectroscopic "end-group" method using the known extinction coefficient for ε-DNP-lysine. Peak IV (ε-DNP-lysine) was identified by chromatography, mixture melting point, and infrared spectrum.

Peak III. Hydrolysis and amino acid analysis showed that there were 9.7 alanine residues/ε-DNP-L-lysine residue in the crude peak and 9.9 alanine residues after rechromatography (see Table II). ε-DNP-lysine residue concentration was measured by absorption at 360 mμ. The assumption made here is that the extinction coefficient of free ε-DNP-lysine is the same in the free amino acid as when the α-amino group is bound in peptide linkage. This assumption was checked on an ε-DNP-L-lysyl-L-alanine dipeptide by measuring the optical density before and after alkaline hydrolysis. There was no change in the molar extinction coefficient after hydrolysis of the peptide bond.

The molecular weight of the compound was checked by determining accurately the optical absorption at 360 mμ of an aliquot of the undecapeptide dissolved in water, drying at 45° *in vacuo*, and weighing the residue. This spectrogravimetric analysis gave 9.9 alanine residues/ε-DNP-lysine residue and a molecular weight of 994.6. The calculated molecular weight for the undecapeptide is 1005. This represents an error of 0.9%. The over-all yield of the pure undecapeptide was 18% based on the first residue attached to the resin.

Racemization of the Undecapeptide Monomer

Since it is necessary to establish whether the immediate environment of the ε-DNP-hapten is homogeneous in the final polymer, the degree of racemization in both the alanine residue and the ε-DNP-lysine residue was measured. Since the ε-DNP-lysine residue was chiefly at risk during the activation and polymerization procedures no attempt was made to study this residue in the undecapeptide monomer, but instead a model of the hapten in its environment was built, and the racemization of the ε-DNP-lysine residue was studied in the model (see below).

In order to show absence of racemization in the alanine residues, the ratio of D to L residues was shown to be 1:1 (see Table III). The undecapeptide monomer was hydrolyzed for 72 hr in 12 N HCl at 110° *in vacuo*. A control containing an equivalent amount of free D- and L-alanine was added to measure hydrolysis losses by the acid. Duplicate hydrolysates were neutralized, desalted, and treated for 12 hr with an excess of D-amino acid oxidase derived from hog kidney (1.43.3) (Worthington Biochemical Corp., Freehold, N. J.) using the conditions of Burton (1955). The

TABLE III: Racemization in the Alanine Residues of the Monomer [D-Ala-L-Ala]$_5$-ε-DNP-L-Lys.[a]

Sample	μmoles of Alanine Recovd after Hydrolysis and Digestion by D-Amino Acid Oxidase
(1) 0.30 μmole of monomer containing 0.274 μmole of alanine	0.132
(2) Duplicate of expt 1	0.130
(3) 0.274 μmole of, L-alanine	0.263

[a] Monomer (0.30 μmole) was hydrolyzed with 2.0 ml of 5.7 N HCl *in vacuo* at 110° for 24 hr and then digested with D-amino acid oxidase (Worthington) under the conditions of Burton (1955) for 24 hr. After precipitation of the protein with trichloroacetic acid (TCA) (final concentration 5%) and extraction of the excess TCA with ether, the sample was analyzed for alanine by automatic amino acid analysis. A standard solution of D-alanine was also digested with D-amino acid oxidase under the same conditions and the figures given are compensated for incomplete digestion.

enzymic hydrolysate was adjusted to a 5% final concentration with 25% trichloroacetic acid, the precipitate was collected and washed three times with 5% trichloroacetic acid, and the washings were returned to the supernatant. The trichloroacetic acid was removed by repeated ether extraction. The aqueous supernatant was dried *in vacuo* and subjected to automatic amino acid analysis. Table III shows that within experimental error and allowing for incomplete digestion by the D-amino acid oxidase, one-half the total alanine residues were digested by the enzyme.

However, the presence of equal quantities of D- and L-amino acid residues does not exclude racemization, since it might be expected that equal quantities of L-alanine might racemize to D-alanine and D-alanine to L-alanine. To rule this out, the pyruvic acid produced by the D-amino acid oxidase from the D-alanine residues was isolated by chromatography in the presence of 0.5 mg of carrier pyruvic acid. Chromatography was performed in two dimensions on silica gel thin layer plates using 1-butanol (60 parts), glacial acetic acid (15 parts), and water (25 parts) as the first solvent and ethanol (80 parts, 0.88 sp gr), ammonium hydroxide (5 parts), and water (15 parts) as the second solvent (Smith and Smith, 1960).

The isolated pyruvic acid contained no detectable quantity of ^{14}C. Hence, no significant racemization from L-alanine to D-alanine occurred and because of the 1:1 ratio of D- and L-alanine, the reverse process could also not have taken place.

Polymerization of the Undecapeptide

The ε-DNP-L-Lys-(Ala)$_{10}$ undecapeptide subunit was polymerized *via* the active ester pentachlorophenol intermediate (Kovacs and Kapoor, 1965). The undecapeptide was dissolved in a minimal volume of dry, freshly distilled dimethylformamide or 2-dichloroethanol and a 1.1 molar excess of pentachlorophenol was added. The mixture was cooled to −20° and a 1.1 molar excess of *N,N'*-dicyclohexylcarbodiimide was added. It was then stirred gently at −20° for 12 hr. The solution was dried with dry nitrogen gas at 20° and the peptides were redissolved in distilled water. The *N,N'*-dicyclohexylurea was filtered off. The peptide polymer was then filtered through a Sephadex G-50 column. The polymeric products of the reaction were found as a single peak in the excluded volume, indicating an increase in molecular volume. The degree of polymerization was checked by reacting both the monomer and the polymeric products with an 8 molar excess of Rhodamine isothiocyanate which absorbs light strongly both at 360 and 470 mμ in the free state and when coupled to polyalanine. The Rhodamine-coupled ε-DNP-L-Lys-(Ala)$_{10}$ compounds, both monomer and polymer, could be separated from free excess Rhodamine isothiocyanate on a CM-cellulose CM23 column, 1.9 × 60 cm, by eluting with distilled water. The degree of substitution of the free amino groups was estimated by measuring light absorption at 360 and 470 mμ and calculating the relative contribution to the absorbance at 360 mμ derived from ε-DNP-L-lysine residue. Substitution of the free amino group on the monomer was 95% of the maximum. Light absorption at 470 mμ relative to that at 360 mμ on the polymeric material showed that an average degree of polymerization of 10.2 had been achieved. This corresponds to an average molecular weight of 10,089 for the polymeric products.

Racemization of the Polymer ε-DNP-Lysine Residue Studied on Model Peptides. The studies of Berger and his colleagues have demonstrated a simple way in which the racemization of a single amino acid residue may be studied in a peptide (Schechter *et al.*, 1965). The principle of this method depends on the complete resistance of an α-DL-amino acid peptide linkage to hydrolysis by various proteolytic enzymes. An enzyme such as carboxypeptidase A will act quantitatively on a tripeptide L-Ala-ε-DNP-L-Lys-L-Ala, splitting off the C-terminal L-alanine residue as long as the substituted lysine is the L stereoisomer. Hence, small degrees of racemization of the ε-DNP-Lys residue may be detected by measuring the free alanine produced after digestion of the tripeptide with carboxypeptidase A. The actual sequence forming the immediate haptenic environment in our polymer is - - - - -L-Ala-ε-DNP-L-Lys-D-Ala - - - - - and is not suitable for this method of detecting racemization since Schechter *et al.* (1965) have shown that the presence of a D residue in the neighborhood of the LL linkage may also affect the rate of hydrolysis. The simple tripeptide L-Ala-ε-DNP-L-Lys-L-Ala does not have this disadvantage and represents a good model of the hapten in its environment. This tripeptide was prepared by making the dipeptide ε-DNP-L-Lys-L-Ala by the same method

which was used for making the large polymer (see section on addition of peptide residues). The α-amino group of this dipeptide was acetylated and treated with a 1 molar equiv of benzoyl-(L-Ala)$_9$-*p*-toluenesulfonate under conditions identical with those used for polymerizing the undecapeptide. After isolation of the tripeptide, the benzoyl ester was hydrogenated in the presence of palladium black (Greenstein and Winitz, 1961). The resulting tripeptide was incubated for 12 hr with carboxypeptidase A (Worthington Corp.) using the conditions of Folk and Schirmer (1963). The free alanine was estimated by automatic amino acid analysis. Between 94 and 96% of the C-terminal free alanine residue was liberated. It could be shown that, under the same conditions, no free alanine was liberated from an L-alanyl-ϵ-DNP-D-lysine dipeptide. Thus the maximum amount of racemization which could have taken place at the ϵ-DNP-lysine residue of the model *N*-acetyl-L-Ala-ϵ-DNP-L-lys-L-Ala was 4–6%. There was apparently little difference in the degree of racemization whether DMF at $-20°$ or 2-dichloroethanol was employed as solvent during the activation and coupling step.

Conformation of the Polymer. Optical rotatory dispersion studies on the polymer in aqueous solution at pH 6.6 were undertaken on the Cary 60 spectropolarimeter. Extrinsic Cotton effects were not observed at 360 mμ, the absorption maximum of the chromophore, nor were the troughs of the α-helical or β-structure intrinsic Cotton effects observed at 233 and 230 mμ. The mean residue rotation at both 233 and 230 mμ was -1500 as compared to -2000 for a random coil form of poly-L-lysine at these wavelengths. For poly-L-lysine the α helical and β forms have mean residue rotations of $-14{,}500$ at 233 mμ and -6300 at 230 mμ, respectively (Sakar and Doty, 1966). Because of the strong absorption of the chromophore in the ultraviolet region, observations below 220 mμ could not be made. It seems likely, therefore, that the polymer in aqueous solution has a "random-coil" structure rather than the β conformation. In view of the alternating D and L residues the polymer would not be expected to have an α-helical structure.

Antigenicity of the Polymer. Ten male and female New Zealand white rabbits were immunized with 1.0 mg of polymer in 0.5 ml of complete Freund's adjuvant. The mixture was injected subcutaneously into all four toepads. Eight or twelve weeks later the animals were given a further dose of 0.5 mg of polymer in 0.15 N saline intravenously.

All ten animals developed circulating precipitating antibodies between 2.5 and 4 weeks after the first immunization. The mean titer at 4 weeks was 280 μg of antibody/ml of serum and the range was from 150 to 510 μg of antibody/ml of serum. After boosting, the titers rose; the highest titer, recorded at 20 weeks after immunization, was 1.0 mg/ml of serum and the lowest titer was 30 μg/ml of serum with a mean of 560 μg of antibody/ml of serum. Estimations of antibody concentration were performed using the gel filtration radioimmunoassay described by Haber *et al.* (1965). The radioactive antigen used was ϵ-DNP-[^{3}H]lysine of a specific radioactivity of 1.2 c/mmole.

Discussion

The literature records a number of investigations with antibodies against hapten–carrier systems simpler than the randomly coupled hapten–protein systems introduced by Landsteiner (1945). In a study by Eisen *et al.* (1964) the DNP group was attached to the ϵ-amino group of lysine residue 41 of ribonuclease and this complex was used as an antigen. Binding studies showed clearly that the antibodies produced were heterogeneous. Another study by Kantor *et al.* (1963) demonstrated heterogeneity of antibody response as judged by heterogeneity of light chain pattern on starch gel electrophoresis when polylysine substituted with DNP in a "random" manner was injected into guinea pigs. Another experiment in which short DNP polypeptides were coupled to proteins also gave rise to antibodies with a heterogeneity function of less than unity (Parker *et al.*, 1966).

Singer (1964) points out some probable reasons why a simple hapten attached to a carrier in different places may not always function as the same antigen. An hydrophobic determinant may bond with hydrophobic areas of the backbone, producing a different "hybrid determinant" than the same hapten attached to a hydrophilic portion of the antigen (see for instance, Weber, 1953). We feel (F. F. Richards, R. W. Sloane, Jr., and E. Haber, in preparation, 1967) that this principle extends more widely; that not only the polarity of the environment but also the variety of the haptenic environment gives rise to a series of hybrid antigens. It is likely that binding affinity is the product of the affinities of the hapten plus those of the surrounding groups. Since the antibody combining site is in all probability larger than the commonly employed haptens, a number of neighboring groups may enter into the binding. Parker *et al.* (1966) has produced evidence that where the DNP determinant has aromatic residues as neighbors such as phenylalanine, these residues may contribute one order of magnitude to the binding affinity of the whole site.

A monosubstituted 41 ϵ-DNP-lysine derivative of ribonuclease has been used as an antigen (Eisen *et al.*, 1964). Since full details of the three-dimensional structure of ribonuclease are not yet available (F. M. Richards, private communication, 1966), a similar but hypothetical situation has been drawn with lysozyme. Figure 2 shows a relief drawing of the structure of lysozyme (after J. L. Bragg in Blake *et al.*, 1965). Superimposed on the drawing are two hypothetical antibody combining sites 25 $\times$ 11 A (Sage *et al.*, 1964; Kabat, 1966). They are centered on lysine residue 13, which is depicted here as if specifically substituted with the DNP group. The large number of combinations which can be obtained from this arrangement may be easily seen. It must also be considered that since the DNP determinant may be anywhere in the site, an even larger series of combinations are possible. When

a protein is substituted by more than one specific determinant it is clear that even more heterogeneity is possible unless the specific determinant residues are separated by distances greater than the size of the antigenic site.

Perhaps a general objection against all macromolecular antigens which have a defined sequence is, that although there is some knowledge of the conformation of the chains and the environment of the hapten *in vitro*, nothing is known about these factors *in vivo*. It could be that *in vivo* the chains associate with themselves or with other proteins (Maurer, 1964) producing just the complexity of antibody response which the definition of the antigen seeks to avoid. This view would render vain any further work on the definition of antigens. The authors feel that this view is too pessimistic, and are encouraged by the fact that antibodies to the polypeptide described in this paper show features which are quite different from those produced against DNP-bovine γ-globulin. This forms the subject of a following paper (Richards *et al.*, 1967).

The polypeptide described in this article represents a compromise between the ideal structure set out in the introduction and what we found attainable in practice. Thus the environment of the DNP determinant is reasonably homogeneous, being composed of the alternate D- and L-alanine residues of the backbone. This arrangement produces symmetrical variation of the position of the alanine methyl groups along the long axis of the chain; yet a chain of relatively uniform diameter (between 3 and 4 A) is retained. If a single alanine stereoisomer polymer had formed the backbone, this could have given rise to an α-helical structure (Pauling *et al.*, 1951; Bamford *et al.*, 1954) with a pitch of 5.4 A containing about 3.6 residues/turn of the helix. Thus, about 20 residues would have been needed to give a separation of the hapten equal to that in the extended form of our polymer. A polypeptide subunit of this size of defined structure is not easy to produce. Poly L-alanine may exist also in the β conformation (Bamford *et al.*, 1954) which corresponds to the "pleated-sheet" structure of Pauling and Corey (1951). Optical rotatary dispersion studies on our polymer gave no evidence of any β conformation and thus it seems that the structure is a "random" coil. An additional important reason for not employing any structure with strong intra- or interchain hydrogen bonding is that such molecules are insoluble under physiological conditions.

In the fully extended form our polymer has a spacing of about 30 A between the individual ϵ-DNP-lysine residues. The hapten rises about 5.5 A above the polypeptide backbone which varies between 3 and 4 A in diameter. These dimensions were obtained by building and measuring atomic models (CPK Atomic Models, Ealing Corp., Cambridge, Mass.) which have accurate Van der Waal's radii. It seems that the separation of the hapten is larger than present estimations of the size of the antibody combining site (Kabat, 1966). However, if the polymer is not fully extended the average linear separation of the haptens may be decreased. One further advantage of using alternating D- and L-alanine residues in the backbone is that poly-DL-alanine has been shown to be only weakly antigenic *per se* (Sela *et al.*, 1965).

Two imperfections mar the homogeneity of the haptenic environment in our polymer. The existence of about 5% racemization on the α carbon of the ϵ-DNP-lysine residue introduces some uncertainty. It would appear that the effect of such racemization would be to rotate the DNP group with respect to its vertical axis on the polymer backbone. Also, since the ϵ-DNP-L-lysine residue is at the carboxy-terminal end of the undecapeptide subunit, the final polymer will have an "incomplete" haptenic environment at the carboxy-terminal end. Both these difficulties are theoretically avoidable by placing the ϵ-DNP-L-lysine group away from the ends of the chain. Initially we tried to synthesize the subunit by adding to the growing chain an L-lysine derivative with both amino functions substituted by bulky protecting groups. However, addition was incomplete and the synthesis was abandoned.

The antigen which has been synthesized is an approach to a homogeneous antigen although as presently constituted it is not completely homogeneous. Sufficient order has, however, been introduced into the molecule to render worthwhile an exploration of the antibodies which it evokes.

Many of the complexities which add to the heterogeneity of macromolecular systems in theory are avoidable by the use of low molecular weight antigens. Thus, Schlossman *et al.* (1965) and Leskowitz and Jones (1965) have demonstrated that low molecular weight substances are antigenic. Some doubt has remained whether all of these antigens are antigenic *per se* or only in combination with some protein or other macromolecular fraction. Schlossman *et al.* (1965) have, however, produced persuasive evidence sustaining their view that an α-DNP-L-lysine nonapeptide is antigenic *per se*. The main difficulty that this system has for us, is that it is only weakly antigenic in guinea pigs and nonantigenic in rabbits. For future analytical work on the structure of the evoked antibodies rather large quantities of antibody are required. Our polymer has proved to be strongly antigenic, producing as much as 1.0 mg/antibody per ml of serum. It is interesting to note that the amount of antibody formed by these rabbits was of the same order as rabbits injected with 10 mg of DNP-bovine γ-globulin in Freund's Adjuvant. Therefore, there seems to be no justification for the view that as the total number of antigenic sites on the antigen is reduced, so is the yield of antibody against the antigen.

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