Preparation and Immunologic Properties of Stereospecific α-Dinitrophenylnonalysines*

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ABSTRACT: α-*N*-Dinitrophenylnonalysine (L_4DL_4) was prepared by solid-phase synthesis (Merrifield, R. B. (1964), *J. Org. Chem. 29*, 3100; *Biochemistry 3*, 1385). By-products formed by partial cleavage of the ε-*N*-benzyloxycarbonyl-protecting groups were detected and conditions for limiting their formation were investigated. Pure α-dinitrophenylnonalysine was obtained from the crude product by preparative ion-exchange chromatography. Digestion of the α-dinitrophenylnonalysine (L_4DL_4) with carboxypeptidase B yielded α-dinitrophenylhexalysine and lysine. Trypsin produced α-dinitrophenyltrilysine, dilysine, and tetralysine. In contrast, digestion of the α-dinitrophenylnona-L-lysine, the all-L stereoisomer, with carboxypeptidase B yielded α-dinitrophenyldilysine and lysine, whereas trypsin digestion produced α-dinitrophenyltrilysine, dilysine, and trilysine. α-Dinitrophenylnona-L-lysine, as previously reported (Schlossman, S. F., Yaron, A., Ben Efraim, S., and Sober, H. A. (1965), *Biochemistry 4*, 1638), was found to be immunogenic in guinea pigs of inbred strain 2 and in random-bred Hartley strain. α-Dinitrophenylnonalysine (L_4DL_4), on the other hand, was completely devoid of the capacity to induce an immune response. Although nonimmunogenic, α-dinitrophenylnonalysine (L_4DL_4) provoked Arthus type but not delayed cross-reactions in animals sensitized to the all-L immunogenic isomer, suggesting that configuration of the oligolysine carrier is of major importance in determining the specificity of the delayed response, but not in hapten-specific antibody-dependent reactions. Thus, the substitution of one central L-lysine residue of α-dinitrophenylnona-L-lysine by the corresponding D residue leads to a major change in the immunologic behavior of this compound. These results suggest the participation of a stereospecific receptor for lysine oligopeptide conjugates both in the induction of the immune response and in the eliciting f the established cell-mediated delayed hypersensitivity reactions.

The relative simplicity and the well-defined structure of synthetic polypeptide antigens permitted systematic investigations of the chemical basis of antigenicity (Sela, 1966). While polyamino acids are much simpler than proteins, they are polymerization products and as such are heterogeneous with respect to sequence and chain length. The availability of still simpler antigens of known amino acid composition and sequence is therefore of advantage (Axelrod *et al.*, 1963; Abuelo and Ovary, 1965; Schlossman *et al.*, 1965; Borek *et al.*, 1965; Dietrich, 1966; Salvin and Liauw, 1967; Richards *et al.*, 1967). A sharp transition from complete lack of immunogenicity to full immunogenicity was found in a homologous series of α-*N*-DNP-lysine oligopeptides, prepared by ion-exchange chromatography of α-*N*-DNP-poly-L-lysine of low average molecular weight, when progressing from α-*N*-DNP-hexa-L-lysine to α-*N*-DNP-octa-L-lysine (Schlossman *et al.*, 1965). The α-*N*-DNP-octa-L-lysine possessing the minimal size necessary for full immunogenicity is small enough to be prepared by stepwise synthetic techniques. This makes it possible to introduce predetermined modifications such as changing the optical configuration of certain lysine residues and to study the effect of such modifications on the immunologic behavior of the compound. In this report, the synthesis of α-*N*-DNP-nonalysine by the solid-phase method (Merrifield, 1962) is described and the immunologic behavior of the all-L compound as well as of the optical isomer α-*N*-DNP-nonalysine (L_4DL_4) is investigated.

Experimental Section

ε-N-Z-D-Lysine. Optically pure ε-*N*-Z-D-lysine was prepared from crude ε-*N*-Z-D-lysine by stereospecific oxidation with snake venom as described by Parikh *et al.* (1958).

Snake Venom Solution. Dried *Crotalus adamanteus* venom (19 mg) purchased from Ross Allen Reptile Institute, Silver Spring, Fla., was dissolved in 0.5 ml of 0.2 M Tris buffer (pH 7.2) and dialyzed against distilled water at 4° for 5 hr. The solution was centrifuged and used in the following oxidation reaction.

Oxidation with Snake Venom. ε-*N*-Z-D-lysine (1 g) was dispersed in 0.1 M Tris buffer (pH 7.2) (13 ml) by efficient mechanical stirring at 37.5°. Snake venom was

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added and oxygen was supplied above the surface of the reaction mixture. After 24 hr at 37.5° a second portion of freshly dialyzed venom (29 mg/0.5 ml) was added and the reaction was continued overnight. The reaction mixture was adjusted with acetic acid to pH 5.0 and filtered, and the precipitate of purified ε-*N*-Z-D-lysine was washed with ice water. (The filtrate and washings were kept for analysis of ε-aminovaleric acid; see below.) The purified ε-Z-D-lysine was dissolved in water (250 ml) by heating to 100°, and filtered through charcoal and Celite. The solution was cooled and the crystalline product was collected, washed with cold water, and dried *in vacuo* over concentrated H_2SO_4. The yield was 0.5 g. The amount of aminovaleric acid detected in the filtrate on reoxidation of this product showed that it contained 0.01% of the L isomer.

Analysis of ε-Aminovaleric Acid Resulting from ε-N-Z-L-lysine. For analysis of the L-lysine originally present, an aliquot (40 ml) of the combined filtrate (160 ml) was evaporated to dryness and 45% HBr in acetic acid (10 ml) was added to the dry residue. The reaction mixture was shaken at room temperature for 1 hr and evaporated to dryness, and the residue was dissolved in 0.2 M citrate buffer (pH 2.2) (100 ml). A 0.5-ml sample was analyzed on a Technicon analyzer (Cadavid and Paladini, 1964), with a column (0.9 × 20 cm) of Technicon Chromo beads (8% cross-linked type A, lot no. 113A). The sample was applied under pressure to a column preequilibrated with 0.35 M citrate (pH 5.28). The eluent was passed at a flow rate of 75 ml/hr (30 lb/in²). Authentic aminovaleric acid emerged at 35 min with an integration constant per micromole of 13; lysine emerged at 65 min with an integration constant per micromole of 7.0. Aminovaleric acid (0.266 μmole) was found in the applied sample, corresponding to 6.0% ε-*N*-Z-L-lysine in the original ε-*N*-Z-D-lysine.

α-N-Nitrophenylsufenyl-ε-N-Z-D-lysine was prepared as its dicyclohexylamine salt as described by Zervas *et al.* (1963) for the L isomer. Before use, the free acid was obtained (Najjar and Merrifield, 1966) by distributing the DCHA[1] salt between dilute H_2SO_4 and ethyl acetate, drying the organic phase, evaporating the ethyl acetate *in vacuo*, and dissolving the residue in dimethylformamide. The concentration of α-*N*-nitrophenylsufenyl-ε-*N*-Z-D-lysine was determined by anhydrous titration with sodium methylate (Fritz and Lisicki, 1951).

Chloromethylcopolystyrene–2% Divinylbenzene. The method described by Merrifield (1964b) was followed, except that 3.8 ml of $SnCl_4$ was used/25 g of copolymer. The product contained 1.0 mmole of Cl/g.

Esterification Step, α-t-BOC-ε-N-Z-L-lysyl Polymer. The following amounts of material were used: 15.9 g of the chloromethyl polymer, 3.7 g of α-*N*-*t*-BOC-ε-*N*-Z-L-lysine (Anderson and McGregor, 1957), and 1.2 ml of triethylamine in 20 ml of ethanol. The substituted polymer contained 0.62 mequiv of nitrogen/g (micro-Dumas) corresponding to 0.31 mmole of ε-*N*-Z-L-lysine/g (or 0.35 mmole/g of unsubstituted copolymer).

The Reaction Vessel. The reaction vessel was a glass filter funnel (medium porosity, 7-cm diameter, 13 cm high) which was fitted at the top with a $CaCl_2$ tube. The vessel was rotated slowly (20 rpm) during reactions and washings at an inclination where the suspension reached about halfway up the filter disk and side wall. During rotation the stem was closed. Liquids were withdrawn by placing the funnel vertically on a suction flask.

Deprotection Step. ε-*N*-*Z*-LYSYL POLYMER. α-*N*-*t*-BOC-ε-*N*-Z-L-lysine polymer (16.5 g, 5.11 mmoles of Z-Lys) was introduced into the above vessel and 85 ml of 0.5 N HCl in acetic acid was added. The suspension was mixed by rotating the vessel at room temperature for 30 min, filtered, and washed as described by Merrifield (1964b). The same procedure was followed after each addition of a lysine residue, except when α-*N*-nitrophenylsufenyl-ε-*N*-Z-lysine was used in the coupling reaction, in which case 0.3 N HCl in acetic acid was used at room temperature for 5 min. Dimethylformamide (82.5 ml) containing triethylamine (10%) was used for neutralization of the hydrochloride.

Addition of Peptide Residues. The second and following residues were added as described by Merrifield (1964b) by using a threefold excess of α-*N*-*t*-BOC-ε-*N*-Z-lysine and of dicyclohexylcarbodiimide in each step, starting with a 10-g portion of the ε-*N*-Z-L-lysyl polymer (3.0 mmoles). After assembling four L-lysyl residues, one ε-*N*-Z-D-lysine was added to the tetra-Z-lysyl polymer by using a threefold excess of α-*N*-nitrophenylsufenyl-ε-*N*-Z-D-lysine, liberated from its DCHA salt before the reaction as described above. After adding four more L-lysine residues, the nona-ε-*N*-Z-lysyl polymer (L_4DL_4) was obtained (13.6 g). A sample of the protected nonalysine-resin was washed and dried *in vacuo* over concentrated sulfuric acid. Nitrogen analysis by the Dumas method gave 2.64 mequiv of nitrogen/g, corresponding to 1.32 mmoles of lysine/g, or 2 mmoles/g of unsubstituted polymer; expected: 3.06 (= 9 × 0.34) mmoles/g of unsubstituted polymer.

α-N-Dinitrophenylnona-ε-N-Z-lysyl Polymer. The above-protected nonapeptide polymer (2.3 g) was dispersed in dimethylformamide (40 ml), fluorodinitrobenzene (1.2 g) and triethylamine (0.6 ml) were added, and the suspension was mixed at room temperature overnight. The reaction solution was removed by filtration and the solid was washed three times each with dimethylformamide, ethyl alcohol, and acetic acid with a 3-min mixing period at each wash. The compound was protected from light during handling and storage.

Cleavage Step. For the cleavage of the peptide from the resin with hydrogen bromide, the conditions of Merrifield (1964b) were followed. Not all of the peptide was liberated from the resin by the HBr treatment. In several preparations it was found, by nitrogen analysis of the resin after the cleavage step, that about 25% of the nitrogen-containing material remained bound to the support. After evaporating the trifluoroacetic acid, the residue was dissolved in water, neutralized with lithium hydroxide, and fractionated by ion-exchange chromatography (Sober, 1962; Stewart and Stahmann,

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: *t*-BOC, *t*-butyloxycarbonyl group; DCHA, dicyclohexylamine; BPA, bovine plasma albumin; HSA, human serum albumin.

1962a,b) on a CM-cellulose column (Peterson and Sober, 1956). A chromatogram of the crude product obtained showed that the crude product contained 70% of α-*N*-DNP-nonalysine. This corresponds to 36% yield based on the first residue attached to the resin.

Fractionation of the Crude α-N-DNP-nonalysine Hydrobromide (L_4DL_4) (Yaron *et al.*, 1964a). The solution obtained from the above cleavage step was diluted to contain a halogen concentration less than 0.2 M. It was applied to a 4.5 × 150 cm CM-cellulose column in Li^+ form, preequilibrated with 0.2 M LiCl. A 0.3 M LiCl solution was pumped on top of the column from a 32-l. reservoir at a 300-ml/hr rate. The well-stirred solution removed from the reservoir was continuously replaced by 1.0 M LiCl pumped in at the same rate. Fractions of 20 ml were collected, the concentration being determined by the absorption at 360 mμ. Aliquots from the extreme parts of the main peak were rerun on a 1.3 × 40 cm CM-cellulose column with a constant-volume (300 ml) gradient from 0.2 to 1.0 M NaCl. The ninhydrin color of the effluent was developed and recorded using the Technicon analyzer according to Cadavid and Paladini (1964). Fractions of the main peak were pooled on the basis of the obtained analytical chromatograms. About 20% of the nonalysine was lost by cutting 10% of the material from each side of the peak to get pure DNP-nonalysine. The pooled fractions were diluted three times with water and passed under gravity through a 3.5 × 10 cm CM-cellulose column in Li^+ form, preequilibrated with 0.1 M LiCl. The yellow top of the column was transferred into a sintered-glass funnel, washed with water, and extracted with 0.2 M HCl (about 20 ml) and water until all yellow color was removed from the adsorbent. Water was added to 200 ml and the solution was lyophilized. The residue was dissolved and the lyophilization repeated. The HCl-free residue was then dissolved in 98% methyl alcohol, filtered if necessary, and precipitated with acetone. The precipitate was collected on a sintered-glass funnel, washed with acetone, and dried *in vacuo* over concentrated sulfuric acid and KOH. The yield was 100 mg. The over-all yield of the pure DNP-nonapeptide was 13% based on the first residue attached to the resin. The Lys to DNP ratio (calcd, 9.0; found, 9.0) was calculated from the spectrophotometrically determined molar amount of α-*N*-DNP-lysine, 0.5 μmole/mg (using an $E_{3600\,Å}$ value of 16,800), and the amount of lysine recovered after hydrolysis, 4.0 μmoles/mg. The chloride content was 5.4 μmoles/mg. These results indicate that the preparation contained 4.5% LiCl and 12.4% water. A 0.5-mg sample chromatographed on a 1.5 × 40 cm CM-cellulose column in the Li^+ form with a constant-volume (300-ml) gradient from 0.2 to 1.0 M LiCl migrated as a single component. It chromatographed as a single peak also when mixed with an equal amount of α-DNP-nona-L-lysine (Schlossman *et al.*, 1965). The optical purity was checked by exhaustive digestion of the material in a 0.07 M collidine–hydrochloric acid buffer (pH 7.5) (2 μmole/ml) with carboxypeptidase B (0.091 mg/ml, purchased from Worthington) at 37°. After 20-hr incubation another portion of 25 μl of the enzyme solution (1 mg/ml) was added and again incubated for 20 hr at the same temperature. The digest was analyzed by paper chromatography (Whatman No. 3MM) in *n*-butyl alcohol–acetic acid–water (25:6:25). The DNP peptides were identified by the yellow color and the ninhydrin-positive reaction. The migration of DNP-oligolysine markers relative to lysine, R_{Lys}, was: DNP-Lys_4, 1.0; DNP-Lys_3, 1.5; and DNP-Lys_2, 2.8. The identity of the main digestion product was established by high-voltage electrophoresis on CM-cellulose paper (Whatman, CM82) (Yaron and Sober, 1965) in 0.062 M pyridine acetate (pH 3.5), 64 V/cm, for 2 hr. Under these conditions the DNP-Lys_5, DNP-Lys_6, and DNP-Lys_7 markers are well separated. The main digestion product migrated identically with DNP-Lys_6.

The digest contained very small amounts of products migrating on paper chromatogram similar to DNP-tetralysine and DNP-trilysine and a spot migrating at a position between DNP-Lys_2 and DNP-Lys_3. These yellow spots were barely observable and comprised less than 3% (by visual comparison with known amounts of α-DNP-Lys_2 run on the same sheet) of the applied amount (300 mμmoles) of the tested digested peptide. No spot corresponding to DNP-Lys_2 was observed. DNP-nona-L-lysine digested and analyzed under similar conditions was shown to produce DNP-Lys_2 and lysine as the final products.

The digestion products obtained from α-DNP-nonalysine (L_4DL_4) were examined also by quantitative ion-exchange chromatography on a 1.3 × 40 cm CM-cellulose column with a constant-volume (300-ml) gradient from 0.2 to 1.0 M NaCl at a 40-ml/hr rate. The ninhydrin color of the effluent was developed and recorded using the Technicon analyzer according to Cadavid and Paladini (1964). Authentic α-DNP-hexa-L-lysine and α-DNP-nonalysine were used as standards. α-DNP-hexalysine emerged at 300 min with an integration constant per micromole of 235; α-DNP-nonalysine emerged at 400 min with an integration constant per micromole of 372. The amount of lysine formed was determined in a separate run on the amino acid analyzer. The applied digest was derived from 0.164 μmole of α-DNP-nonalysine (L_4DL_4). Two major products were obtained, namely lysine (0.49 μmole) and α-DNP-hexalysine (0.15 μmole). A small amount of α-DNP-Lys_7, α-DNP-Lys_8, and α-DNP-Lys_9 were also observed on the chromatogram. Their amounts were estimated from the heights of the very small peaks to be approximately 0.006, 0.003, and 0.002 μmole, respectively.

Tryptic Digestion. α-DNP-nonalysine (L_4DL_4) in 0.07 M collidine buffer (pH 7.5) (5.1 mg/ml) was incubated with trypsin (36 μg/ml, purchased from Worthington) at 37° for 2 hr. The digest was analyzed by high-voltage electrophoresis on CM-cellulose paper (Yaron and Sober, 1965) in 0.062 M pyridine acetate (pH 3.5), 63 V/cm, 45 min. The products obtained, detected by their yellow color and by the positive ninhydrin reactions, were well separated and migrated as DNP-Lys_3, Lys_4, and Lys_2. The position of the yellow spot of DNP-Lys_3 was between Lys_3 and Lys_4.

Poly-ε-p-nitrobenzyloxycarbonyl-L-lysine. Poly-L-lysine hydrochloride ($\overline{DP}$ = 20, 270 mg) was dissolved in ice-cold sodium hydroxide (2 M, 4.6 ml) and a solution

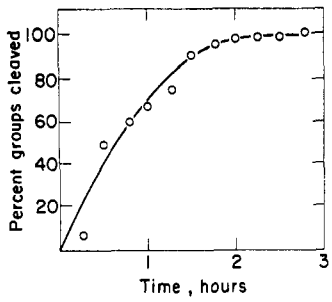


FIGURE 1: Cleavage of ε-*p*-nitrobenzyloxycarbonyl groups from poly-*p*-nitrobenzyloxycarbonyl-L-lysine with $SnCl_2$ in 45% NBr–acetic acid. The reaction was followed by determination of carbon dioxide evolved; blank values of acidity titrated at identical conditions without the polymer are substracted. Details given in text.

of *p*-nitrobenzyloxycarbonyl chloride (Carpenter and Gish, 1952) in dioxane (700 mg in 0.5 ml) was added. The reaction mixture was stirred for 10 min in an ice bath, collected in a sintered-glass filter funnel, and washed with water and a small amount of alcohol and ether; yield, 460 mg (90% of theory) of the material dried *in vacuo* over concentrated H_2SO_4. The product gave a negative ninhydrin test.

Determination of the Extent of Cleavage of ε-N-Benzyloxycarbonyl Groups by HCl in Acetic Acid. Poly-ε-*N*-Z-L-lysine ($\overline{DP}$ = 1200, 200 mg) was dispersed in 0.8 M HCl in acetic acid (10 ml) and then shaken at room temperature for different time intervals. The reaction mixture was freed of most HCl *in vacuo* on a rotary evaporator at 30° for 1 min and then freeze dried. The dry residue (128 mg) was dissolved in dimethylformamide (4.0 ml), fluorodinitrobenzene (0.05 ml) and triethylamine (0.05 ml) were added, and the reaction solution, protected from light, was left at room temperature for 2.5 hr. Dilute HCl was added to the solution cooled in an ice bath. The yellow solid formed was collected on a filter, washed with water to neutral reaction, with small amounts of alcohol and ether, and dried *in vacuo* over concentrated H_2SO_4. The absorption of the product at 350 mμ in dichloroacetic acid (0.4 mg/ml) was measured on a Beckman DB-G spectrophotometer in a 1.0-cm cell. The absorption coefficient for ε-*N*-DNP-L-lysine-HCl ($E_{3500\,Å}$ 14,700 in dichloroacetic acid at 350 mμ) was used for calculating the amount of DNP groups in the poly-Z-lysine. The procedure described was followed also to determine the extent of cleavage in 0.5 M HCl in acetic acid and the cleavage extent of *p*-nitrobenzyloxycarbonyl groups from poly-ε-*p*-nitrobenzyloxycarbonyllysine in 0.8 M HCl in acetic acid.

Cleavage of p-Nitrobenzyloxycarbonyl Groups with Stannous Chloride in HBr–Acetic Acid. Poly-*p*-nitrobenzyloxycarbonyl-L-lysine (11.5 mg, 37.5 base μmoles) was dissolved in dichloroacetic acid (0.5 ml) and a solution of anhydrous $SnCl_2$ in 45% HBr–acetic acid reagent (100 mg/ml, 2 ml) was introduced into the reaction vessel at room temperature and the reaction was followed by determination of CO_2 evolved as described by Yaron *et al.* (1964b). A blank was run at identical conditions without the polymer. From Figure 1 it can be seen that the reaction was complete after 2 hr.

Immunization. Guinea pigs of the Hartley and inbred strain 2 weighing 300–400 g were used. The α-DNP-nonalysines were diluted in buffered saline and emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.). Animals were injected with a total of 0.4 ml containing 0.1 mg of the material to be tested. The injections were distributed equally in the hind footpads. At the end of 1 week animals were reinjected with 0.1 mg of the antigen made up in Freund's incomplete adjuvant (0.5 ml) and administered subcutaneously in the dorsum of the neck. In addition to the standard immunization schedule, Hartley and strain 2 animals were injected, as outlined above, with α-DNP-nonalysine (L_4DL_4) at doses varying from 2 μg to 1.5 mg of material.

Skin Tests. The flanks of guinea pigs were carefully shaved and 0.1 ml of a buffered saline solution containing 10 μg of the material used for immunization was injected intradermally. Animals immunized with 2 μg of antigen were initially skin tested with 1 μg of antigen. The test sites were observed and measured at 3–6, 24, and 48 hr. In general, immediate reactions (Arthus type) were noted at 3–6 hr and they showed edema, erythema, and hemorrhage and necrosis, whereas delayed reactions (24–48 hr) were characterized only by erythema and induration. In control guinea pigs (animals not injected or injected with complete adjuvant alone) or in nonresponding animals, the test injection produced a slight erythematous reaction generally no larger than 5 mm which was apparent by 3 hr and disappeared by 24 hr. Guinea pigs were first skin tested 2–3 weeks after immunization. Nonresponding animals were checked again at 1 month and every 2 weeks for a period of 3 months. Some animals were followed for a period of up to 6 months. In addition to the skin tests with α-DNP-nonalysines all animals were skin tested on one occasion with 1:5000 old tuberculin in 0.1 ml of buffered saline. Blood samples were obtained at 3–4 weeks and at the end of 3 months by cardiac puncture. Animals shown not to respond to α-DNP-nonalysine (L_4DL_4) were then injected with the L_9 stereoisomer in the front footpads with an amount of antigen in complete Freund's adjuvant comparable with that used with the L_4DL_4 compound. Skin tests were then repeated in these animals 3 weeks following immunization in the front footpads.

Circulating Antibody Studies. Passive cutaneous anaphylaxis was performed with selected sera from responding animals (Ovary, 1958). Albino guinea pigs (250 g) of the Hartley strain were injected intradermally at five sites with 0.1 ml of serum or serum dilutions and injected intracardially 4–5 hr later with 100–200 μg of the test antigen (α-DNP-Lys_9-L_9, or α-DNP-Lys_9-L_4DL_4) in 1.0 ml of buffered saline containing 0.5% Evans blue. The responses were measured at 30 min. Positive reactions were in general larger than 10 mm in diameter and were only accepted if the saline site and control site in the same animal were negative. All reactions recorded as negative occurred in animals which had at least one additional site which was positive. Quantitative precipitin determinations were performed with α-DNP-$(Lys)_{11}$-BPA and DNP-HSA as the precipitating anti-

gen as previously outlined (Schlossman and Levine, 1967).

Results

Synthesis. SOLID-PHASE SYNTHESIS OF α-*N*-DNP-NONALYSINE (L_4DL_4). α-*N*-DNP-nonalysine with the stereospecific sequence L_4DL_4 was synthesized by the solid-phase method developed by Merrifield (1964b). Nine ϵ-benzyloxycarbonyllysine residues were assembled in a linear sequence while bound through the carboxyl of the C-terminal lysine to the solid-phase resin. The α-amine of the N-terminal residue was then dinitrophenylated and the peptide released from the resin with HBr in trifluoroacetic acid. The product was analyzed quantitatively by ion-exchange chromatography (Sober, 1962; Stewart and Stahmann, 1962a,b) on CM-cellulose (Peterson and Sober, 1956) for the presence and amounts of any homologous lysine oligomers formed as by-products. The presence of the main peak of α-DNP-nonalysine (35%) of shorter peptides (22%) and of peptides containing more than nine lysine residues (43%) can be observed in the chromatogram (Figure 2).

These findings may be interpreted as follows. Short peptides result from incomplete reactions. Molecules with more than nine residues are a consequence of a side reaction in which a small number of ϵ-benzyloxycarbonyl-protecting groups are cleaved off during the deblocking of α-*t*-BOC groups with HCl in acetic acid. Under such circumstances the ϵ-amino groups released can react with α-*N*-*t*-BOC-ϵ-*N*-Z-lysine (threefold excess) in the subsequent coupling step. Branched peptides of chain length $n = 2(9 - m) + m = 18 - m$ are formed. Here m is the residue number at which the branching started in a given molecule, the lysine residue next to the resin being counted as number one. (Double branching is negligible under the present conditions; see below.) It follows that the shortest branched molecule (formed when branching occurs at the eighth residue) will have 10 residues, and the longest (formed when the branching occurs at the first residue), 17 residues. In support of this assumption is the observation that lysine oligomers up to Lys_{17} are found by chromatography (Figure 2). It follows also that the Lys_9 fraction is free of branched molecules. The amount of branched peptides from Lys_{10} to Lys_{17} decreases as seen in Figure 2. This confirms the above mechanism since formation of the heavier branched molecules is initiated at the earlier stages of the synthesis, when the number of potential branching sites is still small.

The sensitivity of ϵ-benzyloxycarbonyl groups to hydrochloric acid in acetic acid was demonstrated by treating poly-ϵ-Z-lysine with hydrochloric acid in acetic acid for different time intervals at two concentrations (0.5 and 0.8 M) of hydrochloric acid. The number of ϵ-amino groups liberated was determined by measuring the optical density of the dinitrophenylated product in dichloroacetic acid at 350 mμ. A molar extinction coefficient E_{3500A} of 14,700 for ϵ-DNP-lysine monohydrochloride in dichloroacetic acid was determined and used for the calculations. The fraction of Z groups cleaved as a function of time is presented in Figure 3. It can be seen that

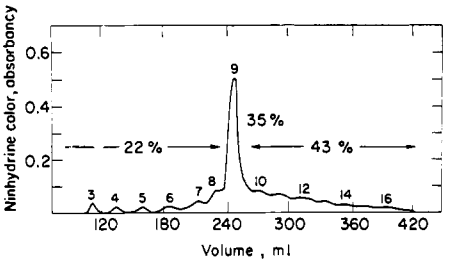


FIGURE 2: Chromatography of crude α-*N*-DNP-nonalysine (2.0 mg) obtained by the solid-phase synthesis method. The protecting *t*-BOC groups were cleaved with 1.0 M HCl in acetic acid. A CM-cellulose column, 1.3 × 40 cm in Na^+ form, equilibrated with 0.1 M NaCl was used with a constant-volume (300-ml) gradient from 0.2 to 1.0 M NaCl at a 30-ml/hr flow rate. The effluent was monitored by developing and recording the ninhydrin color automatically, using the Technicon amino acid analyzer under conditions described by Cadavid and Paladini (1964).

0.78% of the ϵ-Z groups are cleaved in 0.8 M HCl in acetic acid during 30 min. Calculation shows that even this is enough to account for a product containing 35% branched peptides. The extent of decarbobenzoxylation in 0.5 M HCl in glacial acetic acid was 3% after 5 hr corresponding to 0.3% during 30 min. This should lead to 12% of branched oligomers after nine deblocking steps. In view of this result nonalysine was prepared by the solid-phase method with α-*t*-BOC-ϵ-Z-lysine, using 0.5 M HCl in acetic acid in the deblocking reactions. Analysis by ion-exchange chromatography showed that indeed very little branched peptides Lys_{10}–Lys_{17} were formed. The content of low molecular weight peptides was 20% as with the original system, so that the percentage of Lys_9 rose to 75%.

The use of *O*-nitrophenylsulfenyl groups (Zervas *et al.*, 1963; Najjar and Merrifield, 1966; Kessler and Iselin, 1966), for the protection of the α-amines was also investigated. This protecting group can be removed under very mild acid conditions (5 min, 0.3 M HCl in acetic acid was used here) so that the branching reaction does not occur. However, it was observed that a higher proportion of low peptides was obtained (43%). Nevertheless, because of the ease of preparation of the α-*N*-NPS-ϵ-*N*-Z-lysine and because its DCHA salt is crystalline, this method was used for the introduction of the D-lysine residue.

An alternative solution to the branching problem is the use of more stable groups for the protection of the ϵ-amines. With the *p*-nitrobenzyloxycarbonyl group (Carpenter and Gish, 1952), negligible cleavage from poly-ϵ-*N*-*p*-nitro-Z-lysine in 1.0 M HCl in acetic acid was observed after 5 hr. This group can be cleaved from the final product by catalytic hydrogenation or with anhydrous $SnCl_2$ in HBr–acetic acid (see Experimental Section). Although the synthesis of α-*N*-DNP-nonalysine with α-*N*-*t*-BOC-ϵ-*N*-NO_2-Z-lysine was not undertaken, it seems reasonable to use it for the preparation of lysine-containing peptides by the solid-phase method.

In view of the described heterogeneity of the product obtained by the solid-phase synthesis of α-DNP-nonalysine, pure material could be obtained only after effective fractionation. This was achieved by preparative

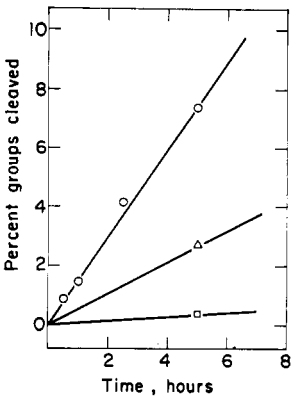


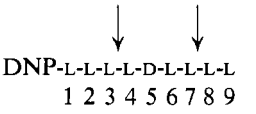
FIGURE 3: Cleavage of benzyloxycarbonyl groups from poly-benzyloxycarbonyl-L-lysine in 0.8 (○) and 0.5 M (△) HCl in acetic acid and of *p*-nitrobenzyloxycarbonyl groups from poly-*p*-nitrobenzyloxycarbonyllysine in 1.0 M HCl in acetic acid (□). The values are corrected by substracting blanks obtained with dinitrophenylated polymer that has not been treated with HCl in acetic acid. Details in text.

ion-exchange chromatography on CM-cellulose (Yaron *et al.*, 1964a). The pure α-*N*-DNP-nonalysine (L_4DL_4) migrated as a single component on a CM-cellulose column under conditions sufficient to separate completely the octa-, nona-, and decalysine. It is pertinent to note that under the same conditions an artificial mixture of α-DNP-nonalysine (L_4DL_4) and α-DNP-nona-L-lysine migrated in a single band.

For the preparation of α-*N*-DNP-nonalysine (L_4DL_4) one D-lysine residue was introduced in position four *via* ε-*N*-Z-α-*N*-NPS-D-lysine (Najjar and Merrifield, 1966). High optical purity of this compound was essential; even small amounts of the L antipode could not be tolerated, because of the antigenicity of α-*N*-DNP-nona-L-lysine. Based on the work of Parikh *et al.* (1958) an analytical procedure was worked out by which we could detect 0.01% of Z-L-lysine in Z-D-lysine. The Z-lysine was oxidized in suspension with whole venom of *Crotalus adamanteus*. The Z-ε-*N*-aminovaleric acid formed from the L form was recovered in the filtrate, decarbobenzoxylated and determined quantitatively on the amino acid analyzer. The oxidation procedure was also used for purification of commercial D-lysine. The purified product contained 0.01% ε-*N*-Z-L-lysine.

DIGESTION WITH CARBOXYPEPTIDASE B. Exhaustive digestion of α-DNP-nonalysine (L_9) was found to produce α-DNP-dilysine (1 mole) and lysine (7 moles). α-DNP-nonalysine (L_4DL_4), on the other hand, produced α-DNP-hexalysine (1 mole) and lysine (3 moles). Quantitative ion-exchange column chromatography of the digestion products obtained from α-DNP-nonalysine (L_4DL_4) showed that 91% of the α-DNP-nonalysine was converted into α-DNP-hexalysine. Though racemization cannot be regarded as completely absent, major contamination of α-DNP-nonalysine (L_4DL_4) by stereoisomers seems to be excluded. Product inhibition of the enzyme by lysine formed during the hydrolysis and the inhibitory influence of the D-lysine residue on neighboring peptide bonds might reduce the hydrolysis rate sufficiently to account, at least partially, for the presence of small amounts of α-DNP-Lys_7 (4%), α-DNP-Lys_8 (2%), and α-DNP-Lys_9 (2%) in the enzymatic digest. The absence of α-DNP-Lys_2 in the digest of α-DNP-nonalysine (L_4DL_4) demonstrates that no appreciable racemization of the D residue occurred, since this would have led to the formation of an all-L product. About 2% racemization can be detected by this method.

DIGESTION WITH TRYPSIN. Exhaustive tryptic digestion of α-DNP-nonalysine (L_9) produced α-DNP-trilysine, dilysine, and trilysine. α-DNP-nonalysine (L_4DL_4) with trypsin produced α-DNP-trilysine, tetralysine, and dilysine in equimolar quantities. The bonds cleaved are indicated in the formula. It is interesting to observe that the bond between residue six and seven,



positioned between two L residues, is not cleaved and subsequently trilysine is not formed as the result of the influence of the D configuration of lysine residue five. The absence of trilysine in the digest of DNP-nonalysine (L_4DL_4) supports the conclusion that racemization of the residue does not occur during the synthesis.

RESPONSE OF GUINEA PIGS TO INJECTION OF α-DNP-NONALYSINES. As is indicated in Table I none of 28 guinea pigs injected with α-DNP-nonalysine (L_4DL_4) in doses varying from 2 μg to 1.5 mg developed either skin sensitivity or circulating antibody to this compound. In contrast, 100% of strain 2 and 75% of Hartley guinea pigs readily responded to α-DNP-nona-L-lysine, the all-L stereoisomer. This latter compound provoked Arthus-type and delayed skin reactions averaging 15–20 mm in diameter, respectively. Sera from animals sensitized to α-DNP-nona-L-lysine were strongly positive in passive cutaneous anaphylaxis tests with either the all-L compound or its stereoisomer as the eliciting antigen. In addition, these sera could be shown in quantitative precipitin tests to have between 80 and 120 μg of antibody/ml. In an effort to determine whether prior immunization with α-DNP-nonalysine (L_4DL_4) prevented subsequent immunization with the all-L stereoisomer, 20 animals unresponsive to α-DNP-nonalysine (L_4DL_4) were injected after 3 months in the front footpads with a comparable amount of α-DNP-nona-L-lysine. Thir-

TABLE I: Response of Hartley and Strain 2 Guinea Pigs to Injection of Stereospecific α-*N*-DNP-nonalysines

Antigens	No. of Animals Responding	
	Strain 2	Hartley
α-*N*-DNP(L-Lys)$_4$-D-Lys-(L-Lys)$_4$	0/14	0/14
α-*N*-DNP(L-Lys)$_9$	12/12	8/12

teen of such animals responded to the all-L compound by the development of both skin sensitivity and circulating antibody. Further, although unresponsive to α-DNP-nonalysine (L_4DL_4), all 28 animals had on at least one occasion a positive delayed reaction to old tuberculin (averaging 20 mm), a component present in the complete Freund's adjuvant mixture. To test the role of hapten and oligolysyl carrier in the specificity of Arthus-type and delayed skin responses, eight guinea pigs sensitized to α-DNP-nona-L-lysine were skin tested with α-DNP-nonalysine (L_4DL_4). While α-DNP-nona-L-lysine provoked both Arthus-type and delayed reactions averaging 15–20 mm, the D-containing stereoisomer elicited only an Arthus-type reaction (12 mm) but no delayed reactions. These findings are consistent with the view that the hapten alone provides sufficient binding energy to react with circulating antibody and provoke an Arthus-type reaction. It is also apparent that the chemical nature of the antigenic determinant and not its size alone will determine whether a substance can elicit a delayed response. The exquisite specificity of the delayed response to both the DNP group and the oligolysyl carrier supports earlier observations demonstrating that only an immunogenic molecule can provoke a delayed response in this system (Schlossman *et al.*, 1966). Further, it can be concluded that the reaction between antigen and the sensitized cells mediating the delayed response involves the participation of a highly stereospecific receptor for the oligolysyl peptide carrier of the immunizing antigen.

Discussion

In the present study it is shown that α-DNP-nona-L-lysine as reported previously (Schlossman *et al.*, 1965) is immunogenic in inbred strain 2 and random bred Hartley guinea pigs. α-DNP-nonalysine (L_4DL_4), on the other hand, cannot induce the immune response. Thus, the substitution of one central L-lysine residue of α-DNP-nona-L-lysine by the corresponding D residue leads to a major change in the immunogenicity of this compound. Similarly, it has been shown that both hapten-substituted homopolyamino and copolyamino acid antigens composed exclusively of D-amino acids were extremely poor immunogens when compared with their all-L counterparts (Gill *et al.*, 1963, 1967; Maurer, 1964; Sela, 1966). In contrast to the L polymers, the usual amounts of D-amino acid polymers used for immunization produced immunologic paralysis in rabbits or mice. However, small amounts of D polymers did provoke the formation of D-specific antibody in these animals (Gill *et al.*, 1967; Janeway and Sela, 1967). In an attempt to correlate the *in vivo* fate of polypeptide antigens with their immunogenic capacity, it was demonstrated that D polymers were slowly degraded and retained in the tissues of the host animal, whereas the corresponding L polymers were rapidly degraded and eliminated (Gill *et al.*, 1964; Carpenter *et al.*, 1967). These findings supported the hypothesis that the diminished immunogenicity of D-polypeptide antigens resulted from persistence of undigested antigen and induction of immunologic paralysis at a critical phase of antibody biosynthesis (Zubay, 1963). The importance of antigen degradation to immunologically active fragments as a preliminary processing step in antibody formation has been the subject of much discussion (Garvey and Campbell, 1957; Rittenberg and Nelson, 1960; Fishman, 1961; Benacerraf *et al.*, 1963; Fishman and Adler, 1963; Robbins *et al.*, 1963). Although such theories are attractive, they are difficult to reconcile with the accumulating immunochemical evidence demonstrating that the antibody formed to numerous protein, carbohydrate, and polypeptide antigens is specific for the conformation and fine structure of the immunizing antigen (see Haber *et al.*, 1964; Kabat, 1966). It seems unreasonable to expect that an extensively degraded antigen still retains the capacity to provoke the formation of antibody specific for native antigen. Other indications suggesting that antigen degradation need not be a preliminary step in antibody formation have been obtained in the α-DNP-oligolysine system. It was previously observed that peptides equal to or larger in size than α-DNP-hepta-L-lysine were immunogenic; smaller α-DNP-oligo-L-lysines were not immunogenic even after prolonged immunization. Further, the antibody formed to α-DNP-$(Lys)_9$ or α-DNP$(Lys)_{11}$ was maximally inhibited in quantitative inhibition studies by the heptamer (Schlossman and Levine, 1967; Schlossman *et al.*, 1968). These data were interpreted to indicate that the heptamer was the largest segment of the immunizing antigen recognized by anti-α-DNP$(Lys)_9$ and α-DNP$(Lys)_{11}$ antibody. Numerous studies aimed at determining the maximal size for an antigenic determinant with similar techniques but with polysaccharide, polynucleotide, and polypeptide antigens have yielded results comparable with those obtained in the α-DNP-oligolysine system (Kabat, 1966). Although such studies do not permit an estimation of the smallest size of an antigenic determinant, they do indicate that the antibody-forming apparatus processed a determinant at least equal in size to α-DNP-hepta-L-lysine. Of considerable interest was the observation that the maximal size for an antigenic determinant, as determined by quantitative inhibition studies, was identical in size with the smallest antigen capable of inducing the immune response. While such studies may be interpreted as supporting the hypothesis that antigen degradation to a small immunogenic fragment occurs, it was shown in a subsequent study that the antibody provoked by α-DNP$(Lys)_{\overline{60}}$ and α-DNP$(Lys)_{\overline{1200}}$ was specific for the conformation of these larger α-DNP-oligolysines and differed in its specificity from anti-α-DNP$(Lys)_9$ or α-DNP$(Lys)_{11}$ antibodies (Schlossman *et al.*, 1968). In addition, it was shown that *in vitro* hydrolysis of α-DNP$(Lys)_{60}$ yielded fragments which were immunogenic, but no longer capable of inducing the formation of antibody specific for the conformation of the parent molecule. However, the antibody formed to hydrolyzed α-DNP$(Lys)_{\overline{60}}$ was identical in its behavior with antisera to α-DNP$(Lys)_9$ or α-DNP$(Lys)_{11}$. Under such circumstances it did not appear that *in vivo* degradation of larger α-DNP-oligolysines to smaller ones occurred prior to the induction of the immune response. If the antibody-forming cell can process a large determinant with conformation or a small one as the hep-

tamer, the dramatic change in the immunogenicity caused by the introduction of a single D-lysine residue at the fifth L-lysine position of α-DNP-nona-L-lysine cannot be solely attributed to slow digestion by host enzymes to an immunogenic fragment. In fact, both trypsin and carboxypeptidase B readily digest these peptides into a size too small to induce the immune response in the α-DNP-oligolysine system. Although various quantities of α-DNP-nonalysine (L_4DL_4) did not provoke a detectable immune response, it can be argued that more sensitive techniques would have demonstrated antibody and that the compound is, indeed, weakly immunogenic. Granting this latter assumption, we still have to account for the marked difference in immunologic "behavior" of these stereoisomers in both the induction of the immune response and the eliciting of an established cell-mediated delayed hypersensitivity reaction. The failure of α-DNP-nonalysine (L_4DL_4) to provoke an immune response would then imply the existence of a stereospecific receptor on an immunologically competent cell or some carrier molecule capable of reacting with the oligolysine backbone of the immunizing antigen. This first nondegradative recognition step would in a yet unknown manner set the stage for subsequent induction of the immune response, immunologic paralysis, or eliciting of the established delayed response. While it would appear that the postulated receptor is highly specific in its requirements for a minimum of seven L-lysines and appears to be inherited as a genetic Mendelian dominant trait in the guinea pig (Levine *et al.*, 1963), the nature of the hapten, its position, and the bond by which it is coupled to the oligolysine carrier seem to be less critical. Any postulated receptor would have to account for the fact that α-DNP-L$(Lys)_9$, L$(Lys)_8$-ε-DNP-L-(Lys), L$(Lys)_9$-butylamide, and L-$(Lys)_4$-ε-DNP-L-Lys-L-$(Lys)_4$ are all immunogenic (Schlossman *et al.*, 1965, 1966; S. F. Schlossman and A. Yaron, unpublished observations), whereas α-DNP-L$(Lys)_6$ and α-DNP-nonalysine (L_4DL_4) cannot induce the immune response. Although the genetic constitution of the host animal and the chemical characteristics of the immunizing antigen are clearly crucial for the induction of the immune response, the exact mechanism by which antigen is processed and in turn induces the production of antibody and delayed hypersensitivity or in fact immunologic paralysis is at present far from clear.

Solid-Phase Synthesis. This method developed by Merrifield (1962) was chosen for the step-by-step synthesis of nonalysines because of the advantage in terms of yield and speed in preparation. The simple way of removing by-products, namely filtering off the reaction solutions, reduces the tedious purification procedures performed after each step in classical peptide synthesis. We found, however, that a considerable amount of by-products, which are attached to the insoluble matrix, can be formed. Such by-products can be removed only after the whole required sequence has been assembled and cleaved from the insoluble polymer. Because of the similarity of molecules with incomplete sequence, or with branched chains, to the main product, very effective fractionation procedures are required. In our case ion-exchange chromatography on CM-cellulose was very efficient in detecting and removing by-products. The observed partial cleavage of ε-benzyloxycarbonyl groups which led to the formation of branched peptides was not noted previously because in those instances in which lysine peptides have been prepared by the solid-phase method (Merrifield, 1964a) the lysine residue was incorporated near the N-terminal of the molecule. There was therefore only limited exposure of the ε-benzyloxycarbonyl group to hydrochloric acid and consequently a very limited decarbobenzoxylation occurred. It is, however, advisable to take the branching reaction into account when incorporation of lysine is followed by a number of additional amino acids. Avoiding the formation of molecules with incomplete sequence would require absolute purity of reactants and completeness of all reactions involved. No side reactions can be tolerated if synthesis of long-chain peptides is attempted. As the synthesis proceeds, short peptides, as observed here, soon become a considerable part of the product, even when they are formed to a very minute extent at each step. It may perhaps be worthwhile to consider modifying the solid-phase synthesis in such a way that preformed peptides are added at each step. It should then be easier to remove by-products arising from side reactions, since these would be fewer in number, if not in quantity, and also differ more in their properties from the main product.

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