Watkins, W. M. (1960), *Bull. Soc. Chim. Biol. 42*, 1599.
Watkins, W. M. (1962), *Immunology 5*, 245.
Watkins, W. M. (1964), The Red Blood Cell, New York, Academic, chapt. 10.
Whistler, R. L., and BeMiller, J. N. (1958), *Advan. Carbohydrate Chem. 13*, 289.

Immunogenicity of a Series of α,*N*-DNP-L-Lysines*

Stuart F. Schlossman,† Arieh Yaron,‡ Shlomo Ben-Efraim,§ and Herbert A. Sober

ABSTRACT: A homologous series of α,*N*-DNP-oligo-L-lysines, ranging in size from the tetramer to the nonamer, were prepared from poly-ϵ,*N*-carbobenzoxy-L-lysine ($\bar{n}$ = 5.7) by reaction with dinitrofluorobenzene to substitute the single α-NH_2 position of each chain. The ϵ,*N*-carbobenzoxy groups were removed, the mixture of α,*N*-DNP-oligo-L-lysines was resolved according to peptide chain length by CM-cellulose chromatography, and the separated materials were used for immunization in Hartley, strain 2, and strain 13 guinea pigs.

This homologous series of compounds, because of the chemical definition in both positions of the hapten and peptide chain length, provide obvious advantages over previously used materials in studies attempting to elucidate the chemical basis of immunogenicity. Immunogenicity was observed in Hartley and strain 2 guinea pigs with α,*N*-DNP-hepta-, octa-, and nona-L-lysine, whereas smaller α,*N*-DNP-oligo-L-lysines were not immunogenic. The L configuration and the presence of a hapten were also required for immunogenicity in this system. The same antigen, e.g., α,*N*-DNP-octa-L-lysine, induced the formation of both delayed and immediate sensitivity.

Synthetic polypeptide antigens have provided a powerful tool for studying the chemical basis of the antigenicity of proteins (Stahmann *et al.*, 1955; Sela, 1962, 1965; Maurer, 1964). For the most part, these studies have indicated that homopolymers of α-amino acids are not antigenic, whereas both linear and branched random copolymers or DNP- or other hapten-substituted polymers of amino acids may be antigenic. While the above-mentioned studies have provided important information as to the chemical basis of antigenicity, most studies were performed with materials which, though less complex than proteins, were in themselves still heterogeneous with respect to sequence and chain length.

Advances in the chromatography of proteins have recently been applied to the purification of lysine oligopeptides (Stewart and Stahmann, 1962; Sober, 1962) and now enable one to prepare oligolysines of known chain length (Yaron *et al.*, 1964). These peptides, when conjugated with haptens in a defined position, provide simple, chemically defined molecules whose immunogenicity can be studied.

The studies to be reported in this paper describe the preparation and immunogenic properties of a homologous series of α,*N*-DNP-substituted L-lysine oligopeptides. These materials were prepared so as to preclude antigen heterogeneity, either in peptide chain length or in hapten position, since each member of this series contains a DNP group in the single α-amino position of the oligopeptide; the ϵ-amino groups remain unsubstituted.

Materials

Poly-ϵ,*N*-benzyloxycarbonyl-L-lysine (LY-50) was prepared for us by Yeda, Rehovoth, Israel. The *N*-carboxy-α-lysine anhydride was polymerized in dioxane using butylamine as the initiator. This preparation had an average degree of polymerization ($\bar{n}$) of 5.7 as determined by anhydrous titration with perchloric acid. Poly-ϵ,*N*-benzyloxycarbonyl-L-lysine ($\bar{n}$ = 60) was also prepared for us by Yeda. Poly-ϵ,*N*-benzyloxycarbonyl-D-lysine ($\bar{n}$ = 80) was provided by Dr. M. Sela, Weizmann Institute, Rehovoth, Israel.

1-Fluoro-2,4-dinitrobenzene and triethylamine were obtained from Eastman Organic Chemicals and dimeth-

* From the Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare, U.S. Public Health Service, Bethesda, Md. *Received May 10, 1965.* A preliminary report (Schlossman *et al.*, 1965) has been published. Correspondence should be directed to this address.

† Present address: Department of Medicine, Harvard Medical School, Beth Israel Hospital, Boston, Mass.

‡ Visiting Scientist from Department of Biophysics, Weizmann Institute of Science, Rehovoth, Israel.

§ Visiting Scientist, National Institute of Allergy and Infectious Diseases, N.I.H., from the Israel Institute for Biological Research, Nes-Ziona, Israel.

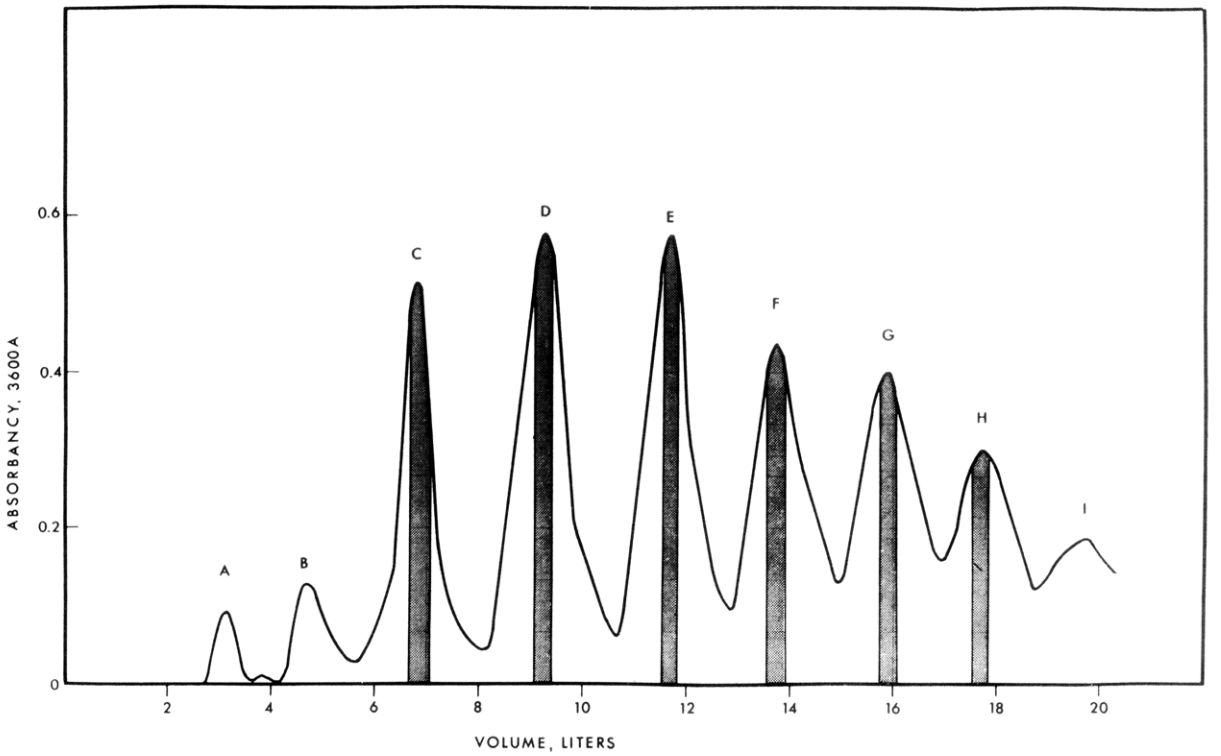

FIGURE 1: Preparative chromatography of α,*N*-DNP-oligo-L-lysines. See text under Methods for experimental details.

ylformamide, Reagent Grade, was obtained from Fisher Scientific Co.

Trypsin, three-times crystallized, was purchased from Worthington Biochemical Corp., and carboxypeptidase B was generously made available to us by Dr. J. E. Folk, National Institute of Dental Research, N.I.H.

CM-cellulose ("Standard" Selectacel, lot 1264, 0.6 meq/g) was obtained from Carl Schleicher and Schuell Co. The material sieved to mesh size 100–230 was used. The adsorbent was successively washed with 0.25 M LiCl, 0.25 M LiOH, 1 M HCl, ethyl alcohol, and 0.25 M LiCl–0.25 M LiOH according to the method of Peterson and Sober (1956), and finally washed with starting buffer until the effluent corresponded in conductivity and *p*H to the starting buffer. During the washing procedure fines were discarded.

Freund's Complete and Incomplete Adjuvant were purchased from Difco Laboratories, Detroit, Mich.

Freund's Complete Adjuvant was also prepared with killed mycobacteria strain H 37RV, 10 mg/ml in Bayol F and Arlacel A (85:15). Bayol F and Arlacel A were obtained from Atlas Chemical Industries.

Spectrophotometry. A Beckman DU spectrophotometer with silica cells of 1 cm light path was used for all spectrophotometric determinations which were made in 0.01 M sodium phosphate buffer, *p*H 7.0, at 3600 A.

Thin-layer chromatography was performed according to standard methods using a cellulose support (cellulose MN 300, Macherey, Nagel and Co., Düren, Germany) and either solvent I (butanol–pyridine–acetic acid–water, 30:20:6:24) or solvent II (0.05 M sodium borate buffer, *p*H 9.2).

Methods

Preparation of α,N-DNP-oligo-L-peptides. Poly-ε,-*N*-carbobenzoxy-L-lysine butylamide (1.04 g) was dissolved in 40 ml dimethylformamide, and 0.4 ml dinitrofluorobenzene and 0.4 ml triethylamine were added. The reaction was allowed to proceed overnight at room temperature. A yellow product was precipitated by the addition of 50 ml of 3 M HCl, washed extensively with 3 M HCl and water on a sintered glass filter, and then dried *in vacuo* over H_2SO_4.

The ε,*N*-carbobenzoxy groups were then removed from α,*N*-DNP-poly-ε,*N*-carbobenzoxy-L-lysine by reaction with 30% HBr in acetic acid for 40 minutes at room temperature (Ben-Ishai and Berger, 1952). The yellow, water-soluble product was isolated by ether precipitation and purified by dissolving in water, filtration, repeated precipitation from water with ethyl alcohol, and by precipitation from a methyl alcohol solution with acetone-ether. Finally, the isolated material was thoroughly washed with ether and dried *in vacuo* over KOH and concentrated H_2SO_4. The yield was 670 mg (70%). An average chain length ($\bar{n}$) of 8.4 was obtained from the molar extinction of α,*N*-DNP-lysine and the lysine content (*vide infra*).

Preparative Chromatography. The α,*N*-DNP-poly-L-lysine–HBr mixture (500 mg) was resolved into its

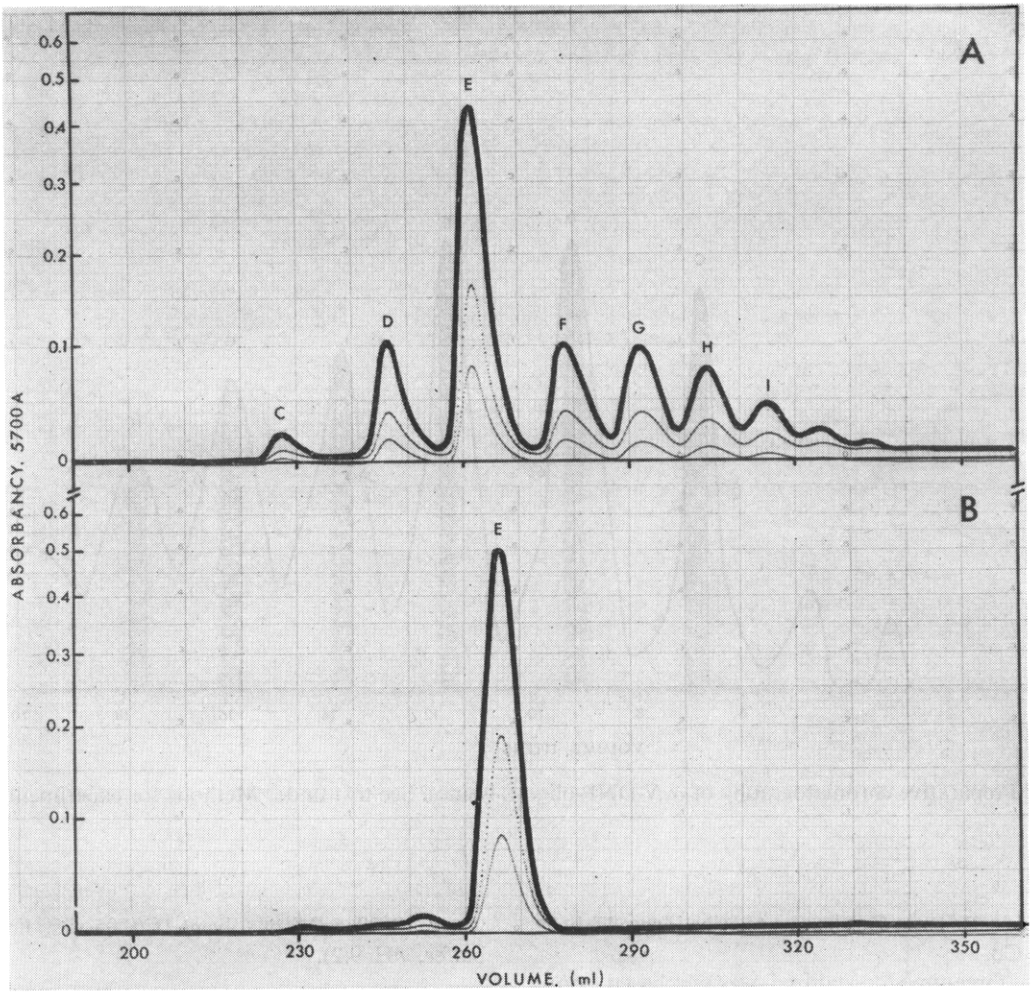

FIGURE 2: Analytical chromatography of peak E of Figure 1. (A) A mixture of the original, unfractionated α,*N*-DNP-oligo-L-lysine preparation (used in Figure 1) with an aliquot of peak E of Figure 1. (B) An aliquot of peak E alone. See text under Methods for experimental details.

individual oligopeptide components in a manner analogous to that used by Yaron *et al.* (1964). The material was neutralized with LiOH, dissolved in 10 ml of 0.05 M LiCl, *p*H 7.5, and applied to a large CM-cellulose column (5.8 × 66 cm) which previously had been equilibrated with 0.05 M LiCl. The sample was washed in under gravity with two portions of 0.05 M LiCl and followed by a 24-liter linear gradient of LiCl from 0.2 to 0.6 M at constant *p*H. The gradient was pumped onto the column at a flow rate of 540 ml/hr established by a Sigmamotor pump (Model 78). Fractions of 180 ml were collected and the effluent was examined at 3600 A. The resolution obtained is shown in Figure 1.

Desalting. Pooled fractions C (6698–7040 ml), D (9099–9439 ml), E (11,549–11,883 ml), F (13,566–13,904 ml), G (15,749–16,089 ml), and H (17,391–17,715 ml) were individually diluted with water to a conductivity corresponding to 0.05 M LiCl. The diluted solutions (2–3 liters) were passed through a small CM-cellulose column (1 × 10 cm), previously equilibrated with 0.05 M LiCl, at a flow rate of 150 ml/hr and washed copiously with water. The product was visible on the column as a yellow band occupying no more than one-half the column height and was recovered quantitatively in a volume of approximately 5 ml by elution with 0.1 M HCl. The material was lyophilized to remove HCl. The yield was 8–12 mg per pooled fraction as determined by its absorption at 3600 A in 0.01 M phosphate buffer, *p*H 7.0.

Analytical Chromatography. A sample from desalted fraction E (0.75 mg) was dissolved in 10 ml of 0.01 M lithium acetate buffer, *p*H 5.0, and applied to a small CM-cellulose column (0.9 × 50 cm) which had previously been equilibrated with 0.01 M lithium acetate. A linear gradient of 600 ml in 0.01 M lithium acetate, *p*H 5.0, to 0.1 M LiCl was used for elution. The procedure was performed in a Beckman Model 120 amino acid analyzer at a flow rate of 30 ml/hr, and the effluent was continuously monitored by ninhydrin determination and recorded (Figure 2B). Similar runs were carried out with fraction E (0.58 mg) added to the original, unfractionated α,*N*-DNP-poly-L-lysine mixture (4 mg) ($\bar{n}$ = 8.4) (Figure 2A).

Determination of α,N-DNP-Oligo-L-lysine Chain Length. The absorption at 3600 A was determined prior to hydrolysis for each of the α,*N*-DNP-oligo-L-lysines prepared and the quantities of α,*N*-DNP-lysine residue calculated using an E_{3600A} value of 16,800. No change in the absorbance of α,*N*-DNP-nona-L-lysine at 3600 A was noted during and after complete enzymatic digestion with trypsin and carboxypeptidase B. The final digest contained products which migrated as α,*N*-DNP-dilysine and lysine by thin-layer chromatography with solvent I.

An aliquot of the α,*N*-DNP-oligo-L-lysine peptide was hydrolyzed in 6 N HCl for 16 hours at 105° in a sealed ampule. The hydrolysate was dried *in vacuo* over KOH and reconstituted to known volume with water, and the lysine content was determined on a Beckman Model 120 amino acid analyzer using the column procedure for basic amino acids (Moore *et al.*, 1958). Examination of the hydrolysates of each of the purified α,*N*-DNP-oligolysines revealed four peaks which emerged at 77, 112, 140, and 360 ml. These peaks correspond in position to lysine, ammonia, α,*N*-DNP-lysine, and butylamine, respectively. Hydrolysis of the monamer, α,*N*-DNP-lysine, under the same conditions for 14 or 24 hours did not yield lysine. One peak, corresponding in position to α,*N*-DNP-lysine (86% recovery), was seen after hydrolysis of α,*N*-DNP-lysine for 14 hours, whereas hydrolysis for 24 hours yielded 2 peaks, the major one corresponding in position to ammonia and the minor one to α,*N*-DNP-lysine (5% recovery). Since the recovery of α,*N*-DNP-lysine varied markedly with the length of hydrolysis, chain lengths were calculated from the spectrophotometrically determined molar amount of α,*N*-DNP-lysine (A) and the amount of lysine recovered after hydrolysis (B) according to the following formula: (A + B)/A = chain length.

The progressive increase in α,*N*-DNP-oligopeptide chain length is related to the order of emergence of the peaks as shown in Figure 3, and the plot indicates that the values obtained best fit the middle line *n*, even though occasional points are closer to the line *n* + 1. The scatter of the points result from an uncertainty of ±5% in the determination of the amount of lysine recovered following hydrolysis. The chain lengths of the purified α,*N*-DNP-oligopeptides shown in Figures 1 and 2 are as follows: peak C, α,*N*-DNP-tetralysine; peak D, α,*N*-DNP-pentalysine; peak E, α,*N*-DNP-hexalysine; peak F, α,*N*-DNP-heptalysine; peak G, α,*N*-DNP-octalysine; peak H, α,*N*-DNP-nonalysine.

α,*N-DNP-L-Lysine* was prepared from ε,*N*-carbobenzoxy-L-lysine by dinitrophenylation according to standard methods (Neuberger and Sanger, 1943; Sanger, 1945): by removal of the ε,*N*-carbobenzoxy-protecting group with 30% HBr in acetic acid, ether precipitation, and reprecipitation from water solution by pyridine. The isolated material moved as a single component identically with authentic α,*N*-DNP-lysine and differently from ε,*N*-DNP-lysine or dinitrophenol in thin-layer chromatography with solvent II. The melting point was 265° (dec); the literature value was 260° (dec)

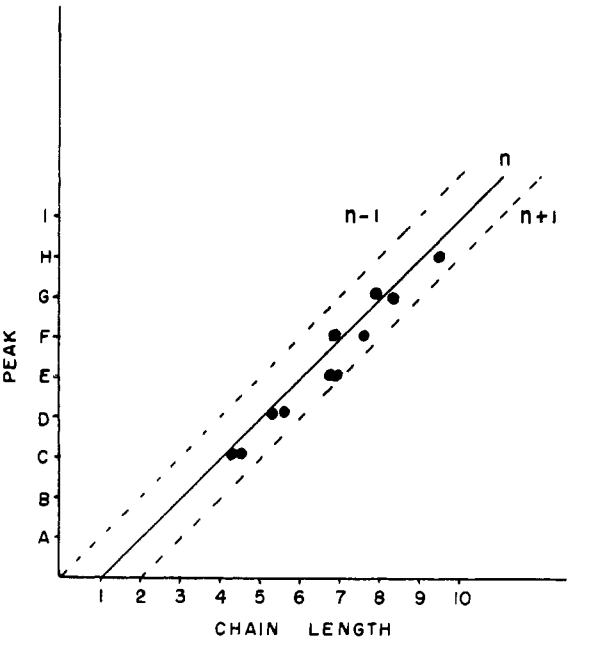

FIGURE 3: Assignment of chain length. Chain length is calculated from the amount of lysine plus DNP-lysine divided by the DNP-lysine content. See text under Methods for experimental details.

(Sanger, 1945). Analytical amino nitrogen (Van Slyke) yielded 15.5 μmoles N with theory calling for 15.2 μmole N. Ninhydrin CO_2 determinations with 3.26 mg yielded no CO_2; none would be expected. The molar extinction (E_{3600A}) was 16,800 in 0.01 M sodium phosphate, *p*H 7.0.

Preparation of α,N-DNP-Poly-L-lysine ($\bar{n}$ = *60*) *and α,N-DNP-Poly-D-lysine* ($\bar{n}$ = *80*). Samples of poly-ε,*N*-benzyloxycarbonyl-L-lysine ($\bar{n}$ = 60) and poly-ε,*N*-benzyloxycarbonyl-D-lysine ($\bar{n}$ = 80) were dissolved in dimethylformamide. Dinitrofluorobenzene and triethylamine were added as indicated above under preparation of α,*N*-DNP-oligo-L-lysines. Removal of the ε,*N*-carbobenzoxy groups and purification followed the above procedure.

Immunization

Guinea pigs of the Hartley strain and of inbred strains 2 and 13 weighing 300–400 g were used. The α,*N*-DNP-oligopeptides were diluted in buffered saline and emulsified with an equal volume of Freund's Complete Adjuvant. Each animal was 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 foot pads.

The Hartley strain was reinjected at the end of 1 and 2 weeks 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. Blood samples were obtained at 3, 4, and 5 weeks by cardiac puncture.

Inbred strains 2 and 13 were reinjected in the dorsum

of the neck with 0.1 mg of the material mixed in 0.5 ml of Freund's Complete Adjuvant approximately 3 weeks after the first injection and following a second intradermal skin test. Blood samples were obtained by cardiac puncture 10 and 20 days after the first injection and 10 days following the second injection.

Skin Tests. The flanks of guinea pigs were carefully shaved and depilated with Nair. One hour later, 0.1 ml of a buffered saline solution containing 10 μg of the material used for immunization was injected intradermally. The test sites were observed at 3–6 hours, 24 hours, and 48 hours. The immediate (Arthus) reactions were graded according to diameter as follows: 0, absent; ±, less than 5 mm; +, 5–10 mm; ++, 10–15 mm; +++, 15–20 mm; and ++++, greater than 20 mm. In general, immediate reactions showed edema, erythema, hemorrhage, and necrosis, whereas delayed reactions were characterized only by erythema and induration. The reactions were classified as "pure, delayed" if no immediate reactions were observed and if no appreciable change occurred in the extent of the reactions between 24 and 48 hours.

In control Hartley guinea pigs (animals not injected at all or injected with adjuvant alone) or in nonresponding animals, the test injection produced a pale erythematous reaction no larger than 5 mm at 3 hours and which disappeared by 24 hours. Such reactions were not observed in control guinea pigs of strains 2 and 13.

Hartley guinea pigs were first skin tested 2–3 weeks after the beginning of immunization. Nonresponding animals were checked again after additional immunizing injections. Guinea pigs of inbred strains 2 and 13 were skin tested 10 and 20 days after the beginning of immunization. Strain 13 animals were rechecked after one or two additional immunizing injections in Freund's Complete Adjuvant.

Passive Cutaneous Anaphylaxis. Albino guinea pigs (250 g) of the Hartley strain were injected intradermally at five sites with 0.1 ml serum or serum dilutions as outlined by Ovary (1958) and injected intracardially 4–5 hours later with 100–200 μg of the test antigen in 1 ml buffered saline containing 0.5% Evans Blue. The responses were measured at 30 minutes. Positive reactions were, in general, larger than 10 mm in diameter and were accepted only if a saline site and a control serum 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.

Systemic Anaphylaxis. Immunized and control animals were injected intracardially with 0.5 ml of a solution containing 0.5 mg of the antigen to be tested in buffered saline. Symptoms of anaphylaxis developed in positive animals within 5 minutes of injection and, in general, the animals died in anaphylactic shock within 10 minutes of injection.

Results

Preparative Chromatography. Figure 1 shows the chromatographic pattern of the α,*N*-DNP-oligolysyl peptides obtained on a preparative CM-cellulose column using a linear LiCl gradient. The elution pattern shows a series of successive peaks, labeled A to H, which emerge with increasing salt concentration in a manner analogous to that obtained by chromatography of oligolysines (Yaron *et al.*, 1964). Pooled fractions, indicated by the shaded areas, were desalted, concentrated, and used for further studies.

Analytical Chromatography. Figure 2B shows the elution pattern obtained on rechromatography of peak E from Figure 1 on a small CM-cellulose column with a linear LiCl gradient. The material appears essentially homogeneous, as it contains not more than 3% of the next smaller chain length and none of larger chain length. Figure 2A shows the elution pattern of an aliquot of peak E mixed with the original material and analyzed on an analytical CM-cellulose column under the same conditions. The elution diagram reveals that peak E corresponds to the third peak on the analytical but the fifth peak on the preparative chromatogram. This reflects the smaller amount of material used on the analytical column, the first two peaks of Figure 1 not being detected. Confirmation is afforded by analytical scale chromatography of peak C from the preparative column, under which conditions it appears as the first peak on the analytical-scale chromatogram.

Response of Hartley Strain Guinea Pigs to Injection of α,N-DNP-Oligolysylpeptides. The results in Table I indicate that fourteen out of sixteen animals tested developed immediate hypersensitivity (Arthus) when immunized with α,*N*-DNP-oligo-L-lysine ($\bar{n}$ = 8.4). The sera from all animals which developed immediate skin reactivity showed positive passive cutaneous anaphylaxis reactions in dilutions as high as 1:1000, whereas sera from nonresponding or control animals always gave negative passive cutaneous anaphylaxis reactions.

Passive cutaneous anaphylaxis reactions were usually performed with the homologous antigen, but could also be elicited with purified α,*N*-DNP-peptides ranging in size from the tetra- to the nonalysine.

Eight animals were tested for systemic anaphylaxis to intracardial injection of 500 μg of the antigen. Animals which had shown positive immediate skin reactivity either developed moderately severe anaphylactic symptoms ($^1/_8$) or died in anaphylaxis with 10 minutes of injection ($^7/_8$). Nonresponding animals or control animals developed no anaphylactic symptoms from this dose of antigen. From the presence of skin reactions at 3–6, 24, and 48 hours, it is probable that, in addition to immediate hypersensitivity, all sensitized animals also possessed the delayed type of skin reactivity.

With these Hartley strain guinea pigs, either α,*N*-DNP-nona-L-lysine or α,*N*-DNP-octa-L-lysine was able to sensitize 66% of the animals tested. The animals developed both immediate and delayed skin reactivity and their sera gave positive passive cutaneous anaphylaxis reactions. With α,*N*-DNP-hepta-L-lysine, only one of six animals could be sensitized, even after prolonged immunization. On the other hand, none of the animals immunized with homologous α,*N*-DNP-peptides smaller than the heptamer could be sensitized, even after pro-

TABLE I: Response of Hartley Strain Guinea Pigs to Injection of α,*N*-DNP-*O*ligolysines.

Antigens	Number of Animals			Passive Cutaneous Anaphylaxis Positive
	Tested	Skin Reaction		
		Immediate (3–6 hr, av)	Delayed (24 hr, av)	
α,*N*-DNP-oligo-L-lysine, $\bar{n}$ = 8.4	16	14 (4+)	14 (4+)	14
α,*N*-DNP-nona-L-lysine	6	4 (4+)	4 (4+)	4
α,*N*-DNP-octa-L-lysine	6	4 (3+)	4 (3+)	4
α,*N*-DNP-hepta-L-lysine	6	1 (2+)	1 (2+)	1
α,*N*-DNP-hexa-L-lysine	6	0	0	0
α,*N*-DNP-penta-L-lysine	6	0	0	0
α,*N*-DNP-tetra-L-lysine	6	0	0	0
α,*N*-DNP-poly-D-lysine, $\bar{n}$ = 80	10	0	0	0

TABLE II: Response of Strain 2 and 13 Guinea Pigs to Injection of α,*N*-DNP-oligolysines.

Antigens	Number of Animals			Passive Cutaneous Anaphylaxis
	Tested	Skin Reaction		
		Immediate (3–6 hr, av)	Delayed (24 hr, av)	
Strain 2				
α,*N*-DNP-oligo-L-lysine, $\bar{n}$ = 8.4	30	0	30 (2+)	0
α,*N*-DNP-nona-L-lysine	6	0	6 (1+)	0
α,*N*-DNP-octa-L-lysine	9	0	6 (1+)	0
α,*N*-DNP-hepta-L-lysine	10	0	0	0
α,*N*-DNP-hexa-L-lysine	5	0	0	0
α,*N*-DNP-penta-L-lysine	5	0	0	0
α,*N*-DNP-tetra-L-lysine	5	0	0	0
α,*N*-DNP-poly-D-lysine, $\bar{n}$ = 80	5	0	0	0
Strain 13				
α,*N*-DNP-oligo-L-lysine, $\bar{n}$ = 8.4	5	0	0	0

longed immunization (at least 3 months) and repeated skin testing.

In order to test the role of optical configuration in this system, ten animals were immunized with α,*N*-DNP-poly-D-lysine ($\bar{n}$ = 80). Even after prolonged immunization and skin testing no animals developed evidence of either delayed or immediate sensitivity whereas, in another group of animals, six of eight animals could be sensitized to a comparable antigen of the L configuration, α,*N*-DNP-poly-L-lysine ($\bar{n}$ = 60).

Response of Strain 2 and 13 Guinea Pigs to Injection of α,N-DNP-Oligolysines. All guinea pigs of strain 2 developed delayed skin reactivity after one injection of either α,*N*-DNP-oligo-L-lysine ($\bar{n}$ = 8.4) or α,*N*-DNP-nona-L-lysine in complete Freund's adjuvant, but only 66% of the guinea pigs could be similarly sensitized with α,*N*-DNP-octa-L-lysine (Table II). None of the strain 2 guinea pigs (0/25) could be sensitized with α,*N*-DNP-oligomers of shorter chain length. Even after repeated immunization and skin testing, animals did not develop sensitivity to peptides smaller than α,*N*-DNP-octa-L-lysine.

Animals which initially developed delayed skin reactivity after one injection of antigen ultimately developed (7–10 weeks) immediate skin reactivity, following two booster injections and three intradermal test injections. At the time these animals showed immediate skin reactivity, their sera also showed a positive passive cutaneous anaphylaxis reaction.

The role of optical configuration was also tested in strain 2 animals and five animals were immunized with α,*N*-DNP-poly-D-lysine ($\bar{n}$ = 80). Even after prolonged immunization and skin testing these animals did not develop evidence of either delayed or immediate sensi-

tivity, whereas in another group of animals all twelve could be sensitized with a comparable antigen of the L configuration, α,N-DNP-poly-L-lysine ($\bar{n}$ = 60). Strain 13 animals could not be sensitized to α,N-DNP-oligo-L-lysine ($\bar{n}$ = 8.4) even after repeated immunization and skin testing for periods up to 3 months.

Discussion

The use of individual members of a homologous series that progresses from no activity to full immunochemical activity affords an obvious advantage in studies of the chemical basis of antigenicity. The results obtained with the particular series used in this study, a homologous series of α,N-DNP-lysine oligopeptides, wherein each member of the series differs only in the number of lysine residues in the peptide chain, have shown that the chain length of the polypeptide is of critical importance for immunogenicity. In this system, the smallest antigen capable of inducing the immune response in guinea pigs was the heptamer, α,N-DNP-hepta-L-lysine (mw 1136). Smaller α,N-DNP-oligolysines were not antigenic even after prolonged immunization, whereas hapten-lysine oligopeptides, larger than the heptamer, e.g., α,N-DNP-$(\text{L-lys})_8$, α,N-DNP-$(\text{L-lys})_9$, α,N-DNP-$(\text{L-lys})_{\bar{60}}$, etc., were good antigens, producing antisera with about 50 μg of antibody nitrogen per milliliter. Previous investigators have reported (Sela *et al.*, 1962; Sela, 1965) that amino acid copolymers as small as 4000 in molecular weight are antigenic. In addition, low molecular weight hapten-substituted polypeptides, such as DNP-bacitracin (Abuelo and Ovary, 1964) and lightly azobenzenearsonylated-L-tyrosine peptides (F. Borek, Y. Stupp, and M. Sela, personal communication), have also been found to be antigenic. The minimal size requirement for immunogenicity may not always relate to the same chain length or molecular weight in every system. It may rather be related to the three-dimensional structure of the antigen molecule and probably varies from system to system.

In any case, the results obtained with the α,N-DNP-oligolysine system indicate that the addition of one lysyl residue (or two) to the six lysyl residues of the α,N-DNP-hexamer is sufficient to convert an immunogenically inactive molecule into an antigen capable of eliciting both immediate and delayed sensitivity in guinea pigs. Hence, with an oligomeric series differing only in the number of repeating residues per antigen molecule, one has a unique opportunity to study the transition between lack of antigenicity and full antigenicity.

Preliminary observations in this transition area have been obtained with strain 2 guinea pigs, which, in confirmation of Levine *et al.* (1963), have all shown immunochemical response to hapten-substituted polylysine antigens. However, as the chain length of the antigen is reduced not all of the individuals in the strain 2 population exhibit the same behavior, i.e., only 66% of the animals responded to the octamer whereas all strain 2 guinea pigs were sensitized by the nonamer, α,N-DNP-$(\text{L-lys})_9$. The partial response in this strain of guinea pigs to the octamer suggests the possibility of genetic heterogeneity even in these highly inbred animals, but may merely reflect the biological uncertainty of any limited assay procedure.

An additional observation is that both delayed and immediate sensitivity were induced by the same well-characterized, chemically defined structure. In view of the inherent heterogeneity of many other antigen systems, it has been possible that different antigens in the mixture were separately responsible for induction of delayed and immediate sensitivity. Strain 2 guinea pigs, because of the marked lag in their development of immediate sensitivity (6 weeks) as opposed to the prompt appearance of delayed skin reactions (10 days), have permitted observations of delayed sensitivity uncomplicated by the presence of circulating antibody. Strain 2 guinea pigs developed delayed sensitivity to the α,N-DNP-octa- or -nona-L-lysine which persisted for weeks prior to the onset of demonstrable immediate sensitivity (development of circulating antibody). Therefore, because of the homogeneity of the antigen and the biological separation of the two responses in strain 2 but not in Hartley strain guinea pigs, one can conclude that these two different biological manifestations of reaction to an antigen are not necessarily related to different antigens but rather to the animal's response to a single antigen, either as a maturation of the same process or reflecting specificity to different portions of the same antigen molecule. One must also consider that the antigenic determinants may be identical, but that differences in affinity to the antibody may regulate the appearance of the delayed or immediate response (Karush and Eisen, 1962).

It should be emphasized that every animal that showed the delayed sensitivity reaction ultimately developed circulating antibody. No immediate sensitivity was ever obtained in an animal that did not show a concomitant delayed reaction or had not shown the delayed reaction earlier in its postimmunization period.

Two other requirements for antigenicity have been noted in these experiments in confirmation of previous work (Maurer, 1963; Gill *et al.*, 1963; Kantor *et al.*, 1963): the L configuration and the presence of a hapten. We have found that neither α,N-DNP-D-polylysine ($\bar{n}$ = 80) nor unsubstituted poly-L-lysine were antigenic in either the Hartley strain or strain 2 guinea pigs.

The difference between antigenicity and nonantigenicity in the α,N-DNP-oligo-L-lysine series is, at most, two lysyl residues. The presence of these two additional ϵ-amino groups with the resultant increase in positive charge may be just enough to provide sufficient stability of electrostatic binding to some specific molecule or receptor site. However, it seems reasonable to exclude the electrostatic attachment of the α,N-DNP-oligo-L-lysine to a high molecular weight tissue or blood component as the major part of the immunogenic process since, in that case, one would have expected that the compound with the D configuration would have been antigenic as well. Amino acid copolymers of D- and L-amino acids have been shown to be immunogenic and D-specific and L-specific antibodies have been obtained

(Sela and Fuchs, 1964; Maurer, 1965). The requirement for the L configuration coupled with the lack of any immunogenic response in the strain 13 guinea pigs readily suggests that the ability of the host to metabolize the antigen is an essential part of the immunogenic process (Benacerraf *et al.*, 1963; Levine and Benacerraf, 1964). However, the nature of (or, in fact, the necessity of) binding to some other molecule has not been shown.

The inability of the pentamer and higher oligomers of lysine and arginine to penetrate the envelope of *Escherichia coli* mutants requiring lysine or arginine for growth has recently been reported by Gilvarg and Katchalski (1965). The relation of peptide chain length and permeability in mammalian systems is not yet known, but it is possible that the immunogenic activity of the α,*N*-DNP-oligo-L-lysines depends on their failure to penetrate the cell wall and their subsequent phagocytosis by macrophages, thus becoming capable of initiating the immune response (Fishman, 1961; Fishman and Adler, 1963).

It is rather provocative to learn from this study that the transition range from no activity to full immunogenic activity lies between the α,*N*-DNP-hexa- and -octa-L-lysine, since octalysine is the first member of the oligolysine series in which molecular conformation ("helicity") could be shown by optical rotatory dispersion measurements (unpublished results). Although Gill and Doty (1962) have concluded from studies with larger and more complex polypeptides that helicity was not necessary for immunogenicity, in view of the short oligopeptide sequence found to be immunogenic in this study, tertiary configuration or, at least, the ability to develop an ordered conformation may well be a requirement for immunogenicity.

References

Abuelo, G., and Ovary, Z. (1964), *Federation Proc. 23*, 247.

Benacerraf, B., Ojeda, A., and Maurer, P. H. (1963), *J. Exptl. Med. 118*, 945.

Ben-Ishai, D., and Berger, A. (1952), *J. Org. Chem. 17*, 1564.

Fishman, M. (1961), *J. Exptl. Med. 114*, 837.

Fishman, M., and Adler, F. L. (1963), *J. Exptl. Med. 117*, 595.

Gill, T. J., III, and Doty, P. (1962), *Polyamino Acids, Polypeptides, Proteins, Proc. Intern. Symp., Madison, Wis., 1961*, 367.

Gill, T. J., III, Gould, H. J., and Doty, P. (1963), *Nature 197*, 746.

Gilvarg, C., and Katchalski, E. (1965), *J. Biol. Chem. 240*, 3093.

Kantor, F. S., Ojeda, A., and Benacerraf, B. (1963), *J. Exptl. Med. 117*, 55.

Karush, F., and Eisen, H. N. (1962), *Science 136*, 1032.

Levine, B. B., and Benacerraf, B. (1964), *J. Exptl. Med. 120*, 955.

Levine, B. B., Ojeda, A., and Benacerraf, B. (1963), *J. Exptl. Med. 118*, 953.

Maurer, P. H. (1963), *Proc. Soc. Exptl. Biol. Med. 113*, 553.

Maurer, P. H. (1964), *Progr. Allergy 8*, 1.

Maurer, P. H. (1965), *J. Exptl. Med. 121*, 339.

Moore, S., Spackman, D. H., and Stein, W. H. (1958), *Anal. Chem. 30*, 1185.

Neuberger, A., and Sanger, F. (1943), *Biochem. J. 37*, 515.

Ovary, Z. (1958), *Progr. Allergy 5*, 459.

Peterson, E. A., and Sober, H. A. (1956), *J. Am. Chem. Soc. 78*, 751.

Sanger, F. (1945), *Biochem. J. 39*, 507.

Schlossman, S. F., Yaron, A., Ben-Efrain, S., and Sober, H. A. (1965), *Federation Proc. 24*, 184.

Sela, M. (1962), *Polyamino Acids, Polypeptides, Proteins, Proc. Intern. Symp., Madison, Wis., 1961*, 347.

Sela, M. (1965), *Advan. Immunol. 5* (in press).

Sela, M., and Fuchs, S. (1964), *in* Proc. Symposium on Molecular and Cellular Basis of Antibody Formation, Prague (in press).

Sela, M., Fuchs, S., and Arnon, R. (1962), *Biochem. J. 85*, 223.

Sober, H. A. (1962), *Polyamino Acids, Polypeptides, Proteins, Proc. Intern. Symp., Madison, Wis., 1961*, 105.

Stahmann, M. A., Tsuyuki, K., Weinke, K., Lapresle, C., and Grabar, P. (1955), *Compt. Rend. 241*, 1528.

Stewart, J. W., and Stahmann, M. A. (1962), *Polyamino Acids, Polypeptides, Proteins, Proc. Intern. Symp., Madison, Wis., 1961*, 95.

Yaron, A., Berger, A., Katchalski, E., Otey, M. C., and Sober, H. A. (1964), *Proc. Intern. Congr. Biochem. 6th, New York, 1964, 2*, 190.