Synthesis and Immunological Properties of the Oligolysyl-N^{ϵ}-dinitrophenyllysine and Oligolysylalanylalanylalanyl-N^{ϵ}-dinitrophenyllysine Peptide Series†

Arieh Yaron,* Edward K. Dunham, III, and Stuart F. Schlossman

ABSTRACT: A new series of 2,4-dinitrophenyllysyl oligopeptides containing a trialanyl sequence was prepared for investigation of the minimal size requirements and influence of charge density on immunogenicity and the specificity of the immune response to those defined antigens. The Lys_n-Ala_3-N^{ϵ}-Dnp-Lys series of peptides was synthesized by: (1) stepwise synthesis of the tetrapeptide Ala_3-N^{ϵ}-Dnp-Lys benzyl ester; (2) polymerization of N^{ϵ}-Z-Lys-*N*-carboxyanhydride using the above tetrapeptide as the initiator; (3) removal of protecting groups; and (4) fractionation of the resulting Lys_n-Ala_3-N^{ϵ}-Dnp-Lys series into its individual components by ion exchange chromatography. All inbred strain 2 guinea pigs, when immunized with the unfractionated Lys_n-Ala_3-N^{ϵ}-Dnp-Lys ($\bar{n}$ = 10), Lys_4-Ala_3-N^{ϵ}-Dnp-Lys, or Lys_7-Ala_3-N^{ϵ}-Dnp-Lys, responded by the development of delayed hypersensitivity and its *in vitro* correlate, antigen-induced DNA synthesis, and with antibody production. The tetrapeptide Ala_3-N^{ϵ}-Dnp-Lys, on the other hand, was not immunogenic. In addition, none of the peptides could sensitize strain 13 animals. These and other observations with Hartley guinea pigs indicated that only peptides possessing the specific immune response gene (polylysine positive) respond. The requirement for an octapeptide to induce the immune response in the Lys_n-Ala_3-N^{ϵ}-Dnp-Lys series was similar to that found with dinitrophenyloligolysines where peptides equal to or larger in size than the heptapeptide were immunogenic. Thus, despite a reduction in the peptide charge density and an absence of a linear sequence of seven lysyl residues, peptides of comparable size were immunogenic.

Synthetic polypeptide antigens of defined chemical structure have been useful tools of immunochemical investigation (Sela, 1970). The dinitrophenyl (Dnp)-oligolysine[1] system has afforded a means of studying the chemical requirements for immunogenicity as well as the specificity of antibody and of cell-mediated immunologic reactions (Schlossman and Yaron, 1970; Paul *et al.*, 1970; Schlossman, 1972). It was established that the minimal chain length required for immunogenicity of polylysine and Dnp-oligolysines in guinea pigs is seven L-lysine residues (Schlossman and Yaron, 1970). We wanted now to find out whether it is possible to replace some of the lysines by other amino acid residues such as alanine. A series of oligopeptides of the general chemical structure of Lys_n-Ala_3-N^{ϵ}-Dnp-Lys was prepared. These immunogens consist of an oligolysine part of varying chain length *n* and of a constant part Ala_3-N^{ϵ}-Dnp-Lys. The trialanyl sequence separates the oligolysine carrier from the Dnp-lysine hapten.

It was expected that if a sequence of seven lysine residues is a prerequisite for immunogenicity, the smallest member of the series to be found immunogenic will be Lys_7-Ala_3-N^{ϵ}-Dnp-Lys. If, however, one, two, or three of the lysine residues can be replaced by alanine without impairment of immunogenicity, the smallest active oligopeptide will be Lys_6-Ala_3-N^{ϵ}-Dnp-Lys, Lys_5-Ala_3-N^{ϵ}-Dnp-Lys, or Lys_4-Ala_3-N^{ϵ}-Dnp-Lys, respectively. We find (1) that peptides of this family having at least eight amino acid residues in all (*i.e.*, $n \geq 4$) are immunogenic in guinea pigs possessing the polylysine gene but not in others (Levine *et al.*, 1963); (2) that despite this control of responsiveness by the polylysine gene, peptides containing the Ala_3 sequence are distinguished from oligolysines and their Dnp conjugates both by antibody and in reactions of cell-mediated immunity; and (3) that immunogenicity and elicitation of cellular responses do not require the presence in the molecule of a sequence of seven lysyl residues which would be immunogenic in itself. A preliminary report of this work was presented at the 4th Annual Meeting of the Israel Immunological Society (Yaron *et al.*, 1972a).

Experimental Section

Analytical Procedures. Melting points are uncorrected. Anhydrous titrations were performed according to Fritz and Lisicki (1951). Microanalyses were performed by the Microanalytical Laboratory of the Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot. Samples for microanalysis were dried in an Abderhalden drying pistol with P_2O_5 at 77° for 16 hr at 0.3 mm. Thin layer chromatography was carried out on silica plates (DC-Karten SI-Riedel de Haen A.G., Seelze, Hannover) at room temperature. The solvents used were 1-butanol–acetic acid–water (4:1:1) (BAW); acetone–chloroform (1:1 and 1:9) (AC); and methanol–chloroform (1:1) (MC), all ratios by volume. Peptide spots were located with the ninhydrin reagent or by charring.

† From the Department of Biophysics, The Weizmann Institute of Science, Rehovot, Israel (A. Y.), and from the Department of Medicine, Harvard Medical School, Beth Israel Hospital, Boston, Massachusetts (E. K. D. and S. F. S.). *Received June 4, 1973.* This work was supported by research grants from the National Institutes of Health (AI 09684-04 and AI 09003) and the National Science Foundation (GB 25474).

[1] Abbreviations used are: Boc, *tert*-butyloxycarbonyl; CM, carboxymethyl; Dnp, 2,4-dinitrophenyl; OBzl, benzyloxy; ONBzl, *p*-nitrobenzyloxy; ONSu, succinimidooxy; tlc, thin-layer chromatography; Z, benzyloxycarbonyl; BAW, 1-butanol–acetic acid–water; AC, acetone–chloroform; MC, methanol–chloroform. All amino acids are of the L conformation unless stated otherwise.

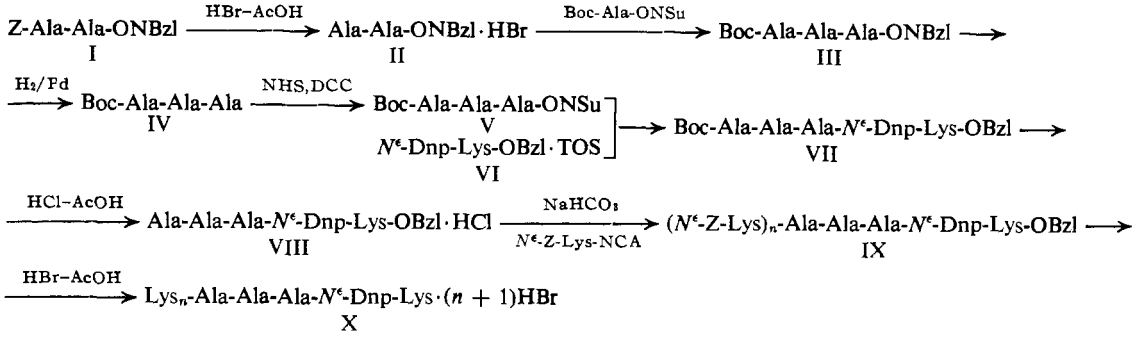

FIGURE 1: Schematic representation of the synthesis of Lys_n-Ala_3-N^{ϵ}-Dnp-Lys. Abbreviations used in the figure are: NHS, *N*-hydroxysuccinimide; DCC, *N*,*N*^{1}-dicyclohexylcarbodiimide; TOS, *p*-toluenesulfonic acid; NCA, *N*-carboxyanhydride.

Amino acid composition of acid hydrolysates of peptides and polymers was determined by automatic amino acid analysis (Spackman *et al.*, 1958).

Synthesis. The synthesis of the sequential polypeptide Lys_n-Ala_3-N^{ϵ}-Dnp-Lys is depicted schematically in Figure 1.

N^{α}-tert-Butyloxycarbonylalanylalanylalanine p-Nitrobenzyl Ester (III). N^{α}-*tert*-Butyloxycarbonylalanine hydroxysuccinimide ester (Anderson *et al.*, 1964) (28.6 g, 0.10 mol) and alanylalanine *p*-nitrobenzyl ester hydrobromide (39 g, 0.104 mol), prepared from I (Schechter and Berger, 1966) in a 70% yield by the hydrogen bromide method of Ben Ishai and Berger (1952), were dispersed in dimethylformamide (100 ml) and triethylamine (14 ml) was added. The mixture was stirred overnight, water was added, and the precipitate obtained was isolated by filtration and washed with water. It was dissolved in chloroform, and the solution obtained was extracted successively with a cold 10% solution of citric acid, water, sodium bicarbonate solution, and water. The organic phase was dried (Na_2SO_4), and the product precipitated with petroleum ether, filtered, and dried to yield 32 g (69%) of III: after crystallization from ethyl alcohol, mp 172–173°; R_F BAW 0.88. *Anal.* Calcd for $C_{21}H_{30}N_4O_8$ (mol wt 466.5): N, 12.0. Found: N, 11.9.

N^{α}-tert-Butyloxycarbonylalanylalanylalanine (IV). III (32 g, 68 mmol) in dimethylformamide (350 ml) was hydrogenated in the presence of Pd (10%) on charcoal at atmospheric pressure and isolated by ether precipitation of the product, yielding 22.5 g of IV: mp 177.5–178° (dec); R_F BAW 0.77, AC (1:9) a single spot at origin; titration equivalent calcd 331, found 335. *Anal.* Calcd for $C_{14}H_{25}N_3O_6$ (mol wt 331.4): C, 50.7; H, 7.6; N, 12.7. Found: C, 51.0; H, 7.4; N, 12.5.

N^{ϵ}-Dinitrophenyllysine Benzyl Ester p-Toluenesulfonate (VI). To a solution of N^{ϵ}-Dnp-lysine (Sanger, 1945) (5.85 g, 19.7 mmol) in benzyl alcohol (75 ml), *p*-toluenesulfonic acid monohydrate (3.64 g, 19.1 mmol) and benzene (75 ml) were added. The reaction mixture was subjected to azeotropic distillation for 4 hr, after which period it solidified. The solid was treated with ether and collected by filtration, yielding 9.3 g (85%) of VI. For analysis VI was crystallized from alcohol–water: titration equivalent calcd 574, found 560. *Anal.* Calcd for $C_{26}H_{30}N_4O_9S$ (mol wt 574.6): C, 54.3; H, 5.3; N, 9.75; S, 5.6. Found: C, 54.3; H, 5.1; N, 10.0; S, 5.8.

Alanylalanylalanyl-N^{ϵ}-dinitrophenyllysine Benzyl Ester Hydrochloride (VIII). IV (5.4 g, 16.3 mmol) and *N*-hydroxysuccinimide (8.55 g, 16.3 mmol) were dissolved in a mixture of dioxane (60 ml) and dimethoxyethane (30 ml) and cooled to 0°. *N*,*N*′-Dicyclohexylcarbodiimide (3.36 g, 16.3 mmol) was added and the reaction mixture stirred at 4° for 16 hr. *N*,*N*′-Dicyclohexylurea was filtered off and the solvent removed *in vacuo*. The oily residue was triturated with ether until solidification occurred. The product was isolated by filtration and crystallized from isopropyl alcohol. The yield of active ester V was 5.2 g (74%): mp 158–164°; titration equivalent calcd 428, found 414 (Wilchek *et al.*, 1970).

A solution of VI (2.05 g, 3.57 mmol) in ethyl acetate was washed with aqueous sodium bicarbonate. The organic phase was dried (Na_2SO_4) and evaporated *in vacuo*. The residue was dissolved in dimethylformamide (32.5 ml) and the concentration of VI as the free base was determined by anhydrous titration with perchloric acid. V (1.5 g, 3.5 mmol) was added to 30.5 ml of the above solution (3.43 mmol of VI) and the mixture left at room temperature overnight. The solution was concentrated by evaporation and the product was precipitated with ether. The yield of compound VII was 1.9 g (77.5%); R_F MC 0.87. Under identical conditions N^{ϵ}-Dnp-Lys-OBzl had R_F MC 0.73. Analysis of Boc groups (Kutassy *et al.*, 1968) gave a calculated titration equivalent of 7.15; found, 730.

VII (1.7 g, 2.38 mmol) was deprotected with HCl in acetic acid (0.6 M, 30 ml, 30 min) at room temperature. The reaction solution was concentrated and the product precipitated with ether and crystallized by dissolving in boiling ethanol (800 ml), cooling in an ice bath, and adding 1 l. of ether. The hydrochloride VIII was washed with ether and dried, yielding 1.45 g (88%); R_F BAW 0.35. *Anal.* Calcd for $C_{28}H_{37}N_7O_9Cl \cdot 2H_2O$ (687): N, 14.3; Cl, 5.16. Found: N, 14.13; Cl, 5.17. The molar ratio of alanine residues (from amino acid analysis) to Dnp (from the absorbance at 360 nm in water, using ϵ_{360} 17,400 M^{-1} cm^{-1}) was Ala/Dnp = 2.9 (calcd, 3.0).

(N^{ϵ}-Benzyloxycarbonyllysyl)$_n$-(alanyl)$_3$-N^{ϵ}-dinitrophenyllysine benzyl ester (IX) was prepared by polymerization of N^{ϵ}-benzyloxycarbonyllysine *N*-carboxyanhydride (Katchalski, 1957) with VIII in the free base form as the initiator in a mixture of benzene and dimethylformamide. The hydrochloride VIII (480 mg, 0.7 mmol) was distributed between solutions of saturated aqueous $NaHCO_3$ and chloroform. The organic phase was separated and the aqueous phase extracted with another portion of chloroform. The organic phases were combined, dried (Na_2SO_4), and evaporated *in vacuo*, and the residue was taken up in 4 ml of dimethylformamide. The concentration of the initiator (0.16 M) was determined by measuring the absorption at 350 nm of an aliquot diluted 4000 times with dichloroacetic acid, using an ϵ_{350} of 14,700 (Yaron and Schlossman, 1968). The initiator solution (3.6 ml) together with 1.2 ml of dimethylformamide were added to a suspension of Z^{ϵ}-Lys-*N*-carboxyanhydride (2.0 g, 6.5 mmol, *N*-carboxyanhydride:initiator = 11.3) in benzene (40 ml, final solvent composition, benzene–dimethylformamide, 8.3:1). The polymerization started immediately (as judged from the evolution of CO_2) and within 15 min a gel was

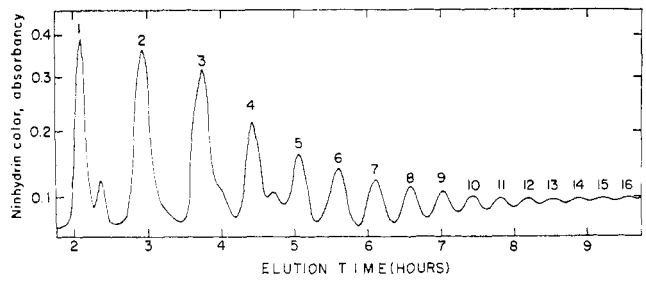

FIGURE 2: Elution diagram showing the distribution of individual oligopeptides in Lys_n-Ala_3-N^ϵ-Dnp-Lys (X) obtained by polymerization in dimethylformamide (N^ϵ-Z-Lys-*N*-carboxyanhydride, 0.18 M; Ala_3-N^ϵ-Dnp-Lys-OBzl, 0.02 M; *N*-carboxyanhydride/initiator = 9.2). X (3 mg) was dissolved in water and sodium hydroxide was added to pH 6–7. The solution was applied to a CM-cellulose column (1.3 × 47 cm) in Na^+ form and eluted with a convex gradient of increasing NaCl concentration produced with a constant volume (300 ml) device, initially 0.1 M and fed with 0.6 M NaCl. The effluent was monitored by ninhydrin analysis (Technicon analyzer); flow rate, 43 ml/hr.

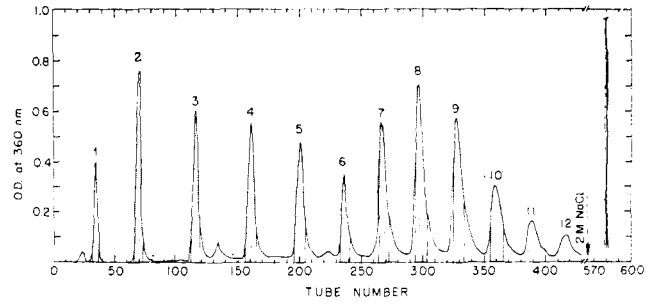

FIGURE 3: Elution diagram showing the distribution of individual oligopeptides in Lys_n-Ala_3-N^ϵ-Dnp-Lys obtained by polymerization in dimethylformamide–benzene (for conditions of polymerization see Experimental Section). The sample (100 mg) was applied to a CM-cellulose column (2.3 × 66 cm) in the Na^+ form, preequilibrated with 0.02 M NaCl. A constant volume (5 l.) gradient of 0.02–0.8 M NaCl was used for elution at a flow rate of 105 ml/hr; 21-ml fractions were collected. All solutions contained 0.5% butyl alcohol. The effluent was monitored by recording the absorption at 360 nm. Fractions pooled for isolation of the individual oligopeptides are indicated as shadowed areas.

formed. After 20 hr at room temperature 20 ml of dimethylformamide was added and the homogeneous solution obtained was poured into 0.01 M HCl containing 5% KCl. The precipitate was collected by filtration, washed with water, and dried *in vacuo* over concentrated H_2SO_4 and NaOH, yield 1.8 g (87%).

$(Lysyl)_n$-$(alanyl)_3$-N^ϵ-dinitrophenyllysine Hydrobromide (X). IX (1.5 g) was dispersed in acetic acid (7.5 ml) and 94 ml of HBr in acetic acid (43%) containing anisole (10%) was added to the mixture. The material dissolved completely and a precipitate began to form after 15 min. The reaction was left to proceed at room temperature for 15 hr. Ether was added to the mixture and the product washed by decantation with more ether. After drying *in vacuo* over KOH and concentrated H_2SO_4, 1.3 g of the polymer X was obtained (93%).

Analytical ion exchange chromatography of X was performed as described in the legend to Figure 2.

Preparative Chromatography of X. X (100 mg) was dissolved in water, adjusted to pH 7, and subjected to ion exchange column chromatography. A typical chromatogram is shown in Figure 3; the experimental details are described in the legend.

In order to remove most of the salt, the pooled fractions were diluted five times and adsorbed on CM-cellulose columns. The peptides were eluted with 0.2 M HCl and obtained in solid form by freeze drying. The amount of salt still present in the samples sufficiently small for use of the peptides in immunologic experiments. Purity of the peptides was examined by rechromatography (Figure 4) and by high-voltage ion exchange paper electrophoresis (Yaron and Sober, 1965).

Table I lists yields of pure peptides obtained and the molar ratios of lysine residues to Dnp-lysine as determined by lysine analysis of solutions of known (from absorption at 360 nm) Dnp-lysine content (Schlossman *et al.*, 1965).

Determination of Benzyl Groups by Gas Chromatography. Fractions from ion exchange chromatography of X containing about 0.4 mmol of oligopeptide were made 1 M in alkali by adding an appropriate amount of 5 M NaOH. The solution was kept at 50° for 30 min and the benzyl alcohol formed was extracted with three 0.25-ml portions of ether. The ether solution was washed with water, dried (Na_2SO_4), and transferred into a boiling flask using more ether to ensure quantitative transfer. The solvent was removed by evaporation *in vacuo* and the residue was dissolved in a known volume of ether just before application to the gas chromatographic column (usually 0.25 ml). Benzyl alcohol in ether (0.07 mmol/ml) was used as a standard and γ-benzyl glutamate as a model compound in working out the conditions for complete hydrolysis and quantitative extractions of the benzyl alcohol. Analysis was performed on a Varian Aerograph Model 1200-2, provided with a flame ionization detector, using a E GSS-X column under He at a flow rate of 70 ml/min at 125°.

$(Lysyl)_n$-N^ϵ-dinitrophenyllysine (XI) was prepared by polymerization of N^ϵ-benzyloxycarbonyllysine *N*-carboxyanhydride, using N^ϵ-benzyloxycarbonyllysine benzyl ester as the initiator. Conditions for polymerization, deblocking, and fractionation by ion exchange chromatography (Figures 5 and 6) were as described for the oligomers X. Table I gives yields of the pure peptides, and molar ratios Lys/Dnp-Lys for some of the isolated oligopeptides.

Oligolysines and N^α-Dnp-oligolysines were prepared as described previously (Schlossman *et al.*, 1965; Yaron *et al.*, 1972b). The random copolymer of glutamic acid and lysine (60:40), mol wt 50,000, was purchased from Pilot Chemicals, Watertown, Mass.

Animals. Guinea pigs of inbred strains 2 and 13 were used. Hartley-strain guinea pigs of a line producing about 30% responders to polylysine were purchased from Camm Research Laboratories, Wayne, N. J.

Immunization. Guinea pigs were immunized with 50–100 nmol of antigen in complete Freund's adjuvant containing *Mycobacterium tuberculosis* $H_{37}Ra$ (1 mg/ml of emulsion),

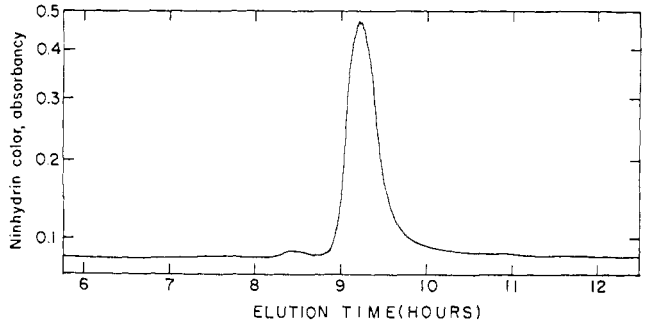

FIGURE 4: Recromatography of Lys_8-Ala_3-N^ϵ-Dnp-Lys (0.2 μmol from peak 8 in Figure 3). Chromatography conditions as in Figure 2.

TABLE I: Preparative Fractionation of Lys_n-N^ϵ-Dnp-Lys (XI) and Lys_n-Ala_3-N^ϵ-Dnp-Lys (X).

	XI				X			
	Content of Oligomers in	Yield of Pure Peptide[c]		Lys/	Content of Oligomers in	Yield of Pure Peptide[c]		Lys/
n[a]	Polymer (%)[b]	μmol	%	Dnp-Lys[d]	Polymer (%)[b]	μmol	%	Dnp-Lys[d]
0	0.36			No Lys	3.3			No Lys
1	2.50	1.6	1.4	1.0	1.9	0.5	1.3	0.96
2	6.40	4.0	3.6	1.8	4.2	1.0	2.6	1.88
3	6.60	3.7	3.3	2.9	3.3	0.7	1.8	
4	7.10	4.2	3.7		3.0	0.7	1.8	
5	6.90	3.8	3.4		7.4	1.7	4.5	
6	5.50	2.8	2.5		18.0	4.4	11.6	
7	9.70	4.7	4.2	6.8	18.0	4.1	10.8	6.7
8	14.50	7.1	6.3		10.4	2.2	5.8	7.9
9	12.30	5.5	4.9		5.4	2.7	7.1	
10	6.80	3.4	3.0		4.5	0.8	2.1	
11	4.80				3.7			
12	2.80				2.7			
13	2.30				2.5			
14	2.20							
15	1.70							
16	1.10							
17	0.60							
>20	4.70				5.0			

[a] n is the number of unsubstituted lysine residues in X and XI. [b] The percentage of a single oligomer was calculated from the optical density (OD) at 360 nm of its peak area (see Figures 3 and 6). The total OD eluted was taken as 100% (which is 70% of the amount applied, by weight). [c] The amount of each peptide after desalting was determined from the OD at 360 nm; the weight of the sample applied initially to the CM-cellulose column was taken as 100%. [d] Lys was determined by amino acid analysis after total hydrolysis. Dnp-Lys was calculated from the OD at 360 nm.

each animal receiving 0.8 ml of emulsion distributed among footpads.

Skin Testing. Animals were shaved and injected intradermally with 0.1 ml of saline containing 15 nmol of antigen 12–14 days after immunization. The sites of injection were inspected after 4 and 24 hr. Delayed reactions were considered positive if there was induration more than 5 mm in diameter after 24 hr.

DNA Synthesis by Cultures of Lymph Node Cells. Antigen-stimulated synthesis of DNA, as indicated by incorporation of thymidine-2-^{14}C by cultures of lymph node cells in the presence of various concentrations of antigen, was determined as described previously (Stulbarg and Schlossman, 1968). Purified protein derivative (PDD) prepared from *Mycobacterium tuberculosis* was obtained from the National Institute of Allergy and Infectious Diseases, Bethesda, Md.

Results

Synthesis of Antigens. In our previous studies Dnp-oligolysine immunogens were prepared basically by polymerizing N^ϵ-Z-lysine *N*-carboxyanhydride and fractionating the resulting polylysine by ion exchange chromatography (Schlossman *et al.*, 1965). The series of oligomers of the general chemical structure Lys_n-Ala_3-N^ϵ-Dnp-Lys used in this study was prepared by an essentially similar procedure. The tetrapeptide initiator Ala_3-N^ϵ-Dnp-Lys was prepared stepwise (Figure 1) and used, in the free base form, to initiate the polymerization of N^ϵ-Z-Lys-*N*-carboxyanhydride. The analogous series Lys_n-N^ϵ-Dnp-Lys was also prepared, the initiator used in this case being N^ϵ-Dnp-Lys-OBzl.

Polymers obtained under various polymerization conditions were deblocked and analyzed by ion exchange chromatography on CM-cellulose. Typical elution diagrams are shown in Figures 2, 3, 5, and 6. When dimethylformamide or dioxane was used as the solvent for the polymerization, a bimodal distribution of chain length was observed with one area in the low molecular weight region (n = 1–7) and another in the range higher than n = 20 (Figure 5). A similar bimodal distribution has been observed previously (Stewart and Stahmann, 1962; Sober, 1962). Two different mechanisms of propagation are most probably responsible for this distribution (Shalitin, 1969). Changing the molar ratio of *N*-carboxyanhydride to initiator influenced the relative amounts of peptides in the two respective regions rather than the distribution in the low molecular weight mode. In polymers obtained by polymerization in dimethylformamide or in dioxane at a molar ratio of *N*-carboxyanhydride to initiator of 8 or 4, the low molecular weight area contained oligolysines of chain length n = 1–7 with very little of the n = 7 and practically no n = 8 or 9 (Figure 5). On increasing the *N*-carboxyanhydride to initiator ratio to 20 or 40 the yield of peptides in the n = 1–7 region became very small. Since it was initially expected that in the series Lys_n-Ala_3-N^ϵ-Dnp-Lys the smallest peptide still being immunogenic will be Lys_7-Ala_3-N^ϵ-Dnp-Lys, we looked for polymerization conditions which would produce a polymer containing sufficient amounts of peptides in the chain length range n = 5–9. Such a distribution was achieved when

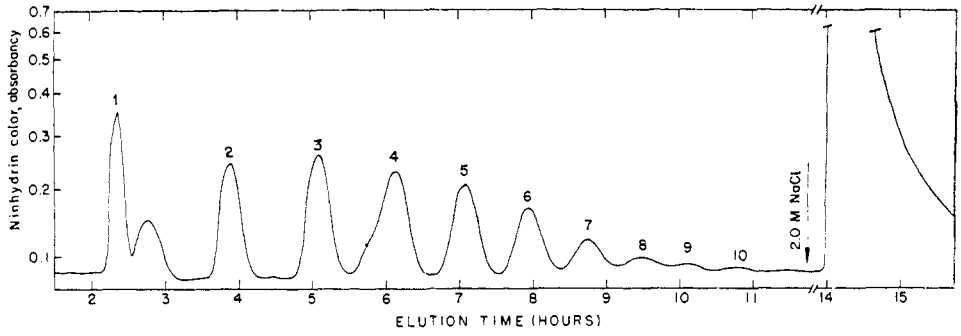

FIGURE 5: Elution diagram showing the distribution of individual oligopeptides Lys_n-N^{ϵ}-Dnp-Lys in a polymer (2.3 mg) obtained by polymerization in dimethylformamide (N^{ϵ}-Z-Lys-*N*-carboxyanhydride, 0.18 M; N^{ϵ}-Dnp-Lys-OBzl, 0.012 M; *N*-carboxyanhydride/initiator = 15) Chromatography conditions as in legend to Figure 2, except that 0.8 M NaCl was used as the limiting solution.

the polymerization was carried out in a mixture of dimethylformamide and benzene (Figures 3 and 6). In this solvent a gel is formed soon after beginning of the polymerization reaction. The low solubility of poly(Z-lysine) in this solvent mixture obviously restricts the formation of high molecular weight fractions with a concomitant increase in the amount of peptides in the low molecular weight region.

The chain length distribution obtained by polymerization in the dimethylformamide–benzene solvent seemed satisfactory and was therefore used on the preparatory scale. A typical elution diagram of a fractionation of 100 mg of Lys_n-Ala_3-N^{ϵ}-Dnp-Lys is shown in Figure 3. Fractions were pooled as indicated in the diagram and homogeneity of the peptides prepared was ensured by rechromatography. A typical diagram obtained with the peptide Lys_8-Ala_3-N^{ϵ}-Dnp-Lys is shown in Figure 4. The chain length of the individual peptides was calculated from the molar ratio of Lys to Dnp-Lys. This ratio was determined for a number of peaks, the remaining peaks being assigned accordingly. Yields of pure peptides and ratios of Lys to Dnp-Lys are given in Table I.

In the tetrapeptide initiator (VIII), the carboxyl group is blocked in the form of a benzyl ester. This group is ultimately removed together with the protecting benzyloxycarbonyl groups by HBr in acetic acid. Since, however, benzyl esters are not cleaved as readily as the Z groups, we analyzed the final oligomers for presence of benzyl groups by the very sensitive gas chromatographic method. It was found that after a 90-min treatment with HBr–AcOH, 5% of the benzyl groups were still present in Lys_2-Ala_3-N^{ϵ}-Dnp-Lys. None could be detected after a 12-hr treatment (0.5% would be detected). These conditions were therefore used in all the preparations.

Possible racemization during peptide synthesis has to be considered. The coupling methods used (Figure 1) are known to give very little racemization if at all. The only step bearing a danger of racemization is the preparation of the activated peptide ester V and its subsequent coupling with VI. Fragment condensation *via* the synthesis and isolation of an activated carbonyl component is known to be racemization prone. It was shown, however, that activation of alanyl peptides by succinimide ester formation and subsequent coupling in dimethylformamide proceed with only about 1% racemization (Bosshard *et al.*, 1973). Moreover, this method was successfully used (Yaron *et al.*, 1972c) in a synthesis of poly(Lys-Ala-Ala) which has been shown by gas chromatography (Gil-Av *et al.*, 1966, 1967) to contain less than 0.5% D-alanine. The quantitative digestion of the polymer by hydrolytic enzymes was an additional proof for absence of major racemization. Gas chromatographic analysis of Lys_7-Ala_3-N^{ϵ}-Dnp-Lys under similar conditions showed that less than 1% of alanine is present in the D configuration.

During polymerization of N^{ϵ}-Z-Lys-*N*-carboxyanhydride using Ala_3-N^{ϵ}-Dnp-Lys-OBzl as the initiator some spontaneous polymerization of the *N*-carboxyanhydride could be expected to occur, which would cause the formation of poly(Z-lysine). The presence of polylysine should be observable by analyzing a peptide by ion exchange electrophoresis after digestion with carboxypeptidase B, since peptides with an N^{ϵ}-Dnp-Lys residue at the C-terminal end are not attacked by the enzyme while oligolysines are hydrolyzed rapidly (Yaron *et al.*, 1972c). A solution containing Lys_7-Ala_3-N^{ϵ}-Dnp-Lys was incubated in collidine buffer with carboxypeptidase B for 16 hr at 37° and subjected to ion exchange paper electrophoresis. A very weak spot of lysine was observed, indicating that a small amount of an oligolysine with a free α-carboxyl was present in the mixture. It is pertinent to note that the appearance of lysine on incubation with carboxypeptidase B is a very sensitive test since a number of lysine residues are split off for every oligolysine molecule. In this particular case, the contaminating oligolysine was shown to be Lys_7 as follows. A mixture of poly(L-lysine), Lys_8, and Lys_7-Ala_3-N^{ϵ}-Dnp-Lys was chromatographed on a CM-cellulose column. The usual pattern of oligolysine separation was obtained, but with increased size of the third and seventh peaks, corresponding to trilysine and heptalysine, respectively. The trilysine served as a marker and it could be con-

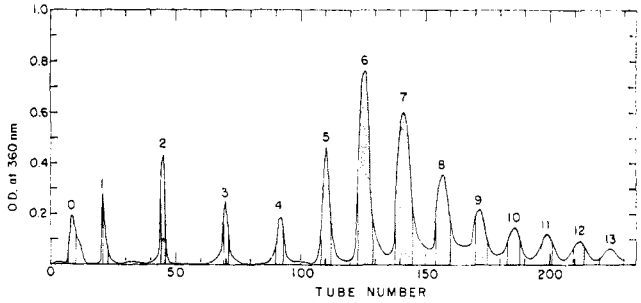

FIGURE 6: Elution diagram showing the distribution of individual oligopeptides Lys_n-N^{ϵ}-Dnp-Lys obtained by polymerization in dimethylformamide–benzene (for conditions of polymerization see Experimental Section). The sample (300 mg) was applied to a CM-cellulose column (2.7 × 90 cm) in Na^+ form, preequilibrated with 0.02 M NaCl. A constant volume (10 l.) gradient of 0.02–0.83 M NaCl was used for elution at a flow rate of 165 ml/hr; 22-ml fractions were collected. The effluent was monitored by recording the absorption at 360 nm. Fractions pooled for isolation of the individual oligopeptides are indicated as shadowed areas.

TABLE II: Immunogenicity of Lys_n-Ala_3-N^ϵ-Dnp-Lys in Guinea Pigs.

Strain	Antigen	Fraction Responding[a]	Thymidine Incorp.[b]
2	Ala_3-N^ϵ-Dnp-Lys	0/4	1.2 ± 0.1 (6)
	Lys_4-Ala_3-N^ϵ-Dnp-Lys	3/3[c]	9.0 ± 0.8 (4)
	Lys_7-Ala_3-N^ϵ-Dnp-Lys	3/3	24.0 ± 2.5 (6)[d]
	$Lys_{\bar{10}}$-Ala_3-N^ϵ-Dnp-Lys	6/6	8.9 ± 1.5 (4)[d]
13	$Lys_{\bar{10}}$-Ala_3-N^ϵ-Dnp-Lys	0/3	ND
Hartley responders[e]	$Lys_{\bar{10}}$-Ala_3-N^ϵ-Dnp-Lys	3/3	ND
Hartley nonresponders[e]	$Lys_{\bar{10}}$-Ala_3-N^ϵ-Dnp-Lys	0/5	ND

[a] Number of animals with positive delayed skin tests at 9–12 days/number tested. One unimmunized animal was included in each group tested and in each case gave no reaction. [b] Ratio of counts per minute of thymidine-2-^{14}C incorporated by 10^7 lymph node cells cultured in the presence of maximally stimulatory concentrations of antigen to counts per minute incorporated by unstimulated cultures from the same animal: mean ± standard error (number of cultures). [c] Skin tests equivocal at 9 days, positive when repeated at 16 days. [d] Stimulated with Lys_8-Ala_3-N^ϵ-Dnp-Lys. [e] Responder status determined by prior immunization and skin-testing with random copolymer of glutamic acid and lysine (Kantor *et al.*, 1963).

cluded that Lys_7-Ala_3-N^ϵ-Dnp-Lys migrated identically with Lys_7.

Since digestion with carboxypeptidase B results in the formation of oligolysines of shorter chain lengths and of lysine, all of which can be separated by ion exchange chromatography from the intact Lys_n-Ala_3-N^ϵ-Dnp-Lys oligopeptide, this represents a method for efficient removal of contaminating oligolysines. In most preparations, however, we did not find such contaminants and, if found, they were present in barely detectable quantities. Still, for experiments in which the absence of a contaminating oligolysine was important, the peptide was purified. Separation was also achieved when subjecting contaminated oligopeptides to prolonged high-voltage ion exchange electrophoresis on CM-cellulose paper. The slower migrating yellow band which was separated from the ninhydrin positive band of the unsubstituted oligolysine was eluted with 0.2 M HCl and obtained in dry form by lyophilization. The separation was found possible up to the seventh member of the series. In the last case a 15-hr electrophoresis at 4000 V was necessary to achieve separation.

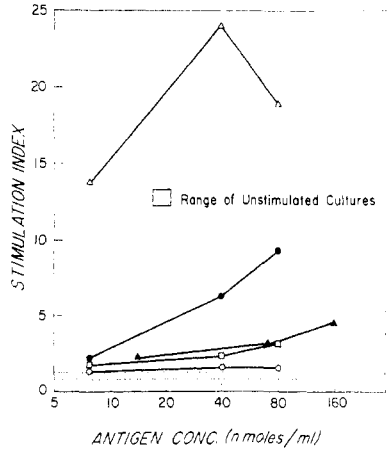

FIGURE 7: Incorporation of thymidine-2-^{14}C by lymph node cells from strain 2 guinea pigs immunized to Lys_7-Ala_3-N^ϵ-Dnp-Lys cultured in the presence of related antigens: (△) Lys_8-Ala_3-N^ϵ-Dnp-Lys; (●) Lys_{11-14}-N^ϵ-Dnp-Lys; (▲) Lys_9; (□) N^α-Dnp-Lys_{6-16}; (○) Ala_3-N^ϵ-Dnp-Lys. Stimulation index: ratio of counts per minute of thymidine-2-^{14}C incorporated by 10^7 lymph node cells cultured in the presence of antigen, to counts per minute incorporated by unstimulated cultures from the same animal.

Immunological Studies. Groups of guinea pigs were immunized with the unfractionated mixture (Lys_n-Ala_3-N^ϵ-Dnp-Lys, $\bar{n} = 10$) and with individual members of the series. Their responses were assessed by skin testing 9–12 days after immunization and in some cases by antigen-induced incorporation of thymidine-2-^{14}C by cultures of their lymph node cells (Table II). All strain 2 and Hartley responder animals immunized with the unfractionated mixture gave a positive response. On the other hand, animals immunized with the tetrapeptide component of the mixture gave a negative response. All strain 2 animals immunized with two larger members of this series, Lys_4-Ala_3-N^ϵ-Dnp-Lys and Lys_7-Ala_3-N^ϵ-Dnp-Lys, gave positive responses. The peptides of intermediate size, $n = 1, 2$, and 3 (penta-, hexa-, and heptapeptides), were recovered in insufficient yield for immunization, although enough Lys_3-Ala_3-N^ϵ-Dnp-Lys was obtained to use in a few lymph node cell cultures (see below). The unfractionated mixture was not immunogenic in strain 13 or Hartley nonresponder animals (as identified by lack of response to the random copolymer of glutamic acid and lysine).

In order to define the capacity of the immunocompetent cell to distinguish between closely related antigens, we performed studies of antigen-induced thymidine-2-^{14}C incorporation using lymph node cells from strain 2 animals immunized with several members of the Lys_n-Ala_3-N^ϵ-Dnp-Lys series and with mono-N^ϵ-Dnp-oligolysine. As stimulating antigens, four members of the Lys_n-Ala_3-N^ϵ-Dnp-Lys series, representative α-Dnp-oligolysines, mono-ϵ-Dnp-oligolysines, and unsubstituted oligolysines were used, and cultures stimulated with purified protein derivative and with no antigen were included in some cases as positive and negative controls. The results obtained are shown in Table III and Figure 7; Table III shows the responses to maximally stimulatory concentrations of antigens, while Figure 7 shows dose–response curves for a number of antigens of cells from strain 2 animals immunized with Lys_7-Ala_3-N^ϵ-Dnp-Lys. In each case the most effective antigen was the immunizing antigen or a larger peptide of the same series. Lys_n-Ala_3-N^ϵ-Dnp-Lys and Lys_n-N^ϵ-Dnp-Lys, sharing an N-terminal oligolysyl sequence and a C-terminal N^ϵ-Dnp-lysine and differing only in the presence or absence of the interposed trialanyl sequence, showed the greatest degree of cross-reactivity but were nevertheless reliably distinguished. Lys_n, the oligolysine without the Dnp group, stimulated cultures from animals immunized to Lys_n-

TABLE III: Cross-Reactions between Lys_n-Ala_3-N^ϵ-Dnp-Lys, N^α-Dnp-Lys_n, Lys_n-N^ϵ-Dnp-Lys, and Lys_n in Antigen-Induced Thymidine Incorporation *in Vitro*.[a]

	Immunizing Antigen				
Stimulating Antigen	Ala_3-N^ϵ-Dnp-Lys	Lys_4-Ala_3-N^ϵ-Dnp-Lys	Lys_7-Ala_3-N^ϵ-Dnp-Lys	$Lys_{\overline{10}}$-Ala_3-N^ϵ-Dnp-Lys	Lys_n-N^ϵ-Dnp-Lys
Ala_3-N^ϵ-Dnp-Lys	1.2 ± 0.1 (6)	1.1 ± 0.1 (4)	1.4 ± 0.3 (2)	1.0 ± 0.1 (4)	1.1 ± 0.1 (6)
Lys_3-Ala_3-N^ϵ-Dnp-Lys				2.4 ± 0.7 (2)	
Lys_4-Ala_3-N^ϵ-Dnp-Lys		9.0 ± 0.6 (2)		5.7 ± 1.4 (2)	
Lys_8-Ala_3-N^ϵ-Dnp-Lys		9.0 ± 1.3 (4)	24.0 ± 2.5 (6)	8.8 ± 1.5 (3)	
$Lys_{\overline{10}}$-Ala_3-N^ϵ-Dnp-Lys					2.3 ± 0.2 (6)
N^α-Dnp-Lys_n	1.2 ± 0.1 (4)	1.4 ± 0.0 (4)		2.4 ± 0.1 (4)	2.7 ± 0.0 (2)
Lys_{13}-N^ϵ-Dnp-Lys		2.0 ± 0.3 (4)	9.2 ± 0.8 (2)		16.9 ± 1.8 (2)
Lys_n		6.2 ± 0.7 (4)	4.5 ± 1.0 (4)	1.8 ± 0.1 (2)	8.0 ± 0.5 (2)
Purified protein derivative	3.3 ± 0.7 (4)		10.0 ± 1.2 (4)		3.6 ± 0.2 (2)
No antigen	1.0 ± 0.0 (10)	1.0 ± 0.0 (10)	1.0 ± 0.1 (12)	1.0 ± 0.1 (18)	1.0 ± 0.0 (10)

[a] Ratios of counts per minute of thymidine-2-^{14}C incorporated by cultures of 10^7 lymph node cells from immunized animals incubated with maximally effective concentrations of antigen to counts per minute incorporated by like cultures from the same animals incubated without antigen; mean ± standard deviation; number of cultures in parentheses.

Ala_3-N^ϵ-Dnp-Lys and to Lys_n-N^ϵ-Dnp-Lys less well though significantly; N^α-Dnp-Lys_n was scarcely effective in stimulating in cultures from animals immunized to Lys_n-Ala_3-N^ϵ-Dnp-Lys or Lys_n-N^ϵ-Dnp-Lys. The irregularity of the response to purified protein derivative, which was included as a positive control in some cultures from animals expected to give negative responses to other antigens, probably reflects deterioration of the purified protein derivative with storage and the variability yet undefined of the test system.

Discussion

It is clear that for $n \geq 4$, Lys_n-Ala_3-N^ϵ-Dnp-Lys is immunogenic in certain guinea pigs. The apparent discrepancy between this finding and the earlier finding that a larger molecular weight random copolymer of lysine and alanine is not immunogenic (Maurer *et al.*, 1964) may be due to the Dnp group, to the ordered nature of the copolymer, or to differing conditions of immunization. The minimum size of the peptide chain needed for immunogenicity or to elicit cell-mediated immune responses appears to be eight amino acid residues, in keeping with previous findings in the Dnp-oligolysine system where seven–eight were required (Schlossman and Yaron, 1970). However, the finding that Lys_4-Ala_3-N^ϵ-Dnp-Lys, an octapeptide containing only five lysines and only four in unbroken sequence, is immunogenic argues against the importance of charge density in the vicinity of the immunodominant group and against the view that immunogenicity requires the presence of an unbroken oligo(L-lysyl) sequence which would be immunogenic in itself. The earlier finding that N^α-Dnp-Lys_8 and N^α-Dnp-Lys_9 (L_4DL_4) with a D-lysine at the fifth position were not immunogenic whereas N^α-Dnp-Lys_9 (all L), N^α-Dnp-Lys_9 (L_7DL), and N^α-Dnp-Lys_9 (LDL_7) were immunogenic, demonstrated that size alone is not sufficient for immunogenicity but that a minimum unbroken sequence of seven L-amino acids is needed. We have now shown that this minimum sequence need not be made up solely of lysyl residues.

In all the animals we studied, the capacity to respond to Lys_n-Ala_3-N^ϵ-Dnp-Lys was associated with possession of the polylysine gene: all animals possessing the gene (strain 2 and Hartley responders) responded to Lys_n-Ala_3-N^ϵ-Dnp-Lys while all animals lacking the gene (strain 13 and Hartley nonresponders) failed to respond to Lys_n-Ala_3-N^ϵ-Dnp-Lys. Thus the capacity to respond to Lys_n-Ala_3-N^ϵ-Dnp-Lys appears to be governed by this specific immune response gene or by one closely linked to it; the number of animals we tested is too small to distinguish between these alternatives.

Immune cells reliably distinguish between Lys_n-Ala_3-N^ϵ-Dnp-Lys and oligolysines not containing the trialanyl sequence, whether or not dinitrophenylated. Longer chain peptides of the homologous series may be as effective as the immunizing antigen or more so, but peptides of other series differing from the immunizing antigen with respect to the presence or position of the Dnp group or the presence of the trialanyl "spacer" are uniformly less effective than the immunizing antigen in stimulating thymidine incorporation. The determinant of antigenic recognition in this system thus appears to involve nothing less than the entire molecule; the ϵ-Dnp-lysine moiety and the length and amino acid sequence of the peptide backbone all contribute, in keeping with earlier demonstrations of the exquisite specificity of cell-mediated immune reactions. If this specificity resides in a single receptor molecule, then that molecule has specificity different from that of circulating antibody, as has been demonstrated in the Dnp-oligolysine system (Schlossman *et al.*, 1969; Levin *et al.*, 1970, 1971), and as is borne out by our studies of antibody raised to Lys_n-Ala_3-N^ϵ-Dnp-Lys (E. K. Dunham *et al.*[2]); the pattern of specificity may be explicable in terms of two or more interacting receptors specific for different aspects of the antigen molecule, in terms of an antibody receptor of a class different from the classes of circulating antibody, or in other terms. Further speculation about the nature of the receptor must await additional experimental evidence.

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[2] E. K. Dunham, A. Yaron, and S. F. Schlossman, manuscript in preparation.

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