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Polylysine-specific Antibodies and Their Reaction with Oligolysines*

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ABSTRACT: Polylysyl rabbit serum albumin and polylysyl rabbit γ-globulin were synthesized via polytrifluoroacetyllysyl derivatives. The average chain length of the lysine peptides attached to rabbit serum albumin was 5.5 residues. Antibodies to polylysyl rabbit serum albumin were obtained in rabbits, their specificity was investigated, and the homologous precipitin reaction was inhibited by oligolysines of increasing chain length. The data obtained indicate that the increase in the efficiency of inhibition rises steeply only up to peptides containing 5–6 lysine residues, even though the extent of inhibition continued to increase up to nonalysine.

The inhibition of antigen-antibody reaction serves as a very useful tool in immunochemical investigations. By means of this technique information may be obtained about the character of the reacting sites on the antigen and antibody molecules, as well as on the nature of the antigen-antibody reaction. Checking smaller and smaller segments of an antigenic molecule as possible inhibitors of the homologous reaction may lead to the elucidation of the size of the combining site. Thus Kabat (1954, 1956) showed, for the dextran-antidextran system, that a series of glucose oligosaccharides up to the isomaltoheptaose inhibited the precipitin reaction of several human antidextran sera. Although individuals have been shown to produce heterogeneous populations of antibodies which vary in the size of their combining sites, in most of the sera tested the relative inhibitory capacity of the oligosaccharides, on a molar basis, increased with increasing chain length up to the hexasaccharide (Kabat and Bezer, 1958). The isomaltoheptaose was not significantly better as an inhibitor than isomaltohexaose, indicating that the upper limit in size for most of the antibody combining sites may be complementary to a hexasaccharide (Kabat, 1960). The same approach was used to determine the size of the antigenic determinants of a DNA preparation that reacted with the serum of a lupus erythematosus patient (Stollar *et al.*, 1962). When oligothymidylic acids of varying chain length were tested for the inhibition of the complement fixation by the denatured DNA-lupus system, it was found that the pentathymidylate was only slightly more effective than the tetrathymidylate.

The availability of a series of purified oligolysine peptides (Stewart and Stahmann, 1962a,b; Sober, 1962; Yaron *et al.*, 1964) prompted us to investigate whether, and to what extent, the different oligopeptides will inhibit a specific polylysine-antipolylysine system. Poly-L-lysine as such is nonimmunogenic (Maurer *et al.*, 1959), and in order to obtain polylysine-specific antibodies, an antigen had to be prepared in which polylysine chains would serve as haptenic groups. This could be achieved by preparing a polylysyl protein. Polypeptidyl proteins have been used extensively in recent years for various investigations (Sela *et al.*, 1963; Katchalski *et al.*, 1964). In order to prepare polylysyl proteins under conditions

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TABLE I: Content of Basic Amino Acids of Rabbit Serum Albumin, Rabbit γ-Globulin, and Their Poly-L-lysyl Derivatives.

Amino Acid (μM/mg)	Rabbit Serum Albumin	Poly-L-lysyl Serum Albumin	Deaminated Poly-L-lysyl Serum Albumin	γ-Globulin	Poly-L-lysyl γ-Globulin	Deaminated Poly-L-lysyl γ-Globulin
Lysine	0.7	1.87	0.258	0.31	0.99	0.16
Histidine	0.26	0.21	0.229	0.08	0.082	0.082
Arginine	0.25	0.21	Trace	0.18	0.2	Trace
Lysine/histidine ratio	2.7	8.9	1.13	3.9	12.1	1.96
Average chain length		5.5			4.2	

that would not denature[1] the protein moiety, it was necessary to replace in the reacting monomer the carbobenzoxy group, which is usually used to block the ε-amino function of lysine and is removed with anhydrous hydrogen bromide, with the trifluoroacetyl group. This group may be removed from poly-ε,*N*-trifluoroacetyl-L-lysine, and from polytrifluoroacetyllysyl proteins under mildly alkaline conditions (1 M piperidine), to yield poly-L-lysine and poly-L-lysyl proteins (Sela *et al.*, 1963). For an immunological investigation to be carried out in rabbits, it was desirable to use rabbit serum albumin as the protein carrier, since its use would limit the production of antibodies with specificities directed against determinants other than polylysine.

Antibodies to lysyl derivatives of rabbit and bovine serum albumins were reported by Stahmann *et al.* (1955, 1959). These derivatives were prepared from the respective protein and the reaction product of ε,*N*-carbobenzoxy-α,*N*-carboxy-L-lysine anhydride with anhydrous hydrogen bromide. The product of the last reaction is most probably lysyl bromide hydrobromide (Brenner and Photaki, 1956). As the lysine derivative reacting with the protein has a free ε-amino group, the lysine-protein derivatives obtained contained probably branched peptide side chains. The lysine residues attached formed approximately 2% of the molecule.

We report in this paper the preparation, characterization, and antigenicity of a poly-L-lysyl rabbit serum albumin, as well as the inhibition of the homologous poly-L-lysyl serum albumin–antipoly-L-lysyl serum albumin system by a series of oligolysines up to decamer. The data obtained indicate that the increase in the efficiency of inhibition rises steeply only up to peptides containing 5–6 lysine residues. After this study was completed, an investigation using alanine peptides for the inhibition of a homologous poly-L-alanyl bovine serum albumin system was reported (Sage *et al.*, 1964). The inhibitory molar effectiveness increased as the size of the hapten was increased to the pentamer. Similarly, Maurer mentions in a review article (1964) that penta- and hexapeptides of L-glutamic acid are good inhibitors of the homologous precipitin reaction of several synthetic polypeptide antigens with a high content of glutamic acid.

Materials and Methods

Poly-L-lysyl Rabbit Serum Albumin. The material used for this study was the derivative described in a previous communication (Sela *et al.*, 1963). The lysine peptides attached to the albumin represented 16.3% of this preparation. We have now characterized it in more detail. The analysis for the number of polypeptide chains added and the average chain length was performed according to Anfinsen *et al.* (1962). The enrichment in lysine residues was determined by amino acid analysis according to the method of Spackman *et al.* (1958), and the results are reported in Table I. The deamination of poly-L-lysyl serum albumin was performed as follows: 5 mg of poly-L-lysyl serum albumin was dissolved in 0.5 ml of water. To this was added 1.5 ml of a freshly prepared saturated solution of sodium nitrite and 0.5 ml of glacial acetic acid. The reaction was allowed to proceed overnight at room temperature, after which the entire sample was dialyzed exhaustively against distilled water and lyophilized. The amino acid analysis of the hydrolysate of the deaminated substance gave the number of the undeaminated lysine residues per poly-L-lysyl serum albumin molecule, i.e., the number of polypeptide chains added. The average chain length of the lysine peptides attached to serum albumin was 5.5 residues. Poly-L-lysyl γ-globulin was analyzed in a similar way.

Poly-L-lysine, Free Base. The preparation of the material has been described previously (Sela *et al.*, 1963). The material used in this study was of a degree of polymerization $n = 55$ (determined for the ε,*N*-trifluoroacetyl derivative).

Poly-L-lysyl Rabbit γ-Globulin. This material was

[1] Bovine pancreatic ribonuclease and insulin completely lost their biological activities after exposure for 2 minutes to a saturated solution of cold anhydrous hydrogen bromide in glacial acetic acid.

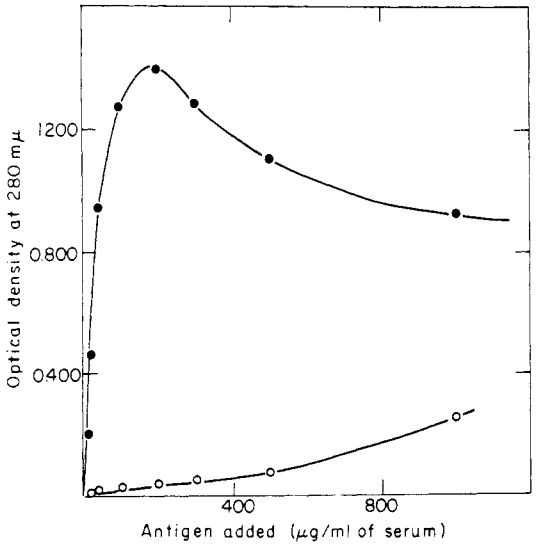

FIGURE 1: Extinction at 280 mμ of solutions in 0.1 N sodium hydroxide of precipitates obtained by addition of poly-L-lysyl serum albumin to antipoly-L-lysyl serum albumin (●), and to normal rabbit serum (○).

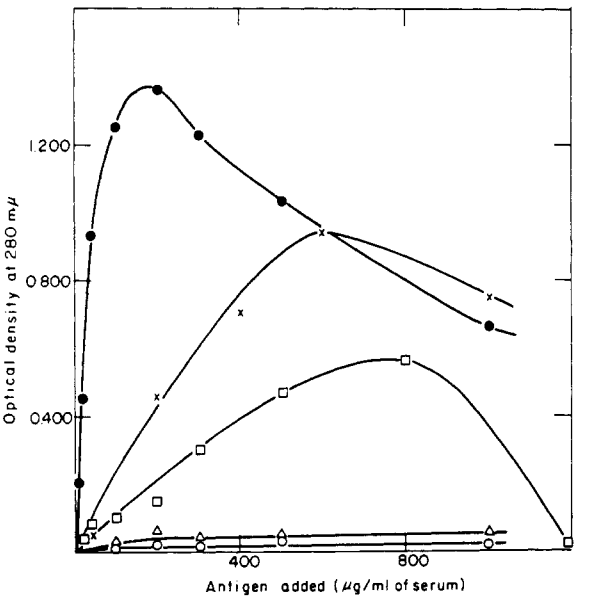

FIGURE 2: Extinction at 280 mμ of solutions in 0.1 N sodium hydroxide of precipitates obtained by addition to antipoly-L-lysyl serum albumin of: poly-L-lysyl serum albumin (●), poly-L-lysyl γ-globulin (×), poly-L-lysine (□), piperidine-treated serum albumin (△), and poly-L-tyrosyl serum albumin (○). All the curves were obtained after substracting the values from parallel experiments with normal serum.

prepared in analogy to poly-L-lysyl serum albumin: γ-Globulin (0.125 g) (prepared on DEAE-cellulose column according to Levy and Sober, 1960) in 0.05 M sodium phosphate buffer of *p*H 7.0 (20 ml) was treated at 2° with ε,*N*-trifluoroacetyl-α,*N*-carboxy-L-lysine anhydride (0.125 g) dissolved in dioxane (5 ml). After 24 hours at 2° the reaction mixture was dialyzed against distilled water for 3 days at 2°, the insoluble material formed was separated by centrifugation, and the supernatant solution was lyophilized. The fluorine content of this material was 9.8%. The protecting trifluoroacetyl groups were removed from the poly-ε,*N*-trifluoroacetyllysyl γ-globulin by the action of aqueous piperidine; 100 mg of the protected derivative was suspended in 7 ml of 1 M aqueous piperidine and the reaction was allowed to proceed for 30 hours, after which the solution was dialyzed and lyophilized.

Poly-L-tyrosyl Rabbit Serum Albumin. This was a gift from Dr. I. Schechter. It contained 17.1% tyrosine, while rabbit serum albumin contains 6.55% tyrosine.

Oligolysyl Peptides. The first ten members of the polylysine series were prepared from partial HCl hydrolysates (16 g) of high molecular weight poly-L-lysine (n = 1000) (Yaron *et al.*, 1964 and in preparation). Adsorption onto CM-cellulose at *p*H 5–6 and elution by a combination of buffered stepwise and linear gradients of LiCl were used to resolve the individual oligolysyl peptides. The higher members of the series required rechromatography. Desalting of the resolved oligomers was achieved by dilution, adsorption on CM-cellulose, elution in 0.05 N HCl, and lyophilization. The oligopeptides were finally precipitated from methanolic solutions by acetone to give 0.25 to 1.0-g amounts of the individual oligolysines. The isolated oligomers contained less than 1% contamination by other members of the series.

Immunization Procedure. Three rabbits were given three intramuscular injections at 10-day intervals. Each injection contained 15 mg of poly-L-lysyl serum albumin in Freund's complete adjuvant. Blood was collected weekly from the marginal ear vein, beginning 8 days after the last injection, and the positively reacting sera from the separate bleedings were pooled. If the titer of antibodies decreased considerably in the course of the bleedings, a booster injection of a saline solution of 2 mg poly-L-lysyl serum albumin was given intravenously.

Quantitative Precipitin Test. Increasing amounts of the homologous antigen or the heterologous materials tested for cross-reaction, dissolved in 0.5 ml of aqueous 0.9% sodium chloride, were added to test tubes containing 0.5 ml of the antiserum. In control experiments aqueous 0.9% sodium chloride was added in the absence of the antigen. Since some of the polylysyl derivatives cause precipitation upon reacting with normal rabbit serum, experiments using normal serum instead of the immune serum were carried out in parallel with each precipitin test. The solutions were mixed and the tubes were placed in a water bath at 37° for 30 minutes and then in the cold room for 24 hours. The resultant precipitates were centrifuged, washed twice with chilled 0.9% sodium chloride, and dissolved in 1.1 ml of 0.1 N NaOH. The extinction of these solutions was read within

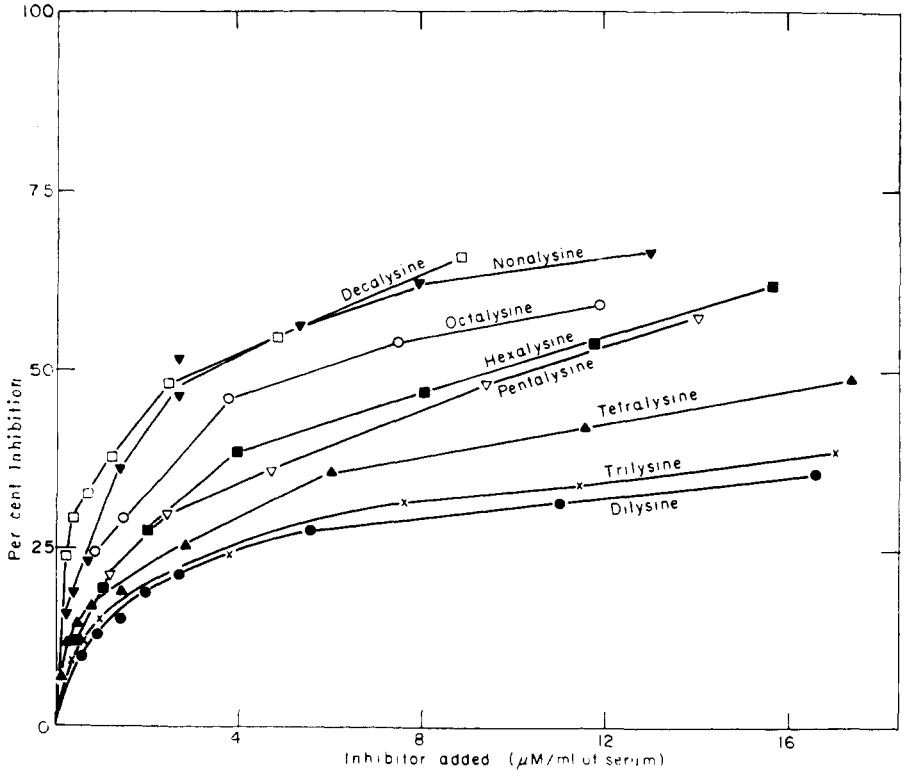

FIGURE 3: Inhibition by oligolysine peptides of the precipitation of antipoly-L-lysyl serum albumin by poly-L-lysyl serum albumin.

10 minutes at 280 mμ, in a Zeiss Model PMQ II spectrophotometer.

Inhibition Studies. Inhibition was measured by decrease in the amount of the antigen-antibody precipitation as a result of previous reaction of the antiserum with the oligopeptide. The quantitative studies were carried out as follows: Solutions of oligolysine peptides in 0.9% sodium chloride were added in increasing amounts (50–3000 μg) to 0.5 ml of the antiserum. After 30 minutes' incubation at 37° the mixtures were maintained overnight in the cold room, and, provided no precipitation had occurred, 100 μg of the homologous antigen in 0.1 ml of 0.9% sodium chloride was added. The mixtures were incubated at 37° for 30 minutes and allowed to stay in the cold room overnight. Any precipitates formed were investigated quantitatively as described. In a control experiment four samples of the antiserum were treated with the same amount of the homologous antigen after addition of aqueous 0.9% sodium chloride in the absence of any oligolysyl peptides. The mean value of the precipitates obtained, expressed as optical density after dissolving them in 0.1 N sodium hydroxide, was used throughout for the calculation of the extent of inhibition.

Results

In the poly-L-lysyl albumin preparation used in this study, 105 lysine residues were attached on the average per albumin molecule (assuming a molecular weight of 69,000). Antisera were prepared in rabbits, and characterized for their serological specificity by precipitation experiments with chemically related macromolecules and by the inhibition of the homologous reaction with oligolysine haptens.

Specificity of the Antisera to Poly-L-lysyl Serum Albumin. Antisera to poly-L-lysyl serum albumin are capable of reacting, in addition to the homologous antigen, with poly-L-lysine and with poly-L-lysyl γ-globulin. The homologous precipitin curve is shown in Figure 1. The nonspecific precipitation of plasma proteins by the antigen, as demonstrated by the precipitin curve with normal serum, is hardly significant below 500 μg/ml of serum. The cross-precipitants polylysine and poly-L-lysyl γ-globulin gave, on the other hand, a stronger reaction with normal serum. Figure 2, therefore, represents the immunospecific cross-precipitin curves obtained after subtracting the values that were obtained with normal serum. As is shown in Figure 2, poly-L-tyrosyl serum albumin did not react with the antiserum, nor was any precipitation obtained upon treating the antiserum with rabbit serum albumin that was subjected to the action of aqueous piperidine. (This treatment is one of the stages involved during the preparation of poly-L-lysyl serum albumin.)

Hapten Inhibition Studies. Having demonstrated the specificity of the antiserum obtained toward poly-L-lysine, its reaction with oligolysine peptides from the dimer to the decamer was investigated. These peptides do not cause any precipitation of the antibodies, but

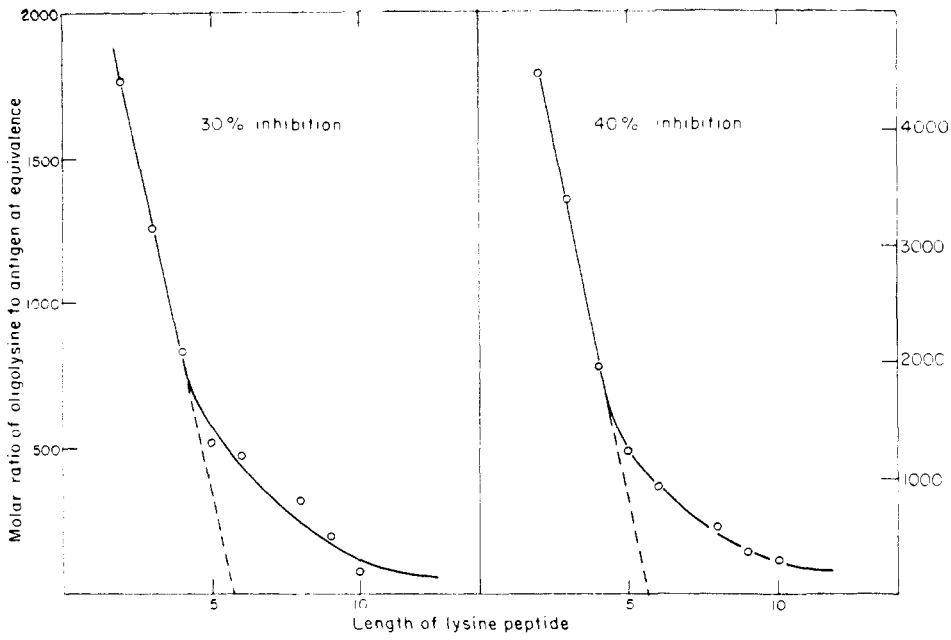

FIGURE 4: The molar ratio of the oligolysine required for 30% (left) or 40% (right) inhibition to the homologous antigen at the equivalence point, as a function of the length of the inhibitory peptide.

are capable of inhibiting the specific homologous reaction. The results are given in Figure 3. The degree of inhibition increases with increase in the length of the peptide up to the nonalysine. The decamer does not seem to be a better inhibitor than the nonamer. No significant inhibition was obtained with L-lysine.

In order to ascertain the efficiency of the various inhibitors, the data of Figure 3 were replotted so as to compare the molar quantities required for an arbitrary degree of inhibition, or rather the ratio between the molar quantities of the inhibitors and the homologous antigen, and its relation with the chain length of the peptide. A plot of this kind is shown in Figure 4, both for the 30% and for the 40% inhibition. In both cases the first part of the curve is a straight line, which on extrapolation intersects the abscissa at the same point, suggesting that oligolysines composed of 5–6res idues are the most effective inhibitors per lysine residue.

Discussion

All homopolymers of α-amino acids tested until now are nonimmunogenic. In order to obtain antibodies specific toward poly-L-lysine recourse was made, therefore, to immunization with poly-L-lysyl serum albumin. The antibodies obtained were directed indeed toward lysine peptides as apparent both from the immunospecific cross-precipitation with poly-L-lysine and poly-L-lysyl γ-globulin and from the inhibition of the homologous reaction with peptides of L-lysine.

While the extent of inhibition increases up to the nonamer (Figure 3), it seems from the analysis of the data given in Figure 4 that the increment in the efficiency of inhibition upon adding one lysine to the peptide chain is constant for the first few lysine residues, but decreases after the fifth lysine is added. This might imply that pentalysine or hexalysine corresponds to the size of the combining site of the antipoly-L-lysyl serum albumin as has been suggested in an analogous study with antibodies to poly-L-alanine by Sage *et al.* (1964).

The average chain length of the lysine peptides attached to serum albumin is 5.5 residues, and thus it seems that the size of the antigenic determinant and that of the combining site on the antibody are closely similar. Nevertheless, peptides up to the nonalysine are slightly better inhibitors than hexalysine. This might be because oligomers greater than the pentamer may have slightly higher combining affinities since they present more combinations of five residues (suggestion made in footnote 4 to the paper of Mage and Kabat, 1963). On the other hand, while the distribution of the lysine peptides on the serum albumin is Poissonian (Katchalski *et al.*, 1955, 1964), the possibility that some antigenic determinants contain 9 lysine residues cannot be excluded. Thus, in view of the possible variations in the length of the lysine peptide determinants, heterogeneity might be reflected also in the antibodies formed. In order to be able to draw more definite conclusions about the size and shape of combining sites on the antibody molecules complementary to polypeptides, it will be necessary to use antigens containing polypeptide determinants of a unique size (synthesized by stepwise methods) rather than those prepared by polymerization techniques.

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