Biochemistry

 Volume 7, Number 1 January 11, 1968

Studies on the Specificity of Antibody to 2,4-Dinitrophenyl-poly-L-lysines*

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ABSTRACT: The influence of hapten position and peptide chain length of the immunizing antigen on the specificity of antibody elicited in guinea pigs has been studied. Antibodies were prepared to α-DNP(Lys)$_{11}$, α-DNP(Lys)$_{1-13}$, α-DNP(Lys)$_{\overline{60}}$, α-DNP(Lys)$_{\overline{1200}}$, and ε-DNP$_{44}$(Lys)$_{\overline{1250}}$ and evaluated by quantitative precipitin and inhibition tests with use of chemically defined α-DNP-oligolysines (n = 1–9), oligolysines, and ε-DNP-lysine as inhibitors. Antisera to α-DNP(Lys)$_{11}$ was maximally inhibited on a molar basis by α-DNP-hepta-L-lysine. In contrast, highly polymerized α-DNP(Lys)$_{\overline{60}}$ and α-DNP(Lys)$_{\overline{1200}}$ provoked the formation of antisera which were more difficult to inhibit by small α-DNP-oligolysines and maximally inhibited on a molar basis by α-DNP-tri-L-lysine. This change in antibody behavior did not reflect specificity for internal lysine sequences or unknown ε-dinitrophenyl (ε-DNP) substitutions of the larger immunizing antigens since oligolysines without the DNP group were poor inhibitors and antisera to randomly substituted ε-DNP$_{44}$(Lys)$_{\overline{1250}}$ was specific for the ε-DNP determinant. To test the hypothesis that antibody specificity was influenced by the chain length of the immunizing antigen, α-DNP(Lys)$_{\overline{60}}$ was partially hydrolyzed with 6 N HCl, and α-DNP-oligopeptides of a low degree of polymerization (n = 1–13) were isolated and used for immunization. Antisera to these oligomeric fractions of α-DNP(Lys)$_{\overline{60}}$ was maximally inhibited on a molar basis by the heptamer and behaved similarly to anti-α-DNP(Lys)$_{11}$ sera.

It was concluded that chain length and conformation of the immunizing antigen as well as hapten position influence the specificity of the antibody formed to DNP-polylysines.

The discovery by Landsteiner (1945) that hapten-specific antibodies are formed against haptenic groups covalently coupled to proteins and the demonstration that these antibodies combine with hapten in solution provide a useful tool for studies of the nature of an antigenic determinant and of the reacting site on an antibody molecule. More recent studies with antisera to hapten-conjugated proteins, dinitrophenylated peptidyl proteins, and randomly substituted hapten polypeptides clearly indicate that the antibody combining site may be complementary in size to an area larger than the haptenic group (Eisen and Siskind, 1964; Parker *et al.*, 1966; Paul *et al.*, 1966). However, the chemical complexity of these conjugated peptides and proteins prevents elucidation of the role played by the amino acid environment of a haptenic group in determining the size and shape of the antibody reactive site. Substantial simplification of an antigenic determinant may be achieved by the use of chemically defined immunizing antigens wherein the amino acid sequence and position of the haptenic determinant are defined and where segments of the immunizing antigen are available (Schlossman *et al.*, 1965; Borek *et al.*, 1965; Richards *et al.*, 1967). This approach, in the dextran-

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antidextran system, with the use of quantitative inhibition of antigen–antibody reactions by oligomeric segments of the immunizing antigen, was initially shown by Kabat (1956) to be a powerful tool for investigation of the antibody combining site and the size of the antigenic determinant. Thus, Kabat (1960) showed that the capacity of a series of isomaltose oligosaccharides to inhibit precipitation of antidextran on a molar basis increased with increasing chain length and reached an upper limit at isomaltohexaose to isomaltoheptaose. These studies demonstrated that the upper limit for most human antidextran combining sites was complementary in size to isomaltohexaose. Numerous studies with use of a similar approach to this with polysaccharide, polynucleotide, and polypeptide determinants for other antigen–antibody systems have given comparable values to isomaltohexaose as the upper limit for combining site size (see Kabat, 1966).

The availability of a series of chemically defined α-DNP-oligolysyl peptides wherein there is a single haptenic determinant on the N-terminal α-amino position of the oligolysyl chain permitted us to investigate the nature of an antigenic determinant in a hapten-substituted system. Earlier studies demonstrated that antibody could be induced in guinea pigs with chemically defined α-DNP-oligolysyl peptides provided the peptide was equal to or larger in size than the heptamer (Schlossman *et al.*, 1965). The antibody formed to small α-DNP-oligolysines was shown in quantitative hapten inhibition studies to be maximally inhibited on a molar basis by α-DNP-hepta-L-lysine (Schlossman and Levine, 1967). This observation prompted us to investigate further the effect of chain length and hapten position on the specificity of the antibody formed to DNP-polylysines. In our studies animals were sensitized to α-DNP-polylysyl peptides with varying sized polylysyl backbones and randomly substituted dinitrophenylated polylysines. Serum antibodies were obtained and studied by quantitative precipitation and inhibition tests with the use of chemically defined α-DNP-oligolysines, oligolysines, and ε-DNP-lysine. Our results demonstrate the profound effects of hapten position and of peptide size of the immunizing antigen on the specificity of anti-DNP-polylysyl antibodies.

Material and Methods

Hapten-Substituted Poly-L-lysines. Poly-ε-benzyloxycarbonyl-L-lysines with an average degree of polymerization of 11, 60, and 1200 ($\bar{n}$ = 11, 60, and 1200) were described in previous publications (Schlossman *et al.*, 1965–1967). The blocked polymers (200 mg) were dissolved in 8 ml of dimethylformamide, and 0.1 ml of 1-fluoro-2,4-dinitrobenzene and 0.1 ml of triethylamine were added (Schlossman *et al.*, 1965). The reaction proceeded overnight at room temperature. The yellow α-DNP-poly-ε-*N*-benzyloxycarbonyl-L-lysyl peptides were isolated and the ε-*N*-benzyloxycarbonyl groups were removed by reaction with 30% HBr in acetic acid. The yellow water-soluble products (α-DNP(Lys)$_{\overline{11}}$, α-DNP-(Lys)$_{\overline{60}}$, and α-DNP(Lys)$_{\overline{1200}}$) were isolated by ether precipitation and purified by dissolving in water and repeated precipitation from water with ethyl alcohol and from methyl alcohol solution with acetone–ether. The material was dried *in vacuo* over KOH and concentrated H_2SO_4.

Poly-L-lysine·HBr ($\bar{n}$ = 1250) was purchased from Yeda, Rehovoth, Israel. (Lys)$_{\overline{1250}}$ (200 mg) was dissolved in 5 ml of 10% sodium bicarbonate. The polypeptide was randomly substituted by the addition of 5 mg of 1-fluoro-2,4-dinitrobenzene in 0.2 ml of dioxane. The mixture was stirred at room temperature for 2 hr, acidified with 1 N HCl, and extracted four times with ether. The dinitrophenylated polypeptide was dialyzed exhaustively against distilled water and lyophilized. The substituted polypeptide had an average of 44 ε-DNP-lysyl residues/1250 lysyl residues (ε-DNP$_{44}$-(Lys)$_{\overline{1250}}$) as determined by use of the known absorbancy of ε-DNP-lysine at 3600 Å.

α-DNP(Lys)$_{\overline{60}}$ (50 mg) was dissolved in 2 ml of 6 N HCl and hydrolyzed in a sealed glass vial in a water bath for 1 hr at 70°. The vial was then chilled, and the HCl was removed *in vacuo* over KOH. The dried material was dissolved in water and neutralized with lithium hydroxide, and the α-DNP(Lys)^{1} hydrolysate was fractionated by CM-cellulose chromatography.

Chromatography. Individual members of the α-DNP-oligo-L-lysine series of peptides were prepared for quantitative inhibition studies and immunization from α-DNP(Lys)$_{\overline{11}}$ by preparative chromatographic separation on CM-cellulose, desalting, and concentration (Schlossman *et al.*, 1965; Schlossman and Levine, 1967). Chain length of individual purified peptides was determined from chromatographic position as compared to known compounds. Peptides were designated in the following fashion: α-DNP-tri-L-lysine = α-DNP(Lys)$_3$ (n = degree of polymerization). The following chromatographically pure haptenic derivatives of oligolysines were prepared: α-DNP(Lys)$_3$, α-DNP(Lys)$_4$, α-DNP(Lys)$_5$, α-DNP(Lys)$_6$, α-DNP-(Lys)$_7$, α-DNP(Lys)$_8$, α-DNP(Lys)$_9$, α-DNP(Lys)$_{10}$, and α-DNP(Lys)$_{11}$. α-DNP-lysine, ε-DNP-lysine, and (Lys)$_{8-10}$ were previously described (Schlossman *et al.*, 1965, 1966).

α-DNP(Lys)$_{\overline{60}}$ (5 mg) was placed on a CM-cellulose column (1.8 × 18 cm) which was equilibrated with 0.05 M lithium chloride. Linear gradients of 600 ml each from 0.05 to 1.0 M lithium chloride and from 1.0 to 2.0 M lithium chloride were used for elution. The effluent was monitored at 3600 Å and recorded on a Gilford Model 2000 automatic spectrophotometer with 1-cm light-path flow cells. Lithium chloride concentration of the effluent was determined by conductivity meter. Figure 1A shows the elution pattern obtained on chromatography of α-DNP(Lys)$_{\overline{60}}$ with linear lithium chloride gradients. The material is seen to emerge at about 700 ml which corresponded to an effluent salt concentration of approximately 1.2 M lithium chloride. For comparison, 5 mg of α-DNP-(Lys)$_{\overline{11}}$ was placed on a similar CM-cellulose column and eluted with identical gradients. The elution diagram for α-DNP(Lys)$_{\overline{11}}$ peptides is shown in Figure 1B.

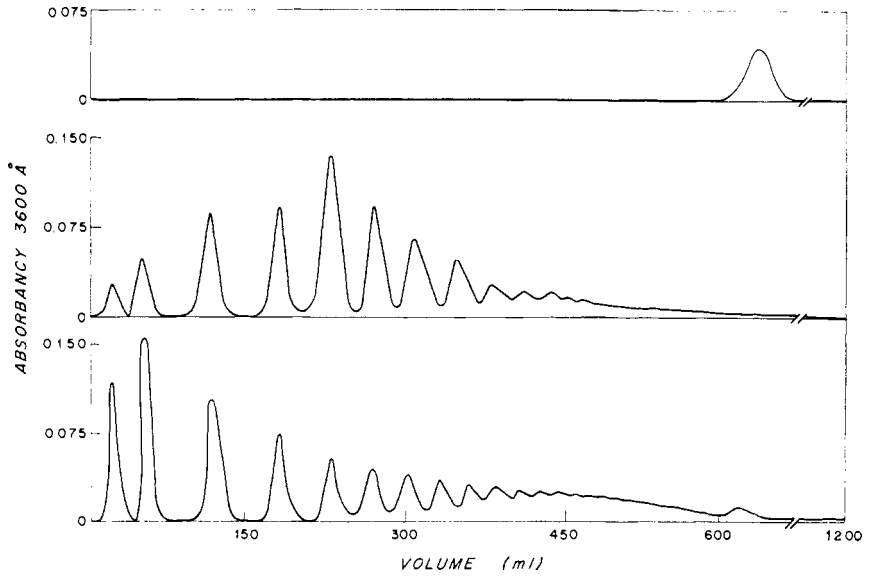

FIGURE 1: Chromatography of α-DNP-poly-L-lysines on CM-cellulose with linear lithium chloride gradients (0–600 ml = 0.05–1.0 M LiCl and 600–1200 ml = 1.0–2.0 M LiCl). (A) Top: α-DNP(Lys)$_{\overline{60}}$. (B) Middle: α-DNP(Lys)$_{\overline{11}}$. (C) Bottom: hydrolyzed α-DNP(Lys)$_{\overline{60}}$. See text under Methods for experimental details.

Figure 1C shows the elution pattern obtained on chromatography of hydrolyzed α-DNP(Lys)$_{\overline{60}}$. The elution pattern shows a series of successive peaks which emerge with increasing salt concentration in a manner analogous to that obtained for the α-DNP-(Lys)$_{\overline{11}}$ (Figure 1B). The first peak seen to emerge corresponds to α-DNP-lysine and each successive peak differs from the preceding peak by the addition of one lysyl residue (Schlossman *et al.*, 1965). The 0.05 M lithium chloride to 0.7 M lithium chloride fraction (0–450 ml) obtained on chromatography of hydrolyzed α-DNP(Lys)$_{\overline{60}}$ and corresponding to α-DNP-lysine to α-DNP(Lys)$_{13}$ (α-DNP(Lys)$_{1-13}$) was pooled, desalted, concentrated, and used for immunization.

Guinea pig anti-α-DNP(Lys)$_{\overline{60}}$ serum (3 ml) and and 3 ml of guinea pig anti-α-DNP(Lys)$_{1-13}$ serum were individually dialyzed in 0.1 M Tris-HCl plus 1 M sodium chloride and applied to a Sephadex G-200 (Pharmacia, Uppsala, Sweden) column (2.5 × 65.0 cm) (Flodin, 1962). The sera were eluted with 0.1 M Tris-HCl plus 1 M sodium chloride at a flow rate of approximately 20 ml/hr. The effluent was collected in 3-ml fractions and its absorbancy recorded at 2800 Å. The elution diagram revealed three distinct peaks. These were individually concentrated by ultrafiltration, dialyzed against 0.15 N sodium chloride, and set up for quantitative precipitin determinations as described below. Approximately 95% of the antibody activity was found in the second peak with both anti-α-DNP-(Lys)$_{\overline{60}}$ and α-DNP(Lys)$_{1-13}$ sera. This peak corresponded to the 7S fraction of antibody, so that most if not all of the precipitable anti-α-DNP(Lys)$_{\overline{60}}$ and anti-α-DNP(Lys)$_{1-13}$ antibodies were considered to be 7S immunoglobulins (Flodin, 1962).

Spectrophotometry. A Gilford spectrophotometer with silica cells of 1-cm light path was used for all spectrophotometric determinations. The α-DNP-lysine content of the peptides was determined in 0.01 M sodium phosphate–saline buffer (pH 7.0) at 3600 Å ($E_{3600\,Å}$ 16,800) (Schlossman *et al.*, 1965). The ε-DNP-lysine content was similarly determined ($E_{3600\,Å}$ 17,400) (Eisen, 1964).

Immunization. Guinea pigs of the Hartley strain weighing approximately 400 g were used. Groups of eight guinea pigs were immunized with each of the following five antigens: (1) α-DNP(Lys)$_{11}$, (2) α-DNP(Lys)$_{\overline{60}}$, (3) α-DNP(Lys)$_{\overline{1200}}$, (4) ε-DNP$_{44}$(Lys)$_{\overline{1250}}$, and (5) α-DNP(Lys)$_{1-13}$. The DNP polypeptides were diluted in buffered saline and emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.). Each animal was injected with a total of 0.4 ml containing 0.1 mg of the material to be tested. Injections were distributed equally in the hind foot pads. The animals were injected subcutaneously in the dorsum of the neck at the end of 1 week with 0.1 mg of the antigen in 0.25 ml of saline and emulsified with an equal volume of Freund's incomplete adjuvant (Difco Laboratories, Detroit, Mich.). Blood samples were obtained by cardiac puncture 4–5 weeks following immunization. Serum from responding animals were pooled and methiolate 1:10,000 was added to all sera. In general, each pool of antisera was obtained from five to seven responding animals.

Quantitative Precipitin and Inhibition Studies. Quantitative precipitin assays (Kabat, 1961) were performed with pools of guinea pig antisera. A multivalent antigen was prepared by coupling α-DNP(Lys)$_{\overline{11}}$ (see Figure 1B for distribution of peptide size) to succinylated bovine plasma albumin by the carbodiimide procedure

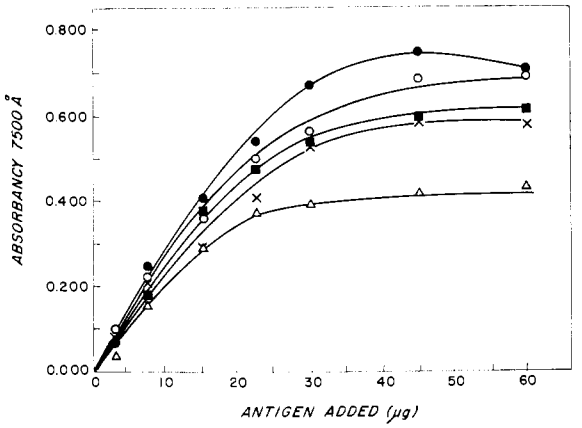

FIGURE 2: Quantitative precipitin curves of anti-DNP-polylysine sera with increasing quantities of α-DNP-$(Lys)_{\overline{11}}$-BPA. (×-×-×) α-DNP$(Lys)_{11}$ sera, (○-○-○) α-DNP$(Lys)_{\overline{60}}$ sera, (●-●-●) α-DNP$(Lys)_{\overline{1200}}$ sera, (△-△-△) α-DNP$(Lys)_{1-13}$ sera, and (■-■-■) ε-$DNP_{44}(Lys)_{\overline{1250}}$ sera.

as previously described (Goodfriend *et al.*, 1964; Schlossman and Levine, 1967). The resultant α-DNP-$(Lys)_{\overline{11}}$-BPA[1] with an average of five α-DNP-oligolysyl residues of varying size per succinylated bovine plasma albumin molecule was used for quantitative precipitin assays. Antiserum (0.2–0.3 ml) was added to a series of 3.0-ml conical centrifuge tubes containing known quantities of α-DNP$(Lys)_{\overline{11}}$-BPA. Saline was added to a volume of 0.75 ml, and the tube contents were mixed. After 1 hr at 37° and 48 hr in the refrigerator, during which time the tubes were mixed several times, the tubes were centrifuged in a refrigerated centrifuge, the precipitates were determined by the use of a modified Lowrey–Folin method (Mage and Dray, 1965) (phenol reagent, 2 N, Fisher Scientific, Fairlawn, N. J.), and the extinction coefficients of these solutions were recorded at 7500 Å.

Inhibition assays were carried out on a microscale with the use of approximately 0.2–0.3 ml of antisera (Kabat, 1961). Known concentrations of inhibitor and antibody were mixed and incubated for 30 min at 37°. A quantity of α-DNP$(Lys)_{\overline{11}}$-BPA which was determined from the precipitin curves to give maximum precipitation of the antibody was added to these tubes. Contents of the tubes were again mixed, incubated for 1 hr at 37°, refrigerated 48 hr, washed, and analyzed. Inhibition assays were performed with α-DNP$(Lys)_n$ (n = 3–9), α-DNP-lysine, ε-DNP-lysine, $(Lys)_{8-10}$, α-DNP$(Lys)_{\overline{60}}$, and α-DNP$(Lys)_{\overline{1200}}$. Per cent inhibition was calculated by dividing the amount of antibody precipitated in the presence of the inhibitor by the amount of antibody precipitated without inhibitor. The points plotted represent the average of duplicate and in some instances triplicate determinations done on separate days.

Results

The behavior of guinea pig antisera from animals sensitized to α-DNP$(Lys)_{11}$, α-DNP$(Lys)_{\overline{60}}$, α-DNP-$(Lys)_{\overline{1200}}$, α-DNP$(Lys)_{1-13}$, and ε-$DNP_{44}(Lys)_{\overline{1250}}$ on addition of increasing quantities of α-DNP$(Lys)_{\overline{11}}$-BPA is given in Figure 2. Very large quantities of α-DNP$(Lys)_{\overline{11}}$-BPA caused a small amount of nonspecific precipitation from normal serum, but none is seen at the range of antigen concentration used for these studies. Further, chemically defined α-DNP-oligo-L-lysines (n = 3–9), oligo-L-lysines, ε-DNP-lysine, and α-DNP-lysine did not produce precipitation from these sera. In contrast, α-DNP$(Lys)_{\overline{60}}$ and α-DNP-$(Lys)_{\overline{1200}}$ react nonspecifically with serum from normal as well as sensitized guinea pigs to produce a precipitate. The amount of precipitate formed with these compounds varied sufficiently to prevent their use in quantitative inhibition studies.

The relative inhibiting powers of α-DNP$(Lys)_n$, $(Lys)_{8-10}$, and ε-DNP-lysine were compared using guinea pig anti-α-DNP$(Lys)_{11}$ sera. As may be seen in Figure 3A, inhibiting power per α-DNP-oligopeptide molecule increased with increasing number of lysyl residues so that on a molar basis α-DNP$(Lys)_7$ > α-DNP$(Lys)_6$ > α-DNP$(Lys)_5$ > α-DNP-$(Lys)_4$ > α-DNP$(Lys)_3$ > α-DNP-lysine. The relative increase in inhibiting power reached a maximum at the heptamer, so that no additional inhibition was obtained with α-DNP$(Lys)_8$ or α-DNP$(Lys)_9$. Further, the immunodominant nature of the DNP group is evident from the inability of $(Lys)_{8-10}$ to produce a significant degree of inhibition.

Although the DNP hapten is conjugated to the α-amino group of the N-terminal lysyl residue, it has been found with all antisera to α-DNP$(Lys)_n$ peptides that ε-DNP-lysine of all the DNP-amino acids tested (Schlossman and Levine, 1967) is a slightly better inhibitor than α-DNP-lysine itself. The difference in binding and thus inhibition may be related to the charged carboxyl group adjacent to the DNP group in α-DNP-lysine as compared to the more distant charged carboxyl group present in ε-DNP-lysine. This negative effect on binding may then contribute to more efficient binding of ε-DNP-lysine. Similarly, it has been shown that protonated 2,4-dinitrophenol (Eisen and Siskind, 1964) and DNPNS (Metzger *et al.*, 1963) have a relatively greater affinity for anti-DNP binding sites than do 2,4-dinitrophenolate or the ionized form (naphtholate ion group) of DNPNS.

The behavior of anti-α-DNP$(Lys)_{\overline{60}}$ and α-DNP-$(Lys)_{\overline{1200}}$ sera is represented in Figure 3B,C, respectively. With these antisera, α-DNP$(Lys)_3$ to α-DNP-$(Lys)_9$ were equally potent inhibitors on a molar basis. However, as compared to anti-α-DNP$(Lys)_{11}$ sera (Figure 3A), approximately ten times the quantity of trimer or higher oligomers was required to reach 50% inhibition. Further, the maximum amount of inhibition

[1] Abbreviations used: BPA, succinylated bovine plasma albumin; DNPNS, 2-(2,4-dinitrophenylazo)-1-naphthol-3,6-disulfonate.

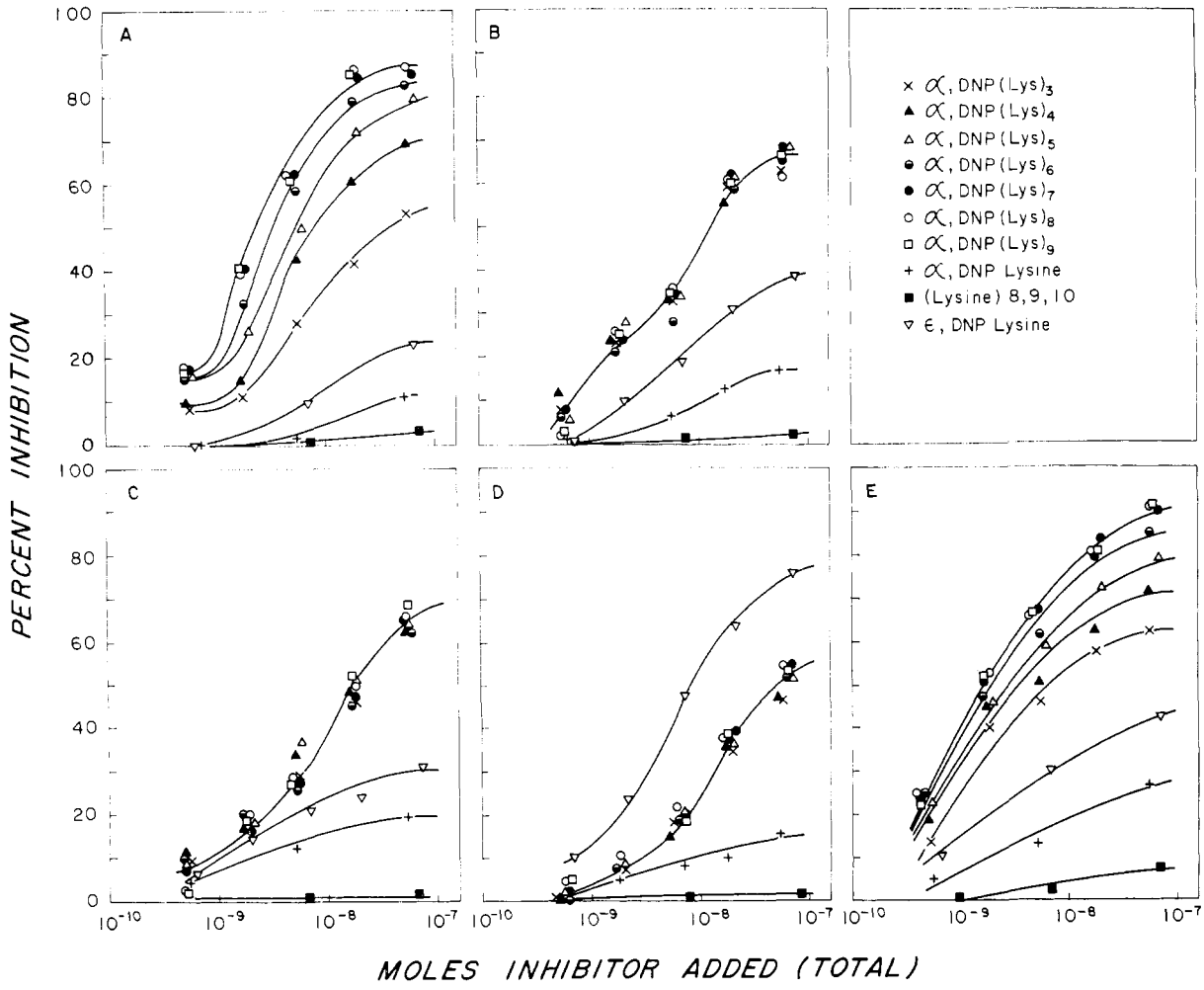

FIGURE 3: Quantitative inhibition of precipitation of anti-DNP-polylysine sera. (A) α-DNP(Lys)$_{11}$ sera, (B) α-DNP-(Lys)$_{\overline{60}}$ sera, (C) α-DNP(Lys)$_{\overline{1200}}$ sera, (D) ε-DNP$_{44}$(Lys)$_{\overline{1250}}$ sera, and (E) α-DNP(Lys)$_{1-13}$ sera.

obtained is less. This change in the inhibitory capacity of various α-DNP-oligo-L-lysines could not be attributed to specificity for internal oligolysyl portions of the larger immunizing antigen since (Lys)$_{8-10}$ produced insignificant inhibition.

These findings raise the possibility that unknown ε-DNP-lysine substitutions on α-DNP(Lys)$_{\overline{60}}$ or α-DNP(Lys)$_{\overline{1200}}$ account for the change in specificity of the antisera. To test this possibility, antibody was prepared to ε-DNP$_{44}$(Lys)$_{\overline{1250}}$ and its specificity for α- and ε-DNP groups studied (Figure 3D). The results obtained indicate that ε-DNP-lysine is a significantly better inhibitor than α-DNP-oligo-L-lysines or oligo-L-lysines without the DNP group despite the use of α-DNP(Lys)$_{\overline{11}}$-BPA as the precipitating antigen. These data suggest that the previously noted specificity for larger α-DNP-oligolysines neither reflected nonspecific binding to areas adjacent to the antibody binding site nor was a consequence of the small α-DNP-oligo-L-lysyl residues of the precipitating antigen.

As further evidence that anti-α-DNP(Lys)$_{\overline{60}}$ and α-DNP(Lys)$_{\overline{1200}}$ specificity was influenced by the larger poly-L-lysyl chain of the immunizing antigen, α-DNP(Lys)$_{\overline{60}}$ was partially hydrolyzed with 6 N HCl and α-DNP-oligopeptides with a degree of polymerization of 1–13 were isolated and used for immunization. Quantitative inhibition tests with anti-α-DNP-(Lys)$_{1-13}$ sera are depicted in Figure 3E. Although α-DNP(Lys)$_{1-13}$ was prepared from α-DNP(Lys)$_{\overline{60}}$, the antibody formed to these oligomeric segments differed significantly from that elicited by α-DNP(Lys)$_{\overline{60}}$. The inhibiting power per molecule increased with increasing number of lysyl residues and reached a maximum at α-DNP(Lys)$_7$. These curves are similar to those found with antisera to α-DNP(Lys)$_{11}$ (Figure 3A) and supply a crucial test for the validity of the hypothesis that the chain length of the immunizing antigen influences the specificity of the antibody formed to various DNP-polylysines. The demonstration that the antibody activities of both α-DNP(Lys)$_{1-13}$ and α-DNP(Lys)$_{\overline{60}}$ antisera were found exclusively in the second peak of the Sephadex G-200 separation indicates that the difference in behavior of these antibodies was not related to differences in behavior of 7S and 19S

antibodies with specificity toward a common antigenic determinant. However, different classes of guinea pig 7S antibodies may still account for these findings and further studies with purified fractions of 7S antibody will have to be undertaken to resolve this point.

Discussion

Although antibodies recognize a well-defined upper limit in size for an antigenic determinant which is an estimate of antibody combining site size, much less is known about the shape and conformation of the antigenic determinant and its influence on the dimensions of the antibody reactive site. It has been known for some time that alterations in the three-dimensional structure of proteins have a profound effect on the antigenic specificity of these materials. For example, conversion of ribonuclease to a random coil markedly diminishes the reactivity of this material to antibody made against native ribonuclease (Brown *et al.*, 1959; Harrington and Sela, 1959). Similarly, numerous studies of carbohydrate, protein, and polypeptide antigens attest to the capacity of antibody to recognize a three-dimensional conformation of the immunizing antigen (see Haber *et al.*, 1964; Kabat, 1966). If the α-DNP group and adjacent oligolysines are not sufficient to account for the difference in behavior of anti-α-DNP-polylysine antibodies, the likely explanation for these differences may reside in the capacity of antibody forming cells to recognize and produce antisera to specific conformations of these peptides. Although both large and small polylysines would be expected to exist in solution as randomly coiled forms under the conditions of immunization, that is neutral pH, the fate and physical interactions of these antigens following injection are not known. It has been shown (Rosenheck and Doty, 1961; Sarkar and Doty, 1966) that large polylysines can assume under appropriate alkaline conditions an ordered α-helical or β configuration, and a β configuration at neutral pH with addition of a detergent. Thus, immunization with a highly polymerized polylysine may present the antibody forming apparatus with a random coil, α helix, β structure, or a multitude of "transitions" between these configurations. In contrast, the chain length of smaller oligolysines may preclude the development of a stable three-dimensional structure (Schellman and Schellman, 1964). It therefore must be considered that anti-α-DNP$(\text{Lys})_{\overline{60}}$ and α-DNP$(\text{Lys})_{\overline{1200}}$ antibody have a reactive site which recognizes a "conformation" (or "conformations") of the immunizing antigen. That the trimer causes maximal inhibition of such an antibody binding site may then only reflect the inability of larger α-DNP-oligolysines under the conditions of quantitative inhibition to assume the appropriate "conformation" of the antigenic determinant. Under such circumstances the site may be physically larger but still maximally inhibited by the trimer. The observation that the trimer is a less efficient inhibitor of α-DNP-$(\text{Lys})_{\overline{60}}$ and α-DNP$(\text{Lys})_{\overline{1200}}$ antisera than the heptamer is with α-DNP$(\text{Lys})_{11}$ antisera would support the conclusion that small α-DNP-oligo-L-lysines cannot be used to discriminate the binding site size of antibodies formed to highly polymerized DNP-polylysines. In this regard, it is unfortunate that α-DNP$(\text{Lys})_{\overline{60}}$ and α-DNP$(\text{Lys})_{\overline{1200}}$ could not be used in quantitative inhibition studies. In contrast, antibody produced to smaller α-DNP-oligolysines may have specificity for the random coil peptide. In the latter case, the peptides may be more complementary to the antibody combining site and thus yield a truer estimate of the binding site size.

Two alternative but less plausible explanations for these findings are: (1) the combining site formed to α-DNP$(\text{Lys})_{\overline{60}}$ and α-DNP$(\text{Lys})_{\overline{1200}}$ is smaller in size than that formed to α-DNP$(\text{Lys})_{11}$, or (2) the magnitude of the binding for the DNP group and the adjacent two to three lysyl residues may be such that hapten inhibition studies are not sufficiently sensitive to discriminate between the inhibitory capacity of the trimer and larger α-DNP-oligolysyl peptides. Both considerations require that highly polymerized α-DNP-polylysines can select out populations or antibody forming cells which either respond to a smaller portion of the immunizing antigen or produce antibody whose binding sites are largely if not exclusively directed in terms of binding energy to the DNP group and an adjacent two to three lysyl residues. Equilibrium dialysis, fluorescence quenching, and inhibition assays with various inhibitors at different pH values are currently being undertaken with a view toward exploring these alternatives.

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Immunochemistry of Polyribonucleotides. Study of Polyriboinosinic and Polyriboguanylic Acids*

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ABSTRACT: The immunological behavior of polyriboinosinic (poly I) and polyriboguanylic acids (poly G), studied with antiribonucleic acid antibodies, depends on the conformation of these polyribonucleotides and has been employed as a tool for the determination of the number of strands per molecule in solution. In the absence of added electrolytes, and up to a concentration of 10^{-3} M NaCl or 10^{-5} M $MgCl_2$, poly I and poly G appear to precipitate as single-stranded molecules, as judged by comparison with poly U, chosen as the reference for a single-stranded system. At higher salt concentration, the weight of polyribonucleotide precipitable by a given weight of antibody is in direct proportion to the number of strands per molecule. In 5×10^{-4} M $MgCl_2$ and at room temperature, an ordered structure of poly I and poly G is precipitated, immunologically four-stranded. This ordered structure is also present in a 1 M NaCl solution for poly I and 0.25 M NaCl for poly G. At lower salt concentration some multistranded structure persists in solution, to an extent varying with the ionic strength of the solvent. At 45°, poly I precipitates as a single-stranded molecule. Poly-N^7-methylinosinic acid and poly-N^1,N^7-dimethylinosinic acid, which do not form ordered structures, have precipitin curves like those of single-stranded poly U, even in the presence of salts.

The conformation of polyribonucleotides in solution has been studied by various physicochemical methods. In previous investigations, we initiated studies of the role of the conformation of polyribonucleotides in their precipitin reactions with anti-RNA antibodies, isolated by specific precipitation with poly A from the sera of animals hyperimmunized with ribosomes (Barbu and Panijel, 1960; Panijel and Barbu, 1961; Panijel, 1963; Panijel *et al.*, 1963).

These antibodies (to which we refer as NG I antibodies) can precipitate all polyribonucleotides and RNA, but do not cross-react with DNA. These results show that some antigenic determinants are common to all ribonucleic acids and synthetic polyribonucleotides, which cannot serve as immunizing antigens but can act as reactive antigens.

In contrast to most of the antinucleic acid antibodies whose antigenic determinants involve the bases (Tanenbaum and Beiser, 1963; Sela *et al.*, 1964; Seaman *et al.*, 1965; Butler *et al.*, 1965; Plescia *et al.*, 1965) the horse NG I antibodies are capable of precipitating multistranded structures as well as single-stranded structures (Panijel *et al.*, 1966a). However, in the case of multistranded structures the immunological reaction is modified. The amount of antibodies precipitable decreases as compared with the corresponding single-stranded system, while the amount of polynucleotide used as reactive antigen precipitable increases proportionally to the number of strands of the molecule.

This relationship was confirmed in several cases where the number of strands in the molecule was known with certainty, for example, in the case of the

* From the Department of Physiopathology of Immunity, Institut Pasteur, Paris, France. *Received December 21, 1966.* This work was supported in part by a grant from the Délégation à la Recherche Scientifique et Technique (Biologie Moléculaire) and by grants from the Centre National de la Recherche Scientifique.

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