Immunochemical Studies on Bacteriophage Deoxyribonucleic Acid. IV. Hapten Inhibition*

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ABSTRACT: Two antisera to each of the following deoxyribonucleic acids (DNA's), T_2, T_4, and T_6, were found to crossreact with heterologous T-even DNA's. They did not react with forty-two other DNA's from a wide variety of sources. When measured by the technique of hapten inhibition, the anti-T_6 sera were found to contain antibodies directed to the α-gentiobioside residues of T_6 DNA.

The reactive site extends at least to hydroxymethylcytosine but its limit is not known. The specificities of the two anti-T_4 sera, which differ in their cross reactions with T_2 DNA, are attributed to the preponderance of antibodies to α-monoglucosyl hydroxymethylcytosine in one serum and β-monoglucosyl hydroxymethylcytosine in the other. The anti-T_2 sera were found to be quite different in that the antibody of one serum is directed toward α-monoglucosyl hydroxymethylcytosine residues, while the second may contain antibodies directed toward a β-glucosyl linkage as well as to α-glucosyl linkages.

Antibodies reacting with T-even bacteriophage DNA have been demonstrated in the serum from rabbits immunized with ruptured bacteriophage (Levine *et al.*, 1960; Murakami *et al.*, 1961, 1962). The presence of glucosidic residues on T-even bacteriophage DNA (Jesaitis and Goebel, 1953; Sinsheimer, 1954; Volkin, 1954) was adduced as a possible source of antigenic specificity. The cross-reactivity of T_2, T_4, and T_6 DNA's and their heterologous antisera appears to be related to the distribution and steric orientation of the glucosidic residues on these DNA's (Murakami *et al.*, 1962). Furthermore, DNA's from a wide variety of sources have been examined for serological reactivity and the lack of reactivity of these DNA's in which glucosidic residues have not been demonstrated lends support to the hypothesis that the glucosidic residues are involved in the antigenic site(s). It is known from the data of Lehman and Pratt (1960) and Kuno and Lehman (1962) that the extent of glucosylation of the deoxyhydroxymethylcytidylic acid in the T-even phages is as follows:

Glucosylation of dHMP[1]	T_2	T_4 (in per cent)	T_6
None	25	0	25
α-Glucosyl	70	70	3
β-Glucosyl	0	30	0
β-Glucosyl-α-glucosyl	5	0	72

Six antisera have been analyzed with respect to cross-reactivity (Murakami *et al.*, 1962). The degree of cross-reaction is expressed as the percentage of the homologous reaction in the summary given in Table I. The present report describes experiments in which the specificity of these six antisera to ruptured bacteriophage was investigated using the technique of inhibition by haptens of known structure. A preliminary report of this work has appeared (Townsend *et al.*, 1961).

TABLE I: Cross-Reactivity of Six Antisera.

Anti-serum	Rabbit No.	Serological Activity T_2 (%)	T_4 (%)	T_6 (%)
Anti-T_2	815-2	100	100	20
Anti-T_2	730-2	100	108	50
Anti-T_4	165	13	100	12
Anti-T_4	851	94	100	6
Anti-T_6	159-1	20	31	100
Anti-T_6	67A	22	20	100

Materials and Methods

DNA and antisera to ruptured bacteriophage were prepared as described by Levine *et al.* (1960). All studies were carried out using the complement-fixation inhibition assay of Wasserman and Levine (1961). The concentrations of antigen and antibody were such as to

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[1] Abbreviation used in this work: dHMP, deoxyhydroxymethylcytidylic acid.

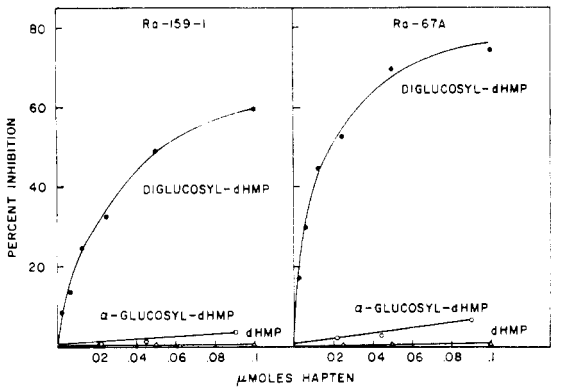

FIGURE 1: Inhibition of two anti-T_6 DNA systems by dHMP, α-monoglucosyl dHMP, and diglucosyl dHMP.

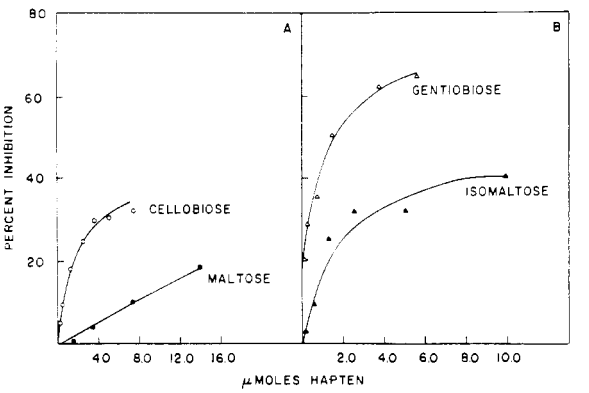

FIGURE 3: Inhibition of T_6 system (Rabbit-67A) by the anomers, cellobiose and maltose, and gentiobiose and isomaltose.

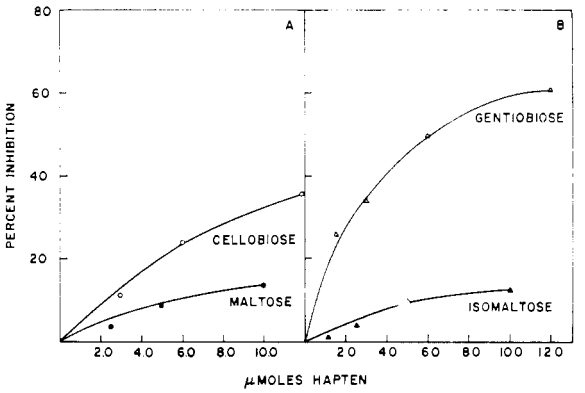

FIGURE 2: Inhibition of T_6 system (Rabbit-159-1) by the anomers, cellobiose and maltose, and gentiobiose and isomaltose.

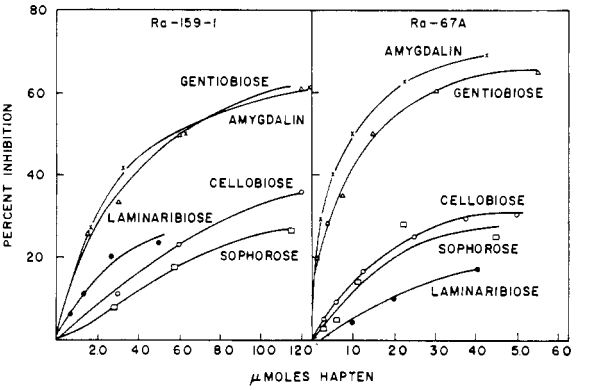

FIGURE 4: Inhibition of the T_6 systems (Rabbits-159-1 and 67A) by amygdalin, a gentiobioside, and four β-diglucosides varying in the position of their glucosidic linkage. The linkages are described in the text.

be in the equivalence zone where complement fixation is maximum. Slight variations in the level of maximal inhibition were found occasionally but in those cases the data for comparison are presented from a single experiment. In no case was the relative order of inhibition by different haptens changed. In order to maintain optimal conditions for complement fixation, seasonal variations in complement titer were counteracted by varying the antiserum dilution.

Maltose, trehalose, and α-methyl glucoside were obtained from Pfanstiehl Laboratories, Waukegan, Ill. Cellobiose, gentiobiose, and amygdalin were obtained from California Corp. for Biochemical Research, Los Angeles, Calif. All the sugars were examined by paper chromatography and all were found to be homogeneous with the exception of amygdalin, which contained a minor impurity.

Results

Hapten Inhibition of T_6 DNA Anti-T_6 DNA. The low cross-reactivity of T_2 and T_4 DNA's with anti-T_6 sera indicated that the antigenic specificity of these sera is directed in part to the high proportion of diglucosylated residues in T_6 DNA (Murakami *et al.*, 1962). The predominant participation of the diglucosyl residues in the antigenic sites is shown in Figure 1, which depicts the inhibitory effect of diglucosyl dHMP, α-monoglucosyl dHMP, and dHMP in two T_6 DNA anti-T_6 immune systems. Diglucosyl dHMP is the more effective inhibitor.

The nature of the linkage of the diglucoside involved in the antigenic sites was examined by comparing the relative inhibitory effect of diglucoside haptens varying in the position and orientation of their glucosidic linkage. In Figures 2 and 3 are presented the results on the orientation of the linkage. Cellobiose and gentiobiose, the β isomers, are more effective inhibitors than maltose and isomaltose, the corresponding α isomers, indicating that the diglucoside residue of the antigenic determinants has a β-glucoside linkage. The position of the linkage in the diglucoside determinant was resolved by

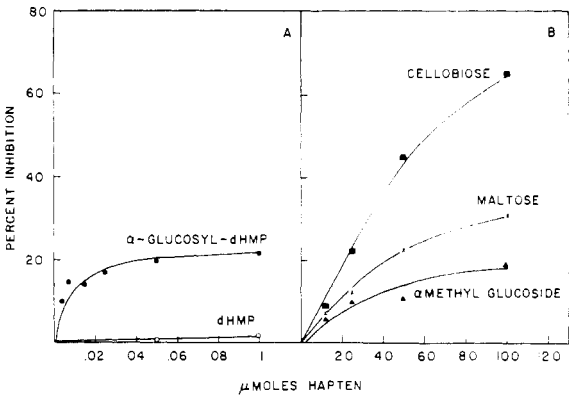

FIGURE 5: Inhibition of the anti-T_4 DNA system (Rabbit-165) by dHMP and α-monoglucosyl dHMP, by the anomers, cellobiose and maltose, and by α-methyl glucoside.

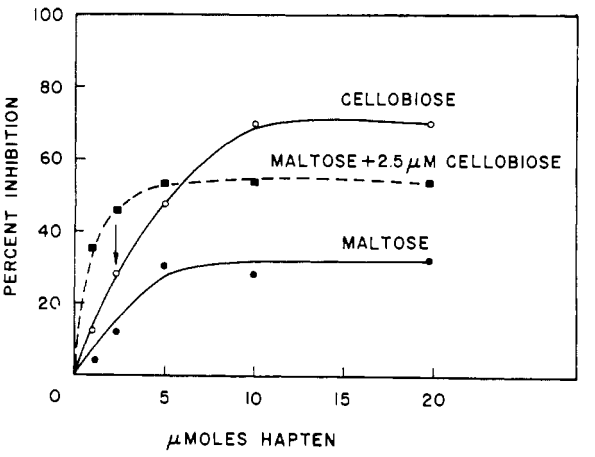

FIGURE 6: Inhibition of the anti-T_4 DNA system (Rabbit-165) by increments of maltose, cellobiose, and maltose plus a constant amount (2.5 μmoles) of cellobiose.

examining the relative inhibitory effect of four β-diglucosides of 1,6-(gentiobiose), 1,4-(maltose), 1,3-(laminaribiose), and 1,2-(sophorose) linkage. The specificity of the receptor site(s) for the 1,6-diglucoside linkage is shown in Figure 4. Gentiobiose is the most effective diglucoside inhibitor of both anti-T_6 systems, indicating that the diglucosyl residue of T_6-dHMP is a gentiobioside. Amygdalin, mandelonitrile-β-gentiobioside, is no more effective than gentiobiose itself, indicating that the glucosidic linkage to dHMP is α.

Although the antibody specificity is directed in part to the gentiobioside residue, it is important to note that diglucosylated dHMP is 50 to 100 times more effective as an inhibitor than gentiobiose alone. Some of the antibody receptor sites presumably extend beyond the diglucoside residue into the nucleotide structure.

Hapten Inhibition of T_4 DNA Anti-T_4 DNA. Two antisera to ruptured T_4 bacteriophage, containing antibodies reacting with T-even DNA, have been examined by hapten inhibition. The varying specificities of these sera are revealed in the cross-reaction data of Murakami *et al.* (1962) summarized in the introduction. The presence of β-monoglucoside residues on T_4 dHMP leads to the prediction that antiserum Rabbit-165, which reacts weakly with T_2 and T_6 DNA's, contains antibodies directed mainly to these substituents unique to T_4. The second antiserum (Rabbit-851) has a strong reaction with T_2 DNA and apparently contains antibodies directed toward α-glucoside residues.

The contribution of the β-glucoside residues of T_4 DNA to the specificity of Rabbit-165 antibodies is illustrated in Figure 5. The glucosylated nucleotide, α-monoglucosyl dHMP, does inhibit this T_4 DNA anti-T_4 DNA reaction but only to the extent of approximately 25%. The nonglucosylated nucleotide, at concentrations at which the glycosylated nucleotide is inhibitory, is ineffective. Cellobiose, a β-diglucoside, inhibits the immune reaction to a greater extent than the α-glucosides, maltose and α-methyl glucoside, indicating the high affinity of the antibody population for

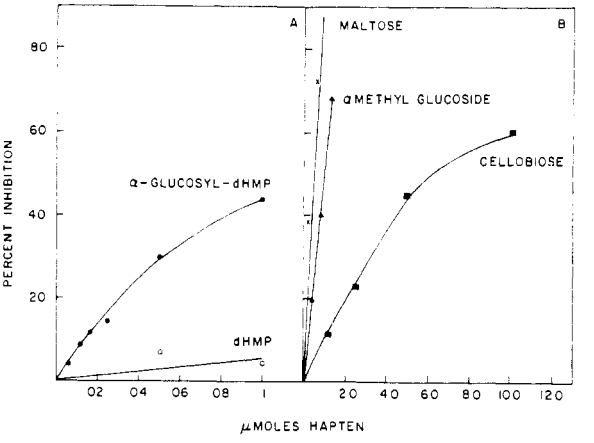

FIGURE 7: Inhibition of the anti-T_4 DNA system (Rabbit-851) by dHMP and α-monoglucosyl dHMP, and by α-methyl glucoside.

the β-glucoside configuration. It is of interest to note that the addition of both α- and β-glucosides to this system results in summation of the inhibitory capacities of each saccharide (Figure 6).

In contrast to the Rabbit-165 immune system, the reaction between T_4 DNA and Rabbit-851 antiserum is inhibited to a greater extent by maltose and α-methyl glucoside than by the β-glucoside-containing saccharide cellobiose (Figure 7). Maltose is approximately thirty times as effective as cellobiose. α-Monoglucosyl dHMP is five times as effective as maltose and is a better inhibitor of this system than of the Rabbit-165 system. Again, the nonglucosylated dHMP, at concentrations at which the glucosylated nucleotide is inhibitory, is relatively ineffective.

Hapten Inhibition of T_2 DNA Anti-T_2 DNA. Two antisera to ruptured T_2 bacteriophage exhibit a com-

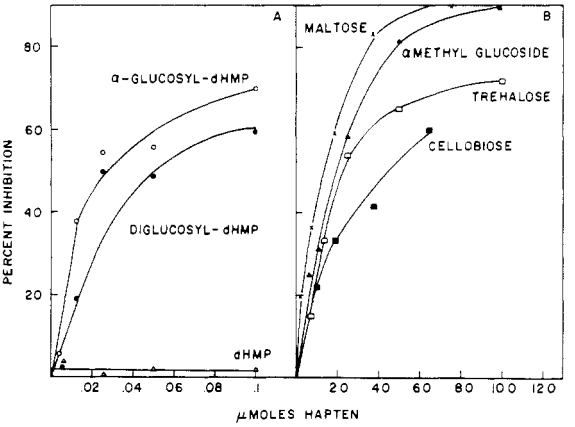

FIGURE 8: Inhibition of the anti-T_2 DNA system (Rabbit-815-2) by dHMP, α-monoglucosyl dHMP, and β-glucosyl-α-glucosyl dHMP, by the anomers, maltose and cellobiose, and by α-methyl glucoside and trehalose.

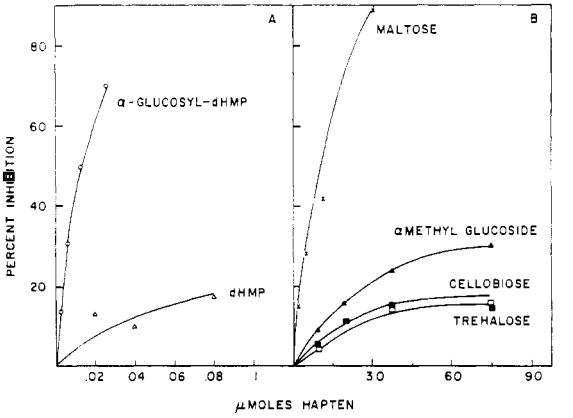

FIGURE 9: Inhibition of the anti-T_2 DNA system (Rabbit-730-2) by dHMP and α-monoglucosyl dHMP, by the anomers, maltose and cellobiose, and by α-methyl glucoside and trehalose.

plete reaction with T_4 DNA while the reaction with T_6 DNA is only partial (summary of data of Murakami *et al.*, 1962, in the introduction). T_2 DNA contains dHMP which is predominantly α-monoglucosylated; a small percentage is diglucosylated (Lehman and Pratt, 1960). The complete cross-reactivity of T_4 DNA leads to the assumption that the antibodies are directed in large part to the α-monoglucosyl residues. The hapten inhibition data obtained with Rabbit-815-2 antiserum support this assumption. The nucleotide, α-monoglucosyl dHMP, is the most effective inhibitor tested and is approximately eighty times more effective than maltose (Figure 8). The difference in inhibitory capacity of the anomers, maltose and cellobiose, is not marked; maltose is approximately four times as effective as cellobiose. Diglucosyl dHMP from T_6 DNA is almost as effective an inhibitor as α-monoglucosyl dHMP. It is noteworthy that little difference is apparent between the anomers of the disaccharides, between maltose and α-methyl glucoside, and between the glucosylated dHMP derivatives.

A second T_2 DNA anti-T_2 DNA system is also inhibited most effectively by α-monoglucosyl dHMP (Figure 9). The nucleotide is almost 100 times more effective than the best saccharide tested, maltose. In contrast to all the other systems examined, dHMP inhibits this system to a significant extent. There is a marked difference between the inhibitory capacity of the anomers, maltose and cellobiose, and between maltose and α-methyl glucoside in this system as compared with the Rabbit-815-2 system. T_6 DNA reacts more strongly in cross-reaction with this serum than with any of the other heterologous sera. This high cross-reactivity appears somewhat anomalous in view of the apparent high specificity of this antiserum for the α-monoglucosylated residues of the DNA antigen and the low content of these residues in T_6 DNA.

Discussion

The apparent specificity of the serological reactivity of the closely related T-even bacteriophage DNA's and their antisera was revealed in studies of their cross-reactions (Murakami *et al.*, 1962). Whereas forty-two DNA preparations of bacterial, viral, plant, and animal origin failed to react with anti-T_4 serum, DNA's isolated from T_2, T_4, and T_6 bacteriophages were active. In contrast, the sera of certain lupus erythematosus patients containing antibodies to DNA react with little qualitative selectivity as to source, indicating the widespread distribution of similar serologically reactive groups among DNA's of varying origin (Stollar *et al.*, 1962). The high serological specificity characteristic of the anti-T-even DNA sera is attributed to the presence of glucosyl residues on these DNA's. Goebel *et al.* (1934) established that disaccharides coupled to proteins can serve as antigenic determinants, and found, in their study, that much of the antibody specificity was directed against the terminal nonreducing hexose moiety. Indeed, the cross-reactions obtained with the T-even antisera can be related qualitatively to the nature of the glucoside substituents on the respective DNA antigens.

In view of the limitations in elucidating polysaccharide fine structure from cross-reaction data alone, it was necessary to investigate the same population of antibodies to DNA by inhibition with hapten groups of known structure. Based on the assumption that the most effective inhibiting hapten would be the structure most similar to the antigenic determinant (Kabat, 1961, p. 241), inhibition studies could yield information on whether the glucosidic groups compose all or part of the antigenic determinants in these DNA antigens. In all six DNA anti-DNA systems studied, the antigenic determinant appears to be much larger than the terminal glucosidic residue alone. Glucosylated dHMP is 10 to 100 times more effective in inhibiting each immune system than the most effective saccharide. It is not pos-

sible to deduce from the present data whether the antibody sites react solely with the glucosylated base or whether the sites encompass the deoxyribose residue, since we have not tested the relative inhibitory effect of the glucosylated base or the glucosylated nucleoside. That the terminal glucose residues contribute greatly to free energy of binding of the anti-DNA is shown by the more effective inhibition by glucosyl dHMP when compared with dHMP in the T_2 and T_4 systems (Figure 5,7,8) or diglucosyl dHMP when compared with dHMP and monoglucosyl dHMP in the T_6 systems (Figure 1). The large contribution of the glucoside to the free energy of binding in this nucleic acid–immune system is in agreement with previous studies on dextrans (Kabat, 1961, p. 249) and the phenyl (*p*-nitrobenzoylamino)-acetate–immune system (Karush, 1956). However, the contribution of the terminal residues to the free energy of binding is much less in a poly-L-alanine system (Sage *et al.*, 1964) and in determinant 3 of the *Salmonella* E–group O–antigen system (Uchida *et al.*, 1963).

Although it is known that the dHMP of T_6 DNA is mainly diglucosylated, the chemical nature of this diglucosyl residue had not been determined at the time of this study. It was concluded from the data obtained with both T_6 DNA antisera that the two glucose residues were linked β-1,6, as shown by the more effective inhibition by gentiobiose when compared to cellobiose (β-1,4), laminaribiose (β-1,3), or sophorose (β-1,2) (Townsend *et al.*, 1961). The equal inhibitory capacity of the β-gentiobioside, amygdalin, and gentiobiose led to the conclusion that gentiobiose is α-linked to deoxyhydroxymethylcytidine (Townsend *et al.*, 1961). Kuno and Lehman (1962) have identified the diglucosyl residues on T_6 DNA as gentiobioside residues.

Inhibition studies with the two T_4 antisera confirm conclusions deduced from the cross-reaction data summarized in the introduction (Murakami *et al.*, 1962), *i.e.*, that the majority of antibodies in one serum are directed to β-monoglucosyl residues (Figure 5) and those of the second serum to α-monoglucosyl residues (Figure 7). Since T_4 DNA contains both α- and β-glucosyl residues, it is not certain whether in the first serum α-glucosyl dHMP is inhibiting 20% of the anti-β-glucosyl deoxyhydroxymethylcytidine reaction or whether 20% of the serological reaction is due to antibodies directed to α-monoglucosyl residues (Figure 5). We favor the latter interpretation because the addition of both anomers, cellobiose and maltose, to the system results in a summation of their individual inhibitory capacities (Figure 6).

The glucosylation of T_2 DNA is such that 70% of dHMP is α-monoglucosylated and 5% is diglucosylated (Lehman and Pratt, 1960). Cross-reaction studies indicate that the antibodies in both anti-T_2 DNA sera are predominantly directed toward the α-monoglucosyl residues. Inhibition studies, however, reveal differences in the serological specificity of these two sera. Although the Rabbit-815-2 system can be inhibited completely by maltose and α-methyl glucoside, β-diglucosides are relatively potent inhibitors, indicating that the affinities of the antibodies in this serum for the anomers are not mutually exclusive (Figure 8). It is somewhat surprising that this serum cross-reacts weakly with T_6 DNA, yet the homologous system is markedly inhibited by the diglucosylated nucleotides of T_6. Possibly, the diglucosyl residues in T_6 DNA may have a spatial arrangement which restricts the antigen-antibody lattice requisite for direct C′ fixation.

The properties of the other anti-T_2 system (730-2) are different from the T_2 system described. The cross-reaction of this serum with T_4 DNA is complete and there is a 50% cross-reaction with T_6 DNA. The hapten inhibition data indicate that the antibody specificity is directed to the α-monoglucosyl residues (Figure 9). Cellobiose and other β-glucosides are relatively weak inhibitors of this system and are only slightly more effective than glucose itself. Maltose is a much more effective inhibitor than α-methyl glucoside. The nucleotide, α-monoglucosyl dHMP, is a very potent inhibitor and its aglucone, dHMP, significantly inhibits this system (Figure 9). The contribution of the aglucone to the antigenic determinant may account for the low inhibitory capacity of α-methyl glucoside as compared with maltose in which the "aglucone" is much larger. The cross-reaction with T_6 DNA may be attributed to the α-glucosyl dHMP aglucone of the diglucosyl dHMP.

In the present studies, the antibodies to DNA were produced in rabbits immunized with ruptured bacteriophage (Levine *et al.*, 1960). Recently, antibodies to the DNA of T-even phage were prepared using the procedure of Plescia *et al.* (1964), in which purified DNA was complexed to methylated bovine serum albumin prior to immunization. The antibodies produced using this mode of immunization had specificities similar to those produced using ruptured bacteriophage as the antigen.

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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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