Antigenicity of Polyribonucleotides*

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ABSTRACT: Using methylated bovine serum albumin–ribopolymer complexes as immunogens, antibodies directed toward polyadenylic, polyinosinic, and polycytidylic acids were produced in rabbits. All the antisera reacted with their homologous antigens when measured by gel diffusion and C′ fixation. Whereas polyinosinic acid cross reacted with antipolyadenylic acid by gel diffusion and C′ fixation, the reciprocal cross reaction between polyadenylic acid and antipolyinosinic acid was not observed. Only the polyinosinic acid–antipolyinosinic acid system was inhibited to any significant extent by mononucleotides, suggesting that the antigenic determinants of these systems are larger than mononucleotides. Pancreatic ribonuclease destroyed the serological activity of polycytidylic acid but did not affect the serological activities of polyadenylic or polyinosinic acid. DNA preparations reacted with these antisera but at antibody concentrations higher than those used to measure the homologous reactions. Thermally denatured DNA was more effective serologically than native DNA. *Proteus vulgaris* and *Escherichia coli* DNA's were used to obtain immunological thermal transition profiles with each ribopolymer antiserum. The antiribopolymers did not distinguish among the different bacterial DNA preparations. However, the anticytidylic acid failed to react with T_4 DNA which contains glucosylated hydroxymethylcytosine.

In addition to the DNA antibodies found in the sera of some patients with lupus erythematosus (Deicher *et al.*, 1959; Seligmann and Milgrom, 1957; Stollar and Levine, 1961), antibodies which react with nucleic acids have been produced experimentally by immunizing rabbits with: (1) ruptured bacteriophage of the T-even series (Levine *et al.*, 1960; Murakami *et al.*, 1961); (2) *Salmonella typhimurium* DNA (Timakov *et al.*, 1963); (3) bacterial ribosomes (Barbu *et al.*, 1961, 1963); (4) bases and derivatives coupled to serum albumin (Butler *et al.*, 1962; Tanenbaum and Beiser, 1963; Erlanger and Beiser, 1964) or to multichain polypeptides (Sela *et al.*, 1964); and (5) calf thymus and T_4 bacteriophage DNA complexed to methylated bovine serum albumin (Plescia *et al.*, 1964). In addition, it has recently been shown that the sera of rabbits hyperimmunized with Gram-negative bacteria contain antibodies to DNA (Christian *et al.*, 1965).

The method of Plescia *et al.* (1964) was used to elicit antibodies directed toward the polyribonucleotides of adenine, inosine, and cytosine. The characteristics of the antibodies produced in response to the immunization with methylated bovine serum albumin[1] (MBSA) complexes of these homopolymers are discussed in the present communication.

Materials and Methods

Polyribonucleotides. The following polyribonucleotides were employed in this study: poly-A, poly-I, and poly-C. The polymers used during the initial stages of this investigation were a generous gift of Dr. J. Fresco of Princeton University. Subsequently, polymers were purchased from Miles Chemical Co., Clifton, N.J. The reported sedimentation coefficients of the commercially obtained polymers were 13.6 S for poly-A, 7.8 S for poly-C, and 6.6 S for poly-I.

Preparation of Immune Sera. MBSA was prepared by the method of Mandell and Hershey (1960). The immunogens were prepared according to the method of Plescia *et al.* (1964), with the following modifications. To 2 ml of a solution containing 250 μg of homopolymer in 0.15 M NaCl was added 25 μl of 1% MBSA. The resulting complex was emulsified with an equal volume of complete Freunds adjuvant and injected into the toepads of a rabbit once a week for 4 consecutive weeks. One rabbit was immunized with each MBSA complex (poly-A, poly-C, poly-I, and poly-U). The rabbits were bled 7 days after the last injection (rabbit No. X-1). At this time, the rabbits were given an intravenous injection of the MBSA-polynucleotide complex without Freunds adjuvant and again bled 7 days later (rabbit No. X-2). After a 3-week interval, another intravenous injection was given and the rabbits were bled 1 week later (rabbit No. X-3). Each bleeding was studied separately. All the rabbits with the exception of the one immunized with poly-U produced antibody when measured by rise in C′ fixation titer

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[1] Abbreviations used in this work: BSA, bovine serum albumin; MBSA, methylated bovine serum albumin; AMP, adenosine monophosphate; IMP, inosine monophosphate; CMP, cytidine monophosphate; GMP, guanosine monophosphate; and TMP, thymidine monophosphate.

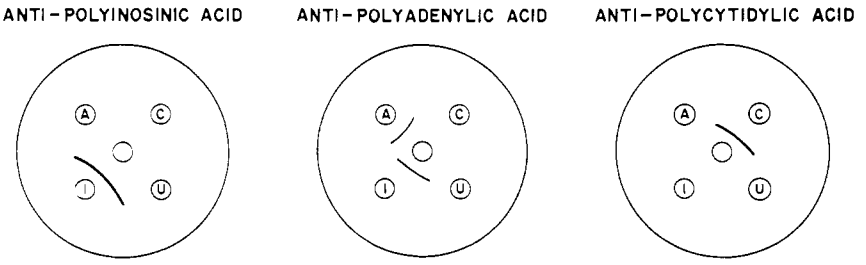

FIGURE 1: Reactions of poly-A (A), poly-C (C), poly-I (I), and poly-U (U) with antipoly-A, antipoly-C, and antipoly-I. Tracings are from double-diffusion plates after 24-hour incubation. Undiluted antisera are in center wells. The outside wells contained 10 μg of ribopolymer antigens.

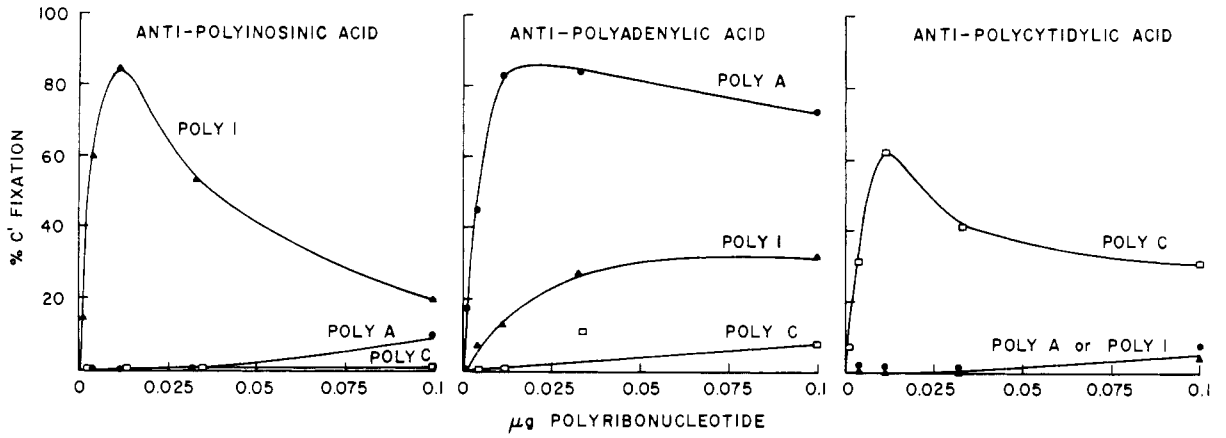

FIGURE 2: Fixation of C′ by varying quantities of poly-I (▲), poly-A (●), and poly-C (□) with antipoly-I (left), antipoly-A (center), and antipoly-C (right). Antiserum dilutions were $^1/_{10,000}$, $^1/_{150}$, and $^1/_{1500}$, respectively.

during the three courses of immunization. For example, with poly-A, the C′ fixation titers were $^1/_{50}$, $^1/_{150}$, and $^1/_{300}$, respectively; with poly-I, the titers were $^1/_{3000}$, $^1/_{5000}$, and $^1/_{12,000}$, respectively; and with poly-C, the titers were $^1/_{300}$, $^1/_{1500}$, and $^1/_{1500}$, respectively. Although preimmunization rabbit sera were not collected, each antigen has been tested by gel diffusion and C′ fixation with about 15 sera from nonimmunized rabbits and sera from rabbits immunized with proteins. A reaction was observed only with the homologous polyribonucleotide antiserum. The rabbit immunized with poly-U-MBSA complex did not produce antibodies to poly-U. Subsequently, four more rabbits have been immunized with poly-U-MBSA. No antipoly-U has been detected in any of these rabbit antisera.

DNA Isolation. Bacterial DNA was isolated according to the procedure of Marmur (1961). Phage DNA was isolated by the phenol method of Gierer and Schramm (1956).

RNAase Digestion. Bovine pancreatic ribonuclease (RNAase) was obtained from Worthington Biochem. Corp., Freehold, N.J. Polyribonucleotides were incubated at 10 μg/ml with 0.1 μg of RNAase for 30 minutes at 37°. At the end of the incubation the reaction mixtures were diluted tenfold into cold buffer and assayed for serological activity.

Denaturation of DNA. DNA at concentrations of 3 μg/ml in 0.145 M NaCl-0.01 M Tris buffer, *p*H 7.4, was immersed in a bath at the desired temperature for 10 minutes and immediately quenched by immersion in an ice bath and tenfold dilution into chilled buffer.

Complement fixation was performed according to the procedure of Wasserman and Levine (1961).

Results

The antibodies, which were produced experimentally by immunizing rabbits with the poly-A-, poly-I-, and poly-C-MBSA complexes, were screened using the agar-diffusion method (Ouchterlony, 1949). With all three antisera a precipitating band developed with the homologous antigen (Figure 1). Only with antipoly-A was a precipitating band observed with the heterologous antigen, poly-I.

The specificity of the sera was further demonstrated by C′ fixation (Figure 2). Antipoly-I serum, when used at a $^1/_{10,000}$ dilution, fixed C′ with poly-I. At this dilution of antiserum, neither poly-A nor poly-C was active. Only the homologous reaction could be demonstrated with antipoly-C serum used at a $^1/_{1500}$ dilution. Antipoly-A serum at a $^1/_{150}$ dilution, however, did react with the heterologous antigen but less effectively than

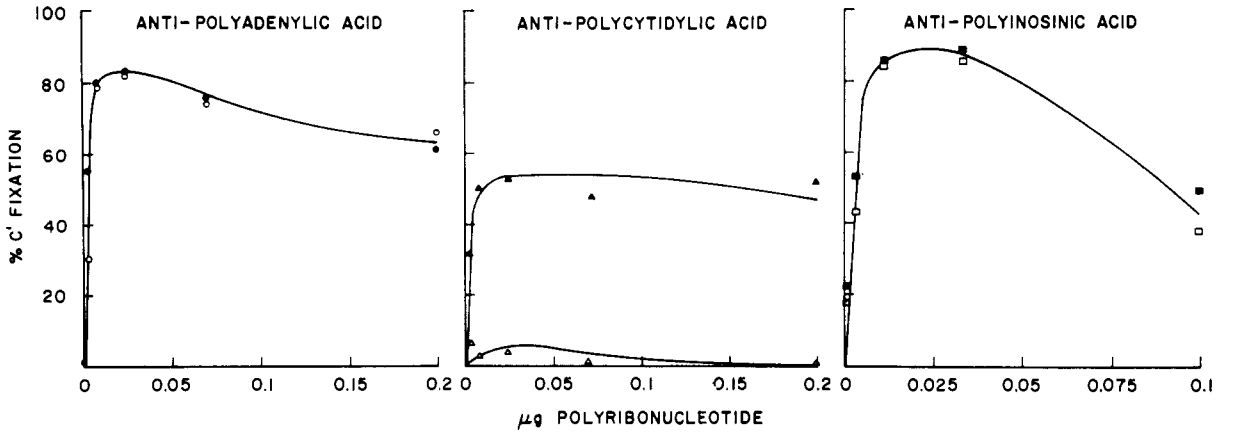

FIGURE 3: Effects of RNAase treatment on the serological activities of poly-A (left), poly-C (center), and poly-I (right). Ten μg of each polynucleotide were incubated with 0.1 μg of RNAase. Immediately after the addition of RNAase (closed symbols) and after a 30-minute incubation at 37° (open symbols), aliquots were diluted in Tris buffer and assayed by C′ fixation. The antiserum dilutions were antipoly-A ($^1/_{150}$), antipoly-C ($^1/_{1000}$), and antipoly-I ($^1/_{10,000}$).

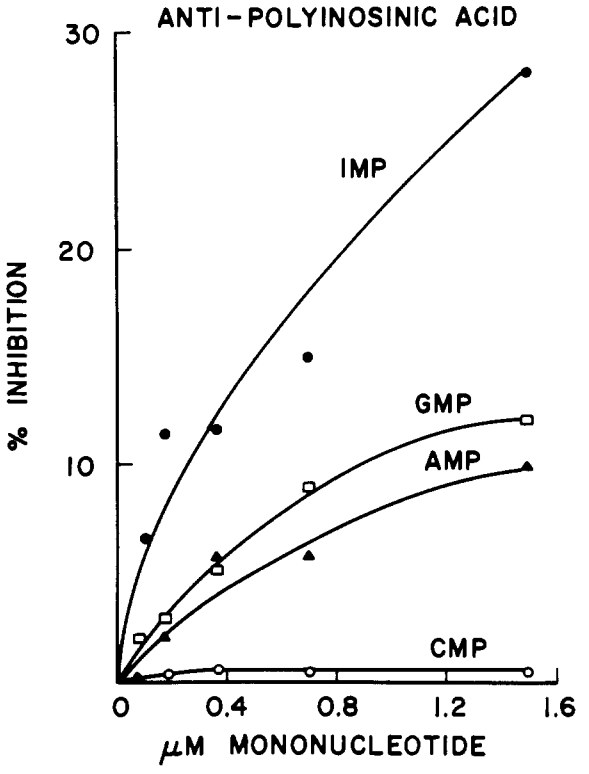

FIGURE 4: Inhibition of C′ fixation of poly-I (0.01 μg) with antipoly-I diluted $^1/_{10,000}$ with varying concentrations of mononucleotides.

the homologous poly-A, in agreement with the results of the agar-diffusion technique.

With all three polyribonucleotide-immune systems, maximum C′ fixation was obtained with about 0.01 μg of ribopolymer. With antipoly-I, the characteristic inhibition of C′ fixation with an excess of antigen was observed. This inhibition zone was not marked in the poly-A or poly-C immune systems. It should be pointed out that these homopolymers, especially poly-I, inhibit C′ activity (Yachnin, 1963). Our serological analyses use these homopolymers at levels well below their C′ inhibiting concentrations.

Pancreatic RNAase is specific for the phosphodiester bonds next to pyrimidine residues (Schmidt *et al.*, 1951). Of the three homopolymers, therefore, only poly-C should be hydrolyzed by RNAase. When poly-A, poly-I, and poly-C were incubated with RNAase (0.1 μg of enzyme to 10 μg of polymer) a decrease in the serological activity of poly-C was observed (Figure 3). The enzyme had no effect on the serological activities of poly-A or poly-I.

It might be expected that the products resulting from the digestion of poly-C by RNAase would inhibit the intact poly-C-antipoly-C reaction. This has been tested but the RNAase remaining in the reaction mixture is sufficient to destroy the 0.01 μg of intact poly-C used as the antigen in the test system. Experiments designed to separate the cytidylic acid oligonucleotides and to assess their specific C′ fixation inhibitory properties have been started. By this procedure the size of the antigenic determinant of polycytidylic acid might be established (Kabat, 1961).

The poly-A and poly-C immune systems could not be inhibited with any of the mononucleotides tested (AMP, IMP, CMP, GMP) in concentrations ranging up to 1.6 μM of nucleotide. The poly-I-antipoly-I system, however, was partially inhibited by IMP (30% inhibition of C′ fixation in the presence of 1.6 μM IMP) and less effectively by GMP and AMP (Figure 4). CMP was not inhibitory in this system. The partial inhibition of the poly-I reaction by AMP and GMP could be explained by the similarity in the purine structure of the monomers.

The failure to demonstrate inhibition with the monomers in the poly-A and poly-C immune systems

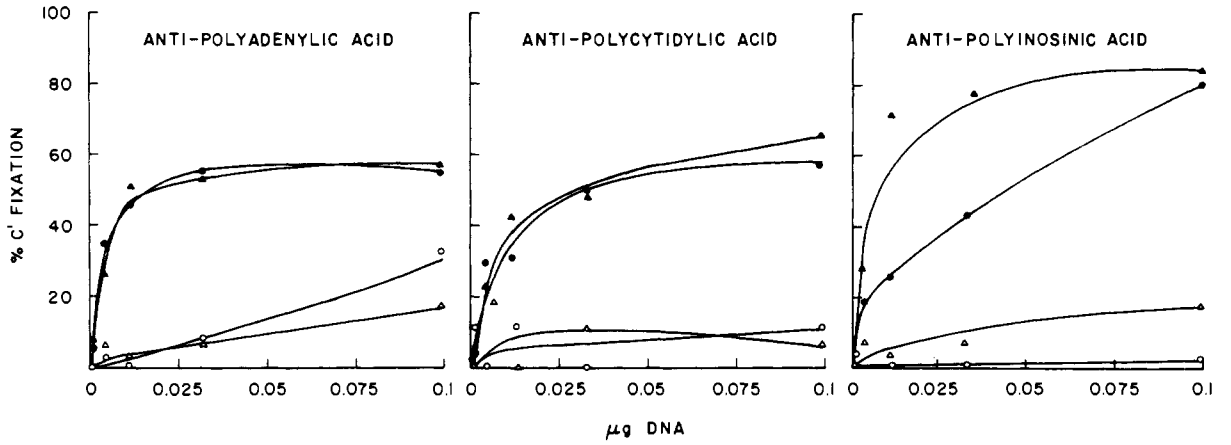

FIGURE 5: Fixation of C′ by varying quantities of thermally denatured (closed symbols) and native (open symbols) *E. coli* (●, ○) and *M. lysodeikticus* (▲, △) DNA. Antiserum dilutions were: antipoly-A ($^1/_{60}$), antipoly-C ($^1/_{60}$), and antipoly-I ($^1/_{80}$).

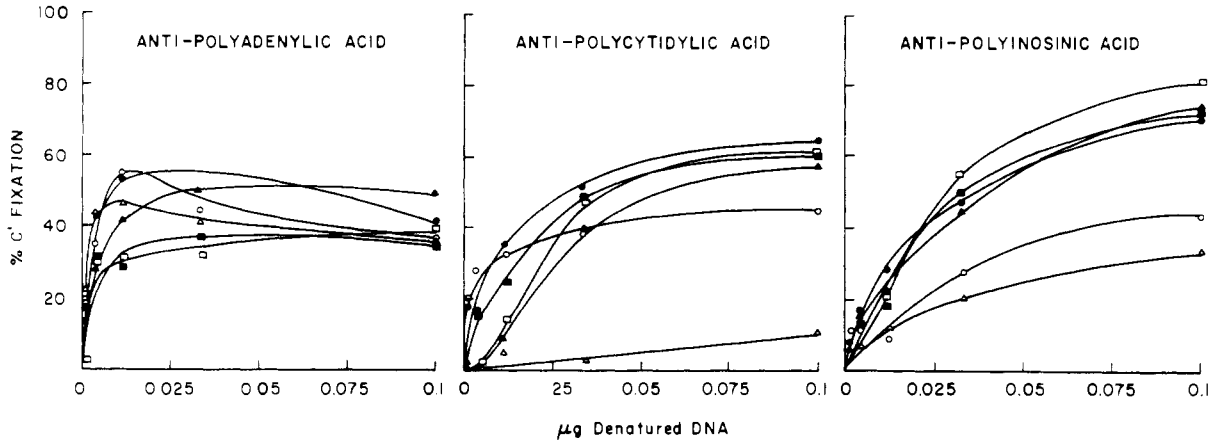

FIGURE 6: Fixation of C′ by varying quantities of thermally denatured DNA from *B. subtilis* (●), *P. vulgaris* (○), *E. coli* (▲), T_4 coliphage (△), *Ps. aeruginosa* (■), and *M. lysodeikticus* (□). Antiserum dilutions were: antipoly-A ($^1/_{60}$), antipoly-C ($^1/_{60}$), and antipoly-I ($^1/_{80}$).

suggests that the antigenic determinants of the ribopolymer antigens are oligonucleotides of as yet undetermined length. In immune systems where the antigenic determinants were found to be a penta-L-alanine (Sage *et al.*, 1964) and a pentathymidylic acid (Stollar *et al.*, 1962), the monomers, L-alanine and TMP, were not inhibitory. In contrast to these systems, a lupus serum containing antibodies to a small DNA antigenic determinant was inhibited by low concentrations of adenine (Stollar and Levine, 1963).

Antibodies produced to uracil-BSA conjugates (Tanenbaum and Beiser, 1963) and uracil-polypeptide conjugates (Sela *et al.*, 1964) react with heat-denatured DNA. This cross reaction has been attributed to the failure of the antibody to distinguish between uracil and thymine. The ability of the antipolyribonucleotide sera to cross react with DNA was tested. It can be seen (Figure 5) that all three sera do react with DNA. Thermally denatured DNA was more effective serologically than native DNA. Two features of this reaction should be noted. The antibody concentration required to demonstrate the cross reaction is much higher than that used in the homologous reaction. The C′ fixation curves do not go into antigen excess inhibition. The absence of a maximum fixation zone has been also observed in other DNA-anti-DNA systems (Stollar and Levine, 1961; Butler *et al.*, 1962; Plescia *et al.*, 1964).

The extent of reaction between each of these three antisera and DNA's isolated from five different bacterial sources as well as T_4 phage were compared. All the DNA's tested (Figure 6) showed a similar extent of cross reaction with antipoly-A serum. While all the bacterial DNA's reacted in the antipoly-C system, T_4 DNA, which contains glucosylated hydroxymethylcytosine instead of cytosine, failed to react. When

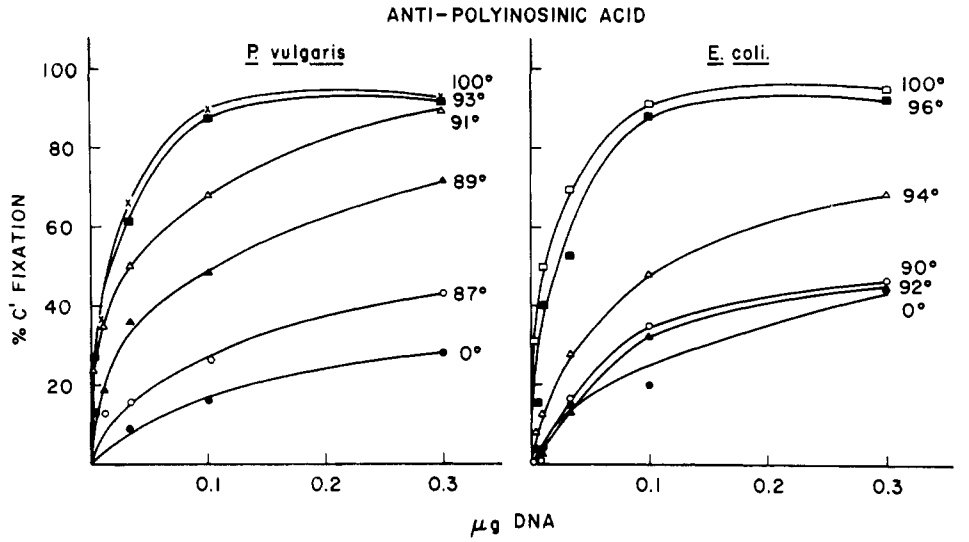

FIGURE 7: Fixation of C′ by varying quantities of *P. vulgaris* and *E. coli* DNA after incubation of the DNA samples (3 μg/ml) for 10 minutes at the designated temperatures in 0.15 M NaCl–Tris buffer followed by fast cooling and appropriate dilution. Antipoly-I was used at a $^1/_{80}$ dilution.

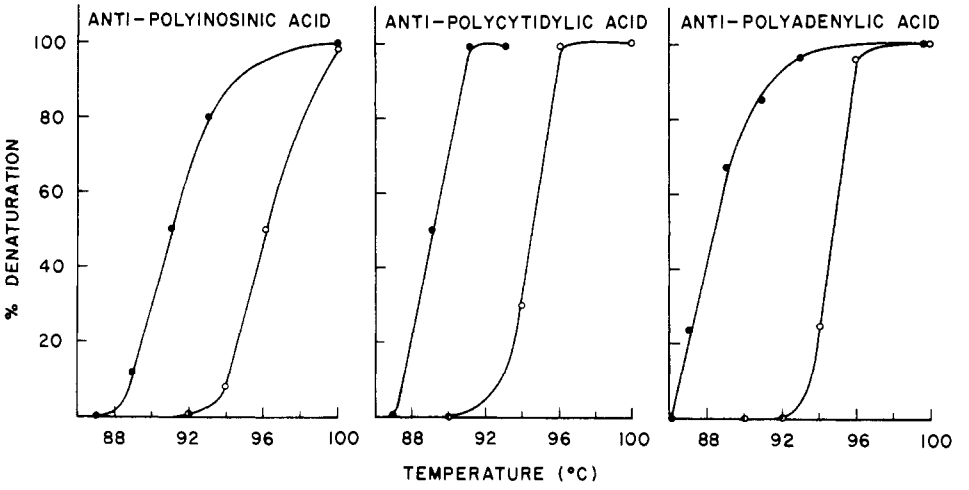

FIGURE 8: Thermal denaturation of *P. vulgaris* (●) and *E. coli* (○) DNA measured immunologically.

tested in the antipoly-I system, *B. subtilis*, *E. coli*, *Ps. aeruginosa*, and *M. lysodeikticus* DNA's showed a comparable and extensive (70–80%) amount of cross reaction, whereas *P. vulgaris* and T_4 DNA reacted much more weakly (43 and 33%, respectively). The reason for the less effective activity of *P. vulgaris* and T_4 DNA in this system is not known.

Stollar and Levine (1961) have used the C′ fixation reaction to estimate the extent of thermal denaturation of T_4 bacteriophage DNA. Using antipoly-A, antipoly-I, and antipoly-C as the indicator of the denatured state, we have performed the same experiments to follow the thermal transition of native *P. vulgaris* and *E. coli* DNA's. Figure 7 shows the results obtained using the antipoly-I serum. The amount of fixation observed with the unheated samples is probably indicative of single-stranded ends of DNA molecules resulting from the isolation procedure. As the temperature is raised, the amount of C′ fixation increases until a temperature is reached at which the molecules are fully denatured.

The melting temperature profiles of *P. vulgaris* and *E. coli* DNA presented in Figure 8 are calculated from a series of curves (Figure 7). The per cent denaturation at each specific temperature is calculated from the ratio of antigen concentration required to give 50% C′ fixation to the antigen concentration giving 50% C′ fixation at 100° (where all molecules are completely denatured). The T_m of *P. vulgaris* DNA as measured with the three different antisera varies within 3°, giving the lowest T_m of 88.5° with antipoly-A and highest

T_m of 91° with antipoly-I. The T_m of *E. coli* DNA showed a narrower range of 1.5° with an average of 95°. It should be noted that the T_m for both DNA samples is higher than T_m determined by absorbance at 260 mμ (*P. vulgaris* T_m 85°; *E. coli* T_m 90.5°; Marmur and Doty, 1962). The absorbance measurements are performed at ambient temperatures and measure both reversible and irreversible denaturation, while the samples used in this study were quenched prior to assay and thus measure only irreversible denaturation (Levine *et al.*, 1963).

Discussion

The data presented show that antibodies specific for poly-A, poly-C, and poly-I can be produced in rabbits immunized with ribopolymer-MBSA complexes. These results extend the findings of Plescia *et al.* (1964) who demonstrated the immunogenicity of calf thymus DNA and T_4 bacteriophage DNA when injected in the form of MBSA complexes. Past attempts to elicit specific antibody production in rabbits immunized with DNA, RNA, and polynucleotides complexed to native and heat-denatured BSA were not successful (Yachnin, 1962). In our own laboratory, we have failed to produce antibodies to denatured or native DNA complexed to streptomycin or protamine. Presumably, the denatured nucleic acid–MBSA complexes are more stable and better able to survive the immunization procedures.

The sera of rabbits immunized with the ribopolymer-MBSA complexes contain only a small amount of antibodies to MBSA. For example, the dilution at which significant C′ fixation was obtained with MBSA was $^1/_{50}$ with each of the three antisera. The data in Figure 2 show that C′ fixation is obtained with the homologous ribopolymers at dilutions of $^1/_{150}$ for antipoly-A, $^1/_{1500}$ for antipoly-C, and $^1/_{10,000}$ for antipoly-I. The MBSA-ribopolymer complexes give comparable C′ fixation with the respective antisera at dilutions of $^1/_{300}$, $^1/_{1600}$, and $^1/_{3000}$. Thus, rabbits produced antibody primarily to the polyribonucleotide. It is interesting to note that native BSA does not react with anti-MBSA. Progressive esterification of carboxyl groups in BSA also decreased the immunological response with anti-BSA (Sri Ram and Maurer, 1959).

The antibodies produced against poly-C, poly-I, and poly-A reacted with the homologous antigens; a slight cross reaction was found only between antipoly-A and poly-I. Thus, while antipoly-A recognized the similarities in structure between A and I, antipoly-I was able to differentiate between the amino and hydroxyl groups on C-6 of adenine and inosine, respectively. Since only one antiserum of each specificity was studied, it is not possible to state whether the broader specificity of the antipoly-A is an inherent property of this class of antibody or a unique case observed with this particular serum.

When studied by optical rotatory dispersion techniques, the secondary structure of polyribonucleotides have been shown to undergo several changes as a function of temperature and *p*H. For example, poly-C undergoes a transition from a single-stranded helix at *p*H 7.0 to a double-stranded one at *p*H 4.1 (Fasman *et al.*, 1964). The helical content of the single-stranded poly-A at neutral *p*H continually changes to a more random conformation as the temperature is raised from 30 to 80° (Holcomb and Tinoco, 1965). Thus the role of antigenic conformation and serological specificity can be investigated by carrying out quantitative precipitin analyses with these homopolymer and their homologous antibodies at ambient temperatures and varying *p*H. It should be noted that the experiments described here were performed at low temperatures and neutral *p*H, thus eliminating any ambiguities which may arise due to conformational changes.

These antisera provide a useful tool for detecting the structural changes which the homopolymers undergo during enzymatic or chemical modifications. As an example of the latter, deamination of poly-A using nitrous acid causes a decrease in its homologous reaction but increases its reaction with antipoly-I (F. Chaslow, unpublished data). This is an expected finding since A is known to be converted to I during deamination. This and other chemical modifications will be described in detail in another communication.

The antihomopolymer sera, like the antibodies directed toward other nucleic acid systems, cross react best with denatured DNA. This differential reactivity has been explained in the light of what is known about the structural states of native and denatured DNA: i.e., groups that are involved in maintaining the double-stranded helix in native DNA are exposed and become capable of reacting with the antibody when the molecule is denatured (Levine *et al.*, 1960). Antipoly-I and antipoly-A react with all the denatured DNA's. While antipoly-C reacts with the denatured DNA of five bacterial species, it cannot react with denatured DNA of T_4 phage which contains glucosylated hydroxymethylcytosine in place of cytosine. This antibody is either capable of distinguishing between a cytosine and glucosylated hydroxymethylcytosine or the glucose moiety in the T_4 DNA sterically prevents the antibody molecule from coming into the necessary proximity with the HMC unit.

Antibodies to *E. coli* ribosomes react with purified RNA preparations (Barbu *et al.*, 1963). Sela *et al.* (1964) demonstrated precipitating antibody to RNA in antisera of rabbits immunized with a uracil-polypeptide conjugate. However, the RNAase activity of the rabbit serum had to be neutralized by anti-RNAase before direct serological activity with RNA could be shown. Inhibition with RNA was noted by Stollar and Levine (1962) in a DNA-lupus antibody system and by Butler *et al.* (1965) in the purin-6-oyl immune system. Antibodies to polyribonucleotides could prove extremely useful in probing the structure of RNA. In preliminary experiments there was no reaction with our poly-A and poly-C antisera using up to 3 μg of ribosomal and soluble RNA isolated from mouse kidney cells (the generous gifts of Dr. W. T. Murakami). When antipoly-I (at a $^1/_{100}$ dilution) was used, direct C′

fixation could be observed with 3 μg of ribosomal but not with an equivalent amount of soluble RNA. The positive cross reaction with ribosomal RNA and antipoly-I is probably due to the higher concentration of homologous antibody in this antiserum as compared to the antipoly-C and antipoly-A. Experiments are being undertaken to ascertain whether the observed lack of reaction with soluble RNA is the effect of molecular weight or whether it is a reflection of the highly ordered secondary structure of soluble RNA.

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References

Barbu, E., Panijel, J., and Quash, G. (1961), *Ann. Inst. Pasteur 100*, 725.

Barbu, E., Quash, G., and Dandeu, J.-P. (1963), *Ann. Inst. Pasteur 105*, 849.

Butler, V. P., Beiser, S. M., Erlanger, B. F., Tanenbaum, S. W., Cohen, S., and Bendich, A. (1962), *Proc. Natl. Acad. Sci. U.S. 48*, 1597.

Butler, V. P., Tanenbaum, S. W., and Beiser, S. M. (1965), *J. Exptl. Med. 121*, 19.

Christian, C. L., Desimone, A. R., and Abruzzo, J. L. (1965), *J. Exptl. Med. 121*, 309.

Deicher, H. R. G., Holman, H. R., and Kunkel, H. G. (1959), *J. Exptl. Med. 109*, 97.

Erlanger, B. F., and Beiser, S. M. (1964), *Proc. Natl. Acad. Sci. U.S. 52*, 68.

Fasman, G. D., Lindblow, C., and Grossman, L. (1964), *Biochemistry 3*, 1015.

Gierer, A., and Schramm, G. (1956), *Nature 177*, 702.

Holcomb, D. N., and Tinoco, I. (1965), *Biopolymers 3*, 121.

Kabat, E. A. (1961), *in* Experimental Immunochemistry, 2nd ed., Kabat, E. A., and Mayer, M. M., eds., Springfield, Ill., Thomas.

Levine, L., Gordon, J. A., and Jencks, W. P. (1963), *Biochemistry 2*, 168.

Levine, L., Murakami, W. T., Van Vunakis, H., and Grossman, L. (1960), *Proc. Natl. Acad. Sci. U.S. 46*, 1038.

Mandell, J. D., and Hershey, A. D. (1960), *Anal. Biochem. 1*, 66.

Marmur, J. (1961), *J. Mol. Biol. 3*, 208.

Marmur, J., and Doty, P. (1962), *J. Mol. Biol. 5*, 109.

Murakami, W. T., Van Vunakis, H., Grossman, L., and Levine, L. (1961), *Virology 14*, 190.

Ouchterlony, O. (1949), *Acta Pathol. Microbiol. Scand. 26*, 507.

Plescia, O. J., Braun, W., and Palczuk, N. C. (1964), *Proc. Natl. Acad. Sci. U.S. 52*, 279.

Sage, H. J., Deutsch, G. F., Fasman, G. D., and Levine, L. (1964), *Immunochemistry 1*, 133.

Schmidt, G., Cubiles, R., Zollner, N., Hecht, L., Strickler, N., Seraidarian, K., Seraidarian, M., and Thannhauser, S. J. (1951), *J. Biol. Chem. 192*, 719.

Sela, M., Ungar-Waron, H., and Schechter, Y. (1964), *Proc. Natl. Acad. Sci. U.S. 52*, 285.

Seligmann, M., and Milgrom, F. (1957), *Compt. Rend. 245*, 1472.

Sri Ram, J., and Maurer, P. H. (1959), *Arch. Biochem. Biophys. 83*, 223.

Stollar, D., and Levine, L. (1961), *J. Immunol. 87*, 477.

Stollar, D., and Levine, L. (1963), *Arch. Biochem. Biophys. 101*, 417.

Stollar, D., Levine, L., Lehrer, H. I., and Van Vunakis, H. (1962), *Proc. Natl. Acad. Sci. U.S. 48*, 874.

Tanenbaum, S. W., and Beiser, S. M. (1963), *Proc. Natl. Acad. Sci. U.S. 49*, 662.

Timakov, V. D., Skavronskaya, A. G., Borisova, N. B., and Zamchuk, L. A. (1963), *Zh. Mikrobiol. Epidemiol. i Immunobiol. 40*, 5 (cf. *Federation Proc. Translation Suppl.* (1963) *22*, T 1028).

Wasserman, E., and Levine, L. (1961), *J. Immunol. 87*, 285.

Yachnin, S. (1962), *J. Clin. Invest. 41*, 1414.

Yachnin, S. (1963), *J. Clin. Invest. 42*, 1947