Immunochemical Studies on Bacteriophage Deoxyribonucleic Acid. V. Specificity of Antibodies to Deoxyribonucleic Acid after Immunization with Methylated Bovine Serum Albumin–Deoxyribonucleic Acid Complexes*

Edna Seaman, Lawrence Levine, and Helen Van Vunakis

ABSTRACT: The serologic specificities of antibodies produced in rabbits by immunization with methylated serum albumin complexes of T_4 deoxyribonucleic acid, T_4 apurinic acid, and T_4 deoxyribonucleic acid in which 50% of the guanine had been destroyed during irradiation with visible light in the presence of methylene blue have been studied. All of the antisera contained antibodies directed primarily toward the α-glucosylhydroxymethylcytidylic acid of the T_4 deoxyribonucleic acid. All of the antisera, with the exception of antiphotooxidized T_4 deoxyribonucleic acid, showed specificity for T-even coliphage deoxyribonucleic acid. The antiphotooxidized T_4 deoxyribonucleic acid contained additional antibodies which reacted with heterologous deoxyribonucleic acids lacking glucosylhydroxymethylcytidylic acid. This cross reaction was used to measure the thermally produced helix-to-random coil transition of five deoxyribonucleic acid preparations varying in their guanine and cytosine content from 37 to 72%. These antibodies were inhibited most effectively by deoxyguanosine and deoxycytidine monophosphates and less effectively by deoxyadenosine and thymidine monophosphates.

Among the mono-, di-, and tricytosine derivatives tested by hapten inhibition, cytidylyl-3′,5′-cytidylic acid was the most effective inhibitor. Removal of the terminal phosphates decreased its effectiveness as an inhibitor. In addition, the antiphotooxidized T_4 deoxyribonucleic acid contained antibodies directed toward the product which resulted from the exposure of deoxyribonucleic acid to visible light in the presence of methylene blue.

Immunization of rabbits with ruptured T-even bacteriophage resulted in the production of antibodies directed not only to the protein coat and tail of the bacteriophage particle but also to the internal contents of the virus, *i.e.*, the internal protein (Levine *et al.*, 1958) and deoxyribonucleic acid (DNA) (Levine *et al.*, 1960; Murakami *et al.*, 1961). Cross reactions among the T_2, T_4, and T_6 bacteriophage DNA's and the lack of reaction with DNA preparations of animal, bacterial, and viral origin suggested that the antibodies were directed in part to the glucosyl residues of the hydroxymethylcytosine unique to the T-even bacteriophage DNA's (Murakami *et al.*, 1961). Subsequent studies by hapten inhibition (Townsend *et al.*, 1965) demonstrated that these antibodies recognized not only the glucose moieties and their specific configuration but also the nucleotide hydroxymethylcytidylic acid.

Attempts to immunize rabbits with purified bacteriophage DNA or other viral, bacterial, or animal DNA's were unsuccessful. Thus, only the ruptured bacteriophage, presumably its protein–DNA complex, was immunogenic. Recently, antibodies to purified T_4 bacteriophage DNA and calf thymus DNA have been produced in rabbits immunized with methylated bovine serum albumin–DNA complexes (Plescia *et al.*, 1964).

We have confirmed the finding with T_4 bacteriophage DNA and have demonstrated that these antibodies are directed toward glucosylhydroxymethylcytidylic acid (dHMP[1]). If T_4 DNA which is photooxidized in the presence of methylene blue is used as an immunogen, additional antibodies directed toward guanine, cytosine, adenine, and thymine residues are produced. These latter antibodies react with DNA's which do not contain glucosylated dHMP. In addition, antibodies directed toward the specific photooxidation product have been detected.

Materials and Methods

The methods used for growth, isolation, and purifica-

*From the Graduate Department of Biochemistry, Brandeis University, Waltham, Mass. *Received June 18, 1965.* Publication No. 381 from the Graduate Department of Biochemistry, Brandeis University. Aided by grants from the National Institutes of Health (AI-01940 and AI-02792) and the American Cancer Society (E-222). L. L. is an American Cancer Society Professor and H. V. V. is a recipient of a Public Health Service Research Career Award (5-K6-AI-2372) from the National Institute of Allergy and Infectious Disease.

[1] Abbreviations used in this work: GMP, CMP, AMP, TMP, guanosine, cytidine, adenosine, and thymidine monophosphates, respectively; dGMP, etc., refers to the deoxy derivative; dHMP, hydroxymethylcytidylic acid; MBSA, methylated bovine serum albumin; dCp, nucleotide of cytidine; dC, cytidine nucleoside; dCp_2, 3′,5′-cytidylic acid; dC_3p_4, 3′,5′-trinucleotide.

TABLE I: Reaction of Antisera with Various DNA Preparations.

Immune System		Extent of Reaction[a]				
Rabbit No.	Immunogen	T_4	T_2	T_6	*B. subtilis*	*Ps. aeruginosa*
824	Ruptured T_4 phage	$^1/_{50,000}$	$^1/_{10,000}$	$^1/_{10,000}$	None[f]	None[f]
528	MBSA[b]-T_4 DNA complex	$^1/_{40,000}$	$^1/_{20,000}$	$^1/_{20,000}$	None[f]	None[f]
568	MRSA[c]-T_4 DNA complex	$^1/_{30,000}$	$^1/_{10,000}$	$^1/_{10,000}$	None[f]	None[f]
549	MBSA-MB treated T_4 DNA, denatured[d]	$^1/_{100,000}$	$^1/_{30,000}$	$^1/_{50,000}$	$^1/_{2000}$	$^1/_{2000}$
559	MBSA-MB treated T_4 DNA, native[e]	$^1/_{30,000}$	$^1/_{10,000}$	$^1/_{15,000}$	$^1/_{300}$	$^1/_{300}$
556	MBSA-apurinic T_4 DNA	$^1/_{900}$	$^1/_{400}$	$^1/_{600}$	None[g]	None[g]

[a] Dilution of antiserum giving the same maximum C′ fixation. [b] MBSA, methylated bovine serum albumin. [c] MRSA, methylated rabbit serum albumin. [d] Native T_4 DNA was photooxidized in the presence of methylene blue, boiled and fast cooled, and complexed with MBSA. [e] Native T_4DNA was photooxidized and complexed with MBSA without prior boiling. [f] No reaction at $^1/_{200}$. [g] No reaction at $^1/_{20}$.

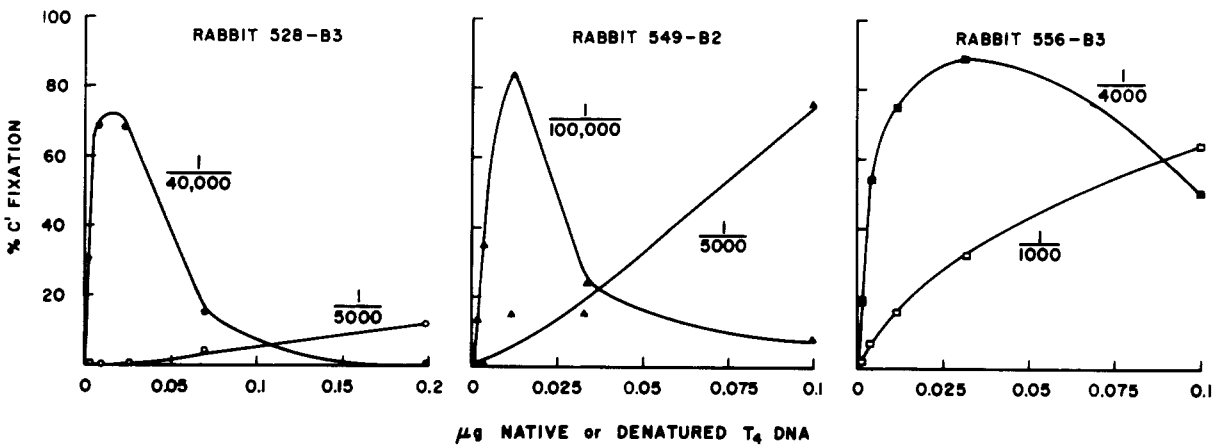

FIGURE 1: Fixation of C′ by increments of native (open symbols) and denatured (closed symbols) T_4 DNA with anti-T_4 DNA (Ra-528-B3), antiphotooxidized T_4 DNA (Ra-549-B2), and anti-T_4 apurinic acid (Ra-556-B3).

tion of T_2, T_4, and T_6 phage were described previously (Levine *et al.*, 1958). Phage DNA was isolated by rupturing the phage with cold phenol buffered with 0.05 M phosphate, pH 7.4 (Gierer and Schramm, 1956). Following three phenol extractions, the DNA was extracted twice with chloroform–isoamyl alcohol and dialyzed against 0.15 M NaCl. Bacterial DNA was isolated according to the procedure of Marmur (1961). Preparation of methylated bovine serum albumin (MBSA) DNA complexes and immunization were performed as described by Seaman *et al.* (1965).

T_4 apurinic acid was prepared according to the method of Tamm *et al.* (1952). The DNA solution was acidified to pH 1.6 using 0.1 N HCl and dialyzed against ten volumes of HCl at the same pH for 24 hr at 37°, followed by dialysis against H_2O in the cold. The product of the reaction was hydrolyzed with concentrated perchloric acid and chromatographed using 2-propanol–HCl solvent system (Wyatt, 1951). No adenine and guanine remained after this treatment.

Partial destruction of guanine was accomplished by photooxidation of T_4 DNA in the presence of methylene blue as described by Simon and Van Vunakis (1962). Base ratio analysis of the photooxidized DNA showed that 50% of the guanine had been destroyed.

For serological analyses, the macro complement (C′) fixation of Mayer *et al.* (1948) and the micro C′ fixation method of Wasserman and Levine (1960) were used.

Results

Rabbits were immunized with MBSA complexes of T_4 DNA or T_4 DNA that had been subjected to different chemical modifications. Table I lists the antisera used in this study and shows the extent of cross reactions with the DNA's obtained from the T-even phages and from two bacterial sources, *B. subtilis* and *Ps. aeruginosa*. One antiserum (Ra-824), prepared against ruptured T_4 phage and whose properties have been

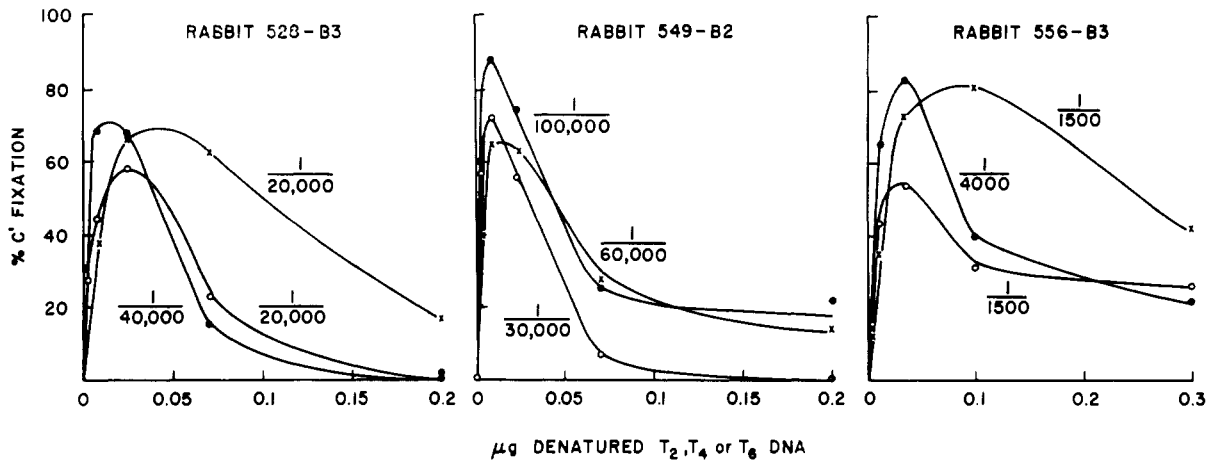

FIGURE 2: Fixation of C′ by increments of T_2 (O), T_4 (●), and T_6 (X) thermally denatured DNA with anti-T_4 DNA (Ra-528-B3), antiphotooxidized T_4 DNA (Ra-549-B2), and anti-T_4 apurinic acid (Ra-556-B3).

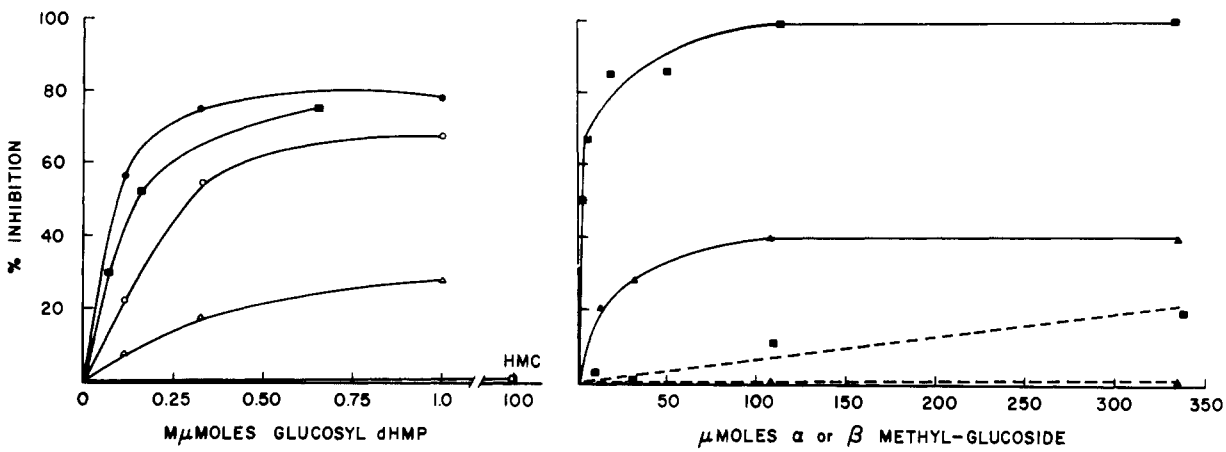

FIGURE 3: Inhibition, by α-glucosyl-dHMP and HMC (left side) and α- (solid line) or β- (dashed line) methyl glucoside (right side), of 0.01 μg of thermally denatured T_4 DNA with antiphotooxidized denatured T_4 DNA (●, Ra-549-B2), antiphotooxidized native T_4 DNA (O, Ra-559-B2), anti-T_4 DNA (▲, Ra-528-B3), and anti-T_4 apurinic acid (■, Ra-556-B2). HMC (□) did not inhibit any of the antisera.

described previously (Levine *et al.*, 1960; Murakami *et al.*, 1961), is listed for comparison.

All of the antisera reacted extensively with the glucosylated DNA's from T_2 and T_6 phage. The antisera prepared against unmodified T_4 DNA (Ra-528 and Ra-568) and against apurinized T_4 DNA (Ra-556) did not react with either of two bacterial DNA samples, indicating that the antibodies were directed primarily toward the glucosyl dHMP residues. Antisera (Ra-549 and Ra-559) obtained after immunization with photooxidized T_4 DNA did react with bacterial DNA's.

Of the three antisera selected for a detailed study, Ra-528 was prepared using unmodified DNA as immunogen. Ra-549 was prepared using DNA in which 50% of the guanine had been destroyed by photooxidation, and Ra-556 was produced after immunization with a DNA from which all of the purine moieties had been removed by acid treatment. The apurinic acid used as immunogen in this study probably possessed a molecular weight of approximately 10,000 (Tamm *et al.*, 1952). Plescia *et al.* (1965) have reported that tetranucleotides with a molecular weight of approximately 1500 can be immunogenic when complexed to MBSA prior to immunization.

As with the other DNA–anti DNA reactions, the single strand conformation is the serologically active form of the antigen (Figure 1). Even with 4 to 20 times more antibody, C′ fixation was observed with native DNA only at high antigen concentrations. This reaction with native DNA probably represents less than 1% single-strand areas in the purified native DNA preparations. Treatment of native DNA with an endonuclease specific for single-strand DNA (Healy *et al.*, 1963) has eliminated this native DNA activity without affecting

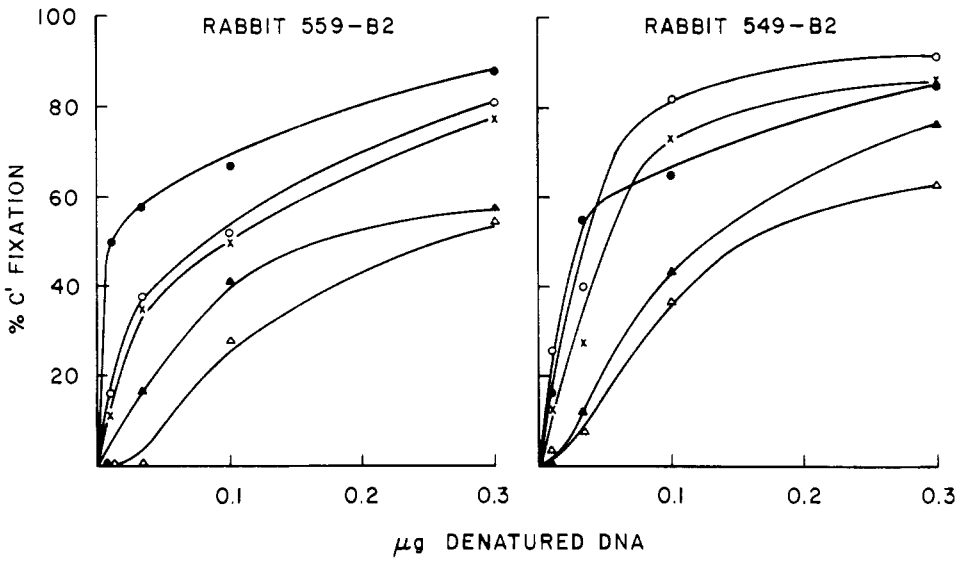

FIGURE 4: Fixation of C′ by thermally denatured *E. coli* (●), *B. subtilis* (○), *Ps. aeruginosa* (▲), *M. lysodeikticus* (△), and *P. vulgaris* (x) DNA with antiphotooxidized native T_4 DNA (Ra-559-B2 diluted $^1/_{300}$) and antiphotooxidized denatured T_4 DNA Ra-549-B2 diluted $^1/_{1000}$.

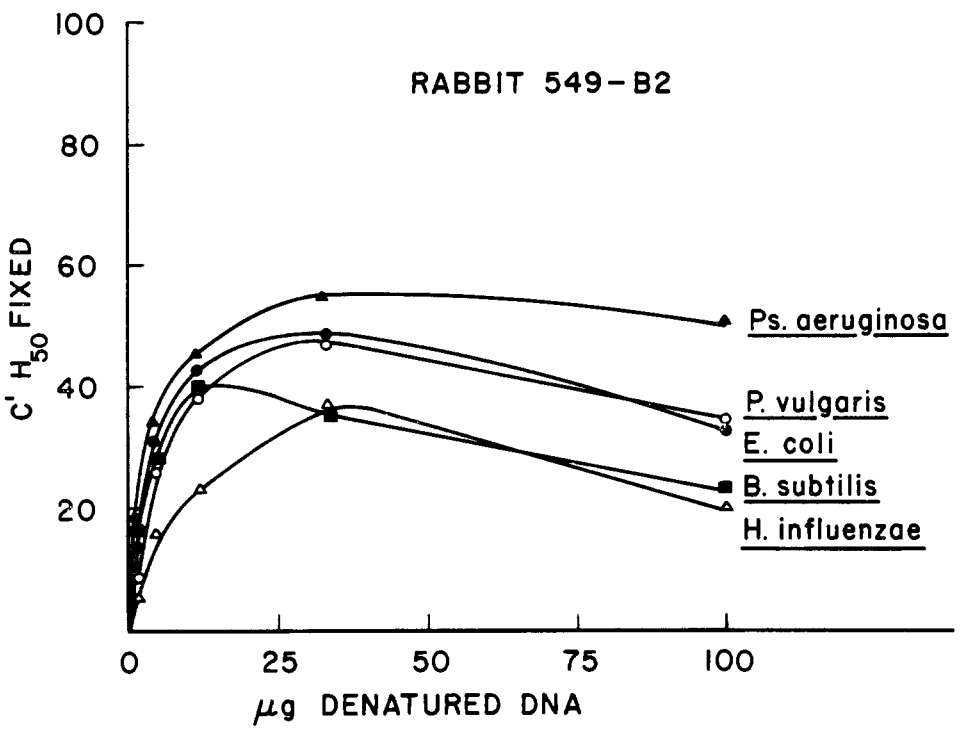

FIGURE 5: Macro-C′ fixation by thermally denatured DNA's with antiphotooxidized DNA (Ra-549-B2 diluted $^1/_{10}$).

its serological activity after thermal denaturation. The differences in reactivity with native DNA observed with different antisera may reflect the size of the antigenic determinants being measured.

The reaction of these antisera with the homologous T_4 DNA and the cross-reacting T_2 and T_6 DNA's is shown in Figure 2. With each antiserum T_4 DNA was the most effective antigen. The heterologous T_2 and T_6 DNA's also reacted if more antibody was used. In general, T_6 DNA was a more effective cross-reacting antigen than T_2 DNA.

The specificity of these antisera was also investigated

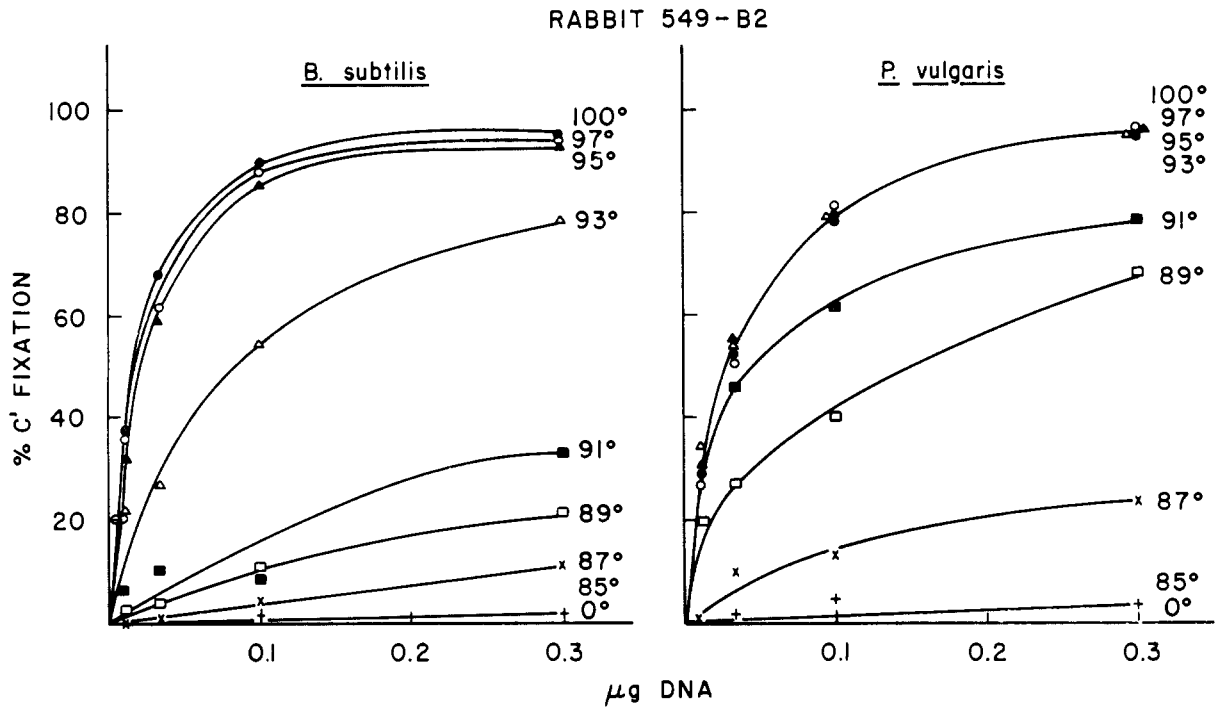

FIGURE 6: Fixation of C′ by increments of *B. subtilis* and *P. vulgaris* DNA after incubation of the samples (3 μg/ml) for 10 min at the designated temperature in 0.15 M NaCl–Tris buffer (pH 7.4) followed by fast cooling and appropriate dilution. Antiserum was antiphotooxidized T_4 DNA (Ra-549-B2 diluted $^1/_{1000}$).

using the technique of hapten inhibition (Figure 3). α-Glucosyl dHMP[2] proved to be an inhibitor of all the T_4 DNA-anti T_4 DNA systems. With three antisera, 50% inhibition was obtained with 0.1, 0.15, and 0.3 mμmole of α-glucosyl dHMP, while 1.0 mμmole was required to give 27% inhibition with another anti T_4 DNA serum. The free base, hydroxymethylcytosine, was not inhibitory in any of these systems in a concentration up to 100 μmoles.

To assess the role of the glucosyl moieties and the configuration of substituted hydroxymethylcytosine, experiments were carried out using α- and β-methyl glucosides as inhibitors. The T_4 apurinic acid immune system was inhibited 50% by 2.0 μmoles of α-methyl glucoside, but only 20% by 350 μmoles of β-methyl glucoside. Another anti-T_4 DNA system (Ra-528-B3) was inhibited 40% by the α-methyl glucoside, while the β-methyl glucoside was ineffective. The other two antisera tested (Ra-549, Ra-559) were not inhibited by concentrations of up to 350 μmoles of either saccharide.

Earlier studies with anti-bacteriophage DNA produced by immunization with ruptured bacteriophage showed a strict specificity for the T-even coliphage DNA's. Forty-two DNA's of bacterial, animal, or viral origin did not react or inhibit these T-even coliphage DNA immune systems. With the MBSA–T_4 DNA immunogens, this narrow specificity toward the glucosylated coliphage DNA was also observed (Table I). However, when the coliphage DNA was irradiated with visible light in the presence of methylene blue, the DNA carrying the resulting lesion produced antibodies which now reacted with DNA's other than coliphage DNA.

Cross reactions were obtained with five DNA's from bacterial sources and the two antisera directed toward photooxidized T_4 DNA (Figure 4). It was difficult to determine whether any correlation existed between the extent of cross reaction and the guanine + cytosine content of these DNA's. The C′ fixation curves could not be completed in antigen excess since larger concentrations of DNA became anticomplementary in the micro C′ fixation technique. In order to circumvent this technical difficulty, the extent of cross reaction was determined using the quantitative macro C′ fixation technique (Mayer *et al.*, 1948). No correlation was found between the guanine + cytosine content of these bacterial DNA's and their extent of cross reaction with this antiserum (Figure 5).

While the immunogenicity of DNA is potentiated by complex formation with MBSA (Plescia *et al.*, 1964), the immunogenic expression of impurities (polysaccharides, teichoic acids, etc.) accompanying the DNA may also be more pronounced. One criterion for determining whether the antibodies are directed toward DNA is to subject native DNA to thermal denaturation. As shown by Marmur and Doty (1962), the characteris-

[2] We are deeply indebted to Dr. I. R. Lehman for α-glucosyl-dHMP and to Dr. K. Burton for the cytidylic acid derivatives.

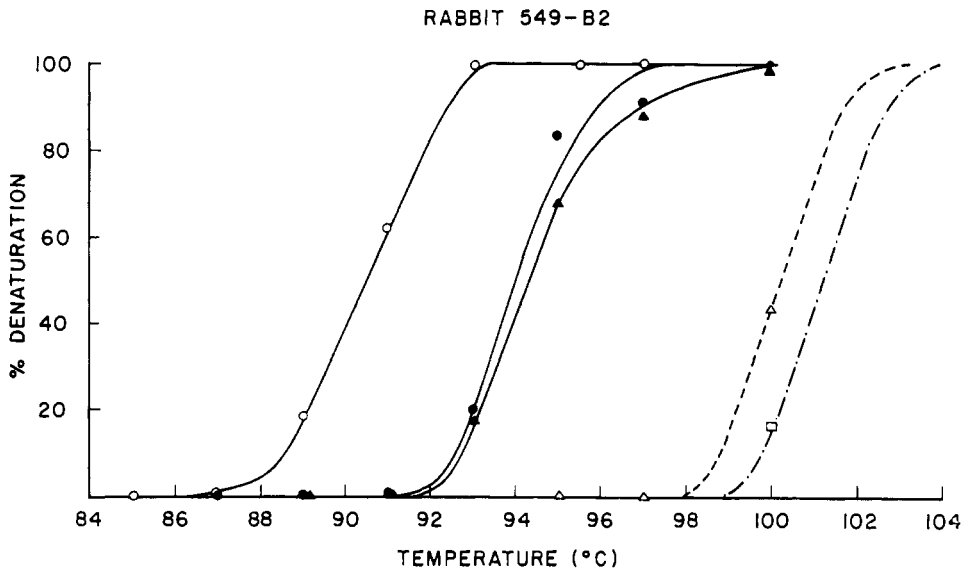

FIGURE 7: Temperature melting curves of *P. vulgaris* (○), *B. subtilis* (●), *E. coli* (▲), *Ps. aeruginosa* (△), and *M. lysodeikticus* (□) DNA.

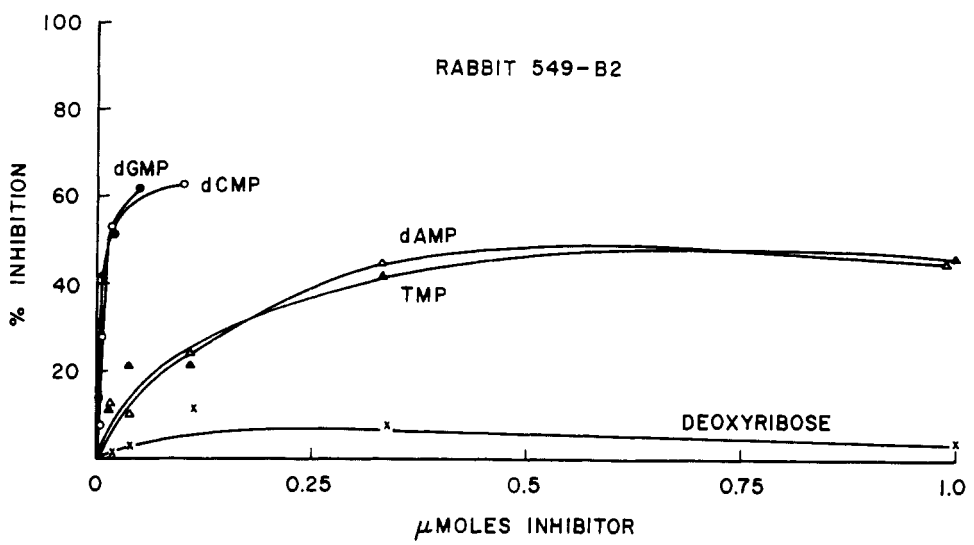

FIGURE 8: Inhibition by dGMP, dCMP, dAMP, TMP, and deoxyribose of C′ fixation of 0.02 μg of *Ps. aeruginosa* thermally denatured DNA and antiphotooxidized T_4DNA (Ra-549-B2 diluted $^1/_{1000}$).

tic temperature of helix-to-random coil transition (T_m) under a given set of experimental conditions depends on the guanine + cytosine content of the DNA. The antiserum to photooxidized T_4 DNA was used to determine the extent of reaction of *B. subtilis* and *P. vulgaris* heated to different temperatures (Figure 6). The ratio of the extent of reaction at a specified temperature to the extent of reaction of DNA which had been heated at 100° to denature all the molecules was used to calculate the T_m curves for *Bacillus subtilis*, *Proteus vulgaris*, and *Escherichia coli* DNA (Figure 7). Since the salt concentration in these experiments did not favor dissociation of the two DNA's with the highest guanine + cytosine content, *i.e.*, *Pseudomonas aeruginosa* and *Micrococcus lysodeikticus*, the point of 100% denaturation for these DNA's was calculated by carrying out additional experiments in low salt and correcting back to the set of experimental conditions.

Hapten inhibition of a heterologous DNA (*Ps. aeruginosa* DNA) by deoxynucleotides and deoxyribose was investigated. dGMP and dCMP proved to be the most potent inhibitors, while dAMP and TMP were less effective (Figure 8). Deoxyribose was not inhibitory at the concentrations tested. Since tracts of cytidylic acid obtained by apurinizing salmon sperm DNA (Burton and Petersen, 1960) were available,[2] they were used as

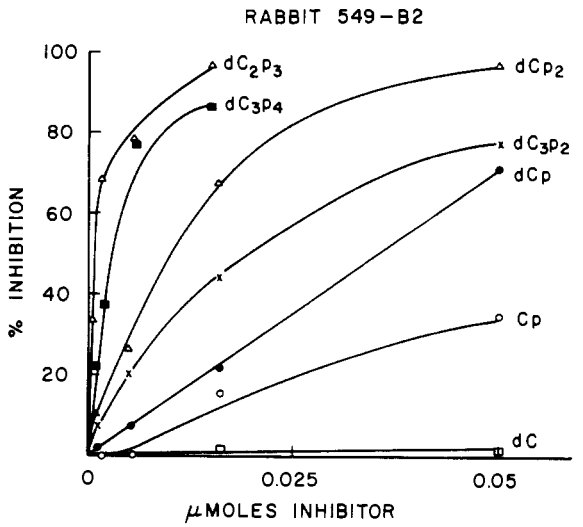

FIGURE 9: C′ fixation inhibition of 0.02 μg of *Ps. aeruginosa* thermally denatured DNA and antiphotooxidized T_4DNA (Ra-549-B2 diluted $^1/_{1000}$).

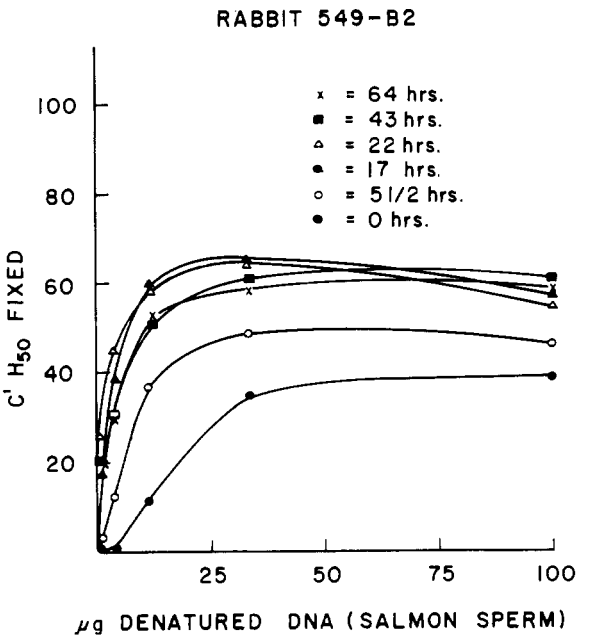

FIGURE 10: Macro-C′ fixation by thermally denatured salmon sperm DNA (photooxidized in the presence of methylene blue for varying periods of time) with antiphotooxidized T_4 DNA (Ra-549-B2 diluted $^1/_{10}$).

inhibitors. These inhibition data (Figure 9) demonstrate that: (1) the ribonucleotide (Cp) inhibits but not as effectively as the deoxyribonucleotide (dCp); (2) the presence of the terminal phosphates increases inhibitory properties; *e.g.* (a) the nucleotide (dCp) is effective whereas the nucleoside (dC) is not; (b) the 3′,5′-cytidylic acid (dCp_2) is more effective than the 5′-cytidylic acid (dCp); and (c) the 3′,5′ trinucleotide (dC_3P_4) is about six times more effective than the trinucleotide from which the 3′,5′-phosphates have been removed (dC_3p_2); and (3) the most effective size for inhibition is the dinucleotide.

Thus, T_4 DNA that has been photooxidized to destroy 50% of the guanine residues produced antibodies directed primarily toward glucosyl dHMP and, to a lesser degree, toward dC_2p_3, dGMP, dAMP, and TMP. Our conclusion with respect to the latter three mononucleotides is limited because of the unavailability of their tracts. The possibility also existed that the antiserum contained antibodies toward the photooxidation product of the altered guanine residues. In order to demonstrate such an antibody, denatured salmon sperm DNA was photooxidized in the presence of methylene blue. At various time intervals, aliquots were removed and assayed for macro C′ fixation. The resulting C′ fixation curves are shown in Figure 10. It can be seen that the serologic activity of denatured salmon sperm DNA increases with time of photooxidation. Identical control reactions, kept in the dark, showed no increase in serologic activity.

Discussion

The antibodies to T_4 DNA present in the antisera of rabbits immunized with MBSA–T_4 DNA complexes are directed toward glucosyl dHMP and in this respect resemble those found in rabbits immunized with ruptured T-even coliphage (Murakami *et al.*, 1961; Townsend *et al.*, 1965). Since the dHMP of T_4 DNA contains 70% α-glucosyl and 30% β-glucosyl residues (Lehman and Pratt, 1960), it is possible to have antibodies directed toward either of these steric configurations (Townsend *et al.*, 1965). With four of the antisera to MBSA-T_4 DNA or to MBSA-modified T_4 DNA complexes, α-glucosyl dHMP was an effective inhibitor. α-Methyl glucoside, but not β-methyl glucoside, also inhibited two of these sera, although compared to the glucosylated nucleotide approximately 1000 times more of the α-methyl glucoside was required. The other two antisera were not inhibited by 350 μmoles of either methyl glucoside. The data from these hapten inhibitions and from the extent of cross reactivity of the heterologous glucosylated DNA's of T_2 and T_6 indicate that the antibodies are primarily directed toward the α-glucosyl dHMP of T_4 DNA.

Antibodies to the ruptured T-even coliphages displayed strict specificity for T-even DNA's. Even when ten times more antiserum was used no cross reactions, as measured by direct fixation with 42 other DNA's lacking glucosyl dHMP, was observed (Murakami *et al.*, 1961). Furthermore, ten other DNA's did not inhibit the homologous T_4 DNA reaction. With the exception of the photooxidized T_4 DNA, all our antisera to MBSA-T_4 DNA complexes displayed this same strict specificity and did not react with *B. subtilis* and *Ps. aeruginosa* DNA even when 200 times more antibody was used.

On the other hand, Plescia *et al.* (1964), using anti-

MBSA-T_4 DNA at the dilutions of antiserum used to measure the homologous reaction, observed cross reactions with *Diplococcus pneumoniae* and *Brucella suis*, but not with calf thymus, *Salmonella enteritidis*, or Rous Sarcoma DNA. Their antibodies to MBSA-T_4DNA complexes may have different specificities or *Diplococcus pneumoniae* and *Brucella suis* DNA may, as Plescia *et al.* (1964) suggest, contain glucosylated dHMP. A third possibility is that these two DNA preparations contained a cross-reacting polysaccharide.

The introduction of a lesion in T_4 DNA by photooxidation resulted in the production of antibodies with broad specificities. Although about 50 to 100 times more antiserum than that used for the homologous reaction is required, C′ fixation was demonstrated with several DNA's which lack glucose. That these were DNA–anti DNA reactions was shown by helix-to-random coil transition curves measured by immunological methods. In addition, these heterologous reactions were inhibited by all four nucleotides. Surprisingly, dGMP and dCMP were the more effective inhibitors although the immunogen contained no unsubstituted cytosine residues and had only half of its original guanine residues intact. It is possible that dCMP is more effective than dAMP and TMP because of its greater structural similarity to glucosyl dHMP. The greater effectiveness of the 3′,5′-cytosine dimer and equal or even decreased effectiveness of the 3′,5′ trimer probably reflects the size of the antibody receptor site.

Although the terminal phosphates are important in enhancing the inhibitory activity of cytidylic acid tracts, it is not yet known whether they function through a charge effect or are a part of the antigenic site. The presence of the phosphate groups was also important in enhancing the inhibitory power of the Pneumococcus SVI system (Rebers *et al.*, 1961). The repeating unit, a galactoglucorhamnoribitol, was four to five times more inhibitory when it possessed a phosphate monoester group.

The partially photooxidized T_4 DNA's proved to be interesting immunogens in still another respect. While photooxidation of DNA in the presence of methylene blue results in the specific destruction of guanine (Simon and Van Vunakis, 1962), the product of the reaction is unknown. Nevertheless, antibodies specific for this product have been formed. Their characterization and application will be the subject of another communication.

References

Burton, K., and Petersen, G. B. (1960), *Biochem. J. 75*, 18.

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

Healy, J. W., Stollar, D., Simon, M. I., and Levine, L. (1963). *Arch. Biochem. Biophys. 103*, 461.

Lehman, I. R., and Pratt, E. A. (1960), *J. Biol. Chem. 235*, 3254.

Levine, L., Barlow, J. L., and Van Vunakis, H. (1958), *Virology 6*, 702.

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

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

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

Mayer, M. M., Osler, A. G., Bier, O. G., and Heidelberger, M. (1948), *J. Immunol. 59*, 195.

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

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

Plescia, O. J., Palczuk, N. C., Braun, W., and Cora-Figuerua, E. (1965), *Science 148*, 1102.

Rebers, P. A., Hurwitz, E., and Heidelberger, J. (1961), *J. Bacteriol. 82*, 920.

Seaman, E., Van Vunakis, H., and Levine, L. (1965), *Biochemistry 4*, 1312.

Simon, M. I., and Van Vunakis, H. (1962), *J. Mol. Biol. 4*, 488.

Tamm, C., Hodes, M. E., and Chargaff, E. (1952), *J. Biol. Chem. 195*, 49.

Townsend, E. E., Van Vunakis, H., and Levine, L. (1965), *Biochemistry 4*, 943.

Wasserman, E., and Levine, L. (1960), *J. Immunol. 87*, 290.

Wyatt, G. (1951), *Biochem. J. 48*, 584.