

constant supply of reduced Fd. The Michaelis constants (K_m) for H_2 and N_2 are 0.28 and 0.17 atm, respectively.

Evidence is presented for the hypothesis that ATP activates "reduced" nitrogenase which in turn reacts with N_2 .

A method is described for measuring N_2 fixation in this system by following the uptake of N_2 and H_2 manometrically.

The technical assistance of Miss Patricia K. Walters is gratefully acknowledged.

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PRODUCTION OF ANTIBODIES TO DENATURED DEOXYRIBONUCLEIC ACID (DNA)*

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In 1956, when we initiated our study of DNA as an antigen, relatively few decisive data were available,^{1, 2} and there was no evidence that chemically pure DNA by itself could induce the formation of antibody. However, the possibility remained that some natural complexes containing DNA, or DNA coupled to a suitable carrier, might elicit the formation of antibodies as a hapten. Initially, therefore, we employed DNA-rich preparations from *Brucella* that had been isolated by the phenol procedure of Braun *et al.*³ These contained DNA-complexes that, in rabbits, elicited the formation of precipitins against several antigens in the DNA-rich preparation. At least one of these was sensitive to the action of DNA-ase⁴⁻⁶ and was found to be a species-specific complex of DNA and polysaccharide.⁶

In the meantime, evidence accumulated that antibodies capable of reacting with pure DNA are formed under certain conditions. Thus, antibodies that react

primarily with single-stranded, thermally denatured DNA were found in sera of individuals with lupus erythematosus and in sera of rabbits injected with lysates of T4 bacteriophage.⁷⁻¹⁰ The nature of the actual immunogens remains unknown. More recently, Butler *et al.*¹¹ and Tanenbaum *et al.*¹² showed that purine and pyrimidine bases are haptens when coupled to a protein such as bovine serum albumin and that antibodies to the haptens react with denatured DNA. These observations, in conjunction with Goebel and Avery's¹³ earlier demonstration that pneumococcal polysaccharides, which are nonantigenic in the rabbit by themselves, could act as haptens when coupled to a protein, motivated us to further immunochemical studies of chemically defined DNA-protein complexes. Because DNA is an acidic polymer, the use of a basic protein was explored, in this instance methylated bovine serum albumin (MBSA). Mixing of solutions of native DNA and MBSA resulted in a compact fibrous precipitate. However, since we desired accessible nucleotides in the potential antigens, we used complexes of heat-denatured, single-stranded DNA and MBSA, a mixture of which formed fine particles which did not dissociate under physiological conditions and were highly resistant to the action of DNAase. As herein shown, rabbits injected with such complexes (heat-denatured calf thymus DNA-MBSA or heat-denatured T4 phage DNA-MBSA) formed antibodies that reacted with thermally denatured DNA from various sources.

Materials and Methods.—Materials: High polymerized, relatively pure calf thymus DNA, and also crystalline DNAase, were obtained from Worthington Biochemical Corporation, Freehold, N. J. Methylated bovine serum albumin (MBSA) was prepared from crystalline bovine serum albumin according to Sueoka and Cheng.¹⁴ Complete Freund's adjuvant was obtained from the Difco Co., Detroit, Michigan. Deoxyribonucleotides were obtained from Sigma and were chromatographically pure. Highly polymerized, relatively pure T4 bacteriophage DNA was obtained from Dr. E. Bautz, and type III pneumococcal capsular polysaccharide (SIII) from Dr. M. Heidelberger. A pool of guinea-pig sera was used as complement (C'). Sheep blood was obtained at a local slaughterhouse, and sensitized erythrocytes for C'-fixation were prepared according to Plescia *et al.*¹⁵

Preparation of immunizing antigens: Calf thymus DNA and T4 bacteriophage DNA were dissolved in 0.15 M NaCl to a concentration of 500 $\mu\text{g/ml}$, based on absorption at 260 $m\mu$ with calf thymus DNA as standard. The DNA was denatured by the method of Doty *et al.*¹⁶ by boiling the solution at 100°C for 10 min and chilling it rapidly in ice-water, after which a 1% solution of MBSA in water was added with mixing until the final weight ratio of MBSA to DNA was 1. A volume of the complex was then emulsified with an equal volume of complete Freund's adjuvant. A complex of MBSA and SIII was obtained in the same way except that the SIII solution was not boiled.

Preparation of antisera: New Zealand white male rabbits (5-6 lb) were given at weekly intervals three injections of freshly prepared antigen containing 0.25 mg DNA/ml or 0.25 mg SIII/ml, administered into the foot pad (0.4 ml) and intramuscularly (1.0 ml), with each rabbit getting a total of 1 mg DNA or SIII. The rabbits were bled by cardiac puncture 7-10 days following the last injection.

Analysis of antisera: The complement-fixation method used was previously described.⁶ The quantitative precipitin method of Heidelberger and Kendall¹⁷ was also employed.

Results.—Analysis of anti-calf thymus DNA serum: Although several antisera were analyzed and yielded similar results, the following data refer to tests with only one (A 1). This serum was analyzed for C'-fixing antibodies against native and thermally denatured calf thymus DNA, MBSA, and the complex of MBSA with denatured DNA. The results, shown in Figure 1, were typical for a protein-

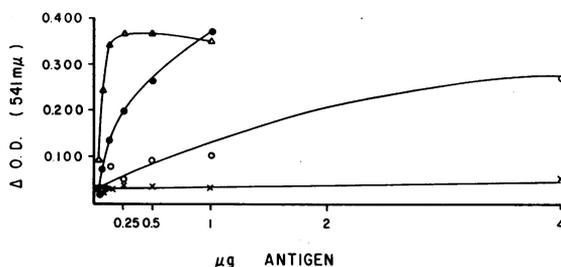


FIG. 1.—Analysis of rabbit anti-calf thymus DNA serum (A 1) for C'-fixing antibodies against methylated bovine serum albumin, Δ — Δ ; thermally denatured calf thymus DNA, \circ — \circ ; methylated bovine serum albumin-thermally denatured calf thymus DNA complex, \bullet — \bullet ; native calf thymus DNA, \times — \times . The ordinate is the difference between the optical densities of the antiserum control (antiserum + C') and of the reaction mixture (antiserum + C' + antigen) and is proportional to the amount of C'-fixing antibody.

hapten complex in that antibodies were formed against the protein, the complex, and the hapten.

The remainder of this report will be restricted to reactions with the hapten alone. While native DNA was inactive (Fig. 1), heat-denatured DNA fixed C', an indication that the antibody was directed against the nucleotide bases which became accessible for reaction on denaturation. The presence in this serum of antibody against denatured calf thymus DNA was also demonstrated by the quantitative precipitin method (cf. Fig. 5).

Deoxyribonucleotides as inhibitors: Each of the four principal constituent deoxyribonucleotides of calf thymus DNA inhibited C'-fixation between rabbit anti-calf thymus DNA and heat-denatured calf thymus at equivalence (Fig. 2). This suggests that all four deoxyribonucleotides may represent portions of the antigenic determinant groups. The amount of inhibitor needed for a given percentage of inhibition can be regarded as a measure of the affinity of inhibitor for the antibody, and it therefore should be noted that deoxycytidylic and thymidylic acids are better inhibitors than deoxyguanylic and deoxyadenylic acids. It remains to be established whether or not the fact that each deoxyribonucleotide gave a maximum inhibition of about 25 per cent reflects an equal probability for

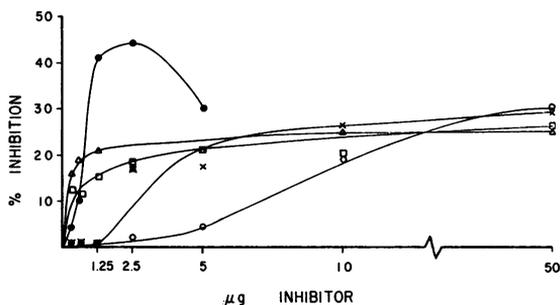


FIG. 2.—Inhibition of C'-fixation between rabbit anti-calf thymus DNA serum (A 1) and thermally denatured calf thymus DNA by deoxyribonucleotides: deoxycytidylic acid-5', Δ — Δ ; thymidylic acid-5', \square — \square ; deoxyguanylic acid-5', \times — \times ; deoxyadenylic acid-5', \circ — \circ ; enzymatic digest of calf thymus DNA, \bullet — \bullet .

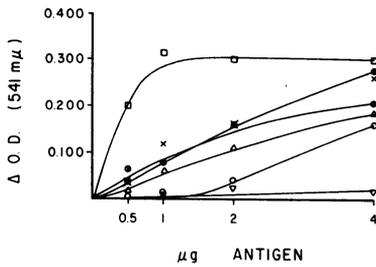


FIG. 3.—Analysis of rabbit anti-calf thymus DNA serum (A 1) for C'-fixing antibody against homologous and heterologous thermally denatured DNA: calf thymus DNA, ●—●; *B. subtilis* DNA, ×—×; *Diplococcus pneumoniae* DNA (streptomycin-resistant strain), Δ—Δ; *Diplococcus pneumoniae* DNA (A 66), □—□; monkey heart cell DNA, ○—○; *Brucella* DNA (smooth strain), ●—○; T4 bacteriophage DNA, ▽—▽.

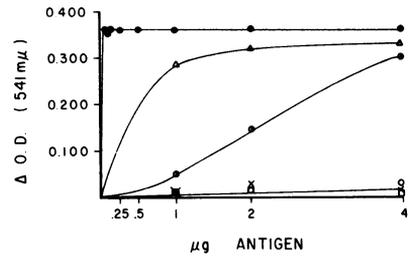


FIG. 4.—Analysis of rabbit anti-T4 phage DNA serum (A 13) for C'-fixing antibody against homologous and heterologous thermally denatured DNA: T4 phage DNA, ●—●; *Diplococcus pneumoniae* DNA (A 66), Δ—Δ; *Brucella suis* DNA, ●—○; calf thymus DNA, ×—×; *Salmonella enteritidis* DNA, ○—○; Rous sarcoma DNA, □—□.

any single nucleotide to be a part of the antigenic reactive sites. The corresponding ribonucleotides did not inhibit; neither did deoxyribose nor the bases by themselves.

As might have been expected, an enzymatic digest of calf thymus DNA, which contains oligonucleotides that may represent larger portions of the antigenic reactive sites, inhibited the reaction more extensively than mononucleotides. Absence of complete inhibition may have been due, at least in part, to the presence in the digest of large fragments of DNA that carried multiple or complete antigenic sites and were still capable of fixing C' when added to antibody. This is supported by the decrease of inhibition with amounts of digest in excess of 2.5 μg.

Specificity of antibody to denatured calf thymus DNA: The specificity of rabbit anti-calf thymus DNA was examined with heat-denatured DNA from *Bacillus subtilis*, *Brucella abortus*, *Brucella suis*, *Salmonella enteritidis*, *Diplococcus pneumoniae*, monkey heart cells, and T4 bacteriophage. With the exception of the last, all DNA preparations reacted, albeit to different degrees (Fig. 3). The extensive cross-reactivity of our antisera resembles that of the antinuclear factors in sera of patients with lupus erythematosus.⁷ The inactivity of T4 phage DNA may be due to its content of glucosylated 5-hydroxymethyl cytosine instead of cytosine which, as shown in Figure 2, strongly inhibits C'-fixation and therefore is an important constituent of the antigenic determinants of denatured calf thymus DNA. The amount of DNA needed for a given extent of C'-fixation varies with the source of DNA (Fig. 2), possibly owing to differences in the distribution, number, and accessibility of antigenic determinants per fragment of DNA as a result of species-specific variations in the sequence of nucleotides, extent of intramolecular hydrogen-bonding, and the length of individual chains formed during preparation.

Fractionation of calf thymus DNA: In order to obtain additional evidence as to whether the reactive antigen was DNA or some contaminant, calf thymus DNA was fractionated by ultracentrifugation in a CsCl density gradient according to Meselson *et al.*¹⁸ The peak fraction at the center of the single band of DNA was heat-denatured and tested as reactive antigen in C'-fixation. It was as reactive as the starting material. The DNA was also fractionated by column chromatography

on DEAE-cellulose. Three distinct fractions, differing in molecular size, were obtained with a sodium chloride gradient; each had the same antigenic activity as the starting material. The DNA was also purified by precipitation with cetyl trimethyl ammonium bromide. The precipitate was extracted successively with 0.15 *M* NaCl and 0.3 *M* NaCl to solubilize any contaminating protein or polysaccharide. The residue, containing essentially all of the DNA, was dissolved in 1.5 *M* NaCl and the DNA precipitated with 2 volumes of ethanol, denatured by heating, and tested. It, too, showed unchanged reactivity.

Analysis of an anti-T4 phage DNA serum: An antiserum produced against a complex of MBSA with heat-denatured T4 phage DNA was analyzed for C'-fixing antibodies against homologous and heterologous heat-denatured DNA, as was the anti-calf thymus DNA serum. The results in Figure 4 show that fixation was complete over a wide range of concentration of denatured T4 phage DNA as antigen, an indication that the system is highly reactive. Also, the specificity is sharper than that of anti-calf thymus DNA. The absence of cross-reaction between denatured calf thymus DNA and anti-T4 phage DNA serum parallels the lack of cross-reactivity between denatured T4 phage DNA and anti-calf thymus DNA serum. The specificity of denatured T4 phage DNA is probably due largely to its glucosylated 5-hydroxymethyl cytosine, which may be the reason for its much better antigenicity than that of calf thymus DNA and the limited cross-reactivity of anti-T4 phage DNA serum. If anti-T4 phage DNA serum is indeed directed primarily against glucosylated 5-hydroxymethyl cytosine, the prediction could be made, on serological grounds, that the cross-reacting pneumococcal DNA and Brucella DNA also would contain this base.

MBSA as a carrier for other nonimmunogenic acidic polymers: As stated earlier, the type-specific capsular polysaccharides of *Diplococcus pneumoniae*, like DNA, fail to stimulate the production of antibody in rabbits when used in chemically pure form, but the rabbit forms antibodies to the polysaccharides if injected with whole pneumococcal cells. MBSA was therefore tested as an immunogenic carrier for one of the pneumococcal polysaccharides, in this instance SIII, a polymer of repeating units of cellobiuronic acid. Like heat-denatured DNA, the type III polysaccharide is acidic and complexes readily with MBSA on mixing to form a stable suspension. The antisera obtained from rabbits injected with this complex in Freund's adjuvant contained measurable amounts of precipitating antibody against SIII (Fig. 5). It is thus evident that MBSA is an adequate immunogenic carrier for acidic polymers such as DNA and polysaccharides. For comparison, two anti-calf thymus DNA sera were also analyzed for precipitins against heat-denatured calf thymus DNA (Fig. 5).

Discussion.—The present data clearly show that thermally denatured DNA acts as a hapten when injected as a complex with MBSA. Since all four deoxyribonucleotides inhibit the antigen-antibody reaction, it can be inferred that the antigenic determinant groups comprise sequences of deoxyribonucleotides. While it now has become possible to induce the synthesis of antibodies against denatured DNA, the question remains: why do nucleic acids have to be coupled to an immunogenic carrier as haptens in order to induce such antibodies? Three possible answers are: (1) DNA's, regardless of species, differ principally in their sequence of nucleotides rather than in their molecular configuration and hence are not recognized as suffi-

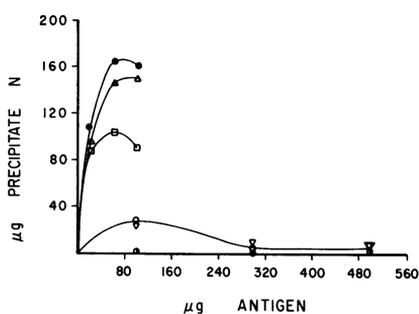


FIG. 5.—Quantitative precipitin analysis of rabbit anti-SIII type-specific capsular polysaccharide sera (A 8, A 9, A 10) and rabbit anti-calf thymus DNA sera (A 1, A 6), with SIII polysaccharide and thermally denatured calf thymus DNA, respectively, as antigen. Sera were obtained from rabbits injected with complex of antigen and MBSA. ●—●, A 8; △—△, A 9; □—□, A 10; ○—○, A 1; ▽—▽, A 6; ●—●, normal rabbit serum + thermally denatured calf thymus DNA. Precipitate N is for 1.0 ml serum.

ciently “foreign” by antibody-forming cells; (2) “unprotected,” noncomplexed DNA may be reduced too rapidly to nonimmunogenic fragments by host nucleases; and (3) DNA by itself may be unable to enter and thus stimulate appropriate antibody-producing cells.

The fact that pneumococcal capsular polysaccharide, as well as denatured DNA, becomes immunogenic in the rabbit when complexed to MBSA suggests that MBSA might be a general immunogenic carrier for acidic polymers composed of suitable antigenic determinant groupings. We expect to know shortly whether or not mRNA and tRNA complexed to MBSA are also antigenic. The availability of a simple, general method for producing antibodies that are presumably specific either for unique bases, such as glucosylated 5-hydroxymethyl cytosine, or for nucleotide sequences might enable the molecular biologist to use precise and sensitive immunochemical methods as an aid in probing the structure of nucleic acids.

Summary.—Thermally denatured calf thymus DNA and T4 phage DNA act as haptens in the rabbit when injected as complexes with methylated bovine serum albumin (MBSA). The antigenic determinant groups of calf thymus DNA seem to comprise sequences of the four common deoxyribonucleotides because (1) each of the deoxyribonucleotides but not ribonucleotides, deoxyribose, or the bases, inhibits the C'-fixation reaction between antiserum and denatured calf thymus DNA; (2) the antiserum cross-reacts with denatured DNA from various mammalian and bacterial species; (3) native DNA is unreactive; (4) the reactive antigen is found in fractions of highly purified DNA and cannot be dissociated from it. The antibodies against denatured calf thymus DNA do not react with single-stranded T4 phage DNA. Similarly, anti-T4 phage DNA serum shows little cross-reactivity with heat-denatured heterologous DNA. This relative specificity of the phage DNA may be due to its unique base content. Pneumococcal polysaccharide, like denatured DNA, also becomes immunogenic in rabbits when complexed to MBSA. Thus, MBSA appears to be a suitable carrier for antigenic acidic polymers.

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URIDINE-SPECIFIC ANTIBODIES OBTAINED WITH SYNTHETIC ANTIGENS*

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The problem of the antigenicity of nucleic acids has been approached from several directions with varying degrees of success.¹ Reports in the older literature^{1, 2} on antibodies with specificity directed toward nucleic acids have been challenged because of doubts concerning the purity of the nucleic acid used either for immunization or for specific reaction with the antibodies formed. Neither RNA¹ nor DNA^{1, 3, 4} nor synthetic polynucleotide⁵ preparations were found to be immunogenic by the serological methods employed. On the other hand, positive results were reported with DNase-sensitive antigens from *Brucellae*^{6, 7} and with a soluble RNA preparation from yeast.⁸ Antibodies directed toward thermally denatured DNA have been detected in rabbit antisera to ruptured T-even coliphage⁹ and in sera of patients with lupus erythematosus.¹⁰⁻¹² In the case of the coliphage the antibodies were shown to be directed, in part, toward the glucosylated 5-hydroxymethylcytosine.¹³ Antibodies with specificity toward RNA were also detected in antisera to bacterial ribosomes.¹⁴⁻¹⁶

An alternative approach to the elucidation of immunological properties of nucleic acids consists of efforts to bind, chemically, their components to well-defined antigens, and to study the specificity of antibodies elicited by means of such artificial conjugates. Thus, antibodies with purine or pyrimidine specificities, reacting with heat-denatured DNA, were obtained in rabbits upon injection of purinoyl-¹⁷ or uracil-conjugates¹⁸ of serum albumins.

This report describes the chemical binding of a uridine derivative to two different multichain synthetic polypeptides, one antigenic and the other nonantigenic.^{19, 20} The injection into rabbits of these synthetic nucleoside-polypeptide conjugates