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*SPECIFIC ANTIBODIES TO THERMALLY DENATURED DEOXYRIBONUCLEIC ACID OF PHAGE T4**

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Immunochemical analyses[1] of antisera to ruptured T4 bacteriophage suggested that an antibody directed against deoxyribonucleic acid (DNA) might be present. In this paper we show that antibodies specific for DNA are indeed present in our antisera, and that they react chiefly with thermally denatured DNA.

Previous workers have reported immunological reactivity of native bacterial and mammalian DNA.[2-4]

Materials and Methods.—Bacteriophage T4 was grown on *Escherichia coli*, strain B, in synthetic media and purified by methods described by Herriott and Barlow.[5]

Immunizing antigen was prepared by freezing and thawing phage preparations containing 10^{13} particles/ml in 0.1 *M* NaCl. The ruptured phage were treated with pancreatic deoxyribonuclease (DNase) to reduce the viscosity. An alum precipitate of the ruptured phage solution was formed, and increasing quantities (5×10^{12} to 4×10^{13} phage equivalents) were injected intravenously into rabbits on alternate days for a period of five weeks. The rabbits were bled seven days after the last injection. After a month, a second and similar course of immunization was repeated. Seven days after the last injection, the rabbits were exsanguinated by cardiac puncture. Many of the antisera were prepared in 1957 and have been stored frozen. The antisera were absorbed with whole phage.

For extraction of DNA, phage preparations were stirred at pH 9.0 in 0.001 *M* pyrophosphate at room temperature. The pH was then adjusted to 7.5, and NaCl added to a final concentration of 0.15 *M*. Three detergent treatments[6] and three phenol extractions[7] were used to remove protein. The DNA was then dialyzed against isotonic saline.

DNA determinations were made by the procedure of Burton,[8] using a standard DNA of known phosphorus content.

For amino acid analysis, 20 mg of DNA were hydrolyzed for 20 hours with 6 *N* HCl at 105°C in an evacuated tube. The amino acids in the hydrolysate were determined on the Spackman, Stein, and Moore automatic amino acid analyzer.[9] The total amino acid content of several preparations was 0.5 per cent by weight of the DNA.[10] The 0.5 per cent value does not include glycine since a large quantity of this amino acid results from the breakdown of adenine and guanine during the hydrolysis.

Antigenic activity was determined by use of quantitative complement (C′) fixation[1] or by a micro-complement fixation technique.[11]

The synthesis of DNA in T4-infected cells was studied as follows: *E. coli* B were grown to 1.3×10^8 cells/ml in nutrient broth containing 0.5 per cent NaCl and infected with 5 T4 phage particles per cell. Aliquots of 500 ml, withdrawn at various times during the infective cycle, were chilled and harvested by centrifugation. Cell lysis was accomplished by suspending the pellets in 5 ml of 0.15 *M* NaCl containing 0.45 per cent of recrystallized sodium dodecyl sulfate and 0.001 *M* pyrophosphate. The solutions were then diluted to 25 ml with saline and centrifuged at $12,000 \times g$ for 30 min to remove cellular debris. Cell number recovered in each sample was corrected as previously described on the basis of RNA content.[12] DNA was determined chemically and immunologically, the latter analysis being carried out on both native and thermally denatured samples.

Results.—Figure 1 illustrates the reaction of native and thermally denatured DNA (100°C—10 min) with absorbed antiserum. Thermally denatured DNA yielded a typical complement fixation curve, showing maximum fixation with 2 μg of DNA. About 30 times more native DNA was required to fix equal quantities of C′. This reaction may be due to a small amount of denatured DNA contaminating the native DNA. Preliminary experiments suggest that the same antibody reacts with denatured and native DNA.

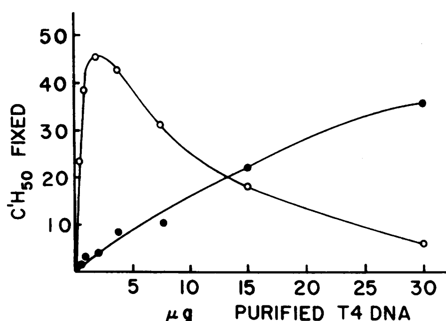

FIG. 1.—Fixation of complement by increasing quantities of native and thermally denatured T4 DNA with 1.0 ml of antiserum to ruptured T4 absorbed with whole T4 and diluted 1:200. ● = Native DNA; ○ = thermally denatured DNA.

As noted above, 2 μg of denatured DNA or 0.01 μg of a hypothetical contaminating protein gave a maximum fixation of C′. In Table 1 we show that, in eleven known

TABLE 1

AMOUNTS OF ANTIGEN REQUIRED FOR PEAK FIXATION OF COMPLEMENT WITH HOMOLOGOUS ANTIBODY

Antigen	Molecular weight	μg Antigen giving peak fixation with homologous antibody
Bovine pancreatic ribonuclease	1.3×10^4	0.35
T2 Phage internal protein	2.0×10^4	1.2
Rhodospirillum heme protein	3.6×10^4	1.8
Swine pepsinogen	4.2×10^4	0.6*
Human serum albumin	6.8×10^4	3.0
E. coli alkaline phosphatase	8.0×10^4	5.0
Human high density lipoprotein	2.0×10^5	9.0
Human low density lipoprotein	1.6×10^6	24.0
T2 Bacteriophage	5.0×10^8	35.0
Pneumococcus polysaccharide (II)	Not determined	1.0
Pneumococcus polysaccharide (XIV)	Not determined	1.2
Denatured T4 DNA	Not determined	2.0

All antisera were diluted so as to fix approximately 50 out of 100 $C'H_{50}$ units at the maximum.
* Determined as pepsin activity.

antigen-antibody systems that we have investigated by C′ fixation, the smallest quantity of antigen giving peak fixation was 0.35 μg of ribonuclease. The reaction with denatured DNA probably cannot be ascribed, therefore, to contaminating protein.

The nature of the antigen is indicated by enzymic tests. Three μg per ml of native or thermally denatured DNA were incubated at 37°C with 0.004 μg pancreatic DNase, 0.02 μg trypsin, or 0.02 μg chymotrypsin per ml. The extent of hydrolysis by DNase was followed by determining the release of acid soluble material absorbing at 260 mμ. Aliquots of the reaction mixtures were removed at various times and heated in a boiling water bath for 10 min (to inactivate the enzymes and denature DNA).

The antigenic activity was destroyed by pancreatic DNase, but was not significantly altered by trypsin or chymotrypsin (Fig. 2*a*). The loss in antigenic activity coincided with the release of acid soluble material (Fig. 2*b*) during DNase digestion and under the given conditions proceeded more rapidly when native rather than denatured DNA was used as substrate.[13] These results are in contrast to those obtained with *E. coli* phosphodiesterase, which is known to digest denatured DNA but not native material.[14] We find that this enzyme destroys the antigenicity of denatured DNA but not that of native DNA.

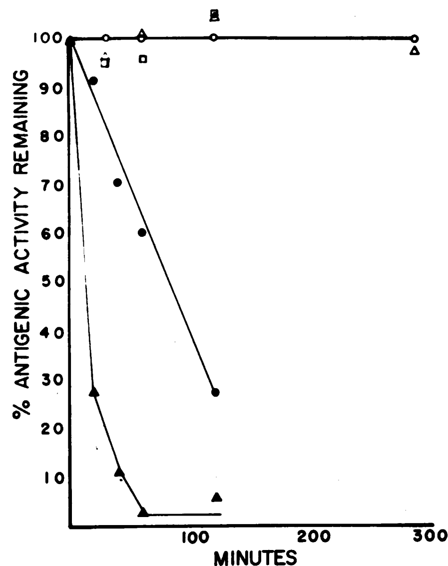

FIG. 2a.—Action of trypsin, chymotrypsin and DNase on the serological reactivity of T4 DNA. ○ = Thermally denatured DNA; △ = trypsin-treated thermally denatured DNA; □ = chymotrypsin-treated thermally denatured DNA; ● = pancreatic DNase treated thermally denatured DNA; ▲ = pancreatic DNase treated native DNA.

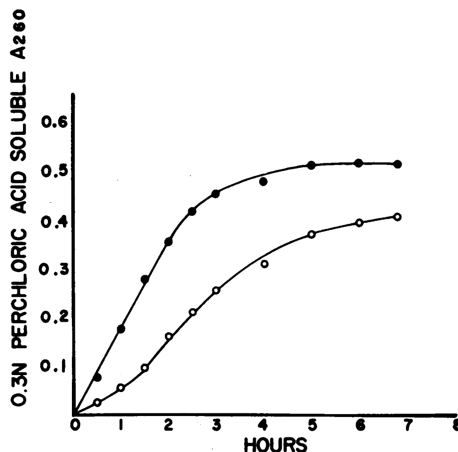

FIG. 2b.—Release of acid-soluble ultraviolet light-absorbing material through the action of pancreatic DNase (0.04 μg/ml) on native and thermally denatured DNA (30μg/ml.) ● = native DNA; ○ = thermally denatured DNA. Measurements of optical density per cm. at 260 mμ (A_{260}) were made after precipitation with an equal volume of 0.6 N perchloric acid.

Recently, it has been shown that denatured DNA can regain, under favorable conditions, several properties of native DNA.[15–17] Since the antibody described above reacts with denatured DNA, DNA renaturation should be accompanied by loss of antigenic activity. The appropriate experiment is described in Figure 3. Three hundred μg of thermally denatured DNA was diluted 1 to 20 into 0.15 M NaCl at 55°C and incubated at this temperature. Aliquots were taken at various times, diluted into ice cold buffer and assayed for antigenic activity. After nine hr, approximately 65 per cent of the activity had been lost, presumably by renaturation. Full recovery of antigenic activity was obtained when an aliquot of the 9-hr sample was reboiled.

Thermal denaturation of DNA presumably ruptures hydrogen bonds holding complementary polynucleotide chains together, thereby exposing groups which were formerly masked.[16,18] In the T-even bacteriophages, such components include thymine, adenine, guanine, 5-hydroxymethylcytosine, and glucosylated 5-hydroxymethylcytosine. It is therefore possible that the antigenic determinants of thermally denatured DNA are glucose and/or the nitrogen bases. With respect to glucose, the antibody may be directed toward repeating glucose residues. Since the extent of glucosylation differs in the T-even phages, immunochemical analyses of cross reactions with DNA from different T2, T4, and T6 phage strains should evaluate this possibility.

DNA was isolated from T4, T2, and $\overline{T2}$. The latter strain of phage was developed by Streisinger and Weigle[19] by crossing T2 with T4 in such a way as to obtain a

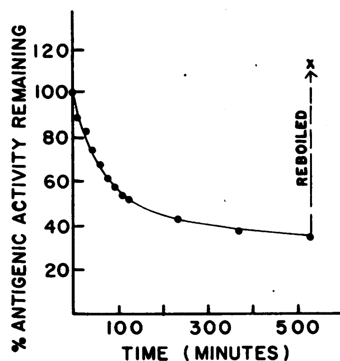

FIG. 3.—Renaturation of thermally denatured T4 DNA at 55°. Thermally denatured DNA was diluted to a final concentration of 15 μg/ml in 0.15 *M* NaCl preheated to 55° and maintained at that temperature. Aliquots were taken at various times, diluted into ice cold buffer and assayed for antigenic activity. After 9 hr at 55°, a sample was reboiled for 10 min prior to assay.

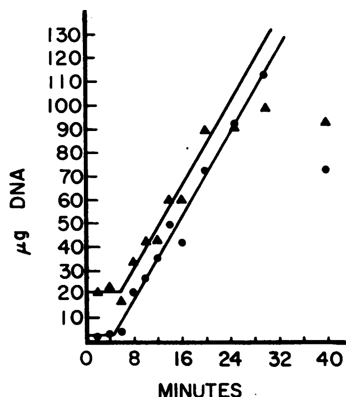

FIG. 4.—Synthesis of phage DNA by *E. coli* B infected with 5 phage particles per bacterium. ▲ = DNA measured by the diphenylamine reaction; ● = phage DNA measured by complement fixation assay of boiled samples. For use in these experiments, antisera were absorbed with phage, then with ruptured phage digested with DNase (previously treated with diisopropylfluorophosphate) and snake venom diesterase.

phage with the glucose content of T4 but otherwise largely isogenic with T2. Fixation of complement was greater with $\overline{T2}$ DNA in the presence of T4 antiserum than with T2 DNA. This property of $\overline{T2}$ DNA was not found in four mutant phages derived from T2. It seems probable, therefore, that glucose residues contribute to the serological reactivity.

The products resulting from the hydrolysis of DNA by pancreatic DNase are potent inhibitors of the antigen-antibody reaction. Some inhibition can be observed with 5-hydroxymethylcytosine but not with glucose. Perhaps the antigenically effective repeat unit is glucosylated 5-hydroxymethylcytosine.

The course of viral DNA synthesis in a culture of *E. coli* B infected with 5 T4 particles per bacterium is shown in Figure 4. Net increase of DNA as determined by the diphenylamine procedure was detectable 6–8 min after infection. Net increase of T4 DNA determined immunologically on heated samples occurred at the same time. *E. coli* DNA[20] did not react with the T4 antiserum nor did lysates of uninfected bacteria.

Discussion and Summary.—In this study, a reaction between thermally denatured DNA and antibody present in antisera of rabbits immunized with ruptured T4 phage was demonstrated.

Evidence that the antigenicity resides in the DNA molecule is manifold. The protein content of the DNA preparation is too low to account for the observed fixation of C′. In infected cells, the times of synthesis of DNA and of the immunologically reactive substance coincide. Pancreatic DNase and *E. coli* phosphodiesterase, enzymes that hydrolyse DNA in entirely different ways, destroy the antigenicity in parallel with their known enzymatic action. The serological re-

activity is low with native DNA and with DNA that has been first denatured and then restored by heating at 55°C. The effects of varied temperature, concentration, ionic strength and pH all indicate that serological reactivity manifests itself when the DNA strands are separated and disappears when the strands recombine.

Several observations suggest that the repeat unit responsible for the serological specificity is glucosylated 5-hydroxymethylcytosine. The antibody found in T4 antiserum cross reacts with DNA from T2 and T6, but not with *E. coli* DNA or with DNA from calf thymus. Among derivatives of T2, only one with a high glucose content yields a DNA serologically equivalent to that of T4. Glucose itself does not compete in the reaction with DNA, but 5-hydroxymethylcytosine does to some extent. The possibility that DNA contains other determinants of serological specificity is not, of course, excluded by these observations.

Antibodies to thermally denatured DNA have also been found in anti-T2 and anti-T6 sera. Immunization with nonglucosylated DNAs has not yet been successful.

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