

*PURINE-SPECIFIC ANTIBODIES WHICH REACT WITH
DEOXYRIBONUCLEIC ACID (DNA)**

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Substances that have the properties of antibodies and react with DNA from various sources are frequently found in the sera of patients with systemic lupus erythematosus.^{1, 2} They appear to be formed spontaneously, perhaps as autoantibodies, but the stimulus for their production is not known.

There have been many attempts to induce the formation of antibodies with specificity towards DNA,³⁻⁵ but those which react with purified DNA have been conclusively demonstrated in only one instance.⁶ In this example, the antigen, a partially denatured DNA from T4 bacteriophage, was unusual in that it contained a glucosylated pyrimidine, and the antibody produced was found to be specific towards this antigenic component.⁷ DNA from sources other than T-even phages did not react with this antibody.

Although antibodies specific for a variety of haptens have been obtained following the classical technic of Landsteiner,⁸ no record has been found of the use of nucleic acid components for such studies. Accordingly, purines and pyrimidines were covalently linked to protein carriers to determine whether they might stimulate the formation of antibodies. Bovine serum albumin conjugates containing the [6-pur-nyl]-sulfonyl, orotic acid, and [6-puriny]- β -alanyl moieties were prepared, but antibodies to these antigens gave equivocal results when examined for purine or pyrimidine specificity.⁹ This report describes the coupling of 6-trichloromethyl-purine¹⁰ to serum albumin, and the use of the resulting conjugate as an antigen to induce the formation of antibodies which exhibit a purine specificity and react with DNA.

Materials and Methods.—Purinoyl-protein conjugates: These substances were synthesized by a method which was derived from a previously described technic¹¹ for the preparation of aroyl-protein conjugates. A chilled solution containing 750 mg bovine serum albumin (BSA; Pentex Fraction V) and 170 mg 6-trichloromethylpurine in 70 ml of 4% tetrahydrofuran-water was stirred at room temperature for 3 hr. The pH was maintained throughout at 10-10.5 by the addition of 0.1 N NaOH and the solution was dialyzed overnight against running tap water. Upon acidification to pH 4.5 with 0.1 N HCl, the conjugated protein precipitated. It was re-dissolved in 25 ml of 0.15 M NaHCO₃, dialyzed against running water, and lyophilized. The product (Pur-BSA) thus obtained dissolved readily in water or buffer solutions and migrated in agar gel electrophoresis on a microscope slide as a single component toward the anode at pH 8.2 more rapidly than did unconjugated BSA. From spectrophotometric measurements, Pur-BSA was estimated to contain approximately 24 purine groups per mole of BSA (molecular weight, 67,000).¹² In the same way, the conjugate (Pur-HSA) with human serum albumin (HSA, Pentex Fraction V) was prepared. It contained about 27 moles of purine per mole. Spectral properties of these derivatives determined in a Cary spectrophotometer in 1 cm cells are given in Table 1.

Immunochemical procedures: Rabbits were immunized by injection, into the foot-pads,¹³ of Pur-BSA or Pur-HSA in complete Freund's adjuvant mixture weekly for three weeks. The animals were bled three times by cardiac puncture, beginning seven days after the last injection, and were exsanguinated 14 days after the final injection. The bleedings from individual rabbits were pooled.

TABLE 1
SPECTROPHOTOMETRIC PROPERTIES OF PROTEINS AND CONJUGATES

Substance	Conc. ($\mu\text{g}/\text{ml}$)	λ max, $m\mu$; optical density		
		pH 1	pH 6.2	pH 12
BSA	1540	277; 0.905	278; 0.965	292; 1.170
Pur-BSA	310	282; 1.240	286; 1.260	292; 0.950
HSA	1328	277; 0.865	278; 0.790	291; 1.020
Pur-HSA	244	283; 1.030	284; 1.040	292; 0.805
(N-[Purin-6-oyl]-glycine) ¹⁸	13.6	277; 0.580	289.5; 0.620	297; 0.430

All animals (17) immunized produced antibodies which precipitated with both Pur-protein conjugates.

Quantitative precipitin data were obtained using a procedure described by Kabat¹⁴ which employs the Folin-Ciocalteu color reaction for analysis of specific precipitates. The complement-fixation method of Stollar and Levine¹⁵ was used to study the reaction between antibody and DNA.

DNA: Pneumococcal DNA was prepared by the procedure of Hotchkiss,¹⁶ which involves ethanol precipitation and deproteinization by shaking with chloroform-*iso*amyl alcohol. Denaturation was effected by heating in isotonic sodium chloride-veronal buffer¹⁷ for 10 min in a boiling water bath followed by immediate chilling in an ice bath.¹⁵

Results.—Quantitative precipitin values obtained upon addition of several purine-containing antigens to a representative antiserum (E22-30, Pur-BSA) are depicted in Figure 1. The small amount of antibody precipitable by BSA or HSA compared

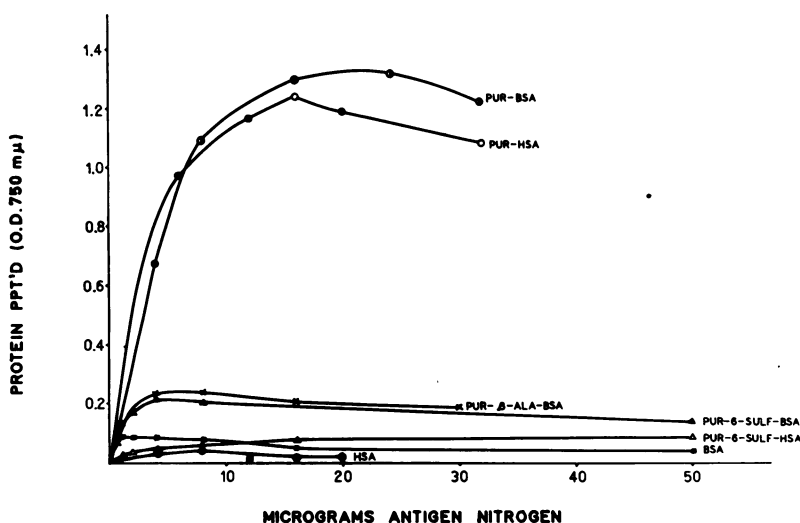


FIG. 1.—Precipitin reaction between antiserum E-22-30 (1 ml of a 1:5 dilution) and various antigens. Ordinate, optical density at 750 $m\mu$ after Folin-Ciocalteu reaction on precipitate. Abbreviations: Pur-BSA, [Purin-6-oyl]-bovine serum albumin; Pur-HSA, [Purin-6-oyl]-human serum albumin; Pur- β -ala-BSA, Purinyl-6- β -alanyl-bovine serum albumin; Pur-6-Sulf-BSA, purinyl-6-sulfonyl-bovine serum albumin; Pur-6-Sulf-HSA, Purinyl-6-sulfonyl-human serum albumin; BSA, bovine serum albumin; HSA, human serum albumin.

with the much larger precipitates obtained with Pur-BSA or Pur-HSA suggests that most of the antibody in this serum has purine specificity. The low reactivity of [purinyl]- β -alanyl-BSA, [purine-6-sulfonyl]-BSA, and [purine-6-sulfonyl]-HSA may either be due to structural differences of the antigens which contain purinyl, purinesulfonyl, and purinoyl residues, or to differences in the extent of incorporation of purine residues. The nature and specificity of these cross-reactions are under investigation.

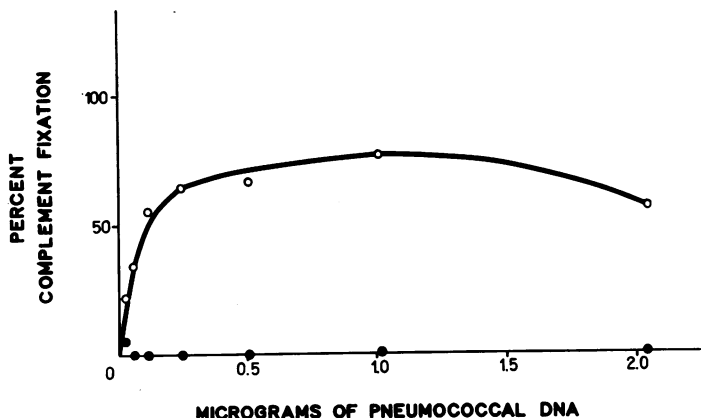


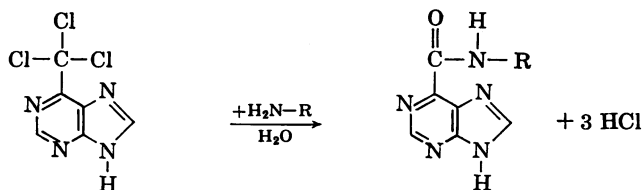
FIG. 2.—Complement-fixation reaction between serum E-22-30 (dilution 1:400) and native (closed circles) and denatured (open circles) pneumococcal DNA.

The ability of native and heat-denatured pneumococcal DNA to fix complement by reacting with E-22-30 at 1:400 dilution is shown in Figure 2. It is apparent that the ability of native DNA to fix complement was negligible, whereas 1 μ g of denatured DNA fixed 75% of the complement added. Fixation of complement was observed when native pneumococcal DNA was added to a 1:100 dilution of this antiserum; 4 μ g of native DNA was required for 70% fixation, a level attained at this antiserum dilution with 0.03 μ g denatured DNA. It remains to be ascertained whether this represented a reaction of antibody with native DNA or with traces of denatured DNA present in the preparation used. A 1:50 dilution of the pre-immunization serum from this rabbit did not fix complement with native or denatured DNA. Precipitin reactions between the pneumococcal DNA and antiserum E-22-30 have not been observed.

Antiserum E-22-30 was also observed to fix complement in the presence of DNA from bacteriophage $\phi\chi$ -174 and denatured DNA from *E. coli*, *B. subtilis*, *B. natto*, and *H. influenzae*. There was no reaction between antipurinoyl sera and RNA from rat liver by either complement-fixation or precipitin technics.

The effectiveness of various purines, pyrimidines, and imidazole compounds in inhibiting the complement-fixation reaction between DNA and antibody is shown in Table 2. All determinations were carried out with 1 μ g of heat-denatured pneumococcal DNA, a concentration which gave maximal fixation of complement with the dilution of antiserum used.

Discussion.—The antigens derived from the serum albumins and 6-trichloromethylpurine were formed in a reaction similar to that observed between this purine derivative and amines or amino acids,¹⁸ to give amide linkages between the purine



R = amino acid or protein residue.

TABLE 2
ORDER OF HAPTEN INHIBITION

Micromoles of hapten required for 50% inhibition of complement-fixation in the reaction of 1 μ g denatured pneumococcal DNA and 1.0 ml of a 1:400 dilution of antiserum E-22-30. (Except where otherwise noted, the compounds were commercial products of standard purity.)

Hapten	Micromoles
N-[Purin-6-oyl]- ϵ -aminocaproic acid ^a	<0.01 (100%) ^b
N-[Purin-6-oyl]-glycine ^a	<0.02 (89%) ^b
Purine-6-carboxamide ^{c, d}	0.01
Purine-6-carboxylic acid methyl ester ^e	<0.06 (85%) ^b
Purine-6-carboxylic acid	0.07
8-Methylpurine ^f	<0.10 (69%) ^b
Hypoxanthine	<0.11 (75%) ^b
4-Amino-5-imidazole-carboxamide	<0.29 (81%) ^b
Xanthine	0.10
6-Methylpurine	0.12
2'-Deoxyguanosine	0.12
7-Methylpurine ^g	0.15
Adenine	0.30
Purine	0.40
Guanosine	0.60
2'-Deoxycytidylic acid	0.60
Adenosine	0.70
2'-Deoxyadenosine	0.70
9-Methylpurine ^g	1.00
Thymine	1.50
Cytidylic acid	>1.25 (42%) ^h
Cytosine	5.4
Uracil	>4.5 (40%) ^h
4-Amino-5-formylamino-6-methylpyrimidine ⁱ	>6.8 (18%) ^h
Imidazole	>7.4 (0%) ^h

^a Cohen, S., E. Thom, and A. Bendich, manuscript in preparation.

^b Per cent inhibition in parentheses represents value at lowest concentration tested.

^c McKay, L. B., and G. H. Hitchings, *J. Am. Chem. Soc.*, **78**, 3511 (1956).

^d Giner-Sorolla, A., and A. Bendich, *J. Am. Chem. Soc.*, **80**, 3932 (1958).

^e Cohen, S., E. Thom, and A. Bendich, *J. Org. Chem.*, **28**, Oct. 1962 (in press).

^f Gift from Dr. G. B. Brown.

^g Bendich, A., P. J. Russell, and J. J. Fox, *J. Am. Chem. Soc.*, **76**, 6073 (1954).

^h Fifty per cent inhibition not achieved, actual values given in parentheses.

ⁱ Gabriel, S., and J. Colman, *Ber.*, **34**, 1234 (1901).

nucleus and most probably the ϵ -amino residues of lysine. That an acylation of the latter residues occurred is inferred from two experimental observations. First, the ultraviolet absorption spectrum of the serum albumin shifted in neutral solution from a maximum of 278 $m\mu$ to 284–286 $m\mu$ in agreement with values expected for the spectra of N-[purin-6-oyl]-amino acids.^{10, 18} Second, the most potent inhibitor of the complement-fixation reaction (Table 2) was N-[purin-6-oyl]- ϵ -aminocaproic acid, which structurally most closely resembles purinoyl-lysine.

The complement-fixation experiments (Fig. 2) demonstrate that antipurinoyl antibody reacts with denatured DNA at a dilution which does not show any reaction with native DNA. This finding is similar to those of Levine *et al.*^{6, 15} on the reaction of rabbit antibodies to bacteriophage DNA and of sera from lupus erythematosus patients with native and denatured DNA. These observations can be explained by current concepts of the structure of DNA^{19, 20} which postulate that heat denaturation exposes the bases by disruption of the hydrogen bonds between the paired strands of native DNA. However, the underlying basis for the reaction between the antibody studied here and DNA is puzzling since the purine in the antigen was coupled via position 6 whereas, in DNA, the purines are linked to deoxyribose at position 9.

The relative effectiveness of various compounds as haptens in inhibiting the reaction between the denatured DNA and antipurinoyl antibody (Table 2) is in agree-

ment with the postulated structure of the conjugated antigen. As noted previously, N-[purin-6-oyl]- ϵ -aminocaproic acid was the most effective inhibitor, followed closely by N-[purin-6-oyl]-glycine and purine-6-carboxamide. All other purines and purine derivatives tested were more effective inhibitors than the pyrimidines. Imidazole, which together with the pyrimidine ring, constitutes purine, was completely ineffective as an inhibitor. 4-Amino-5-formylamino-6-methylpyrimidine, an "open-ring" purine analog, was as ineffective an inhibitor as were other pyrimidines. However, 4-amino-5-imidazole-carboxamide, the analog with an intact imidazole ring but lacking the 2-carbon of purine provided about as good inhibition as did xanthine.

Substitution of purines at position 9 appeared to decrease their effectiveness as haptens, and the explanation for these results is not readily apparent from the structure of the antigen or of DNA. It is possible that, by using a technic as sensitive as complement-fixation, only a small fraction of the antibodies capable of reacting with purinoyl groups is being studied. A partial solution to the question of whether or not the specificity of the reaction of the antibody with DNA is an accurate reflection of the specificity of the bulk of the antibody may be obtained by a comparison of the various haptens as inhibitors in the reaction studied here with their activity as inhibitors of the precipitation reaction between the antibody and Pur-BSA. Such studies are in progress.

The availability of antibodies with purine specificity makes possible a wide variety of investigations extending the application of the tools of immunochemistry to the problems of DNA structure and denaturation. For example, the specificity of the antibody for denatured DNA may be useful to measure the "nativeness" of a DNA preparation, and may also be used to study the mode of action of various denaturing agents. Immunologic methods have already been used to study the denaturation of bacteriophage DNA by heat and formaldehyde.^{20, 21}

The complex biological activities of DNA also provide areas for investigation. Preliminary results indicate that this antipurinoyl antibody inhibits DNA-induced transformation to streptomycin resistance in pneumococci. Studies of possible effects of the antibody on cellular infection and neoplasia induced by DNA-containing viruses have been initiated.

The possibility of obtaining antibodies with specificities toward different classes of nucleic acids by conjugation of the proper haptenic derivatives to carrier proteins is a logical extension of this work.

Summary.—Antibodies with purine specificity have been elicited in rabbits immunized with purinoyl-protein conjugates. These antibodies react with heat-denatured DNA as judged by complement fixation, and this reaction can be inhibited by various purines and purine derivatives.

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INCORPORATION OF GLUTAMATE INTO RIBONUCLEIC ACID*

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Zubay recently reported in these PROCEEDINGS¹ the results of experiments designed to determine whether glutamate and glutamine are separately coded for protein synthesis. He concluded that "it would appear that an s-RNA exists for glutamine but not for glutamic acid," and that "in protein synthesis glutamine is coded for, while glutamic acid probably is not."² Zubay reported that unlabeled glutamine reduced labeling of RNA by C¹⁴-glutamate by *E. coli* extracts, whereas unlabeled glutamate did not affect incorporation of C¹⁴-glutamine; he stated that when C¹⁴-glutamate was incubated in this system, the labeled RNA gave only C¹⁴-glutamine on hydrolysis.³ It is evident that such results would be expected if the extracts used contained glutamine synthetase and little or no glutamate-activating enzyme. However, Zubay discarded the possibility that the glutamate-activating enzyme was destroyed in the preparation of the extract because the extract catalyzed incorporation of "13 out of 15 amino acids tested."

Several considerations indicate that Zubay's conclusions are not generally applicable. Evidence that glutamate is incorporated into RNA without obligatory