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

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Communicated by E. L. Tatum, March 7, 1963

Progress in a program to synthesize antigens which might be used to elicit antibodies specific for nucleic acid components has been reported.[1] In the foregoing paper, the properties and biochemical applications of an antiserum complementary to purin-6-oyl residues were outlined. It was demonstrated, using the complement-fixation reaction, that this "anti-purinoyl" antibody cross-reacted with denatured DNA, but not with native DNA, from several species.

This communication illustrates the principle of synthesis of polyfunctional antigens containing pyrimidine residues and describes conjugates which contain 5-acetyluracil and uracil residues, respectively, attached through the $N_1(N_3)$ position, to serum albumins. Some of the characteristics of rabbit antibodies to these conjugates are also delineated. The interaction between "anti-pyrimidyl" antisera and DNA can be demonstrated by complement fixation; the reaction between antibody to the 5-acetyluracil conjugate and DNA has been shown in addition by passive cutaneous anaphylaxis in the guinea pig. Possible uses for these antisera in the investigation of the biological functions of nucleic acids are discussed.

Materials and Methods.—Pyrimidine-protein conjugates: These substances were synthesized by adaptation of the elegant methods developed by Shaw and co-workers[2-4] for synthesis of pyrimidines and (N_1)-pyrimidyl amino acids, and for *N*-terminal amino acid analysis of proteins.

Polyfunctional (5-acetyluracil-1-) bovine serum albumin (5-AcU-BSA): "Ethoxyacrylamide," mp 90–91°, was synthesized from urethane, diketene, and triethylorthoformate by following the published[2, 3] procedure. To a cold solution of 2.3 gm BSA (0.03 mMole, Pentex Fraction $\overline{V}$) in 50 ml of water plus 20 ml 0.1 *N* NaOH (pH 11.2), 750 mg "ethoxyacrylamide" (3.6 mMoles) was added in small portions. After agitation for 5 min, 10 ml tetrahydrofuran and another 20 ml of 0.1 *N* NaOH were added, and the mixture was stirred at 37° for an hour. Adjustment of the pH to 10.3 by addition of another 4 ml of NaOH was followed by further stirring for 18 hr at this temperature. The mixture was then dialyzed for 24 hr against running tap water. The clear, orange solution (pH 7.2) was treated dropwise with 1 *N* HCl to bring the pH to 2.2. After standing for 10 min, the mixture was brought to pH 5.0 with the addition of 10% Na_2CO_3. The off-white, precipitated conjugate was centrifuged in the cold, and was dissolved in 25 ml 0.15 *M* $NaHCO_3$ to give a clear, rust-red solution. Addition of 27 ml 0.15 *M* HCl to pH 4.9 caused re-precipitation of the conjugate. After centrifugation in the cold, the conjugate was dissolved in 30 ml 0.1 *M* phosphate buffer, pH 7.0. It was dialyzed against cold tap water for 24 hr, and then lyophilized to yield 2.2 gm of a light tan-colored product.

The conjugate in phosphate buffer, pH 7.0, exhibited an absorption peak at 286 mμ, and at a concentration of 248 μg/ml had an O.D. of 1.38. On the assumption that an average 5-acetyl-

uracil-(N_1)-acetic acid has an ϵm of 13,500 at 286 mμ,[3] it was calculated[1] that this antigen contained approximately 26 5-acetyluracil residues per mole (Mol. wt. = 70,000).

Polyfunctional (5-acetyluracil-1-) human serum albumin (5-AcU-HSA): This conjugate was made by following exactly the foregoing protocol. It was estimated to contain 19 residues of 5-acetyluracil per mole by the spectrophotometric method.

Polyfunctional (uracil-1-) bovine serum albumin (U-BSA): One and two tenths gm of urethane was dissolved in 40 ml absolute ether, and an equimolar amount (0.31 gm) of sodium was added. After 5 hours reflux, 1.35 ml ethylpropiolate (13.5 mMole) was added, and the yellow reaction mixture was refluxed overnight.[4] The resultant dark brown solution and sediment were acidified with 75 ml of cold 1 *N* H_2SO_4, and the ether phase was washed seven times with water to remove excess acid. After drying over Na_2SO_4, the ether was flash-evaporated to give a reddish brown oil. A solution of 2.3 gm BSA in 50 ml 0.1 *N* NaOH was added to the foregoing ethoxymethyleneacetylurethane, and the reaction was stirred at 37° overnight. The conjugated protein was isolated, reprecipitated four times, and was lyophilized as described above. By analogy with reported values for 1-methyluracil[5] and for 1-methyl, 5-bromouracil,[6] it was assumed that 1-α-aminocaproyluracil would have an ϵm of *ca.* 9,500 at 280 mμ and pH 7.0. Based on this premise, it was estimated from spectrophotometric measurement that the conjugate contained 9–10 residues of uracil per mole.

Haptens: 5-acetyluracil-(1)-caproic acid—To 5 ml of 2 *N* KOH was added 4 mMole of ϵ-aminocaproic acid (524 mg). After solution, 1 gm "ethoxyacrylamide" was added. An exothermic reaction ensued and the mixture turned yellow. After heating in the waterbath 10 minutes, the reaction was chilled and 0.40 ml 77% H_2SO_4 (11 mMoles) was added. The white precipitate was centrifuged, washed with water, and was recrystallized from boiling water. After a second crystallization, the derivative (800 mg), mp 187–188° ($\lambda 1$ max 286 mμ, $\lambda 2$ max 233 mμ, in phosphate buffer, pH 7.0) was obtained. It was dried over H_2SO_4 *in vacuo* for analysis.

Analysis: Calculated for $C_{12}H_{16}O_5N_2$: C, 53.75; H, 5.99; N, 10.45. Found: C, 53.55; H, 6.17; N, 10.68.

1-Phenyluracil: Following condensation of ethyl propiolate with the sodium derivative of urethane, the reaction mixture was extracted, washed with acid, and was evaporated to an oil which was subjected to fractional distillation at reduced pressure. The fraction boiling at 110–111°/0.3 mm was collected, and crystallized in the cold. Reaction of this β-ethoxy, N-ethoxycarbonylacrylamide in alcohol with aniline at room temperature afforded prisms of β-anilino, *N*-ethoxycarbonylacrylamide (mp 148–149°); which in turn was cyclized by heating with 1 *N* NaOH to give, after acidification, 1-phenyluracil, mp 244–245° (lit. mp, 246°).[4]

5-Acetyluracil, mp 285°, was synthesized by treating "ethoxyacrylamide" with 3 *N* NH_4OH on the steam bath.[3] Other purines and pyrimidines were commercial products of standard purity. DNA preparations were denatured as outlined previously.[1]

Immunochemical procedures: These were carried out essentially as described for the purine-specific antibodies.[1] Passive cutaneous anaphylaxis experiments were performed as outlined by Ovary.[7, 8] Antisera from rabbits immunized with U-BSA were carefully absorbed with BSA until tests with anti-BSA showed that a slight excess of BSA was present in the supernatant fluid.

Complement-fixation reactions were carried out by Levine's method,[9, 10] using the "Isosaver" solution described by these authors. This reaction medium contains veronal buffer, which might conceivably have interfered by competition with certain of the pyrimidines used in the hapten-inhibition experiments. If such were the case, compounds which exhibited negative or negligible activity in this test system might actually have been capable of fitting into the antibody combining region. Thus, the order of hapten inhibitions for pyrimidines presented here is not absolute and may be subject to some future revision. It should be noted (*vide infra*), however, that so far the order of hapten inhibition is roughly the same for those compounds which have been tested with anti-5-acetyluracil antibody by both the precipitin and C′ fixing reactions. Attempts are currently being made to obviate any possible interference, by substituting "tris" for veronal in the buffer used for C′ fixation.

Results.—Quantitative precipitin data depicting the reaction between the globulin fraction prepared from an anti-5-acetyluracil serum and several antigens are shown in Figure 1. The magnitude of the precipitates obtained with 5-AcU-BSA and

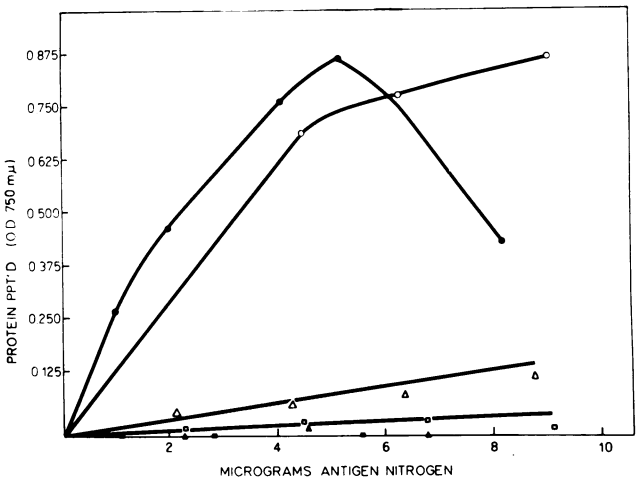

FIG. 1.—Precipitin reaction between the Na_2SO_4-precipitated γ-globulin fraction (1 ml of a 1:10 dilution) of antiserum E-22-54 and various antigens. *Ordinate:* optical density at 750 mμ after Folin-Ciocalteu reaction on precipitate.
●, 5-AcU-BSA; ○, 5-AcU-HSA; △, Pur-BSA; □, Pur-HSA; ■, BSA; ▲, HSA.

5-AcU-HSA as compared with those obtained by adding HSA, BSA, Pur-HSA, or Pur-BSA,[1] demonstrated that this serum exhibits a "pyrimidyl" specificity. The hapten-inhibition experiments in this precipitin system provide further evidence for specificity (Fig. 2). These data indicate that the effectiveness of haptens for fit into the antibody combining region, in decreasing order, is as follows: 5-acetyluracil-1-caproic acid > 5-acetyluracil > purin-6-oyl-ϵ-amino caproic acid > thymine > uracil > cytosine.

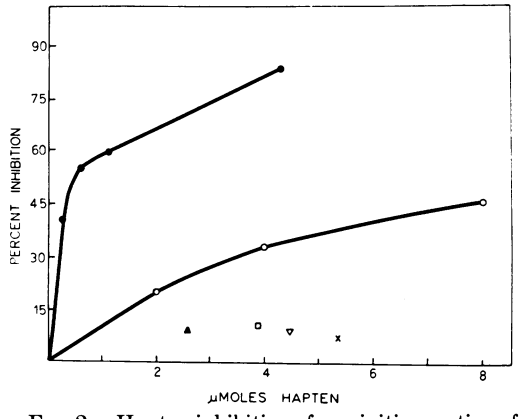

FIG. 2.—Hapten inhibition of precipitin reaction of Na_2SO_4-precipitated γ-globulin fraction (1 ml of a 1:10 dilution) of antiserum E-22-54 and its homologous antigen. ●, 5-Acetyluracil-1-caproic acid; ○, 5-Acetyluracil; ▲, N-[purin-6-oyl]-ϵ-amino-caproic acid; □, thymine; △, uracil; ×, cytosine.

The antisera to 5-acetyluracil, as was found with "anti-purinyl" sera,[1] fix complement with heat-denatured DNA and to a more limited extent with native DNA. The results of typical experiments demonstrating this reaction are shown in Figures 3 and 4. With native and thermally denatured DNA preparations from two different bacterial species it can be seen that antiserum E-22-53 fixes approximately 25% C′ with the former, as opposed to 80% with the latter, at a concentration of *ca.* 0.15 μg. These results are different from those previously obtained with the anti-purin-6-oyl antibodies,[1] which showed an absolute specificity for denatured DNA obtained from various species.

Hapten-inhibition experiments indicated that the C′ fixation reaction of antibody with thermally denatured DNA was as specific as the precipitin reaction, based upon the limited number of haptens tested (Fig. 5.) Further C′ fixation tests gave results from which were plotted values of per cent inhibitions versus log hapten concentrations. From these data extrapolations were made to determine the 50% inhibition points of the reaction between antiserum E-22-53 (1:600 dilution) and heat-denatured pneumococcal DNA. It was thus determined that the amounts of haptens required to cause 50% C′ fixation were: 5-acetyluracil-1-caproic acid (4×10^{-5} μMoles); 5-acetyluracil (2.5×10^{-2} μMoles); thymine (2×10^{-1} μMoles) and purin-6-oyl-ε-aminocaproic acid (1×10^{-1} μMoles). The following haptens were essentially inactive: uracil, cytosine, hydroxymethylcytosine, and ε-aminocaproic acid. 1-Phenyluracil was anticomplementary in this test system. These experiments further demonstrated that the specificity of the antibody is directed at least against the uracil-1-lysyl moiety of the immunizing antigen, and that the same microspectrum of antibodies is involved both in precipitin reactions and in C′ fixing cross reactions with DNA.

It was also determined that there was no demonstrable reaction between yeast RNA or rat liver RNA (concentrations up to 10 μg; native and heated) and antiserum E-22-53, again at 1:600 dilution, under conditions where pneumococcal controls (0.53 μg) gave over 90% C′ fixation.

Antisera against U-BSA, after ab-

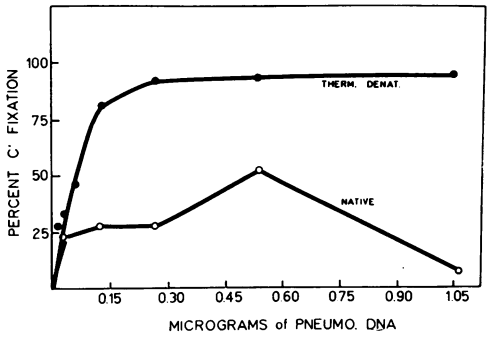

FIG. 3.—Complement fixation reaction between serum E-22-53 (dilution 1:800) and native (open circles) and thermally denatured (closed circles) pneumococcal DNA.

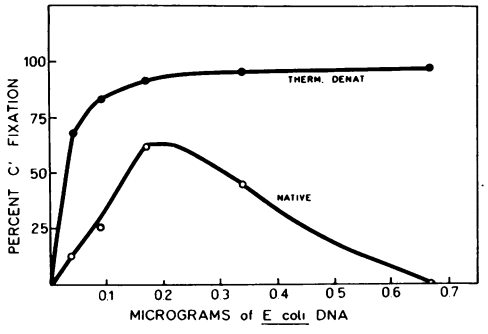

FIG. 4.—Complement fixation reaction between serum E-22-53 (dilution 1:600) and native (open circles) and thermally denatured (closed circles) *E. coli* DNA.

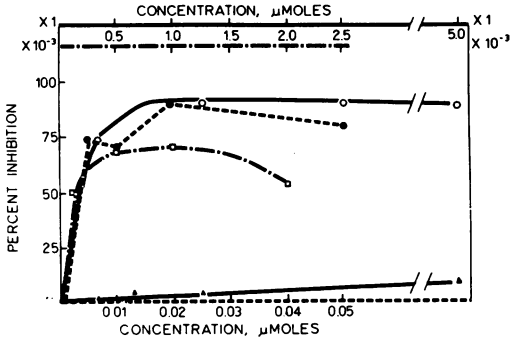

FIG. 5.—Hapten inhibition of complement fixation reaction between antiserum E-22-53 (dilution 1:600) and 0.53 μg pneumococcal thermally denatured DNA. *Ordinate:* per cent inhibition of C′ fixation. □, 5-Acetyluracil-1-caproic acid, concentrations measured on the upper ($\times 10^{-3}$) abscissa; ●, 5-Acetyluracil, concentrations measured on the lower abscissa; O, thymine; ▲, cytosine. Latter two concentrations measured against the uppermost abscissa.

sorption with BSA, gave normal precipitin curves when tested with the homologous antigen. However, the specificity of these antisera could not be demonstrated by hapten inhibition using uracil. Failure to obtain evidence for specificity in these experiments is possibly a function of the heterogeneity of size of the antibody combining regions of these particular antisera and may be comparable to differences in hapten inhibition by glucose from one antidextran serum to another, as discussed by Kabat.[11] The problem may become amenable to experimental solution when syntheses of U-HSA, for use as a cross-reacting protein, and of uracil-1-caproic acid, which hypothetically is a better hapten inhibitor, are completed. Attempts to prepare more highly substituted U-BSA are also contemplated. The absorbed anti-U-BSA antisera, even after heating to 60° and prolonged ultracentrifugation, were still anticomplementary, so that it was not possible to test their specificity by C′ fixation. Experiments with unabsorbed sera and RNA are in progress.

Evidence for a precipitin reaction between "anti-purinoyl" or "anti-pyrimidine" antisera and various species of DNA has been sought. Equivocal data for a precipitation reaction between anti-5-acetyluracil antibody and denatured DNA's has been obtained. These experiments indicate that a search for conditions for such reactions should not be abandoned.

Further evidence that the reaction of anti-purinoyl and anti-5-AcU antisera with DNA is, indeed, an antigen-antibody reaction has been obtained by the method of passive cutaneous anaphylaxis (PCA). Using the technique described by Ovary[7] and allowing a 5-hr latent period, 1:1000 dilutions of these antisera gave positive reactions with calf thymus DNA. It was also found that PCA could be demonstrated using anti-5-AcU serum and the conjugated protein 5-AcU-BSA, and evidence for cross reactivity with Pur-HSA was also obtained. In addition, the anti-purinoyl antibody was also found to react not only with Pur-HSA but also with 5-AcU-HSA. In both cases, the reaction with the homologous antigen could be obtained with at least a 10-fold higher dilution than was necessary for the cross reaction.

Discussion.—The syntheses of polyvalent antigens from serum albumins and β-ethoxy, N-ethoxycarbonylacrylamide and its α-acetyl derivative are based upon the novel method devised by Dewar and Shaw[3] for the determination of *N*-terminal residues in proteins. By using NaOH instead of triethylamine and by maintaining constant alkalinity, it is possible to substitute not only the terminal amino acid but also the ϵ-amino group of many of the lysine residues with 5-acetyluracil and uracil groups (see Fig. 6.) This conclusion is substantiated not only by the spectrophotometric analysis of the conjugates but by the finding that the most effective hapten inhibitor in the reaction between the anti-5-AcU-BSA antibody and its homologous antigen was 5-acetyluracil-1-caproic acid.

Immunization with 5-AcU-BSA elicited essentially no antibody to the protein moiety, and therefore these antisera could be used directly for precipitin determinations without prior absorption with BSA. Conversely, the antisera formed against U-BSA contained large amounts of anti-BSA, and had to be carefully absorbed with BSA before quantitative precipitin determinations were done. This difference in the production of antiprotein antibody is probably related to the difference in the number of pyrimidyl groupings introduced on the carrier protein (i.e., 26 versus 9). It should be possible to introduce a larger number of uracil residues,

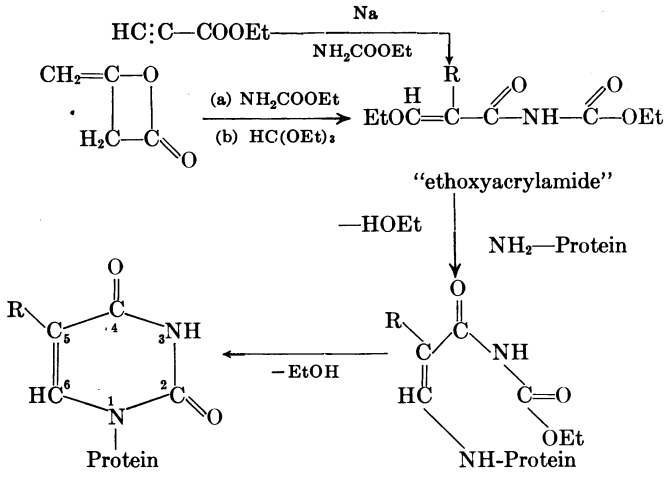

FIG. 6.—Shaw synthesis of pyrimidines as applied to the formation of polyvalent protein antigens. The $-NH_2$ groups on the protein depicted above are most probably the ϵ-amino groups of lysine residues. $R = H$, polyvalent uracil-1-protein; $R = CH_3CO$, polyvalent 5-acetyluracil-1-protein.

and indeed to extend this substitution of carrier proteins with thymine residues, by the use of such reactive intermediates as the ethoxyacryloylisocyanates, described by Warrener and Shaw.[12]

One interesting question that arises in connection with the experimental results to date using the anti-5-AcU antibody is the reason for lack of reaction with RNA. It might have been expected, since uracil is a constituent of RNA, that some cross reaction between an antibody with 5-AcU specificity and this relatively non-helical macromolecule would occur. On the other hand, since the immunizing antigen contained 5-substituted uracil moieties, and since thymine but not uracil acted as an inhibitor in C′ fixation with DNA, it can be argued that these antisera should have been more specific for cross reactions with DNA than with RNA. In this connection it should be pointed out that prior results[1] with anti-purin-6-oyl antibody showed cross reactions also only with various DNA's. Thus, in addition to consideration of the structures of the antigens in order to propose an explanation for interaction or lack of interaction between these antisera and informational macromolecules, one must also consider the relative three-dimensional conformations of RNA and of DNA and how these may relate to the exposure of certain antibody-reactive groups. Another factor bearing on this point may be the relative molecular weights of the RNA's and DNA's used in C′ fixation tests.

A striking difference between this antibody and the antibody to purin-6-oyl BSA is that the latter cross reacts only with the thermally denatured or single-stranded species of DNA. The reason for this difference is not known. Because of intrinsically better complementarity of antibody to the N_1-linked pyrimidine, as opposed to the unnatural conformation presented by antibody to the C_6-linked purine, the suggestion is offered that perhaps anti-5-AcU antibody may influence the equilibrium which possibly exists[13] between double-stranded and single-stranded DNA in solution.

Many DNA's and RNA's contain, as minor constituents, bases other than the usual four, and some of these may be characteristic for a specific biopolymer. It is even conceivable, therefore, that an antibody which reacts with a limited type of informational macromolecules may be obtained by coupling a minor base to a protein. The investigations of Levine,[9] demonstrating an antibody which reacts only with denatured DNA preparations containing glucosylated 5-hydroxymethylcytosine residues, suggest that such specificities might be approached.

Conjugated proteins have now been used to obtain antisera with two distinct specificities which react with DNA. It will be of interest to determine if this specificity is translated into differences in reactivity with DNA's of varying base composition. Such DNA's with differing GC content have been prepared from several bacterial species, and their ability to fix complement with the antisera is being studied. Should the antibodies be able to distinguish different DNA's, an immunological approach to many biological problems involving DNA would be possible.

Fluorescent and ferritin-labeled DNA specific antibodies have potential use in the investigation of viral infection, of viral-induced neoplasia, and of episomic elements. Neutralization and inhibition techniques can be used to investigate the role of DNA in transformation and other genetic phenomena. Exploratory experiments designed to test these applications for "anti-purinoyl" and "anti-pyrimidyl" antisera are in progress.

Summary.—The synthesis of polyfunctional antigens containing uracil-1 (3)- and 5-acetyluracil-1 (3)- residues is described. The latter antigen has been used to elicit specific antibodies in rabbits. Antisera thus obtained cross react with thermally denatured DNA, and to a considerably lesser extent with native DNA, as measured by complement fixation reactions. Their reaction with DNA can also be demonstrated by passive cutaneous anaphylaxis in the guinea pig. Possible uses for these antisera in the investigation of the biological functions of nucleic acids are discussed.

* Supported by funds from the National Science Foundation (NFS-14420), the U.S. Public Health Service (E-1763), and contract (266 (40)) between the Office of Naval Research and Columbia University.

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