[5] Plescia, O. J., J. Noval, N. C. Palczuk, and W. Braun, *Proc. Soc. Exptl. Biol. Med.*, **106**, 748 (1961).
[6] Palczuk, N. C., O. J. Plescia, and W. Braun, *Proc. Soc. Exptl. Biol. Med.*, **107**, 982 (1961).
[7] Deicher, H. R. G., H. R. Holman, and H. G. Kunkel, *J. Exptl. Med.*, **109**, 97 (1959).
[8] Stollar, D., L. Levine, H. I. Lehrer, and H. Van Vunakis, these PROCEEDINGS, **48**, 874 (1962).
[9] Levine, L., W. T. Murakami, H. Van Vunakis, and L. Grossman, these PROCEEDINGS, **46**, 1038 (1960).
[10] Townsend, E., W. T. Murakami, and H. Van Vunakis, *Federation Proc.*, **20**, 438 (1961).
[11] Butler, V. P., Jr., S. Beiser, B. F. Erlanger, S. W. Tanenbaum, S. Cohen, and A. Bendich, these PROCEEDINGS, **48**, 1597 (1962).
[12] Tanenbaum, S. W., and S. M. Beiser, these PROCEEDINGS, **49**, 662 (1963).
[13] Goebel, W. F., and O. T. Avery, *J. Exptl. Med.*, **54**, 437 (1931).
[14] Sueoka, N., and T. Cheng, *J. Mol. Biol.*, **4**, 161 (1962).
[15] Plescia, O. J., K. Amiraian, and M. Heidelberger, *J. Immunol.*, **78**, 147 (1957).
[16] Doty, P., J. Marmur, J. Eigner, and C. Schildkraut, these PROCEEDINGS, **46**, 461 (1960).
[17] Heidelberger, M., and F. E. Kendall, *J. Exptl. Med.*, **54**, 515 (1931).
[18] Meselson, M., F. W. Stahl, and J. Vinograd, these PROCEEDINGS, **43**, 581 (1957).

*URIDINE-SPECIFIC ANTIBODIES OBTAINED WITH SYNTHETIC ANTIGENS**

BY MICHAEL SELA, HANNA UNGAR-WARON, AND YAEL SHECHTER

SECTION OF CHEMICAL IMMUNOLOGY, THE WEIZMANN INSTITUTE OF SCIENCE, REHOVOTH, ISRAEL

Communicated by Christian B. Anfinsen, June 10, 1964

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 RNA[1] nor DNA[1, 3, 4] nor synthetic polynucleotide[5] 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 coliphage[9] 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-conjugates[18] 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

elicited, in both cases, antibodies with specificity toward uridine, and which reacted with single-stranded thymus DNA, heat-denatured *E. coli* RNA, and polyribouridylic acid.

Materials and Methods.—Nucleosides and polyadenylic acid were obtained from Sigma Chemical Company, uracil and d-ribose from Nutritional Biochemicals, and calf thymus DNA from Worthington Biochemical Corp. We are indebted to Dr. S. Ochoa for a gift of polyuridylic acid, and to Dr. U. Z. Littauer for a gift of *E. coli* RNA.

The multichain polymer multi-poly-DL-alanyl—poly-L-lysine (pAla—pLys) was prepared[19] from N-carboxy-DL-alanine anhydride and poly-L-lysine, in a residue molar ratio of Ala:Lys, 7:1. Uridine-5'-carboxylic acid and thymidine-5'-carboxylic acid were synthesized according to Moss *et al.*[21] N,N'-dicyclohexylcarbodiimide (0.5 gm, Fluka, Switzerland) was added to a mixture of pAla—pLys (1.5 gm in water) and uridine-5'-carboxylic acid (0.75 gm in dimethylformamide). The water content of the final reaction mixture was 5%. After 18 hr, it was dialyzed against distilled water (3 days), filtered, and freeze-dried. The resulting U-pAla—pLys (Fig. 1) contained 9% uridine-5'-CO-, determined from the extinction at 260 mμ (corresponding to a molar ratio of U:Ala:Lys, 1:32:4.5), and had a molecular weight of 80,000, calculated from a sedimentation coefficient of $s_{20,w}$ = 4.3 S (1% solution in 0.9% sodium chloride), a diffusion coefficient of $D_{20,w}$ = 4.7×10^{-7} cm^2 sec^{-1}, and a partial specific volume of 0.72. A thymidine derivative, T-pAla—pLys, was prepared analogously (8.5% thymidine-5'-CO-).

In order to obtain a multichain polymer that would contain both uridine and tyrosine, use was made of the observation that U-pAla—pLys still contained unreacted amino groups. It was, therefore, reacted (0.8 gm in 0.05 *M* phosphate buffer, pH 7.0) with N-carboxy-L-tyrosine anhydride (0.2 gm in dioxane); after 24 hr at 2°, the product was dialyzed against distilled water (3 days) and freeze-dried. The resulting (U,pTyr)-pAla—pLys contained 14.5% tyrosine residues (from the extinction at 293.5 mμ and pH 13, as well as ninhydrin colorimetry after hydrolysis and paper chromatography[19]). The molar ratio of U:Tyr:Ala:Lys was 1:3.2:32:4.5. The polymer had a sedimentation coefficient $s_{20,w}$ = 4.7 S.

Calf thymus DNA and *E. coli* RNA were denatured by boiling aqueous solutions for 10 min followed by rapid cooling in an ice bath. DNA was also heat-denatured in 1% formaldehyde.[22]

Immunological procedures: The polymers tested did not give any precipitate with preimmunization sera. Groups of eight rabbits were immunized (using Freund's adjuvant) as described previously.[19] All experiments were carried out with pooled antisera. Precipitin and inhibition tests were performed as described by Fuchs and Sela.[20]

Results.—The homologous precipitin reaction of the system U-pAla—pLys-

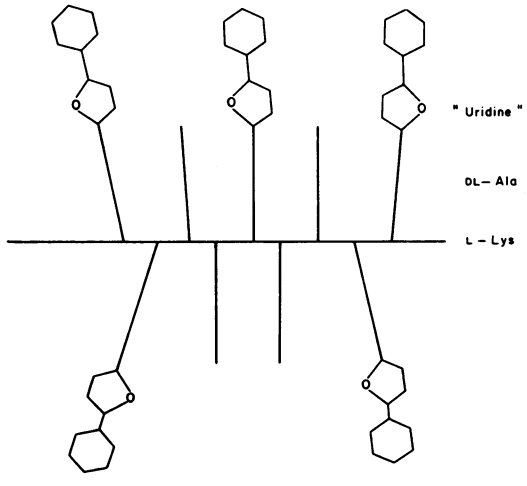

FIG. 1.—Schematic presentation of the multichain nucleoside-polypeptide conjugate U-pAla—pLys.

anti-U-pAla—pLys is shown in Figure 2. The antisera cross-precipitated partially with T-pAla—pLys, and slightly with pAla—pLys. In the last case, the maximal precipitation occurred at a much higher concentration of the polymer. The homologous reaction was also positive when checked by the passive cutaneous anaphylaxis technique.[23]

The specificity of the antibodies obtained is apparent from inhibition studies. Neither uracil (up to 5 mg/ml serum) nor d-ribose (up to 10 mg/ml) nor a mixture of the two had any inhibitory effect on the homologous precipitin reaction. On the other hand, almost total inhibition was observed with uridine, and a partial one with thymidine (Fig. 3). Cytidine, at 5 mg/ml serum, caused 20 per cent inhibition. No inhibition was found with guanosine (0.5 mg/ml) or adenosine (1 mg/ml). The last two nucleosides are not well soluble at higher concentrations. Uridine did not affect the extent of precipitation of egg albumin with antiegg albumin. Nucleoside-5′-carboxylic acids were not used in inhibition studies, as uridine-5′-carboxylic acid inhibited efficiently the homologous systems of both U-pAla—pLys and egg albumin. On the other hand, uridine-3′-phosphoric acid caused 50 per cent inhibition at 1 mg/ml antiserum, but did not inhibit at all the homologous egg albumin-antiegg albumin system.

The cross-reaction of the antiuridine antibodies with nucleic acids was also investigated. Native calf thymus DNA did not cross-precipitate with the antiserum, while heat-denatured DNA gave a typical precipitin reaction, and an even better cross-precipitation was obtained with DNA heat-denatured in the presence of formaldehyde (Fig. 4).

Neither native nor heat-denatured *E. coli* RNA cross-precipitated with the anti-

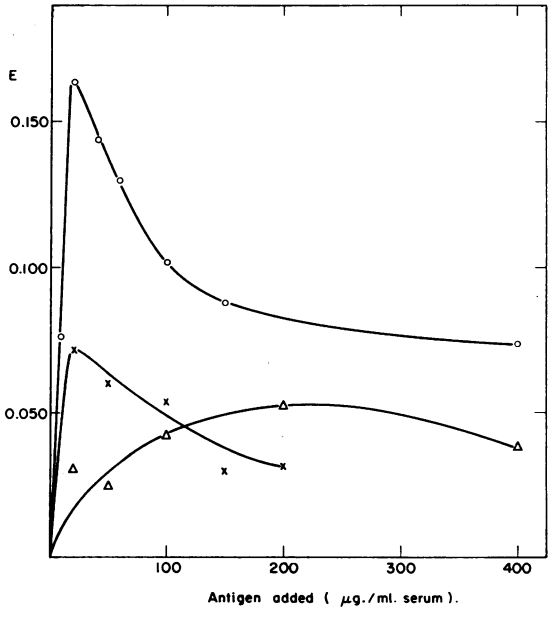

FIG. 2.—Extinction at 2800 A of solutions in 0.1 *N* sodium hydroxide of precipitates obtained by the addition to antiserum against U-pAla—pLys of: O, U-pAla—pLys; ×, T-pAla—pLys; Δ, pAla—pLys.

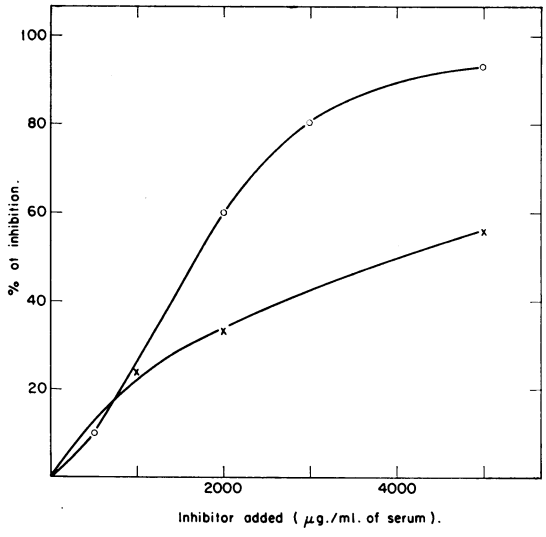

FIG. 3.—Inhibition curves of the homologous reaction of the system U-pAla—pLys and its antiserum by: O, uridine; ×, thymidine (antigen conc. 20 μg/ml serum).

uridine antiserum. This was due to the presence of RNase in the serum (rabbit serum contains RNase equivalent to approximately 0.1 μg bovine pancreatic RNase per ml[24]). The RNase action was inhibited by the addition of 200 μg of γ-globulin isolated on DEAE-cellulose[25] from an anti-RNase antiserum per 1 ml of antiuridine serum. When after 24 hr RNA was added, cross-precipitation was obtained with the heat-denatured material, but not with the native *E. coli* RNA (Fig. 5). Polyuridylic acid gave a precipitate with the antiuridine serum in the presence of anti-RNase γ-globulin, in contrast to polyadenylic acid (Fig. 5). None of the nucleic acid samples mentioned above gave, at the concentrations used, any precipitation with either normal rabbit sera or antiegg albumin sera.

In Figures 6 and 7 are shown, respectively, some precipitin and inhibition reactions of the antiserum to (U,pTyr)-pAla—pLys. In this case, beside specific antiuridine antibodies, antipolypeptide antibodies were also formed, as apparent from the incomplete inhibition of the homologous reaction by uridine (Fig. 7).

Discussion.—The experiments described show that antibodies with specificity

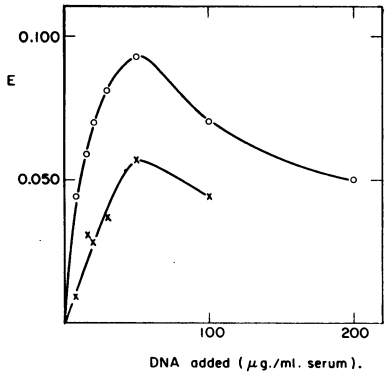

FIG. 4.—Extinction at 2800 A of solutions in 0.1 *N* sodium hydroxide of precipitates obtained by the addition to antiserum against U-pAla—pLys of: ×, heat-denatured DNA; O, DNA heat-denatured in the presence of 1% formaldehyde.

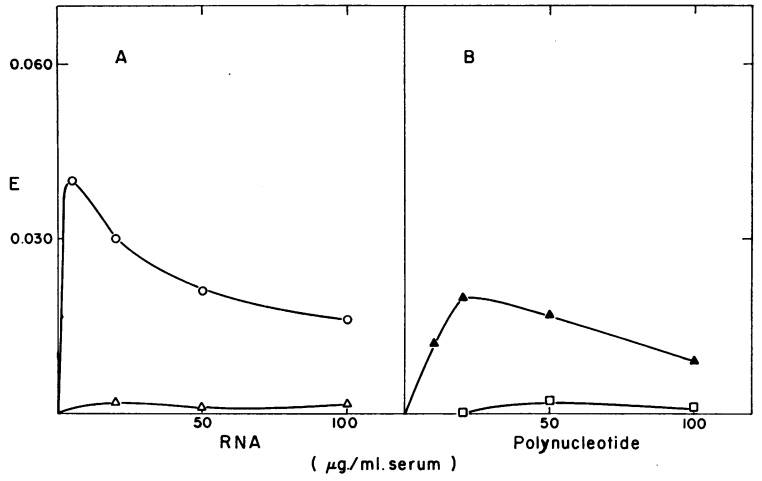

FIG. 5.—Extinction at 2800 A of solutions in 0.1 *N* sodium hydroxide of precipitates obtained by the addition to antiserum against U-pAla—pLys previously treated with 200 μg/ml serum anti-RNAase γ-globulins of: (*A*) O, heat-denatured RNA; Δ, native RNA. (*B*) ▲, polyuridylic acid; □, polyadenylic acid.

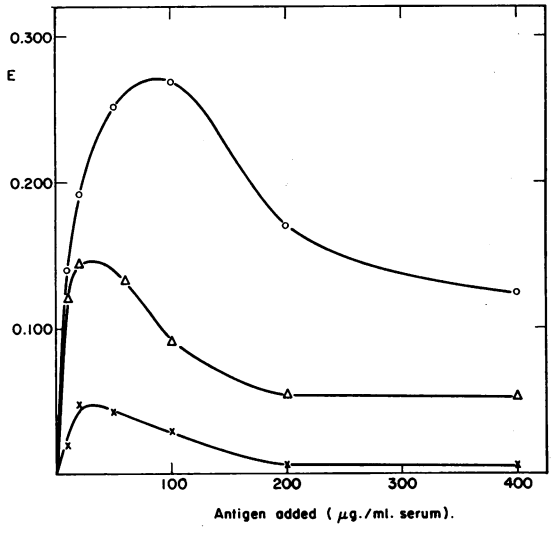

FIG. 6.—Extinction at 2800 A of solutions in 0.1 *N* sodium hydroxide of precipitates obtained by the addition to antiserum against (U, pTyr)-pAla—pLys of: O, (U,pTyr)-pAla—pLys; Δ, U-pAla—pLys; ×, pAla—pLys.

toward uridine may be obtained in rabbits upon injection of synthetic molecules in which uridine-5′-carboxylic acid is bound through an amide bond to the amino-terminal groups of poly-DL-alanyl side-chains of a multichain synthetic polypeptide. The attachment of the uridine-5′-carboxylic acid residues not only changed extensively the specificity of an antigenic synthetic polypeptide (Fig. 6), but also converted the nonantigenic[19] multichain poly-DL-alanine into an immunogen (Fig. 2), with specificity due mostly to uridine. While some anti-poly-DL-alanine was

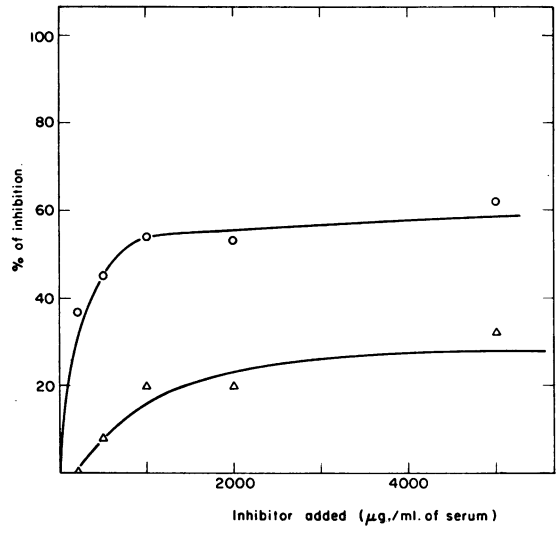

FIG. 7.—Inhibition curves of the homologous reaction of the system (U,pTyr)-pAla—pLys and its antiserum by: O, uridine; Δ, thymidine (antigen conc. 40 μg/ml serum).

formed, the antibodies in the equivalence zone (*ca.* 120 μg/ml serum) consisted almost entirely of antiuridine, as apparent from inhibition studies (Fig. 3).

The antigenic specificity of U-pAla—pLys is due to the nucleoside unit as a whole, since uracil and/or ribose did not inhibit the homologous reaction, while uridine and uridylic acid were efficient inhibitors. The inhibition with thymidine demonstrates that neither the methyl group in position 5 of the pyrimidine ring nor the hydroxyl in position 2 of the ribose are of paramount importance in defining the combining sites of the antibodies formed. The lack of inhibition by the purine nucleosides stresses the role of the pyrimidine ring in the specificity. Nucleoside-5′-carboxylic acids inhibited efficiently both the uridine-specific homologous system and the egg albumin-antiegg albumin system. This is in agreement with a previous report[26] that heterocyclic carboxylic acids inhibit precipitin reactions.

The specificity toward uridine of the antibodies obtained is also apparent from the cross-precipitation with polyuridylic acid, but not with polyadenylic acid. In order to obtain precipitin reactions, RNase activity of the sera[24] had to be neutralized with antibodies against the enzyme. By this technique the reaction of heat-denatured *E. coli* RNA, but not of native RNA, with antibodies toward uridine could also be demonstrated (Fig. 5). The lack of interaction of antibodies to purinoyl and uracil conjugates of proteins with RNA[17, 18] may thus have been due to the presence of RNase in the sera tested. In studies of antiribosomal antibodies, Barbu and Dandeu[27] have used bentonite to remove RNase from the sera. The cross-precipitations, reported here, with RNA and polyuridylic acid may be incomplete, as it is possible that not all RNase activity was removed from the sera tested.

Even though DNA does not contain uridine, a single-stranded calf thymus DNA preparation (but not the double-stranded DNA) cross-precipitated with the test antiserum, probably because of its thymidine content. This observation is similar to the report of cross-reaction of antibodies against a uracil-protein conjugate with

single-stranded DNA.[18] The lack of reactivity of double-stranded DNA with the antiuridine sera is in agreement with previous reports[9, 11, 17, 18] of the preferential reactivity of antisera specific toward nucleic acid or their components, with single-stranded rather than double-stranded DNA. Apparently, the antigenic determinants are not available in the highly ordered structures of double-stranded DNA or the high molecular weight *E. coli* RNA,[28] for the reaction with nucleoside-specific antibodies. While the heat-denaturation of *E. coli* RNA is a reversible phenomenon,[28] the cross-reaction with the antiuridine serum suggests that the renaturation upon quick cooling is not complete. On the other hand, it was reported recently that polyuridylic acid, which cross-reacted with antiuridine, is a randomly coiled polynucleotide.[29]

The induction with fully synthetic antigens of biosynthesis of antibodies specific toward uridine permits a systematic investigation of the role of various molecular parameters in the immunogenicity and antigenic specificity of nucleoside-containing synthetic macromolecules. Preliminary results indicate that antibodies with specificity toward thymidine were obtained upon injection into rabbits of T-pAla—pLys, and the synthesis and immunochemical characterization of other potential nucleoside-specific immunogens is in progress. The availability of antibodies with specificity toward nucleosides and nucleotides should be helpful in investigations of the manifold chemical, physical, and biological properties of nucleic acids.

Summary.—Completely synthetic antigens obtained by the chemical binding of uridine-5′-carboxylic acid to synthetic multichain polypeptides have elicited, in rabbits, antibodies with specificity toward uridine, as apparent from cross-precipitation and inhibition reactions. The attachment of the uridine derivative to a nonantigenic macromolecule converted it into an immunogen. The antibodies formed reacted with polyuridylic acid, heat-denatured RNA and DNA, but not with polyadenylic acid, native *E. coli* RNA, or double-stranded calf thymus DNA.

* This investigation was supported in part by the USPHS research grant AI-04715 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

[1] Pasternak, G., F. Hoffman, H. Bielka, and L. Venker, *Pathol. Microbiol.*, **23**, 392 (1960).

[2] Lackman, D., S. Mudd, M. G. Sevag, J. Smolens, and M. Wiener, *J. Immunol.*, **40**, 1 (1941).

[3] Colter, J. S., *Nature*, **190**, 550 (1961).

[4] Yachnin, S., *J. Immunol.*, **91**, 529 (1963).

[5] Yachnin, S., *Nature*, **195**, 1319 (1962).

[6] Phillips, J. H., W. Braun, and O. J. Plescia, *Nature*, **181**, 573 (1958).

[7] Olitzki, A. L., *Brit. J. Exptl. Pathol.*, **41**, 623 (1960).

[8] Bigley, N. J., M. C. Dodd, and V. B. Geyer, *J. Immunol.*, **90**, 416 (1963).

[9] Levine, L., W. T. Murakami, H. Van Vunakis, and L. Grossman, these PROCEEDINGS, **46**, 1038 (1960).

[10] Deicker, H. R. G., H. R. Holman, and H. G. Kunkel, *J. Exptl. Med.*, **109**, 97 (1959).

[11] Stollar, D., and L. Levine, *J. Immunol.*, **87**, 477 (1961).

[12] Stollar, D., L. Levine, H. J. Lehrer, and H. Van Vunakis, these PROCEEDINGS, **48**, 874 (1962).

[13] Murakami, W. T., H. Van Vunakis, H. J. Lehrer, and L. Levine, *J. Immunol.*, **89**, 116 (1962).

[14] Barbu, E., J. Panijel, and G. Quash, *Ann. Inst. Pasteur*, **100**, 725 (1961).

[15] Barbu, E., G. Quash, and J.-P. Dandeu, *Ann. Inst. Pasteur*, **105**, 849 (1963).

[16] Panijel, J., *Compt. Rend. Acad. Sci.*, **256**, 4540 (1963).

[17] Butler, V. P., S. M. Beiser, B. F. Erlanger, S. W. Tanenbaum, S. Cohen, and A. Bendich, these PROCEEDINGS, **48**, 1597 (1962).

[18] Tanenbaum, S. W., and S. M. Beiser, these PROCEEDINGS, **49**, 662 (1963).

[19] Sela, M., S. Fuchs, and R. Arnon, *Biochem. J.*, **85**, 223 (1962).
[20] Fuchs, S., and M. Sela, *Biochem. J.*, **87**, 70 (1963).
[21] Moss, G. P., C. B. Reese, K. Schofield, R. Shapiro, and Lord Todd, *J. Chem. Soc.*, 1149 (1963).
[22] Stollar, D., and L. Grossman, *J. Mol. Biol.*, **4**, 31 (1962).
[23] Ovary, Z., *Progr. Allergy*, **5**, 459 (1958).
[24] Philipson, L., and M. Kaufman, *Biochim. Biophys. Acta*, **80**, 151 (1964).
[25] Levy, H. B., and H. A. Sober, *Proc. Soc. Exptl. Biol. Med.*, **103**, 250 (1960).
[26] Kleinschmidt, W. J., and P. D. Boyer, *J. Immunol.*, **69**, 247 (1952).
[27] Barbu, E., and J.-P. Dandeu, *Compt. Rend. Acad. Sci.*, **256**, 1166 (1963).
[28] Cox, R. A., and U. Z. Littauer, *Biochim. Biophys. Acta*, **61**, 197 (1962).
[29] Richards, E. G., P. Flessel, and J. R. Fresco, *Biopolymers*, **1**, 431 (1963).

*THE ENZYMATIC METHYLATION OF RNA AND DNA, VIII. EFFECTS OF BACTERIOPHAGE INFECTION ON THE ACTIVITY OF THE METHYLATING ENZYMES**

BY MARVIN GOLD, RUDOLF HAUSMANN,† UMADAS MAITRA,‡ AND JERARD HURWITZ§

DEPARTMENT OF MOLECULAR BIOLOGY, ALBERT EINSTEIN COLLEGE OF MEDICINE

Communicated by B. L. Horecker, June 11, 1964

We, as well as others, have previously reported on the presence in *Escherichia coli* of several enzymes which catalyze the transfer of methyl groups from *S*-adenosylmethionine to sRNA,[2-4] ribosomal RNA,[5, 6] and DNA.[4, 7] Although the biological function of the methylated bases which these enzymes produce is still obscure, the species and strain specificity of the methylation reactions suggest that they provide a basis for a recognition mechanism. The virulent bacteriophage-host cell system is an example of a phenomenon involving recognition by the host of a foreign nucleic acid; in some instances, phage DNA is rapidly synthesized while the host DNA is rapidly degraded. If methylated bases are involved in controlling such a recognition mechanism, then a study of the methylated base content of DNA's of various bacteriophages grown in different hosts might provide a clue as to the biological function of the methylating enzymes. In order to establish a suitable system for further investigation, we have studied the effects of phage infection on the activities of the various methylating enzymes in the host cell. This communication summarizes such studies. It has been found that while the RNA methylases are apparently unchanged, DNA methylation activity increases markedly after infection with T2. In contrast, T3 infection induces an enzyme which cleaves *S*-adenosylmethionine to thiomethyladenosine and homoserine.

Materials and Methods.—(a) *Bacteria and phage:* *E. coli* B, used for infection experiments with the T series of bacteriophage was a strain obtained from Dr. C. Bresch of the University of Cologne. *E. coli* K12 strain W3104 and its lysogenic variant, W3104 (λ) were obtained from Dr. A. D. Kaiser of Stanford University and were used for studies on the effects of infection with or induction of bacteriophage λ, respectively.

Phages T1, T2, T4, T5, and T6 were generously provided from the stocks of the Department of Microbiology, New York University School of Medicine. Phages T3 and T7 were gifts of Drs. R. Latarjet of the Pasteur Institute and C. Bresch, respectively. Bacteriophage λ stocks were prepared from a single plaque isolated after plating the supernatant medium of an *E. coli* K12 (λ) culture on *E. coli* K12.