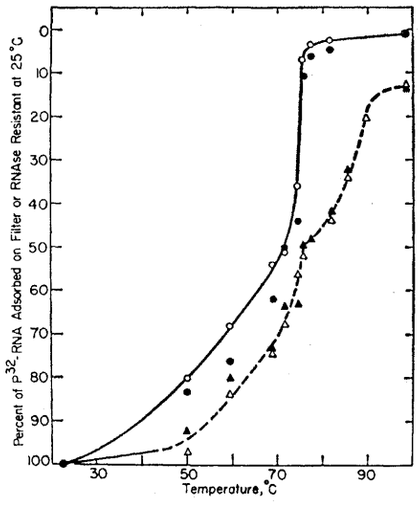

Fig. 4. Thermal dissociation of bacteriophage alpha DNA-RNA hybrid and cRNA, in 0.01*M* PO_4, *p*H 7. The solid line shows the percentage of P^{32}-RNA absorbed by the filter after heating to a given temperature and then cooling rapidly, in the absence (○), and presence (●) of 25 μg of AMD per milliliter. The dashed line shows the percentage of ribonucelase resistant P^{52}-RNA after heating and cooling in the absence (△) and presence (▲) of 25 μg of AMD per milliter.

dium chloride and then against 0.15*M* sodium chloride and 0.015*M* sodium citrate. In the latter solvent, hybrid was made by mixing 26 μg RNA and 160 μg DNA from bacteriophage alpha, and heating for 5 minutes at 100°C; the reaction mixture was rapidly cooled and then annealed at 52°C for 4 hours. Approximately 65 percent of the RNA in the mixture was resistant to ribonuclease; of this resistant fraction, two-thirds was RNA-DNA hybrid and one-third was ordered (helical) RNA. Thermal dissociation of these RNA complexes was measured in two ways. Samples of the annealed mixture were diluted 40-fold by a solution of 0.01*M* phosphate, *p*H 7, with and without AMD, heated to a given temperature for 10 minutes, quenched in ice, and then subjected to the following tests. In the first, the sample is made 0.01*M* in Mg^{++}; then 1 μg of pancreatic ribonuclease per milliliter is added, and the whole is incubated for 10 minutes at 37°C. The sample is chilled, precipitated with 5 percent trichloroacetic acid (TCA), collected on a Millipore filter, and counted. The radioactivity (count/min) in the acid-precipitable fraction represents UMP^{32} (uridylic acid) in the ribonuclease-resistant RNA, which includes both RNA-DNA hybrid and ordered RNA. A second sample, after quenching, is diluted further with 0.5*M* potassium chloride and passed through a coarse paper filter, according to a procedure developed by Hall and Nygaard (*20*). The filter selectively retains RNA-DNA hybrid, which is measured by the radioactivity due to the UMP^{32} in the RNA.

The result of this experiment is shown in Fig. 4. The percentage of P^{32}-RNA trapped by the filter, and consequently a measure of hybrid dissociation, is not affected by AMD. The graph of the percentage of P^{32}-RNA which is ribonuclease-resistant reveals two dissociations, one occurring at the temperature at which hybrid dissociates, and a second about 10°C higher. This second dissociation corresponds to the temperature of irreversible dissociation of cRNA in this solvent. Again, neither dissociation is affected by AMD, as measured by properties of the RNA molecules themselves. The result with DNA-RNA hybrid is of special significance, since it indicates that the DNA strand in hybrid is in a conformation that cannot bind AMD. It is already known that one helical conformation does not bind AMD, the A helix, of which sRNA is a representative. It is tempting to suggest that DNA-RNA hybrid, as well as ordered complementary RNA, are in the A-helical conformation.

ROBERT HASELKORN
Committee on Biophysics, University of Chicago, Chicago, Illinois

References and Notes

1. P. Doty, H. Boedtker, J. Fresco, R. Haselkorn, M. Litt, *Proc. Natl. Acad. Sci. U.S.* **45**, 482 (1959).
2. R. Haselkorn and P. Doty, *J. Biol. Chem.* **236**, 2738 (1961).
3. G. Kalnitsky, J. P. Hummel, H. Resnick, J. R. Carter, L. B. Barnett, C. Dierkes, *Ann. N.Y. Acad. Sci.* **81**, 542 (1959).
4. E. P. Geiduschek, J. Moohr, S. B. Weiss, *Proc. Natl. Acad. Sci. U.S.* **48**, 1078 (1962).
5. M. Grunberg-Manago, *J. Mol. Biol.* **1**, 240 (1959).
6. S. N. Timasheff, J. Witz, V. Luzzati, *Biophys. J.* **1**, 525 (1961).
7. J. M. Kirk, *Biochim. Biophys. Acta* **42**, 167 (1960).
8. H. M. Rauen, H. Kersten, W. Kersten, *Z. Physiol. Chem.* **321**, 139 (1960).
9. I. H. Goldberg, M. Rabinowitz, E. Reich, *Proc. Natl. Acad. Sci. U.S.* **48**, 2094 (1962). J. Hurwitz, J. J. Furth, M. Malamy, M. Alexander, *ibid.*, p. 1222.
10. W. Kersten, *Biochim. Biophys. Acta* **47**, 610 (1961).
11. L. D. Hamilton, W. Fuller, E. Reich, *Nature* **198**, 538 (1963).
12. I. H. Goldberg, M. Rabinowitz, E. Reich, *Proc. Natl. Acad. Sci. U.S.* **49**, 226 (1963).
13. E. Reich, I. H. Goldberg, M. Rabinowitz, *Nature* **196**, 743 (1962).
14. E. Reich, *Science*, this issue.
15. E. P. Geiduschek, *J. Mol. Biol.* **4**, 467 (1962).
16. M. Spencer, W. Fuller, M. H. F. Wilkins, G. L. Brown, *Nature* **194**, 1014 (1962).
17. T. Nakamoto, F. Fox, S. B. Weiss, *J. Boil. Chem.*, in press.
18. E. P. Geiduschek and S. B. Weiss, personal communication.
19. E. Reich, R. M. Franklin, A. J. Shatkin, E. L. Tatum, *Science* **134**, 556 (1961); ———, *Proc. Natl. Acad. Sci. U.S.* **48**, 1238 (1962).
20. A. Nygaard and B. D. Hall, *Biochem. Biophys. Res. Commun.* **12**, 98 (1963).
21. Supported by grant E-4448 from the U.S. Public Health Service. Samples of bacteriophage DNA were provided by M. Stodolsky and G. Tocchini-Valentini; T7 cRNA by E. P. Geiduschek and S. B. Weiss; RNA polymerase and UTP^{32} by S. B. Weiss, W. Robinson, and F. Fox; and actinomycin D by H. B. Woodruff of Merck & Co. I am grateful to E. P. Geiduschek for his helpful suggestions, W. Fuller for a description of the DNA-AMD model prior to publication, and to L. Johnson for expert technical assistance.

15 November 1963

Actinomycin: Correlation of Structure and Function of Its Complexes with Purines and DNA

Abstract. *Actinomycin inhibits strand separation of helical DNA at concentrations corresponding to those which inhibit DNA polymerase activity in vitro. This report proposes first that actinomycin affects DNA polymerase indirectly by way of the changed physical properties of the template and that it functions in the major groove of helical DNA; second, that RNA polymerase functioning in the minor groove is subject to direct steric inhibition by bound actinomycin; and third, that the reaction of actinomycin with free purines is unrelated to its reaction with DNA.*

Actinomycin (AM) (Fig. 1) is a bacteriostatic, tumor-inhibiting chromopeptide antibiotic which inhibits the syntheses of nucleic acids by intact organisms and by enzyme preparations. These effects are of interest in part because they are useful in the study of gene action and related problems, but perhaps more because of the selectivity and specificity of action of this unusual compound. Low concentrations of AM selectively suppress cellular (*1*, *2*) and enzymic DNA-directed RNA synthesis (*3*, *4*, *5*), DNA synthesis being affected only at much higher concentrations (*1*, *2*, *5*). The inhibition of RNA

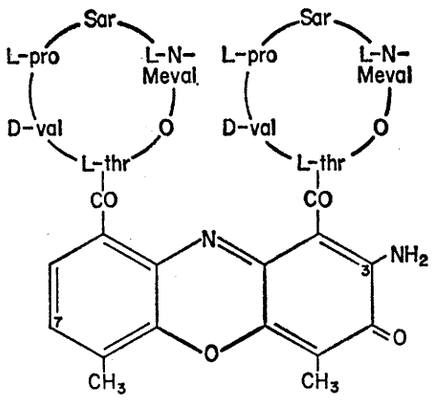

Fig. 1. Structure of actinomycin C_1 (D) (*11*); L-*thr*, L-threonine; D-*val*, D-valine; L-*pro*, L-proline; *Sar*, sarcosine; and L-*N-meval*, L-*N*-methylvaline.

synthesis requires the binding of AM by DNA (*6*) and it is absolutely dependent on guanine-containing binding sites in DNA (*7, 8*). Thus the elucidation of the reaction between AM and DNA may provide insight into the nature of and the differences between the polymerizations leading to the formation of RNA and DNA, respectively, and may perhaps lead to some concrete models for the physiological regulation of template activity.

This report presents some findings pertaining to the reaction of AM with DNA, particularly in relation to a model recently proposed for the structure of AM-DNA complexes (*9*). According to this model, the binding of AM to DNA depends on specific hydrogen bond formation, a mechanism first considered by Hartman (*4*).

Many changes in different portions of the AM molecule may affect its inhibitory properties to a greater or lesser degree (*4, 6, 10, 11*). However, the integrity of several characteristic functional groups is indispensable for maintenance of its biological activity and any hypothesis which proposes to explain the reaction of AM with DNA must take these into account. These functional groups are: (i) The free chromophore amino group. Only one of the hydrogens of this group may be substituted, and then only by a methyl group—even this change is associated with 90 percent loss of activity (*6, 10*). (ii) The unreduced quinoidal ring system (*12*). Reduction of the quinone destroys the planarity of the ring system and converts the quinoidal oxygen, an effective potential acceptor of hydrogen for hydrogen bonding, to a phenolic oxygen. (iii) The lactone rings. These assure a compact form and definite spatial orientation of the peptide-chain constituents. The dicarboxylic acid which results on hydrolysis of the lactones and whose peptide chains presumably have a flexible, random structure, is the only AM derivative which has not been crystallized (*13*); it is also devoid of biological activity (*10*) and cannot form complexes with DNA (*1, 10, 12*). These facts suggest that some AM structures which participate in the binding reaction with DNA may have a reactive configuration imposed on them by the lactones.

The possibility that AM might react with constituents of nucleic acids as well as with DNA itself was first explored by Kersten (*14*). He found that the spectral changes produced in AM solutions by DNA were also produced by several purine compounds in the following decreasing order of effectiveness: deoxyguanosine) guanosine) adenine, AMP, ADP, ATP, adenosine, and deoxyadenosine which reacted equally. Inosine, xanthosine, and normal pyrimidine bases and nucleosides were inactive in this test. Since deoxyguanosine was the most efficient of the compounds tested, Kersten concluded that AM reacts in some way with the guanine in DNA. Despite the proven validity of this conclusion, it appears unlikely that the mechanism of the model reaction is closely related to that of the AM-DNA complex itself for the following reasons. (i) Free deoxyguanosine is 10 to 20 times less effective in causing the characteristic spectral changes in AM solutions than is the deoxyguanosine in DNA. (ii) Although deoxyguanosine was the most efficient model compound of those tested, guanosine and adenine compounds showed appreciable activity; on this basis the adenine in DNA, as well as that in RNA, and the guanine in RNA would be expected to react with AM. However, neither the adenine in DNA (*7, 8*) nor RNA (*9*) can make complexes with AM. Therefore, the structural features in DNA which account for its complex-forming capacity probably cannot be deduced from a study of the model reaction.

To characterize this model reaction more fully, the reactivity with AM of a larger number of purines, their analogs, and derivatives has been investigated. The compounds tested represent structural variations affecting nearly all positions in the purine ring system. With the exception of unsubstituted free purine, which is inactive, all the compounds produced spectral differences in AM solutions closely resembling those caused by DNA and deoxyguanosine, although quantitative differences in reactivity are apparent. From the data shown in Fig. 2, no single structural feature of the purine ring appears indispensable for the reaction of the free compounds with AM. This general lack of specific structural prerequisites for the reaction of free purines and their nucleosides is in marked contrast to the reaction of nucleic acids, where only the guanine in DNA can react with AM.

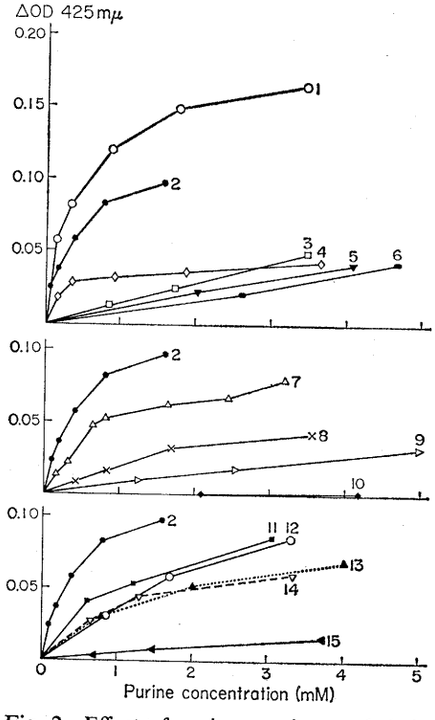

Fig. 2. Effect of various purines and their derivatives on the spectral properties of actinomycin. Control experiments had shown that the difference spectra produced by all active substances were qualitatively identical. The maximum difference is the decrease in absorption seen near 425 mμ. All measurements were made in 0.01*M* tris-HCl, *pH* 7.4, containing 0.01*M* NaCl. The effect of deoxyguanosine (*curve 2*) is charted in each figure for reference. Other curves are as follows: *Curve 1*, 6-thiodeoxyguanosine; *curve 3*, 2-fluoroadenosine; *curve 4*, tubercidin; *curve 5*, 5-aminobenzimidazole; *curve 6*, benzimidazole; *curve 7*, 2,6-diaminopurine; *curve 8*, adenosine; *curve 9*, 2-aminopurine; *curve 10*, purine; *curve 11*, 6-dimethylaminopurine; *curve 12*, puromycin aminonucleoside; *curve 13*, aminopyrazolopyrimidine deoxyriboside; *curve 14*, 6-methylaminopurine; and *curve 15*, 6-methylpurine.

Since the structure of helical DNA is known in detail (*15*), the arrangement of the portions of purines which might be available for reaction with AM molecules is readily defined. The nonspecific reactivity pattern of free purines shows no correlation with the accessible functional groups of DNA-purines. Therefore, the model reaction does not furnish insight into the reason why helical DNA forms complexes with AM, and, from the standpoint of the purine bases, the model reaction appears to have little in common with that occurring between AM and DNA.

The loose specificity of the model reaction is not restricted to purines but it is seen also in the behavior of several actinomycins. This is well illustrated by the behavior of *N*-dimethylene actinomycin, a derivative in which a cyclic dimethylene structure replaces the two hydrogens of the naturally occurring free amino group. This compound does not inhibit growing *Bacillus subtilis* (*10*) and L-cells (*12*) nor does it inhibit the formation of RNA by RNA-polymerase in the reaction in which calf thymus DNA is the primer (*12*).

As shown in Fig. 3, the spectrum in the visible region of *N*-dimethylene actinomycin is unchanged in the presence of DNA; and precipitation of DNA in the presence of this derivative leads to the formation of colorless fibers (*12*) rather than the yellow-orange ones produced in the presence of biologically active actinomycins (*9*). In contrast to its inertness with DNA, *N*-dimethylene actinomycin is very active in the model reaction; as observed also by Müller (*16*), its spectrum changes markedly in the presence of deoxyguanosine, yielding the difference spectrum shown in Fig. 3. This difference spectrum cannot be compared with that observed in the case of biologically active compounds since the spectra of the parent compounds differ; however, the direction and magnitude of the spectral changes shown in Fig. 3 resemble those of the parent AM with DNA or model compounds—depression and shift of the maximum to longer wavelength. *N*-Dimethylene actinomycin reacts also in a similar way with other purine compounds and yields spectral shifts qualitatively identical with those produced by deoxyguanosine. The order of reactivity of these is: deoxyguanosine → diaminopurine → guanosine → adenosine → benzimidazole (*12*). Thus

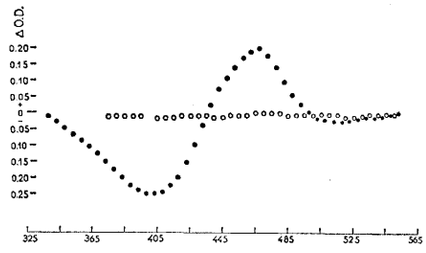

Fig. 3. Difference spectrum of *N*-dimethylene actinomycin C_3 (63.5 mμmole/ml) in the presence of calf thymus DNA (167 mμmole/ml), open circles, and in the presence of deoxyguanosine (3.65 m*M*), closed circles. The solvent was 0.01*M* tris-HCl in 0.01*M* NaCl, *p*H 7.4.

an AM derivative devoid of biological activity and incapable of forming complexes with DNA interacts strongly in solution with the model compounds. It has been shown that biological activity of different actinomycins against intact cells and organisms can be correlated well with the capacity to inhibit enzymic DNA-dependent synthesis of RNA and to form complexes with DNA (*6*). No such correlation exists for the model reaction. Thus the mechanism of the reaction of AM with model compounds differs from that of the AM-DNA reaction, and DNA provides something more than a simple reactive purine nucleoside. It seems reasonable to assume that the three-dimensional structure of DNA furnishes a specific, oriented array of functional groups which together account for the reaction with AM.

The model reaction is of interest even though it provides no solid clue to the nature of the AM-DNA complex, since the complexes formed by

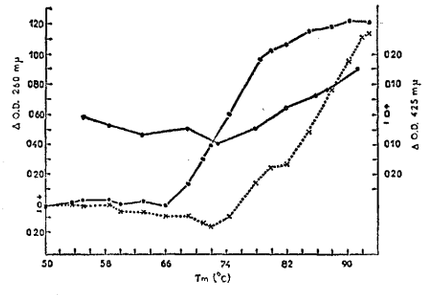

Fig. 4. Heat denaturation of calf thymus DNA with actinomycin C_1 (crosses) and without (dots) actinomycin C_1 (20.9 mμmole/ml). The solvent was 0.01*M* tris-HCl in 0.01*M* NaCl, *p*H 7.4. The concentration of DNA-P was 24.4 mμmole/ml. The open circles show the optical density at 425 mμ. Increase at 425 mμ reflects decreased binding of AM to DNA.

AM with purines show varying and occasionally appreciable stability to heat (*12*) and they may, therefore, participate in the formation of macromolecular structures analogous to those occurring in solutions of guanylic acid (*17*). These complexes, like AM-DNA complexes (*4*), are also dissociated by urea (*12*).

When numerous AM molecules become attached to a DNA helix, changes in physical properties of the helix might be expected and have been observed. For example, the viscosity of DNA solutions is increased somewhat in the presence of AM (*16, 18*), though this increase is considerably less than in the case of proflavine (*16, 18, 19*). In addition, isolated lampbrush chromosomes lose their flexibility, becoming rigid and rod-like, and their characteristic loops shrink rapidly when exposed to the antibiotic (*20*).

For reasons independently deduced and set forth in the accompanying paper by Haselkorn (*21*), it appeared desirable to determine whether AM might increase the resistance of DNA to the denaturing action of heat. As seen in Fig. 4, the "melting" temperature of a sample of calf thymus DNA is raised substantially by AM. Figure 4 also shows the effect of temperature on the binding of AM to DNA. It has been demonstrated recently that the AM-DNA complex can be dissociated at elevated temperatures (*8*). The data in Fig. 4 show that the dissociation of bound AM (reflected by increased absorption at 425 mμ) coincides with the denaturation of the DNA itself. The T_m (transition temperature) of calf thymus DNA in the ionic environment selected for the experiment shown in Fig. 4 was 74.4°C, and this was raised by AM to 86°C. Over a range of NaCl concentrations corresponding to T_m of 54° to 92°C for control samples, the addition of AM always increased the value of T_m; however, the increment in T_m for a given ratio of AM to DNA-P (phosphorus in DNA) is less at high salt concentrations (for example, when the T_m was 92°C the increase due to AM was 7°C) than at ionic strengths giving T_m in the range of 55° to 80°C (raised 12° to 15°C by AM). At all salt concentrations tested, AM remained bound to DNA (judging by difference absorption at 425 mμ) until the DNA began to undergo denaturation. Therefore, the forces which bind AM to native DNA

are stronger than those which act to maintain the DNA structure itself. Furthermore, within the limits of the experimental conditions tested, AM will be bound to native DNA at any temperature, but not to single-stranded DNA. Therefore, the presence of deoxyguanosine in a polynucleotide is not sufficient for AM binding, as is also suggested by the elegant finding of Haselkorn (*21*) that DNA-RNA hybrids probably do not form complexes with AM. Thus several lines of evidence support the conclusion that AM binding to DNA requires, in addition to deoxyguanosine, a helical structure in the B conformation (*9*).

It appears likely that the previously reported (*7, 8*) reaction of AM with denatured and single-stranded DNA's (though less than native DNA) reflects the AM binding of residual helical regions. It might be anticipated that the AM-binding capacity of DNA from bacteriophage ϕX-174 would be very low or nonexistent if the antibiotic were added under conditions at which (*22*) no ordered structure remains. If added at room temperature, such complexes that occur would have the additional effect of stabilizing helical segments so that dissociation of AM on subsequent heating would be observed only at temperatures well above those which normally produce complete randomness of DNA structure. Current data are insufficient to determine whether AM-binding capacity might be a valid measure of the helical content of DNA preparations.

The increase in T_m is a function of the ratio of AM to DNA-P (Fig. 5), although as noted earlier, the absolute value of the increase in T_m due to AM binding is less at high ionic strength.

The stabilizing effect of AM is dependent on its specific binding to DNA. Thus the T_m of the deoxyadenylic-deoxythymidylic copolymer (*23*), which does not bind AM (*7, 8*), is unaffected by the antibiotic; and AM derivatives whose capacity to form complexes with DNA is partially or totally abolished show a corresponding decrease in the capacity to raise the T_m of DNA (*24*).

A comparison of the thermostability of the AM-DNA complexes with that of AM-deoxyguanosine complexes illustrates another aspect of the difference between them. The persistence of the AM-DNA association was seen to depend on the retention of the DNA double-strandedness, and thermosensitivity of the complex appeared abruptly at the onset of DNA-denaturation. In contrast, the AM-deoxyguanosine complex exhibits a continuous thermolability beginning a few degrees above room temperature (see Fig. 6); this has also been observed by Kersten and Kersten (*25*).

The AM-inhibition of DNA-directed RNA synthesis observed in intact cells appears to be due to interference with the function of the enzyme RNA-polymerase (*3–8*). The DNA synthesis of intact cells is also suppressed by AM, but less readily than RNA synthesis (*1, 2*) is suppressed; correspondingly the activity in vitro of DNA-polymerase is considerably less sensitive to AM than that of RNA-polymerase (*1, 5*). The results shown in Figs. 4 and 5 suggested a possible explanation of the differential AM sensitivity of the two nucleic acid polymerizing reactions. The inhibition of RNA-polymerase by AM could reflect a direct steric interference with enzyme function by AM molecules bound to portions of the DNA template normally participating in RNA formation; on the other hand the inhibition of DNA-polymerase might be attributable to the altered physical properties of the template resulting from the binding of a larger quantity of AM, rather than attributable to a steric effect.

The results of an experiment which

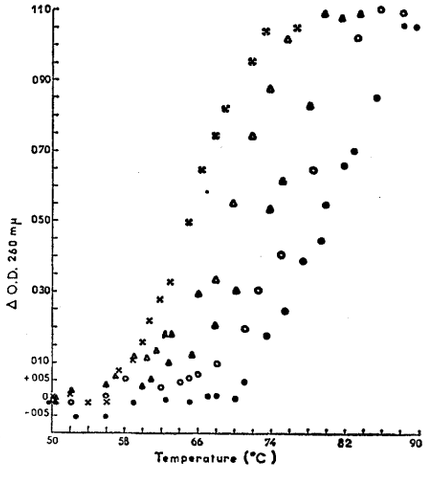

Fig. 5. Heat denaturation of transforming pneumococcal DNA. The solvent was 0.001*M* tris-HCl in 0.005*M* NaCl, *p*H 7.4. The DNA-P concentration was 43.5 mμmole/ml. Crosses indicate no AM; dots, 20.4 mμmoles of AM per milliliter; open circles, 13.6 mμmole/ml; closed triangles, 6.8 mμmole/ml; and open triangles 2.7 mμmole/ml.

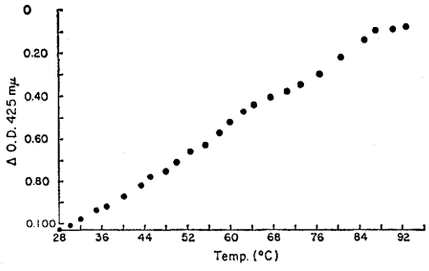

Fig. 6. Effect of temperature on the spectral difference of AM (20.4 mμmole/ml) at 425 mμ produced by deoxyguanosine (3.96 m*M*). The solvent was 0.01*M* tris-HCl in 0.01*M* NaCl, *p*H 7.4.

tests this possibility are shown in Fig. 7. A single concentration of a preparation of native transforming pneumococcal DNA was used as the template for RNA synthesis and for DNA synthesis. The appropriate enzyme and nucleoside triphosphate precursors were added, together with varying AM concentrations. The T_m of the same DNA preparation was measured at ratios of AM to DNA-P corresponding to those in the enzyme reaction mixtures. It can be seen (i) that RNA-polymerase is inhibited at lower concentrations of AM than is DNA-polymerase, as previously shown (*5*); (ii) that the inhibition of RNA-polymerase increases to virtual completion through a range of AM concentrations which do not affect the heat stability of the template; and (iii) that the values of the ratio of AM to DNA-P at which DNA-polymerase is inhibited correspond closely to those which give rise to progressive increases in T_m.

The increase in T_m of a given DNA preparation is a function of the ratio of AM to DNA-P, and it is seen at all salt concentrations tested; thus variations in ionic strength alter the value of $\Delta T_m/\Delta$AM without significantly affecting the ratio of AM to DNA-P at which change is first observed. The ratio of AM to DNA-P required to raise the T_m of pneumococcal DNA has been established for several actinomycins of different biological activity. In each case the ratio of AM to DNA-P required to raise T_m coincided with that inhibiting DNA-polymerase (*24*).

The following interpretation is proposed. Actinomycin bound to guanine-containing sites on helical DNA directly inhibits RNA-polymerase, independently of any observable physical consequence, by blocking those surfaces of the template participating in enzyme

activity. Since a quantity of bound AM which almost completely suppresses DNA-dependent RNA synthesis does not affect DNA-polymerase activity, it may be tentatively concluded that in the course of DNA replication the polymerase functions on surfaces of the template which are essentially unobstructed by any bound AM. In view of the probable homogeneity of the sites of complex formation (*8, 12*) it seems likely that DNA-polymerase never "encounters" AM bound to DNA. The interference with DNA-polymerase activity eventually observed at higher concentrations of AM is an indirect action of the antibiotic, coincides with appreciable increases in T_m, and may be attributed to inhibition of the strand separation normally required for replication of template DNA.

The external portion of the native DNA molecule is separated by the sugar phosphate "backbones" into two helical grooves. As a consequence of the regular pattern of base pairing and the antiparallel polarity of the strands comprising the double helix any functional group of a DNA constituent always has a fixed relation to each of the two grooves. Thus in DNA from T-even bacteriophage the glucose residues and the methyl group of thymine are always located in the major groove, while the 2-amino group of guanine projects only into the minor groove.

In order to account for the established base specificity of AM binding, it can be assumed that a part of the AM molecule must be oriented in a distinctive way with respect to some aspect of the DNA guanine, and this implies a binding of the antibiotic in one of the DNA grooves rather than along the sugar-phosphate backbone.

Intercalation of the AM chromophore between adjacent DNA base pairs in the manner demonstrated for proflavine (*26*) is unlikely (*9*) since (i) it does not account for the need for guanine, the AM lactones, or the AM amino group; (ii) the bulk of the peptide chains prevents the intercalation of more than a minor part of the chromophore and specifically the intercalation of the amino group; (iii) the presence of the bulky stearylamino group in position 7 of the actinomycin chromophore is incompatible with intercalation, whereas 7-stearylaminoactinomycin (*16*), like 7-bromoactinomycin (*10*), reacts quite normally with DNA.

The following considerations may be added to those cited elsewhere (*9*) in support of the proposal that AM lies in the minor groove of helical DNA. (i) Actinomycin binding is not impaired by the presence of glucose in the major groove of T-even phage DNA's. Every hydroxymethylcytosine (HMC) residue (and thus every G-C base pair) of T4 DNA is glucosylated (*27*), and the sugar residues would interfere with intercalation of the chromophore by preventing the approximation of the AM peptide chains to the helix surface. Despite glucosylation and its lower guanine content, T4 DNA is slightly more efficient than calf-thymus DNA in binding AM (*12*). (ii) Actinomycin bound to one strand of DNA suppresses the template activity, for RNA synthesis, of both strands (*7, 8*). This effect could most easily be accomplished in the minor groove which can be completely occluded by the antibiotic. (iii) The synthetic homopolymer mixture of polydeoxyinosinic and polydeoxycytidylic acids (dIdC) (*28*) resembles synthetic dGdC (homopolymer mixture of deoxyguanylic and deoxycytidylic acids) (*29*) except for the absence, in dIdC, of the 2-amino group of guanine. The dIdC does not form complexes with AM (*12*). (iv) The postulated binding in the minor groove would account for the role of all AM and DNA structures so far known to be required for complex formation.

The hydrogen bonds envisioned as the links between the AM chromophore and deoxyguanosine of DNA (*9*) would tend to immobilize a guanine residue with respect to the sugar-phosphate backbone of its own DNA strand and thereby they would increase also the stability of the hydrogen bonding of the affected G-C base pair. The anticipated rise in T_m associated with the increased rigidity of the polymer has been found, but the magnitude of the observed changes appears surprising, since it far exceeds on a molar basis that recorded for any reversible stabilizing agent examined to date. This impressive effect may be due to the fact that when the AM chromophore forms the complex with deoxyguanosine in one DNA strand four hydrogen bonds can be formed between the two free hydrogens of each peptide chain and the phosphate oxygens of the opposite DNA strand (*9*). These bonds provide an additional cross-linking influence which extends over at least three base pairs for each AM molecule bound. Such a hydrogen-bonding system could

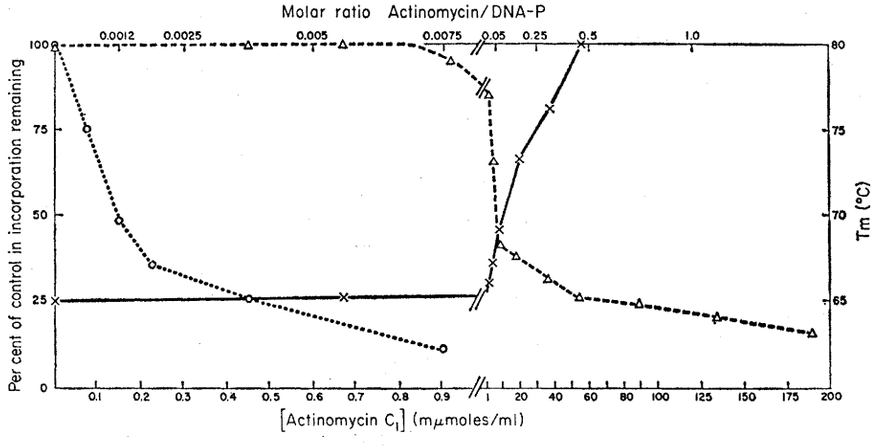

Fig. 7. Effect of AM on RNA polymerase (open circles), DNA polymerase (open triangles), and T_m of pneumococcal DNA (crosses). Ribonucleic acid polymerase was prepared (*31*) and asayed (15 min, 37°C) with ATP-C^{14} as radioactive precursor; DNA polymerase was prepared (*32*) and assayed (*5*) with deoxy-ATP-C^{14} as radioactive precursor. Incorporation in the absence of AM (shown as 100% of control) was 1.52 mμmole of adenylic acid with RNA polymerase and 1.2 mμmole of deoxyadenylic acid with DNA. The reaction mixture for RNA polymerase contained 3.02 μg of enzyme protein in 0.25 ml, that for DNA polymerase contained 0.6 μg enzyme protein in 0.3 ml. The concentration of DNA-P in the enzyme reactions was 120 mμmole/ml. For the heating experiments the concentration of DNA-P was 42 mμmole/ml, and the solvent was 0.001*M* tris-HCl in 0.005*M* NaCl, *p*H 7.4. The results are presented as follows: the incorporation of nucleotide in both polymerase reactions is plotted as a function of AM concentration (lower abscissa), while the T_m is plotted as a function of the ratio of AM to DNA-P (upper abscissa). The scale of this ratio is identical for both the heating and incorporation experiments, although the absolute concentrations of both reagents is different in the two systems.

be responsible for the large observed changes of T_m and might explain the role of the lactones in the activity of the AM molecule.

It seems appropriate to consider the possibility that the invariable restricted distribution of DNA functional groups might underlie the enzymatic specificity of the nucleic acid polymerases. All available evidence concerning the activity of these enzymes points to the likelihood that the replication of templates occurs by way of a mechanism in which sequences are determined by the type of base pairing which occurs in DNA. If this is the case, the hydrogen-bonding system of the native helix would have to be disrupted, at least at the site of enzyme action. The hydrogen bonds linking the base pairs are centrally located around the helix axis; and the hydrogen bonds between any base pair could be severed by an enzymatic approach from either groove. Denaturation of a short segment would allow the affected bases to rotate, permitting normal base pairing with incoming nucleotides to occur in either groove. It may be significant that proflavine, which is thought to intercalate between successive base pairs (*26*), thus affecting the structure of both DNA grooves, inhibits both nucleic acid polymerases unselectively (*3, 5*), whereas AM, which is assumed to bind only in the minor groove (*9*), exhibits striking selectivity in its effect on the same enzymes. Therefore, it seems reasonable to propose, as a working hypothesis, that each nucleic acid polymerase normally "sees" the DNA base sequence from only one groove. RNA-polymerase is displaced from DNA by AM (*30*). If AM is assumed to lie in the minor groove, it would seem logical to expect that this groove is the specific template site for RNA-polymerase and thus the site of RNA synthesis and perhaps of its regulation. DNA replication would then be postulated to proceed in the major groove. These possibilities can be tested experimentally.

E. REICH

Rockfeller Institute, New York 21

References and Notes

1. I. J. Slotnick, *Ann. N.Y. Acad. Sci.* **89**, 342 (1960); J. M. Kirk, *Biochim. Biophys. Acta* **42**, 167 (1960).
2. E. Reich, R. M. Franklin, A. J. Shatkin, E. L. Tatum, *Science* **134**, 556 (1961); *Proc. Natl. Acad. Sci. U.S.* **48**, 1238 (1962).
3. I. H. Goldberg and M. Rabinowitz, *Science* **136**, 315 (1962).
4. G. Hartmann and U. Coy, *Angew. Chem.* **74**, 501 (1962); G. Hartmann, U. Coy, G. Kniese, *Z. Physiol. Chem.* **330**, 227 (1962).
5. J. Hurwitz, J. J. Furth, M. Malamy, M. Alexander, *Proc. Natl. Acad. Sci. U.S.* **48**, 1222 (1962).
6. E. Reich, I. H. Goldberg, M. Rabinowitz, *Nature* **196**, 743 (1962).
7. I. H. Goldberg, M. Rabinowitz, E. Reich, *Proc. Natl. Acad. Sci. U.S.* **48**, 2094 (1962); *ibid.* **49**, 226 (1963).
8. E. Kahan, F. Kahan, J. Hurwitz, *J. Biol. Chem.* **238**, 2491 (1963).
9. L. Hamilton, W. Fuller, E. Reich, *Nature* **198**, 538 (1963).
10. W. Müller, *Naturwiss.* **49**, 156 (1962).
11. H. Brockmann, *Fortschr. Chem. Org. Naturstoffe* **18**, 1 (1960).
12. E. Reich, unpublished observations.
13. H. Muxfeldt, personal communication.
14. W. Kersten, *Biochim. Biophys. Acta* **47**, 610 (1961).
15. R. Langridge, D. Marvin, W. Leeds, H. Wilson, C. Hooper, M. Wilkins, L. Hamilton, *J. Mol. Biol.* **2**, 38 (1960).
16. W. Müller, personal communication.
17. M. Gellert, M. Lipsett, D. Davies, *Proc. Natl. Acad. Sci. U.S.* **48**, 2013 (1962).
18. L. Lerman, personal communication.
19. J. D. Leith, Jr., *Biochim. Biophys. Acta* **72**, 643 (1963).
20. M. Izawa, V. Allfrey, A. E. Mirsky, *Proc. Natl. Acad. Sci. U.S.* **49**, 544 (1963).
21. R. Haselkorn, *Science*, this issue.
22. R. L. Sinsheimer, *J. Mol. Biol.* **1**, 43 (1959).
23. H. K. Schachman, J. Adler, C. M. Radding, I. R. Lehman, A. Kornberg, *J. Biol. Chem.* **235**, 3242 (1960).
24. E. Reich and I. H. Goldberg, in preparation.
25. W. Kersten and H. Kersten, *Z. Physiol. Chem.* **330**, 21 (1962).
26. L. S. Lerman, *J. Mol. Biol.* **3**, 18 (1961).
27. S. R. Kornberg, S. Zimmerman, A. Kornberg, *J. Biol. Chem.* **236**, 1487 (1961).
28. A. Kornberg, *Enzymatic Synthesis of DNA* (Wiley, New York, 1961).
29. C. M. Radding, J. Josse, A. Kornberg, *J. Biol. Chem.* **237**, 2869 (1962).
30. I. H. Goldberg, E. Reich, M. Rabinowitz, *Nature* **199**, 44 (1963).
31. M. Chamberlin and P. Berg, *Proc. Natl. Acad. Sci. U.S.* **48**, 81 (1962).
32. I. R. Lehman, M. J. Bessman, E. S. Simons, A. Kornberg, *J. Biol. Chem.* **233**, 163 (1958).
33. I thank E. L. Tatum for encouragement; A. Kornberg and R. Inman for dIdC; M. Roger for pneumococcal DNA; D. L. Hamilton for *N*-dimethylene actinomycin; S. Huang for a sample of DNA-polymerase; H. Moroson for the use of a Beckman spectrophotometer; W. Fuller and R. Haselkorn for helpful discussion, and the Helen Hay Whitney Foundation and National Institutes of Health for support.

15 November 1963

Diabetes Mellitus in the Sand Rat Induced by Standard Laboratory Diets

Abstract. *During an attempt to establish a laboratory colony of the sand rat* (Psammomys obesus) *we found that this animal invariably became obese and developed severe diabetes mellitus when fed on commercial laboratory rat feed, but remained normal when fed on fresh vegetables only. The signs of diabetes included elevated blood glucose, excessive glucose and ketone bodies in the urine, and cataracts. The diabetic animals showed degeneration of the pancreatic insulin-producing tissue (beta-cells).*

The North African rodent *Psammomys obesus,* or the sand rat, inhabits areas where the vegetation consists of fleshly salt-loving plants (for example, *Salicornia*). The sand rat seems to eat exclusively these succulent plants in which the sap has a salt content often in excess of that in sea water (*1*). Our interest in establishing a laboratory colony of sand rats was based on their exceptional tolerance to salt and the high concentrating capacity of their kidneys.

Adult sand rats imported from Egypt were mated in the laboratory and produced seemingly normal litters. The young grew well until weaning but soon afterward developed cataracts and during the following months most of them died. There was seldom any outward indication of the reason for death, and there was no general infection, except in cases where a wound or broken tooth was present. We did record, however, that one or more of the following signs of diabetes mellitus existed in approximately 60 of these laboratory-reared sand rats: cataracts, elevated blood and urine sugar, ketonuria, and degeneration of the pancreatic beta-cells as shown by aldehyde-fuchsin staining.

The animals were fed on a standard laboratory diet (Purina Laboratory Chow), supplemented with occasional carrots; either water or a 5-percent NaCl solution was provided for drinking. To discover possible nutritional deficiencies we supplemented the usual diet of certain animals with various combinations of the following additives: salts and trace elements, the common vitamins, increased protein, and liberal amounts of fresh vegetables (*2*). Mixed grains (cracked corn, oats, millet, and sorghum) were provided, either alone or in addition to the usual diet, to some of the sand rats so that preferred items might be selected. None of these various nutritional measures improved the condition of the animals. It then appeared reasonable that the signs we had observed were caused either by the standard laboratory diet (or some dry, high-energy food such as the mixed grains) or they re-