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# Biochemical Studies of the Purine Analogues, 2-Aminopurine and 2, 6 Diaminopurine

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# Biochemical Studies of The Purine Analogues, 2-Aminopurine and 2,6 Diaminopurine

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## ABSTRACT

An alternating copolymer of deoxy 2,6 diamino purine and thymine ($d\overline{DAP}$-T) has been synthesized by substituting 2,6 diamino purine deoxyriboside triphosphate for dATP in a dAT primed synthesis with DNA polymerase. This polymer differs from dAT in having an amino group in the minor groove, and in several physical properties. For example, the additional amino group allows a third hydrogen bond of the Watson-Crick type to be formed with thymine, and the expected increase in the stability of the helix is reflected in a transition temperature (Tm) which is higher by 25°C. than that of dAT. Also, the buoyant density of $d\overline{DAP}$-T in CsCl differs appreciably ($\rho$ = 1.718) from that of dAT ($\rho$ = 1.679).

The antibiotic actinomycin has been shown to inhibit RNA polymerase by binding to helical DNA. The specificity of this interaction is thought to be determined by the 2-amino group of guanine since neither dAT nor dI:dC interact with the antibiotic. dAT-like polymers containing 2,6 diaminopurine can be shown to interact with actinomycin by the following criteria:

1) The buoyant density of the polymer in CsCl gradients is altered in the presence of the antibiotic.

2) The Tm of the dAT-like polymers containing DAP is increased in the presence of actinomycin.

3) The visible spectrum of actinomycin is changed in the presence of $d\overline{DAP}$-T.

4) The template function of dAT-like polymers containing DAP for RNA polymerase is strongly inhibited by actinomycin.

Thus the introduction of an amino group at the 2 position of the purine in the minor groove of helical DNA suffices to convert the A-T base pair from antibiotic indifference to antibiotic sensitivity.

The second purine analogue studied in this thesis is 2-aminopurine. Like 2,6 diaminopurine, 2-aminopurine is a substrate analogue of only dATP with DNA polymerase. Also dAT polymers containing 2AP are found to interact with actinomycin. However, the substrate properties and the physical characteristics of polynucleotides containing 2-aminopurine are unusual.

1) Significant utilization of d2APTP by DNA polymerase with all templates (exonuclease III-treated DNA, dAT, dA:dT, dAC:dTG) tested requires the simultaneous presence of dATP.

2) The thermal denaturation profile of dAT (12.5% 2AP) is non-cooperative and occurs at a lower temperature than dAT.

3) The degree of hyperchromicity of polymers containing 2AP is appreciably lower than that of dAT.

To explain these unusual properties, it is proposed that the 2AP-T base pair is fully denatured under the conditions used for enzymatic synthesis and for the study of polymers containing 2AP.

A twofold theory to explain the mutagenic action of 2-aminopurine is as follows:

1) Since the 2AP-T base pair is believed to be denatured, the presence of this unwound segment of DNA corresponds to the events which follow U.V. irradiation of DNA. The mutation occurs as a result of a mistake during the course of repair.

2) At an acid pH the N-1 of dCTP is protonated. Therefore, a 2AP-C base pair containing two hydrogen bonds can be formed at an acid pH. Since the pH of C in polynucleotides is displaced to lower pH values than that of the monomer, 2AP would be expected to pair with protonated dCTP more frequently than d2APTP pairing with a protonated C residue in the template DNA.

These hypotheses are currently under investigation.

# ACKNOWLEDGEMENTS

The work reported in this thesis has been completed in collaboration with Mr. David C. Ward of The Rockefeller University. It has been a rewarding experience to have had the benefit of his expert laboratory skills and scientific integrity. Several others have given their time, suggestions, and encouragement to guide the work of this thesis: Dr. Irving H. Goldberg of Harvard University; Dr. George Acs of the Institute for Muscle Disease, New York; and Dr. David Luck of The Rockefeller University.

I am indebted to the entire faculty and administration for the opportunity to study at The Rockefeller University. In particular, I would like to thank Dr. Detlev Bronk, Dr. Frank Brink, Jr., and Dr. Edward Tatum.

Dr. Edward Reich, my research advisor, has made my graduate education a rich and satisfying experience. I sincerely appreciate his vast knowledge, ardent interest, availability for consultation, and respect for my development.

Finally, I would like to thank my wife, Kathy, for her generous help in many aspects of this thesis.

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# PREFACE

Only a few analogue deoxynucleoside triphosphates have been studied with DNA polymerase of E. coli. These are dBrUTP, Trautner et al. (1962); dBrCTP, Bessman et al. (1958); dFUTP, Kornberg (1963); dUTP, Bessman et al. (1958); dITP, Bessman et al. (1958); d6-methyl ATP, Novogrodsky et al. (1966), Lazarus and Swartz (1964); and d8BrGTP; Kapuler et al. (1967). This thesis describes the substrate properties of the purine analogues 2,6-diaminopurine and 2-aminopurine. The physical properties of polymers containing these analogues are described.

These analogues were selected for study for several reasons:

1) Assuming their incorporation into polymers, the structure of these analogues provides a test of the model proposed for the structure of actinomycin-DNA complexes.

2) These analogues are known to have mutagenic properties and a biochemical investigation may help to yield insight into the mechanism of their mutagenicity.

3) These analogue nucleotides and polynucleotides provide a test for the necessity of Watson-Crick base pairing in the specification of sequences by nucleic acid polymerases.

4) The presumed structure of base pairs containing these analogues is likely to be correlated with interesting physical properties.

# CHAPTER I

## MATERIALS

*E. coli* B harvested in mid-log growth were purchased from the Grain Processing Company of Muscatine, Iowa. Nucleoside triphosphates were obtained from P-L Biochemicals, Inc., Milwaukee, Wisconsin. Radioactive nucleoside triphosphates were purchased from Schwarz Bioresearch Inc., Orangeburg, New York. 2,6-Diaminopurine deoxyriboside and 6-thiodeoxyguanosine were prepared by P-L Laboratories using the trans N-deoxyribosylase of *Lactobacillus helveticus*. 2,6-Diaminopurine riboside and thioguanosine were obtained from the Cyclo Corporation. $\alpha P^{32}$-labeled rATP and rUTP were obtained from the ICN Company. All other chemicals were obtained from standard commercial sources.

## METHODS

### Preparation of 2-aminopurine deoxyriboside from 6-thiodeoxyguanosine.

6-Thiodeoxyguanosine was refluxed with twice its weight of Raney nickel W-2 in 100 mls. of water/gm. of nucleoside. After two hours, the spectrum was read to determine the extent of the desulfurization. Thiodeoxyguanosine has a characteristic absorption maximum at 320 mμ at neutral pH; whereas d2AP has an absorption maximum at 303 mμ When the ratio O.D. 303/O.D. 320 reached 23, the reaction mixture was filtered and the Raney nickel extracted several times with boiling water. Quantitative recovery of d2AP was realized at this step.

### Preparation of the monophosphates of 2-aminopurine deoxyriboside and 2,6-diaminopurine deoxyriboside.

The monophosphates were synthesized using the technique of Tener (1961). In essence, the unprotected deoxynucleoside is reacted with one molar equivalent of cyanoethyl phosphate and dicyclohexyl-carbodiimide (10-20 molar equivalents) in dry pyridine. The 5' hydroxyl of the deoxyribose is much more reactive than the 3' hydroxyl so that a 20-30% overall yield of the 5' diester of cyanoethyl phosphate is obtained. No detectable 3' phosphate is produced. The cyanoethyl phosphate diester is cleaved by heating at 60°C for two hours in 9M $NH_4OH$. Following extraction of dicyclohexylurea with ether, the deoxynucleoside monophosphate was purified by column chromatography on D.E.A.E. cellulose ($HCO_3^-$). The monophosphate was eluted by means of a linear gradient (total of 20 column volumes) of triethyl ammonium bicarbonate with limiting concentrations of 0 and 0.3M. The peak corresponding to monophosphate was concentrated and freed of triethyl ammonium bicarbonate by repeated flash evaporations. The purity was assayed by electrophoresis in .05M sodium citrate buffer, pH 3.5. The electrophoretic mobility of both d2APMP and dDAPMP is similar to that of dAMP.

Conversion of 2-aminopurine deoxyriboside monophosphate and 2,6-diaminopurine deoxyriboside monophosphate to the corresponding triphosphate.

The triphosphates were synthesized from the monophosphates by the method of Smith and Khorana (1958). The yield of triphosphate varied but usually about 30-40% based on monophosphate was obtained.

Preparation of $\alpha P^{32}$-labeled 2-aminopurine deoxyriboside triphosphate and 2,6-diaminopurine deoxyriboside triphosphate.

The $\alpha P^{32}$-labeled deoxynucleotides were synthesized in the usual way using cyanoethyl phosphate-$P^{32}$ (Nuclear Chicago) at a specific activity of 100-200 μc/μmole.

Synthesis of dAT.

The synthesis of de novo dAT was carried out according to the procedure of Schachman et al. (1960). The concentration of each dNTP was 400 mμmoles/ml and polymer synthesis was followed viscometrically (Ostwald). When the viscosity reached a maximum, the reaction was terminated by the addition of phenol. The polymer was dialyzed against .01M Tris-HCl pH 7.9,.01M KCl and stored in the freezer.

Synthesis of dG:dC.

dG:dC was synthesized in a primed reaction as described by Radding et al. (1962). Synthesis was followed and the polymer isolated as in the case of dAT.

Synthesis of dAC:dTG.

A small amount of dAC:dTG was obtained from Mr. P.J. Cassidy of Harvard University. Substantial net synthesis of polymer was found to occur in glycine buffer (0.07M) pH 9.2. Under these conditions de novo synthesis of dAT was not observed. The kinetics of synthesis can be

seen in Fig. 1. The lag period varied with different enzyme preparations. This lag could be shortened by the addition of small amounts of crude preparations of DNA polymerase (Fraction IV). The nature of this lag period is currently under investigation.

Synthesis of dAG:dTC.

dAG:dTC was synthesized as described by Byrd et al. (1965).

Synthesis of dI:dC.

dI:dC was synthesized as described by Inman and Baldwin (1964).

Determination of radioactive polymer synthesis.

The rate of synthesis of the various polymers as well as routine assays of DNA and RNA polymerase were determined by acid precipitation of the radioactive polymer product. An aliquot of the reaction varying from 5 $\lambda$ to 250 $\lambda$ was added to 5 mls. of cole 5% (W/V) trichloroacetic acid and filtered through a Millipore filter (HAWP 002500). After the filter was washed with 30-40 mls. of cold 5% trichloroacetic acid and dried, the amount of radioactivity was determined with a Packard scintillation counter.

Synthesis of $\overline{\text{dDAP}}$-T and dAT containing 5% DAP.

The polymer $\overline{\text{dDAP}}$-T was prepared as follows: a reaction mixture (5mls.) containing 300 mμmoles/ml. $\alpha P^{32}$ dDAPTP; 300 mμmoles/ml. TTP; .07M Tris-HCl pH 7.0; .007M $MgCl_2$; .01M β-mercaptoethanol; 150 units DNA polymerase and 30 mμmoles/ml. dAT was incubated at 37°C. The rate of polymer synthesis was followed by measuring the accumulation of acid insoluble radioactivity as seen in Fig. 2. Since it had been observed that the polymer was soluble in phenol, the reaction (ten-fold synthesis above primer input in 8 hours) was terminated by the addition of 2 volumes of absolute ethanol and the precipitated polymer washed several times with ethanol. The polymer was then dissolved and dialyzed against .01M Tris-HCl pH 7.9, .01M KCl.

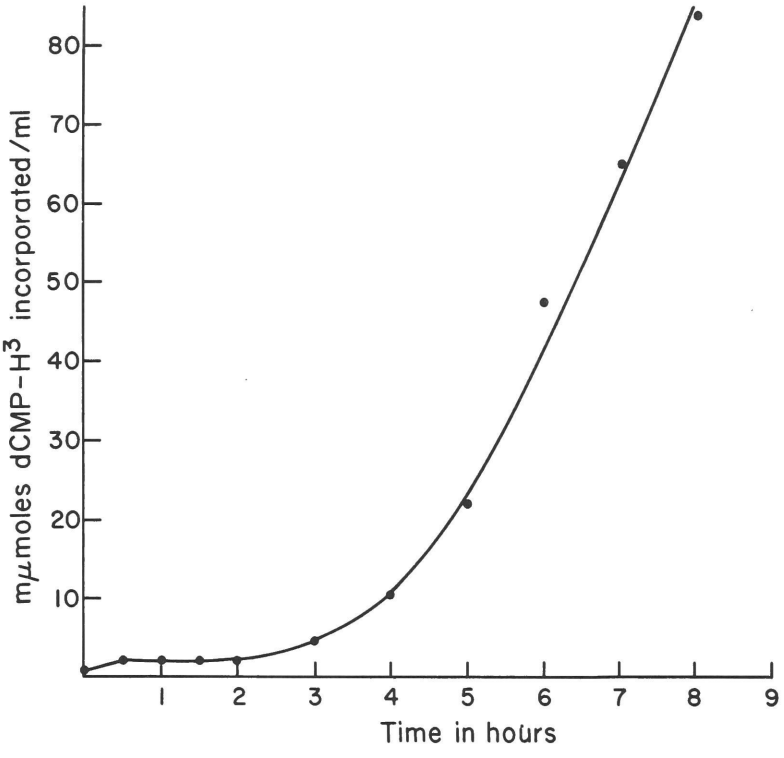


Fig. 1. Synthesis of dAC:dTG. The complete reaction contained: 60 μmoles/ml. glycine buffer, pH 9.2; 6 μmoles/ml. $MgCl_2$; 500 mμmoles/ml. of dATP, dCTP, dGTP and TTP; 15 mμmoles/ml. of dAC:dTG primer, and 30 units/ml. of DNA polymerase, Fraction VII. The incubation was carried out at 37°C.

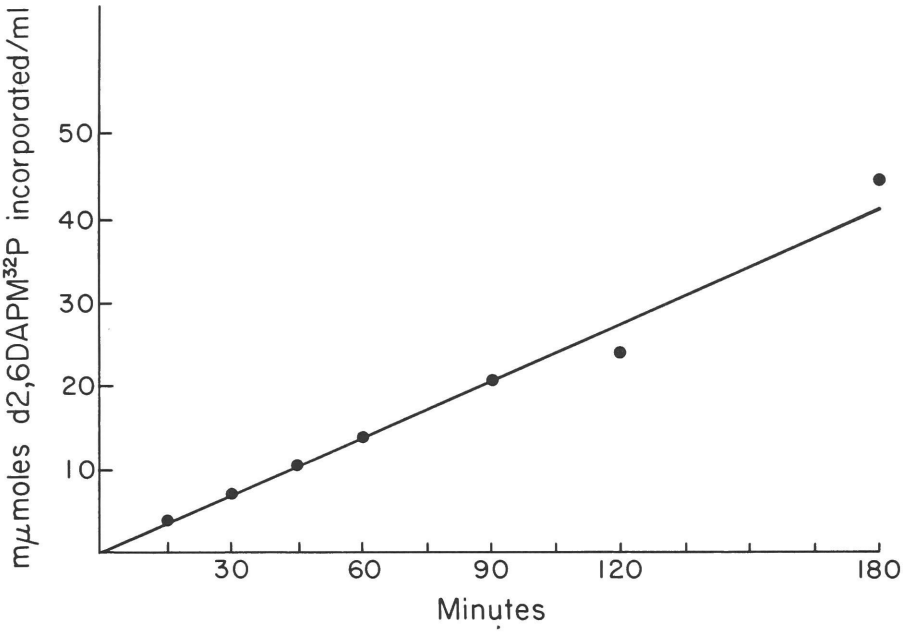


Fig. 2. Synthesis of $\overline{\text{dDAP}}$-T. The complete reaction contained: 70 μmoles/ml. Tris-HCl buffer, pH 7.0; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 30 mμmoles/ml. dAT primer; 500 mμmoles/ml. of dDAPTP and TTP; and 50 units/ml. DNA polymerase, Fraction VII. The incubation was carried out at 37°C.

A dAT-like polymer in which 10% of the purines consisted of DAP was also prepared. For this preparation the following conditions were used: 3 mls. containing .07M Tris-HCl, pH 7; .007M $MgCl_2$; .01M β-mercaptoethanol; 500 mμmoles/ml. TTP; 500 mμmoles/ml. dATP-$H^3$ (specific activity 10 μc/μmole); 50 mμmoles/ml. dDAP$^{32}$TP (specific activity .4 μc/μmole); 20 mμmoles/ml. dAT primer; and 90 units DNA polymerase. The progress of the reaction was followed by the acid precipitation of the radioactive polymer (Fig. 3). The reaction was terminated at 80 min. by the addition of 2 volumes of ethanol and the precipitate washed with ethanol. The washed precipitate was then dialyzed against .01M Tris-HCl pH 7.9, .01M KCl.

Synthesis of dAT polymers containing 5% 2AP and 12.5% 2AP.

A dAT-like polymer containing 10% of its purines as d2AP was prepared under the following conditions: 3 mls. containing .07M Tris-HCl pH 7.0; .007M $MgCl_2$; .01M β-mercaptoethanol; 100 mμmoles/ml. TTP; 60 mμmoles/ml. dATP-$H^3$ (specific activity 10 μcuries/μmole); 60 mμmoles/ml. d2AP$^{32}$TP; 20 mμmoles/ml. dAT primer; and 90 units DNA polymerase. Synthesis was followed by the acid precipitation of the polymer (Fig. 4). The reaction was terminated at 35 min. by the addition of phenol. The polymer was then dialyzed as described for dAT.

A dAT-like polymer containing 25% of the purines as 2AP was prepared as follows: 2 mls. contained .07M potassium phosphate, pH 7.0; .007M $MgCl_2$; .01M β-mercaptoethanol; 100 mμmoles/ml. TTP; 100 mμmoles/ml. d2AP$^{32}$TP; 10 mμmoles/ml. dATP-$H^3$ (specific activity 10 μcuries/μmole); 15 mμmoles/ml. of dAT; and 15 units/ml. of DNA polymerase, Fraction VII. After 2 hours incubation, 10 mμmoles/ml. of dATP-$H^3$ (specific activity 10 μcuries/μmole) were added (Fig. 5). At the end of six hours the reaction was terminated by the addition of phenol. The polymer was then dialyzed against .01M Tris-HCl pH 7.9 and .01M KCl.

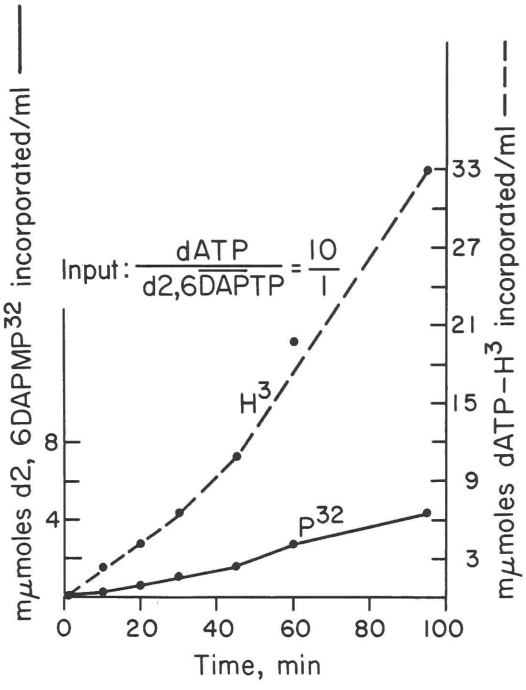


Fig. 3. Synthesis of dAT (5% DAP). The complete reaction contained: 70 μmoles/ml. Tris-HCl buffer, pH 7.0; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 30 mμmoles/ml. of dAT primer; 500 mμmoles/ml. of dATP-$H^3$ (2 x $10^3$ c.p.m./mμmole) and TTP; 50 mμmoles of dDAPTP$^{32}$ (1 x $10^3$ c.p.m./mμmole); and 50 units/ml. DNA polymerase. The incubation was carried out at 37°C.

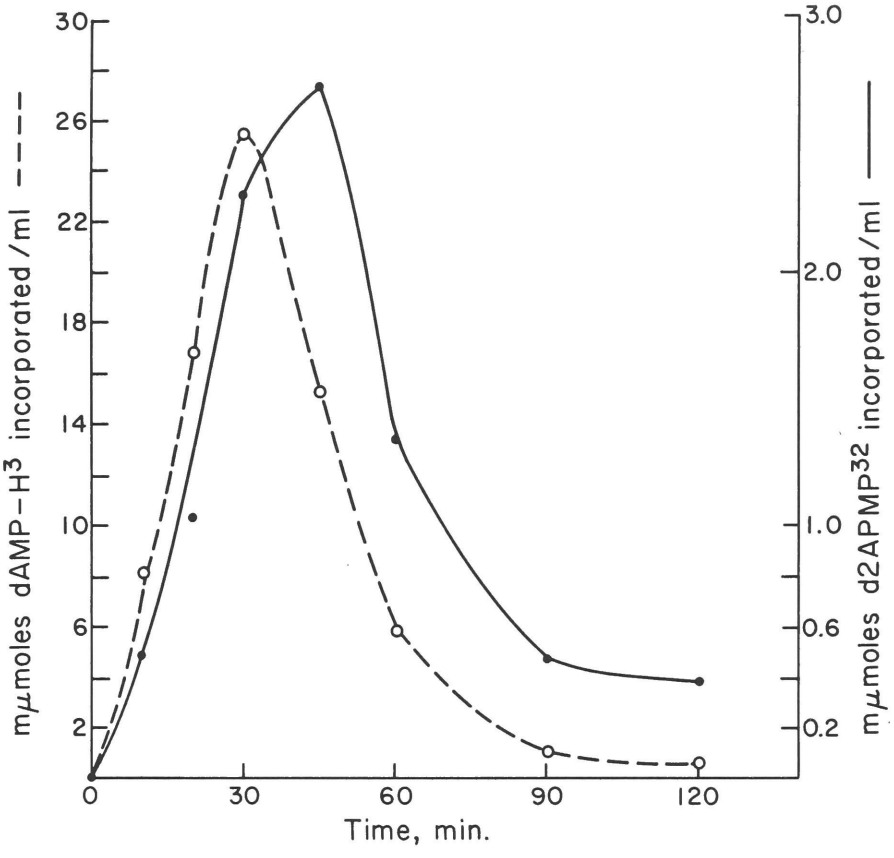


Fig. 4. Synthesis of dAT (5% 2AP). The complete reaction contained: 70 μmoles/ml. Tris-HCl buffer, pH 7.0; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 30 mμmoles/ml. dAT primer; 100 mμmoles/ml. TTP; 60 mμmoles/ml. dATP-$H^3$ (2 x $10^3$ c.p.m./mμmole) and d2APTP$^{32}$ (2 x $10^4$ c.p.m./mμmole); and 50 units/ml. DNA polymerase. The incubation was carried out at 37°C.

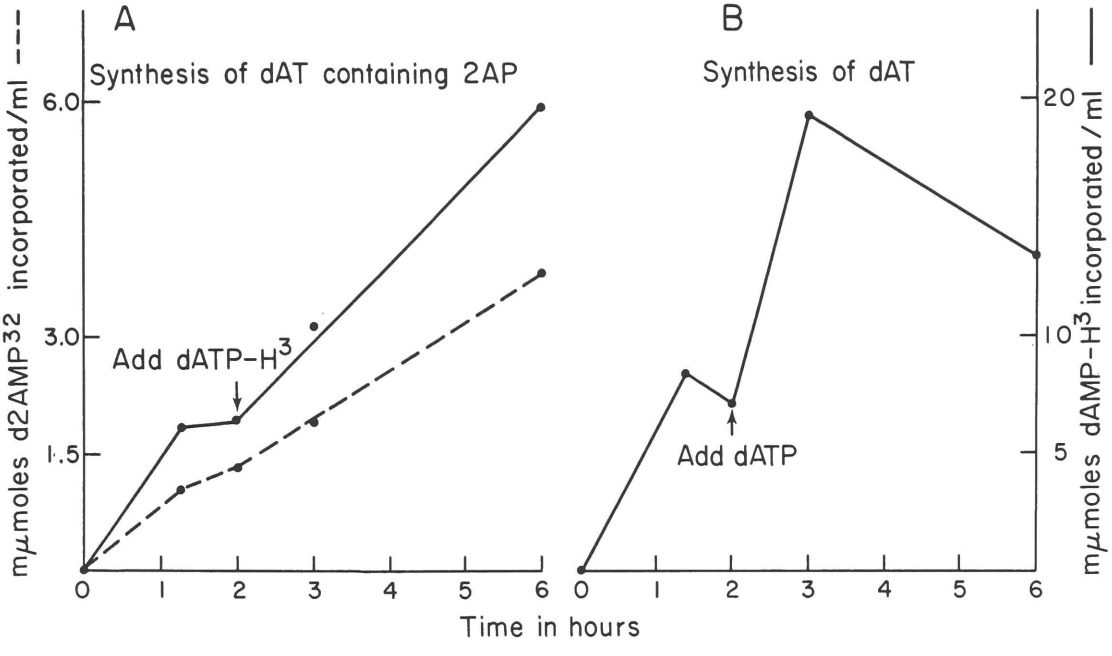


Fig. 5. Synthesis of dAT (25% 2AP). A. The complete reaction contained 70 μmoles/ml. potassium phosphate buffer, pH 7.0; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 15 mμmoles/ml. dAT primer; 100 mμmoles/ml. TTP; 100 mμmoles/ml. $d2APTP^{32}$ ($4 \times 10^4$ c.p.m./mμmole); 10 mμmoles/ml. $dATP-H^3$ ($3 \times 10^4$ c.p.m./mμmole) and 9 units/ml. DNA polymerase, Fraction VII. The incubation was carried out at 37°C. After 2 hours 10 mμmoles/ml. more of $dATP-H^3$ ($3 \times 10^4$ c.p.m./mμmole) was added. B. The conditions as described in part A except that the $d2APTP^{32}$ was omitted.

Purification of dATP, dGTP, dCTP, and dTTP.

Although the commercially available deoxynucleoside triphosphates are adequate for most purposes, it was found that each dNTP was contaminated with the others. The triphosphates were purified effectively by paper chromatography in a system containing isobutyric acid -- 1M $NH_4OH$ (100:60).

Isolation of enzymes.

RNA polymerase

RNA polymerase was isolated from E. coli B according to Chamberlin and Berg (1962) except that the homogenate was treated directly with streptomycin without preceding centrifugation. The purified enzyme was stored in 50% glycerol containing bovine serum albumin (1 mg./ml.) at -20°C. The activity obtained by this procedure was regularly equal to 2000-3000 units/mg. protein; the preparations were free of detectable contaminating ribonuclease or polynucleotide phosphorylase.

DNA polymerase

Most of the experiments reported herein were performed with an enzyme corresponding to Fraction VII of Richardson et al. (1964). The collaboration of Mr. Peter J. Leininger in the preparation of the enzyme is gratefully acknowledged.

This enzyme fraction was found to contain several nuclease activities including endonuclease, exonuclease I, and exonuclease III. To obtain DNA polymerase which was free of these nucleases, Fraction VII enzyme was chromatographed on G-75 Sephadex (.05M potassium phosphate, pH 7.4, .01M β-mercaptoethanol). All glassware and solutions were sterilized and sterile technique was employed in the manipulations. DNA polymerase was totally excluded from the gel; whereas the nucleases were retained. Exonuclease I and exonuclease III could not be detected in the DNA polymerase

fraction. The absence of endonuclease was shown as follows: 1) 100 dAT units/ml. of DNA polymerase were incubated with DNA (50 mμmoles/ml.) from bacteriophage f-1 for three hours. At the end of this period no decrease in infectivity of the DNA was observed. I thank Dr. June Rothman for carrying out this biological assay. 2) When DNA (50 mμmoles/ml.) from Pneumococcus carrying marker for streptomycin resistance was incubated with 60 dAT units/ml. of DNA polymerase for three hours, no detectable loss in transforming ability was observed. I thank Mr. Thomas Easton for performing this biological assay.

Exonuclease III

Exonuclease III free of endonuclease, exonuclease I, and exonuclease II was prepared by Mr. William Beers according to an unpublished procedure of Basch. I wish to thank Mr. Beers for preparing the various exonuclease III treated DNA's which have been used in this work.

Assay of analogues of dNTP as substrates for DNA polymerase.

In the presence of four dNTPs, $Mg^{++}$, and a DNA primer, DNA polymerase from E. coli catalyses the synthesis of a polymer product which resembles primer DNA in base composition and nearest neighbor frequency. If one of the dNTPs is omitted from the enzyme reaction, a very limited amount of synthesis occurs; this is assumed to reflect the addition of the radioactive dNTP to the termini of primer DNA (Adler et al. 1958). This requirement for four dNTPs allows the examination of the ability of various analogues of dNTPs to replace the normal dNTP in DNA synthesis.

Since it is usually difficult to synthesize radioactive analogues of dNTPs, one of the three normal dNTPs in the reaction mixture is radioactive. Thus, the utilization of the analogue is followed by the synthesis of a polymer containing the normal radioactive dNTP. In practice, it is possible to determine if an analogue of dNTP is a good substrate for DNA polymerase, but it is very difficult to establish whether an inefficient substrate is really utilized at all. This ambiguity arises as a result of the limited synthesis which occurs in the presence of three dNTPs without the analogue dNTP.

# CHAPTER II

The first part of this thesis is concerned with the purine analogue 2,6 diaminopurine. The results to follow describe:

1) The substrate properties of dDAPTP with DNA polymerase;
2) The physical properties of synthetic polymers containing DAP;
3) The template properties of polymers containing DAP;
4) The interaction of actinomycin with polymers containing DAP.

## The substrate properties of dDAPTP with DNA polymerase.

As seen in Fig. 6, the structure of dDAPTP is similar in some ways to both dGTP and dATP. This similarity may result in uncertainty as to whether dDAPTP functions as an analogue of A or G during DNA synthesis. In its hydrogen bonding properties, DAP resembles A, since a DAP-T pair of the Watson-Crick type permits the formation of three H-bonds; whereas a DAP-C pair yields only one (Fig. 7). Thus, if hydrogen bonds of the Watson-Crick type are important in the selection of nucleotides by the nucleic acid polymerases, then dDAPTP would be expected to behave as an analogue of dATP. This possibility was tested as follows: in a calf-thymus DNA-primed reaction, dDAPTP was substituted successively for each of the normal dNTPs. The data in Fig. 8 show that measurable synthesis proceeded only when dDAPTP replaced dATP. This indicates that under these conditions, dDAPTP is a substitute only for the corresponding adenine nucleotide, and that dDAPTP does not behave as an analogue of G. It is noted (Fig. 9) that the affinity of DNA polymerase for dDAPTP equals that for dATP.

The time course of utilization of dDAPTP with DNA polymerase was determined for each of five DNA templates: heat-denatured calf thymus DNA (Fig. 10), native *E. coli* DNA (Fig. 11), heat-denatured *E. coli* DNA (Fig. 12), heat-denatured $T_4$ DNA (Fig. 13), and native $T_4$ DNA (Fig. 14).

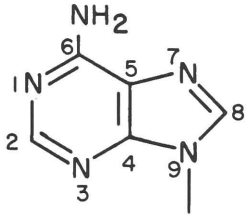


Fig. 6. The structure of DAP.

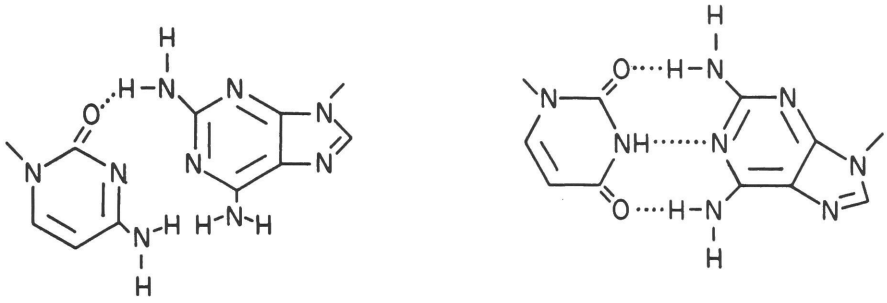

Fig. 7. The hydrogen bonding properties of DAP-T and DAP-C.

| dNTP present | mμmoles TMP-$H^3$ inc./ml. |
|---|---|
| 1. dATP, dCTP, dGTP, TTP-$H^3$ | .93 |
| 2. dDAPTP, dCTP, dGTP, TTP-$H^3$ | .33 |
| 3. dATP, dCTP, dDAPTP, TTP-$H^3$ | .02 |
| 4. dATP, dDAPTP, dGTP, TTP-$H^3$ | .02 |

The complete reaction contained: 60 μmoles/ml. glycine buffer, pH 9.2; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 33 mμmoles/ml. of each dNTP; 100 mμmoles/ml. heat-denatured calf thymus DNA; and 100 units/ml. DNA polymerase. The incubation was carried out at 37°C for 30 minutes.

Fig. 8.

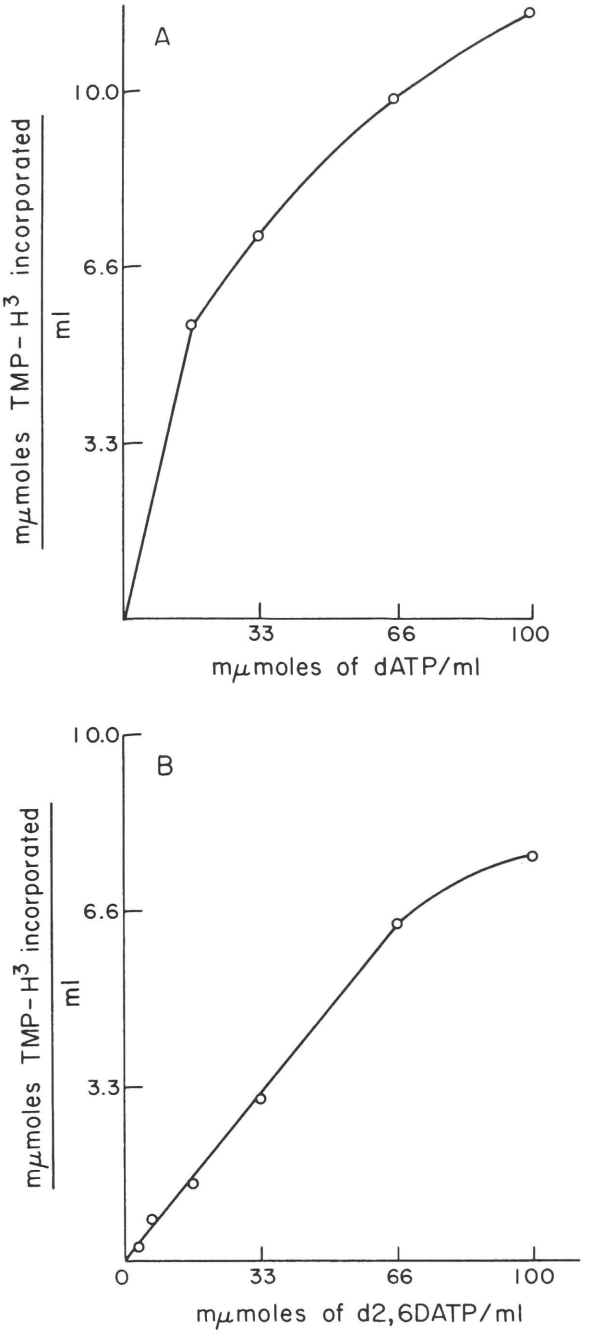


Fig. 9. Affinity of DNA polymerase for dATP and dDAPTP. The complete reaction shown in the upper figure contained, in addition to the amount of dATP present, the following: 66 μmoles/ml. glycine buffer, pH 9.2; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 100 mμmoles/ml. of dGTP, dCTP, and TTP (1 x $10^3$ c.p.m./mμmole); 100 mμmoles/ml. heat-denatured calf thymus DNA; and 100 units/ml. DNA polymerase. The incubation was carried out at 37°C. The lower figure had the same reaction conditions as above except that dDAPTP replaced dATP.

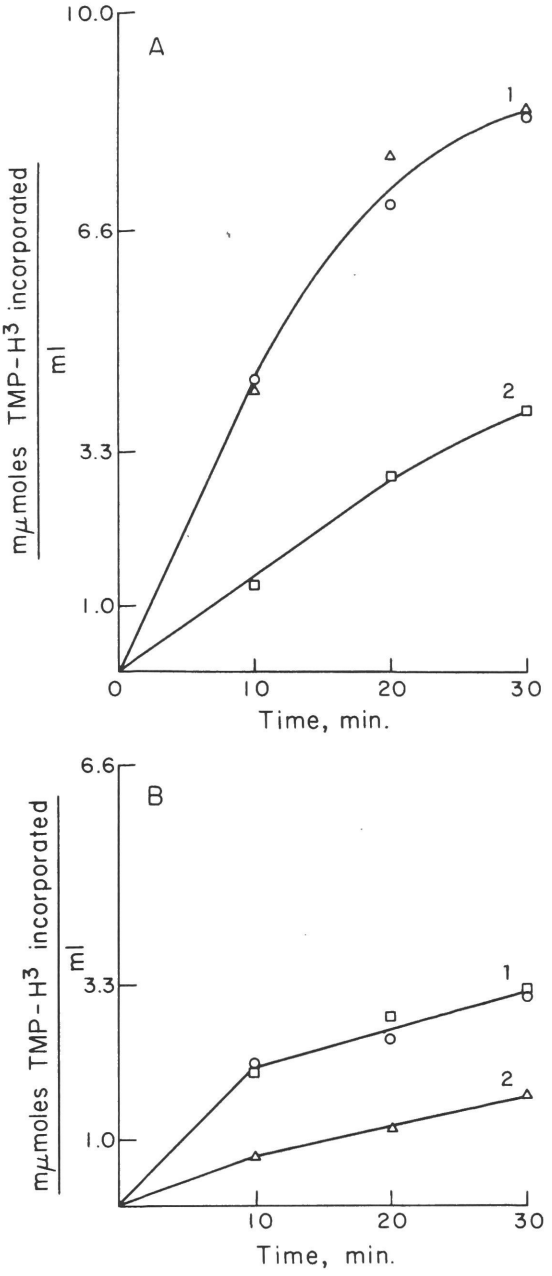


**Fig. 10. Utilization of denatured calf thymus DNA by DNA polymerase. A. The complete reaction contained 66 μmoles/ml. glycine buffer, pH 9.2; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 33 mμmoles of dATP, TTP (1 x $10^3$ c.p.m./mμmole); dCTP, dGTP; 100 mμmoles heat-denatured calf thymus DNA; in addition, 1 contained 200 units/ml. DNA polymerase and 2 contained 50 units/ml. DNA polymerase. B. The reaction conditions are the same as in part A except that dDAPTP replaced dATP.**

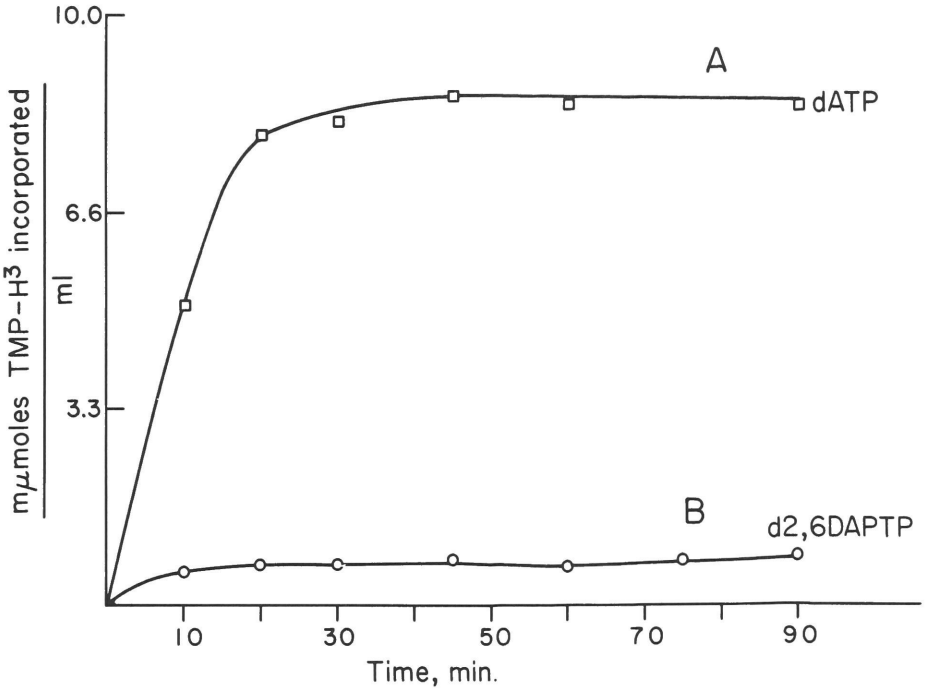


Fig. 11. Utilization of native E. coli DNA by DNA polymerase. A. The complete reaction contained: 66 μmoles/ml. glycine buffer, pH 9.2; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 33 mμmoles/ml. of dATP, dCTP, dGTP, and TTP ( 1 x $10^3$ c.p.m./mμmole); 100 mμmoles/ml. native E. coli DNA; and 100 units/ml. DNA polymerase. The incubation was carried out at 37°C. B. The reaction conditions were the same as in part A, except that dDAPTP replaced dATP.

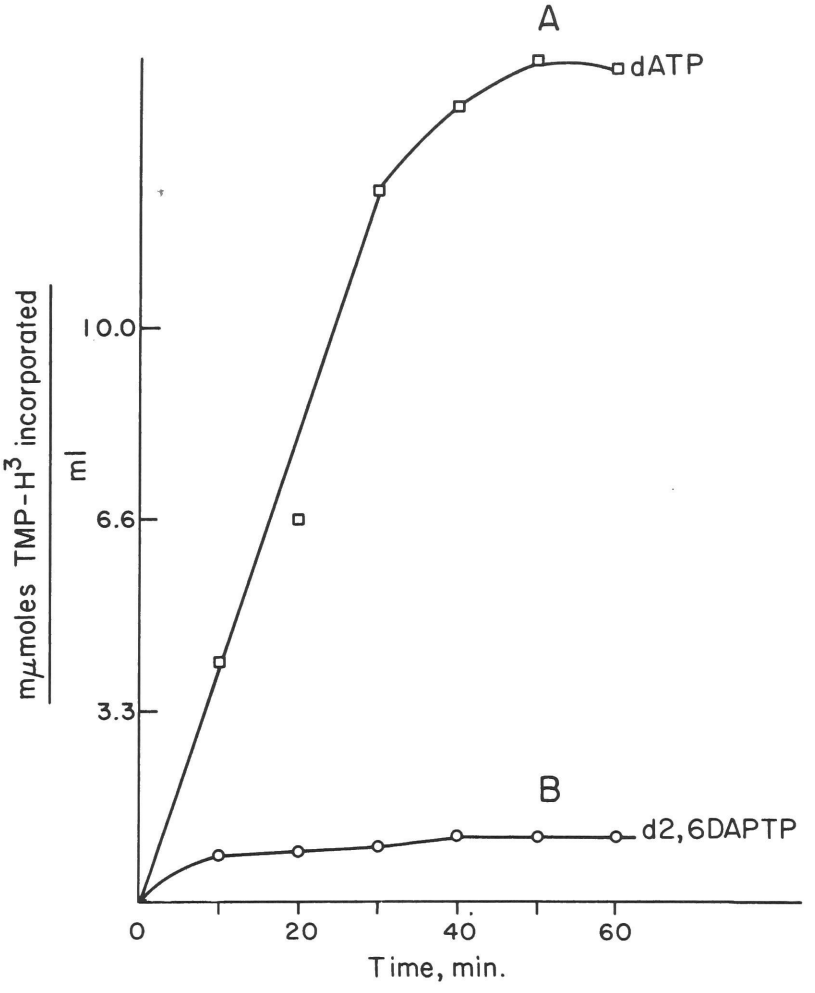


Fig. 12. Utilization of denatured E. coli DNA by DNA polymerase. A. The complete reaction contained: 66 μmoles/ml. glycine buffer, pH 9.2; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β -mercaptoethanol; 33 mμmoles/ml. of dATP, dCTP, dGTP, and TTP ($1 \times 10^3$ c.p.m./mμmole); 100 mμmoles/ml. of heat denatured E. coli DNA; and 100 units/ml. DNA polymerase. The incubation was carried out at 37°C. B. The reaction conditions were the same as in part A. except that dDAPTP replaced dATP.

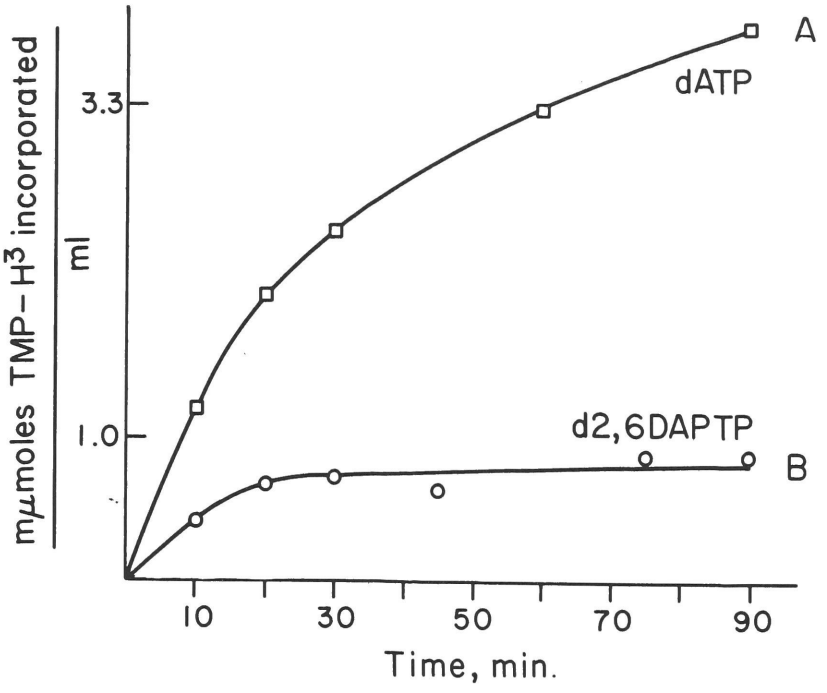


Fig. 13. Utilization of heat denatured T4 DNA by DNA polymrease. A. The complete reaction contained: 66 μmoles/ml. glycine buffer, pH 9.2; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 33 mμmoles/ml. of dATP, dCTP, dGTP, and TTP (1 x $10^3$ c.p.m./mμmole); 100 mμmoles/ml. heat denatured T4 DNA; and 100 units/ml. DNA polymerase. The incubation was carried out at 37°C. B. The reaction conditions were the same as in part A, except that dDAPTP replaced dATP.

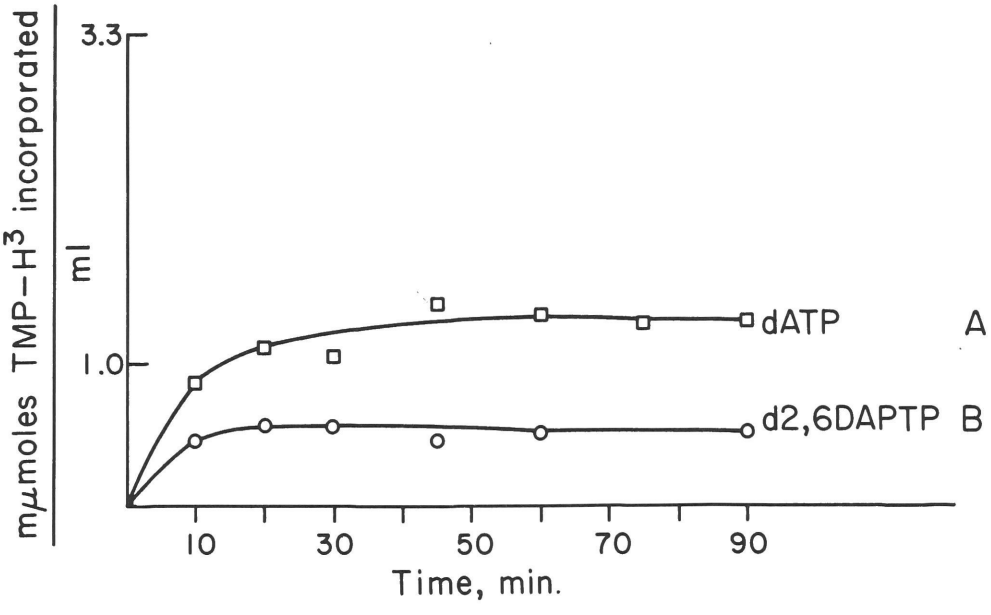


Fig. 14. Utilization of native T4 DNA by DNA polymerase. A. The complete reaction contained: 66 μmoles/ml. glycine buffer, pH 9.2; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 33 mμmoles/ml. of dATP, dCTP, dGTP, and TTP ( 1 x $10^3$ c.p.m./mμmole); 100 mμmoles/ml. native T4 DNA; and 100 units/ml. DNA polymerase. The incubation was carried out at 37°C. B. The reaction conditions were the same as in part A, except that dDAPTP replaced dATP.

Although dDAPTP can replace dATP in all cases tested, the relative effectiveness of the analogue was greatest with calf thymus DNA.

Exonuclease III-degraded DNA as a template for DNA polymerase.

Exonuclease III, isolated from E. coli is a nuclease which degrades from the 3'OH end of helical polynucleotides (Fig. 15). The DNA product of this degradation was found to be an excellent template for DNA polymerase (Richardson, et al. 1964). The data in Fig. 16 are from an experiment in which $T_7$ DNA, previously degraded to the extent of 25% by exonuclease III, functioned as a template for DNA polymerase. The pattern of synthesis characteristically has two slopes. The initial rapid synthesis corresponds to the repair of the exonuclease III degraded portion of the DNA. The second, slower process, corresponds to the synthesis of new strands of DNA.

Under these conditions, dDAPTP effectively replaced dATP in the repair and subsequent synthesis of the degraded $T_7$ DNA (Fig. 16).

Utilization of dDAPTP by DNA polymerase for synthesis of dAT-like polymers.

When dATP and TTP are incubated with DNA polymerase, a polymer (dAT) with a perfectly alternating sequence of A and T residues is produced de novo (Schachman et al. 1960). No polymer synthesis de novo was observed when dDAPTP was used in place of dATP. In fact, when equal concentrations of dATP and dDAPTP were used, the lag period preceding polymer synthesis was increased (Fig. 17). However, when the synthesis was primed with dAT, dDAPTP effectively replaced dATP, yielding a polymer containing DAP and T residues.

The sequence of DAP-containing polymers was established by the nearest neighbor method using RNA polymerase (Fox, C.F. et al. 1964).

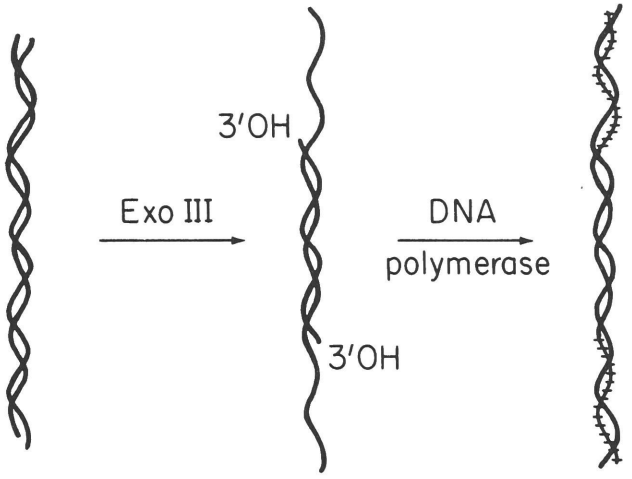


Fig. 15. Exonuclease III degradation of DNA. Exonuclease III isolated from E. coli degrades from the 3'OH end of helical polynucleotides. This degraded DNA can function as a template for DNA polymerase.

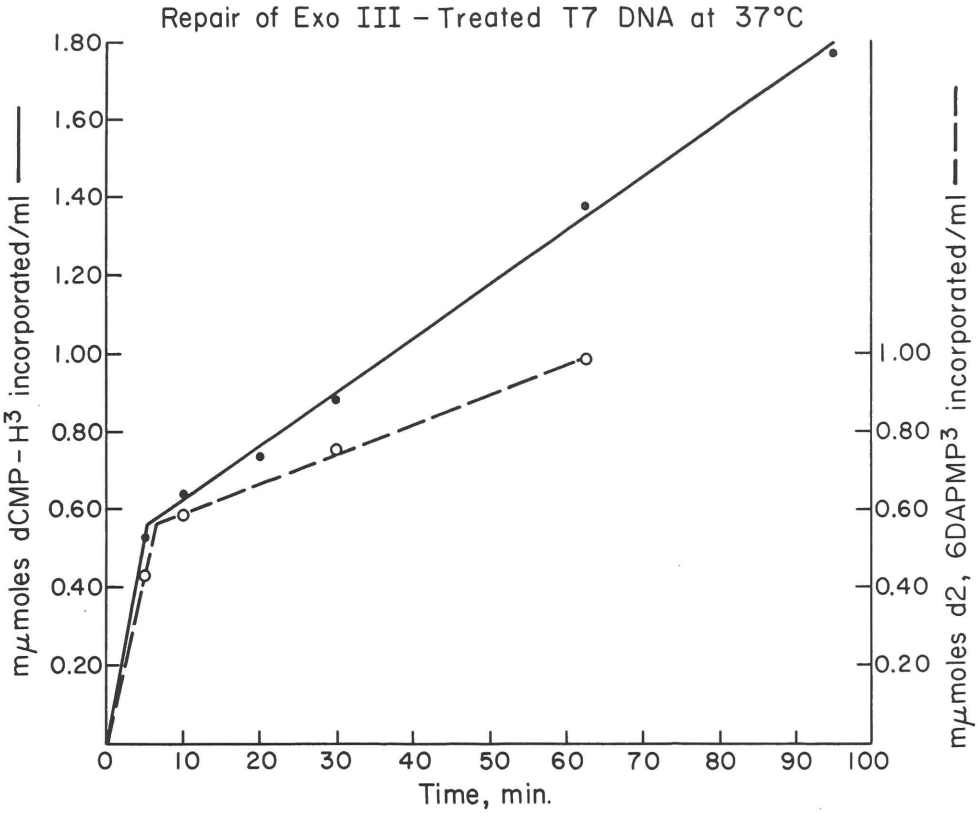


Fig. 16. Exonuclease III degraded DNA as a template for DNA polymerase. A. The complete reaction contained 70 μmoles/ml. potassium phosphate buffer, pH 7.0; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 100 mμmoles/ml. of dATP, TTP, dGTP, and dCTP-$H^3$ ($7.5 \times 10^4$ c.p.m./mμmole); 15 mμmoles/ml. of exo III-degraded T7 DNA (25% degraded); and 30 units/ml. DNA polymerase, Fraction IX. The incubation was carried out at 37°C. B. The reaction conditions were the same as in part A except that 100 mμmoles/ml. of dDAPTP$^{32}$ ($4 \times 10^3$ c.p.m./mμmole) replaced dATP.

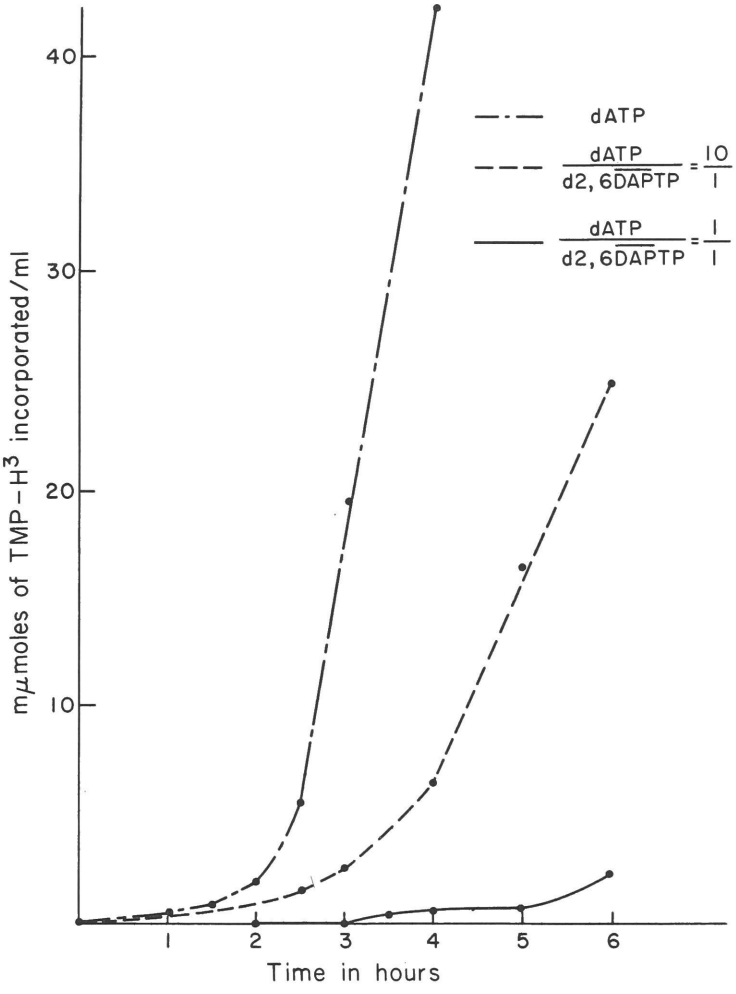


Fig. 17. De novo synthesis of dAT. The three experiments shown above contained the following: 70 μmoles/ml. Tris-HCl buffer, pH 7.0; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; and 30 units/ml. DNA polymerase. In addition, —— - —— contained 400 mμmoles/ml. of TTP-$H^3$ and dATP; —— —— —— contained 400 mμmoles/ml. of TTP-$H^3$ and dATP; and 40 mμmoles/ml. d2,6APTP; ———— contained 400 mμmoles/ml. of TTP-$H^3$ and 200 mμmoles/ml. of dATP and dDAPTP. This incubation was carried out at 37°C for 6 hours.

These polymers were found to have a perfectly alternating sequence of purine and pyrimidine residues.

dAT polymers containing varying amounts of DAP could be obtained by adjusting the ratios of dATP and dDAPTP in the reaction mixture. As seen in Fig. 18, the input ratio is reflected in the composition of the polymer. For example, a polymer containing 50% of the purines as DAP can be obtained by conducting the synthesis with equimolar amounts of dATP and dDAPTP.

Physical characteristics of $\overline{\text{dDAP}}$-T.

The ultraviolet spectrum of $\overline{\text{dDAP}}$-T, isolated as described in Methods, is shown in Fig. 19. The extinction coefficient of the polymer at 260 mμ is 6.8 O.D./μmole nucleotide. A hyperchromicity of 30% at 260 mμ was observed on thermal denaturation. The parent polymer, dAT, showed a hyperchromicity of 45% under identical conditions. Like dAT, $\overline{\text{dDAP}}$-T undergoes apparently complete renaturation on cooling. This is additional evidence for the perfectly regular structure of $\overline{\text{dDAP}}$-T.

The Tm of DNA has been shown by Doty et al. (1959) to be a function of the base composition of the DNA; the higher the G-C content of the DNA, the higher the Tm. This extra stability of the G-C base pair is believed to arise from its ability to form three hydrogen bonds. This is best illustrated in the pair of polymers dG:dC (Tm = 82°) and dI:dC (Tm = 27°). (Inman and Baldwin, 1964). The additional hydrogen bond which can be formed due to the presence of the 2-amino group of guanine yields additional stabilization of 55°C.

If the number of hydrogen bonds per base pair is an important determinant of the stability of DNA helices, then $\overline{\text{dDAP}}$-T, with three assumed hydrogen bonds per base pair should have a higher transition temperature than dAT. Fig. 20 shows that $\overline{\text{dDAP}}$-T (Tm = 66.2°) has a substantially higher Tm than that of dAT (Tm = 41.5°). However, the

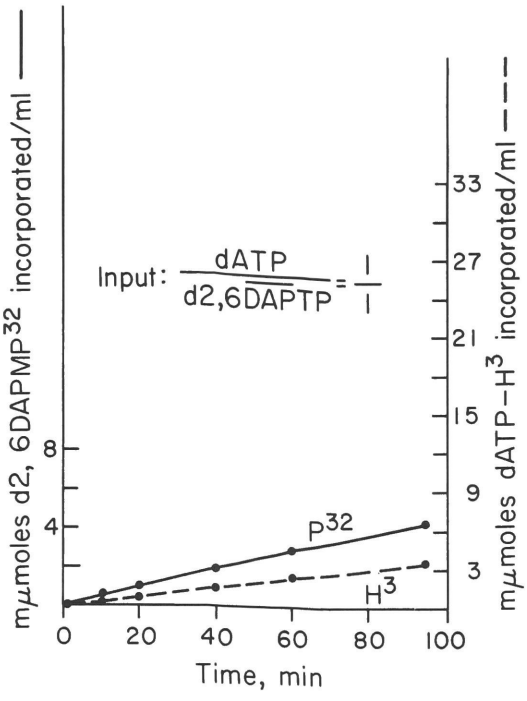


Fig. 18. Synthesis of dAT (50% DAP). The complete reaction contained 70 μmoles/ml. potassium phosphate, pH 7.0; 7 μmoles/ml. $MgCl_2$; 10 mμmoles/ml. dAT primer; 300 mμmoles/ml. TTP; 150 mμmoles/ml. dATP-$H^3$ (3 x $10^4$ c.p.m./mμmole); 150 mμmoles/ml. dDAPTP$^{32}$ (4 x $10^3$ c.p.m./mμmole); and 30 units/ml. of DNA polymerase, Fraction VII. The incubation was carried out at 37°C. for 3 hours.

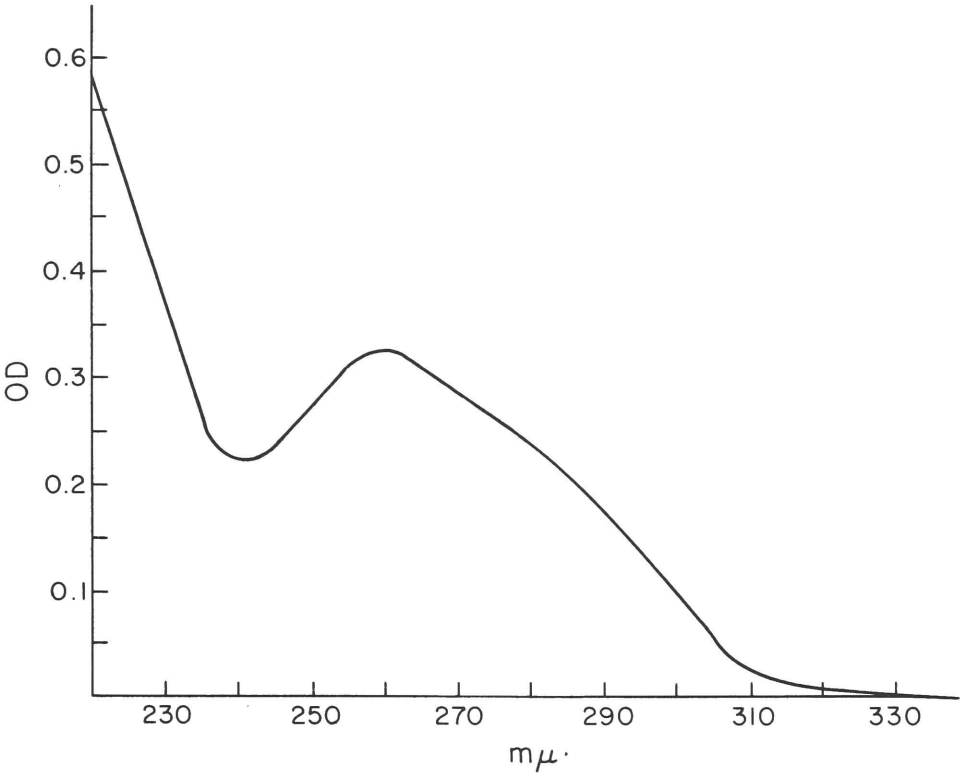


Fig. 19. U.V. spectrum of $d\overline{DAP}$-T.

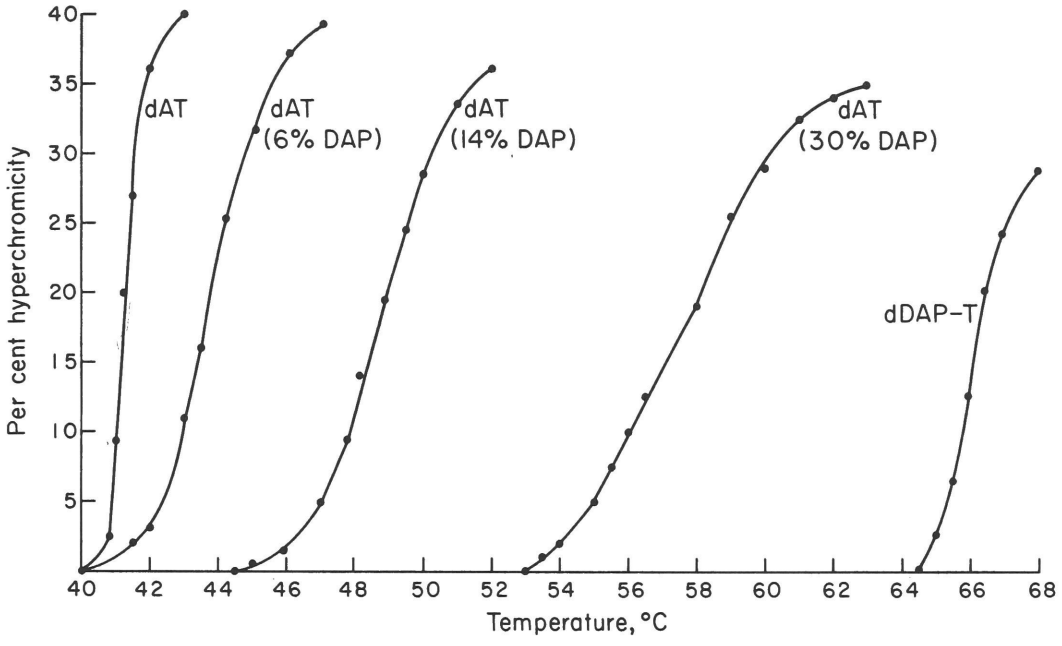


Fig. 20. Thermal denaturation of dAT-like polymers containing DAP: The various dAT-like polymers were dissolved in .01M Tris-HCl buffer, pH 7.9.

absolute increase in Tm due to the third hydrogen bond is not as great as in the case of the dG:dC vs. dI:dC pair. Thus, an increase in the number of hydrogen bonds results in a higher Tm, but the magnitude of this increase cannot be predicted.

A series of dAT-like polymers containing different proportions of DAP were synthesized. The thermal denaturation profiles of this group of polymers is shown in Fig. 20. Two facts are noteworthy: 1) The breadth of the transition increases as the amount of DAP in the polymer increases from 0 - 30%, and then decreases. It is assumed that the width of the transition reflects the heterogeneity of the base pair sequences. 2) The Tm of the polymer is a function of the proportion of DAP. In fact, as seen in Fig. 21, a plot of Tm vs. per cent of purines in dAT as DAP yields a straight line. A similar observation was reported by Doty et al. (1959) for naturally occuring DNAs. However, in contrast to the situation described above, the synthetic polymer dAT does not fall on the line with the native DNAs. This may be attributed to the fact that the polymers being compared in Fig. 21 all have perfectly alternating purine-pyrimidine sequences; whereas the results of a comparison of natural DNAs with dAT are effected by the heterogeneity of the sequences.

The buoyant density in CsCl of $d\overline{DAP}$-T was determined by analytical centrifugation according to Luck and Reich (1964). $d\overline{DAP}$-T, dAT and the bacteriophage DNA SP-8 were dissolved in a solution of CsCl ( $\rho = 1.710$). After 18 hours centrifugation at 44,000 r.p.m., the contents of the analytical cell were examined with the UV optical system. A densitometric tracing of the photograph is shown in Fig. 22. The density of $d\overline{DAP}$-T ( $\rho = 1.717$) differs significantly from that of dAT ( $\rho = 1.679$).

Template properties of dAT-like polymers containing DAP with RNA polymerase.

When dAT-like polymers containing DAP function as templates with RNA polymerase, they direct the synthesis only of rAU. Furthermore, as seen

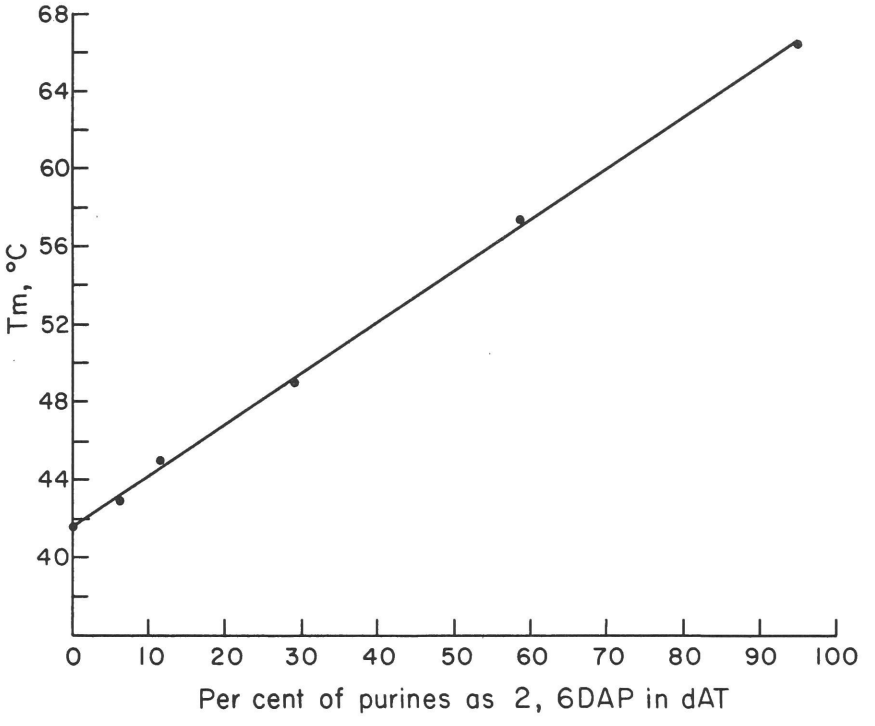


Fig. 21. Tm of dAT-like polymers containing DAP vs. the DAP content of the polymer.

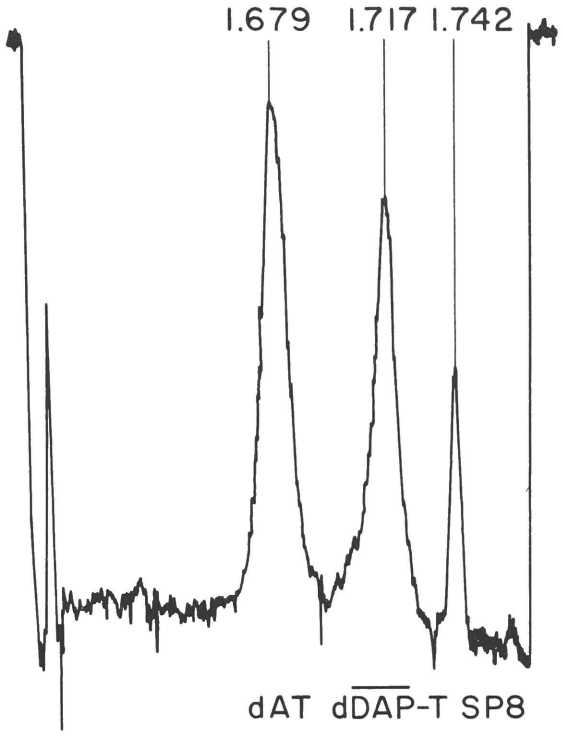


Fig. 22. Analytical equilibrium centrifugation of dDAP-T in CsCl density gradients. The reference markers are bacteriophage SP-8 DNA and synthetic dAT.

in Fig. 23 if rGTP or rCTP was present during the synthesis or rAU directed by $d\overline{DAP}$-T, no significant incorporation of CMP or GMP resulted.

The above results are in disagreement with a model of RNA polymerase function proposed by Margolin et al. (1966). According to this model, the functional groups located in the minor groove direct the selection of the incoming nucleotide. For example, the 2-amino group of guanine and the 2-keto group of cytosine are proposed to direct the incorporation of either GTP or CTP depending on the polarity of synthesis. However, as seen above, DAP which also possesses a 2-amino group in the minor groove, does not direct the incorporation of either C or G. The Margolin model is also inconsistent with the template behavior of dI:dC. Although the I-C base pair resembles an A-T base pair in the functional group distribution in the minor groove, dI:dC directs the synthesis only of poly G, poly I, or poly C.

As will be seen in chapter 3 of this thesis, dAT-like polymers containing 2-amino purine also direct the synthesis only of rAU. Consequently, the functional groups in the major groove do not alone direct the selection of incoming nucleotides. On the contrary, the enzyme allows the full hydrogen bonding capabilities of the template bases to be expressed. In view of these findings, it follows that the selection of bases by RNA polymerase is not directed by the functional groups of the template base which are restricted to either of the two grooves; conversely, the enzyme appears to exploit the full Watson-Crick pairing potential of every template base.

The data in Fig. 24 reveal the template efficiency for RNA polymerase of dAT-like polymers as a function of DAP content. The template efficiency of these polymers has a linear, inverse, correlation with increasing proportion of DAP. (Fig. 25). Since the Tm of these polymers is also a linear function of DAP content, it follows that a similar but inverse relationship exists between template efficiency and Tm.

| | Substrate | Product |
|---|---|---|
| 1. | ATP, UTP-$H^3$ | 26 mμmoles of rAU/ml |
| 2. | ATP, UTP, CTP-$H^3$ | < 0.012 mμmoles CMP incorporated/ml ( < 1 residue of CMP/2150 residues of polymer synthesized) |
| 3. | ATP, UTP, GTP-$H^3$ | < 0.016 mμmoles GMP incorporated/ml ( < 1 residue ofGMP/1600 residues of polymer synthesized) |

**Fig. 23.** **$\overline{\text{dDAP}}$-T as template with RNA polymerase. Enzyme incubations were performed at 37°C for 30 minutes in a final volume of 0.25 ml. containing 10 mμmoles/ml. $\overline{\text{dDAP}}$-T as template.**

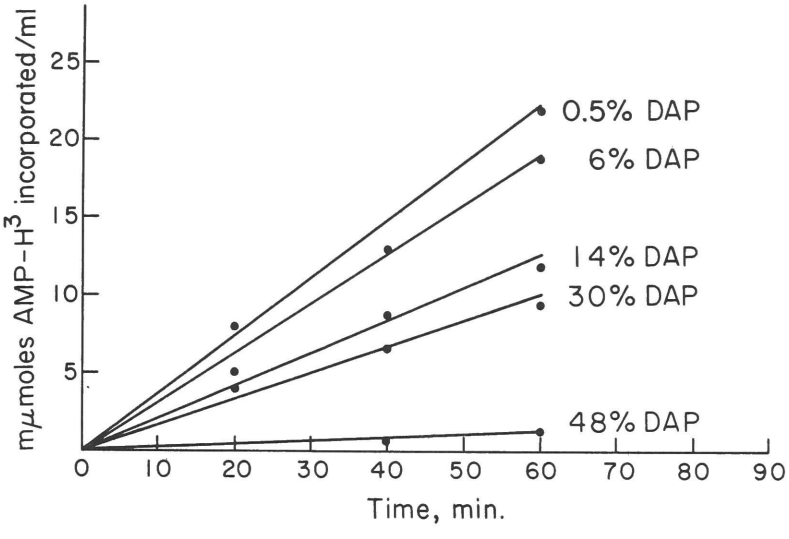


Fig. 24. Template efficiency with RNA polymerase of various dATs containing DAP. The complete reaction contained 40 μmoles/ml. of Tris-HCl buffer, pH 7.9; 4 μmoles/ml. $MgCl_2$; 1 μmole/ml. $MnCl_2$; 10 μmoles/ml. β -mercaptoethanol; 10 mμmoles/ml. of each of the dAT-like polymers containing DAP; and 15 units/ml. RNA polymerase. The incubation was carried out at 37°C for one hour.

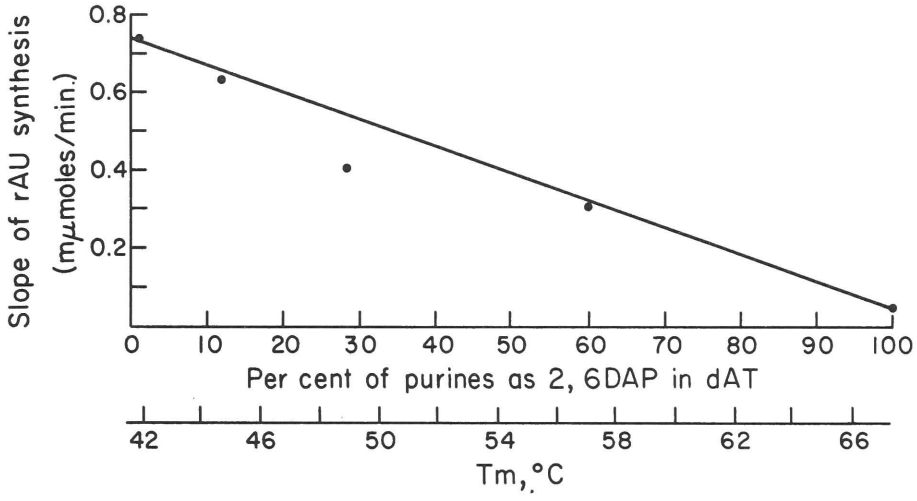


Fig. 25. Template efficiency of dAT-like polymers as a function of DAP content.

Interaction of actinomycin, mithramycin, and nogalamycin with DAP-containing polymers.

The formation of complexes between actinomycin (Fig. 26) and DNA is known to depend on specific structures in both the antibiotic and the polydeoxynucleotide (Reich and Goldberg, 1964). These include the base guanine in the helical conformation of DNA, and the amino group, the quinoidal oxygen and the intact peptide lactones of the antibiotic. A model for the structure of AM-DNA complexes which accounts for the participation of these functional groups has been proposed (Hamilton, et al., 1963 -- Fig. 27). According to this model, AM is located in the minor groove of the DNA helix, where three hydrogen bonds may be formed between the chromophore of the antibiotic and deoxyguanosine in DNA. The specificity of the interaction is attributed to a hydrogen bond between the quinoidal oxygen of AM and the 2-amino group of guanine.

The model has already been subjected to a variety of tests. For example, several lines of evidence show that the N-7 of guanine is probably not involved in complexing AM, since extensive substitution of this position by mustard gas (Reich, 1964) or other alkylating agents (Michelson, 1967) does not diminish the AM binding capacity of DNA; conversely, AM does not affect the rate of alkylation of DNA by mustard gas (Reich, 1964). Another experimental test of the model has been conducted with the synthetic DNA polymer dI:dC (Reich, 1964). The structure of this polymer corresponds exactly to that of dG:dC except for the absence of the 2-amino group of guanine. As judged by the results of spectral and enzymatic assays, the removal of this amino group is correlated with a loss of the ability to interact with AM. These and other findings are therefore fully in accord with predictions which can be derived from the model.

As noted above, the removal of the 2-amino group of guanine from the AM-sensitive G-C base pair yields the AM-resistant I-C pair. The A-T base pair is known not to interact with AM (Reich and Goldberg, 1964); however, the structure of this base pair permits the insertion of an

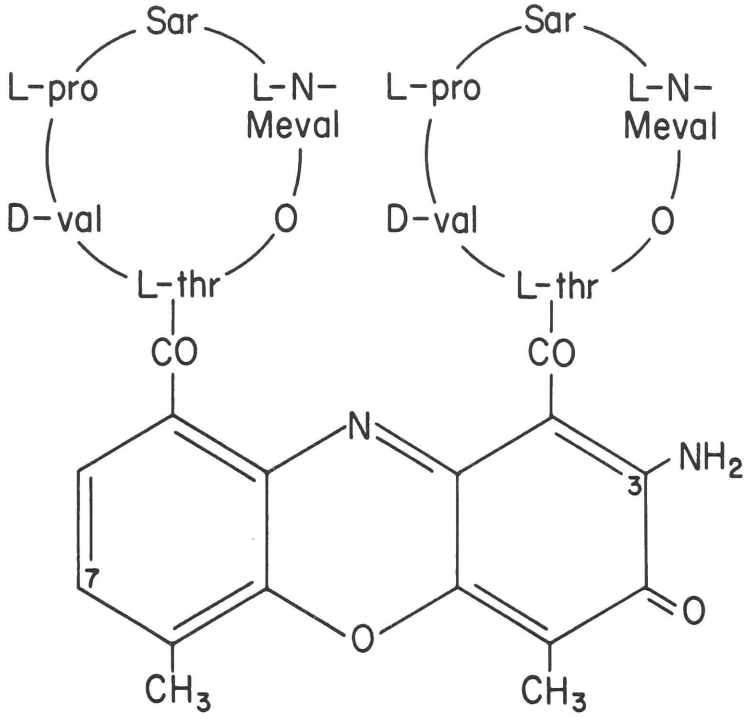


Fig. 26. Structure of actinomycin D: L-thr = L-threonine; D-val = D-valine; L-pro = L-proline; sar = sarcosine; and L-N-meval = L-N-methyl-valine.

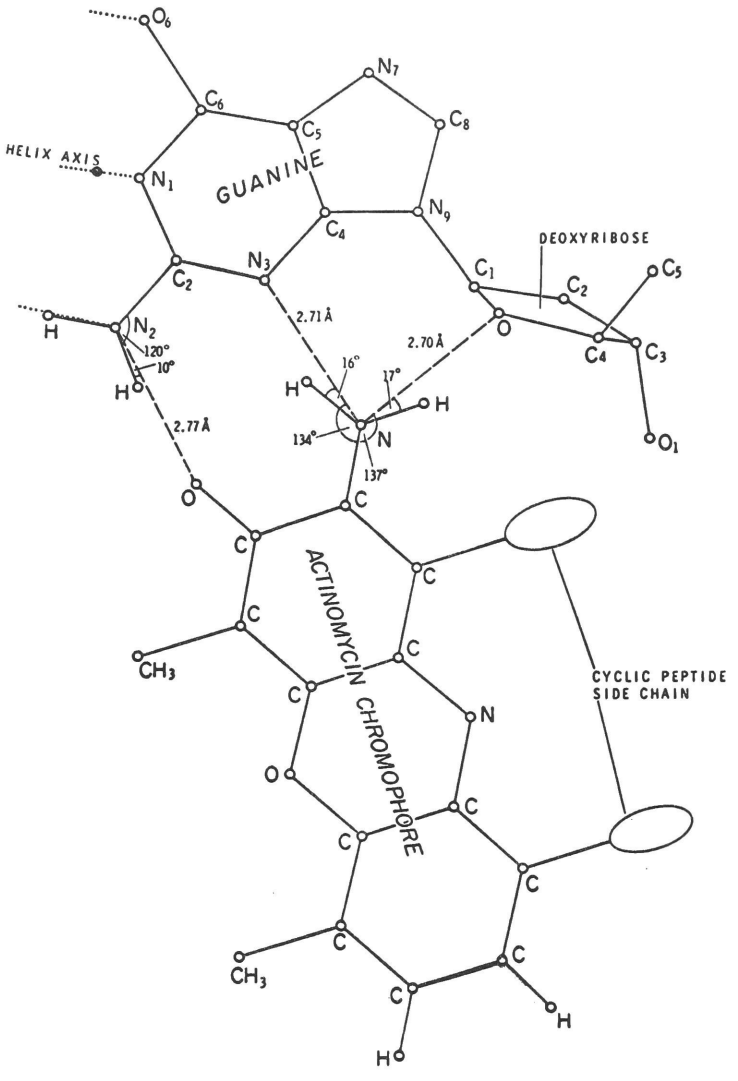


Fig. 27. Proposed model of AM binding to deoxyguanosine of DNA in the B conformation. Hydrogen bond lengths and angles calculated from coordinates measured on skeletal wire models. Hydrogen bonds between AM and DNA indicated by -----. Hydrogen bonds between guanine and cytosine in DNA by ····· Reproduced from Hamilton, et al. (1963).

amino group at the position in the helix normally occupied by the amino group of guanine. Such an insertion can be accomplished by substituting DAP or 2AP for adenine. If the specificity of AM binding is determined simply by a purine amino group suitably located in the minor groove, $\overline{\text{dDAP}}$-T should interact with AM. As predicted by the model, the formation of complexes between $\overline{\text{dDAP}}$-T and AM can be demonstrated by means of a variety of techniques.

Previous work (Ward, et al., 1965) has shown that the mithramycin group of antibiotics, like AM, requires the 2-amino group of guanine for DNA binding; $\overline{\text{dDAP}}$-T also interacts with these.

Effect of actimomycin on buoyant density of $\overline{\text{dDAP}}$-T.

Since binding of AM is associated with significant decreases in the buoyant density of DNA (Kersten, et al., 1966) (Reich and Luck, 1966), a sample of $\overline{\text{dDAP}}$-T containing AM was centrifuged under conditions identical with those used for the experiment illustrated in Fig. 22. No band corresponding to the $\overline{\text{dDAP}}$-T polymer could be seen in the cell containing AM, and this unexpected finding was confirmed on repetition of the run. Since the polymer was radioactive, its presence in the analytic cell in acid precipitable form at the end of centrifugation was verified. This established that no significant degradation had occurred.

In order to characterize more fully the effect of AM, preparative centrifugation in CsCl gradients was performed. The results of such an experiment are shown in Fig. 28. In the absence of AM, the radioactivity of the polymer, which was due to incorporated dDAP-MP$^{32}$, was distributed in a single symmetrical band with a density corresponding to $\rho = 1.715$. The addition of AM caused a remarkable alteration in the sedimentation of the polymer; one half the radioactivity was located at the very top of the gradient where AM normally forms a micelle; the other half was found in small yellow droplets which adhered to the centrifuge tube at the level of the meniscus.

CsCl Density Gradient of d2,6$\overline{DAP}$-T($^{32}$P)

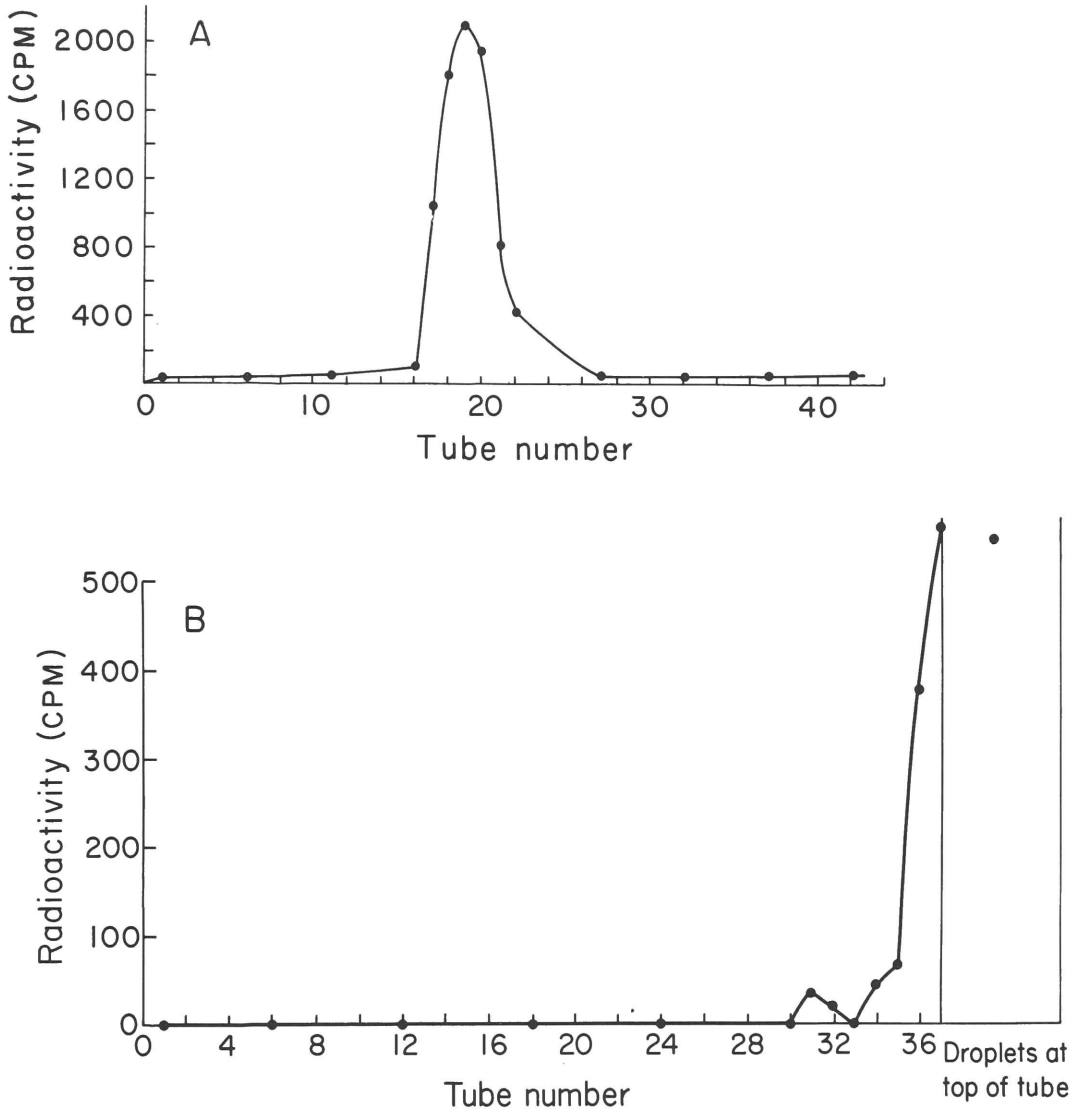


Fig. 28. Preparative equilibrium centrifugation in CsCl density gradients. Each tube contained 5 mls. CsCl ( $\rho$ = 1.710), and 0.14 O.D. units (260 m$\mu$) of radioactive d$\overline{DAP}$-T, a total of 20 m$\mu$moles of polymer nucleotide. A. Control. B. As in A, but containing a total of 20 m$\mu$moles of actinomycin D. The specific radioactivity of the polymer in A was twice that in B. Following 120 hrs. of centrifugation (Spinco SW-39 rotor, 33,000 r.p.m., 25$^{o}$) the tubes were punctured, 2-drop fractions were collected, and the acid insoluble radioactivity of the indicated samples was determined. The density of fraction 19 (Fig. A) corresponded to $\rho$ = 1.715.

As a result of these observations, analytical density gradient centrifugation was performed on a polymer with a purine ratio of A/DAP = 10. As seen in Fig. 29, the buoyant density of this polymer is indistinguishable from that of dAT. The addition of AM to the centrifuge cell leads to the appearance of a new, symmetrical band at a density $\rho = 1.661$ which differs significantly from that of the control specimen. This is additional evidence for the interaction of AM with DAP incorporated in a polydeoxynucleotide.

Effect of AM on thermal denaturation of $d\overline{DAP}$-T.

Previous work (Haselkorn, 1964), (Reich, 1964), has shown that bound AM significantly stabilizes DNA to denaturation by heat; elevations of the transition temperature (Tm) due to AM of up to $12^{o}$-$15^{o}$C have been observed with naturally occurring native DNAs. The data in Fig. 30 show a substantial increase in Tm of dAT containing 10% DAP on addition of AM; the corresponding increase for $d\overline{DAP}$-T (Fig. 31) is almost $40^{o}$ -- a change far greater than that previously recorded for any DNA.

Spectral changes in actinomycin solutions produced by $d\overline{DAP}$-T.

The change in the spectrum of AM solutions which occurs on addition of guanine-containing DNAs is a characteristic parameter of the AM-DNA interaction (Goldberg, et al., 1962). The difference spectrum shown in Fig. 32 demonstrates that $d\overline{DAP}$-T, like DNA, produces alterations in the spectrum of AM.

Template function of $d\overline{DAP}$-T with RNA polymerase: effect of antibiotics.

Although a much less effective template than dAT, $d\overline{DAP}$-T directs the formation of RNA containing only A + U in perfectly alternating sequence with E. coli RNA polymerase. The synthesis of RNA is linear for several hours; the concentration of polymer selected for the experiment reported here was on the linear portion of the velocity/template relationship.

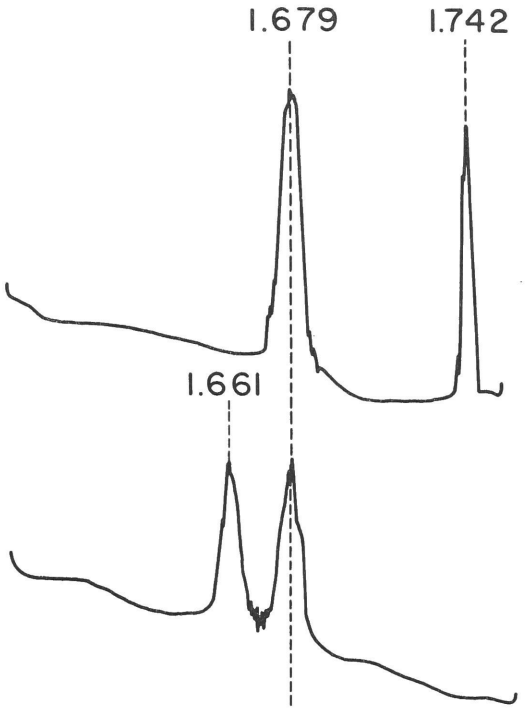


Fig. 29. Analytical equilibrium centrifugation of dAT (5% DAP) in CsCl density gradient. The analytic cell corresponding to the upper densitometer tracing contained dAT (5% DAP) 0.01 O.D. unit (260 mμ); pure dAT and bacteriophage SP-8 DNA were present as reference standards. The buoyant density of dAT (5% DAP) $\rho$ = 1.679 is not detectably different from that of pure dAT. The cell corresponding to the lower tracing contained dAT (5% DAP) and pure dAT as above, with added actinomycin (4 mμmoles/ml.). A new band is seen at $\rho$ = 1.661. This band is considered to represent dAT (5% DAP) with bound actinomycin. Actinomycin is known not to effect the buoyant density of dAT.

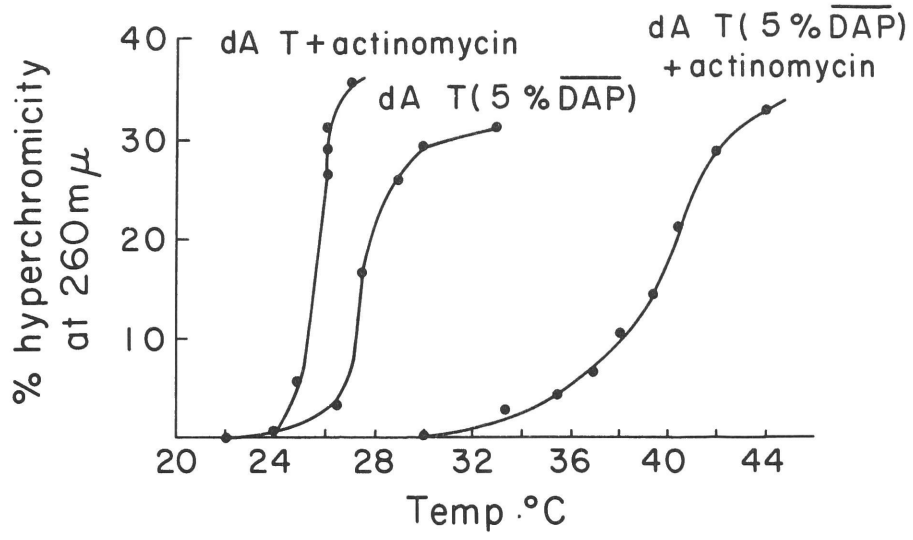


Fig. 30. Thermal denaturation of dAT (5% DAP): Effect of actinomycin. dAT (5% DAP) 36 mμmoles/ml. was dissolved in 0.001M $Na^+$. AM, where present, was added to a final concentration of 6.1 mμmoles/ml; this concentration of AM did not alter the Tm of pure dAT.

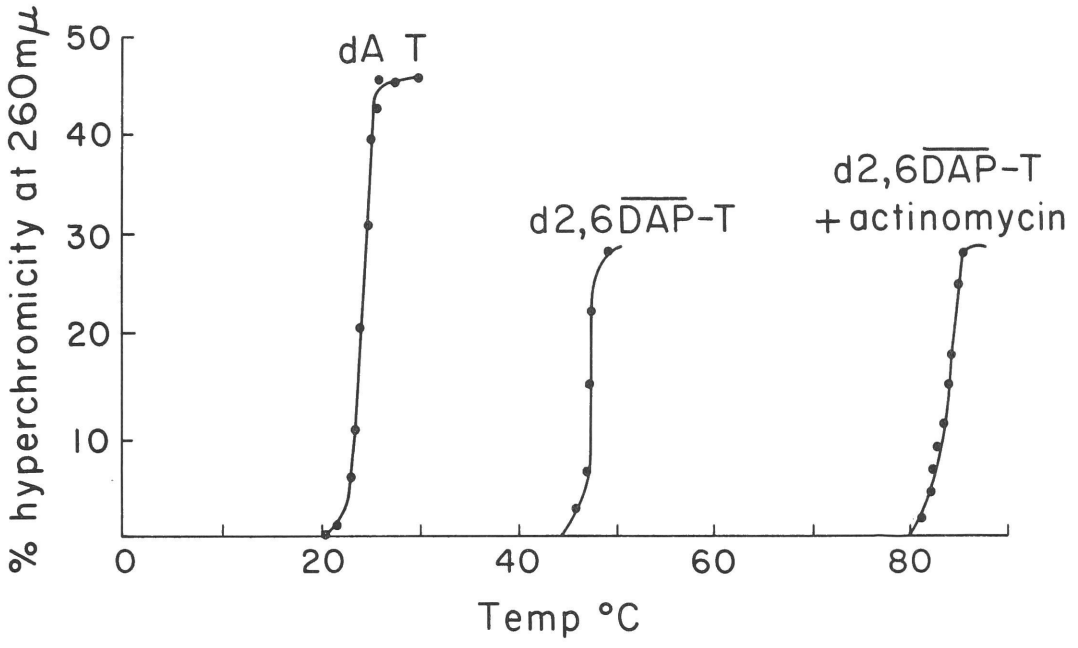


Fig. 31. Thermal denaturation of dDAP-T: Effect of actinomycin. dDAP T, 54 mμmoles/ml., was dissolved in 0.001 M $Na^+$. Where present, AM was added to a final concentration of 10.5 mμmoles/ml.

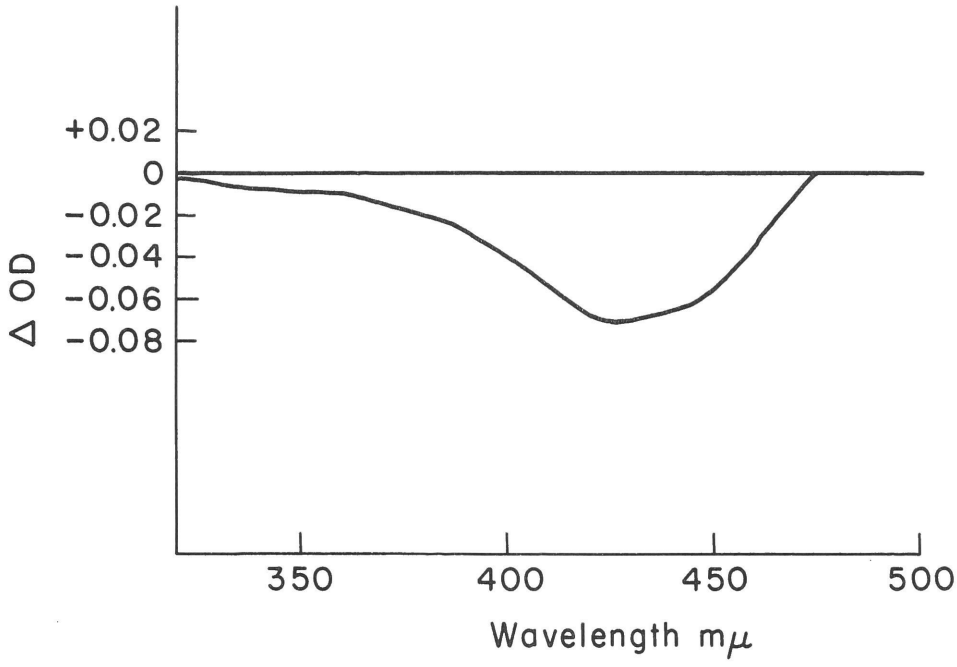


Fig. 32. Difference spectrum of AM in the presence of $\overline{\text{dDAP}}$-T. A solution of AM (0.85 O.D./ml. at 440 mμ) containing $\overline{\text{dDAP}}$-T (24 mμmoles/ml.) was read against a control solution of AM in Cary Model 14 spectrophotometer.

The effect of AM on RNA synthesis directed by $\overline{\text{dDAP}}$-T and by dAT (5% DAP) is shown in Fig. 33. The template function of both polymers is progressively inhibited by increasing concentrations of AM. The ratio of AM/DNA-nucleotide which provides 50% inhibition of synthesis is 1:100 for $\overline{\text{dDAP}}$-T, and 1:100 for dAT (5% DAP); whereas this ratio for native pneumococcal DNA is 1:800. Two points concerning the effect of AM on $\overline{\text{dDAP}}$-T may be noted. The first is that a small fraction of RNA synthesis appears absolutely resistant to AM. Secondly, the inhibition curve shows a single, very steep slope as the concentration of AM is increased. With naturally occurring native DNAs the effect of AM is usually biphasic -- an initial steep decline is seen at low AM concentration followed by a more gradual inhibition (Goldberg, et al., 1962), (Hurwitz, et al., 1962). It is suggested that this difference may be due to the greater heterogeneity of the structure, sequences and configuration of pneumococcal DNA as compared with the perfectly regular structure of $\overline{\text{dDAP}}$-T.

Since the specificity of the mithramycin-DNA interaction closely resembles that previously established for AM (Ward, et al., 1965), the effect of mithramycin on the template functions of $\overline{\text{dDAP}}$-T was examined. As seen in Fig. 34, mithramycin strongly inhibits RNA synthesis directed by $\overline{\text{dDAP}}$-T. As in the case of AM, a single, steep slope of inhibition is observed, and a totally antibiotic-resistant component remains.

Nogalamycin binds to A-T base pairs in native DNA, particularly when these are arranged in alternating sequences characteristic of dAT (Bhuyan and Smith, 1965). It was of interest to determine whether the DAP-T base pair, which resembles the G-C pair in its stability, in having a purine amino group in the minor groove, and in the presumed formation of three hydrogen bonds, would interact with nogalamycin. The data in Fig. 35 show that nogalamycin strongly inhibits the template function of $\overline{\text{dDAP}}$-T. In contrast to the results with AM and mithramycin, no antibiotic-resistant synthesis is apparent. Since nogalamycin complexes strongly

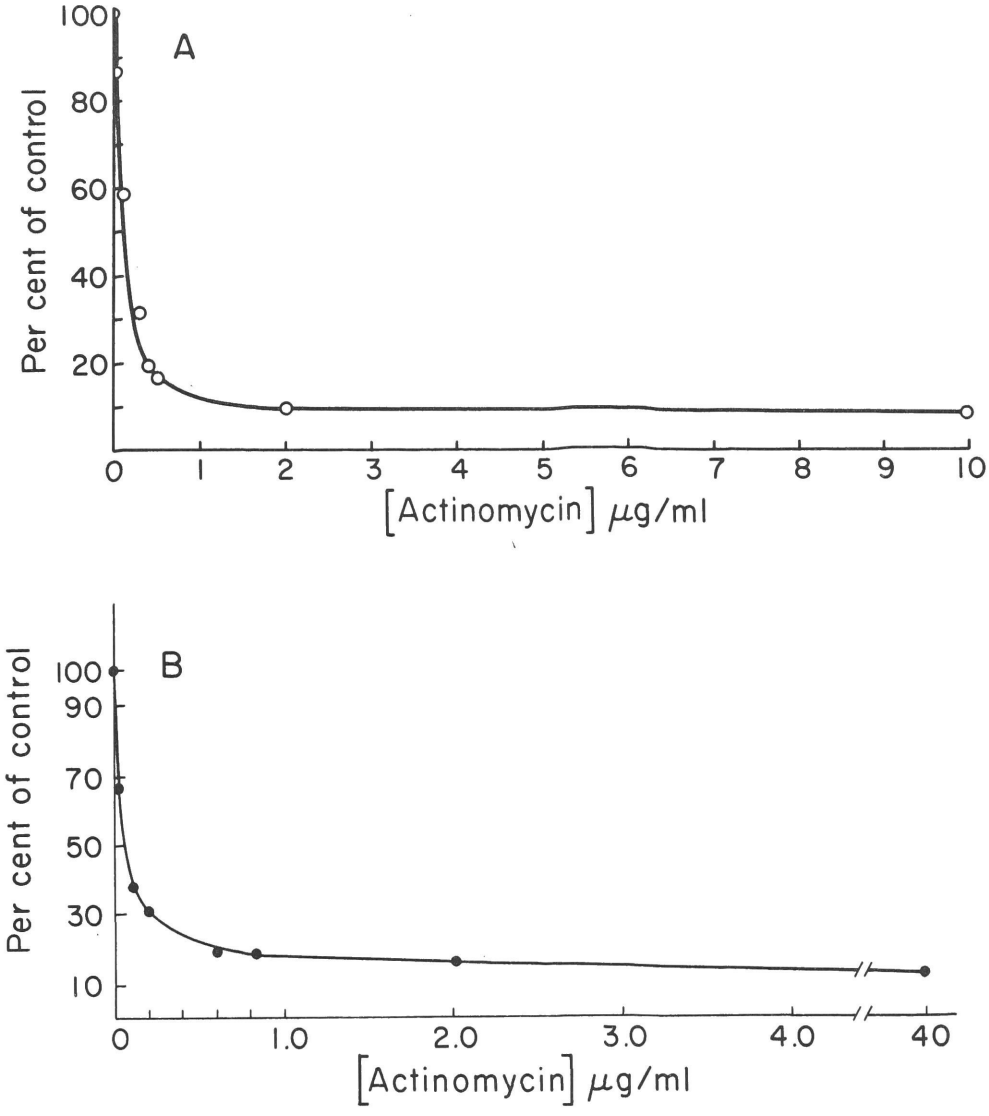


Fig. 33. Effect of AM on RNA synthesis directed by (A) $\overline{\text{dDAP}}$-T and (B) dAT (5% DAP). Enzyme incubations were performed at 37°C for 30', in a final volume of 0.125 ml., containing 9 mμmoles/ml. of the respective polymer templates. 100% incorporation corresponds in (A) to 11.6 mμmoles/ml. of $H^3$-UMP and in (B) to 39.2 mμmoles/ml. $H^3$-AMP.

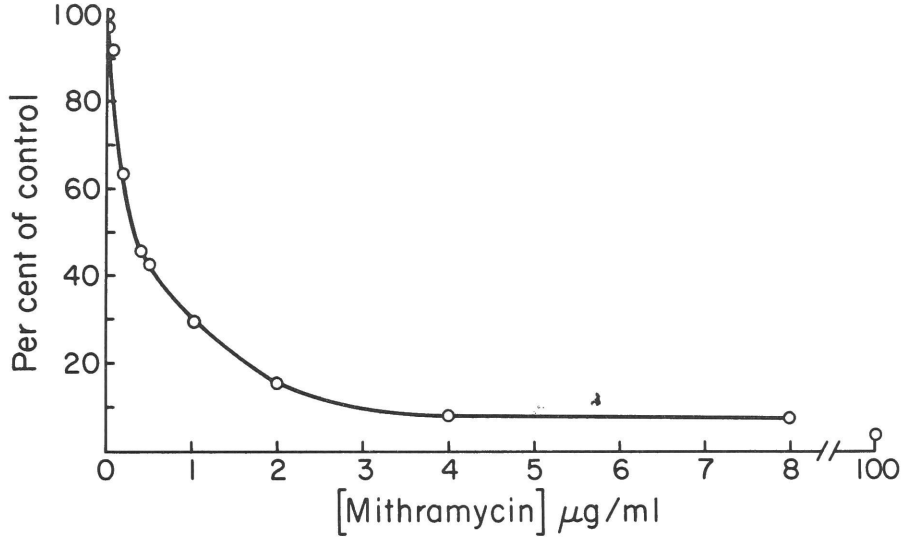


Fig. 34. Effect of Mithramycin on RNA synthesis directed by $\overline{\text{dDAP}}$-T. Conditions as for Fig. 33. 100% incorporation corresponds to 10.7 mμmoles/ml. of $H^3$-UMP.

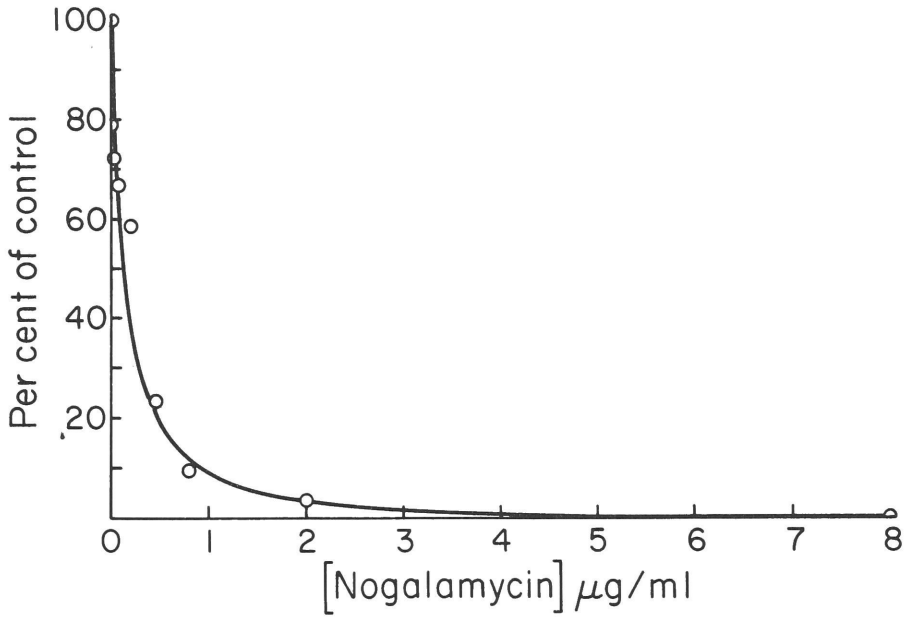


Fig. 35. Effect of Nogalamycin on RNA synthesis directed by $d\overline{DAP}$-T. Conditions as for Fig. 33. 100% incorporation corresponds to 11.9 mμmoles/ml. of $H^3$-UMP.

with dAT, whereas AM and mithramycin do not, the AM-resistant and mithramycin-resistant syntheses are considered to represent the template activity of the residual dAT which was used to prime dDAP-T formation.

Discussion and summary.

Due to the location of its two amino groups, DAP is structurally similar in some respects to both adenine and guanine. However, in its substrate behavior with both DNA polymerase and RNA polymerase (Ward, et al., 1967), DAP is found to function only as an adenine analogue. The resulting DAP-T base pair, like the G-C pair, might be expected to form three hydrogen bonds. The higher Tm of dDAP-T, as compared with dAT, is consistent with this expectation. In fact, a linear relation is observed between the Tm and the DAP content of dAT-like polymers. It is of interest that the formation of the additional hydrogen bond in the DAP-T base pair, as in the G-C base pair, is correlated also with a substantial increase in buoyant density of the polymer.

dAT-like polymers containing DAP function as templates with RNA polymerase and direct the formation of RNA containing only A and U in perfectly alternating sequences. Moreover, the template efficiency of dAT-like polymers is inversely related to their DAP content. Thus the template efficiency is a linear function of the Tm of the polymer.

The outstanding property which DAP-T, 2AP-T and G-C base pairs have in common is a purine 2-amino group and its location in the minor groove of helical DNA. The presence of this amino group permits these dAT-like polymers to interact with actinomycin. The following properties are characteristic of the interaction of AM with naturally occurring DNAs:

1) The spectrum of the antibiotic changes.
2) The buoyant density of the polymer changes.
3) The thermal stability of the polymer is increased.
4) The template function of the polymer with RNA polymerase is inhibited.

The alternating polymers which contain either DAP or 2AP faithfully reproduce all the above parameters of actinomycin-DNA complexes.

dAT does not interact with actinomycin; thus, the introduction of a purine 2-amino group into the minor groove of helical DNA is sufficient for converting a base pair from actinomycin resistance to actinomycin sensitivity.

# CHAPTER III

The second part of this thesis is concerned with the purine analogue 2-amino purine. This purine was selected for study for two reasons. First it was hoped that the incorporation of 2AP into dAT-like polymers would provide a test of the model of AM-DNA complexes as in the case of DAP. Because 2AP shares with DAP and G an amino group at the two position, binding of AM to 2AP-containing polymers would provide strong evidence in favor of the model. The second reason is based on the established mutagenic action of this analogue. Since Gottschling and Freese (1961) had found that 2AP was incorporated into bacterial and bacteriophage DNA, it seemed feasible to study the substrate behavior of d2APTP with D. coli DNA polymerase with the hope of obtaining polymers containing this base.

The data below show that the substrate properties of d2APTP for DNA polymerase and some of the physical characteristics of polynucleotides containing 2AP are unusual. The interaction of actinomycin with dAT-like polymers containing 2AP will also be described.

## The substrate properties of d2APTP with DNA polymerase.

As seen in Fig. 36, 2AP can pair with T to form two hydrogen bonds of the Watson-Crick type, but only one hydrogen bond can be formed with C. Since the incorporation of nucleotides by nucleic acid polymerases is known to require the formation of at least two hydrogen bonds, 2AP would be expected to behave exclusively as an analogue of A. This expectation is fulfilled as shown by the data in Fig. 37a. With denatured calf thymus DNA as primer for DNA polymerase, d2APTP could substitute effectively for dATP. However, with other primers such as E. coli or $T_4$ DNA, d2APTP was a much less efficient substrate (Fig. 37 b,c,d; Fig. 38).

An interesting aspect of d2APTP utilization is the lag period seen in Fig. 37 a, B. Such a lag period was also observed with denatured calf thymus

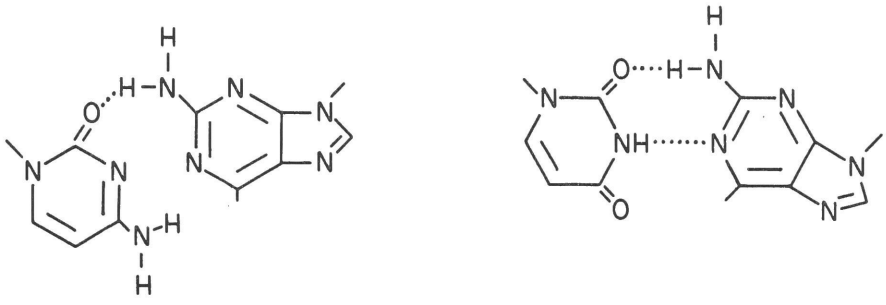

Fig. 36. Hydrogen-bonding properties of 2AP-T and 2AP-C.

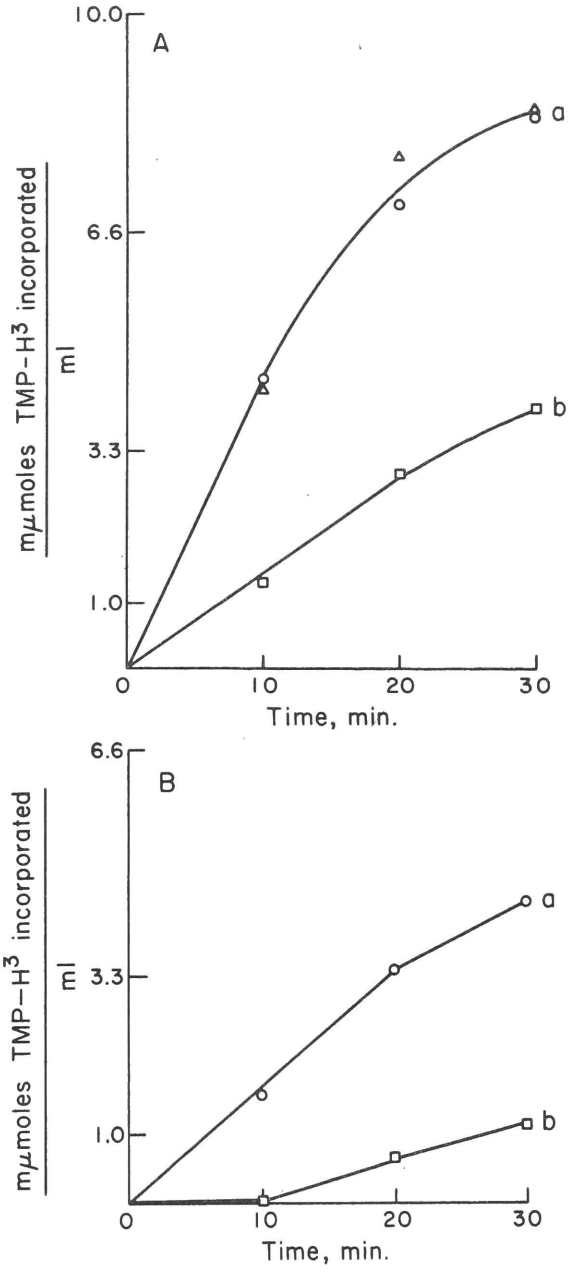


Fig. 37a. Utilization of denatured calf thymus DNA by DNA polymerase. A. The complete reaction contained 66 μmoles/ml. glycine buffer, pH 9.2; 7 μmoles $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 33 mμmoles of dATP, TTP, ($1 \times 10^3$ c.p.m./mμmole), dCTP, dGTP; 100 mμmoles heat-denatured calf thymus DNA; in addition (a) contained 200 units/ml. DNA polymerase and (b) contained 50 units/ml. DNA polymerase. B. The reaction conditions are the same as in part A except that d2APTP replaced dATP.

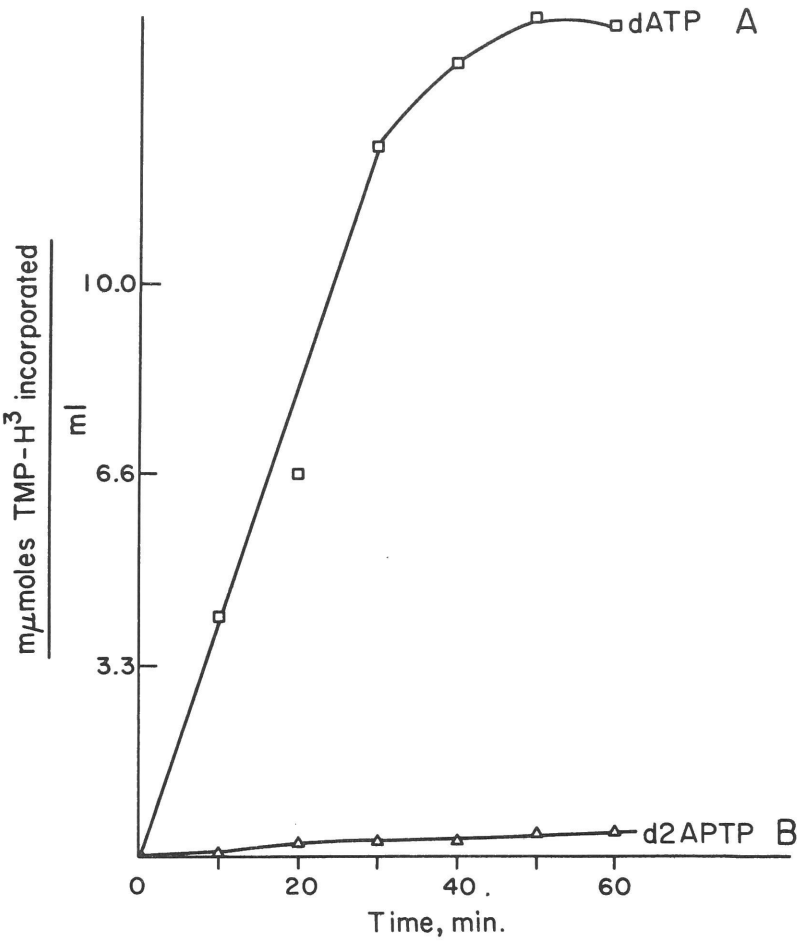


Fig. 37b. Utilization of denatured E. coli DNA by DNA polymerase. A. The complete reaction contained: 66 μmoles/ml. glycine buffer, pH 9.2; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 33 mμmoles/ml. of dATP, dCTP, dGTP, and TTP ($1 \times 10^3$ c.p.m./mμmole); 100 mμmoles/ml. heat-denatured E. coli DNA; and 100 units/ml. DNA polymerase. The incubation was carried out at 37°C. B. The reaction conditions were the same as in part A, except that d2APTP replaced dATP.

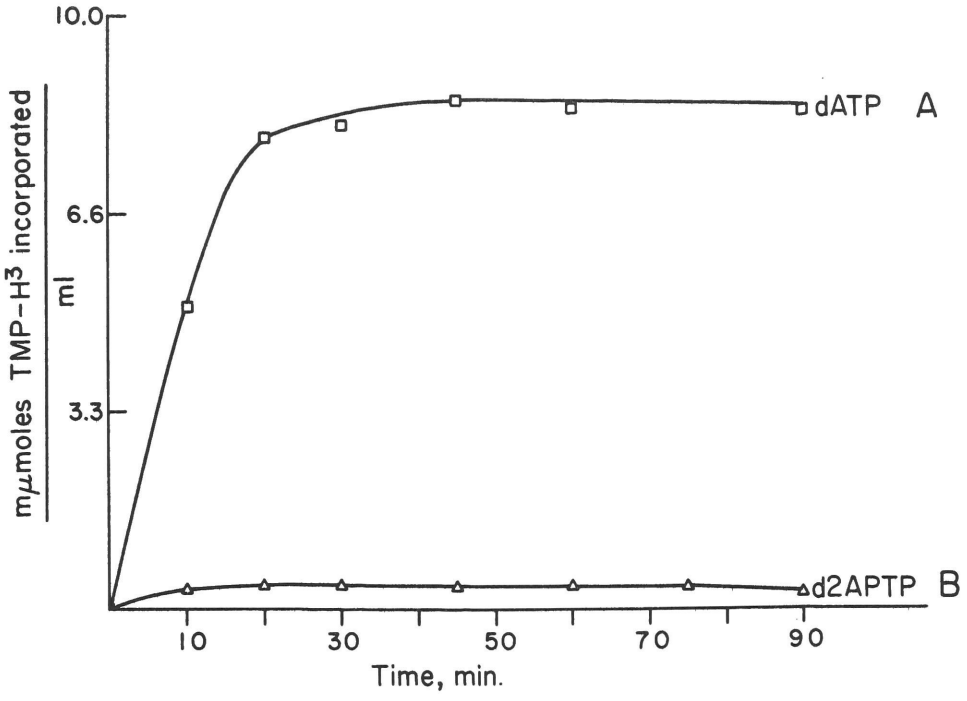


Fig. 37c. Utilization of native E. coli DNA by DNA polymerase. A. The complete reaction contained: 66 μmoles/ml. glycine buffer, pH 9.2; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 33 mμmoles/ml. of dATP, dCTP, dGTP and TTP-$H^3$ (1 x $10^3$ c.p.m./mμmole); 100 mμmoles/ml. native E. coli DNA; and 100 units/ml. DNA polymerase. The incubation was carried out at 37°C. B. The reaction conditions were the same as in part A, except that d2APTP replaced dATP.

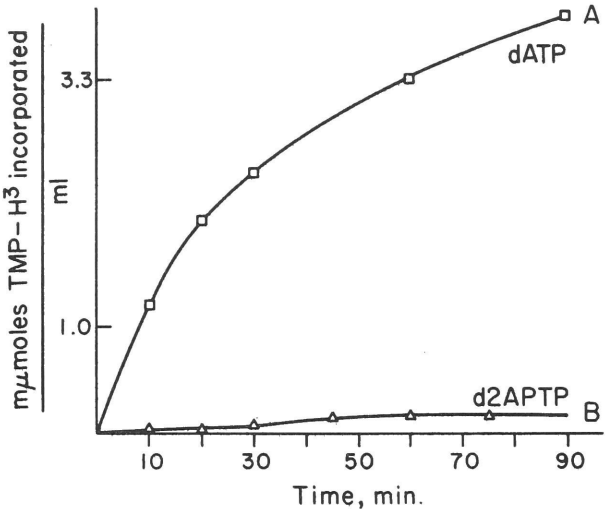


Fig. 37d. Utilization of heat denatured $T_4$ DNA by DNA polymerase. A. The complete reaction contained: 66 μmoles/ml. glycine buffer, pH 9.2; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 33 mμmoles/ml. of dATP, dCTP, dGTP, and TTP ($1 \times 10^3$ c.p.m./mμmole); 100 mμmoles/ml. heat denatured $T_4$ DNA; and 100 units/ml. DNA polymerase. The incubation was carried out at 37°C. B. The reaction conditions were the same as in part A, except that d2APTP replaced dATP.

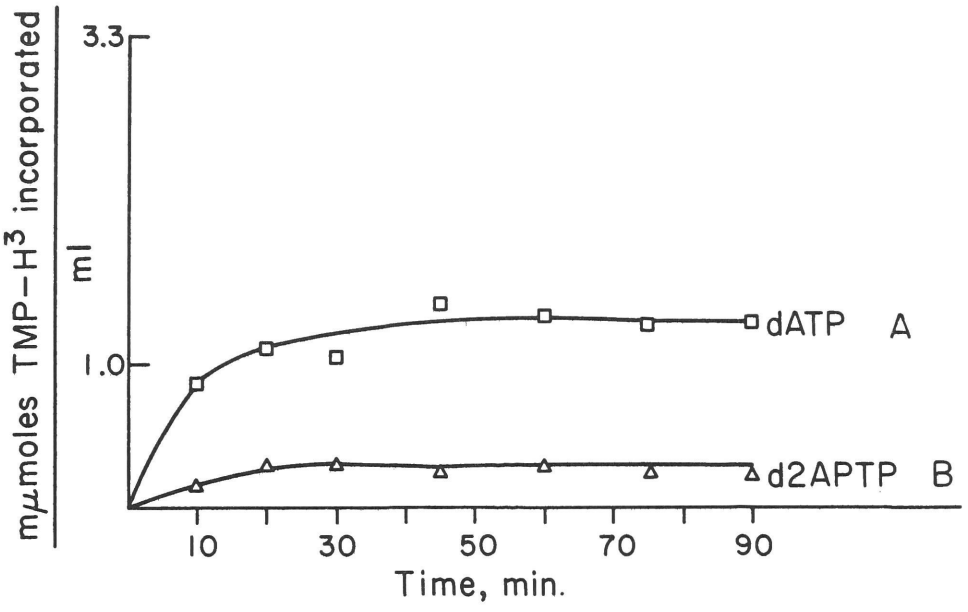


Fig. 38. Utilization of native $T_4$ DNA by DNA polymerase. A. The complete reaction contained: 66 μmoles/ml. glycine buffer, pH 9.2; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 33 mμmoles/ml. of dATP, dCTP, dGTP and TTP ($1 \times 10^3$ c.p.m./mμmole); 100 mμmoles/ml. native $T_4$ DNA; and 100 units/ml. DNA polymerase. The incubation was carried out at 37°C. B. The reaction conditions were the same as in part A, except that d2APTP replaced dATP.

DNA primer when the enzyme concentration was lowered. This suggests that some enzyme activity other than DNA polymerase is required for incorporation of d2APMP.

Another unexpected finding was observed when an attempt was made to replicate the synthetic polynucleotide dAT using d2APTP instead of dATP. Under conditions where dAT was replicated manyfold, no product was formed with d2APTP and TTP. No product was formed de novo from d2APTP and TTP under conditions which permitted the rapid de novo synthesis of dAT.

In crystallographic studies of purine-pyrimidine complexes, Sobel (1967) was unable to obtain complexes of A-substituted 2AP with either U or T. However, complexes were formed between 2AP derivatives and 5-halogenated pyrimidines; and the analysis showed that the complexes consisted of base pairs of the Watson-Crick type. With this in mind, several attempts were made to produce 2AP polymers using templates containing 5-bromo dUMP, or 5-bromo dUTP as substrates together with d2APTP, or both. The incorporation of 2AP into polymers was not observed in any case in the absence of dATP (Fig. 39).

Although no polymers were formed in which 2AP was the only purine, alternating dAT-like polymers containing both A and 2AP could be isolated in low yield. By adjusting the ratio d2APTP:dATP, polymers in which 2AP represents respectively 10% of the purines (input -- 2AP/A = 1) and 25% of the purines (input -- 2AP/A = 10) were obtained. Thus, the synthesis of polymers by DNA polymerase in the presence of high concentrations of d2APTP is very poor. All attempts to increase the yield of polymer failed. For example, as shown in Fig. 5, the addition of a further quantity of dATP following exhaustion of the initial input of dATP permitted further synthesis. However, a second addition of dATP did not stimulate polymer formation.

These results show 1) that d2APTP functions as a substrate analogue of dATP for DNA polymerase; and 2) that its utilization is dependent on

| Template | dNTP present | mμmoles dAMP inc./ml. | mμmoles d2APMP inc./ml. |
|---|---|---|---|
| dABrU | dATP, TTP | 13.5 mμmoles | -- |
| dABrU | d2APTP, TTP | -- | .07 |
| dABrU | dATP, d2APTP, TTP | 11.5 | .23 |
| dABrU | dATP, dBrUTP | 12.5 | -- |
| dABrU | d2APTP, dBrUTP | -- | .06 |
| dABrU | d2APTP, dATP, dBrUTP | 8.3 | .07 |
| dAT | dATP, dBrUTP | 9.5 | -- |
| dAT | d2APTP, dBrUTP | -- | .01 |
| dAT | dATP, d2APTP, dBrUTP | 10 | .32 |

The complete reaction contained 70 μmoles/ml. potassium phosphate buffer, pH 7.0; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 30 units/ml. DNA polymerase (Fraction IX); 20 mμmoles/ml. of the respective template, and 100 mμmoles/ml. of the respective dNTP.

Fig. 39.

the DNA primer in the reaction. One possibility is that d2APTP functions well only in certain base sequences since it has been argued, though with little experimental support, that incorporation of bases is subject to the influence of nearest neighbors. To test this idea polymers containing various defined sequences were used as primers. Fig. 40 shows that dA:dT, like dAT, directs the incorporation of d2APTP when dATP is present. No d2APTP is incorporated in the absence of dATP. A similar result was obtained with dAC:dGT (Fig. 41) as primers. Thus in all the cases, significant utilization of d2APTP requires the simultaneous presence of dATP.

It has been proposed that 2AP can substitute for G as well as A during DNA synthesis in vivo. However, no significant replacement of dGTP by d2AP-TP could be seen with dAC:dTG or dAG:dTC primers. To determine whether d2APTP can act as an analogue of dGTP in the presence of dGTP, the synthetic polymers dG:dC and dI:dC were selected as primers. As seen in Fig. 42, the presence of d2APTP during dG:dC synthesis does not yield any significant incorporation of $d2APMP^{32}$ (one residue of $d2APMP^{32}$/2400 residues of CMP-$H^3$). The same level of incorporation of $d2APMP^{32}$ is found during replication of the synthetic polymer dI:dC (Fig. 43).

Properties of dAT-like polymers containing 2AP.

The U.V. spectrum of dAT(12.5% 2AP) is shown in Fig. 44. The presence of 2AP is established by its characteristic absorption maximum at 303 mμ. Upon heat denaturation a hyperchromicity of only 20% is observed with dAT (12.5% 2AP); this may be compared with the value of 45% found for dAT. In addition to reduced hyperchromicity, the denaturation profile of the polymer is altered. In contrast to the normal narrow sigmoid curve obtained with alternating polynucleotides, a broad linear increase in absorption occurs at a lower temperature (Fig. 45).

In order to characterize the sequences containing 2AP, dAT (12.5% 2AP), a nearest neighbor analysis (Josse, et al., 1961) was performed

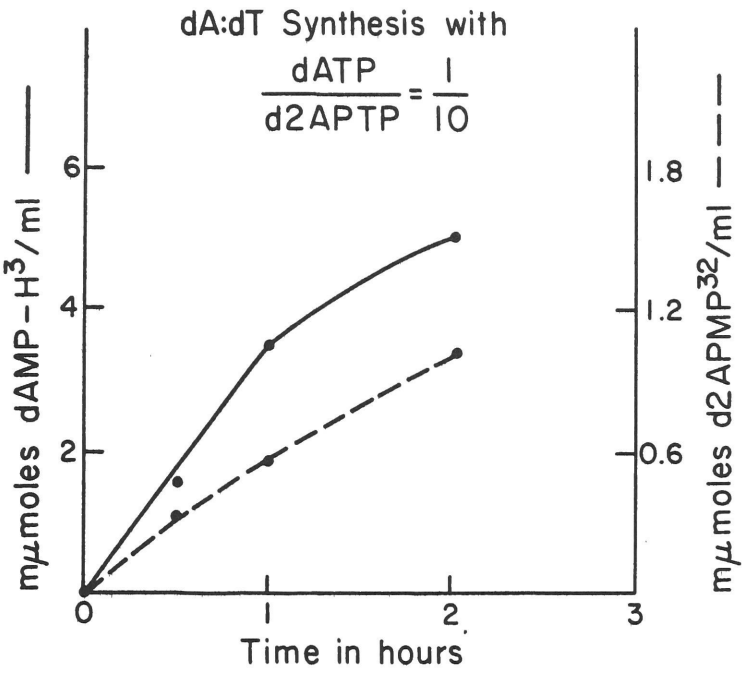


Fig. 40. Synthesis of dA:dT containing 2AP. The complete reaction contained 70 μmoles/ml. potassium phosphate buffer, pH 7.0; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 20 mμmoles/ml. dA:dT primer; 100 mμmoles/ml. $d2APTP^{32}$ ($1 \times 10^5$ c.p.m./mμmole), and TTP; 10 mμmoles/ml. $dATP\text{-}H^3$ ($3 \times 10^4$ c.p.m./mμmole); and 60 units/ml. DNA polymerase (Fraction VII). The incubation was carried out at 37°C for 3 hours.

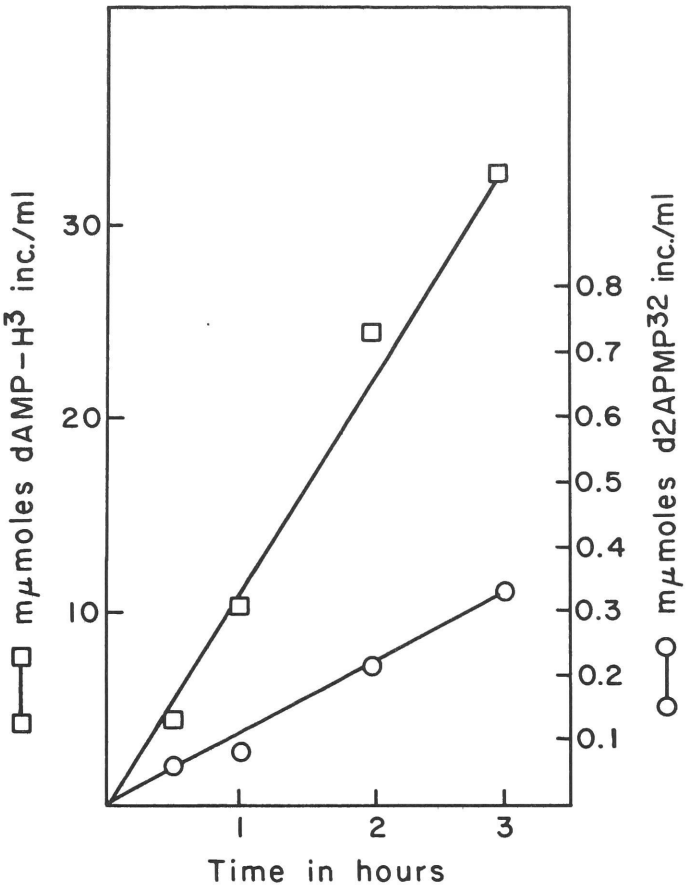


Fig. 41. Synthesis of dAC:dTG containing 2AP. The complete reaction contained 70 μmoles/ml. $K_2HPO_4$; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 100 mμmoles/ml. dATP ($3 \times 10^4$ c.p.m./mμmole); $d2APTP^{32}$ ($6 \times 10^4$ c.p.m./mμmole), TTP, dCTP, and dGTP; 15 mμmoles/ml. of dAC:dTG primer; and 60 units/ml. DNA polymerase (Fraction VII). The incubation was carried out at 37°C for 3 hours.

| Template | dNTP present | mμmoles dCMP-$H^3$ inc./ml. | μμmoles d2APMP$^{32}$ inc./ml. |
|---|---|---|---|
| dG:dC | dGTP, dCTP-$H^3$ | 19.5 | -- |
| dG:dC | dCTP-$H^3$, d2APTP$^{32}$ | 10.4 | 20 |
| dG:dC | dGTP, dCTP-$H^3$, d2APTP$^{32}$ | 47.0 | 20 |
| dG:dC | dITP, dCTP-$H^3$ | 16.5 | -- |
| dG:dC | dITP, dCTP-$H^3$, d2APTP$^{32}$ | 21.5 | 20 |

The complete reaction contained 70 μmoles/ml. Tris-HCl, pH 7.0; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β -mercaptoethanol, 30 units/ml. DNA polymerase (Fraction VII), 20 mμmoles/ml. dG:dC primer, 100 mμmoles/ml. of the respective dNTP. The specific activity of dCTP-$H^3$ and d2APTP$^{32}$ was $4 \times 10^4$ c.p.m./mμmole and $1.8 \times 10^5$ c.p.m./mμmole respectively. The incubation was carried out at 37°C for one hour.

Fig. 42.

| Template | dNTP present | mμmoles dCMP-$H^3$ inc./ml. | μμmoles d2APMP$^{32}$ inc./ml. |
|---|---|---|---|
| dI:dC | dGTP, dCTP-$H^3$, d2APTP$^{32}$ | 66 | 4 |
| dI:dC | dITP, dCTP-$H^3$, d2APTP$^{32}$ | 75 | 10 |

The complete reaction contained 70 μmoles/ml. Tris-HCl, pH 7 0; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 30 units/ml. DNA polymerase (Fraction VII); 12.5 mμmoles dI:dC primer, 100 mμmoles/ml. of the respective dNTP. The specific activity of dCTP-$H^3$ and d2APTP$^{32}$ was 4 x $10^4$ c.p.m./mμmole and 1.8 x $10^5$ c.p.m./mμmole respectively. The incubation was carried out at 37°C for one hour.

Fig. 43.

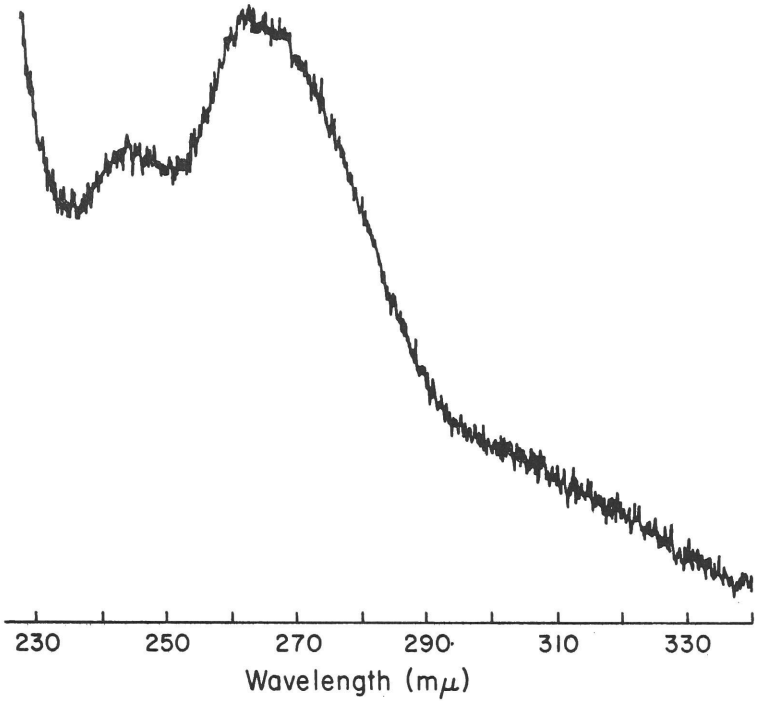


Fig. 44. The V.U. Spectrum of dAT (12.5% 2AP).

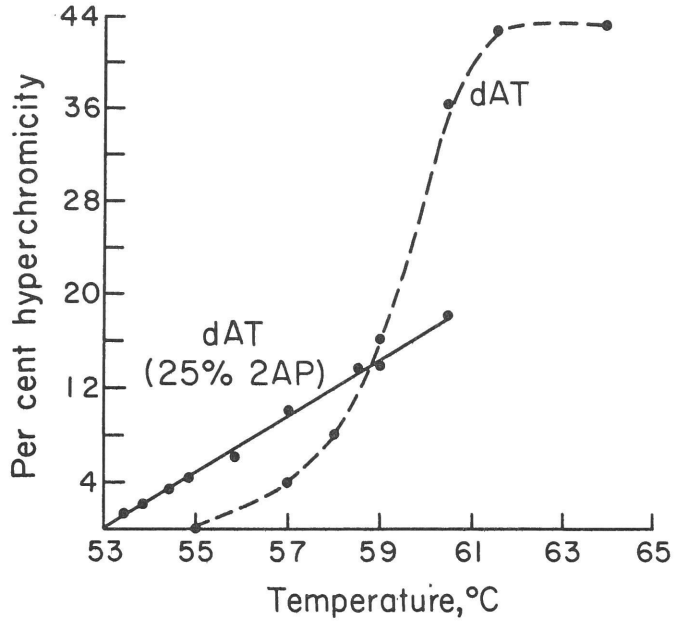


Fig. 45. Thermal denaturation of dAT and dAT (12.5% 2AP). The polymers were dissolved in .1M Tris-HCl buffer, pH 7.9.

on a product synthesized with $\alpha P^{32}$-d2APTP. The chromatographic analysis of the enzymatically degraded polymer is shown in Fig. 46. The peaks correspond to the following:

1) Peak A - 3' dAMP-$H^3$
2) Peak B - 3' $P^{32}$dTMP
3) Peak I - dinucleotides $ApTp^{32}$ and $2APpTp^{32}$ (All $P^{32}$ in this peak was susceptible to alkaline phosphatase, which excludes all other dinucleotide combinations.)
4) Peak II -$\alpha$ $P^{32}$-d2APTP

The sequences (-$Ap^{32}$ 2APp-) and (-$2APp^{32}$ 2APp-) can be excluded since neither $Ap^{32}$ nor $2APp^{32}$ were observed. Also the dinucleotides ($Ap^{32}$ 2APp) and ($2APp^{32}$ 2APp) can be excluded since the $P^{32}$ in these dinucleotides would not be susceptible to alkaline phosphatase. It is also of interest to note that micrococcal nuclease and spleen diesterase degrade $2APpTp^{32}$ sequences poorly. A perfectly alternating sequence of purine-pyrimidine was also found with dAT (5% 2AP) using RNA polymerase with $\alpha$ $P^{32}$ - ATP. These experiments demonstrate that 2AP residues, like their normal A counterparts, always occur next to pyrimidines, and that the incorporation of 2AP does not distort the normal base sequence of the polymer.

Interaction of actinomycin with dAT-like polymers containing 2AP.

In order to test the proposal that actinomycin binding requires 2-amino groups of purines in DNA, dAT-like polymers containing 2AP were tested for their ability to interact with actinomycin. Fig. 47 shows the result of a preparative CsCl centrifugation of dAT (5% 2AP) in the presence and absence of actinomycin. The buoyant density of the polymer centrifuged in the presence of AM has been shifted, suggesting an interaction with the antibiotic. (It is known that the density of dAT, which does not form complexes with AM is unaffected by the antibiotic.)

It has been shown that complex formation with AM increases the transition temperature of DNA polymers. Fig. 48 shows the result of such an

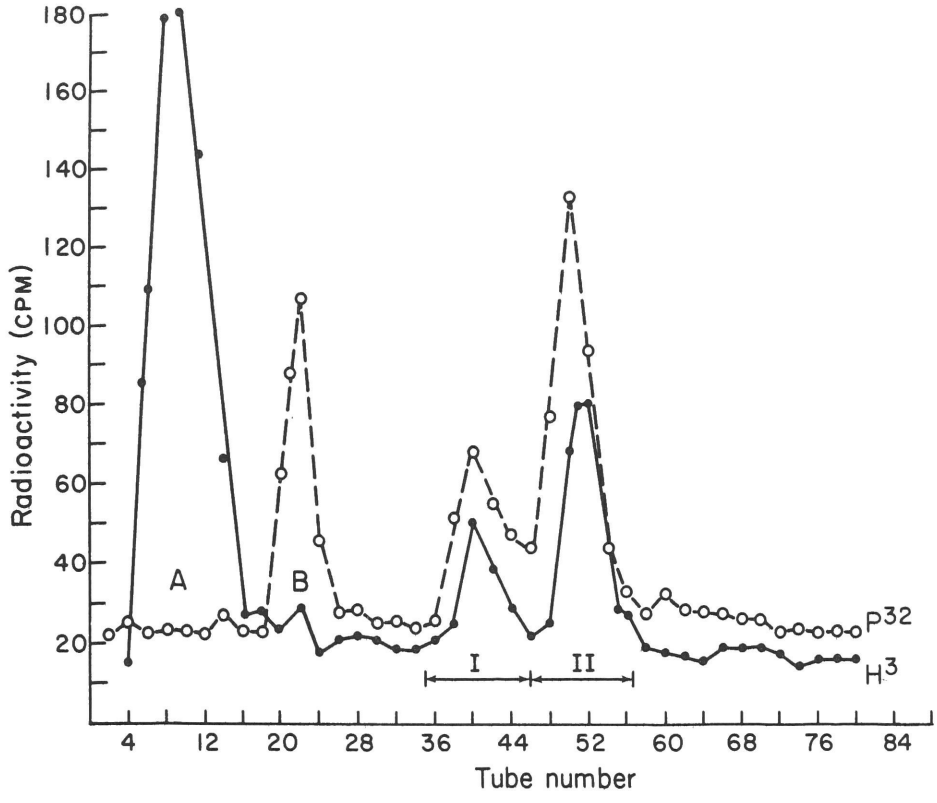


Fig. 46. Enzymatic degradation of dAT (12.5% 2AP). The polymer dAT (12.5% 2AP) in a volume of one ml. was incubated with 46 units of micrococcal nuclease containing 4 µmoles Tris-HCl buffer, pH 8.6 and 2 µmoles $CaCl_2$. At the end of three hours, 15 units of spleen phosphodiesterase were added every hour over a period of 4 hours. The degraded polymer was applied to a D.E.A.E. cellulose column (.9 x 13 cm.). The nucleotides were then eluted by means of a gradient of triethyl ammonium bicarbonate with limiting concentrations of 0 and .6M. 2-ml. samples were collected and the peak tubes were combined and concentrated by flash evaporations. Peak I and II were then treated with bacterial alkaline phosphatase. The $P^{32}$ in both of these fractions was not charcoal adsorbable after phosphatase treatment.

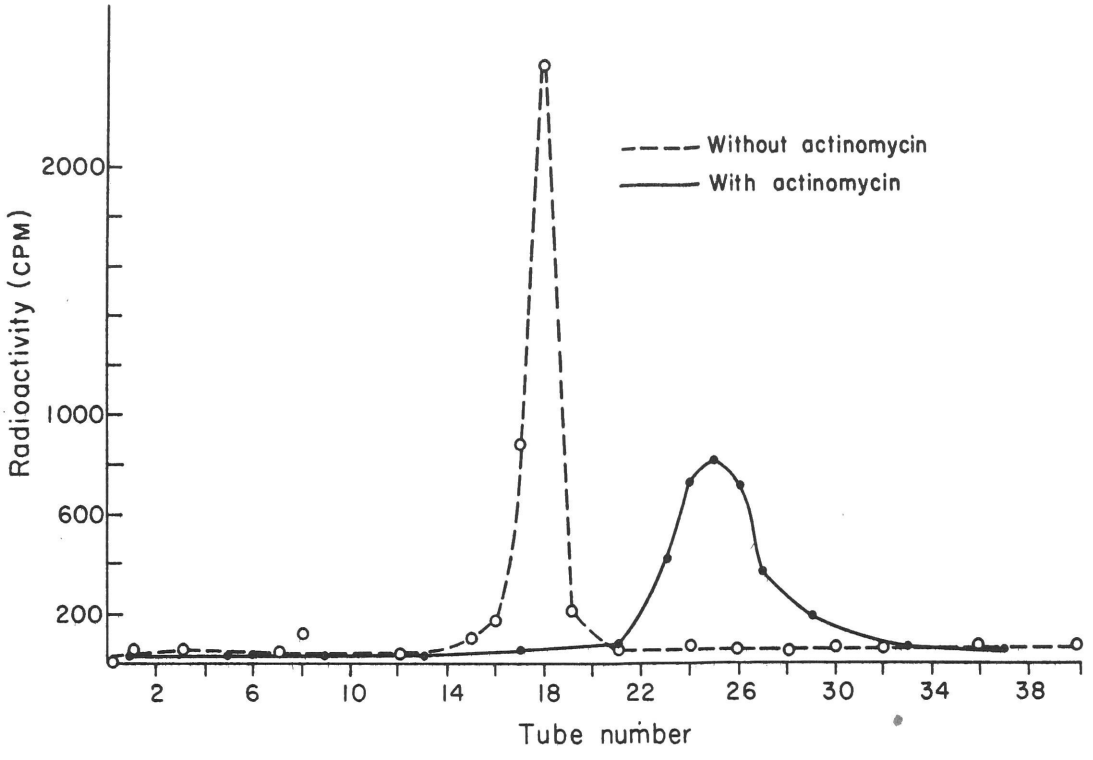


Fig. 47. Preparative equilibrium centrifugation in CsCl density gradients. Each tube contained 5 mls. CsCl ( $\rho = 1.710$) and 0.08 O.D. units of radioactive dAT (5% 2AP), a total of 13 mμmoles of polymer nucleotide. (A) Control. (B) As in A, but containing 20 mμmoles of actinomycin D. Following 120 hours of centrifugation (Spinco SW - 39 rotor, 33,000 r.p.m., 25°), the tubes were punctured, 2-drop fractions were collected and the acid insoluble radioactivity of the indicated samples was determined.

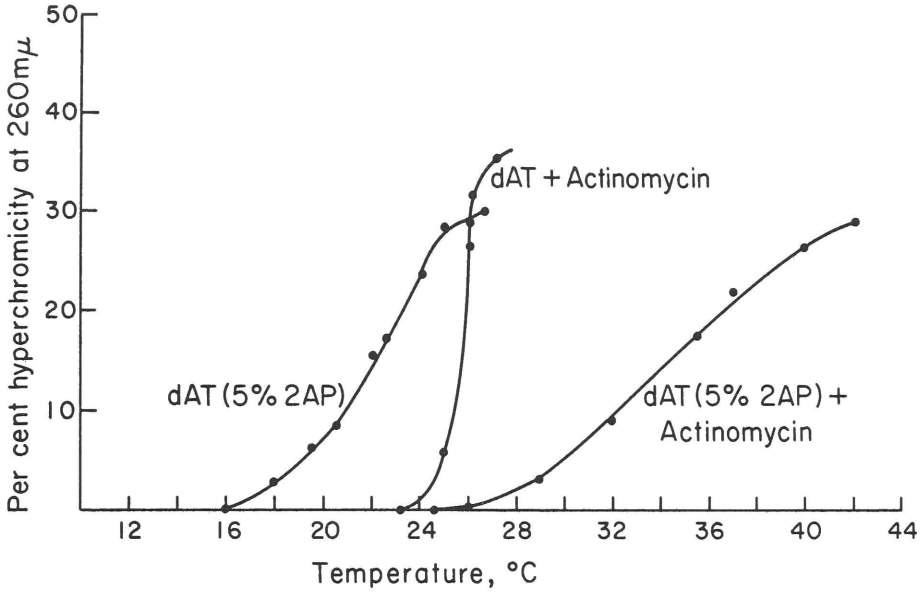


**Fig. 48. Thermal denaturation of dAT (5% 2AP): Effect of actinomycin.** dAT (5% 2AP) 30 mμmoles/ml. was dissolved in .001M $Na^+$. Where present, AM was added to a final concentration of 6.1 mμmoles/ml.; this concentration did not alter the Tm of dAT.

experiment conducted with dAT (5% 2AP). The Tm of the polymer is increased by 13° in the presence of AM. It may be recalled that the initial Tm of the dAT (5% 2AP) is below that of the parent polymer dAT.

As in the case of dAT-like polymers containing DAP, dAT (5% 2AP) can serve as a template for RNA polymerase. Under these conditions, the RNA product contains only A and U. Neither CMP nor GMP is incorporated at detectable levels in the presence or absence of ATP and rUTP (Fig. 49).

Since complex formation with AM is known to inhibit RNA polymerase function, it was of interest to establish whether the antibiotic could affect RNA synthesis directed by the template dAT (5% 2AP). The data in Fig. 50 show this to be the case. The ratio of AM/DNA-P which yields 50% inhibition is 1:100 -- the same value found for dAT (5% DAP).

These results show that the presence of either 2AP or DAP in a dAT-like polymer is sufficient to promote binding of AM. DAP and 2AP nucleotides behave as substrate analogues of A with DNA polymerase and RNA polymerase (Ward, et al., 1967); however, in polydeoxynucleotides containing these bases, DAP and 2AP resemble G-containing polymers in the location of a 2-amino group in the minor groove of DNA. These polymers resemble G-containing polymers also in their ability to interact with AM.

Substitution of d2APTP for dATP in repair of exonuclease III-treated DNA.

A further suggestion that the incorporation of 2AP into DNA results in structural abnormalities was obtained when d2APTP was used to replace dATP in the repair of exonuclease III-treated DNA by DNA polymerase.[1] (See page 24 for a description of this reaction.) Fig. 51 shows the results of a repair reaction with d2APTP. Complete repair does not occur when

[1] In the experiments to be described, E. coli DNA was used as the substrate for exonuclease III. This DNA had been isolated from bacteria grown in the presence of $P^{32}$ of high specific activity. The purified DNA was stored so that $P^{32}$ decay could proceed, yielding DNA with many chain scissions. These provide starting points for the action of exonuclease III.

| Substrate | Product |
|---|---|
| 1. ATP, UTP-$H^3$ | 48.8 mμmoles of rAU/ml. |
| 2. ATP, UTP, CTP-$H^3$ | <.01 mμmole/CMP inc./ml. (< 1 residue of CMP 4800 residues of polymer synthesized) |
| 3. ATP, UTP, GTP-$H^3$ | <.010 mμmoles of GMP inc./ml. (< 1 residue of GMP/4800 residues of polymer synthesized) |

Enzyme incubations were performed at 37°C. for 30' in a final volume of 0.250 ml., containing 10 mμmoles/ml. of dAT (5% 2AP) as template.

Fig. 49. dAT (5% 2AP) as a template with RNA polymerase.

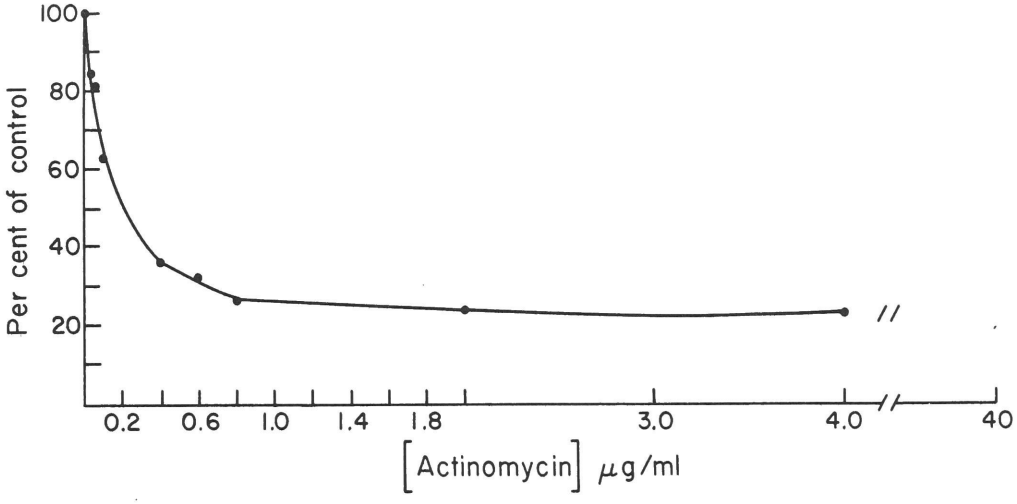


Fig. 50. Effect of AM on RNA synthesis directed by dAT (5% 2AP). Enzyme incubations were performed at 37° for 30', in a final volume of 0.125 ml. containing 10 mμmoles/ml. of dAT (5% 2AP). 100% incorporation corresponds to 40 mμmoles/ml. AMP-$H^3$.

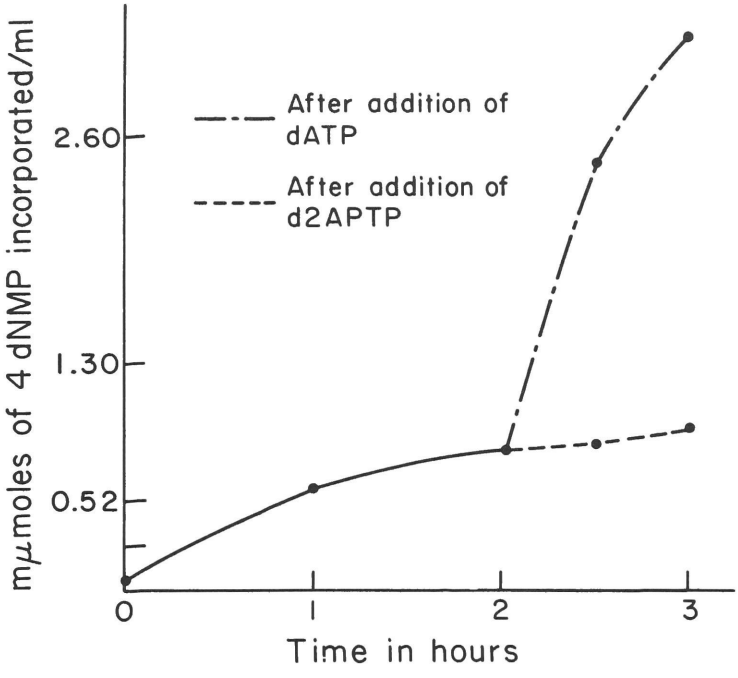


Fig. 51. Repair of Exonuclease III-treated E. coli DNA with DNA polymerase. The complete reaction contained 70 μmoles/ml. potassium phosphate buffer, pH 7.0; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 100 mμmoles/ml. d2APTP, TTP, dCTP ($2 \times 10^3$ c.p.m./mμmole), dGTP; 50 mμmoles/ml. exonuclease III-treated E. coli DNA (25% degraded); and 60 units DNA polymerase. The incubation was carried out at 20°C. At the end of 3 hours, the reaction was divided into three parts. Part A remained the same. 100 mμmoles/ml. more of d2APTP was added to part B and 100 mμmoles/ml. of dATP was added to part C.

d2APTP replaces dATP. This is not due to the depletion of d2APTP or other dNTP, for the addition of dATP permits completion of the repair, whereas the addition of more d2APTP does not (Fig. 51).

The degree of repair which occurs when d2APTP substitutes for dATP is not influenced by increasing the concentration of the enzyme (Fig. 52). The total repair may be increased by providing additional primer, (Fig. 53), but the ratio nucleotide repair/primer nucleotide is not thereby affected. This indicates that the use of d2APTP limits the degree of chain lengthening of any primer strand.

In order to determine the absolute amount of repair which may occur with d2APTP, a homogenous exonuclease III-treated DNA, the DNA of bacteriophage $T_7$, was studied. In the experiment shown in Fig. 54, complete repair with dATP-$H^3$ was equivalent to 540 μμmoles or 11,500 nucleotides per $T_7$ -DNA equivalent, or 5750 nucleotides in each strand of a $T_7$ - DNA molecule. The corresponding amounts for repair with 2AP replacing A are: 57 μμ moles, or 1050 nucleotides per $T_7$ DNA double helix, and 525 per $T_7$ DNA strand -- a value equal to 9% of the synthesis with dATP, or 1.3% of the length of the DNA molecule.

Discussion.

The results described indicate that the biochemical and physical properties of d2APTP and polymers containing 2AP are abnormal. The hypothesis offered below is believed to be consistent with the observations recorded to date, and can account for the unusual substrate behavior of d2APTP and the unexpected physical properties of polynucleotides containing 2AP. It is proposed that the 2AP-T base pair is fully denatured under the conditions used for enzymatic synthesis and for the study of physical properties of 2AP-containing polymers. Some of the consequences of this proposal are as follows:

1) The Tm should occur at a lower temperature and the thermal denaturation profile should be non-cooperative. As seen in Fig. 45, these predictions are confirmed with the polymer dAT (12.5% 2AP).

| Amount of DNA polymerase | Amount of Product |
|---|---|
| 1. 20 units DNA polymerase (Fraction IX) | 1.7 mμmoles of all 4 dNTP inc./ml. |
| 2. 60 units DNA polymerase (Fraction IX) | 1.7 mμmoles of all 4 dNTP inc./ml. |
| 3. 120 units DNA polymerase (Fraction IX) | 1.7 mμmoles of all 4 dNTP inc./ml. |

The above reactions contained, in addition, 70 μmoles/ml. potassium phosphate buffer, pH 7.0; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 100 mμmoles/ml. of d2APTP, dCTP-$H^3$ ($2 \times 10^3$ c.p.m./mμmole), dGTP, and TTP; and 50 mμmoles/ml. of exonuclease III-treated E. coli DNA (degraded 25%). The incubation was carried out at 20°C. for 3 hours.

Fig. 52. Repair with d2APTP of Exo III-treated E. coli DNA with increasing amounts of DNA polymerase.

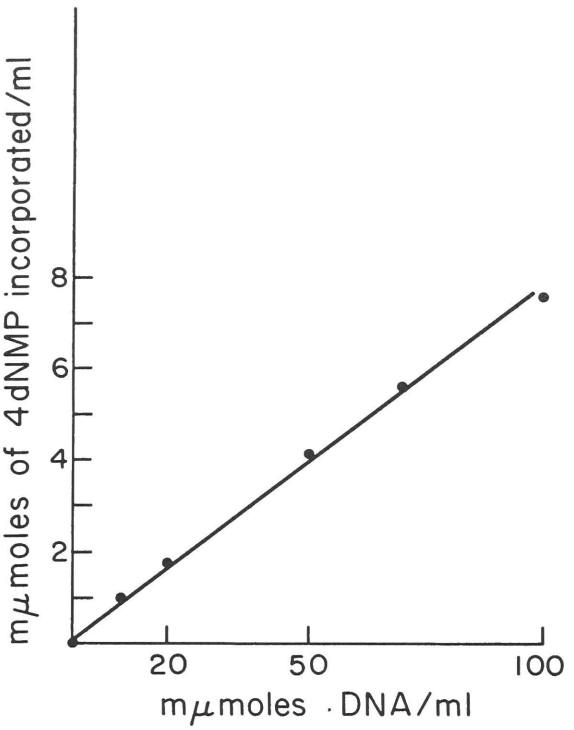


Fig. 53. Amount of d2APTP utilized as a function of the amount of exonuclease III-treated DNA. In addition to the amount of template indicated above, the reaction contained: 70 μmoles/ml. potassium phosphate buffer, pH 7.0; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 100 mμmoles/ml. d2APTP, dCTP, TTP-$H^3$ ($2 \times 10^3$ c.p.m./mμmole), and dGTP; and 60 units/ml. DNA polymerase (Fraction IX). The incubation was carried out at 20°C. for 2.5 hours.

| Template | dNTP present | Product |
|---|---|---|
| Exonuclease III-treated $T_7$ DNA (20% degradation) | dATP-$H^3$, dCTP, dGTP, TTP | 530 μμmoles dAMP-$H^3$ inc./ml. |
| Exonuclease III-treated $T_7$ DNA (20% degradation) | d2APTP$^{32}$, dCTP, dGTP, TTP | 57 μμmoles d2APMP$^{32}$ inc./ml. |

The reaction contained 70 μmoles/ml. potassium phosphate buffer, pH 7.0; 7 μmoles/ml. $MgCl_2$; 10 μmoles/ml. β-mercaptoethanol; 60 units/ml. DNA polymerase (Fraction IX); 15 mμmoles/ml. of exonuclease III-treated $T_7$ DNA (20% degraded); and 100 mμmoles/ml. of the respective dNTP.

Fig. 54. Repair of Exonuclease III-treated $T_7$ DNA with d2APTP$^{32}$ and dATP-$H^3$.

2) The degree of hyperchromicity of polymers containing 2AP should be appreciably lower than dAT. The hyperchromicity of dAT in the ionic conditions used in these experiments is 45%; that of dAT (5% 2AP) is 30%; and that of dAT (12.5% 2AP) is 20% -- a decrease of 38% and 55% respectively.

3) Since template efficiency is inversely related to polymer stability, RNA polymerase should utilize 2AP containing polymers as templates more effectively than dAT. Fig. 55 shows the utilization of dAT (5% 2AP), dAT, and dAT (5% DAP) as templates with RNA polymerase at 10°C. As anticipated, dAT (5% 2AP) is the most effective template under these conditions.

4) It should not be possible to synthesize large polynucleotides with all the A replaced by 2AP. Two lines of evidence are in agreement with this expectation: 1) d2APTP is utilized by DNA polymerase with the primers dAT, dA:dT, or dAC:dTG, only when dATP is present. 2) An incomplete repair of exonuclease III-treated DNA occurs when d2APTP replaces dATP.[1]

The following additional predictions can be made and are now being tested experimentally:

1) The 2AP-T base pair might not be denatured at very high ionic strength (> 2M salt).

2) There should be no hyperchromism at 303 mμ, the maximum of 2AP, on thermal denaturation.

3) Amino groups of native DNA do not react with either formaldehyde or nitrous acid. However, 2AP in the polymer dAT (12.5% 2AP) should be accessible to these agents.

4) The limited polymerase product which is synthesized by DNA polymerase using d2APTP and naturally occurring DNA primers might be partially susceptible to a nuclease which specifically degrades single stranded DNA.

[1] A similar incomplete repair occurs when dITP and dUTP replace dGTP and dTTP. Since polymers containing I and U have lower Tms than polymers containing G or T, the hypothesis of weak base pairs is substantiated.

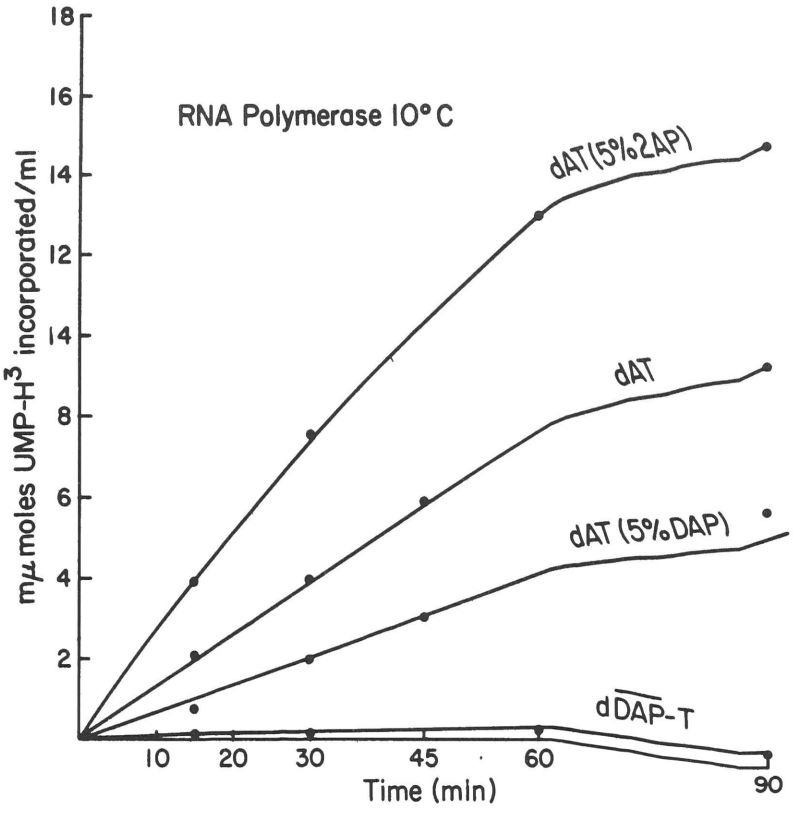


Fig. 55. Template efficiency with RNA polymerase of dAT, dAT (5% DAP), dAT (5% 2AP) and $d\overline{DAP}$-T at 10°C. The complete reaction contained 40 μmoles/ml. of Tris-HCl buffer, pH 7.9; 4 μmoles/ml. $MgCl_2$; 1 μmole/ml. $MnCl_2$; 10 μmoles/ml. β-mercaptoethanol; 10 mμmoles/ml. of each of the dAT-like polymers; and 15 units/ml. RNA polymerase. The incubation was carried out at 10°C for 90 minutes.

As noted above, both the 2AP-T base pair and the A-T base pair may form two hydrogen bonds. The large apparent difference in stability of these base pairs is not at present understood. It is of interest that in contrast to DNA polymerase, RNA polymerase can catalyse the formation of an alternating polymer of r2AP-U (Ward, et al., 1967). The Tm and the denaturation profile of this polymer closely resembles that of rAU. It is known that the Tm of helical ribopolymers is higher than that of the corresponding deoxypolymers. However, the difference between the ribo-2AP-U and the deoxy-2AP-T base pairs is apparently greater than anticipated on the basis of previous comparative observations (Ward, et al., 1967).

An important question is whether the hypothesis presented above can account for the outstanding biological property of 2AP -- namely its mutagenic action in bacteria and bacteriophage. On the basis of evidence which will not be reviewed here, Freese (1959) has proposed that the mutagenic action of 2AP can be explained by the ability of 2AP to pair without a tautomeric shift either with two hydrogen bonds with T or with one hydrogen bond with C.

At first, several reservations can be attached to this proposal.

1) The major evidence is derived from observations with BrU. Therefore, the hypothesis of 2AP action is itself based on hypothetical assumptions concerning BrU (Freese, 1963).

2) It is difficult to justify a comparison of the action of BrU and 2AP since the former is extensively incorporated into DNA (replacing up to 100% of the thymidine in a strand of DNA in vivo, Dunn and Smith, 1954, and in vitro, Trautner, et al., 1962); whereas the latter is incorporated very poorly indeed (Gottschling and Freese, 1961; Rudner, R., 1961; and Wacker, et al., 1960). Under in vivo conditions with E. coli, Gottschling and Freese (1961) found less than one 2AP in 272 A residues.

3) At least 40% of the mutations caused by 2AP are non-revertible by BrU (Kreig, 1963). In view of the fact that the in vivo methods are

not precise, it seems reasonable to conclude that one half of the mutations do not conform to the scheme proposed by Freese; therefore, the Freese hypothesis cannot adequately account for the mutagenicity of 2AP.

4) Under all conditions and with every primer and template tested, d2APTP behaves as an analogue only of dATP. As seen above (Figs. 42 and 43), d2APTP does not function as an analogue of dGTP in the replication of dG:dC and dI:dC. Moreover, when 2AP functions in a template, the polymer in which it is incorporated directs the formation of polynucleotides containing only A and U or A and T. Thus, in its template function as in its substrate properties, 2AP behaves exclusively as an analogue of A.

Since the evidence presented in this thesis is not consistent with the Freese hypothesis, the search for alternative explanations appears to be justified.

To replace the Freese hypothesis a twofold theory is offered for the mutagenicity of 2AP.

1) The first element is based on the experimental evidence described in the present work. As indicated above, it is proposed that the 2AP-T base pair is denatured under normal in vitro and in vivo conditions. The presence of a locally unwound segment of a helical DNA molecule corresponds to the events which follow the irradiation of DNA with ultra-violet light. In the latter instance the denaturation is caused by the formation of thymine-thymine dimers between adjacent T residues in one of the DNA strands. Studies of Boyce and Howard-Flanders, 1964, and Setlow and Carrier, 1964, of the events which follow irradiation make it appear likely that the following events take place: A.) The damaged region containing the thymine dimer is excised. B.) The resulting gap is repaired by a repair enzyme -- probably DNA polymerase.

It seems most reasonable to equate the mutagenic event with the mistake which occurs during the repair process of the damaged segment (Setlow, 1965). With the denaturation of the 2AP-T base pair and the resulting similarity

with U.V. irradiated DNA, the predominant mutagenic action of 2AP can be visualized as resembling that due to U.V., namely, excision, trimming and repair -- the mutagenic effect being a mistake during the course of repair.

2) In addition to the above, a second form of 2AP mutagenicity can be formulated. There is no indication of base analogue utilization by polymerases under conditions which permit the formation of fewer than two hydrogen bonds between the substrate and the template bases. This fact is an additional weakness of the Freese hypothesis. Under the proper conditions two hydrogen bonds might in fact be formed between 2AP and C. Such a situation could be selected by choosing a pH at which the C is protonated. As shown in Fig. 56, such a situation would permit a hydrogen bond situation which is found in the 2AP-T base pair. Since the pH of C in polynucleotides is displaced to lower pH values than that of the monomer, this scheme would preferentially operate under conditions in which 2AP in polynucleotides is being replicated rather than permitting the incorporation of 2AP under the direction of a protonated C residue in the template DNA. It may be noted that the absence of the amino function in the 6 position of 2AP permits the pairing with a protonated C residue; whereas the corresponding pair with A would be inhibited by the 6-amino group.

One prediction that can be derived from this proposal is that the mutagenic action of 2AP might be enhanced by growth of bacteria at pH 5.5 -- assuming the intracellular pH reflects the external medium. A second prediction which follows from this model is that an alternating polymer of 2AP and C should assume a helical conformation below pH 4.5; whereas the corresponding polymer containing A in place of 2AP should not. Both of these predictions are currently under investigation.

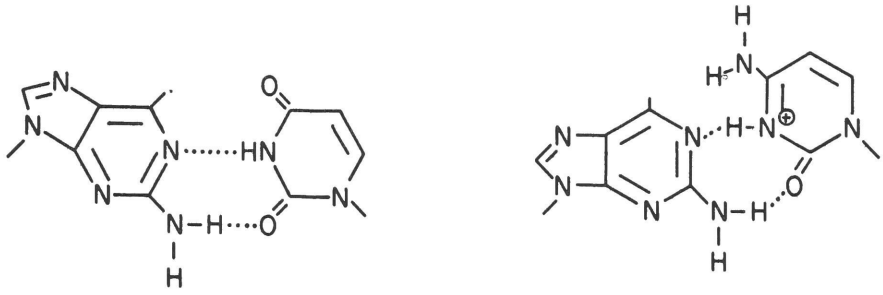

Fig. 56. Hydrogen-bonding properties of 2AP-T and 2AP-C-$H^+$.

# ABBREVIATIONS

The following abbreviations are used: A = adenine; G = guanine; C = cytosine; I = hypoxanthine; DAP = 2,6 diaminopurine; 2AP = 2 aminopurine; BrU = 5 bromouracil; dDAP = 2,6 diaminopurine deoxynucleoside; d2AP = 2 aminopurine deoxynucleoside; dNTP = deoxynucleoside 5' triphosphate; dDAPTP and dDAPMP = the 5' triphosphate and monophosphate respectively of 2,6 diaminopurine deoxynucleoside; d2APMP and d2APTP = the 5' monophosphate and triphosphate respectively of 2-aminopurine deoxynucleoside; dATP, dCTP, dGTP, and TTP = the 5' triphosphate of deoxyadenosine, deoxycytidine, deoxyguanosine, and thymidine respectively; AMP and UMP = adenylic acid and uridylic acid respectively; DNA = deoxyribonucleic acid; RNA = ribonucleic acid; dAT = alternating deoxynucleotide copolymer of A + T residues; $d\overline{DAP}$-T = deoxynucleotide polymer in which 90% of the residues are arranged in alternating sequence of DAP + T, the remaining 10% consists of alternating A + T residues; dAT (5% DAP) = a dAT copolymer in which 10% of the purine residues are occupied by DAP, 90% by A; dG:dC = homopolymers of the deoxynucleotides G and C; dI:dC = homopolymers of the deoxynucleotides I and C; AM = actinomycin; Tm = melting temperature or midpoint of optical density transition.

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