THE ENZYMATIC SYNTHESIS OF A COPOLYMER OF 6-METHYL DEOXYADENYLATE AND DEOXYTHYMIDYLATE*

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The presence of the methylated base, 6-methyl adenine, in trace amounts in certain bacterial and bacteriophage DNA's was first noted by Dunn and Smith (3). The origin from methionine of the methyl groups found in the "trace bases" in S-RNA has been demonstrated by Borek et al (2) and Biswas et al (1). The enzymatic synthesis of 6-methyl adenine in DNA has been shown by Gold et al (4) to occur through the methylation of adenine residues at the polydeoxynucleotide level.

The introduction of a methyl group at the 6 amino position of adenine might possibly exert an effect on the hydrogen bonding relation with thymine in the DNA double helix. Since 6-methyl adenine is present in only very small amounts in bacterial DNA, its effect on the melting curve and other physical properties of the molecule would be extremely difficult to detect. Several lines of evidence, summarized by Richardson et al (9), indicate that

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the DNA polymerase functions in assembling DNA by sequentially ordering the deoxynucleotide substrates in accordance with base pairing to the bases in the template. Thus, it might be possible to employ the DNA polymerase to examine the hydrogen bonding capacity of the 6-N-methyl analogue of deoxyadenosine triphosphate (d6MATP).

We wish to report that d6MATP can substitute for dATP as a substrate for the DNA polymerase in the presence of a DNA template. Evidence will also be presented for the de novo enzymatic synthesis of an alternating copolymer of 6-methyl deoxyadenylate and deoxythymidylate (d6MAT) analogous to the dAT copolymer reported by Schachman et al (10).

Materials and Methods. The E. Coli polymerase* was carried through fraction VII and then refractionated on DEAE cellulose (7). Salmon sperm DNA was prepared by a modification of the procedure of Kay, Simmons, and Dounce (6). DNA synthesis was assayed by the incorporation of labeled deoxynucleoside triphosphates into an acid-insoluble form, as described by Lehman et al (7).

d6MATP was prepared from the DNA of E. Coli ($15T^-$), grown in the presence of 5-amino uracil so as to be enriched approximately 10-12 fold in 6-methyl adenine (6MA) content (3). The DNA was degraded to 5' deoxynucleotides by the successive action of pancreatic DNA'ase and snake venom phosphodiesterase. The mixture of deoxynucleotides was resolved on a Dowex-1 (chloride form) column (7). d6MA5'P was eluted from the column in the dA5'P peak and then separated from it by paper chromatography at 4°C in a solvent system of isopropanol-H_2O (7:3) in saturated NH_3 (3).

*Generously provided by Dr. Arthur Kornberg

Attempts to convert the d6MA5'P to the triphosphate with a purified dA5'P kinase from E. Coli were unsuccessful. d6MA5'P kinase activity could not be demonstrated in a variety of E. Coli extracts utilizing a radioactivity assay (7). Consequently, d6MAPPP was prepared from the deoxynucleoside monophosphate utilizing the chemical synthesis of Smith and Khorana (11). Analysis showed no detectable contamination with adenine nucleotides (<2%). d6MA5'P^{32}PP was prepared in a similar fashion starting with the DNA from E. Coli ($15T^-$) cells grown under conditions of limiting thymine in the presence of inorganic P^{32}.

Optical density melting curves were measured with a Zeiss PMQ II spectrophotometer equipped with a thermostatically controlled cell holder. Evaporation of water from the solutions was prevented by a layer of paraffin oil (pre-washed with hot water).

Results. Enzymatic Incorporation of d6MA5'P^{32}PP into DNA. P^{32}-labeled d6MATP can substitute for dATP in the polymerase reaction and can be incorporated into newly synthesized DNA (Table I). The relative rate of analogue incorporation is about 45% that of the natural substrate. Over 96% of the radioactivity incorporated into DNA from d6MA5'P^{32}PP could be reisolated as d6MA5'P^{32} after successive digestions of the product by pancreatic DNA'ase and snake venom phosphodiesterase and subsequent resolution of the mixture of nucleotides on a Dowex-1 chloride column, followed by paper chromatography in the isopropanol-H_2O (7:3) in saturated NH_3 system. d6MA5'P^{32}PP cannot substitute for any of the other three deoxynucleoside triphosphate substrates for the DNA polymerase, as shown on Table I. Various DNA's and dAT can serve as template for d6MAP^{32}PP incorporation with the DNA polymerase.

TABLE I

Incorporation of d6MA5'$P^{32}PP$ into DNA

Deoxynucleoside triphosphates	cpm incorporated	mμ moles incorporated
1. dCTP, dGTP, dTTP, C^{14}dATP	767	*1.19
2. dCTP, dGTP, dTTP, ----, d6MA5'$P^{32}PP$	828	.54
3. dCTP, dGTP, ----, dATP, d6MA5'$P^{32}PP$	5	.003
4. ----, dGTP, dTTP, dATP, d6MA5'$P^{32}PP$	16	.01
5. dCTP, ----, dTTP, dATP, d6MA5'$P^{32}PP$	12	.007

*Corrected for self absorption.

Synthesis of 6-methyl deoxyadenylate-deoxythymidylate (d6MAT) Copolymer.

There is evidence of polymer formation by the DNA polymerase using dTTP and d6MATP as substrates, analogous to the de novo synthesis of the dAT copolymer. As can be seen in Figure 1, this reaction can be followed by both the hypochromic effect and by the incorporation of radioactivity into an acid-insoluble product. The latter is detectable well before any change in optical density is discernible. The lag period of 12-13 hours is quite long when compared to that of about 5 hours for dAT synthesis under comparable conditions. The lag period of the unprimed reaction can be shortened slightly by increasing the enzyme concentration. In contrast, where a small amount of d6MAT polymer is present as primer, the reaction starts almost immediately. No polymer is formed when either d6MATP or dTTP is used as the only substrate.

In order to determine the nucleotide sequence in the d6MAT polymer, a "nearest neighbor" (5) analysis was performed on the product formed from d6MATP and dT5'$P^{32}PP$. After the de novo synthesis had reached its maximum,

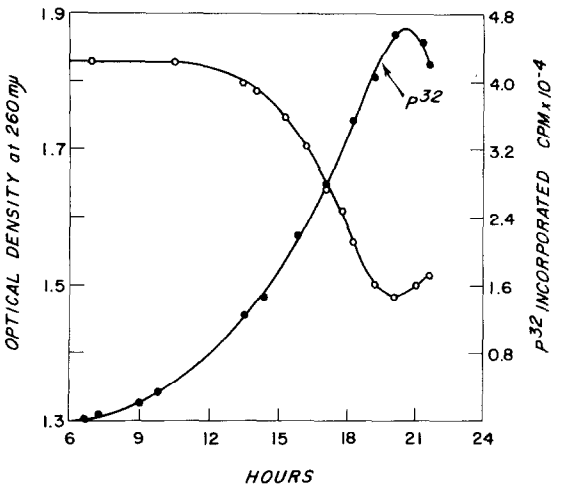

Figure 1

Unprimed synthesis of d6MAT copolymer. The reaction mixture contained in 0.66 ml, 40 μmoles of potassium phosphate buffer at pH 7.5, 4 μmoles of $MgCl_2$, .23 μmoles each of d6MATP and dT5'P^{32}PP (4.7×10^5 cpm/μmole), and 5.4 units of E. Coli polymerase. Absorbency measurements were made at 37°C in the Zeiss PMQ II spectrophotometer with a light path reduced to 2 mm. A thymine solution of similar optical density was employed as a blank during the incubation.

Determination of P^{32} incorporation into an acid-insoluble form was carried out on .01 ml aliquots as described by Lehman et al (7).

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calf thymus DNA was added as carrier and the product reprecipitated with cold 3.5% perchloric acid. After several washings and reprecipitations, the product and carrier were degraded quantitatively to 3' deoxynucleotides by the action in succession of micrococcal nuclease and spleen phosphodiesterase. Resolution of the mixture of five 3' deoxynucleotides was carried out by descending paper chromatography in an isobutyric acid (90 ml) - .5N

NH_3 (54 ml) solvent system, which separates the four principal mononucleotides but does not separate deoxyadenylate from 6-methyl deoxyadenylate. The resolution of dA3'P from d6MA3'P was subsequently carried out by paper chromatography in the isopropanol-H_2O (NH_3) system. Over 99% of the counts incorporated from dT5'P^{32}PP were released, following such a digestion, as d6MA3'P^{32}, suggesting that the polymer synthesized consisted of an alternating sequence of deoxythymidylate and 6-methyl deoxyadenylate residues.

Thermal Denaturation of d6MAT Copolymer. The d6MAT copolymer in 0.2M NaCl-0.1M Na citrate melts sharply with a T_M at 50°C about twenty degrees below the melting temperature of dAT (Figure 2). Like the dAT copolymer, but unlike DNA, the melting of the d6MAT copolymer upon heating, and subsequent reformation upon cooling, appears to be completely reversible. The absence of a hysteresis for the d6MAT copolymer is probably attributable to the alternation of deoxythymidylate and 6-methyl deoxyadenylate units, as has been suggested in the case of the dAT copolymer (10).

Discussion. d6MATP can be incorporated into DNA by the DNA polymerase quite readily in place of dATP, when the three other deoxynucleotide triphosphate substrates are present. However, it should be emphasized that this is not the pathway of synthesis of 6-methyl adenine in DNA. It has already been clearly shown by Gold et al (4) that methylation of adenine occurs at the polydeoxynucleotide level. Further, there appears to be no kinase activity in E. Coli ($15T^-$) capable of converting d6MA5'P to the triphosphate. The N-methyl-5-fluoro-cytosine analogue of deoxycytidine triphosphate has been shown by Okazaki and Kornberg (8) to replace deoxycytidine triphosphate to a limited extent in the DNA polymerase reaction. The fact that the N-methyl analogue of adenine is relatively

more effective as a replacement may be related to the presence of a 5-fluoro substituent on the cytosine analogue.

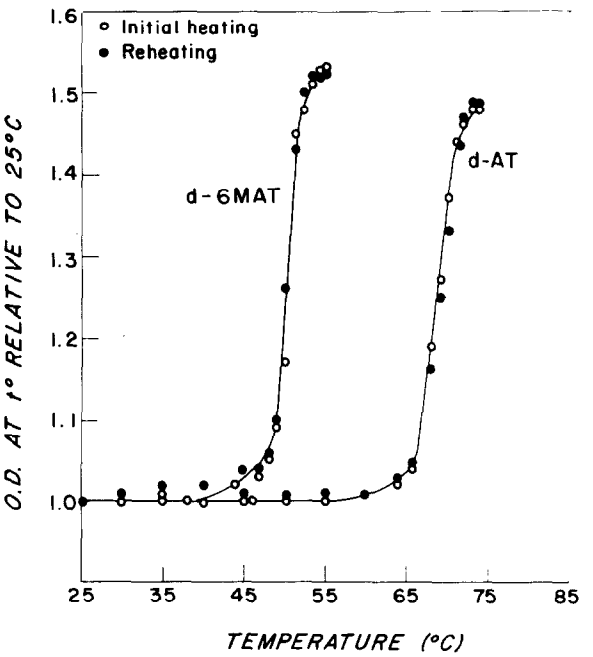

Figure 2

Melting curves of dAT and d6MAT copolymers. The copolymers were prepared from de novo syntheses. When minimal absorbency was attained, the reactions were terminated by adding a mixture of NaCl and sodium citrate to give final concentrations of 0.2M and 0.1M, respectively. Dialysis at 4°C for 36 hours against repeated changes of the NaCl-sodium citrate mixture were then carried out.

Plotted on the abscissa is the relative absorbency at 260 mμ at t° compared to the value at 25°. The first heating cycle for each copolymer is indicated by open circles. After quick cooling, reheating produced the results shown in the closed circles. The absorbency of the copolymer solutions ranged between 0.440 and 0.480 at 25°.

A copolymer consisting of alternating units of 6-methyl deoxyadenylate and thymidylate can be synthesized by polymerase from d6MATP and dTTP after a considerable lag period. Since the mechanism of action of polymerase appears to require the proper recognition of the appropriate deoxynucleotide for incorporation at a given site on the basis of the hydrogen bonding relationship, it would appear that d6MATP can form the necessary hydrogen bonds with thymine. This could still occur at the 6 amino position of 6-methyl deoxyadenylate, since only one of the hydrogen atoms is methyl-substituted. However, this might represent a weaker bond than in the adenine-thymine pair, as evidenced by the lower T_M. On the basis of the melting curve data, it would appear that the regular alternating units of the molecule readily reform a highly hydrogen-bonded structure after melting and cooling.

The reaction mixture (0.3 ml) contained 20 μmoles of Tris buffer, pH 7.5, 2 μmoles $MgCl_2$, 0.3 μmoles 2-mercaptoethanol, 24 mμ moles of salmon sperm DNA, 0.13 units of polymerase, and 5.0 mμ moles of each of the above deoxynucleoside triphosphate substrates. Specific activity of C^{14}dATP was 6.48×10^5 cpm/μmole. Specific activity of d6MA5'P^{32}PP was 1.54×10^6 cpm/μmole. After incubation at 37°C for thirty minutes, 0.2 ml of a solution of calf thymus DNA (2.5 mg/ml) was added as carrier, and the reaction was stopped by the addition of 0.5 ml of cold 7% perchloric acid. The DNA precipitate was then washed, dissolved in 0.2N NaOH, re-precipitated, rewashed, and finally dissolved in 0.5 ml of 2N NaOH, transferred to a planchette and dried. The radioactivity was then measured in an end window gas flow counter.

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