*THE ENZYMATIC TERMINATION OF POLYDEOXYNUCLEOTIDES BY 2',3'-DIDEOXYADENOSINE TRIPHOSPHATE**

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Abstract.—The nucleoside 2',3'-dideoxyadenosine is lethal to *E. coli* and blocks DNA synthesis irreversibly. The hypothesis that a derived dideoxynucleoside triphosphate is incorporated terminally into the cellular DNA has been tested in an *in vitro* system. The triphosphate of dideoxyadenosine was synthesized and shown to inhibit the *in vitro* synthesis of DNA by purified *E. coli* DNA polymerase. The kinetics of inhibition of nucleotide incorporation and pyrophosphate exchange were studied. Early in synthesis the dideoxynucleotide is a competitive inhibitor of the enzyme. Subsequently, synthesis is almost completely arrested.

Radioactive dideoxyadenosine triphosphate was prepared. The compound was shown to be incorporated enzymatically into dAT copolymer to the extent of about one molecule per molecule of template. It is released from such templates by DNA polymerase at less than 1 per cent of the rate of release of other natural nucleotides. The label is released by snake venom phosphodiesterase far more rapidly than total nucleotides. This nucleoside triphosphate has thus been shown to be a competitive inhibitor of DNA polymerase and a terminator of polydeoxynucleotide chains.

Some years ago we had observed that *Escherichia coli* killed by D-arabinosyladenine or thymine deprivation were nevertheless capable of DNA synthesis in the absence of the lethal agent or treatment.[1] It was desirable to have a specific irreversible inhibitor of DNA synthesis and it was supposed that 2',3'-dideoxynucleosides might act in this way.[1, 2] Indeed 2',3'-dideoxyadenosine (Fig. 1) did act in this way and was shown to be lethal to numerous strains of *E. coli*.[1] The structure of the compound had suggested that if the nucleotide were incorporated into DNA, the absence of the 3'-hydroxyl group would prevent further elongation of the polydeoxynucleotide chain.

The very high levels of radioactivity needed to find the few molecules of

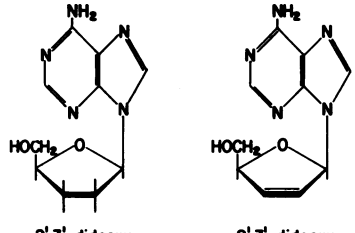

FIG. 1.—Structures of dideoxynucleosides of adenine.

dideoxyadenosine which might be incorporated into the bacteria made it advisable to check the proposed mechanism initially in an isolated enzymatic system. Therefore, the triphosphate of dideoxyadenosine was synthesized and used *in vitro* with *E. coli* DNA polymerase.

Materials and Methods.—Chemicals: The dideoxyadenosine used in our initial experiments was kindly provided by Dr. Roland K. Robins. Additional dideoxyadenosine was synthesized in this laboratory (*a*) according to the procedures of McCarthy *et al.*[3] and Robins *et al.*[4] and (*b*) by reduction of 2′,3′-dideoxy-2′-adenosinene (Fig. 1) provided by the Cancer Chemotherapy National Service Center. Tritiated dideoxyadenosine was prepared by New England Nuclear Corporation by catalytic reduction of 2′,3′-dideoxy-2′-adenosinene in dioxane with tritium gas. The tritiated dideoxyadenosine was purified of any free sugar, adenine, or deoxyadenosine by paper chromatography on Whatman no. 1 paper in water made pH 10 with 1 *M* NH_4OH and by column chromatography on Dowex-1 (OH^-) in 30% methanol (w/w). The latter step was essential for the separation of 2′,3′-dideoxyadenosine from 2′-deoxyadenosine. It was shown after hydrolysis that 99.85% of the radioactivity was present in the dideoxy sugar.

Highly polymerized calf thymus DNA and barium cyanoethylphosphate were purchased from Calbiochem, Inc. The dAT copolymer and dGdC were obtained from Biopolymers, Pinebrook, New Jersey. Deoxynucleoside triphosphates were obtained from Sigma Chemical Co. and Calbiochem, Inc. Thymidine-2-^{14}C-triphosphate, deoxyadenosine-8-^{14}C-triphosphate, deoxycytidine-2-^{14}C-triphosphate, and ^{32}P-pyrophosphate were purchased from New England Nuclear Corp. Triethylamine and dicyclohexylcarbodiimide were purchased from Eastman Organic Chemicals Division.

Assays: Calf thymus DNA was activated by DNase, and DNA polymerase was assayed as described by Richardson.[5] The reaction mixture contained 0.07 *M* potassium phosphate pH 7.4, 0.007 *M* magnesium chloride, 0.001 *M* β-mercaptoethanol, all necessary deoxynucleoside triphosphates at 25 μM except deoxyadenosine triphosphate which was used at 4 μM, either dAT copolymer at 10 mμmoles of nucleotide/ml or activated calf thymus DNA at 30 mμmoles of nucleotide/ml, and 0.02–0.05 unit of DNA polymerase per sample. Pyrophosphate exchange was assayed by the method of Bessman *et al.*;[6] potassium-^{32}P-pyrophosphate was present at 0.002 *M*. Incorporations were measured by collecting the acid-precipitated material on Millipore filters, washing with cold 5% TCA and water, drying, and counting in toluene-Liquifluor scintillation fluid in a Tri-Carb liquid scintillation spectrometer. Aqueous samples were counted in Bray's solution.[7]

Synthesis of ddATP: The monophosphate of dideoxyadenosine was synthesized by the cyanoethylphosphate method of Tener in a 20% yield.[8] Dideoxyadenosine is significantly more labile than 2′-deoxyadenosine. The monophosphate was converted to the triphosphate in a yield of 38–40% with the deoxynucleotide kinases of *E. coli* prepared as described by Lehman *et al.*[9] Complete destruction of ribonucleotides was accomplished by the method of Neu and Heppel.[10] Mono-, di-, and triphosphates were separated on a DEAE-cellulose ($CO_3^=$) column 1 × 4 cm according to Khorana and Smith.[11]

Enzymes: Highly purified *E. coli* DNA polymerase was kindly given to us by Dr. Arthur Kornberg. The enzyme preparation was a portion of the homogeneous enzyme described in his recent paper on the active site of DNA polymerase.[12] Snake venom phosphodiesterase was purchased from Worthington Biochemical Corp.

Isolation of template: After exposure to DNA polymerase, the template was precipitated by acid and collected on glass fiber filters, counted, and extracted as described by Weiss *et al.*[13]

Results.—Inhibition of DNA synthesis: We have studied the inhibition of incorporation of dTTP by the triphosphate of dideoxyadenosine (ddATP) in systems containing dAT copolymer or activated calf thymus DNA. As shown in Table 1, the activity of the polymerase with dAT copolymer was markedly inhibited by ddATP even at equimolar concentrations of dATP and ddATP.

TABLE 1. *Inhibition of E. coli DNA polymerase by dideoxyadenosine triphosphate.*

Polydeoxynucleotide	Ratio ddATP/dATP	Per cent inhibition*
Poly dAT	1	54
	2	79
	4	82
Activated calf thymus DNA	4	35

* Assays were performed as described in *Materials and Methods*. The incorporation of ^{14}C-thymidine triphosphate was studied after a 30-min interval. Controls were 2200 cpm.

Such inhibition is considerably increased by increasing the ratio of ddATP to dATP to 4. With activated calf thymus DNA as template, a comparable ratio of ddATP to dATP is significantly less inhibitory. It is of interest that the incorporation of dCTP by the polymerase with dGdC as template is not inhibited by ddATP (18 μM).

As can be seen in Figure 2, the presence of ddATP sharply reduces the rate of incorporation of dTTP into dAT copolymer very early in the reaction. The inhibition of synthesis by ddATP at varying dATP was therefore examined at early times (5 min) in the reaction with dAT copolymer. Despite the obviously noncompetitive later component of the kinetics of inhibition in the presence of ddATP, the kinetics of the initial phase of the reaction closely approached competitive inhibition as revealed by the Lineweaver-Burk plots. The K_i of ddATP in the reaction was 5.4 μM.

Figure 2 also shows that the incorporation of dTTP was completely dependent on the presence of dATP. In the absence of dATP, ddATP is unable to support a measurable incorporation of dTTP.

Despite the abrupt and relatively early reduction of incorporation in the inhibited system, preincubation for ten minutes with ddATP in the absence of dATP did not totally eliminate the ability to incorporate, as detected after subsequent addition of dATP.

Inhibition of pyrophosphate exchange: As shown in Figure 3, the pyrophosphate exchange reaction of DNA polymerase is inhibited in the complete system by

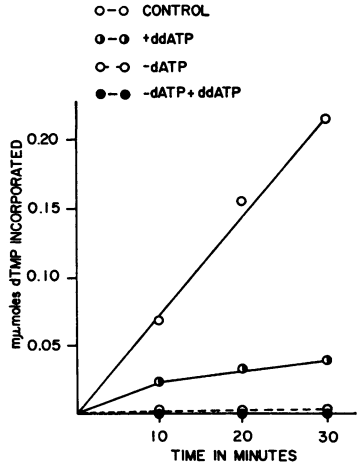

FIG. 2.—Time study of the inhibition of *E. coli* DNA polymerase by ddATP at a ratio of 4 ddATP to 1 dATP, assayed as described in *Materials and Methods* with dAT copolymer as template.

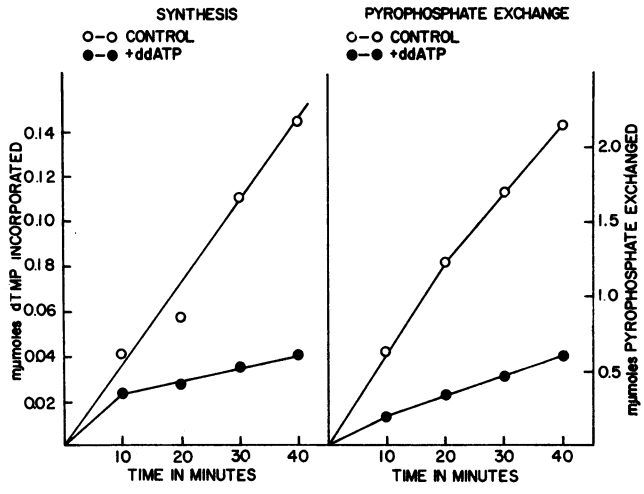

FIG. 3.—Comparison of inhibition by ddATP of synthesis and pyrophosphate exchange by DNA polymerase. (See legend to Fig. 2.)

ddATP, approximately paralleling the inhibition of polymer synthesis. In the absence of either dTTP or dATP, pyrophosphate exchange is reduced about 50 per cent (Fig. 4). The latter figure also shows that in the absence of dATP, pyrophosphate exchange is virtually shut off by ddATP. With ddATP as the only nucleoside triphosphate present, no pyrophosphate exchange was detectable.

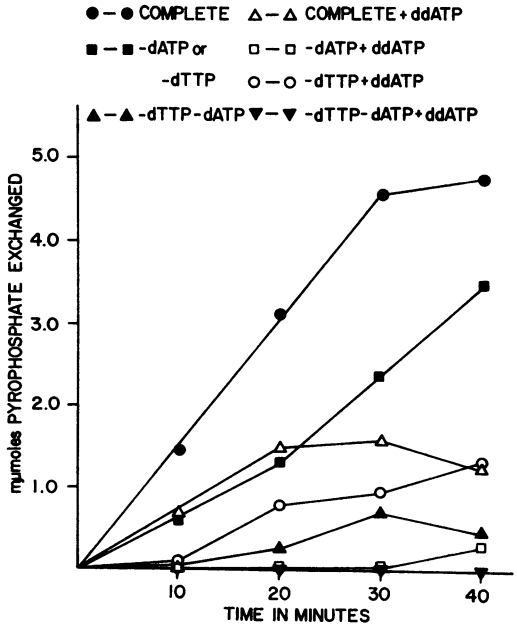

FIG. 4.—Inhibition of pyrophosphate exchange by ddATP in the presence and absence of normal nucleoside triphosphates. The concentration of ddATP was that used in Fig. 2.

Incorporation of ^{3}H-ddATP: In order to determine whether the dideoxyadenosine was actually incorporated into the polydeoxynucleotide, the triphosphate of tritiated dideoxyadenosine was prepared and used with DNA polymerase assayed with both dAT copolymer and activated calf thymus DNA as templates. In both cases ddATP was incorporated into acid-precipitable material. The number of 3′-hydroxyl ends of dAT copolymer was estimated from the sum of single additions of dTTP and dATP. There were 0.90 $\mu\mu$mole of dTTP and 2.54 $\mu\mu$moles of dATP added per 0.90 μg dAT copolymer under these conditions. The sum of 3.35 may be compared to 2.54 ± 0.24 $\mu\mu$moles (four estimates) of ddATP incorporated in the template in the presence of both dTTP and dATP. The observed single additions of dATP and dTTP may be high as a result of the exonuclease activity of the polymerase. In any case, it is evident that the addition of ddATP approached the value for the total accepting ends of the template chain in the complete system.

After exposure to ^{3}H-ddATP in the DNA polymerase system (as in legend, Fig. 2), the dAT template was isolated as described by Weiss *et al.*[13] and centrifuged to equilibrium in CsCl, 40,000 rpm for 40 hours at 20° in the Ti 65 rotor. The tritium label banded with the polymer (Fig. 5) at a density of 1.68 gm/cc.

Additional template was labeled with ^{3}H-ddAMP, isolated, and digested with snake venom phosphodiesterase; the conversion of label and optical density to acid-soluble material was followed with time. As seen in Figure 6, the tritium-labeled ddAMP is far more rapidly released into acid-soluble material than the

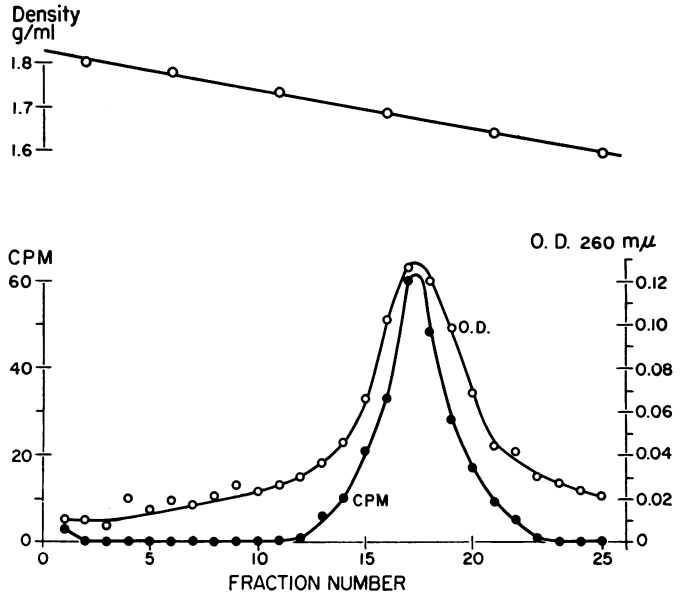

FIG. 5.—dAT copolymer isolated after exposure to ^{3}H-ddATP as in Fig. 2 and centrifuged to equilibrium in CsCl, 40,000 rpm, 40 hr, 20°C. The specific activity of the ^{3}H-ddATP was 3.3 × 10^8 cpm/μmole.

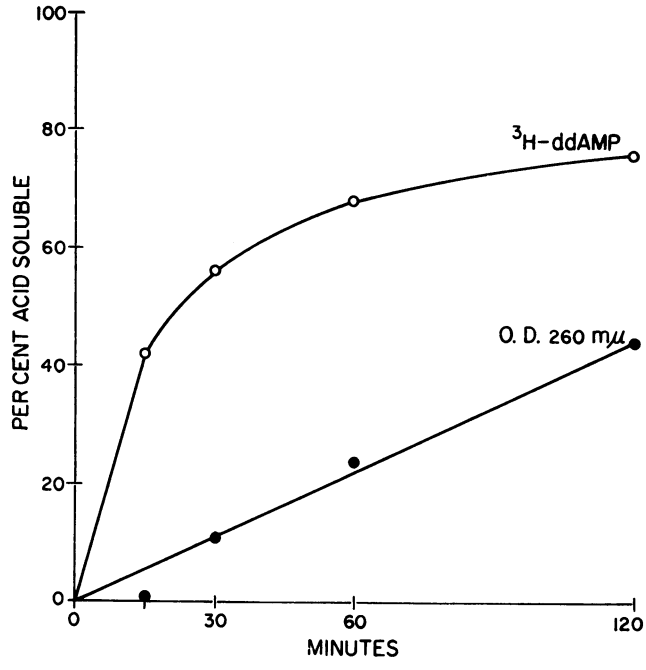

FIG. 6.—Digestion of ^{3}H-ddAMP-labeled template (2500 cpm ^{3}H and 2.12 units OD 260) with snake venom phosphodiesterase 100 μg assayed in 5.5 ml 0.1 *M* tris pH 8.5, 0.03 *M* Mg^{+2}. Samples were precipitated with 3% perchloric acid and the neutralized supernatants were analyzed. At zero time, the released counts were less than 10% of that released in 15 min. Counts released enzymatically were corrected by subtracting the counts at zero time.

unlabeled nucleotides, as measured by optical density. This is consistent with the view that ddAMP is at the end of the polydeoxynucleotide chain.

Isolated template that had been labeled by incubation with ddATP was exposed to DNA polymerase in the complete system for synthesis and the rate of release of radioactive ddAMP from the template compared to that for dAMP. The dideoxynucleotide was released at only 1/200 the rate of the normal deoxynucleotide.

Discussion.—It has been demonstrated that ddATP inhibits the incorporation of dTTP and dATP by DNA polymerase into dAT copolymer by at least two mechanisms. The first is an apparent competition with dATP for the enzyme. The second is an essentially complete termination of the dAT copolymer chain by the nucleotide lacking a 3′-hydroxyl group. Investigations with 2′,3′-dideoxythymidine triphosphate with *E. coli* DNA polymerase[12, 14] have shown that this nucleotide also terminates polydeoxynucleotide chains.

Such terminated polydeoxynucleotides should be of considerable interest in the study of numerous reactions involving DNA. It may be noted that a partially purified DNA polymerase of animal cells, kindly given to us by Dr. John Furth, is also inhibited by ddATP.

At the present time, experiments are in progress to determine whether the com-

pound does kill the bacteria by mechanisms demonstrated in our enzymatic study. If it can be shown that the nucleoside terminates DNA chains in intact cells, it will be of great interest to explore the genetic properties of such cells, e.g., conjugation and transfer of chromosomal markers, inducibility of lysogenic phages, etc. In any case, the effect of the nucleoside in producing and modifying various types of genetic change is an obvious avenue of study.

No effects of this or other dideoxynucleosides have been observed in limited tests with animal cells (unpublished data). It appears that the adenosine kinase of animal cells is quite unable to phosphorylate these compounds to a significant extent. The observation that ddATP inhibits an animal cell DNA polymerase suggests that such a nucleotide might well prevent the multiplication of animal cells. This potentiality once more underlines the importance of learning how to insert nucleotides into animal cells.

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