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Termination of Deoxyribonucleic Acid in *Escherichia coli* by 2',3'-Dideoxyadenosine[1]

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2',3'-Dideoxyadenosine was previously shown to be lethal to *Escherichia coli* and to inhibit deoxyribonucleic acid (DNA) synthesis irreversibly in this organism. It was also shown that triphosphate of this analogue terminates DNA chains in an in vitro system. Data presented here show that the nucleoside is relatively insensitive to *E. coli* adenosine deaminase and is converted intracellularly into the dideoxynucleotide, including the triphosphate. Thymine nucleotide pools were not reduced in inhibited bacteria, nor did preformed DNA break down. Some adenine was liberated from the dideoxyadenosine on incubation, and the latter was incorporated into ribonucleic acid. Nevertheless, about 4,000 molecules of the dideoxynucleoside were incorporated into DNA per cell. The dideoxynucleotide occurred in DNA chains in a terminal position, liberated selectively by venom phosphodiesterase. The possible nature of the lethal event is discussed.

The lethality of 2',3'-dideoxyadenosine to several strains of *Escherichia coli* was demonstrated in 1966 (3). The compound had been tested because the absence of a 3'-hydroxyl group indicated that the derived nucleotide might terminate deoxyribonucleic acid (DNA) chains. Indeed, the metabolic lesion observed in *E. coli* was a relatively specific irreversible inhibition of DNA synthesis (3), a result suggesting the validity of the proposed mechanism of toxicity. More recently, we showed that the triphosphate of this dideoxynucleoside does in fact terminate polydeoxynucleotide chains in vitro when a purified DNA polymerase derived from *E. coli* is used (16). We have now extended this study to demonstrate in vivo incorporation of the nucleotide to the termini of polydeoxynucleotide chains of *E. coli* DNA.

A number of chemical and physiological problems have appeared in the course of this study. We have, therefore, also explored such questions as the stability of the nucleoside to the bacterial deaminase, the sensitivity of T-even phage multiplication to the nucleoside, the formation of the nucleotide in vivo, and the effect of the compound on the pool of thymidine nucleotides and on the stability of the bacterial DNA.

MATERIALS AND METHODS

Bacteria used in these experiments were *E. coli* B and *E. coli* 15 TAU (4). All experiments were performed in the medium 52 previously described (3).

Dideoxyadenosine, kindly supplied by Roland K. Robins, was supplemented with material prepared in this laboratory by catalytic reduction of 2',3'-didehydro-2',3'-dideoxyadenosine. This material had been provided by the Cancer Chemotherapy National Service Center or had been synthesized in this laboratory according to the methods of McCarthy et al. (6) and Robins et al. (12). Radioactive dideoxyadenosine was prepared by catalytic reduction of 2',3'-didehydro-2',3'-dideoxyadenosine with tritium gas. The dideoxyadenosine was purified of free sugar, free adenine, and deoxyadenosine by paper chromatography in water made *p*H 10 with 1 M NH_4OH and by column chromatography on Dowex-1 (OH^-, 2 × 20 cm) in 30% methanol (w/w). Upon acid hydrolysis, 0.15% of the label was found in the adenine portion of the molecule.

Cells were collected on membrane filters (diameter, 47 mm; pore size, 0.45 μm; Millipore Corp., Bedford, Mass.), and nucleotide pool isolations were performed by the method of Neuhard and Munch-Petersen (7). Mono-, di-, and triphosphates were separated on diethylaminoethyl cellulose according to the method of Smith and Khorana (14).

The preparation and cesium chloride centrifugation of cellular DNA were done as described by Carpenter and Binkley (2).

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Adenosine deaminase was induced, extracted, and assayed according to Koch and Vallee (5).

RESULTS

Dideoxyadenosine as a substrate for E. coli adenosine deaminase. The activity of *E. coli* adenosine deaminase in cell extracts was examined. The adenine nucleosides 2'-deoxyadenosine and adenosine are readily deaminated by this enzyme. As is shown in Table 1, 2',3'-dideoxyadenosine was deaminated at only about 4% of the rate for 2'-deoxyadenosine at comparable concentrations. K_m values of 77 and 182 μM were found for deoxyadenosine and dideoxyadenosine, respectively, and V_{max} values were 8.3 and 0.42 μmoles per mg of protein per hr, respectively. The presence of dideoxyadenosine (10^{-4} M) did not alter the rate or the extent of deamination of 2'-deoxyadenosine at an equal concentration.

Adenine is not deaminated by the *E. coli* adenosine deaminase (5). After exposure to the enzyme preparation, the dideoxyadenosine was examined by paper chromatography and was shown to be present as dideoxyadenosine; it had not been cleaved to give enzyme-resistant free adenine.

The dideoxynucleoside was less stable than 2'-deoxyadenosine, and some adenine was freed from the compound on prolonged incubation in a culture of *E. coli* at 37 C. It will be seen subsequently that, despite the relatively low tritium content of the adenine (0.15%) compared with the remainder of the tritium in the dideoxyribosyl moiety, the adenine liberated was incorporated into both ribonucleic acid (RNA) and DNA and tended to complicate the eventual labeling pattern.

Conversion of dideoxyadenosine to the nucleotide level in intact cells. We have shown that 2',3'-dideoxyadenosine-5'-triphosphate can be utilized by *E. coli* DNA polymerase in vitro, and is in fact terminally incorporated into the polydeoxynucleotide chain (16). The nucleotide pool of 15 TAU exposed to tritiated 2',3'-dideoxyadenosine was isolated; a small portion (about 1%) of the radioactive nucleoside taken into the cell was converted to nucleotides. The mono-, di-, and triphosphates were separated (Table 2). Upon removal of the phosphate from the dideoxyadenosine monophosphate and chromatography of the free nucleoside on Dowex-1 (OH^-) in 30% methanol, the label was found in dideoxyadenosine (Fig. 1), indicating that the radioactive material in the monophosphate came from phosphorylation of dideoxyadenosine and not from breakdown of the nucleoside and utilization of the adenine portion. Degradation of the 2',3'-

TABLE 1. *Deamination of adenine nucleosides by E. coli adenosine deaminase (5)*

Nucleoside	Concn (μM)	Rate (μmoles per mg per hr)
2'-Deoxyadenosine	41	4.5
3'-Deoxyadenosine	40	0.32
2',3'-Dideoxyadenosine	40	0.18

TABLE 2. *^{3}H-Dideoxyadenosine in the nucleotide pool of 15 TAU[a]*

Phosphate[b]	Counts per min		
	30 min	60 min	120 min
Monophosphate	8,940	15,530	18,040
Triphosphate	12,010	16,620	17,080

[a] An exponentially growing culture of *E. coli* 15 TAU was harvested by centrifugation, and the cells were resuspended in minimal medium plus tritiated dideoxyadenosine at 1.7×10^7 counts per min per μmole and 1 μmole/ml. At the indicated times, a 5-ml sample was harvested on a membrane filter, and the acid-soluble pool was isolated as described in Materials and Methods.

[b] Only trace amounts of diphosphate were detected.

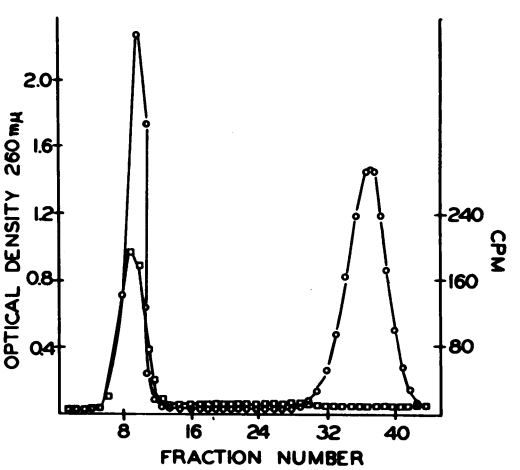

FIG. 1. *Dowex-1 (OH^-) column chromatography of monophosphate nucleosides isolated from ^{3}H-dideoxyadenosine-treated cells. The monophosphate peaks from the nucleotide pools described in Table 2 were digested with alkaline phosphatase. Carrier dideoxyadenosine and deoxyadenosine were added, and the solution was then made 30% in methanol and chromatographed on a Dowex-1 (OH^-) column, previously equilibrated with 30% methanol. Dideoxyadenosine was eluted first.*

dideoxyadenosine-5′-triphosphate isolated from the cells to free nucleoside, followed by chromatography, also indicated that the label was in 2′,3′-dideoxyadenosine.

If one assumes 0.25 mg (dry weight) per 10^9 cells and that turbidity reflects cell mass, and uses the specific activity of dideoxyadenosine, the amount of dideoxyadenosine triphosphate can be calculated and compared with the published values for deoxyadenosine triphosphate. There were 1.88, 1.90 and 1.03 μmoles of dideoxyadenosine triphosphate per g (dry weight) in 15 TAU at 30, 60, and 120 min. The published value for deoxyadenosine triphosphate (7) in 15 TAU in log-phase growth is 0.20 ± 0.06 μmole per g (dry weight). Thus, the concentration of dideoxyadenosine triphosphate in the cells was 5 to 10 times that of deoxyadenosine triphosphate. This value is sufficiently high to cause inhibition of DNA polymerase if the ratio of dideoxyadenosine triphosphate to deoxyadenosine triphosphate required for inhibition in the cell is the same as that required in the in vitro system (16).

Incorporation of tritiated dideoxyadenosine into cellular DNA. The terminal incorporation in vitro of dideoxyadenosine monophosphate residues into polydeoxynucleotides by *E. coli* DNA polymerase (16), the possible existence of numerous short polydeoxynucleotide chains during DNA synthesis in vivo, and the conversion of dideoxyadenosine to the triphosphate in intact cells made it reasonable to look for incorporation of radioactive dideoxyadenosine into cellular DNA.

An exponentially growing culture of 15 TAU was harvested and resuspended in minimal medium containing ^{3}H-dideoxyadenosine at 3.6 × 10^7 counts per min per μmole and 1 μmole per ml. Duplicate samples of 0.5 ml were removed at 0, 30, 60, 90, and 120 min, and incorporation of isotope into total nucleic acids and alkali-resistant nucleic acid was determined as described by Stern et al. (15). Incorporation into alkali-resistant nucleic acid was maximal at 40 pmoles at 60 min, although total incorporation increased to three times this level at 120 min. It was calculated that this amount incorporated into alkali-resistant nucleic acid or DNA was about 4,000 molecules of dideoxyadenosine per cell. The isotope present in alkali-sensitive nucleic acid was suspected to be in adenine liberated from the analogue and then incorporated into RNA.

To study this in greater detail, we fractionated the nucleic acids in a cesium chloride gradient. An exponentially growing culture of 15 TAU was harvested, and the cells were resuspended in three different cultures. These were incubated for 1 hr in mineral medium. In the control, the DNA was labeled by incorporation of ^{14}C-thymine. In the second, ^{3}H-dideoxyadenosine was added as in the previous experiment on incorporation. In the third, ^{14}C-uracil was added to unlabeled dideoxyadenosine. Uracil was incorporated into RNA.

As is shown in Fig. 2, dideoxyadenosine was incorporated into acid-precipitable material, banding in CsCl at a density of 1.710, corresponding to the density of *E. coli* 15 TAU DNA. However, acid-precipitable label also appeared in positions corresponding to those for ribosomal RNA and transfer RNA from a culture exposed to ^{14}C-uracil. It is likely, therefore, that the label appearing in positions for RNA came from cleavage of dideoxyadenosine to free adenine and reutilization of this labeled moiety.

The DNA band was dialyzed to remove CsCl and was digested with snake venom phosphodiesterase; the conversion of total nucleotide and radioactive nucleotide to acid-soluble form was then followed. Because of the possibility that some label in the DNA might be present as 2′-deoxyadenosine, the acid-soluble nucleotides were treated with alkaline phosphatase, and the resulting nucleosides were separated on Dowex-1 (OH^-). The results (Table 3) show clearly that

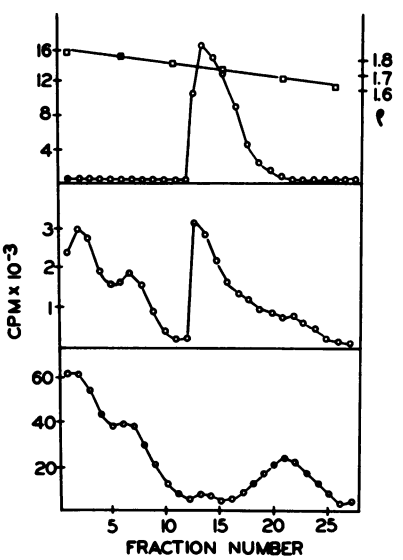

FIG. 2. *Cesium chloride centrifugation of cellular material. Cells were treated by the method of Carpenter and Brinkley (1) and banded in cesium chloride by centrifugation at 29,000 rev/min for 22 hr at 8 C. Top: control cells labeled with ^{14}C-thymine. Middle: cells killed with ^{3}H-dideoxyadenosine (10^7 counts per min per μmole, 1 μmole/ml). Bottom: cells killed for 2 hr with unlabeled dideoxyadenosine while being labeled with ^{14}C-uracil. All incorporations represent acid-precipitable material in the fraction.*

radioactive dideoxyadenosine is incorporated into cellular DNA and, as compared with 2'-deoxyadenosine, is relatively rapidly released by snake venom phosphodiesterase. These results support the hypothesis of chain termination by dideoxyadenosine.

Effect of dideoxyadenosine on the pools of thymine nucleotides. Since dideoxyadenosine accelerates thymineless death (3), it was asked whether the compound did not in some manner reduce the synthesis of thymine nucleotides. As can be seen in Table 4, the presence of a lethal concentration of dideoxyadenosine (1 μmole per ml) did not reduce the concentration of these nucleotides significantly. On the contrary, the intracellular concentration of deoxythymidine triphosphate appeared to be significantly elevated, perhaps as a result of accumulation under conditions of inhibition of DNA synthesis.

Lack of effect of dideoxyadenosine on degradation of cellular DNA. Cells prelabeled with thymine for two generations were washed and resuspended in complete unlabeled medium with and without dideoxyadenosine. The amount of label remaining acid-precipitable in the course of killing over a 2-hr interval was determined. All of the label remained acid-precipitable in the absence or presence of the analogue. Cells prelabeled with thymine for only 10 min gave the same result. Thus, the reduced incorporation of pyrimidines into DNA is not due to a generalized stimulation of degradation of DNA. However, the possibility that there is an increased degradation of newly synthesized DNA or that nicks have been introduced in the DNA, or both, has not been excluded.

TABLE 3. *Release of ^{3}H-dideoxyadenosine from labeled cellular DNA*[a]

Time (min)	Optical density (260 nm)	Counts/min in	
		Dideoxyadenosine	Deoxyadenosine
30	0.040	640	391
120	0.221	970	2,700

[a] The DNA from a culture exposed to ^{3}H-dedioxyadenosine at 8.4×10^7 counts per min per μmole was isolated by the method of Carpenter and Brinkley (2), dialyzed, and digested with snake venom phosphodiesterase. The DNA was precipitated with acid and the optical density of the supernatant fluid was read. The supernatant fluid was digested with alkaline phosphatase. Carrier dideoxyadenosine and carrier deoxyadenosine were added, and the samples were chromatographed on Dowex-1 (OH^-). The amount of enzyme present was low so that it was possible to distinguish the release of the two nucleotides in time.

TABLE 4. *Determination of thymidine nucleotide pools in the presence and absence of 2',3'-dideoxyadenosine in 15 TAU*[a]

Sample	Nucleoside and base		Monophosphate		Triphosphate	
	Counts/min	Per cent	Counts/min	Per cent	Counts/min	Per cent
30-min pool						
Control	13,830	88.4	695	4.4	1,060	6.8
+ Dideoxyadenosine	15,005	83.0	805	4.1	2,110	11.7
60-Minute Pool						
Control	14,790	81.4	1,250	6.9	2,120	11.7
+ Dideoxyadenosine	19,270	76.0	1,540	6.1	4,190	16.5

[a] An exponentially growing culture of 15 TAU was harvested by centrifugation and resuspended in minimal medium containing ^{14}C-thymine at 6×10^6 counts per min per μmole. Samples of 4.5 ml were removed from each culture and harvested rapidly by collection on a membrane filter. The pool was isolated and chromatographed as described in Materials and Methods.

Lack of effect of dideoxyadenosine on T6r$^+$ multiplication. Dideoxyadenosine at 1 μmole per ml did not prolong the latent period or reduce the burst size of T6r$^+$ in single infection of *E. coli* strain B in synthetic medium. The cells were pretreated with nucleoside, and the viable count was reduced to 1% of the original. These cells supported a normal multiplication of phage in the presence or absence of nucleoside. When DNA synthesis was measured in infected cells in the presence or absence of the analogue, no differences were observed in the start of DNA synthesis or in its rate over a 75-min interval, a period which permitted an approximately 10-fold increment in DNA.

DISCUSSION

The work on the incorporation of dideoxyadenosine into *E. coli* DNA was delayed until after studies with the in vitro system (3) because it was suspected that very few ends of the chromosomal polydeoxynucleotide would be available for termination. The experiments with phage were undertaken to attempt to provide a system in which the numbers of such ends would be increased. It is not yet known why T-even phage systems are insensitive to the agent when the host is sensitive. Although the *E. coli* DNA

polymerase is inhibited by the triphosphate of the analogue, it is possible that the T6-induced DNA polymerase is not similarly sensitive. This remains to be studied.

While we were examining the effects of the dideoxynucleoside triphosphate on DNA synthesis with an in vitro system (3), a new hypothesis of discontinuous DNA synthesis was advanced (8–11). One group of experimental consequences of this hypothesis was realized, namely, that numerous short chains of acid-precipitable polydeoxynucleotides were found early in DNA synthesis (8–11). This hypothesis and result suggested that we might detect numerous small pieces of DNA terminated by dideoxyadenosine. The level of incorporation of the analogue into DNA observed in our experiments is in fact consistent with this hypothesis. DNA has been isolated from cells treated with the analogue and was indeed found to be terminated with dideoxyadenosine. This result is consistent with our original hypothesis (3) and with the in vitro data (16). If all of the analogue is assumed to be terminal in acid-precipitable fragments of 100 nucleotide pairs, the level of incorporation in DNA would correspond to a replication of about 8% of the genome.

While this work was in progress, enzymatic studies with dideoxythymidine triphosphate (13) were also being undertaken (1). This work has similarly led to the conclusion that this dideoxynucleotide is a chain terminator of polydeoxynucleotides in cell-free systems (1). However, we have shown that *E. coli* strains are insensitive to dideoxythymidine, dideoxycytidine, and dideoxyuridine at fairly high concentrations (3). It appears then that dideoxyadenosine is the only agent presently available which has been shown to terminate the DNA chains of intact *E. coli* cells.

Although we have observed that lethality with dideoxyadenosine is accompanied by terminal incorporation of the nucleotide into DNA in the cell, the course of killing is far from that of single-hit kinetics. Indeed, many molecules of the analogue are incorporated before a drop in viable count can be detected. The nature of the actual lethal event is, therefore, unclear, although it can be imagined that the presence of multiple breaks in a double-stranded chromosome would kill.

It has not yet been determined whether the molecules terminated with the analogue are multiple copies of the same portion of DNA, which might be the case if terminated chains were detached from the chromosome, or whether these molecules include many unique chains retained on a double helix containing many breaks.

We have learned from M. R. Atkinson and A. R. Kornberg (*personal communication*) that the triphosphates of D-arabinosylcytosine and D-arabinosyladenine also terminate polydeoxynucleotide chains with *E. coli* DNA polymerase. The enzymatic rates of addition of these nucleotides are very low, and it is not yet known whether these compounds indeed terminate DNA in *E. coli* or in eucaryotic cells in which the substances are quite lethal. For the reasons described above, even if the compounds are found at the ends of DNA chains in vivo, it will not be clear whether this termination is actually the lethal event.

ACKNOWLEDGMENT

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