

Polymer Synthesis in Killed Bacteria: Lethality of 2', 3'-Dideoxyadenosine

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ABSTRACT

DOERING, ALICE MCGOVERN (University of Pennsylvania School of Medicine, Philadelphia), MIEKIE JANSEN, AND SEYMOUR S. COHEN. Polymer synthesis in killed bacteria: lethality of 2', 3'-dideoxyadenosine. *J. Bacteriol.* 92:565-574. 1966.—We studied the metabolic capabilities of cells that had lost the ability to multiply under a variety of lethal treatments. Cultures of a polyauxotrophic mutant of *Escherichia coli* strain 15 requiring thymine, arginine, uracil, and adenine for growth were killed to a few per cent survivors by several different methods. These treatments included streptomycin, thymineless death, D-arabinosyladenine (ara-A), and 2', 3'-dideoxyadenosine (DDA). The killed cells were washed and supplied with complete media, and were compared with control cells with respect to the ability to incorporate thymine, arginine, and uracil. Cells killed with streptomycin in the absence of thymine were only partially inhibited in deoxyribonucleic acid (DNA) synthesis; they were markedly inhibited in synthesis of ribonucleic acid (RNA) and protein. Cells that had suffered thymineless death were essentially uninhibited in DNA synthesis, partially so in RNA synthesis, but extensively inhibited in protein synthesis. Killing by ara-A did not prevent DNA synthesis, but markedly inhibited RNA and protein synthesis. The lethality of DDA was studied in the presence of exogenous adenosine; lethality was partially prevented by deoxyadenosine. Dideoxyadenosine was similar to ara-A and thymineless death in killing in a pattern in which RNA and protein synthesis continued while DNA synthesis was inhibited. Cells killed by DDA, however, were markedly inhibited in subsequent thymine incorporation, unlike cells killed by the other methods. In addition, at this time, the DDA-killed cells were more inhibited in incorporation of arginine than of uracil. DDA also potentiated thymineless death; when cells were killed rapidly by the combined treatment, only the ability to synthesize DNA was lost irreversibly. This agent (DDA) may permit the detailed study in *E. coli* of the relation of DNA synthesis to numerous phenomena, such as genetic recombination, sequential transcription, and the number and distribution of chromosome breaks.

The lethality and some metabolic effects of 9- β -D-arabinofuranosyladenine (ara-A) with selected strains of *Escherichia coli* and mouse fibroblasts are described in the accompanying publication (4). It has been shown that mouse cells, inhibited in deoxyribonucleic acid (DNA) synthesis for long periods by either ara-A or D-arabinosylcytosine (ara-C), are capable of normal rates of DNA synthesis for 6 hr after removal of the inhibitory arabinosyl nucleoside (Doering, Keller, and Cohen, *in press*). Although it is not possible to study this phenomenon in bacteria with ara-C, we were able to test this with ara-A in the purine-requiring *E. coli* strain 15 TAUAd.

In effect, then, we studied the polymer-syn-

thesizing ability of killed cells, i.e., cells that had lost the ability to multiply, to form a colony. We had, in fact, used a similar technique in recent studies to show that bacteria killed by streptomycin in the absence of protein synthesis are physiologically dead and incapable of protein synthesis at the time of plating; i.e., loss of physiological function does not occur during incubation on the plate but does occur at the time of the first step in the test of viability (Stern, Barner, and Cohen, *in press*).

In studying the effects of ara-A, we compared these with thymineless death and other toxic agents. It soon became clear, even as in mouse fibroblasts, that a toxic agent that inhibited DNA

synthesis need not produce irreversible damage in the mechanisms of DNA synthesis and that no lethal agent capable of irreversibly jamming this process was in fact known. In fact, no instance of such irreversible damage, capable of serving as a control in our experiments, was known.

It was suspected that a 2'-deoxynucleoside lacking a 3'-hydroxyl might well be lethal, because it might terminate DNA chains, and we therefore tested some recently synthesized compounds of this type for toxicity. We determined that the synthetic nucleoside, 2',3'-dideoxyadenosine (DDA) was indeed lethal. Unlike other lethal treatments, DDA was largely irreversible in inhibiting DNA synthesis in killed cells, as predicted. The structural relations of the five adenine nucleosides used in this study are presented in Fig. 1.

Killed cells were also studied for their ability to synthesize RNA and protein. Some treatments, such as thymineless death and DDA, resulted in the loss of the ability to incorporate amino acids into protein to a greater extent than that of RNA synthesis.

MATERIALS AND METHODS

The isolation of the polyauxotrophic *E. coli* strain 15 TAUAd, designated strain TAUAd, is described in the accompanying paper (4). The supplementation for growth of a mineral medium containing glucose with thymine, arginine, uracil, adenosine or adenine, and histidine is described in that paper, as are the growth and viability of the organism in various complete and deficient media. The organism was seen to die rapidly,

after an initial lag, in a complete medium lacking only thymine (thymineless death), an effect markedly reduced by concomitant adenine deficiency. In the absence of adenine, ara-A was shown to be more toxic than just the thymine deficiency under comparable conditions.

Methods used for the study of the incorporation of thymine-2- C^{14} , arginine-guanido- C^{14} , and uracil-2- C^{14} into strain TAUAd, as well as the sources of those compounds and of ara-A, were described (4). Streptomycin sulfate was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. DDA and its synthetic precursor, 2',3'-dideoxy-2'-adenosinene, were kindly made available by Roland Robins of the University of Utah (6). 2',3'-Dideoxycytidine and 2',3'-dideoxyuridine were given to us by James Hunter of The Upjohn Co.

RESULTS

DNA synthesis in cells killed by ara-A and thymine deficiency. It is difficult to compare cells killed by the two different methods. Thymineless death was carried out in the presence of exogenous adenosine, and the control for that system was a normally growing culture. On the other hand, ara-A killing occurs in the absence of adenosine (4), and the control culture for this system is devoid of adenosine and contains cells slowly growing by depleting its adenine nucleotides from both a soluble pool and messenger ribonucleic acid (RNA). In the latter case, the cells starved for adenosine must restore not only its adenosine triphosphate level and messenger RNA but also the essential deoxyribonucleotides before thymine incorporation and DNA synthesis can occur.

Nevertheless, Fig. 2 shows a comparison of DNA synthesis under the four different conditions. A normal culture was permitted to grow for 90 min in complete medium to about 3×10^8 viable cells per milliliter; a similar culture was exposed to thymine deficiency, and viability fell from 2×10^8 to 10^7 viable cells per milliliter. When the two cultures were washed and re-exposed at 10^8 cells per milliliter to a complete medium containing thymine-2- C^{14} , the dead cells synthesized DNA at an initial rate very similar to that of the control (Fig. 2A). In the dead culture, the rate remained essentially constant after a brief period of increase. Since the thymine incorporation per increment of 10^8 normal cells was about 1.2 μ moles, the dead culture appeared to pass through the equivalent of several cycles of DNA replication.

A similar result was obtained after ara-A killing. A control culture without adenosine showed a 70% increase in turbidity in 3 hr and a similar increment in viable cells (1.5×10^8 to 2.6×10^8 per milliliter). In the same time, a similar culture

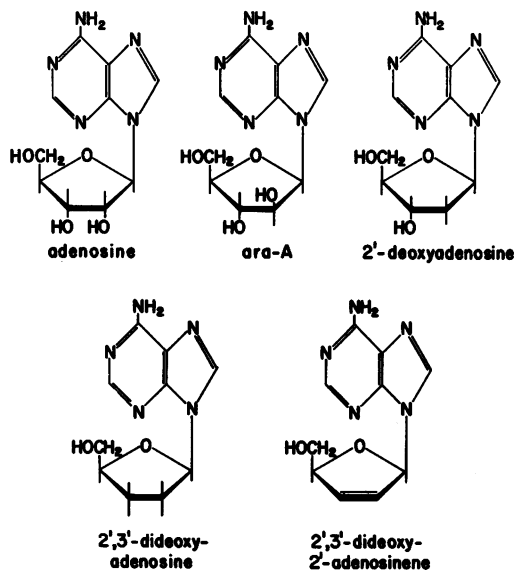


FIG. 1. Structure of adenine nucleosides.

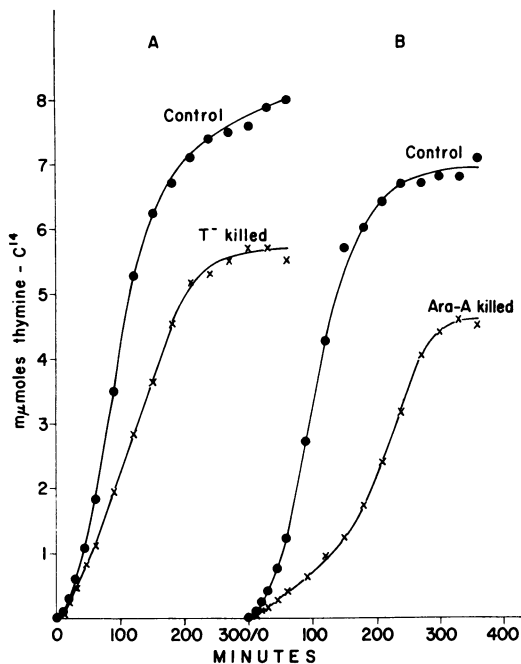


FIG. 2. Incorporation of thymine- C^{14} (5.72×10^5 counts per min per μ mole) into cultures of thymine-deficient or ara-A-killed cells (5 to 7% survivors) and their controls. The killed cells were washed and suspended in complete media. The control for the thymine-deficient cells was derived from a growing culture, whereas that for the ara-A-killed cells was from an adenosine-starved culture. A thymine-deficient culture contains arginine, uracil, and adenosine in the minimal media. Ara-A killing occurs in a medium containing thymine, arginine, and uracil but lacking adenosine.

containing 10^{-4} M ara-A had a 40% increase in turbidity and a 97% decrease in viability. These cultures were washed and exposed to a complete medium at cell numbers essentially similar to those in the normal and thymineless series. The adenosine-starved culture began DNA synthesis at a slightly slower rate than did the normal culture (Fig. 2B). The cells killed with ara-A began DNA synthesis at a rate about one-third that of the adenosine-starved culture, but this rate slowly increased to approach that in the growing culture. The total increment in DNA, measured in terms of thymine incorporation, was several times that necessary for one doubling.

RNA and protein synthesis in cells killed by ara-A and thymine deficiency. In Fig. 3 are presented the data of three experiments for incorporation of uracil, arginine, or thymine into an adenosine-starved paired culture killed with ara-A (and the control of adenosine-starved cells). Survival of the three treated cultures was comparable

(5 to 8%). The rates of uracil and arginine incorporation were very low and remained low for 90 min, in contrast to the relatively high rates of thymine incorporation.

In Fig. 4 data are given for uracil and arginine incorporation in paired cultures, in which thymine deficiency had led to a 1% survival of viable cells. Surprisingly, in cells killed by thymineless death, RNA synthesis was far less inhibited than was protein synthesis. This is in contrast to the ara-A-killed cultures, in which both RNA and protein synthesis were extensively inhibited.

Polymer synthesis in cells killed by streptomycin. It has been shown in earlier papers that the lethality of streptomycin can occur without apparently affecting the course of thymine incorporation into DNA (7). Killing by this antibiotic has been shown to proceed in the absence of protein synthesis, under conditions in which the inception and rate of killing coincide with the appearance and rate of synthesis of RNA. We have demonstrated also that cells that had, without protein synthesis, lost the ability to form colonies by plating had simultaneously lost the ability to incorporate amino acids into protein (Stern, Barner, and Cohen, *in press*).

We studied the ability of cells, killed by streptomycin (60 μ g per ml) in a complete medium, to make nucleic acids after removal of the antibiotic by washing. Such cells (2.4% survivors), killed over a 2-hr period, were unable to incorporate thymine into DNA, or significant amounts of uracil into RNA (Fig. 5). Thus, under these conditions of killing, streptomycin appears to block irreversibly all polymer synthesis.

However, it can be considered that if streptomycin prevents protein synthesis before a cycle of DNA synthesis is completed, as indeed may occur, a bacterium will complete a cycle of DNA synthesis and be unable to begin a new cycle of DNA synthesis. The apparently irreversible inhibition of DNA synthesis as a result of streptomycin killing would then be only a secondary effect of the system.

We avoided this by killing with streptomycin in the absence of thymine. At 1 hr, a culture treated with streptomycin in the absence of thymine had only 1.6% survivors, in contrast to the thymineless culture without antibiotic which had 30% survivors (Fig. 6). Cells killed as in Fig. 6 to similar levels of survivors (0.3 to 2.8%) were then washed and tested for DNA synthesis in a complete medium. It appears that cells killed by streptomycin in the absence of thymine can make DNA at an initial rate approaching that in thymineless death. The cells killed in thymineless death can, in 90 min, make more than one equiva-

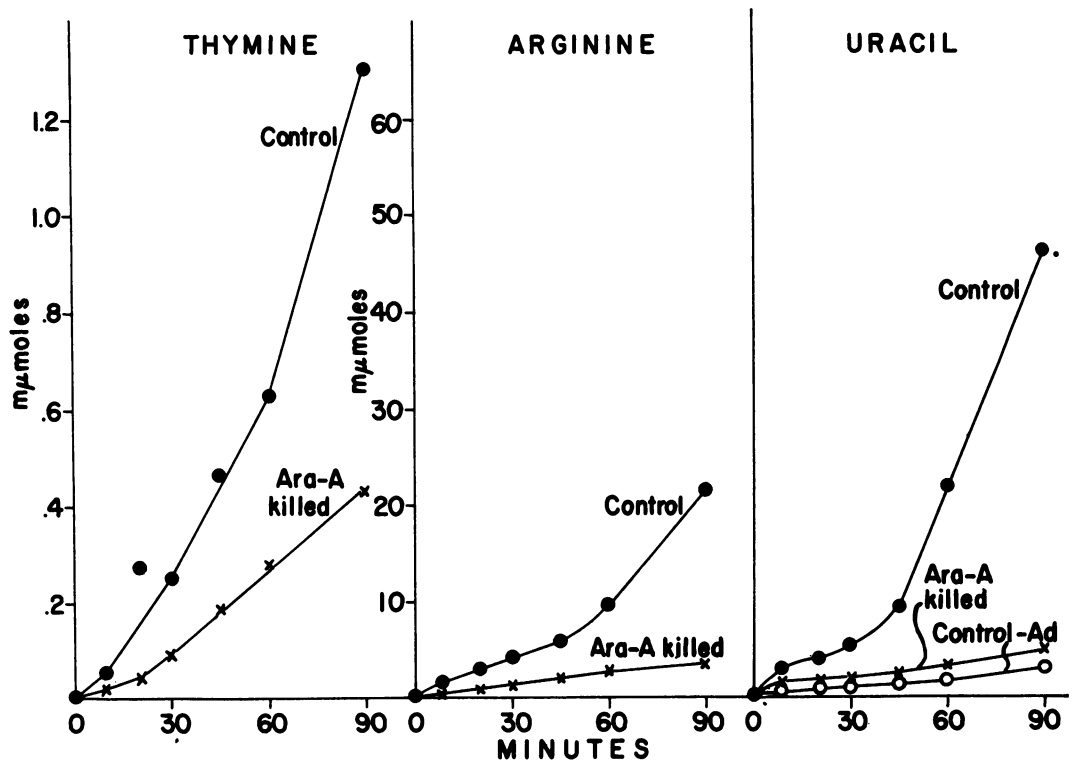


FIG. 3. Incorporation in a complete medium of thymine (5.72×10^6 counts per min per μmole), arginine (7.2×10^4 counts per min per μmole), or uracil (3.4×10^4 counts per min per μmole) into cultures of *ara-A*-killed cells (5 to 8% survivors) and adenosine-starved controls (see legend of Fig. 2). Efforts were made to have the initial cell concentrations at the same level; however, due to the various treatments, small differences did occur.

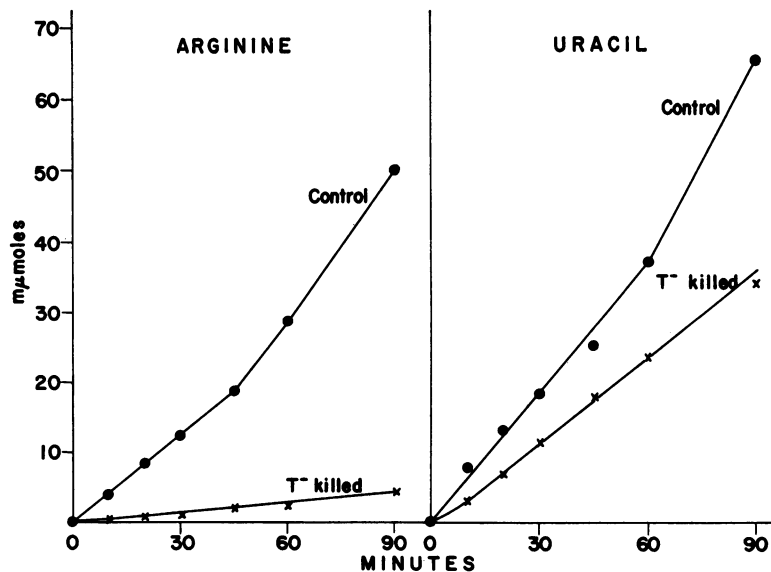


FIG. 4. Incorporation in a complete medium of arginine (7.2×10^4 counts per min per μmole) or uracil (3.4×10^4 counts per min per μmole) into washed cultures of thymine-deficient cells (1% survivors) and the growing control (see legend of Fig. 2). The difference in initial cell concentrations was slight.

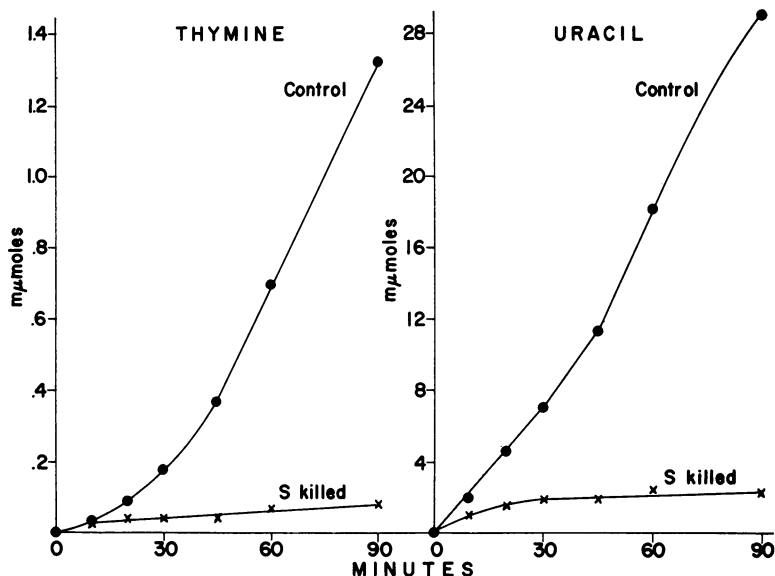


FIG. 5. Incorporation in a complete medium of thymine (5.72×10^5 counts per min per μ mole) or uracil (3.72×10^4 counts per min per μ mole) into cultures of cells killed (2.4% survivors) by $60 \mu\text{g/ml}$ of streptomycin (S) in arginine-deficient medium, compared with incorporation in arginine-starved controls.

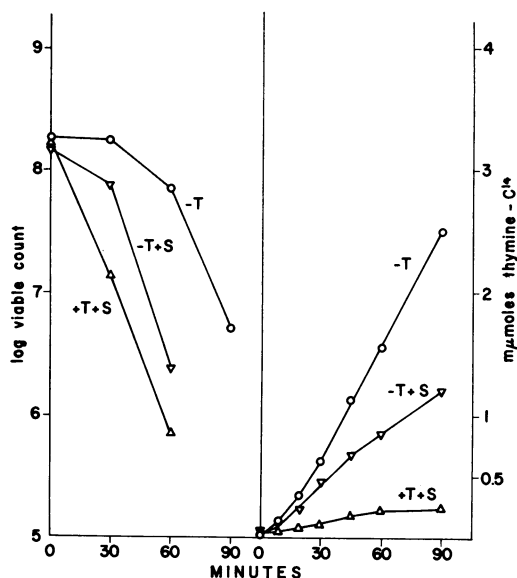


FIG. 6. Viability of cells killed by streptomycin in the presence of arginine with or without thymine (T), and the incorporation of thymine (7.9×10^5 counts per min per μ mole) into the various cultures of killed cells after washing and resuspending in a complete medium.

lent of DNA ($2.2 \mu\text{moles}$ per 1.85×10^8 cells). However, after streptomycin killing, the DNA synthesis in 90 min did not exceed 55% of one equivalent, a value approaching the theoretical value for the completion of one cycle in a random culture (5).

Lethality of DDA. From the preceding data it can be seen that several lethal treatments, two of which prevent DNA synthesis and one of which does not prevent the completion of a cycle, do not, in fact, destroy the ability of the cell to make a polydeoxyribonucleotide. In fact, no agents that can prevent the extension of a DNA chain were known when these experiments were done, and it appeared desirable, if only as a control for these experiments, to have such an agent. For some time, it has appeared desirable to test 2',3'-dideoxynucleosides with this in mind, since the absence of a 3'-hydroxyl might permit addition of the 2',3'-dideoxyribonucleoside-5'-phosphate to a chain but prevent subsequent additions.

Several compounds in this series have recently become available (3, 6) and were tested in our systems. 2',3'-Dideoxycytidine and dideoxyuridine are inactive as inhibitors of growth or multiplication. However, DDA is an interesting lethal agent which appears to prevent DNA synthesis both during and after killing. Both DDA and the unsaturated synthetic precursor, 2',3'-dideoxy-2'-adenosine (Fig. 1), were tested at $0.1 \mu\text{mole}$ per ml in the absence of adenosine in the medium. DDA was shown to be slightly toxic under these conditions, whereas the unsaturated compound permitted growth. The rate of growth in the latter instance, measured turbidimetrically, was slightly less than the rate of growth on 2'-deoxyadenosine and occurred with a proportionate increase in viable cells. The latter rather surprising result suggests a slow addition of water across the double

bond to form 2'-deoxyadenosine, which supports growth as well as does adenosine.

The lethality of DDA was studied at a somewhat higher concentration (0.25 μ mole per ml) in the presence and absence of adenosine (0.05 μ mole per ml) or deoxyadenosine (0.25 μ mole per ml). DDA had similar lethality whether or not adenosine is present, although cell growth, measured turbidimetrically, was much increased in the system containing adenosine (Fig. 7). Thus, lethality is largely independent of cell growth. 2'-Deoxyadenosine is protective against DDA at least as long as 2'-deoxyadenosine can survive deamination and cleavage of the resulting deoxyinosine. We determined that DDA is far less rapidly deaminated than is 2'-deoxyadenosine or ara-A by the *E. coli* adenosine deaminase, although DDA is deaminated at an appreciable rate by mammalian adenosine deaminases (Keller and Cohen, unpublished data).

The effect of DDA concentration in the presence of adenosine is presented in Fig. 8, comparing DDA with dideoxycytidine at 1 μ mole per ml. The latter is seen to be essentially noninhibitory at this concentration, whereas inhibitory effects with DDA are obtained at 0.1 μ mole per ml. Very ex-

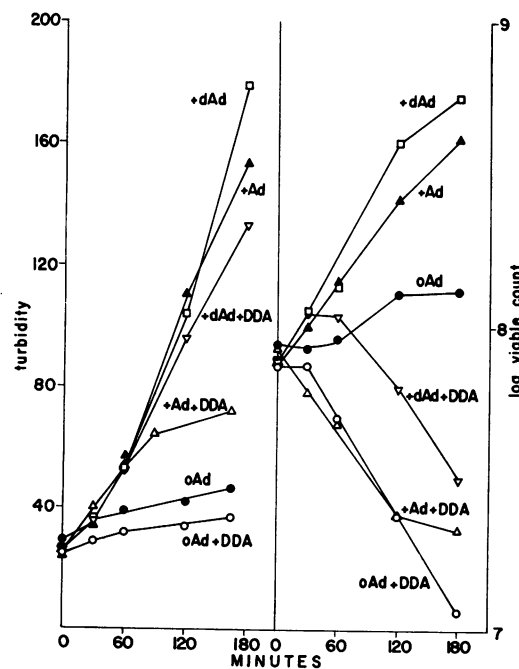


FIG. 7. Turbidity and viability of cultures exposed to deoxyadenosine (dAd), adenosine (Ad), 2',3'-dideoxyadenosine (DDA), or combinations of these nucleosides. These experiments were done in the presence of thymine, arginine, and uracil.

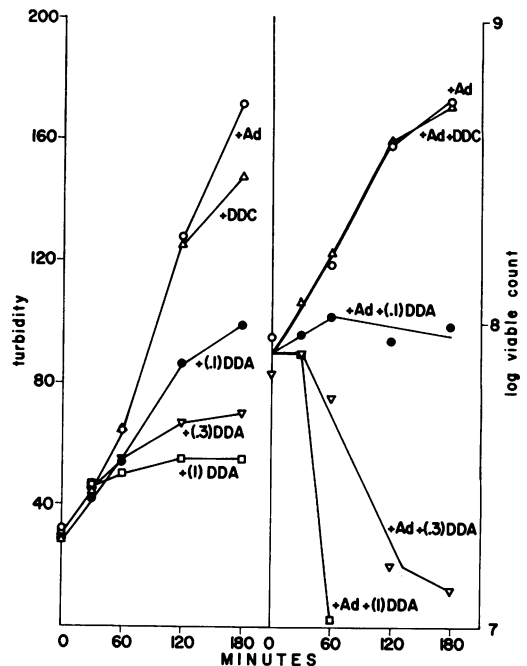


FIG. 8. Turbidity and viability of cultures of cells exposed to various concentrations of DDA and 1 μ mole of dideoxycytidine (DDC) per ml. The figures in parentheses represent micromoles of DDA per milliliter. These experiments were done in the presence of thymine, arginine, uracil, and adenosine.

tensive killing is obtained at 1 μ mole of DDA per ml. It is evident that this compound is not as toxic on a weight basis as are many antibiotics, but this degree of lethality nevertheless warrants our interest.

Lethality with other strains of *E. coli*. DDA at 1 μ mole per ml was lethal to all strains of *E. coli* tested. It killed strain TAU at a rate slightly greater than strain TAUAd. Prototrophic strain B was killed without any detectable effect on turbidity increase over a 3-hr interval. Strain B, uracil-requiring strain W_(e-), and the more slowly growing prototrophic strain K-12 (λ) were killed at similar rates. No lysis was detected in these cultures over a 3-hr interval.

Effect of DDA in other nutritional deficiencies. At 0.3 μ mole per ml, DDA markedly potentiated thymineless death, killing without a detectable lag in a thymine-deficient culture (Fig. 9). A deficiency of arginine did not appear to be significantly inhibitory to the development of lethality, whereas a deficiency of uracil did appear to inhibit killing.

These experiments were repeated at 1 μ mole per ml (Fig. 10). The absence of arginine did not

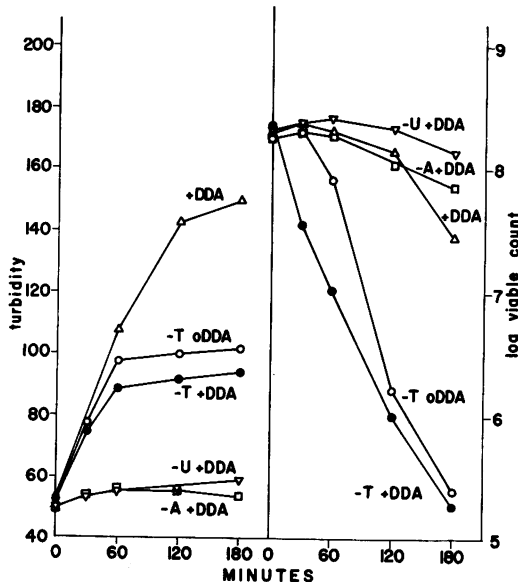


FIG. 9. Turbidity and viability of cultures of cells during killing by $0.3 \mu\text{mole per ml}$ of DDA with or without thymine (T), arginine (A), or uracil (U). The media contained adenosine in all cases.

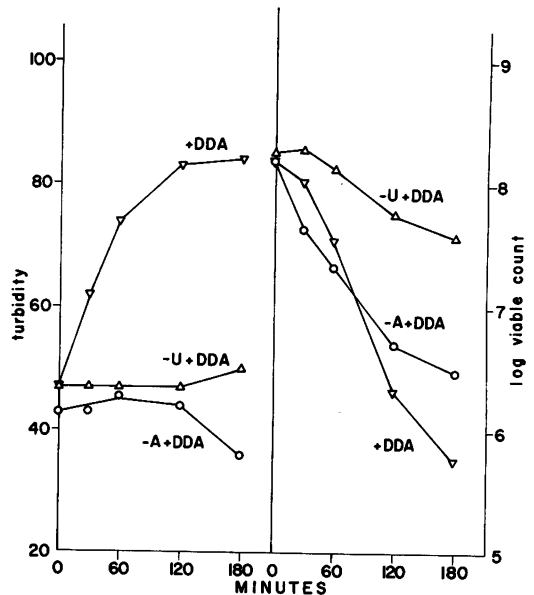


FIG. 10. Turbidity and viability of cultures of cells during killing by $1.0 \mu\text{mole per ml}$ of DDA with or without arginine or uracil. The media contained thymine and adenosine.

greatly change the initial rate of killing. As indicated earlier, however, the absence of uracil was sharply inhibitory to DDA killing.

Incorporation of radioactive nutrients during DDA killing. As can be anticipated from the increase in turbidity in the presence of DDA, RNA and protein synthesis were not inhibited in complete media in the presence of $1 \mu\text{mole}$ of DDA per ml. The initial rates of synthesis of these polymers were the same as in the control, although after growth the control soon exceeded the rates in the DDA-killed cells. On the other hand, a culture of 1.3×10^8 cells incorporated thymine at an initial rate of one-third that of the control; however, incorporation stopped completely between 10 and 30 min, with the cells having incorporated no more than 10% of the thymine required for a doubling of the cells.

Incorporation of thymine, arginine, and uracil in DDA-killed cells. The initial rate of DNA synthesis in the DDA-killed cells (1% survivors after 2 hr in $1 \mu\text{mole}$ of DDA per ml) was much lower than in the control culture (Fig. 11). After 1 hr, the incorporation of thymine was only about 10% that in the control, whereas that of uracil was significantly greater, about 20% of that in the control. Surprisingly, arginine incorporation was very low, a result similar to that of thymineless death.

Since DNA synthesis is so inhibited in DDA-killed cells, it must be asked whether this result is

similar to that with streptomycin-killed cells. In that system, inhibition of DNA synthesis was a secondary effect of other more immediate physiological effects. In the case of streptomycin-killed cells, we have shown that streptomycin killing in the absence of thymine did not in fact produce an irreversible inhibition of DNA synthesis (Fig. 6). Since DDA killing markedly potentiated thymineless death, cells killed by DDA in the absence of thymine can be compared for ability to incorporate thymine, arginine, or uracil with cells killed to a comparable extent by thymineless death alone. Cells killed by DDA in the absence of thymine (Fig. 12) had a slightly greater (4% survivors after 60 min) initial rate of arginine incorporation than did cells killed by thymine deficiency alone (8% survivors after 90 min). The initial rates of uracil incorporation were similar. On the other hand, DDA-killed cells incorporated thymine at only 15% of the rate of the cells killed entirely as a result of thymine deficiency.

Although this value may seem disproportionately high if DDA had actually killed the cells by terminating DNA chains, thymineless death had itself resulted in up to 36% killing in 60 min. Thus, some of the cells killed in the DDA-treated culture had died from a treatment (thymineless death) which did not reduce subsequent thymine incorporation, and we suppose that these had

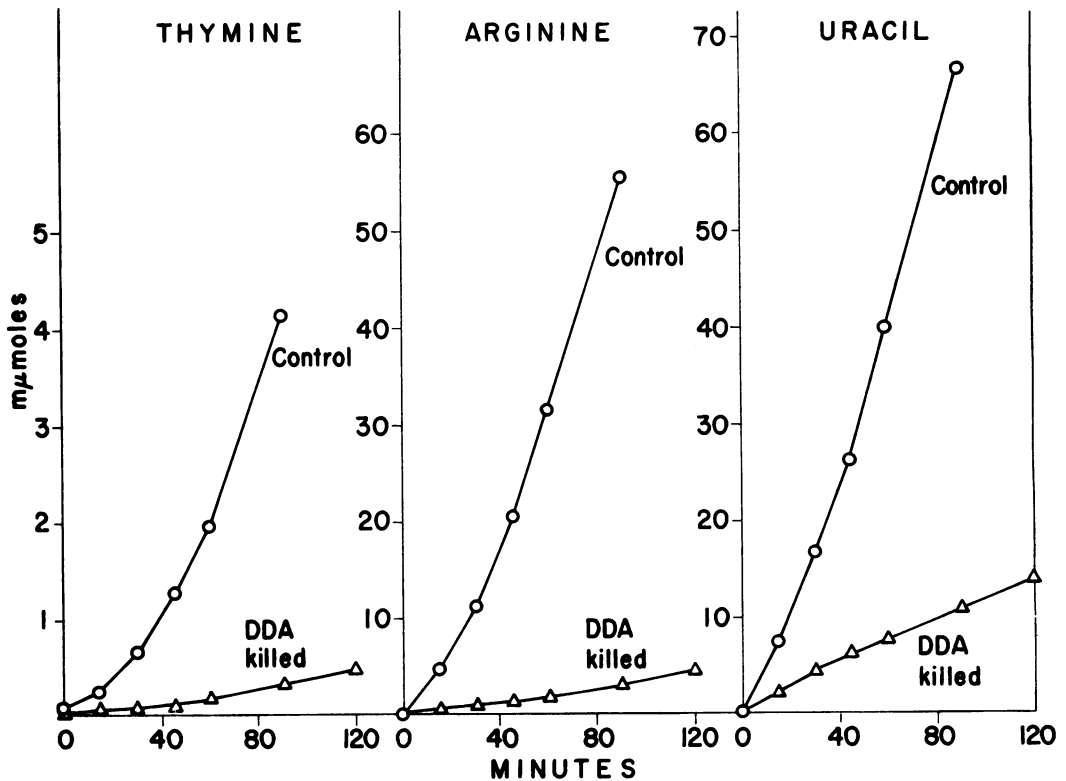


FIG. 11. Incorporation of thymine (7.95×10^6 counts per min per μ mole), arginine (7.62×10^4 counts per min per μ mole), or uracil (3.83×10^4 counts per min per μ mole) into DDA-killed cells (1% survivors) and control cells which had been washed and resuspended in complete media.

contributed to the DNA synthesis observed in the killed culture. It seems clear, therefore, that cells killed by DDA treatment are specifically and irreversibly altered, during and after such treatment, in the ability to make DNA, and in this respect are unlike cells killed by ara-A, thymineless death, or streptomycin.

DISCUSSION

The initial objective in this work was an effort to test the view of Chu and Fischer (2) concerning the incorporation of ara-C into DNA and the possible significance of the postulated incorporation for lethality. It seemed to us that such incorporation into DNA would be lethal only if ara-C terminated a DNA chain and prevented subsequent extension of the chain. This could not be tested in *E. coli*, although the lethal action of ara-A could be studied in the bacterial system. As described elsewhere (Doering, Keller, and Cohen, *in press*), mouse fibroblasts inhibited and killed in some measure by ara-C and ara-A could then nevertheless synthesize DNA for long periods. These two arabinosyl nucleosides had rather

similar effects. Although ara-C can not be studied in this way with *E. coli*, ara-A has been tested in a similar manner and, as described in this paper, the results with the bacterium were similar to those in the animal cells. After inhibition of DNA synthesis and extensive cell killing by ara-A, the DNA chains still provide templates for continuing synthesis, and we infer that ara-A has not blocked such synthesis by terminal addition.

One control in experiments of this type was that of cells killed by thymineless death, since it has been known for 10 years that cells killed in this way can still make DNA (1). It may well be that the DNA made after thymineless and ara-A killing is that of an induced phage and that lethality in both instances is actually due to this induction. We ask whether the inhibition of protein synthesis in both types of dead cells is not relevant to this hypothesis, since it suggests that the induction itself, i.e., a reaction displacing the phage genome from the host genome, might be the lethal event rather than later physiological events related to phage development.

In any case, the rather striking phenomenon of

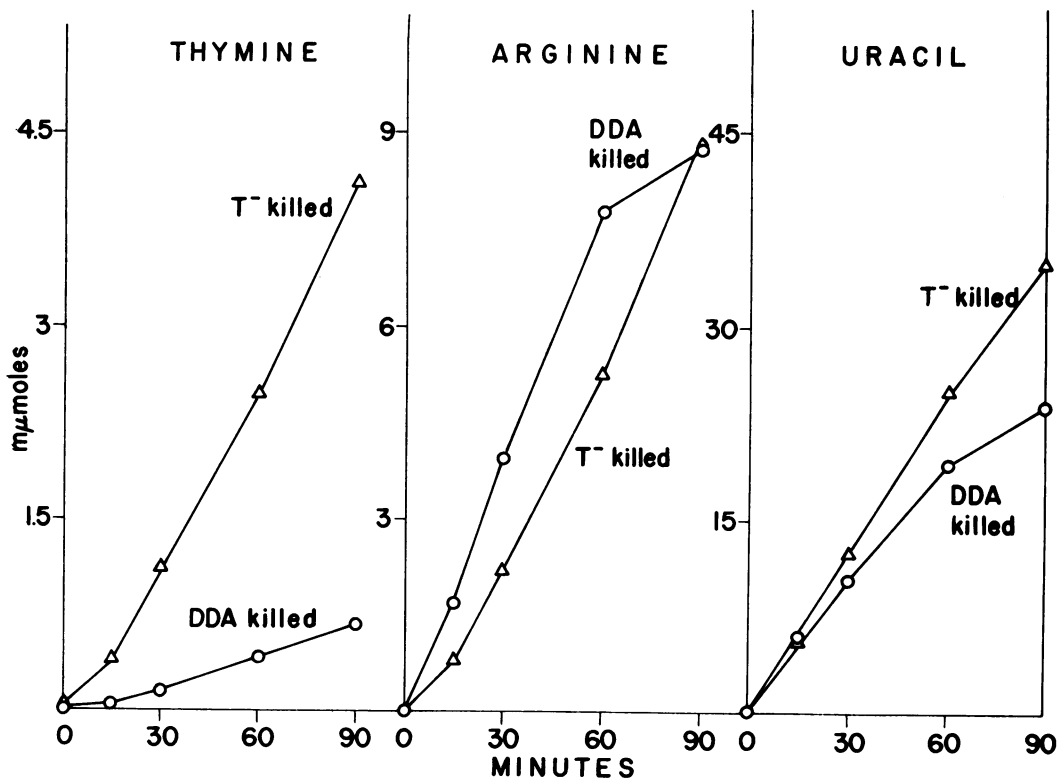


FIG. 12. Incorporation of thymine (7.95×10^5 counts per min per μ mole), arginine (8.84×10^4 counts per min per μ mole), or uracil (3.83×10^4 counts per min per μ mole) into cultures of cells killed by DDA without thymine (4% survivors) and into thymine-deficient cultures as controls (8% survivors). Cultures were washed and resuspended in complete media for the incorporation studies.

DNA synthesis in killed cells emphasized the absence of a rather obvious control, namely, that in which the advent of lethality occurs, as one might expect, with the irreversible termination of DNA synthesis. The lack of an agent which can effect such a complete cessation of DNA synthesis has been a serious deficiency in many types of experiments, of which the study of genetic recombination has been one. It is possible that DDA will be helpful in dissecting such phenomena.

It is obviously not proven in these experiments that DDA adds to the end of a polydeoxynucleotide and blocks further synthesis in this way. The structure of the compound, the lack of inhibition of its effect by adenosine and prevention of effect by deoxyadenosine, the development of lethality in the absence of arginine, and the synergism of thymineless death and the irreversible termination of DNA synthesis, are consistent with this possibility. To prove this point, it will be necessary to study the incorporation of the labeled compound. For maximal specificity, it would be desirable to have a radioactive compound labeled in the dideoxy sugar, perhaps derived from the reduction

with tritium of the adenosine. If DDA actually adds terminally to the bacterial chromosome, it can be calculated that a maximal lethal incorporation to the extent of two to four molecules per cell might be possible, and 1 liter of culture containing 4×10^{11} bacteria might incorporate no more than 10^{-5} to 10^{-6} μ moles. This emphasizes the great specific activity of the DDA needed in this important experiment, and it may not be possible to perform such a test of the postulated mechanism of lethality with intact bacteria. On the other hand, although DDA is incorporated terminally, the bacterial chromosome may continue to be synthesized, albeit with numerous breaks. It will be of interest to test both of these possibilities.

ACKNOWLEDGMENT

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