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# The Mode of Action of 5-Aza-2′-Deoxycytidine in *Escherichia coli*

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5-Azadeoxycytidine is a good donor of deoxyribosyl group in reactions catalyzed by pyrimidine deoxyriboside phosphorylase, promoting the deoxyriboside-dependent incorporation of thymine by wild-type cells of *E. coli* about twice as effectively as either deoxycytidine or deoxyuridine. Prior deamination to 5-azadeoxyuridine is necessary for the utilization of the deoxyribose moiety of 5-azadeoxycytidine, since the deoxyribosyl-donor activity is entirely lost in mutants deficient in cytidine deaminase. Upon infection of wild-type cells with phage T4 the deamination of 5-azacytidine and 5-azadeoxycytidine is strongly depressed; a proportional loss of the deoxyribosyl-donor ability of the latter is observed while the activity of deoxycytidine remains unaffected.

In wild-type cells of *E. coli* 5-azadeoxycytidine inhibits protein synthesis and the replication of phage f2 as effectively as 5-azacytidine, but has much less effect on the replication of phage T4. In deaminase-less mutants 5-azadeoxycytidine has practically no inhibitory effects.

It is concluded that 5-azadeoxycytidine enters the cells of *E. coli via* deamination, followed by phosphorolytic cleavage of the glycosidic bond. Direct utilization of the deoxynucleoside, conserving the glycosidic bond and the 6-aminogroup, does not occur to any appreciable extent. In accord with this finding the biological effects of 5-azadeoxycytidine are similar to those of 5-azauridine but different from the action of 5-azacytidine.

5-Aza-2′-deoxycytidine [1] is a potent bacteriostatic [2] and cancerostatic agent [3]. In mouse leukemic cells this compound competes with the incorporation of deoxycytidine and deoxyuridine into DNA [4]. In cultures of *E. coli*, however, preliminary experiments have indicated that 5-azadeoxycytidine interferes mostly with the messenger function of RNA, but has remarkably little effect on the synthesis of DNA of phage T4, while 5-azacytidine is a strong inhibitor of replication of the DNA of this phage [5]. The present experiments show that these effects of 5-azadeoxycytidine can be interpreted as being due to a peculiar mechanism of entry of the inhibitor into the bacterial cells.

## METHODS

5-Azacytidine and 5-azadeoxycytidine were prepared in the Department of Chemical Syntheses of this Institute, following the methods previously described [1,6]. Cytidine was obtained from Hoffmann La Roche, Basle; deoxycytidine (A grade), thymidine (A grade) and deoxyuridine (B grade) were from Calbiochem.

[2-$^{14}$C]Cytidine (188 mC/mmole), [2-$^{14}$C]uridine (280 mC/mmole), [2-$^{14}$C]thymine (8.7 mC/mmole), [2-$^{14}$C]deoxycytidine (25 mC/mmole) and [$^{14}$C]leucine (uniformly labeled, 83 mC/mmole) were obtained from the Institute of Research and Application of Radioisotopes, Praha. All radioactive compounds were suitably diluted with non-radioactive materials to obtain a convenient specific radioactivity.

Two strains of *E. coli* B deficient in cytidine deaminase were kindly donated to us by Dr. Munch-Petersen, Copenhagen. One of them, 0018, required a pyrimidine for growth; the other, OK 408, had no requirements, but was also deficient in deoxyribomutase and purine nucleoside phosphorylase. An F$^+$ strain was derived from OK 408 by growing it together with F$^+$ strain 2027 (met$^-$, leu$^-$, lac$^-$) obtained from Dr. Werner Arber, Genève. Both strains of bacteria were grown on tryptone medium and the cultures mixed in a ratio donor/acceptor equal to 1/20. After 2 h of incubation at 37° with gentle shaking, the mixed culture was diluted $10^6$ times and plated on a mineral agar with glucose. Single colonies were tested for their ability to give plaques with phage f2. All those isolated gave imperfect and very turbid plaques, but could be

*Enzymes.* Cytidine deaminase or cytidine aminohydrolase (EC 3.5.4.5); deoxycytidylate hydroxymethylase or 10-hydroxymethyltetrahydrofolate:deoxycytidine monophosphate hydroxymethyltransferase; dCTPase or dCTP nucleotidohydrolase (EC 3.6.1.12); endolysine or *N*-acetylmuramide glycanohydrolase (EC 3.2.1.17); β-galactosidase or β-D-galactoside galactohydrolase (EC 3.2.1.23); thymidine phosphorylase or thymidine:orthophosphate deoxyribosyltransferase (EC 2.4.2.4); deoxyribosyl transferase or nucleoside:pyrimidine (purine) deoxyribosyl transferase (EC 2.4.2.6); pyrimidine phosphoribosyl transferase (EC 2.4.2.9).

used for replication of phage f2 in a liquid medium. Lysis of the cells was completed by adding lysozyme (5 μg/ml) and EDTA (5 mM). The lysates were treated with chloroform. The deaminase-less character of the isolates was verified by growing them in a glucose-mineral salts medium in the presence of cytidine (50 μg/ml), measuring the absorbance of the culture fluid, clarified by filtration on nitrocellulose membrane filters and acidified with HCl (0.33 M). From the absorbance at 255 and 280 nm the extent of conversion of cytidine to uridine was calculated.

Most experiments with uninfected bacteria and with phage T4 were performed in liquid cultures in shaken flaks, using the medium of Spizizen as previously described [5]. For replication of phage f2 a Tris-medium with low content of phosphorus [7] was used in order to avoid complications connected with precipitation of calcium ions necessary for the absorption of phage. Potassium dihydrogen phosphate was added to obtain a concentration of 200 μM. Immediately before infection the same amount of calcium chloride was added. In all experiments with phages the multiplicity of infection was at least 5, but usually 10 viable phage particles per bacterial cell. The fraction of uninfected cells was negligible.

The incorporation of labeled bases, nucleosides or leucine into macromolecules was determined by precipitation with trichloroacetic acid and filtration on nitrocellulose membrane filters (Synpor, Synthesia, Uhříněves). The radioactivity was determined with a gas-flow counter Frieseke-Höpfner.

Deoxycytidylate hydroxymethylase was determined according to Dirksen *et al.* [8], deoxycytidine triphosphatase was determined spectrophotometrically according to Wiberg *et al.* [9], fractionating the reaction mixtures on columns of Dowex 1×2. Phage endolysine was determined as described by Salser, Gesteland and Bolle [10].

## RESULTS

### *The Partition of Cytidine and Deoxycytidine in RNA and DNA*

Our previous study concerning the deoxyriboside-dependent incorporation of thymine [11] by prototrophic strains of *E. coli* has shown that 5-azadeoxycytidine may serve as a deoxyribosyl donor similarly as natural deoxynucleosides [12]. This finding indicates that 5-azadeoxycytidine may enter the cells by a mechanism involving the cleavage of the glycosidic bond, liberating a free base and the deoxyribosyl radical, which may be either catabolized or utilized in transdeoxyribosylation reactions [13]. Lichtenstein, Barner and Cohen [14] have postulated a similar mechanism for the utilization of deoxycytidine, observing that added deoxycytidine equilibrated completely with labeled uracil in a pyrimidine-deficient strain of *E. coli*. In order to verify the validity of their conclusions for our wild-type strain not requiring pyrimidines, we performed similar experiments, using cytidine or deoxycytidine labeled in the base, in competition with an excess of unlabeled deoxycytidine and cytidine, respectively. The results of these experiments are given in Tables 1–3. Excess cold deoxycytidine depresses the total amount of cytidine incorporated by the cells but does not selectively affect the portion incorporated into DNA; the percent of cytidine label incorporated into DNA, which is low in uninfected host cells or early after infection with phage T4, but very high later after infection, remains remarkably insensitive to the presence of excess deoxycytidine (Table 1). Conversely, most of the label of deoxycytidine is incorporated into both RNA and DNA (Table 2). Excess cytidine depresses the incorporation into DNA as well as into RNA (Table 3); however, some degree of

Table 1. *Incorporation of cytidine into DNA and RNA and competition with deoxycytidine*
The bacteria were infected with phage T4 Simultaneously with the infection either [$^{14}$C]cytidine alone (1 μg/ml, 0.1 μC/ml) or labeled cytidine with unlabeled deoxycytidine (50 μg/ml) were added

| Time | RNA | | DNA | | Proportion in DNA | |
|---|---|---|---|---|---|---|
| | Cyd | Cyd + dCyd | Cyd | Cyd + dCyd | Cyd | Cyd + dCyd |
| min | counts/min | counts/min | counts/min | counts/min | % | % |
| 5 | 15980 | 6225 | 1340 | 645 | 7.7 | 9.4 |
| 10 | 19500 | 6710 | 8200 | 2280 | 29.6 | 25.4 |
| 15 | 22150 | 6170 | 19350 | 4910 | 46.6 | 44.3 |
| 20 | 20200 | 6820 | 27900 | 6140 | 58.0 | 49.0 |
| 25 | 19100 | 6526 | 32500 | 8975 | 62.8 | 57.8 |
| 30 | 17750 | 5100 | 35450 | 11950 | 66.6 | 70.0 |

Table 2. *Incorporation of deoxycytidine into RNA and DNA*
[$^{14}$C]Deoxycytidine (2.27 μg/ml, 0.005 μC/ml) was added and its incorporation into RNA and DNA was determined

| Bacteria | Time | Incorporation into | | Proportion in DNA |
|---|---|---|---|---|
| | | RNA | DNA | |
| | min | counts/min | counts/min | % |
| Uninfected bacteria | | | | |
| | 5 | 1872 | 328 | 14.9 |
| | 10 | 3836 | 604 | 13.6 |
| | 15 | 6743 | 1037 | 13.3 |
| | 20 | 10142 | 1358 | 11.8 |
| | 25 | 14591 | 1929 | 11.6 |
| Bacteria infected with phage T4 | | | | |
| | 5 | 871 | 131 | 13.1 |
| | 10 | 1876 | 434 | 18.8 |
| | 15 | 2098 | 1612 | 43.5 |
| | 20 | 2050 | 2980 | 59.2 |
| | 25 | 1700 | 4230 | 71.2 |

Table 3. *Incorporation of deoxycytidine into DNA and RNA and competition with cytidine*
[$^{14}$C]Deoxycytidine (2 μg/ml) (0.0044 μC/ml), either alone or together with cytidine (50 μg/ml), was added to both uninfected and T4 phage-infected cultures of *E. coli*. The incorporation into RNA and DNA was determined

| Bacteria | Time | RNA | | DNA | | Proportion in DNA | |
|---|---|---|---|---|---|---|---|
| | | dCyd | dCyd + Cyd | dCyd | dCyd + Cyd | dCyd | dCyd + Cyd |
| | min | counts/min | counts/min | counts/min | counts/min | % | % |
| Uninfected bacteria | 25 | 30837 | 2319 | 3620 | 1733 | 10.5 | 42.7 |
| T4 phage-infected bacteria | 25 | 3555 | 444 | 6292 | 1162 | 63.9 | 72.5 |

Table 4. *Incorporation of labeled cytidine, deoxycytidine and uridine into 5-hydroxymethylcytosine and thymine of T4 phage DNA*
The nucleosides labeled with $^{14}$C were added to a culture infected with phage T4. Phage DNA was isolated, hydrolysed with concentrated formic acid at 170° for 1 h and the bases chromatographed in isopropanol-HCl. The zones of bases, visible under a Mineralite lamp, were cut out and their radioactivity determined in a liquid scintillation counter. Each analysis was run in duplicate

| Nucleoside | Radioactivity of | | Radioactivity in | |
|---|---|---|---|---|
| | 5-hydroxy-methyl-cytosine | thymine | 5-hydroxy-methyl-cytosine | Average |
| | counts/min | | % | |
| [$^{14}$C]Cytidine | 19704 | 32209 | 38.0 | 39.0 |
| | 26700 | 40108 | 40.0 | |
| [$^{14}$C]Deoxy-cytidine | 43792 | 74569 | 37.0 | 36.7 |
| | 72356 | 126746 | 36.3 | |
| [$^{14}$C]Uridine | 94330 | 147746 | 39.0 | 38.5 |
| | 90068 | 146938 | 38.0 | |

selectivity is observed, especially in uninfected cells, where the incorporation of deoxycytidine into DNA is less affected by cytidine than the incorporation into RNA. This result may indicate that a limited amount of deoxycytidine may enter the cells directly upon conservation of the glycosidic bond. This question was not followed further and the experiment represented in Table 3 is not sufficient to prove this possibility.

These experiments indicate that a complete equilibration between the ribo- and deoxyribonucleosides of cytosine is taking place; the ribo- and deoxyribonucleosides mutually compete in some stage preceding this equilibration.

In Table 4 the incorporation of cytidine, deoxycytidine and uridine into 5-hydroxymethylcytosine and thymine of T4-phage DNA is represented. From this experiment, again analogous to former finding of Lichtenstein *et al.* [14], we see that the same ratio of 6-oxo-to 6-aminopyrimidines of DNA is obtained irrespective of the form in which the labeled pyrimidine is administered; cytidine and deoxycytidine are partially deaminated and uridine, or its phosphorylated derivatives, partially aminated before entering DNA.

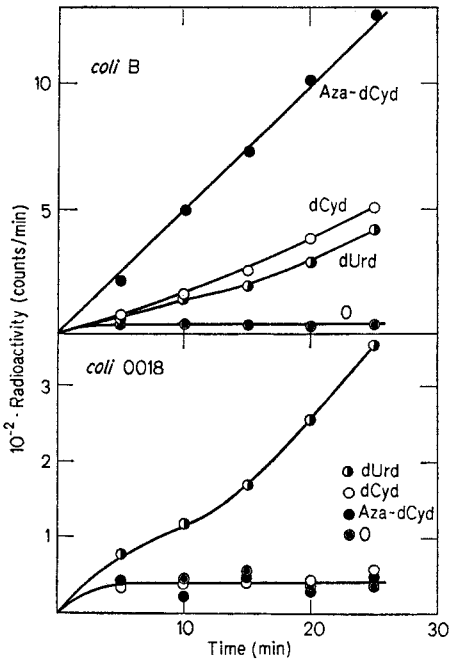


Fig. 1. *Incorporation of thymine by wild-type and deaminase-deficient strains of* E. coli *in the presence of different deoxynucleosides.* The concentration of [$^{14}$C]thymine was 3.3 μg/ml; that of deoxynucleosides, 50 μg/ml. Upper part, strain *E. coli* B; lower part, a deaminase-deficient strain 0018

### *The Inactivity of 5-Azadeoxycytidine in Strains Deficient in Cytidine Deaminase*

To evaluate the significance of deamination for the uptake of 5-azadeoxycytidine the deaminase-less mutants of *E. coli* B were used, incapable of deaminating both cytidine and deoxycytidine. These experiments were prompted by the communication of Munch-Petersen [15], indicating that deoxycytidine cannot act as deoxyribosyl donor in such strains. The data of Fig. 1 confirm this finding and indicate that 5-azadeoxycytidine behaves in a similar manner, promoting the incorporation of thymine by a wild-type strain of *E. coli* about twice as much as cytidine or uridine, but being completely inactive in deaminase-less mutants. Deoxyuridine, however, is

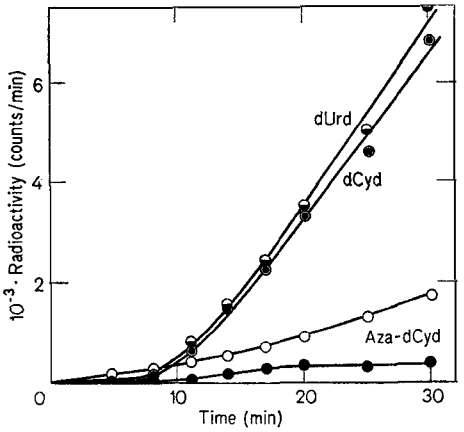


Fig. 2. *Incorporation of thymine in the presence of different deoxynucleosides by a culture of* E. coli *B infected with phage T4.* The conditions were the same as in Fig. 1

Table 5. *The deamination of cytosine and 5-azacytosine nucleosides by normal and T4 phage-infected cultures of* E. coli *B*

To a culture of *E. coli* B ($5 \times 10^8$ cells/ml), growing on glucose-mineral salts medium, L-tryptophan (10 μg/ml) was added and one-half of the culture infected with 10 viable T4 phages per bacterial cell. Immediately after infection the nucleosides (50 μg/ml) were added to both aliquots. Aliquots of the cultures were acidified with HCl (final concentration 0.167 M) and filtered on membrane filters. The extent of deamination of cytidine and deoxycytidine was calculated from absorbancing at 280 and 250 nm, that of 5-azacytidine and 5-azadeoxycytidine from the readings at 255 and 240 nm

| Nucleoside | Deamination after 30 min incubation | |
|---|---|---|
| | Normal culture | T4 phage-infected culture |
| | % | % |
| Cytidine | 90 | 68 |
| Deoxycytidine | 84 | 66 |
| 5-Azacytidine | 70 | 19 |
| 5-Azadeoxycytidine | 74 | 16 |

equally effective in both the deaminase-less and wild-type strains.

If wild-type cells are infected with phage T4, both deoxycytidine and deoxyuridine still promote the incorporation of thymine, but 5-azadeoxycytidine is much less effective (Fig. 2). Table 5 shows that the deamination of 5-azacytidine and 5-azadeoxycytidine is strongly depressed upon infection with phage T4 while the deamination of cytidine and deoxycytidine is slightly affected. This observation provides another example of close connection between the rate of deamination and deoxyribosyl-donor ability of deoxycytidine and its analogs. Assuming that the deoxyribosyl-donor ability is proportional to the rate of uptake of the azapyrimidine moiety of the

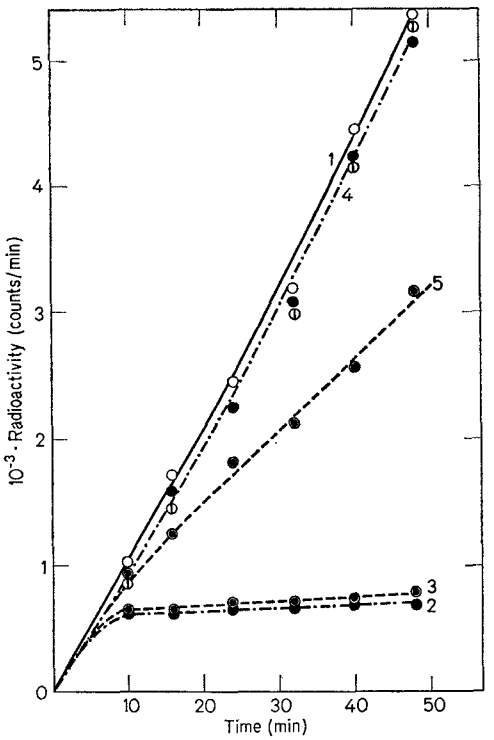


Fig. 3. *Incorporation of leucine by wild-type and deaminase-deficient strains of* E. coli *in the presence of 5-azacytidine and 5-azadeoxycytidine.* ○, control, *coli* B; ⊙, control, *coli* OK 408; ●, 5-azadeoxycytidine, 20 μg/ml; ◉, 5-azacytidine, 20 μg/ml. Curves 1,2,3, wild-type strain *E. coli* B; curves 4 and 5, strain OK 408. The control of *E. coli* OK 408 has not been drawn since it was nearly identical with curve 1

compound, we may understand why 5-azadeoxycytidine is a rather inefficient inhibitor of replication of phage T4. However, the reasons why the infection with phage T 4selectively modifies the properties of host enzymes, remain obscure. Other examples of similar modifications of host enzymes by infection with T-even phages have been described [16,17].

According to these findings 5-azadeoxycytidine enters the cell by deamination followed by trans-deoxyribosylation. We would therefore expect that the biological effects of this compound would resemble those of 5-azauridine. Furthermore, if this way of entry is exclusive and deamination is an obligatory step for the entry of the inhibitor into the cells, we could predict that the compound should be without any effect in a deaminase-less strain. The experiments to be described confirm both these assumptions.

In uninfected cultures of *E. coli* 5-azadeoxycytidine inhibits total protein synthesis as effectively as 5-azacytidine while in a deaminase-less strain the inhibition is hardly significant (Fig. 3). The synthesis of β-galactosidase in a glycerol medium is inhibited by 97% in *E. coli* B while 15% inhibition is observed

Table 6. *Inhibition of synthesis of β-galactosidase by 5-azadeoxycytidine and 5-azacytidine in* E. coli
The culture was induced with isopropylthiogalactoside (0.5 mM) and the enzyme assayed at 10 min intervals. The inhibitors (20 μg/ml) were added simultaneously with the inducer. The slopes of the production curves were expressed in per cent of the control

| Medium | Inhibitor | Rate of synthesis of β-galactosidase | |
|---|---|---|---|
| | | *E. coli* B | *E. coli* OK 408 |
| | | % of control | |
| Glycerol + casamino acids | 5-Azadeoxycytidine | 2.9 | 87.0 |
| | 5-Azacytidine | 2.0 | 11.1 |
| No carbon source | 5-Azadeoxycytidine | 72.5 | 69.0 |
| | 5-Azacytidine | 15.3 | 69 |

completely removed by both cytidine and deoxycytidine, while only cytidine but not deoxycytidine is capable of completely removing the inhibitory effect of 5-azacytidine.

In a deaminase-less $F^+$ strain 5-azacytidine still inhibits the replication of phage f2, although less effectively than in wild-type strains. 5-Azadeoxycytidine, however, has no inhibitory activity (Table 8).

### *The Effect of 5-Azadeoxycytidine on the Replication of Phage T4*

In our earlier study 5-azacytidine has been shown to inhibit specifically the synthesis of T4 phage DNA, probably by interfering with the formation of 5-hydroxymethyl deoxycytidylate. No effect on the

Table 7. *Effect of inhibitors 5-azadeoxycytidine and 5-azacytidine on the production of phage f2 in the presence of cytidine and deoxycytidine*

| Counteractors | | Inhibitor | Effect of inhibitor | | | |
|---|---|---|---|---|---|---|
| | | | 5-azadeoxycytidine | | 5-azacytidine | |
| Nucleoside | Concn. | Concn. | phage | production | phage | production |
| | μg/ml | μg/ml | counts | % control | counts | % control |
| None | 0 | none | $8.8\times10^{11}$ | 100 | $6.4\times10^{11}$ | 100 |
| None | 0 | 10.0 | $1.7\times10^{9}$ | 0.19 | $7.2\times10^{8}$ | 0.11 |
| Cytidine | 2.5 | 10.0 | $8.1\times10^{10}$ | 9.16 | $2.4\times10^{10}$ | 3.75 |
| | 5.0 | 10.0 | $3.2\times10^{11}$ | 36.5 | $2.2\times10^{11}$ | 34.4 |
| | 10.0 | 10.0 | $4.3\times10^{11}$ | 49.4 | $4.7\times10^{11}$ | 73.7 |
| | 20.0 | 10.0 | $6.0\times10^{11}$ | 68.4 | $4.9\times10^{11}$ | 76.5 |
| Deoxycytidine | 2.5 | 10.0 | $4.1\times10^{10}$ | 4.6 | $1.8\times10^{10}$ | 2.8 |
| | 5.0 | 10.0 | $2.6\times10^{11}$ | 29.6 | $9.8\times10^{10}$ | 15.3 |
| | 10.0 | 10.0 | $5.9\times10^{11}$ | 67.0 | $1.4\times10^{11}$ | 21.8 |
| | 20.0 | 10.0 | $6.5\times10^{11}$ | 73.9 | $1.9\times10^{11}$ | 28.9 |

Table 8. *Effect of 5-azadeoxycytidine and 5-azacytidine on the production of phage f2 in a wild-type strain and deaminase-less strain*

| Inhibitor | Production of phage f2 | | | |
|---|---|---|---|---|
| | in strain 2027 | | in strain OK 408 $F^+$ | |
| | counts | % | counts | % |
| None | $3.3\times10^{11}$ | 100 | $2.6\times10^{11}$ | 100 |
| 5-Azadeoxycytidine | $3.3\times10^{8}$ | 0.11 | $2.2\times10^{11}$ | 86 |
| 5-Azacytidine | $1.17\times10^{9}$ | 0.39 | $1.07\times10^{10}$ | 4.1 |

under the same conditions in the deaminase-less strain (Table 6).

5-Azadeoxycytidine inhibits the replication of phage f2 to the same extent as 5-azacytidine. The inhibitory effects of both compounds are different only in their sensitivity to the counteracting action of cytidine and deoxycytidine. Table 7 shows that the inhibition with 5-azadeoxycytidine is nearly

synthesis of early phage-specific proteins has been noted, but in the late period of phage replication the rate of protein synthesis has been found to drop to about 30% of the control. Proteins normally formed at this time, *e.g.* phage endolysine, are not formed in the presence of 5-azacytidine. As a result of these inhibitory effects the burst size of viable phage is reduced to about 0.1% of the control. Cytidine counteracts both inhibitory effects if added earlier than or simultaneously with 5-azacytidine; deoxycytidine, however, is incapable of restoring the normal burst size; in its presence, inhibition of DNA-synthesis persists while the rate of protein synthesis becomes normal (Fig. 4). Proteins formed under these conditions are predominantly of early character; this is evident from Table 9, showing a distinct overproduction of some early enzymes.

5-Azadeoxycytidine is a much weaker inhibitor of replication of phage T4, reducing the burst size to about 10—20% of the control. Synthesis of phage

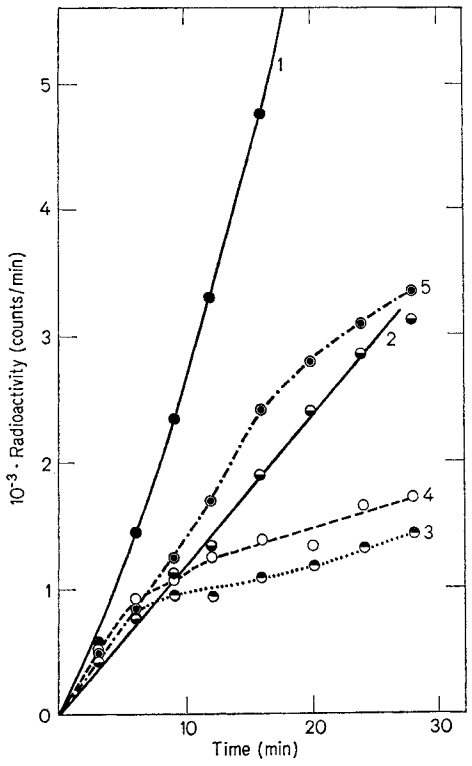


Fig. 4. *Incorporation of leucine by wild-type bacteria infected with phage T4.* 1, uninfected culture; 2, 3, 4, 5, cultures infected with phage T4; 2, no additions; 3, 5-azadeoxycytidine 4, 5-azacytidine; 5, 5-azacytidine and deoxycytidine. All inhibitors (20 μg/ml) were added simultaneously with the phage. The concentration of deoxycytidine (curve 5) was 50 μg/ml

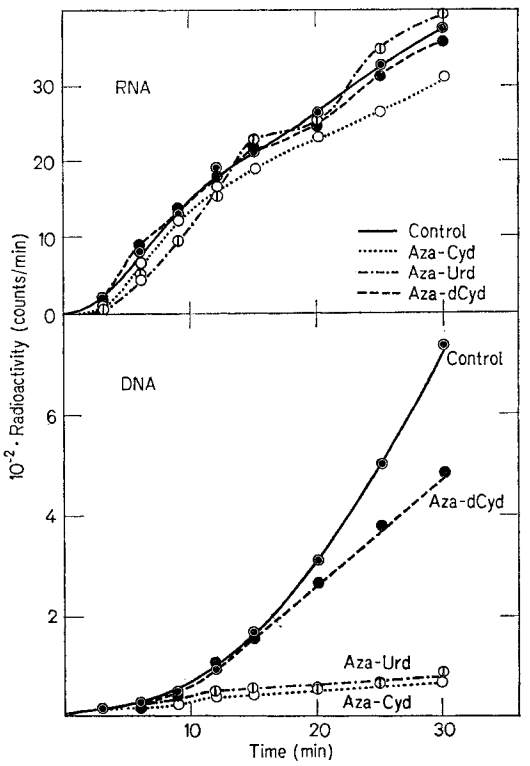


Fig. 5. *Effect of 5-azapyrimidine nucleosides on the synthesis of RNA and DNA in a culture of* E. coli *B infected with phage T4.* The incorporation of $^{32}P$ into alkali-sensitive and alkali-stable fractions was determined [5]. Upper part, RNA; lower part, DNA. The inhibitors (20 μg/ml) were added simultaneously with the infecting phage

Table 9. *Synthesis of some early enzymes of phage T4 in the presence of 5-azacytidine*
The concentration of 5-azacytidine was 10 μg/ml, that of deoxycytidine 50 μg/ml

| Enzyme | Additions | Time | Activity |
|---|---|---|---|
| | | min | % control |
| Deoxycytidylate hydroxymethylase | 5-azacytidine and deoxycytidine | 8 | 98 |
| | | 14 | 132 |
| | | 20 | 172 |
| Thymidylate kinase | 5-azacytidine | 10 | 37 |
| | | 25 | 98 |
| | 5-azacytidine and deoxycytidine | 10 | 177 |
| | | 25 | 234 |
| Deoxycytidine triphosphatase | 5-azacytidine | 10 | 112 |
| | | 25 | 200 |
| | 5-azacytidine and deoxycytidine | 10 | 242 |
| | | 25 | 254 |

Table 10. *Inhibition of synthesis of T4 phage endolysine by 5-azadeoxycytidine*
The concentration of the inhibitor was 50 μg/m

| Additions | Time of addition | Phage titre[a] | Endolysine[b] | |
|---|---|---|---|---|
| | | | 20 min | 30 min |
| | min | | | |
| None | — | $2.4\times10^{11}$ | 56.2 | 113 |
| 5-Azadeoxycytidine | 0 | $3.8\times10^{10}$ | 0.8 | 7.1 |
| 5-Azadeoxycytidine | 11 | $2.7\times10^{10}$ | 50.0 | 68.0 |

[a] Plated 1 hour after infection, completing the lysis with chloroform.
[b] Arbitrary units.

DNA is slightly affected (Fig. 5). However, protein synthesis in the late phase is inhibited in a similar manner as with 5-azacytidine (Fig. 4, Table 10). The rate of production of phage endolysine is reduced to about 10% of the control. Deoxycytidine completely counteracts all these inhibitory effects.

## DISCUSSION

The experiments with strains deficient in cytidine deaminase indicate that deoxycytidine as well as 5-azadeoxycytidine enter the cells *via* deamination and uncoupling of the deoxyribose moiety. 5-Azauracil, transiently formed by deamination and

phosphorolytic cleavage of the glycosidic bond of 5-azadeoxycytidine, may enter the cell metabolism either after ribosylation with ribose-1-phosphate, followed by phosphorylation, or by a direct reaction with 5-phosphoribosyl-1-pyrophosphate catalysed by pyrimidine phosphoribosyltransferase. The latter reaction has been demonstrated to occur [18] in cell-free extracts of *E. coli*.

5-Azadeoxycytidine is taken up by the cells of *E. coli* by a mechanism analogous to the mode of entry of deoxycytidine and other analogs. Barth *et al.* [19] stated that deamination of deoxycytidine must precede its phosphorolysis, since deoxycytidine is not a substrate of pyrimidine deoxynucleoside phosphorylase. Tono and Cohen [20] have shown that arabinofuranosyluracil enters the cells of *E. coli* predominantly by phosphorolysis; direct phosphorylation without a cleavage of the glycosidic bond could be demonstrated to a very small extent with resting cells. In mammalian cells, however, direct phosphorylation of arabinosylcytosine has been found to predominate over deamination [21].

We may ask whether the outlined pathway is the exclusive way of entry of 5-azadeoxycytidine into the cells of *E. coli* or if some small portion may enter the cells with conservation of the glycosidic bond. The marginal inhibitory effects observed in deaminase-less strains cannot be taken as evidence in favor of this possibility. The 14% inhibition of replication of phage f2 in a deaminase-less strain is insignificant in comparison with the thousand-fold inhibition in wild-type strains. We may conclude that the cells of *E. coli* are incapable of taking up biologically significant amounts of 5-azadeoxycytidine by a mechanism not involving deamination.

The proposed pathway is also consistent with an earlier finding [22] that the action of 5-azadeoxycytidine may be counteracted by pyrimidine bases, while only nucleosides but not bases are effective counteractors of 5-azacytidine. This does not mean, however, that the action of 5-azadeoxycytidine is simply equivalent to that of 5-azauracil; we know, in fact, that the latter compound is much less active. This apparent discrepancy may probably be explained by a more efficient uptake of the deoxynucleoside in comparison with the base; evidence has been presented [23] indicating the existence of a transport mechanism, controlling the rate of uptake and transformation of a number of nucleosides and deoxynucleosides. Even if the deaminase and phosphorylase are located in the cell surface, they evidently are not accessible without the mediation of a transport mechanism. Therefore, 5-azauracil formed in the cells by deamination and phosphorolysis is much more effective than the same compound administered externally.

The peculiar biological effects of 5-azadeoxycytidine as well as the counteracting ability of deoxycytidine are in perfect agreement with the proposed pathway. The uncoupling of the deoxyribose moiety accounts for the lack of preferential interference with the metabolism of DNA. The obligatory deamination preceding phosphorolysis implies that the inhibitor enters the cells exclusively in the 6-oxo form unlike 5-azacytidine, which presumably may also be directly phosphorylated with preservation of the 6-amino group. In a later paper [24] evidence will be presented indicating that the strong inhibition of protein synthesis by 5-azacytidine is actually due to 5-azauridine produced by deamination while the inhibition of synthesis of phage DNA is a direct function of 5-azacytosine nucleotides. This finding provides a clue to the understanding of the effects of 5-azadeoxycytidine. Entering the cells exclusively in the form of derivatives of 5-azauracil, this compound strongly interferes with protein synthesis similarly as 5-azauridine, but is without appreciable effect on the synthesis of T4 phage DNA.

5-Azadeoxycytidine is a much weaker inhibitor of replication of phage T4 than 5-azacytidine. However, both compounds inhibit the synthesis of late phage proteins in a quite similar manner. In the case of 5-azacytidine this inhibition is preceded by nearly complete blocking of replication of phage DNA while with 5-azadeoxycytidine the synthesis of phage DNA is sligtly affected. Therefore, the lack of replication of DNA cannot be the cause of inhibition of late protein synthesis and the coincidence of the onset of inhibition of leucine incorporation with the derepression of late phage genes is probably accidental. It seems that the time of the rather sharp onset of inhibition of leucine incorporation is determined by the rate of uptake of the inhibitor by the cells. In uninfected cells mRNA synthesized in the presence of 5-azacytidine cannot be translated into functional protein; in a culture infected with phage T4, protein synthesis remains unaffected by 5-azacytidine for a period as long as 10 min, permitting normal synthesis of early phage enzymes. This difference probably is due to restricted uptake of the inhibitor by infected cells. The rate of phosphorylation and incorporation of 5-azacytidine is severely limited in comparison with uninfected cells [16] and the same is true with 5-azadeoxycytidine (*cf.* Fig. 1 and 2). Deoxyuridine, the uptake of which is probably unaffected by phage infection, was observed to cause a sharp inhibition of leucine incorporation beginning 4 min after phage infection [24].

The decrease of the rate of protein synthesis in cells infected with phage T4 thus seems to be a primary effect, independent of the preceding blockage of replication of phage DNA. On the other hand the quality of proteins synthesized undoubtedly is a function of previous replication of DNA. The overproduction of early enzymes and the lack of synthesis of phage endolysine, taking place in the presence of

5-azacytidine with excess deoxycytidine, seems to be a secondary phenomenon, being due to primary inhibition of DNA-replication. Preventing the replication of DNA by other means, *e.g.* by a genetic block or irradiation of phage with ultraviolet light [8,9], is known to have the same effect.

The ability of deoxycytidine to counteract some but not all inhibitory effects of 5-azacytidine also may be understood on the basis of the mode of entry of deoxycytidine into the cells, involving an obligatory deamination and uncoupling of deoxyribose. While capable of restoring the normal rate of protein synthesis, deoxycytidine cannot counteract the inhibitory effect of 5-azacytidine on the synthesis of DNA in T4-infected cells. This finding may be readily understood knowing that the inhibition of T4-DNA synthesis is due to 5-azacytosine phosphates formed by direct phosphorylation of 5-azacytidine, a pathway inaccessible to deoxycytidine. We may say that 5-azadeoxycytidine and deoxycytidine behave as a perfectly reciprocal pair of inhibitor-counteractor, interfering, negatively or positively, with protein synthesis, but incapable of interference with the synthesis of T4 phage DNA. This finding might seem logical if it were not for the fact that uridine derivatives entering the cells are partially aminated before being incorporated into T4 phage DNA (Table 4). We would therefore expect that even nucleosides entering the cell exclusively in the 6-oxo-form would, to a certain extent, acquire the inhibitory or counteracting properties of the 6-amino-derivatives. However, this type of inhibition or counteraction cannot be demonstrated. The reasons of this are not clear.

The present experiments show that the biological activity of inhibitors in different biological systems may be quite specific, being determined by the mechanism of their uptake by a particular type of cells. It seems that in mammalian cells, capable of direct phosphorylation of 5-azadeoxycytidine, this compound has quite different effects than in bacterial cells.

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