Effect of Hydroxyurea on Replication of Bacteriophage T4 in *Escherichia coli*[1]

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Hydroxyurea inhibited the replication of bacteriophage T4 in *Escherichia coli* B. The concentration of hydroxyurea required to inhibit net deoxyribonucleic acid (DNA) synthesis 50% was about 50-fold less than that required in uninfected cells. Even in the presence of high hydroxyurea concentrations, phage DNA was readily synthesized from the products of breakdown of the *E. coli* DNA, and viable phage were made. Deoxyribonucleotide, but not ribonucleotide, synthesis was strongly inhibited in the presence of hydroxyurea. The data indicate that hydroxyurea specifically inhibits de novo DNA synthesis in *E. coli* infected with bacteriophage T4 by inhibiting the ribonucleoside diphosphate reductase system, but does not affect DNA synthesis at subsequent steps.

Numerous studies have shown that hydroxyurea inhibits deoxyribonucleic acid (DNA) synthesis, not only in mammals and other multicellular organisms (9, 14, 24), but also in *Escherichia coli* and other microorganisms (5, 11, 13). One major difference is that a much higher concentration of hydroxyurea is required to inhibit DNA synthesis in *E. coli* than is required for comparable inhibition in mammalian cells. It is not clear whether this is due to different permeabilities or different mechanisms of action in the two kinds of cells.

Hydroxyurea has been found to be bacteriostatic in *E. coli* for up to 3 hr of exposure, but bactericidal during longer exposure (11). Protein and ribonucleic acid (RNA) synthesis are not inhibited directly by hydroxyurea, and the proteins and RNA made appear to be normal (11). Thus, it appears that hydroxyurea rather specifically inhibits DNA synthesis, but there is disagreement as to the exact site of action of the compound. There is considerable support for the idea that the primary site of action in vivo is inhibition of the reduction of ribonucleotides to deoxyribonucleotides (25), but a number of other effects have also been reported. In bacteria, these include alteration of DNA (12), assembly of T-even coliphages (8), and inhibition of deoxycytidine 5'-diphosphate (dCDP) kinase (H. S. Rosenkranz et al., *unpublished data*). Elford (3) and Y. C. Yeh (*personal communication*) have shown that the *E. coli* and bacteriophage T4-induced ribonucleoside diphosphate reductase systems are inhibited by hydroxyurea in vitro.

The *E. coli* DNA is degraded after infection with T-even bacteriophages, and Kozloff (7) summarized early evidence indicating that the degradation products can be efficiently incorporated into phage DNA. If the primary effect of hydroxyurea in infected *E. coli* is inhibition of the ribonucleoside diphosphate reductase systems, then phage DNA should still be synthesized from the *E. coli* DNA breakdown products in hydroxyurea-treated *E. coli* if hydroxyurea has no other direct effect upon DNA synthesis. We have studied the effect of hydroxyurea in *E. coli* B infected with bacteriophage T4, and found that the reduction of ribonucleoside diphosphates to deoxyribonucleoside diphosphates was inhibited more than 90%, and that infectious phage were formed efficiently from the products resulting from breakdown of *E. coli* DNA. These results suggest that the primary effect of hydroxyurea in these cells is inhibition of the ribonucleoside diphosphate reductase system and that DNA synthesis is not affected at subsequent steps.

METHODS AND MATERIALS

The *amber* (*am*) phage mutants used in this work were generously supplied by R. S. Edgar. These mutants grow in *E. coli* CR63, but not in *E. coli* B (4). The growth of cells and preparation of phage in Casamino Acids-glycerol medium has been described earlier (17). *E. coli* B was used in all the experiments, except for titering *amber* mutants (which were plated on *E. coli* CR63); all cultures were grown at 37 ± 1 C with forced aeration. Growth was followed

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by measuring the turbidity in a Beckman DB spectrophotometer at 650 nm. Cultures were used when they reached 4 × 10⁸ to 5 × 10⁸ cells/ml, and were infected at a multiplicity of four or five phage per cell unless indicated otherwise. When titering for phage, the first dilution tube always contained several drops of chloroform to lyse infected cells.

Uracil-2-^{14}C was purchased from Tracerlab, Richmond, Calif., and thymidine-2-^{14}C from Schwarz BioResearch, Orangeburg, N.Y. Radioactivity was measured on a Nuclear-Chicago gas-flow planchet counting system.

Hydroxyurea was purchased from the Sigma Chemical Co., St. Louis, Mo. Unless indicated otherwise, the hydroxyurea was added to the cells just prior to the phage. It was added either in solid form or as a freshly prepared solution.

DNA was determined by the method of Short et al. (15). Deoxycytidine 5'-phosphate (dCMP) hydroxymethylase and deoxycytidine 5'-triphosphatase (dCTPase) were assayed as described earlier (17, 18), by 30-min incubation at 35 and 30 C, respectively.

DNA in uninfected *E. coli* was labeled by adding 0.02 μmole and 1 μc of ^{14}C-thymidine to 80 ml of *E. coli* (2 × 10⁸ cells/ml). After aeration for 20 min, the cells were harvested by centrifugation and resuspended in fresh medium. The desired amount of hydroxyurea was then added to 12-ml portions of this culture, and the cells were infected with T4 or *am*N122; 1-ml samples were removed at various times and mixed with 0.1 ml of 50% trichloroacetic acid. The precipitates were dissolved in 0.8 ml of 0.1 N NaOH, and reprecipitated with 1.2 ml of 10% trichloroacetic acid. These precipitates were washed with 2-ml portions of cold 95% ethyl alcohol and diethyl ether, dissolved in 1 N NH₄OH, plated, and counted to determine the amount of ^{14}C-thymidine remaining in the DNA.

Nucleotides in infected *E. coli* were labeled by adding 1.5 ml of uracil-2-^{14}C solution (2.66 × 10⁶ counts per min per ml and 24 × 10⁶ counts per min per μmole) to 11 ml of *E. coli* infected with phage. Hydroxyurea, when present, was added 5 min before the phage, and the uracil was added 3 min after the phage. The cells were harvested 15 min after infection, and the intracellular nucleotides were extracted and separated on ion-exchange columns as described earlier (19).

RESULTS

The effect of hydroxyurea concentration on the turbidity and DNA content of uninfected cells and cells infected with bacteriophage T4 is shown in Fig. 1. Several findings are significant. The sensitivity of DNA synthesis to hydroxyurea inhibition was greatly enhanced after phage infection. Whereas 0.01 to 0.02 M hydroxyurea was required to inhibit bacterial DNA synthesis by 50%, 2.6 × 10⁻⁴ M inhibited phage DNA synthesis by 50%. Whereas the turbidity of inhibited uninfected cultures continued to increase, presumably owing to cell elongation (11), the turbidity of inhibited
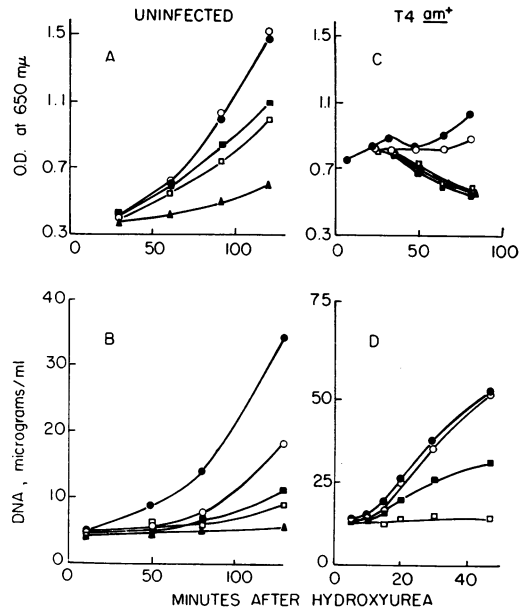

FIG. 1. *Effect of hydroxyurea on cell mass and DNA synthesis. At the times indicated, samples were withdrawn from the cultures of uninfected cells (A, B) and cells infected with phage T4 (C, D) for determination of optical density or DNA content. Hydroxyurea was present at the following concentrations: A, B, none (●), 1.3 × 10⁻² M (○), 4.0 × 10⁻² M (■), 6.6 × 10⁻² M (□), 13 × 10⁻² M (▲), C, none (●), 2.5 × 10⁻⁴ M (○), 2.5 × 10⁻³ M (■), 1.1 × 10⁻² M (□), 4.1 × 10⁻² M (▲); D, none (●), 2.6 × 10⁻⁵ M (○), 2.6 × 10⁻⁴ M (■), 2.6 × 10⁻³ M (□).*

infected cultures increased for only 20 min and then remained constant or decreased, suggesting cell lysis. The uninhibited infected culture showed a temporary decrease in turbidity between 35 and 45 min due to normal cell lysis, but then lysis inhibition was effected by the released phage and the turbidity again began to increase as phage synthesis continued. The premature lysis of the inhibited cultures may be due to the absence of lysis inhibition because of the small phage yields from inhibited cells as shown below.

Previous studies (11) indicated that hydroxyurea does not generally affect protein synthesis directly in *E. coli*, and this was clearly true also in bacteria infected with phage T4 (Fig. 2). Two early enzymes, dCMP hydroxymethylase and dCTPase, are made just as readily in the presence of hydroxyurea as in its absence. In cells infected with T4 mutants unable to induce phage DNA synthesis, early enzyme synthesis does not stop at 15 min but continues for up to 60 min (22). Hydroxyurea neither inhibited this extended early enzyme synthesis in cells infected with *am*B22, a mutant unable to induce phage DNA polymerase
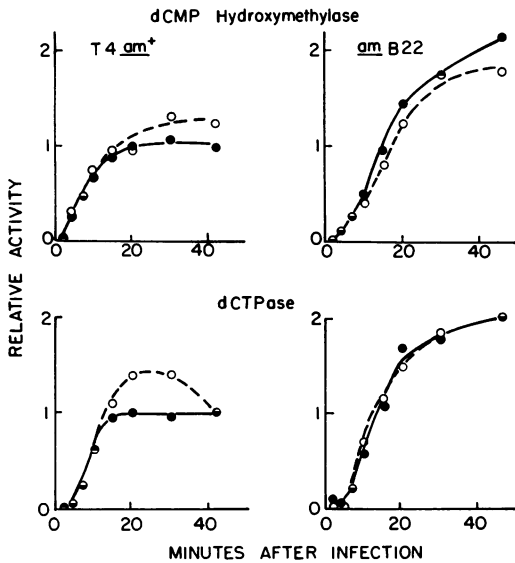
FIG. 2. *Effect of hydroxyurea on synthesis of phage-induced early enzymes. Phage T4 were added to E. coli B in the absence of hydroxyurea (●) or presence of 6.3 × 10⁻² M hydroxyurea (○), and samples were removed at the indicated times. The samples were immediately frozen in dry ice-acetone, and were stored overnight in the freezer. They were then thawed and lysed with chloroform; 0.05- and 0.5-ml portions were assayed for dCTPase and dCMP hydroxymethylase, respectively. A similar experiment was carried out with amB22 and 6.0 × 10⁻² M hydroxyurea.*

and DNA synthesis in *E. coli* B (17), nor induced extended early enzyme synthesis in cells infected with T4 (Fig. 2). This failure of hydroxyurea to extend early enzyme in cells infected with T4 suggests that, although no net DNA synthesis occurs in these cells, phage DNA is being synthesized.

If phage DNA is synthesized in the presence of hydroxyurea, it would indicate either that the primary block is leaky or that phage infection provides a partial bypass of the block. If the primary block in *E. coli* is inhibition of the ribonucleoside diphosphate reductase system, then the breakdown of *E. coli* DNA to deoxyribonucleotides after infection with T-even phages would provide a limited alternate supply of deoxyribonucleotides. If this were the case, phage DNA could be synthesized in the absence of net DNA synthesis. This DNA breakdown is most conveniently observed after infection of *E. coli* B with T4 mutants unable to induce phage DNA synthesis; *am*N122, which induces a defective dCMP hydroxymethylase, is such a mutant. Hydroxyurea did not affect the usual breakdown of *E. coli* DNA in cells infected with *am*N122 as measured by the decrease in trichloroacetic acid-insoluble

¹⁴C-thymidine, whereas little or no loss of acid-insoluble thymidine occurred in cells infected with T4, even in the presence of hydroxyurea (Fig. 3). Thus, in hydroxyurea-treated cells, DNA breakdown occurs, but the products can be efficiently reincorporated into DNA, presumably phage DNA.

If limited phage DNA synthesis can occur in the presence of hydroxyurea, the demonstration that infectious phage synthesis also occurs would indicate that the phage DNA is normal, or nearly so. Some phage synthesis did occur in the inhibited cells (Fig. 4), and the yield (15 to 20 phage/cell) in the presence of enough hydroxyurea to prevent net DNA synthesis (*see* Fig. 1) was close to that expected from degradation of the bacterial DNA to deoxyribonucleotides and the subsequent synthesis of phage DNA from these precursors. The bacterial chromosome has a molecular weight of about 2.5 × 10⁹ daltons (2), whereas the phage chromosome has a molecular weight of about 1.3 × 10⁸ daltons (16). Since the (adenine + thymine)/(guanine + cytosine) ratios are about 0.97 and 1.89 in *E. coli* and T4 DNA, respectively (6, 23), the supply of deoxyadenosine 5′-phosphate or deoxythymidine 5′-phosphate would be limiting. Thus, a maximum of about 14 phage can be synthesized from each bacterial chromosome. Cells grown under the conditions used in this experiment actually contain DNA equivalent to three or four *E. coli* chromosomes (2), but degradation of the host
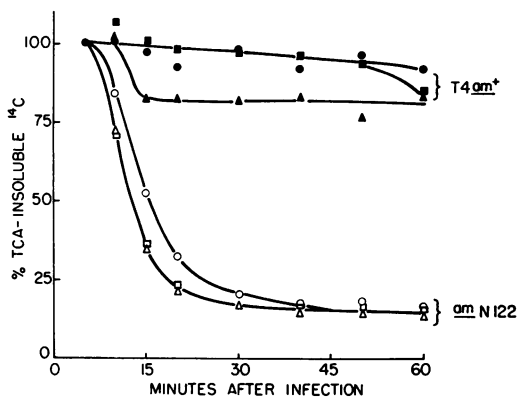

FIG. 3. *Effect of hydroxyurea on breakdown and reutilization of labeled E. coli DNA. After infection of the cells with T4 or amN122 in the presence of hydroxyurea, 1-ml samples were removed at the indicated times and assayed for trichloroacetic acid-insoluble ¹⁴C. The curves represent cells infected with phage in the presence of the following concentrations of hydroxyurea: T4, none (●), 3.2 × 10⁻³ M (■), 6.4 × 10⁻² M (▲); amN122, none (○), 3.0 × 10⁻³ M (□), 6.2 × 10⁻² M (△).*
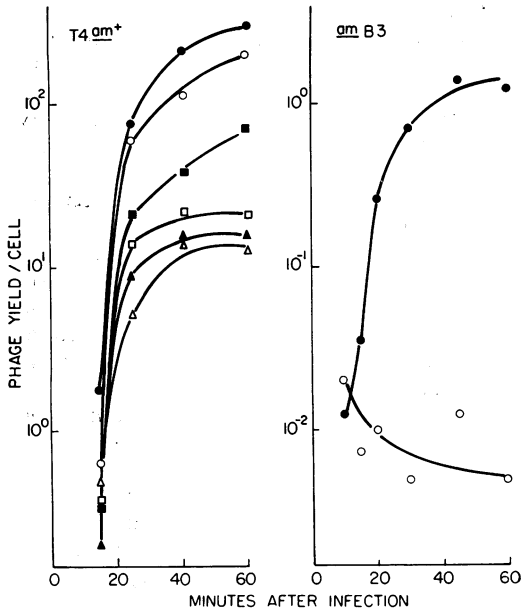
FIG. 4. *Effect of hydroxyurea on phage yield. E. coli was infected with T4 in the presence of the following hydroxyurea concentrations: none* (●), 2.5×10^{-4} M (○), 1.3×10^{-3} M (■), 3.1×10^{-3} M (□), 1.6×10^{-2} M (▲), *and* 6.9×10^{-2} M (△). *Another culture was infected with amB3 at a multiplicity of infection of 1.2 in the absence* (●) *or presence of* 1.5×10^{-2} M (○) *hydroxyurea. Samples were removed at the indicated times for determination of phage titer. T4 was titered on E. coli B; amB3 was titered on E. coli CR63, a permissive host for amber mutants.*

chromosome is incomplete (*see* Fig. 3). The maximum theoretical yield can be roughly estimated to be 30 to 40 phage per cell, indicating that at least 50% of the deoxyribonucleotides produced by breakdown of the *E. coli* DNA are incorporated into phage DNA in the presence of hydroxyurea.

Phage T4 mutants defective in genes 46 or 47 are unable to degrade *E. coli* DNA to acid-soluble form (20). Thus, the limited phage yield observed above in the presence of hydroxyurea should be completely abolished if *E. coli* B is infected with a gene 46 or 47 mutant in the presence of hydroxyurea. The results in Fig. 4 indicate that this is the case. In cells infected with the gene 46 mutant *am*B3, between one and two phages per cell were synthesized in the absence of hydroxyurea; this phage yield was completely eliminated by hydroxyurea.

To determine the extent of inhibition of deoxyribonucleotide synthesis by hydroxyurea in phage-infected cells, two T4 mutants were used which have been previously shown to accumulate

deoxyribonucleotides (19). Mutant *am*E56 cannot induce dCTPase (21) and therefore accumulates dCDP and deoxycytidine 5'-triphosphate (dCTP); *am*B24 cannot induce deoxyribonucleoside monophosphate kinase (20) and accumulates dCMP and deoxyhydroxymethylcytidylic acid (dHMP). The total deoxycytidine and deoxyhydroxymethylcytidine nucleotide pool was decreased more than 10-fold, and the total cytidine nucleotide pool was increased more than 2-fold, by the addition of hydroxyurea to the culture (Table 1). A similar effect was observed with the total thymidine and uridine nucleotide pools. In general, there was a good correlation between the reduced size of the deoxyribonucleotide pools and the increased size of the ribonucleotide pools. These data are good direct evidence that the in vivo reduction of ribonucleotides to deoxyribonucleotides is dramatically inhibited by hydroxyurea. Similar results were obtained with a much lower concentration of hydroxyurea (3.4×10^{-4} M). This rules out the possibility that the hydroxyurea might be inhibiting the reductase only at high hydroxyurea concentrations, and that some other step in DNA replication is inhibited at the lower hydroxyurea concentrations in phage-infected cells.

The hydroxyurea effect was reversible (Fig. 5). When hydroxyurea was removed from the infected culture by 15 min, phage production paralled that observed in the absence of hydroxyurea. When the hydroxyurea was removed at 25 and 35 min after infection, considerable phage synthesis occurred, but the rate decreased with increasing exposure to hydroxyurea. This could be related to the decreasing turbidity of infected cultures observed in Fig. 1C. Cell lysis would reduce the number of phage-yielding cells, and this lysis appears to begin between 20 and 30 min after infecton.

DISCUSSION

The data presented indicate that hydroxyurea dramatically inhibits deoxyribonucleotide synthesis in T4-infected *E. coli*, but allows efficient utilization of preformed deoxyribonucleotides for the synthesis of infectious phage DNA. As shown in the diagram below, when reaction 1 is blocked by hydroxyurea, reaction 3 can take place as long as reaction 2 is taking place. When reaction 2 is blocked by a phage mutation, reaction 3 does not occur, indicating that there is no synthesis of deoxyribonucleotides from some unknown pathway in these phage-infected cells.

Ribonucleotides

$1\downarrow$

E. coli DNA $\xrightarrow{2}$ Deoxyribonucleotides $\xrightarrow{3}$ phage DNA

TABLE 1. *Effect of hydroxyurea on the incorporation of ^{14}C-uracil into deoxyribonucleotides in phage-infected E. coli*

Mutant	Nucleotides accumulated[c]	Hydroxyurea	Total labeled nucleotide pools[a] (pmoles/ml of culture[b])					
			dC + dH	rC	Total C + H	dT	rU	Total T + U
amE56	dCDP-dCTP	None	135	97	232	34	101	135
		0.053 M	14	281	295	2	149	151
amB24	dCMP-dHMP	None	377	354	731	111	647	758
		0.053 M	6	732	738	7	939	946

[a] dC refers to the sum of labeled uracil incorporated into dCMP, dCDP, and dCTP. Similarly, dH, rC, dT, and rU refer to the total label incorporated into the corresponding acid-soluble nucleotides, including nucleoside diphospho sugars.

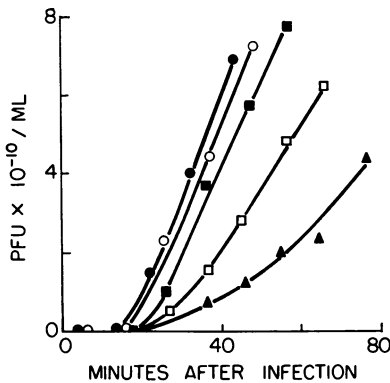[b] Approximately 4×10^8 cells/ml.

[c] See reference 19.

FIG. 5. *Recovery of phage production after removal of hydroxyurea. E. coli was infected with T4, and hydroxyurea was added 3.5 min after infection to give a final concentration of 4.9×10^{-3} M; 0.1-ml samples were removed from this culture and diluted 101 × in fresh medium at the following times after infection: 2 min (●), 5 min (○), 15 min (■), 25 min (□), and 35 min (▲).*

This phage DNA made in the presence of hydroxyurea can be used for transcription of late RNA and is infective, suggesting that it is essentially normal. The data strongly suggest that hydroxyurea may have one primary effect in E. coli, the inhibition of the ribonucleoside diphosphate reductase system. Other defects in nucleic acid structure and metabolism may subsequently occur as a result of this deoxyribonucleotide deficiency.

Cohen and Barner (1) showed that infection of E. coli with T-even phage results in increased ribonucleotide reductase activity, but did not determine whether this increased activity is due to new phage-induced enzymes. Recently, Yeh, Dubovi, and Tessman (Virology, in press) isolated T4 mutants unable to induce this increased

ribonucleotide reductase activity, suggesting that the phage genome does carry one or more structural genes for new ribonucleotide reductase enzymes. Furthermore, Yeh (*personal communication*) has shown that the phage-induced system is sensitive to hydroxyurea in vitro, and our results suggest that this reductase system is also sensitive to hydroxyurea in vivo. The greater sensitivity of phage-infected cells to hydroxyurea as compared to uninfected cells could possibly be an indication of a more sensitive phage-induced enzyme, but more likely is just an indication that phage-infected cells are more permeable to the agent. Phage infection has previously been shown to increase cell permeability (10).

Margaretten et al. (8) did not observe that phage-infected cells are more sensitive to hydroxyurea than are uninfected cells, and the reason for this discrepancy is obscure. They also reported that abnormal T4 phage structures are observed. Their results and ours can be explained by assuming that hydroxyurea-treated cells make enough progeny DNA from the breakdown of E. coli DNA to permit extensive transcription of late genes and synthesis of late proteins in excess of that needed to package the limited quantities of DNA available. Thus, bizarre structures due to DNA deficiency might result. Such structures might also be formed in cells infected with gene 46 or 47 mutants which also induce limited phage DNA synthesis (20).

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