

Relationship Between *Escherichia coli* B Titer and the Level of Deoxycytidylate Deaminase Activity Induced on Bacteriophage T2r⁺ Infection

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The activities of six bacteriophage T2r⁺-induced enzymes (thymidylate synthetase, deoxycytidylate deaminase, thymidylate kinase, deoxycytidylate hydroxymethylase, deoxycytidine pyrophosphatase, and dihydrofolate reductase) were measured after dilution of phage-infected *Escherichia coli* B from 8×10^8 to 2×10^8 cells per ml. The only enzyme activity altered was that of deoxycytidylate deaminase, which increased three- to fourfold. Conversely, the rapid concentration of cells from 2×10^8 to 8×10^8 per ml did not result in a reduction in deaminase activity. Although an enhancement in aeration reduced the response of deoxycytidylate deaminase to cellular dilution, the influence of potential metabolic inhibitors or activators could not be shown. The change in deoxycytidylate deaminase activity appeared to be associated with an altered translational event, since the increase could not be prevented by rifampin but was blocked effectively by chloramphenicol and hydroxylamine. In addition, antibody to the T2 phage-induced deoxycytidylate deaminase demonstrated that the increase in enzyme activity was associated with a corresponding increase in radioactive leucine incorporated into the enzyme antigen.

During studies on the purification and properties of bacteriophage T2r⁺-induced deoxycytidylate (dCMP) deaminase (EC 3.5.4.12), an inverse relationship was observed between the specific activity of the deaminase and the cell titer at which infection occurred. The net effect was a two- to threefold increase in dCMP deaminase specific activity when phage infection took place at 1.7×10^8 as compared with 5×10^8 cells per ml (15). In contrast, the specific activities of other phage-induced enzymes, such as thymidylate (dTMP) synthetase (EC 2.1.1.b) and dCMP hydroxymethylase (EC 2.1.2.b), were unaffected by the cell titer.

Initial attempts to explain the relationship between enzyme activity and cell titer revealed that the level of deaminase activity induced by bacteriophage T2r⁺ in *Escherichia coli* B grown to high cell densities was partially dependent on the oxygen tension of the medium. Under conditions of optimal aeration, the specific activity of phage-induced deaminase was independent of the host cell titer through the exponential phase of growth to about 5×10^8 organisms per ml. However, as the culture passed into the phase of negative acceleration, the dCMP deaminase induced in infec-

tion declined until, at 8×10^8 cells per ml, only one-third of the level induced at lower cell densities was found. The enhanced aeration did not influence the specific activities of the phage-induced dTMP synthetase and dCMP hydroxymethylase, both of which remained independent of the titer.

The selective modulation of phage-induced deaminase activity by factors associated with host cell density suggested that the regulatory effect was imposed at the level of transcription or translation. In this paper, evidence is presented to suggest that the restriction of dCMP deaminase synthesis occurs at the translational level of protein synthesis.

MATERIALS AND METHODS

Organisms, media, and culture conditions. *E. coli* B, maintained on nutrient agar slants (Difco), and bacteriophage T2r⁺ were used in this study. A high-titer phage stock, stored in 0.9% sodium chloride, was prepared from *E. coli* B grown in Davis medium and purified by three cycles of differential centrifugation. The phage concentration was determined essentially as described by Adams (1).

To study the relationship between the host growth level and the activities of several early enzymes in-

duced on infection with phage T2r⁺, 1-liter volumes of M9 minimal medium (1) containing 0.4% glucose were inoculated with 20 ml of an overnight culture of *E. coli* B grown in the same medium containing 0.2% glucose. The inoculated flasks were aerated vigorously at 37 C on a New Brunswick rotary platform shaker (model VS) at 180 to 200 rev/min. Growth was followed by measuring the increase in absorbance at 650 nm with a Coleman Jr. II spectrophotometer. Optical density readings were converted to cell titers with a standard curve prepared against a direct count (Petroff-Hausser counting chamber) of the organism, under identical growth conditions. After the desired level of growth was reached, cultures were infected with T2r⁺ at a multiplicity of 5 per bacterium, and the aeration was continued. At specified time intervals, samples of 4×10^{10} cells were poured over an equal volume of chipped ice containing sufficient chloramphenicol to yield a final concentration of 50 μ g per ml. The chilled cells were harvested by centrifugation at $18,000 \times g$ for 10 min, suspended in 2.0 ml of a solution containing 5 mM potassium phosphate (pH 7.1), 0.04 mM deoxycytidine triphosphate (dCTP), and 2 mM magnesium chloride, and disrupted by 1 min of sonic treatment with the microprobe of a Biosonik II sonifier at 90% intensity. The sonic extracts were centrifuged at $30,000 \times g$ for 30 min, and the supernatant fractions were assayed for the enzyme activities described below.

In experiments where either infected or uninfected high-titer cells were diluted into fresh, spent, or inhibitor-containing medium, the same surface-to-volume ratio was maintained to minimize differences in aeration rates.

E. coli B were concentrated from 2×10^8 to 8×10^8 cells per ml in an Amicon ultrafilter (model 402) containing a PM-30 membrane. At 40-lb air pressure, 200 ml was reduced to 50 ml in less than 3 min. The concentrated samples were returned to the shaker at a surface-to-volume ratio equal to that of the unconcentrated control.

Materials. The chemicals and materials used were obtained from the following suppliers: rifampin, deoxycytidine-5'-triphosphate, and dithiothreitol from Calbiochem Co., Los Angeles, Calif.; chloramphenicol, the 5'-monophosphates of deoxycytidine, deoxyuridine, and thymidine, tris(hydroxymethyl)aminomethane (Tris), and folic acid from the Sigma Chemical Co., St. Louis, Mo.; hydroxyurea and streptomycin sulfate from Nutritional Biochemical Corp., Cleveland, Ohio; L-¹⁴C-leucine (316 mCi/mmole), dCMP-2-¹⁴C (48.5 mCi/mmole), dCTP-2-¹⁴C (42 mCi/mmole), and uracil-2-¹⁴C (54.8 mCi/mmole) from Schwarz Mann, Orangeburg, N.Y.; thymidine-2-¹⁴C (59 mCi/mmole) from Amersham/Searle Corp., Arlington Heights, Ill.; L-leucine-4,5-³H (5 Ci/mmole) and Liquifluor scintillation solution from New England Nuclear Corp., Boston, Mass.; and membrane filters from Millipore Filter Corp., Bedford, Mass. All other chemicals were reagent grade. Actinomycin D was a generous gift of the Merck Sharp and Dohme Research Laboratories, West Point, Pa.

Enzyme assays. Deoxycytidylate deaminase was

assayed in a reaction mixture which contained the following components (in μ moles): dCMP, 0.5; dCTP, 0.06; Tris-hydrochloride (pH 8.0), 5; MgCl₂, 0.5; dithiothreitol, 5; sodium fluoride, 15; and enzyme and water to a final volume of 1.0 ml. Enzyme activity was followed at 30 C by the decrease in absorbance at 290 nm with a Gilford multiple absorbance recording spectrophotometer as described previously (14). Since earlier measurements of dCMP deaminase were made at 37 C, the activity obtained at 30 C was doubled to make them directly comparable. The validity of this correction was determined experimentally.

Thymidylate synthetase activity was assayed spectrophotometrically at 30 C by the method of Wahba and Friedkin (21), as modified by Lorenson et al. (12).

Deoxycytidylate hydroxymethylase activity was assayed at 37 C by the method of Pizer and Cohen (18).

Deoxynucleotide kinase (EC 2.7.4.4), an enzyme that phosphorylates dCMP, 5-hydroxymethyl-dCMP, and dTMP to the corresponding diphosphates (2,5), was assayed by the method of Lembach and Buchanan (11), with the following modification. From reaction mixtures incubated for 10 min at 30 C, 10- μ liter samples were removed and pipetted onto Whatman DE-81 anion exchange paper. The chromatograms were developed, descendingly, with 4 N formic acid, a procedure that separates dTMP from thymidine-5'-diphosphate (dTDP) and thymidine-5'-triphosphate (dTTP), as the latter two remain essentially at the origin (6). The regions containing dTDP and dTTP were cut from the dried chromatograms, immersed in 10 ml of Liquifluor, and counted at 60% efficiency in a Nuclear-Chicago scintillation spectrometer.

Dihydrofolate reductase (EC 1.5.1.3) was measured at 30 C in the presence of Tris-hydrochloride, pH 7.5, with the spectrophotometric assay of Bertino et al. (3).

Deoxycytidine pyrophosphatase (dCTPase, EC 3.6.1.12) was measured by a modification of the radioactive assay described by Warner and Barnes (22). A typical reaction mixture contained the following components (in μ moles): Tris-hydrochloride (pH 8.5), 2.5; magnesium acetate, 1.4; dithiothreitol, 0.25; tetrahydrodeoxyuridylylate (H₄dUMP), 0.25; dCTP-2-¹⁴C (3.4×10^6 counts per min per μ mole), 0.02; and enzyme and water to a final volume of 0.05 ml. The H₄dUMP was added to inhibit the phage-induced dCMP deaminase present in the extracts (16). Reaction mixtures were incubated at 37 C for 10 min and terminated by heating in a boiling water bath for 2 min. After the precipitated protein was removed by centrifugation, 2- μ liter samples were spotted in duplicate on polyethyleneimine (PEI)-cellulose thin-layer sheets (Brinkmann Instruments, Inc., Westbury, N.Y.) containing markers of dCTP, dCMP, and dUMP. Chromatograms were developed with 0.2 N sodium formate, pH 3.6 (19). After the chromatograms were dried, the ultraviolet-absorbing areas were cut out, placed in scintillation vials with 10 ml of Liquifluor, and counted. The counting efficiency of this system was 66%.

For each enzyme, a unit of activity is defined as one μ mole of substrate transformed per min under the assay conditions described, with all the data reported as initial velocities. Specific activities are presented as milliunits per mg of protein.

Radioactive labeling procedures. For pulse-labeling of protein, ribonucleic acid (RNA), or deoxyribonucleic acid (DNA), a sample containing 4×10^8 infected or uninfected cells was pipetted into a pre-warmed conical tube containing one-tenth the final reaction volume of either L - ^{14}C -leucine (0.2 mM; 20 μ Ci/ μ mole), uracil- ^{14}C (0.1 mM; 20 μ Ci/ μ mole), or thymidine- ^{14}C (0.1 mM; 20 μ Ci/ μ mole), respectively. After 30 sec, the incorporation of radioactivity was terminated by the addition of an equal volume of ice-cold 10% trichloroacetic acid containing 2 mM unlabeled precursor. The cells labeled with leucine were heated for 20 min at 90 C, while those labeled with uracil or thymidine were placed on ice for at least 1 hr. Samples were transferred quantitatively to 0.45- μ m Millipore filters with two 5-ml portions of 5% trichloroacetic acid and were washed with an additional 10 ml of 5% trichloroacetic acid followed by four 5-ml portions of distilled water. The filters were dried, placed in scintillation vials with 10 ml of Liquifluor, and counted. The counting efficiency for ^{14}C in this system was 82%.

Phage-induced proteins were labeled by adding L -leucine- $4,5$ - 3H to a final concentration of 0.01 mM (specific activity, 12 μ Ci/ μ mole) in a 1-liter culture of *E. coli* B 2 min after infection with T2r⁺ phage. The extent of 3H incorporation was monitored by removing 4×10^8 cells from the cultures and pipetting them into an equal volume of ice-cold 10% trichloroacetic acid that contained 2 mM unlabeled leucine. After the trichloroacetic acid solution was heated for 20 min at 90 C, the cells were centrifuged and washed twice in the cold with 1-ml portions of 5% trichloroacetic acid. The well drained pellet was dissolved in 0.1 N sodium hydroxide, and samples were counted in 10 ml of a modified Bray's solution (4) at 21% efficiency.

Preparation of antibody to dCMP deaminase. The preparation of rabbit antiserum to T2r⁺ phage-induced dCMP deaminase has been described (G. F. Maley, D. U. Guarino, and F. Maley, *J. Biol. Chem.*, *in press*). A partially purified immunoglobulin G (IgG) fraction was prepared from the immune serum by ammonium sulfate precipitation (20). The final precipitate was dissolved in distilled water, dialyzed against a solution of 5 mM potassium phosphate (pH 7.1) and 0.85% saline, and then diluted to 5 mg of protein per ml with the same buffer and stored at -10 C.

Precipitation of tritium-labeled dCMP deaminase by specific antibodies. One liter of *E. coli* B, grown to 8×10^8 cells per ml, was infected with T2r⁺ phage and 3H -leucine was added 2 min later. Five minutes after infection, 500 ml of the culture was diluted fourfold into fresh medium containing 3H -leucine at the same concentration and specific activity as that added initially to the infected cells. Both control and diluted cultures were poured over ice 10 min after infection, and the cells were centrifuged and washed

once with 0.9% saline. The pellet (1 to 1.2 g wet weight) was suspended in 10 ml of a solution of 5 mM potassium phosphate (pH 7.1), 0.04 mM dCTP, and 2 mM magnesium chloride, and treated sonically for 1 min (Biosonik III, 60% intensity, large probe). The resulting suspension was centrifuged at $30,000 \times g$ for 30 min to remove cellular debris.

The dCMP deaminase was partially purified from the crude extracts by a small-scale version of the first step in the procedure for the preparation of homogeneous enzyme (G. F. Maley, D. U. Guarino, and F. Maley, *J. Biol. Chem.*, *in press*). A 5% streptomycin sulfate solution (1.8 ml) was added dropwise, with stirring, to 9.0 ml of the crude extract at 0 to 4 C. After 10 min of additional stirring, the precipitate containing the deaminase activity was centrifuged at $17,000 \times g$ for 15 min and suspended in 5.0 ml of a solution containing 0.1 M potassium phosphate (pH 7.5), 3 mM magnesium chloride, 0.04 mM dCTP, 20 mM 2-mercaptoethanol, and 0.1% (v/v) chloroform. The suspension was tightly capped and incubated at 37 C for 3 hr and then at room temperature overnight. The autolysate was chilled in ice for 2 hr, centrifuged at $30,000 \times g$ for 30 min, and the enzyme-containing supernatant fraction was assayed. This procedure provided a 55 to 85% recovery of the dCMP deaminase with a three- to fourfold purification. The variability in total recovery resulted from differences in the amount of the enzyme precipitated initially by the streptomycin sulfate.

Antibody (300 μ g of protein per unit of deaminase) and 0.9% saline were added to 2.0-ml portions of each autolysate to a final volume of 2.6 ml. A parallel autolysate without antibody was treated similarly.

To circumvent the problem of nonspecific precipitates, which could amount to 50% of the protein and radioactivity from samples incubated with antibody for 65 hr, the time required for the immune precipitation of the deaminase was decreased by adding five to seven units of pure carrier T2r⁺ dCMP deaminase to each 2-ml portion of autolysate. The immune reactions were incubated at 37 C for 1 hr and then at 0 C overnight. The precipitates were collected by centrifuging for 20 min at $30,000 \times g$, washed twice with 0.5 ml of cold 0.9% NaCl, and dissolved in 0.1 N sodium hydroxide. Protein and radioactivity determinations were performed in triplicate. Under these conditions, the autolysates without antibody yielded precipitates that were only 5 to 10% of those obtained from autolysates incubated with antibody. Addition of carrier dCMP deaminase to autolysates did not alter the equivalence zone for maximal immune precipitation of the dCMP deaminase. The antibody precipitated insignificant amounts of protein or radioactivity from similar autolysates prepared from tritium-labeled uninfected cells.

Other methods. Protein was determined by the method of Lowry et al. (13) with crystalline bovine serum albumin as a standard.

Dihydrofolate was prepared as described by M. Friedkin, E. J. Crawford, and D. Misra (*Fed. Proc.* 21:176, 1962), and tetrahydrofolate by the catalytic reduction of folic acid with hydrogen (12).

For inhibitor studies, a freshly prepared solution

of 0.05 M hydroxylamine hydrochloride was neutralized with solid Tris base just prior to use. Rifampin was dissolved in 95% ethanol and diluted with water to a final concentration of 5 mg per ml in 50% ethanol. Chloramphenicol was dissolved in twice-distilled water at a concentration of 0.05 M.

RESULTS

Effect of culture dilution on T2r⁺-induced enzymes. Table 1 shows the level of several phage-induced enzymes, involved in the biosynthesis of pyrimidine deoxyribonucleotides, that were measured in extracts prepared from undiluted and fourfold diluted cultures of infected *E. coli* B. Of the six enzymes assayed, only dCMP deaminase increased to a significant extent on dilution of the cells. The increase in activity usually varied from three- to fourfold.

Soluble activators or inhibitors of T2r⁺-induced dCMP deaminase. To determine whether the threefold difference in levels of the phage-induced dCMP deaminase activity resulted from the presence of naturally existing activators or inhibitors of the enzyme, cultures of cells were grown to 2×10^8 and 8×10^8 cells per ml, and infected for 12 min with T2r⁺. Cell-free extracts were prepared as described above. When mixtures of low- and high-titer extracts were assayed for deaminase activity, the total number of units of enzyme added to the reaction cuvette could be accounted for, indicating the absence of soluble agents capable of influencing deaminase activity. Similarly, addition of boiled extracts (2 min at 100 C) from phage-infected high- and low-titer cells did not affect the dCMP deaminase activity of unheated controls; this finding, however, could be discounted by the heat lability of a potential activator or inhibitor.

To investigate the possibility that a soluble inhibitor or repressor of phage-induced dCMP deaminase activity was released into the medium during the growth of *E. coli* B, portions of a cell culture grown to 8×10^8 per ml were diluted fourfold into either fresh or spent high-titer medium 5 min before infection with bacteriophage T2r⁺. (The spent medium was obtained by centrifuging the cells from an 8×10^8 cell per ml culture of *E. coli* B and filtering the supernatant fraction through a 0.45- μ m membrane filter.) Twelve minutes postinfection, extracts were prepared from the control and diluted cultures. The dCMP deaminase activity from the undiluted cells was 56 milliunits per mg of protein, whereas that from the cells diluted into fresh or spent medium was increased threefold to 170 and 174 milliunits per mg of protein, respectively.

To determine whether permeability might be a barrier to a potential regulatory agent released

TABLE 1. *Effect of dilution of a high-titer culture of E. coli B on the level of several T2r⁺ phage-induced enzyme activities^a*

Enzyme	Specific activity (milliunits/mg)	
	Undiluted	Diluted
Deoxycytidylate deaminase	49	142
Thymidylate synthetase	6.2	6.6
Dihydrofolate reductase	32	34
Deoxycytidine pyrophosphatase	67	80
Deoxynucleotide kinase	70	93
Deoxycytidylate hydroxymethylase	23	22

^a Five minutes after infection, a portion of a culture of *E. coli* B (8×10^8 cells per ml) was diluted fourfold into fresh medium. Seven minutes later, extracts were prepared and the indicated enzymes were assayed as described in Materials and Methods.

into the medium, the following experiment was performed. A culture of *E. coli* B grown to 4×10^8 cells per ml was harvested by centrifugation, washed with 0.12 M Tris-hydrochloride (pH 8.0), and resuspended in one-tenth volume of the same buffer at 37 C. The cells were sensitized for 2 min with 0.2 mM ethylenediaminetetraacetic acid (EDTA) (10). The treatment was terminated by diluting portions of the cells 10-fold into either fresh or spent medium. Thirty seconds later, each portion was infected for 10 min with T2r⁺, and extracts were prepared as usual. The dCMP deaminase activity in each extract was about 145 milliunits per mg of protein. In both experiments the dTMP synthetase activities, regardless of the cell titer or diluent, were between 4.2 and 4.6 milliunits per mg of protein.

To insure that the EDTA treatment had made the *E. coli* B permeable, 10 μ g of actinomycin D per ml was added 30 sec after infection of the control and EDTA-treated cells. The dCMP deaminase activities in extracts from control and sensitized cells after infection for 12 min with T2r⁺ were 124 and 8 milliunits per mg of protein, respectively, confirming that the EDTA had increased the permeability of the cells to actinomycin.

These results are consistent with the idea that an inhibitor or repressor of phage-induced dCMP deaminase did not accumulate in the medium during the growth of the *E. coli* B cultures.

Effect of altering host titer on phage-induced dCMP deaminase activity. Since a fourfold dilution of *E. coli* B grown to 8×10^8 cells per ml yielded a threefold increase in dCMP deaminase

specific activity subsequent to T2r⁺ phage infection over that induced in the undiluted cells, it was of interest to determine whether the converse applied; that is, does a rapid concentration of cells at 2×10^8 per ml to 8×10^8 cells per ml yield the lower level of phage-induced deaminase specific activity found normally with cells infected at the latter titer? Three 200-ml portions of a culture of *E. coli* B grown to 2×10^8 cells per ml were concentrated to 8×10^8 cells per ml in an Amicon ultrafilter as described above and returned to the rotary shaker. At 0.5, 20, and 40 min after concentration, the cultures were infected with T2r⁺ for 10 min. The dCMP deaminase activity of extracts prepared from the concentrated cells at each time interval, and in an extract prepared from an unconcentrated control, was between 121 and 150 milliunits per mg of protein. The dTMP synthetase activity was between 6 and 7 milliunits per mg of protein.

Thus the level of T2r⁺-induced dCMP deaminase, but not that of dTMP synthetase, appeared to be controlled by a host cell density-related constraint that could be relaxed on dilution of the *E. coli* before infection. Conversely, this restriction could not be imposed by concentrating cells rapidly from a low to a high titer prior to infection. The possibility that this effect was a time-related phenomenon was considered next.

Enzyme activity produced following dilution at various times after infection. The results of several experiments showed that the dCMP deaminase and dTMP synthetase activities in extracts of high-titer *E. coli* B diluted fourfold 20 min before to 5 min after infection were routinely between 130 to 170 and 5.4 to 7.0 milliunits per mg of protein, respectively, providing the assays were conducted with extracts from cells that had been infected for a total of 10 min. As the time interval between infection and dilution was extended beyond 5 min, however, the dCMP deaminase specific activity declined. Thus, a fourfold dilution of infected high-titer cells 15 to 30 sec prior to the termination of protein synthesis at 10 min post-infection yielded phage-induced deaminase activity at a level only slightly elevated over that found in an undiluted control.

To define more clearly the relationship between dilution and the increase in phage-induced deaminase activity, the experiment reported in Table 2 was performed. The change in dCMP deaminase and dTMP synthetase specific activities was measured over several 3-min intervals during the infection cycle, with each interval initiated by the fourfold dilution of a portion of the T2r⁺-infected high-titer cells into fresh medium. As indicated, the dilution of cells at any time after

TABLE 2. Incremental changes in T2r⁺ phage-induced deoxycytidylate (dCMP) deaminase and thymidylate (dTMP) synthetase activities over 3-min periods following dilution of infected *E. coli* B^a

Time after infection (min)	Δ dCMP deaminase activity		Δ dTMP synthetase activity	
	Undiluted	Diluted	Undiluted	Diluted
3-6	27	122	3.3	4.0
5.5-8.5	20	132	2.8	3.2
8-11	10	98	1.2	1.2
10.5-13.5	4	84	0.6	0.6

^a At the beginning of each time interval, 50 ml of a T2r⁺ phage-infected culture of *E. coli* B at 8×10^8 cells per ml was poured over ice while an identical portion was diluted fourfold into fresh medium. Three minutes later, the diluted culture and an additional 50 ml of the infected high-titer culture were poured over ice. Extracts were prepared and enzymes assayed as described in the text. Each value in milliunits per mg of protein represents the difference between the basal enzyme level at the beginning of each interval and that in diluted and undiluted cultures 3 min later.

infection resulted in a marked increase in deaminase activity when compared with that formed in a parallel sample of undiluted cells. The greatest increase in deaminase activity occurred during the 5.5- to 8.5-min interval following dilution, although the cells still retained the capacity to synthesize considerable quantities of enzyme on dilution (64% maximal) even at 10.5 min after infection. In contrast, the synthetic capability of the undiluted high-titer cells diminished rapidly after the 3- to 6-min interval, where it was about one-fourth to one-fifth that of the diluted cells, to about one-twentieth that formed in the 10.5- to 13.5-min interval following dilution. The change in dTMP synthetase activity, however, was essentially unaffected by dilution at each of the time intervals measured.

Effect of inhibitors on the dilution-dependent increase in dCMP deaminase activity. Figure 1 reveals the pattern of pulse-label incorporation of precursors into protein, RNA, and DNA on dilution of *E. coli* B from 8×10^8 to 2×10^8 cells per ml, either before or after infection. After a fourfold dilution of infected or uninfected cells into fresh medium (Fig. 1A), an increase in leucine uptake of 45 to 50% was obtained. When phage-infected high-titer cells were labeled under conditions of continuous incorporation, a similar increase was found in uptake after dilution (*data not shown*). In response to dilution, there was a ninefold increase in uracil uptake before infection

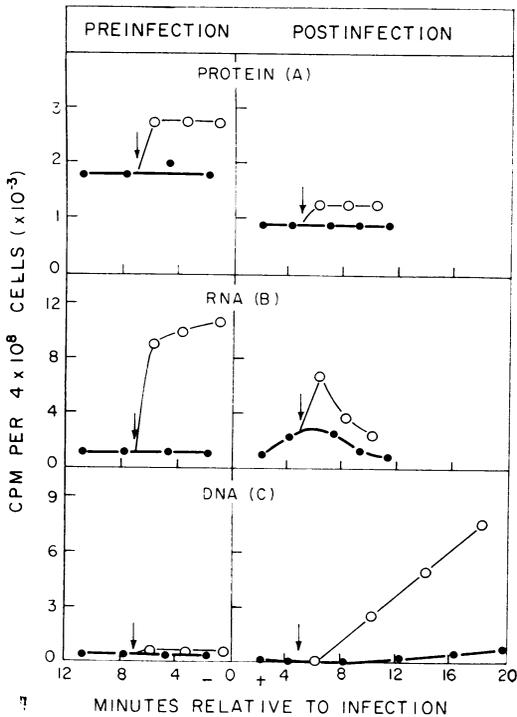


FIG. 1. Pulse-labeling of protein, RNA, and DNA in undiluted and diluted uninfected and T2-infected *E. coli* B grown to 8×10^8 cells per ml. In A, B, and C, 500 ml of cells were infected with T2r⁺ at zero time. At 7 min before infection and at 5 min after infection (\downarrow), 100 ml of the respective cultures were diluted with 300 ml of fresh medium. Samples containing 4×10^8 cells were pulse-labeled for 30 sec with (A) L-¹⁴C-leucine, (B) uracil-2-¹⁴C, or (C) thymidine-2-¹⁴C as described in the text. Symbols: ●, cells at 8×10^8 per ml; ○, cells at 2×10^8 per ml.

of the *E. coli* but only a twofold increase after infection (Fig. 1B). Thymidine incorporation increased by 50% on dilution of the uninfected cells and by 5- to 10-fold in infected cells (Fig. 1C). Since there appeared to be no obvious relationship between the incorporation of any single precursor tested and the threefold increase in dCMP deaminase activity on dilution of the culture, specific inhibitors were used to determine whether a requirement for either protein, RNA, or DNA synthesis could be established.

(i) **Protein synthesis.** Figure 2 shows the time course of appearance of T2r⁺-induced dCMP deaminase and dTMP synthetase in *E. coli* B infected at 8×10^8 cells per ml. The increase in deaminase after dilution of a portion of the higher titer culture at 5 min postinfection is rather evident, and, as anticipated, the dTMP synthetase remained unchanged by the dilution. The addition

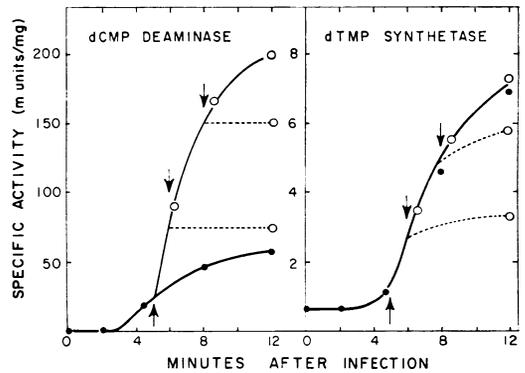


FIG. 2. Effect of chloramphenicol on the increase in deoxycytidylate (dCMP) deaminase and thymidylate (dTMP) synthetase following the dilution of T2-infected *E. coli*. One liter of *E. coli* B grown to 8×10^8 cells per ml was infected with T2r⁺-phage, and 5 min later 200 ml was diluted with 600 ml of fresh medium (\uparrow). At 6 min and at 8 min postinfection (\downarrow), 200 ml of the diluted cells were transferred to flasks containing chloramphenicol at a final concentration of 0.25 mM (\dashrightarrow). Samples were withdrawn from the cultures at the indicated times and assayed for dCMP deaminase and dTMP synthetase. Symbols: ●, 8×10^8 cells per ml; ○, 2×10^8 cells per ml.

of the protein synthesis inhibitor chloramphenicol at 6 and 8 min after infection blocked the normal increase in deaminase and synthetase activities. Similarly, if T2r⁺-infected high-titer cells (8×10^8 cells/ml) were diluted directly into fresh medium containing chloramphenicol, there was no significant increase in deaminase or synthetase activity over that found in the cells at the time of dilution (data not shown).

Hydroxylamine was employed also as an inhibitor of protein synthesis since it blocks in a manner different from chloramphenicol. As illustrated in Fig. 3, the increase in dCMP deaminase activity after dilution of phage T2r⁺-infected *E. coli* was almost completely prevented. In separate experiments, addition of either hydroxylamine or chloramphenicol up to 2 min after infection prevented the appearance of dCMP deaminase and any increase in the level of dTMP synthetase over that already present in the uninfected cells.

(ii) **RNA synthesis.** To determine whether the increase in dCMP deaminase was associated with an elevated synthesis of messenger RNA (mRNA), rifampin, an inhibitor of transcriptional initiation, was employed. Figure 4 reveals that the addition of rifampin to a level of 50 μ g per ml 1.5 min after infection of cells grown to 8×10^8 per ml is ineffective in preventing the dilution increase in deaminase activity. That mRNA synthesis was impaired by rifampin is shown (Fig. 4) by the

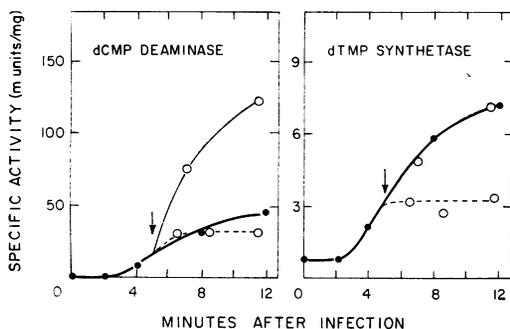


FIG. 3. Effect of hydroxylamine on the increase in deoxycytidylate (dCMP) deaminase and thymidylate (dTMP) synthetase following the dilution of T2-infected *E. coli*. One liter of *E. coli* B grown to 8×10^8 per ml was infected with T2r⁺ and 5 min later (↓) two 150-ml portions were diluted with separate 450-ml volumes of fresh medium, one containing a final hydroxylamine concentration of 0.5 mM (---). Samples were withdrawn at indicated times for enzyme assays. Symbols: ●, cells at 8×10^8 per ml; ○, cells at 2×10^8 per ml.

pulse-label incorporation of uracil-2-¹⁴C into RNA, which was found in several experiments to decrease rapidly until at 7 min after infection the label in the mRNA was less than 1% of that found in untreated controls. Despite the failure of uracil incorporation to increase after dilution of the culture into fresh medium containing rifampin at 7 min (Fig. 4), dCMP deaminase activity increased as usual, suggesting that new transcriptional initiations are not necessarily involved in promoting the enhancement of enzyme activity. In separate experiments, chloramphenicol was found to inhibit the increase in dCMP deaminase activity observed after dilution of rifampin-treated cells with essentially the same kinetics reported for the rifampin-free cells in Fig. 2. That this level of rifampin was effective in blocking the initiation of transcription was revealed by the finding that the inhibitor completely prevented the appearance of the phage-induced enzymes when added 30 sec after infection.

(iii) DNA synthesis. When hydroxyurea, an inhibitor of DNA synthesis, was added to a high-titer culture of *E. coli* B to a final concentration of 50 mM 5 min prior to infection, the subsequent T2r⁺-induced dCMP deaminase and dTMP synthetase activities in extracts from undiluted or fourfold diluted portions of culture were not greatly different from those found in identically treated hydroxyurea-free controls (Table 3). Although the drug had only a marginal effect on enzyme induction, pulse-label thymidine-2-¹⁴C incorporation into DNA was reduced to 30% of the control. As indicated in Table 3, hydroxyurea had no effect on RNA synthesis.

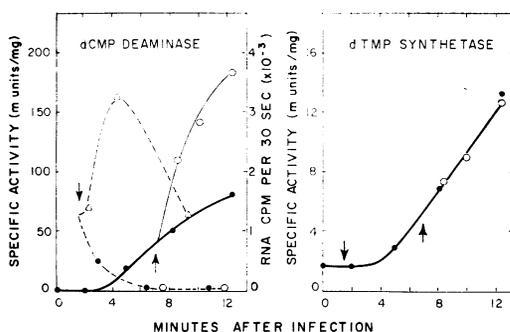


FIG. 4. Effect of rifampin on the increase in deoxycytidylate (dCMP) deaminase and thymidylate (dTMP) synthetase following the dilution of T2-infected *E. coli*. One liter of *E. coli* B at 8×10^8 per ml was infected, and 100 ml was removed to serve as a drug-free control for uracil-2-¹⁴C incorporation. At 1.5 min postinfection (↓), rifampin was added to the remaining 900 ml to give a final concentration of 50 μg/ml, and at 7 min postinfection (↑), 200 ml of this culture was diluted with 600 ml of fresh medium containing 50 μg/ml of rifampin. Samples were withdrawn for pulse-labeling with uracil-2-¹⁴C (---) and for enzyme assays (—) as described in the text. Symbols: ●, cells at 8×10^8 per ml plus rifampin; ○, cells at 2×10^8 per ml plus rifampin; △, cells at 8×10^8 per ml without rifampin.

Evidence that elevated dCMP deaminase activity is due to de novo synthesis. Although the inhibitor studies strongly suggested that the threefold increase in dCMP deaminase activity after the dilution of high-titer infected cells was due to newly synthesized protein, more direct proof would, of course, be preferred. The availability of antibodies to highly purified T2r⁺-induced deaminase (G. F. Maley, D. U. Guarino, and F. Maley, *J. Biol. Chem.*, *in press*) provided the means for obtaining this proof.

Deoxycytidylate deaminase was induced in undiluted and diluted portions of an *E. coli* B culture containing ³H-leucine. If the threefold increase in deaminase activity after dilution was due to de novo enzyme synthesis, the specific radioactivity of the protein precipitated from both high- and low-titer cells with the antibody should be exactly the same. However, if the increased activity on dilution is due not to an increase in enzyme protein but to a threefold increase in activity only, then the specific radioactivity of the protein precipitated from the diluted cultures should be one-third of that precipitated from the high-titer cells.

Preliminary experiments with the antibody method indicated that a one-step, three- to four-fold purification of the enzyme, which is described above, was necessary to enhance both rate and extent of the precipitin reaction. The titration of

TABLE 3. Relationship of DNA and RNA synthesis to T2r⁺ phage-induced deoxycytidylate (dCMP) deaminase and thymidylate (dTMP) synthetase following dilution of infected *E. coli* in the presence of hydroxyurea^a

Titer	Hydroxyurea	Enzyme activity 12 min postinfection (milliunits/mg)		DNA synthesis 10 min postinfection (counts/min/pulse)	RNA synthesis 10 min postinfection (counts/min/pulse)
		dCMP deaminase	dTMP synthetase		
8 × 10 ⁸	—	42	5.8	670	375
2 × 10 ⁸	—	142	5.6	2,900	1,650
8 × 10 ⁶	+	43	5.2	240	230
2 × 10 ⁶	+	110	4.6	980	1,500

^a Duplicate liter cultures of *E. coli* B at 8 × 10⁸ cells per ml, one of which received solid hydroxyurea to a concentration of 50 mM 5 min earlier, were infected with T2r⁺ phage. Five minutes postinfection, a portion of each was diluted fourfold into fresh medium with the hydroxyurea concentration maintained at 50 mM in the treated culture. Twelve minutes after infection, extracts were prepared, and enzymes were assayed as described in the text.

dCMP deaminase in a partially purified autolysate of infected cells is shown in Fig. 5. Complete precipitation of the deaminase activity was obtained when antibody was added at a concentration equal to or greater than 250 μg of protein per unit of deaminase activity. No additional protein was precipitated on the addition of greater amounts of antibody. By adding homogeneous unlabeled dCMP deaminase to the autolysates as carrier, the incubation period for precipitation could be shortened and the formation of a non-specific precipitate avoided (*see above*).

The results of an experiment in which pure dCMP deaminase was added as carrier to two autolysates, one prepared from an infected high-titer culture of *E. coli* B and the other from the same culture diluted fourfold 5 min after infection, are presented in Table 4. During infection and dilution, the cells were continuously labeled with ³H-leucine as described above. As shown in Table 4, the value obtained by dividing the total micrograms of protein recovered in an immune precipitate by the total deaminase units present initially in the corresponding autolysate was exactly the same for both the undiluted and diluted cultures. This value was within a few per cent of that obtained from a saline control in which only homogeneous dCMP deaminase and antibody were present, indicating that the principal material precipitated from the autolysate was deaminase. It is also shown that the specific radioactivity of the dCMP deaminase precipitated, expressed as counts per minute per unit of deaminase, was exactly the same for undiluted and diluted portions of the culture. The increased deaminase activity after dilution must be due, therefore, to *de novo* synthesis.

DISCUSSION

While normal levels of several early enzymes are induced by T2r⁺ bacteriophage in *E. coli* B

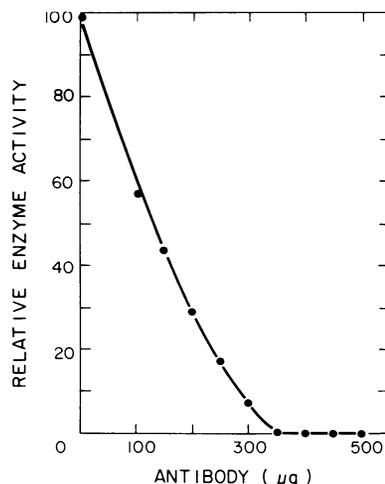


FIG. 5. Titration of T2r⁺-induced deoxycytidylate (dCMP) deaminase with a specific antibody. Each reaction consisted of 0.5 ml of autolysate prepared from a 10-min infected culture of *E. coli* B which contained 1.43 units of enzyme activity, antibody as indicated, and 0.9% saline to a final volume of 1.0 ml. The precipitates were collected by centrifugation, and the supernatant portion was assayed for residual deaminase activity as described in the text.

grown to 2 × 10⁹ cells per ml (9), this was not the case for the phage-induced dCMP deaminase reported here. As indicated, the specific activity of the deaminase (in contrast to the other enzymes in Table 1) was inversely related to the concentration of *E. coli* at which infection was initiated. Although no evidence was obtained for the presence of an activator, inhibitor, or repressor of dCMP deaminase, the reduced levels of enzyme activity in the high-titer cultures appeared to be associated with a host-mediated constraint imposed only after extended metabolism of the culture and not one involved immediately with

TABLE 4. Evidence relating the elevated deoxycytidylate (dCMP) deaminase activity following cell dilution to de novo enzyme synthesis with antibody to the T2r⁺ phage-induced dCMP deaminase^a

Cell titer	Enzyme source ^b (units)		Immune precipitates		Protein (μ g per unit of total deaminase)	Counts/min/unit of deaminase
	Autolysate	Plus carrier	Protein (μ g)	³ H (counts/min)		
8 \times 10 ⁸	2.1	8.7	362	13,700	41.5	6,500
2 \times 10 ⁸	3.4	9.3	372	20,900	40.2	6,200
Control	0	7.5	286	0	38.2	0

^a Two minutes after a 1-liter culture of *E. coli* B, at 8 \times 10⁸ cells per ml was infected, ³H-leucine was added to 0.01 mM (12 μ Ci/ μ mole). At 5 min postinfection, half of the culture was diluted fourfold into fresh medium containing the same amount of ³H-leucine. The dCMP deaminase in extracts prepared from the control and diluted portions of the culture at 10 min postinfection was 65 and 160 milliunits per mg of protein, respectively. Autolysates were prepared from the extracts as described in the text.

^b To 2.0 ml of the high- and low-titer autolysates, 6.6 and 5.9 units of homogeneous dCMP deaminase, respectively, were added; 7.5 units of pure enzyme were added to a 0.9% saline control. Antibody (300 μ g per unit of deaminase) was then added, the volume was adjusted to 2.6 ml with 0.9% saline, and the precipitates were isolated.

cell density. Evidence favoring this proposal was derived from the finding that *E. coli* B, concentrated by ultrafiltration from 2 \times 10⁸ to 8 \times 10⁸ cells per ml prior to infection, supported the induction of dCMP deaminase levels associated with the lower cell titer, even after a 40-min incubation period following concentration. During this incubation period, the culture density increased from 8 \times 10⁸ to about 1.3 \times 10⁹ cells per ml. It would thus appear that artificial concentration of cells does not reproduce the same metabolic conditions associated with the normal growth cycle. Although the inductive effect in cells grown normally to high titers (8 \times 10⁸ cells/ml) could be altered by the degree of aeration, aeration alone was not responsible for the regulation of the deaminase level since five other phage-induced enzymes were unaffected (Table 1).

The increase in pulse-label incorporation of precursors into protein, RNA, and DNA which followed the fourfold dilution of uninfected *E. coli* B from 8 \times 10⁸ to 2 \times 10⁸ cells per ml (Fig. 1) was expected, since the cells were shifted from the phase of negative acceleration back to the logarithmic growth phase. Because the uptake of these precursors also increased following dilution of high-titer infected cells, no quantitative conclusions relating phage macromolecule synthesis to changes in dCMP deaminase activity could be made. Specific inhibitors were used, therefore, in an attempt to delineate the macromolecular events associated with the increase in deaminase activity following dilution.

The immediate block of the increase in dCMP deaminase by chloramphenicol following dilution of high-titer infected cells (Fig. 2) established that protein synthesis was involved. This finding ruled out the possibility that the change in deaminase

activity resulted from a rearrangement or a simple activation of existing dormant molecules or their subunits. To verify the requirement for protein synthesis, hydroxylamine, recently shown to be a specific inhibitor of peptide chain initiation (7, 8, 17), was used. As the results in Fig. 3 reveal, initiation of new peptide chains was essential for the increase in T2r⁺-induced dCMP deaminase activity. Since hydroxylamine does not inhibit peptide chain elongation (8), some increase in deaminase activity representing the completion of chains already initiated was expected. Consistent with this proposal was the finding that deaminase activity increased for about 1 to 1.5 min following dilution into medium containing hydroxylamine (Fig. 3).

While protein synthesis was necessary for the increase in deaminase activity (Fig. 2, 3), this appeared not to be the case for RNA synthesis (Fig. 4). At a time when labeled uracil incorporation in the presence of rifampin was less than 1% of the control, the inhibited cells still contained sufficient information to elicit a 2.5-fold increase in deaminase activity on cell dilution. Chloramphenicol prevented the rise in deaminase activity, which indicated that protein synthesis is required for the observed increase in the rifampin-treated cells.

Hydroxyurea reduced the thymidine-2-¹⁴C incorporated on phage infection by 70% but had no effect on the levels of T2r⁺-induced dCMP deaminase (Table 3), suggesting that a relationship between phage-induced DNA synthesis and the increase in deaminase activity probably does not exist. Although hydroxyurea at the concentration employed was shown to inhibit completely a net increase in the DNA content of infected cells, competent phage could still be made as a result of

the degradation and reutilization of the host cell DNA (23). Thus, even at hydroxyurea concentrations that almost completely impair the conversion of ribonucleotides to deoxyribonucleotides, as much as 20 to 30% of the normal phage yield may be synthesized from host DNA (23). The 30% incorporation of thymidine-2-¹⁴C in the presence of hydroxyurea (Table 3) was therefore not unexpected.

The most convincing evidence for the association of de novo enzyme synthesis with the increase in deaminase activity following dilution was provided by the antibody precipitation experiments (Table 4). It is reasonable to assume from the data in the last column in Table 4 that, since the specific radioactivity of the enzyme precipitated from high- and low-density cultures is the same, despite the presence of three times more deaminase activity in the latter culture, three times more enzyme protein must have been synthesized in the low-density cultures. As indicated, the T2r⁺-induced dCMP deaminase molecules synthesized in control and diluted portions of a culture of *E. coli* B have a specific radioactivity of 6,200 to 6,500 counts per min per unit, which is 83 to 87% of theory (7,450 counts per min per unit). The theoretical value is based on the presence of 72 moles of leucine per mole of pure deaminase (G. F. Maley, D. U. Guarino, and F. Maley, *J. Biol. Chem.*, *in press*). Considering that the ³H-leucine was added 2 min postinfection, the results are well within the experimental limit of the assay.

From the data presented, the intracellular location of the dilution effect is probably at the translational level of protein synthesis. For some as yet unknown reason, dilution of the high-titer cells relaxes a highly specific constraint on protein synthesis, and an increased amount of dCMP deaminase is made. In an effort to delineate the translational step involved in the modulation of deaminase activity, cell-free dCMP deaminase synthesis with T2 phage DNA and mRNA as templates is being studied. Preliminary experiments have shown that, when primed with T2r⁺ DNA, cell-free systems prepared from *E. coli* B grown to 8×10^8 cells per ml synthesize about one-half the protein but only one-eighth the dCMP deaminase made by a similar system prepared from cells grown to 2×10^8 per ml. Mixing experiments using ribosomes and 105,000 \times g supernatant fractions from *E. coli* B grown to 2×10^8 and 8×10^8 cells per ml, respectively, revealed that the cause for the decreased protein and deaminase synthesis resides in the S-100 and not the ribosome fraction.

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