On the Metabolic Role of T6 Phage-induced Dihydrofolate Reductase

INTRACELLULAR REDUCED PYRIDINE NUCLEOTIDES*

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SUMMARY

Infection of Escherichia coli with T-even bacteriophage or T5 leads to the production of large amounts of dihydrofolate reductase. As shown previously, this represents the synthesis of a new, virus-specific protein. Since appreciable levels of this enzyme exist in the cell before infection, an attempt was made to demonstrate a specific metabolic requirement for the viral enzyme. This included (a) a study of the structure of tetrahydrofolate produced enzymatically by both the bacterial and the viral reductase and (b) measurements of levels of reduced pyridine nucleotides in the cell before and after infection. Both the cellular and the phage-specific reductase catalyze the formation of the L-diastereoisomer of tetrahydrofolate, as does the same enzyme isolated from chicken liver. Experiments on pyridine nucleotide concentrations reveal a 2- to 3-fold increase in the reduced forms of both di- and triphosphopyridine nucleotide occurring within about 10 min after infection, followed by a return to preinfection values. These observations are discussed with reference to the reductive biosynthetic steps in the production of deoxycytidylate.

Infection of Escherichia coli with T-even bacteriophages leads to a considerable increase in the rate of deoxycytidylate synthesis (1). To a certain extent this is made possible by the formation in the infected cell of several enzymes which are involved in the production of phage DNA precursors (reviewed in Reference 2). Such enzymes fall into two groups: (a) those which are required for synthesis of the unique viral pyrimidine, 5-hydroxymethylcytosine, and (b) those which are involved in the increased rate of production of “normal” DNA components, notably thymidylate. The first group includes enzymes, such as deoxycytidylate triphosphatase (3) and deoxycytidylate hydroxymethylase (4), which are not found in uninfected cells, while the second group contains enzymes, notably thymidylate synthetase (5) and dihydrofolate reductase (6), which exist in uninfected cells but in which activity is augmented after infection by the formation of virus-specific enzymes.

The activity of thymidylate synthetase, as determined in cell-free extracts, rises some 6-fold after infection of E. coli strain B with T2, T4, or T6 (5). However, mutants of T4 which are unable to initiate the formation of the phage-specific synthetase (7) can utilize the corresponding bacterial enzyme for thymidylate formation. Such cultures can form DNA at about 70% of the rate of cultures infected with wild type T4 (8). Therefore, the synthetase is formed in infected cells in considerable excess over its quantitative requirement for support of DNA synthesis. This raises a question about the metabolic role of the phage-induced dihydrofolate reductase (6, 9), since the activity of the latter enzyme, as determined in extracts of T6-infected E. coli B, is about 10-fold higher than that of the synthetase. Since the methylation of dUMP to form dTMP is the only well known metabolic reaction in which tetrahydrofolate is consumed, by means of oxidation to dihydrofolate (10), it would appear that the virus-specific reductase is almost entirely superfluous; one might expect that the corresponding bacterial reductase could fulfill the quantitative need for tetrahydrofolate production.

There are at least two possible reasons why a phage-specific dihydrofolate reductase might be required in a qualitative sense. (a) A different isomer of tetrahydrofolate might be required for functioning of dCMP hydroxymethylase and phage-induced dTMP synthetase than that utilized by bacterial tetrahydrofolate-requiring enzymes. Dihydrofolate reductase of chicken liver produces tetrahydrofolate having only one of the two possible configurations with respect to carbon 6 of the molecule (11), and it is conceivable that the E. coli and the T6 enzymes could form different stereoisomers. (b) A change in the intracellular concentration of reduced pyridine nucleotides might necessitate the formation of an enzyme with altered properties. Cohen (12), on the basis of experiments with C1-labeled glucose, concluded that the activity of the pentose shunt pathway of glucose utilization is decreased following phage infection. A possible result could be a decrease in the intracellular concentration of reduced triphosphopyridine nucleotide. This is of interest in light of the specificities of the E. coli and the T6 reductases for pyridine nucleotides. At neutral pH the bacterial enzyme is absolutely specific for TPNH, while the viral enzyme can utilize DPNH, although it is a poorer substrate than TPNH (9).

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This paper describes experiments designed to test both of the above suggestions.

EXPERIMENTAL PROCEDURE

Biological Systems—All experiments were carried out with *E. coli* B and T6 bacteriophage. The phage strain, which does not require tryptophan as an adsorption cofactor (13), was obtained from Dr. L. M. Kozloff. M9 medium, an inorganic salts-glucose medium (14), was used throughout for growth of bacteria.

Chemicals—Dihydrofolate and dl-tetrahydrofolate were prepared as described previously (9). Tetrahydrofolate was prepared by enzymatic reduction of dihydrofolate, essentially as described by Mathews and Huennekens (11), except that the source of dihydrofolate reductase was either phage-infected *E. coli* or uninfected bacteria. The enzyme was prepared according to Mathews and Sutherland (9) through the ammonium sulfate fractionation step. Chemically and enzymatically prepared tetrahydrofolates were purified by DEAE-cellulose chromatography as described previously (11).

Pyridine nucleotides and enzyme substrates were obtained from Sigma. Commercial enzyme preparations were purchased from Boehringer. Fluorodeoxyuridine was a gift of Dr. Robert Duschinsky of Hoffmann-La Roche.

Extraction of Pyridine Nucleotides—Pyridine nucleotide extractions were carried out according to an unpublished procedure supplied by Dr. Walter Hempfling of the University of Pennsylvania. Unless otherwise indicated, bacteria were grown to a turbidity of 80, as determined with the Klett colorimeter (No. 420 filter), corresponding to a viable count of about 2.5 \times 10^8 ml^{-1}. Samples of 100 ml were removed at various times after phage infection (approximately 4 phages per bacterium), chilled in ice, and centrifuged at 0–4°C. The precipitated cells were drained thoroughly and resuspended in 2.0 ml each of distilled water. For extraction of oxidized pyridine nucleotides, 1.0 ml of 15% perchloric acid was added to each suspension. After 10 min at room temperature the mixtures were chilled in ice and centrifuged. From each supernatant solution 2.0 ml were withdrawn and neutralized with 0.5 ml of 1 M triethanolamine-HCl buffer, pH 7.4, and sufficient 1 M KOH to bring the pH of the mixture to 7.4. The volume of each mixture was adjusted to 4.0 ml, and the mixtures were chilled for 30 min and centrifuged. The clear supernatant solutions were used for assay of DPN and TPN.

For extraction of reduced pyridine nucleotides, to each 2.0-ml suspension of bacteria in water was added 1.0 ml of an ethanol-10% KOH mixture (0.1 v/v). The mixtures were heated at 50°C for 10 min, then cooled in ice. Neutralization to about pH 8 was accomplished by addition of 1.0 ml of 2 M triethanolamine-HCl buffer, pH 5.5. The tubes were chilled for at least 30 min more, then centrifuged in the cold at 40,000 \times g for 15 min. The clear supernatant fluid was assayed for DPNH and TPNH.

Pyridine Nucleotide Assays—Estimation of the pyridine nucleotide content of extracts was carried out by the methods of Estabrook and Maitra (15), with one modification. The determinations involve measurement of the fluorescence of an extract before and after treatment with a specific enzyme that reduces DPN and TPN, or that oxidizes DPNH or TPNH. Estabrook and Maitra assayed both reduced pyridine nucleotides in the same extract by treatment first with acetaldehyde and alcohol dehydrogenase, which oxidizes DPNH, followed by α-ketoglutarate, ammonia, and glutamate dehydrogenase, an enzymatic system that oxidizes TPNH. The fluorescence change is noted after each addition of enzyme. Because of the presence in my extracts of ethanol, which unfavorably affects the alcohol dehydrogenase equilibrium, I added, first, oxidized glutathione and glutathione reductase, to determine TPNH concentration, followed by the components of the glutamate dehydrogenase reaction, to measure DPNH.

Fluorometric measurements were carried out with a Turner fluorometer, model 111, attached to a 1-ma recorder (Texas Instruments). Incident light was filtered with a Corning No. 7-50 filter, which allows maximal transmission at 360 μm. The secondary filter was a combination of a Wratten 2A filter and a Corning 5-61 filter. This combination transmits light only above 415 μm, with peak transmission at 440 μm. Most assays were carried out with concentrations of pyridine nucleotide in the reaction cuvette in the range of 10^{-7} to 10^{-8} M. Within this range, as determined with standards, fluorescence change is directly proportional to pyridine nucleotide concentration. Standards were run with each assay. Generally, an assay mixture containing 10^{-7} M pyridine nucleotide would show a fluorescence change of about 40 units on the arbitrary 0 to 100 scale of the fluorometer, after the addition of sufficient enzyme to drive the reaction rapidly to completion.

Recovery of pyridine nucleotides was determined by adding known amounts to bacterial suspensions before extraction and assay. Recovery of oxidized pyridine nucleotides was 80 to 85% and that of reduced pyridine nucleotides was 85 to 90%. Assay values for unknown samples were corrected accordingly.

RESULTS

Comparison of Enzymatically Prepared Tetrahydrofolates—If the bacterial and the T6-induced dihydrofolate reductases lead to formation of different isomers of tetrahydrofolate, then it should be possible to distinguish the two forms on the basis of different reactivity in tetrahydrofolate-requiring enzyme systems. For example, if the bacterial enzyme makes the L,L diastereoisomer and the T6 enzyme makes the d,L form, the L,L form might be fully active with *E. coli* thymidylate synthetase, and the d,L form completely inactive. Furthermore, the L,L isomer would be twice as active as the chemically prepared dl mixture (11). On the other hand, if the two enzymatically synthesized products have the same structure, each would be twice as active as the chemically prepared material. In order for the tests to be conclusive, they must be run below the saturating concentration for tetrahydrofolate, so that enzyme reaction rate is proportional to the concentration of the active tetrahydrofolate isomer.

Tetrahydrofolate was prepared by chemical reduction of dihydrofolate and by enzymatic reduction with the reductases from T6-infected and from uninfected *E. coli*. The three preparations were tested for their reactivity in three different enzyme systems: thymidylate synthetase from uninfected *E. coli* and from T6-infected cells, and serine hydroxymethylase from chicken liver. In each case the product of enzymatic reduction, whether prepared with the bacterial or the viral enzyme, was twice as active as the chemically prepared material. Therefore, both of the enzymatically prepared tetrahydrofolates have the same structure. Since these products have the same reactivity...
with an enzyme from chicken liver as does the L,L-tetrahydrofolate produced by chicken liver dihydrofolate reductase (11), it is concluded that both the E. coli and the T6 reductases lead also to formation of the L,L diastereoisomer.

**Pyridine Nucleotide Levels**—At the outset of this study it had been expected that the intracellular TPNH content would decrease after phage infection, since the activity of the pentose shunt pathway is depressed in infected cells (12). The analyses, however, showed that the levels of both TPNH and DPNH increase after infection, rising to maximal values at about 10 to 15 min, and decreasing thereafter to preinfection levels.

Results of a typical experiment are shown in Fig. 1A. In most experiments the rise in reduced pyridine nucleotide took place after a lag of 3 to 5 min, and the extent of increase was 2- to 3-fold.

It was possible that the pyridine nucleotide concentrations were altered during the chilling, centrifugation, and resuspension of the cells. For this reason, it appeared desirable to perform an experiment in which cells are removed directly from a culture and extracted immediately. However, extracts prepared without prior concentration of the cells had such a high back-ground fluorescence relative to the small amount due to reduced pyridine nucleotide that it was impossible to carry out satisfactory determinations. This difficulty was partially circumvented as follows. Cells were concentrated 4-fold by centrifugation immediately before infection. Samples of 2.0 ml each were removed directly to the ethanol-KOH mixture, and extracts were prepared in the usual manner. These extracts were assayed with the components of the glutamate dehydrogenase system only, to give a measure of total reduced pyridine nucleotide. Results of this experiment are in accord with those presented in Fig. 1. Reduced pyridine nucleotide concentration rises after a short lag, reaching a maximal value within 10 min after infection. The level then declines, reaching the preinfection value within 20 min.

Concomitant experiments were carried out to determine the levels of oxidized pyridine nucleotides. Fig. 1B shows that the DPN concentration changes in a fashion essentially complementary to the change in DPNH level; i.e., it decreases at first and finally increases to the initial value. Curiously, it was impossible to detect TPN in these extracts. The assay for TPN involved treatment of the extract with glucose 6-phosphate and glucose 6-phosphate dehydrogenase. The extracts did not contain an inhibitor of the enzyme, since TPN added to an extract was rapidly reduced. Moreover, as mentioned earlier, when TPN was added to a cell suspension just before extraction, about 85% was recovered. The failure to detect TPN by this method is puzzling; it may be related to the possible existence of an acid-labile form of TPN (16). Alternate methods of extraction have not yet been attempted.

The kinetics of the changes in reduced pyridine nucleotide concentration is similar to that observed by Puck and Lee (17) for changes in bacterial permeability following infection of E. coli by T-even phage. These workers found that there was an initial increase in cellular permeability, as determined by leakage of material from cells, followed by a "sealing" reaction which rendered the cells less permeable than they had been before infection. It appeared, therefore, that the changes in reduced pyridine nucleotide content and permeability might be causally related. Consistent with this hypothesis is the fact that the terminal oxidative system of bacteria is located near the cell surface. Moreover, the rate of oxygen uptake in E. coli decreases slightly after infection by T-even phage (18, 19). Thus, it appeared possible that some aspect of the interaction of the phage tail with the bacterial cell surface might result both in a change in the permeability of the cell membrane and in some perturbation of the terminal electron transport system. The latter phenomenon would cause a transient anaerobiosis, resulting in the observed rise in reduced pyridine nucleotide level.

If the above hypothesis is true, then the characteristics of the cyclic permeability change should be similar to those of the change in reduced pyridine nucleotide content. Puck and Lee found, for example, that the extent of leakage increases with increasing multiplicity of infection up to a value of 30. However, the rise in reduced pyridine nucleotide takes place only at relatively low multiplicities. The effect is maximal between multiplicities of 3 and 6, with no discernible effect above a multiplicity of 10.

Another comparison with the leakage phenomenon was made by studying the effect of a synchronized infection. Puck and Lee found that, since the increase in permeability and the sealing reaction are brought about by different factors, the extent of leakage was raised by initiating all infections simultaneously. Since the sealing reaction requires energy, the extent of leakage is greatly increased by carrying out infection in the cold or in the absence of a carbon source. Fig. 2 illustrates the effect of synchronization achieved by carrying out the infection in the absence of glucose. Cells were grown as described above, centrifuged, washed twice, and resuspended in M9 medium minus glucose. The cells were incubated for 10 min and T6 was added at a multiplicity of 4. They were incubated 10 min longer, and glucose was then added at time zero (Fig. 2). Except for the first minute, when normal levels of reduced pyridine nucleotide were reached as a result of glucose addition, the overall kinetics and extent of change were the same as those observed in the random infection illustrated in Fig. 1. Interestingly, the lag phase is still observed. However, it is apparent from
this experiment and from the studies of varying multiplicities that there is no direct relationship between the cyclic permeability change and the cyclic change in reduced pyridine nucleotide concentration.

It was of interest to determine whether the observed rise in reduced pyridine nucleotide level is a specific consequence of phage infection or whether it can be evoked by other agents which are capable of interacting with bacterial surface components. Fig. 3 shows that low concentrations of either EDTA (2 x 10^{-4} M) or calcium ions (5 x 10^{-4} M) are capable of causing a rise in reduced pyridine nucleotide. Although the mechanism of these effects is not known, these experiments do suggest that the phenomenon described in this paper is not a specific result of infection. However, they underline the need for further information on the factors controlling relative concentrations of oxidized and reduced pyridine nucleotides in bacteria.

In the experiments of Fig. 3, no decrease in reduced pyridine nucleotide content occurred 15 to 20 min after treatment, as was consistently observed with phage infection. This suggests that some aspect of the metabolism of the infected cell is responsible for consumption of reduced pyridine nucleotides.

The reductive biosyntheses associated with the formation of thymidylate and the reduction of ribonucleotides to deoxyribonucleotides may well be responsible for this. This idea was given a partial test by following DPNH and TPNH levels in T6-infected cells in the presence of fluorodeoxyuridine. Under these conditions the formation of thymidylate is blocked (20). Presumably, tetrahydrofolate would accumulate, and dihydrofolate reductase would not be as active in vivo as in normal infection. Results of this experiment are shown in Fig. 4. The level of DPNH rose and subsequently dropped, as in normal infection. However, TPNH concentration rose continuously during the entire period examined. This experiment indicates that different mechanisms are responsible for oxidation of DPNH and TPNH in normal infection. Moreover, it suggests that TPNH is the physiological reductant for dihydrofolate in vivo.

**DISCUSSION**

This paper indicates that a 2- to 3-fold increase in concentration of DPNH and TPNH takes place after infection of E. coli by phage T6. The mechanism responsible for this is not known, nor is it yet known whether it is an obligatory feature of the infection process. The phenomenon reported here is somewhat reminiscent of Epel's demonstration (21) of an increase in TPNH levels occurring after fertilization of sea urchin eggs. However, the effect in the eggs appears to be more specific, since the concentration of DPNH does not change appreciably. In addition, the changes are more rapid in the urchin eggs, with maximal levels of TPNH being attained 2 min after fertilization.

The observations reported here do not appear to pertain directly to the finding of Amelunxen and Grisolia (22) that DPNH oxidase activity in E. coli increases after infection with T2. These workers assayed whole cell suspensions for activity of this enzyme. The increase presumably reflected the increase in permeability of the cells, which allowed the externally added DPNH ready access to the intracellular enzyme. Results of the present studies suggest that the activity of DPNH oxidase in vivo is somewhat reduced after infection.

Little work has been reported on pyridine nucleotide levels in
bacteria, and it is of interest to compare the data of the present study with those of experiments on animal systems. Pyridine nucleotide concentrations in log phase bacteria — just before infection — have been computed as micromoles per g of packed cells. These are: DPN, 0.49; DPNH, 0.10; and TPNH, 0.06. The corresponding figures for adult male rat liver (23) are 0.73 for DPN, 0.04 for DPNH, 0.36 for TPN, and 0.38 for TPNH.

As mentioned above, TPN could not be detected in E. coli extracts in this study. This is probably not due to the existence of extremely low levels of TPN, since Hempfling has found that E. coli contains about 15 to 20% as much TPN and DPN, and this amount would certainly be detectable.

The experiments described in this paper do not indicate that there is an absolute metabolic requirement for phage-induced dihydrofolate reductase, and the precise role of the enzyme remains unclear. The need for this enzyme is probably quantitative rather than qualitative; i.e., its presence allows an increased rate of DNA synthesis and phage growth, but these could proceed to a considerable extent in its absence, with the bacterial reductase being utilized. A similar situation has been noted for phage T4-specific thymidylate synthetase (8). The latter studies were carried out with a mutant of T4 which could not initiate the formation of thymidylate synthetase. It would be desirable to carry out similar studies with a dihydrofolate reductaseless phage mutant, but it is difficult to see how one could select for such a mutant or screen for it in large populations of phage particles.

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1 Personal communication.