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Growth of a Dihydrofolate Reductaseless Mutant of Bacteriophage T4

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A mutant of bacteriophage T4 was isolated which was unable to induce virus-specific dihydrofolate reductase in infected cells. The mutant was able to form several other early enzymes of pyrimidine metabolism. Growth of the mutant in a wild-type host, *Escherichia coli* B, was compared with that of the parent strain, T4BO$_1$, and T4*td*8, a mutant which lacks the ability to induce thymidylate synthetase. Growth studies were carried out in minimal medium, which gave higher growth rates and phage yields than the supplemented media used in previous studies. The reductase mutant formed deoxyribonucleic acid and plaque-forming particles at a rate slightly higher than the synthetase mutant but 1.5- to 2-fold lower than that of the wild-type phage under all conditions studied. The addition of thymine to a culture infected by the mutant increased the growth rate significantly, suggesting that the genetic lesion leads to a partial thymidylate deficiency. Like other viral genes controlling steps in thymidylate metabolism, the dihydrofolate reductase gene appears to be useful but not completely essential for growth.

Infection of *Escherichia coli* with T-even bacteriophages results in the formation of several virus-specific enzymes of pyrimidine metabolism. Some of these, including deoxycytidylate hydroxymethylase (4) and a deoxynucleotide kinase which can act upon 5-hydroxymethyldeoxycytidylate (2), are involved in the biosynthesis of the unique phage deoxyribonucleic acid (DNA) component, 5-hydroxymethylcytosine (23), and, as such, do not occur in uninfected cells. Another class of phage-specific enzymes acts to supplement pre-existing pathways for the synthesis of thymine nucleotides. This class includes deoxycytidine monophosphate (dCMP) deaminase, an enzyme undetectable in uninfected bacteria (10), and deoxythymidine monophosphate (dTMP) synthetase (5) and dihydrofolate (DHF) reductase (15), two enzymes which duplicate pre-existing activities of the growing cell. Evidence has been presented that the latter two enzymes can be distinguished, on the basis of physical and catalytic properties, from the corresponding enzymes of the host cell (7, 14, 16, 17).

An increasing body of evidence, from various biological systems, suggests that the enzymes of thymine metabolism play an important, if indirect, role in the regulation of DNA synthesis. In this context, it is of interest to know what roles the phage-specific enzymes of thymine metabolism play in maintaining the greatly increased rate of DNA synthesis which is observed in infected cells relative to uninfected cells. These questions can be most easily approached if one can obtain phage mutants which are unable to form the enzymes of interest in infected cells. In 1963 Simon and Tessman (19) isolated "thymidine-requiring" mutants of phage T4, and Shapiro, Eigner, and Greenberg (18) showed that these mutants were unable to form thymidylate synthetase. Growth studies carried out in this laboratory with one of the mutants of Simon and Tessman (11, 19) showed that under certain conditions the absence of phage-specific thymidylate synthetase in infected *E. coli* B led to a two- to threefold reduction in burst size. Thus, this enzyme appeared to be useful but not absolutely essential for phage growth, since the phage could utilize the bacterial synthetase to a considerable extent. Similarly, Hall and Tessman (8) showed that dCMP deaminaseless mutants of T4 can grow reasonably well on *E. coli* B, suggesting that, in the absence of the deaminase, deoxyuridine monophosphate (dUMP) can be supplied via other pathways, principally the reduction of uridine nucleotides (1). Figure 1 illustrates some of the reactions of interest. Enzymes whose activity appears or is augmented

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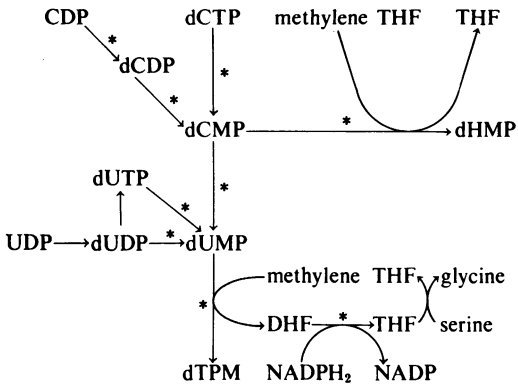

FIG. 1. *Enzyme reactions in thymidylate metabolism. Abbreviations: CDP, cytidine 5'-diphosphate; dCDP, deoxycytidine diphosphate; dCTP, deoxycytidine 5'-triphosphate; dCMP, deoxycytidine 5'-phosphate; THF, tetrahydrofolate; dHMP, 5-hydroxymethyl-deoxycytidylate; UDP, uridine diphosphate; dUDP, deoxyuridine diphosphate; dUTP, deoxyuridine triphosphate; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate; DHF, dihydrofolate; NADP, nicotinamide adenine dinucleotide phosphate; NADPH2, reduced NADP.*

following T-even phage infection are indicated with an asterisk.

Recently, Hall, Tessman, and Karlström (9) devised an ingenious plaque assay for the recognition of phage mutants with defects in pyrimidine metabolism. The assay utilizes a plating host which is auxotrophic for pyrimidines and which lacks deoxycytidine deaminase, and growth conditions which probably result in a deoxyuridine deficiency in uninfected cells. Under these conditions, thymidylate synthetase (*td*) mutations can be distinguished by a white halo surrounding the plaque. This is probably caused by more extensive bacterial growth resulting from excretion of deoxyuridine compounds from mutant-infected cells. A new mutation, *wh*, was recognized by Hall et al. on the basis of formation of a plaque which had a thicker and whiter halo than that formed by *td* mutants. These authors suggested that *wh* mutants might be deficient in their ability to induce DHF reductase.

By use of the techniques of Hall et al., a *wh* mutant of T4 was isolated in this laboratory, and its inability to induce DHF reductase was demonstrated (14). The present paper describes studies on phage growth and DNA synthesis in cells infected by this mutant.

MATERIALS AND METHODS

E. coli B was used as host bacterium for all of the growth experiments. *E. coli* B305, supplied by P. Reichard, was used as plating bacterium for isolation of the *wh* mutant. This is a uracil-requiring, deoxycytidine deaminaseless strain which is presumably the same as strain OK305, used by Hall et al. (9).

$T4BO_1$, a wild-type T4 strain, and T4*td*8, a thymidylate synthetaseless mutant, were kindly supplied by I. Tessman. T4r118, an rII mutant from the collection of Benzer, was obtained from R. Wilhelm. A *wh* mutant was isolated exactly as described by Hall et al. (9). The mutant was back-crossed four times against $T4BO_1$, to eliminate extraneous mutations. The strain is called T4*wh*2, to distinguish it from T4*wh*1, the designation applied by Hall et al. to their purified *wh* strain.

All growth experiments were carried out with the minimal salts-glucose medium M9, for reasons described below. Phage platings in the growth experiments were carried out on nutrient agar plates.

Assays for dCMP hydroxymethylase, dTMP synthetase, and DHF reductase were carried out as previously described (12). dCMP deaminase was assayed by the method of Warner and Lewis (22).

Phage-specific DNA synthesis was followed by incorporation of uracil-2-^{14}C, as previously described (12).

RESULTS

Choice of medium for growth studies. Earlier studies form this laboratory on growth of a T4 thymidylate synthetaseless mutant had employed a glycerol-Casamino Acids medium (11). This medium, although chemically defined, allows rapid growth of *E. coli*, and it was felt that phage multiplication would also be quite extensive in this medium, following Stent's statement (20) that "generally the virus grows more abundantly when the nutrition of its host cell is optimal: what is good for the cell is good for the virus. . ." However, the experience of this laboratory has been that T-even phages and T5 multiply more rapidly, and reach higher final titers, in minimal media than in the enriched glycerol-Casamino Acids medium or in complete media, such as broth. This appears to be related to the phenomenon of lysis inhibition (3), which is more extensive in minimal medium than in an enriched medium. Figure 2 (upper half) compares the growth of $T4BO_1$ in M9 and nutrient broth. Samples were assayed for plaque-forming ability with and without prior chloroform lysis. In the broth culture, lysis was complete by 80 min, whereas in M9 most of the cells had not lysed by 100 min and the total phage concentration was still increasing. The phage yield at 100 min in M9 [1,214 plaque-forming units (PFU) per cell] was nearly five times the total phage yield in broth (263 PFU per cell). This effect of medium composition on lysis inhibition is further illustrated by comparable growth experiments with an rII mutant of T4 (lower half of Fig. 2). With this

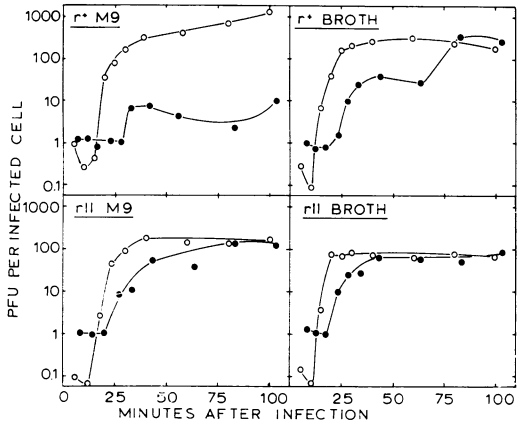

FIG. 2. *Growth of T4r+ and a T4rII mutant in M9 medium and nutrient broth. Bacteria were grown in the indicated media to a concentration of about 2.5 × 10⁸/ml and diluted to about 10⁸/ml immediately prior to infection. In the experiments with M9, 20 μg of L-tryptophan per ml was added just before infection. Phage was added at a multiplicity of 4. Samples were removed at the indicated times and plated either with (○) or without (●) prior chloroform lysis.*

mutant, which does not exhibit lysis inhibition, the extent of difference between the burst sizes in M9 and broth was less than twofold (163 in M9 as opposed to 86 in broth). The reason for this extended lysis inhibition in minimal medium is not known. However, since differences in growth rates of mutant phage as compared to wild-type would presumably be most apparent under conditions giving high growth rates and phage yields, it was decided to use a minimal medium for these studies. In addition, knowledge of the growth properties of T4 in different media is useful in the preparation of high-titer virus stocks.

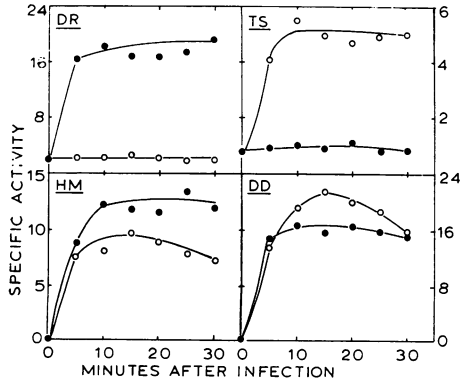

FIG. 3. *Early enzyme synthesis in cells infected with T4td8 (●) or T4wh2 (○). DR, dihydrofolate reductase; TS, thymidylate synthetase; HM, deoxycytidylate hydroxymethylase; DD, deoxycytidylate deaminase. Specific activities are in millimicromoles per minute per milligram of protein.*

Early enzyme synthesis. Figure 3 confirms the suggestion of Hall et al. (9) that the *wh* mutation confers an inability to induce the formation of DHF reductase. However, the *wh*-infected cells can synthesize thymidylate synthetase and deoxycytidylate deaminase. This suggests that the *wh* gene is a structural gene for DHF reductase and not a regulatory gene, for a phage mutated in a regulatory gene affecting DHF reductase might well be expected to show abnormalities in the syntheses of these two closely related enzymes (9).

Growth of T4wh2. Earlier experiments from this laboratory on growth of the dTMP synthetase mutant T4*td*8 showed that significant differences in growth rates between the mutant and the parent strain were observed only in dilute cultures, of the order of 10^4 cells/ml. Therefore, similar experiments were carried out with *wh*2 (Fig. 4). T4*wh*2 grows somewhat more rapidly than *td*8, but not as well as the wild-type strain. In the two experiments whose data are combined in Fig. 4, the average final yields were: wild type, 190; *td*8, 89; *wh*2, 121.

The existence of phage-induced DHF reductase was predicted from the expected need of infected cells to increase the rate at which tetrahydrofolate (THF) was formed for continued synthesis of thymidylate (15). One would expect, therefore, that the absence of the phage reductase would lead to a partial thymidylate deficiency and that the presence of thymine in the culture

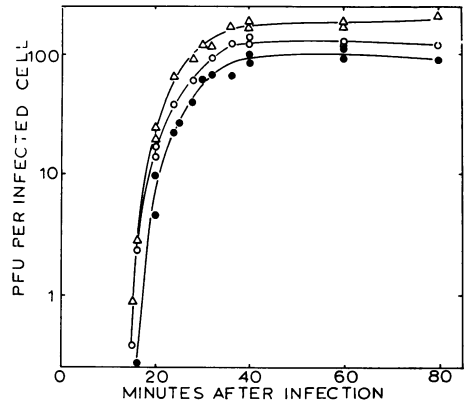

FIG. 4. *Growth of T4BO₁ (△), T4td8 (●), and T4wh2 (○) on Escherichia coli B at low cell density. Infection was carried out at a multiplicity of 4 and a cell concentration of about 10⁸/ml in the presence of 20 μg of L-tryptophan per ml; 3 min after infection, each culture was diluted 10⁴-fold into M9 containing no tryptophan. Samples were removed at the indicated times, lysed with chloroform, and plated.*

would increase the growth rate. This prediction is verified by the data of Fig. 5. These data are plotted linearly, to illustrate the magnitude of the difference between final phage yields for the wild type and *wh*2. Although phage yields were considerably higher in this experiment than in that of Fig. 4, probably owing to delayed cell lysis, the same relative difference was seen between *wh*2 and the wild type. As earlier observed for *td*8 (11), the addition of thymine to a *wh*2-infected culture stimulates the growth rate to a value almost, but not completely, as high as that given by wild-type T4.

In the earlier study from this laboratory on the growth of *td* mutants, it was found that at high cell concentrations (ca. 10^8 per ml) the growth rate of *td*8 on *E. coli* B was not significantly different from that of wild-type T4 (11). This was ascribed to a "cross-feeding" effect, in which excess thymine compounds from the earliest-lysing cells were incorporated into other cells which were still producing phage. However, under the conditions of more extensive phage growth used in the present study, an approximately twofold difference was seen between $T4BO_1$ and T4*td*8, both with respect to rate of DNA synthesis and formation of infectious particles (Fig. 6). As was observed in the growth experiments at low cell density, T4*wh* multiplied and formed DNA slightly more rapidly than *td*8 but nearly twofold below the rates attained by the wild type. It is of interest that, at early stages of infection, the *wh* mutant formed infectious particles at about the same rate as did the wild type and that the biochemical deficiency became manifest only at later stages. This suggests that the intracellular THF supply is adequate at first

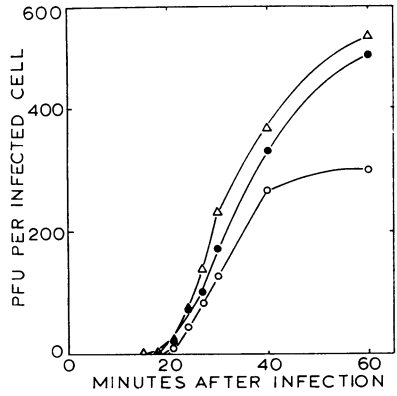

FIG. 5. *Growth of $T4BO_1$ (△) and T4wh2 in the presence (●) and absence (○) of thymine. Protocol was the same as for the experiment of Fig. 4, except that thymine was present in the indicated culture at 10 μg/ml.*

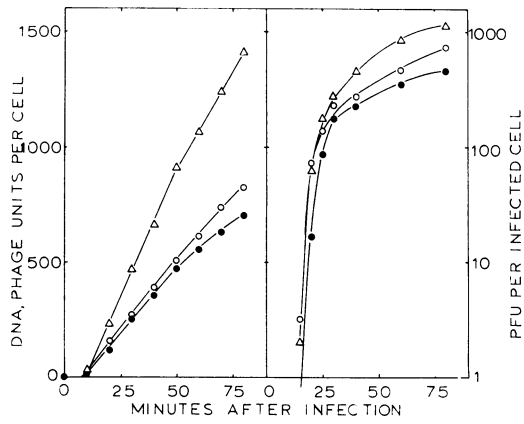

FIG. 6. *DNA synthesis and phage growth in Escherichia coli B infected at high cell density. Cells were infected at a multiplicity of 8 and a cell concentration of about 2.5 × 10^8/ml. (Left) DNA synthesis; (right) phage growth. Symbols: △, $T4BO_1$; ●, T4td8; ○, T4wh2. Samples were assayed for plaque-forming titer after chloroform lysis.*

to support a normal rate of thymidylate synthesis but that it becomes depleted by the sustained high activity of thymidylate synthetase. Under these conditions of rapid oxidation of THF catalyzed by the synthetase (21), the bacterial reductase, acting alone, cannot replenish the THF supply at a rate sufficiently rapid to support the high rate of DNA synthesis characteristic of normal infections.

DISCUSSION

The above data support the idea, previously expressed (13), that the metabolic role of phage-specific DHF reductase is quantitative, rather than qualitative. Like the related enzyme thymidylate synthetase (11), the viral reductase acts to supplement a pre-existing activity and thus helps to maintain the high rate of DNA synthesis characteristic of T-even phage-infected cells. A similar situation has been noted briefly in studies on deoxycytidylate deaminaseless mutants of T4 (8). Thus, these three enzymes, closely related both functionally and from the standpoint of genetic control (9), play quantitatively significant, but not absolutely indispensable, roles in DNA metabolism.

The *wh* mutants were originally isolated by Hall et al. on the basis of a thick white halo formed around plaques when the plating bacteria were grown under conditions of limiting deoxyuridine. The observation that the *wh* mutants give thicker halos than *td* mutants, a finding which has been confirmed in this laboratory, suggests that dUMP or deoxyuridine compounds

accumulate to a greater extent in *wh*-infected cells than in *td*-infected bacteria. This would not be expected *a priori*, on the basis of our present knowledge of the regulation of nucleotide metabolism, and it would be of interest to test this suggestion by direct measurement of pool sizes in *E. coli* under various conditions of phage infection. Such studies are now in progress in this laboratory.

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LITERATURE CITED

1. BERTANI, L. E., A. HÄGGMARK, AND P. REICHARD. 1961. Synthesis of pyrimidine deoxyribonucleoside diphosphates with enzymes from *Escherichia coli*. J. Biol. Chem. **36**:PC67–PC68.
2. BESSMAN, M. J., AND L. J. BELLO. 1961. The enzymology of virus-infected bacteria. III. Evidence for the formation of a trifunctional deoxynucleotide kinase. J. Biol. Chem. **236**: PC72–PC73.
3. DOERMANN, A. H. 1948. Lysis and lysis inhibition with *Escherichia coli* bacteriophages. J. Bacteriol. **55**:257–276.
4. FLAKS, J. G., AND S. S. COHEN. 1959. Virus-induced acquisition of metabolic function. I. Enzymatic formation of 5-hydroxymethyldeoxycytidylate. J. Biol. Chem. **234**:1501–1506.
5. FLAKS, J. G., AND S. S. COHEN. 1959. Virus-induced acquisition of metabolic function. III. Formation and some properties of thymidylate synthetase of bacteriophage-infected *Escherichia coli*. J. Biol. Chem. **234**:2981–2986.
6. FRASER, D., AND E. A. JERREL. 1953. The amino acid composition of T3 bacteriophage. J. Biol. Chem. **205**:291–295.
7. GREENBERG, G. R., R. L. SOMERVILLE, AND S. DEWOLF. 1962. Resolution of phage-initiated and normal host thymidylate synthetases of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S. **48**:242–247.
8. HALL, D. H., AND I. TESSMAN. 1966. T4 mutants unable to induce deoxycytidylate deaminase activity. Virology **29**:339–345.
9. HALL, D. H., I. TESSMAN, AND O. KARLSTRÖM. 1967. Linkage of T4 genes controlling a series of steps in pyrimidine biosynthesis. Virology **31**:442–448.
10. KECK, K., H. R. MAHLER, AND D. FRASER. 1960. Synthesis of deoxycytidine-5′-phosphate deaminase in *Escherichia coli* infected by T2 bacteriophage. Arch. Biochem. Biophys. **86**: 85–88.
11. MATHEWS, C. K. 1965. Phage growth and DNA synthesis in *Escherichia coli* infected by a thymine-requiring bacteriophage. J. Bacteriol. **90**:648–652.
12. MATHEWS, C. K. 1966. DNA metabolism and virus-induced enzyme synthesis in a thymine-requiring bacterium infected by a thymine-requiring bacteriophage. Biochemistry **5**:2092–2100.
13. MATHEWS, C. K. 1966. On the metabolic role of T6 phage-induced dihydrofolate reductase. Intracellular reduced pyridine nucleotides. J. Biol. Chem. **241**:5008–5012.
14. MATHEWS, C. K. 1967. Evidence that bacteriophage-induced dihydrofolate reductase is a viral gene product. J. Biol. Chem. **242**:4408–4412.
15. MATHEWS, C. K., AND S. S. COHEN. 1963. Virus-induced acquisition of metabolic function. VI. Dihydrofolate reductase, a new phage-induced enzyme. J. Biol. Chem. **238**:PC853–PC854.
16. MATHEWS, C. K., AND S. S. COHEN. 1963. Inhibition of phage-induced thymidylate synthetase by 5-fluorodeoxyuridylate. J. Biol. Chem. **238**: 367–370.
17. MATHEWS, C. K., AND K. E. SUTHERLAND. 1965. Comparative biochemistry of bacterial and phage-induced dihydrofolate reductases. J. Biol. Chem. **240**:2142–2147.
18. SHAPIRO, D. M., J. EIGNER, AND G. R. GREENBERG. 1965. Inability of thymine-dependant mutants of bacteriophage T4 to induce thymidylate synthetase. Proc. Natl. Acad. Sci. U.S. **53**:874–881.
19. SIMON, E. H., AND I. TESSMAN. 1963. Thymidine-requiring mutants of phage T4. Proc. Natl. Acad. Sci. U.S. **50**:526–532.
20. STENT, G. S. 1963. Molecular biology of bacterial viruses, p. 30. W. H. Freeman and Co., San Francisco.
21. WAHBA, A. J., AND M. FRIEDKIN. 1961. Direct spectrophotometric evidence for the oxidation of tetrahydrofolate during the enzymatic synthesis of thymidylate. J. Biol. Chem. **236**:PC11–PC12.
22. WARNER, H. R., AND N. LEWIS. 1966. The synthesis of deoxycytidylate deaminase and dihydrofolate reductase and its control in *Escherichia coli* infected with bacteriophage T4 and T4 amber mutants. Virology **29**:172–175.
23. WYATT, G. R., AND S. S. COHEN. 1953. The bases of the nucleic acids of some bacterial and animal viruses: the occurrence of 5-hydroxymethylcytosine. Biochem. J. **55**:774–782.