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Bacteriophage Tail Components

V. Complementation of T4D Gene 28⁻-Infected Bacterial Extracts with Pteroyl Hexaglutamate

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Synthetic pteroyl hexaglutamate (9×10^{-6} M) stimulated the formation of new T4D particles in vitro in extracts of *Escherichia coli* B infected with T4D gene 28⁻. The stimulation was specific for this form of folic acid since neither pteroyl pentaglutamate nor pteroyl heptaglutamate stimulated phage formation. T4D formation in vitro in *E. coli* B extracts prepared after infection with 11 other phage mutants known to be involved in phage tail plate formation (5⁻, 6⁻, 7⁻, 8⁻, 10⁻, 25⁻, 26⁻, 27⁻, 29⁻, 51⁻, 53⁻) was not stimulated by the addition of pteroyl hexaglutamate. It can be concluded that the T4D gene 28 product is involved in the formation of the phage tail plate pteroyl hexaglutamate.

This report is concerned with the identification of the bacteriophage gene or genes responsible for the formation of the dihydropteroyl hexaglutamate used for the assembly of the T4D phage tail plate (5-9) during in vitro complementation experiments. Twelve T4D genes were identified earlier (1-4, 12) as essential for the formation of the T4D tail plate. In addition, the viral-induced dihydrofolate reductase is a tail plate component (9). Dihydropteroyl hexaglutamate, which is not a protein, cannot be the primary product of any of these 13 genes. However, one or more of these genes could be producing a catalytic proteins(s) which participates in the formation of the folate compound. An extract of the nonpermissive host, *Escherichia coli* B, infected with an amber mutant in such a gene would be expected to have all of the phage components necessary for phage assembly, except for the folate compound. The addition of synthetic pteroyl hexaglutamate might then stimulate in vitro T4D formation in such an extract. It should be emphasized that such a stimulation will be limited by the ability of the extract to reduce the added folate compound and by the presence of any competing folate compounds.

Chemical studies of the formation of folate polyglutamate compounds in uninfected and T4D-infected cells show that the metabolism of these compounds is quite complex (6). Although uninfected host cells contain mostly compounds equivalent in size to *pte(glu)*$_3$, they do contain small amounts of larger folate compounds. T4D infection stimulated the conversion of *pte(glu)*$_3$ compounds to *pte(glu)*$_6$ compounds. These studies also showed that infection with T4D 28⁻ caused the production a very large folate polyglutamates containing 7 to 12 glutamate residues.

In agreement with these chemical studies, it has now been found that T4D gene 28⁻ extracts are apparently deficient in pteroyl hexaglutamate and do form small amounts of new phage particles in response to added pteroyl hexaglutamate. A preliminary report of this work has been presented earlier (L. M. Kozloff, Fed. Proc. **30**:1263, 1971).

MATERIALS AND METHODS

Preparation and purification of bacteriophage stocks and extracts of Excherichia B infected with T4 D amber mutants. Most of the biological materials and methods were identical to those used earlier (7-9). Various T4D amber mutants were obtained from R. S. Edgar, W. B. Wood, and their colleagues (1-4, 12). Single plaques of these mutants were picked and grown on the permissive host *E. coli* CR63 and purified by standard procedures. The mutants, all defective in tail plate formation, were gene 5 (*amN135*), gene 6 (*amN102*), gene 7 (*amB16*), gene 8 (*amN132*), gene 10 (*amB255*), gene 25 (*amN62*), gene 26 (*amN131*), gene 27 (*amN120*), gene 28 (*am452*), gene 29 (*amB7*), gene 51 (*amS29*), and gene 53 (*amH28*). Extracts of mutant-infected nonpermissive host *E. coli* B were prepared by the procedures used earlier (7, 9), except that the bacteria at 3×10^8 were usually infected with a multiplicity of infection of 4:1 at 30 C. Occasionally, especially for the preparation of gene 28⁻ extracts, the

cells were superinfected 5 min after the initial infection. This later procedure improved subsequent complementation but resulted in a higher initial phage titer. Some extracts of 28⁻ (*am452*), about 10% of those prepared, were inactive upon subsequent complementation and were discarded.

Extracts were treated with Norit A-activated charcoal by placing the charcoal (100 mg) in a dialysis bag with 0.5 ml of buffer (7) and then immersing the bag in 0.5 ml of the extract. Pteroyl pentaglutamate, pteroyl hexaglutamate (two separate preparations), and pteroyl heptaglutamate were synthesized by the solid-phase method, purified by DEAE-cellulose chromatography, desalted, and suspended in water at a concentration of 2×10^{4} to 4×10^{-4} M (10). Normally these compounds were added to complementation mixtures at a ratio of 2 to 5 vol per 100 vol of extract, giving a final concentration about 10^{-5} M.

RESULTS

Stimulation of in vitro T4D formation by synthetic pteroyl hexaglutamate in extracts of infected host cells. Synthetic pteroyl hexaglutamate was added to separate extracts prepared by infecting *E. coli* B with the different phage mutants known to be involved in tail plate formation. Only gene 28⁻ extracts were stimulated to form small amounts of new T4D phage upon the addition of this compound (Table 1). Gene 28⁻ extracts had starting titer of from 5×10^{8} to 2×10^{9}, and the actual increase in phage titer observed was usually 50 to 400%. No other extract ever responded in this way. The starting titers of the other extracts varied; for example, 5⁻, 7⁻, 10⁻, 25⁻, 27⁻, 29⁻, and 53⁻ had starting titers from 10^{9} to 4×10^{9}, and none of them gave more than a 5% increase upon the addition of pteroyl hexaglutamate. Other extracts such as 6⁻, 8⁻, 25⁻, 26⁻, and 51⁻ had starting titers of about 10^{10}, and the addition of hexaglutamate caused no increase in phage titer. Several experiments were carried out with a preparation of pteroyl hexaglutamate reduced with dithionate and ascorbate. These preparations were found to inhibit complementation, and the inhibitory elements were found to be the residual dithionate and ascorbate. For unknown reasons, occasional 28⁻ extracts, about 10 to 15% of a total of over 50 different preparations, did not appear to respond to added pteroyl hexaglutamate. The properties described below are therefore typical of the bulk of 28⁻ preparations prepared.

Some of the properties of phage formation in T4D gene 28⁻-infected *E. coli* B are shown in Fig. 1. Similar to what was found previously in mixed infected bacterial extracts (7), the stimulation of phage formation in 28⁻ extracts was related to the concentration of added pteroyl hexaglutamate; about 10^{-5} M was optimal (7); higher concentrations inhibited phage assem-

TABLE 1. *In vitro T4D formation in bacterial extracts*

T4D Mutant used to infect *E. coli* B	Effect of 9×10^{-6} M pteroyl hexaglutamate[a]	Role of gene product (11)
Gene 5 (*amN135*)	None (4)[b]	Stoichiometric
Gene 6 (*amN102*)	None (6)	Stoichiometric
Gene 7 (*amB16*)	None (3)	Intermediate-stoichiometric[c]
Gene 8 (*amN132*)	None (1)	Stoichiometric
Gene 10 (*amB255*)	None (2)	Ambiguous-stoichiometric[c]
Gene 25 (*amN62*)	None (2)	Stoichiometric
Gene 26 (*amN131*)	None (2)	Catalytic
Gene 27 (*amN120*)	None (2)	Stoichiometric
Gene 28 (*amA452*)	STIMULATED ~50)	Catalytic (?)
Gene 29 (*amB7*)	None (2)	Stoichiometric
Gene 51 (*amS29*)	None (2)	Catalytic
Gene 53 (*amH28*)	None (2)	Stoichiometric

[a] Stimulation is defined as the formation of at least 10^{9} new T4D particles in 1 to 3 h at 30 C in at least two separate experiments.

[b] Number of experiments.

[c] J. King (personal communication) has found that the products of gene 7 and gene 10 are phage tail plate constituents.

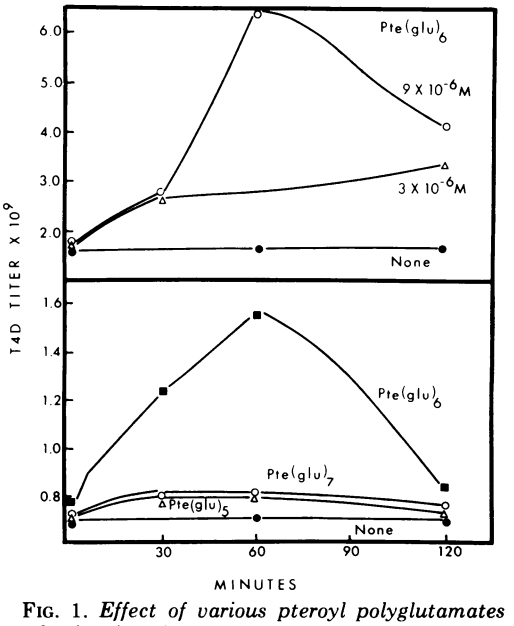

FIG. 1. *Effect of various pteroyl polyglutamates on the in vitro formation of new T4D particles in extracts of E. coli B infected with T4D 28⁻. Top, effect of different concentrations of pte(glu)₆; bottom, effect of different pteroyl polyglutamates, all at* 9×10^{-6} *M.*

bly. Neither the pteroyl pentaglutamate nor the pteroyl heptaglutamate stimulated phage formation in these extracts.

One unusual feature of phage formation in 28^- extracts of Fig. 1 observed many times was the decrease in the apparent number of new phage particles after 1 to 2 h. One possible explanation for the fall in phage titer is that extra glutamyl residues are added to the phage particle pteroyl polyglutamate. When a 28^- cell extract was complemented with pteroyl hexaglutamate, there was an initial rise and then subsequent decrease in phage titer (Fig. 2). If, at this time, a portion of the extract was diluted into acetate buffer (pH 4.5) containing partially purified hog kidney conjugase (5), an exopeptidase which hydrolyses glutamyl residues from folate polyglutamates, there was an apparent transient reactivation of the newly formed phage particles. Since this enzyme is known to be able to attack the phage tail folate compound (and to inactivate phage [5]), it is possible that the original fall in phage titer in these complementation systems is due to the addition of extra glutamyl residues to the phage tail folate compound.

Snustad (11) has pointed out that T4D gene products appear to act either stoichiometrically or catalytically in forming new phage (see Table 1). The results of Snustad suggest a catalytic role for the product of T4D gene 28 and support the conclusion that such a gene product is acting catalytically in the formation of the pteroyl hexaglutamate needed for tail assembly.

Stimulation of in vitro T4D formation by synthetic pteroyl hexaglutamate in mixtures of infected bacterial exracts. An extract of *E. coli* B infected with a T4D mutant containing an amber mutation in gene 7 was depleted of its folate compounds by treatment with activated charcoal. These depleted extracts were then mixed with a second extract. If the second extract was deficient in pteroyl hexaglutamate, new phage particles would not be formed upon addition of charcoal-treated 7^- extracts, but new particles would still be formed upon addition of untreated 7^- extracts. Furthermore, the addition of synthetic pteroyl hexaglutamate should stimulate phage formation only if this compound was the limiting phage component.

Extracts of *E. coli* made after infection with 7^- or with 10^- T4D mutants were used in these experiments, since both of these gene products are structural protein components of the phage tail plates, and these extracts should presumably contain the appropriate folate compounds.

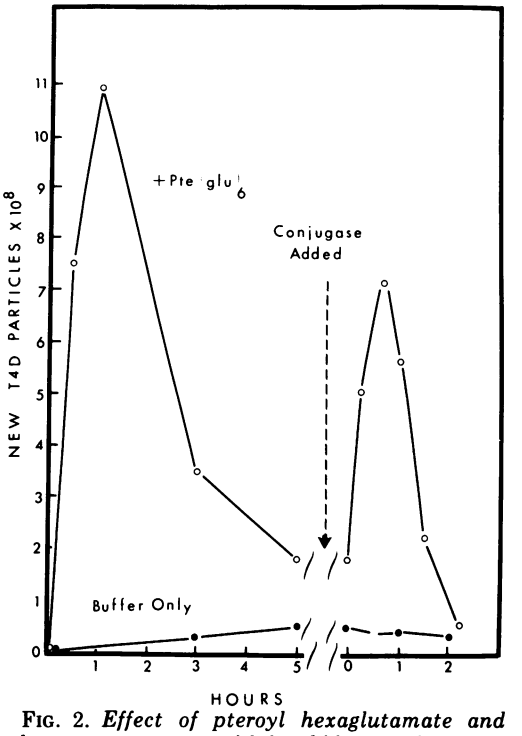

FIG. 2. *Effect of pteroyl hexaglutamate and subsequent treatment with hog kidney conjugase on the in vitro formation of new T4D particles in extracts of E. coli B infected with T4D 28⁻. In this experiment the starting T4D titer was 1.2 × 10⁹, and the maximum titer reached after 1 h was 2.3 × 10⁹.*

Both mutant extracts can be partially depleted by charcoal treatment (7). Three T4D mutants (26^-, 28^-, and 51^-), shown or suspected to make gene products having a catalytic function in tail plate formation (11), were then tested for their ability to form phage with 7^- and 10^- extracts.

Typical experiments with 7^- extracts are shown in Fig. 3 and 4. The formation of new T4D particles in mixtures of untreated 7^- extracts plus 28^- extracts is not stimulated by the addition of pteroyl hexaglutamate, and the rate of phage formation actually was inhibited by the addition of this compound. On the other hand, charcoal treatment of 7^- extracts to partially deplete them of folate compounds greatly inhibited phage formation upon subsequent complementation with a 28^- extract. Furthermore, mixtures of 28^- extracts plus depleted 7^- extracts now responded to the addition of pteroyl hexaglutamate by forming increased amounts of new phage particles. Similar results were obtained when untreated or charcoal-treated 10^- extracts rather than 7^- extracts were complemented with 28^- extracts. In can be concluded that 28^- extracts lack

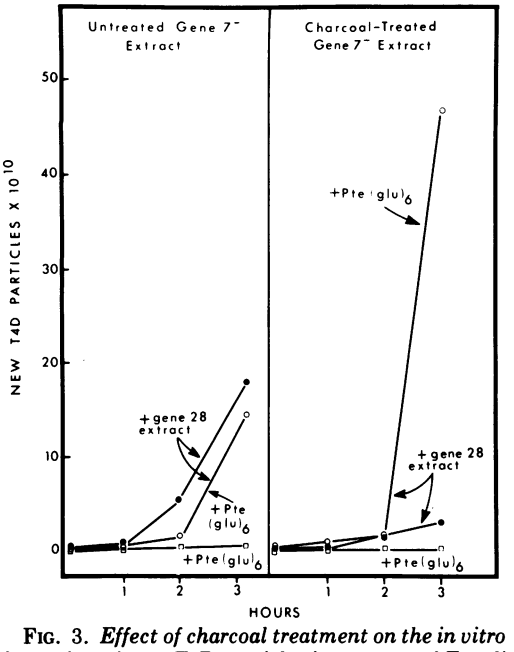

FIG. 3. *Effect of charcoal treatment on the in vitro formation of new T4D particles in extracts of E. coli B infected with T4D gene 7⁻. The 7⁻ extracts are complemented with equal volumes of 28⁻ extracts. The pte(glu)₆ was at a final concentration of 9 × 10⁻⁶ M and amounted to only 3% of the final complementation volume. Starting titer was 2 × 10⁹/ml.*

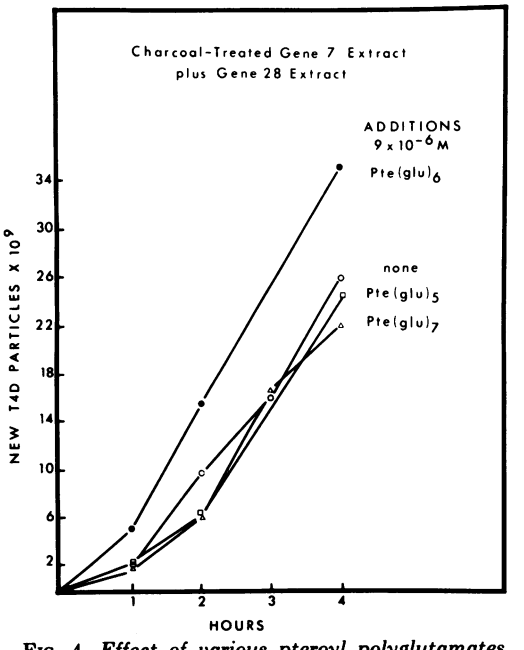

FIG. 4. *Effect of various pteroyl polyglutamates on the formations of new T4D particles in mixtures of charcoal-treated gene 7⁻ extracts and gene 28⁻ extracts. Starting titer was 2 × 10⁹/ml.*

pteroyl hexaglutamate and the gene 28 product participates in the formation of this compound.

Figure 4 shows the specificity of the stimulation due to the addition of the polyglutamate compounds. Gene 28⁻ extracts plus partially depleted 7⁻ extracts form increased amounts of phage in response to the addition of the pteroyl hexaglutamate compound, but the addition of the corresponding penta- or heptaglutamyl derivatives did not increase the amount of new phage particles formed. When these compounds were added to mixtures of 28⁻ extracts and untreated 7⁻ extracts, all inhibited complementation, and the penta- and hepta- were more effective inhibitors than the hexaglutamyl compound. These observations not only support the conclusion that 28⁻ extracts are deficient specifically in the hexaglutamyl compound, but also demonstrate that over a period of several hours these extracts are unable to convert significant amounts of either the pteroyl pentaglutamate or the pteroyl heptglutamate to the pteroyl hexaglutamate.

Although neither 26⁻ nor 51⁻ extracts were stimulated by pteroyl hexaglutamate in single complementation incubation mixtures, experiments were carried out with 26⁻ extracts and 51⁻ extracts to complement untreated and charcoal-treated 7⁻ extracts. Contrary to what was found with 28⁻ extracts, charcoal treatment of the 7⁻ extracts did not inhibit later complementation with either 26⁻ or 51⁻ extracts. This supports the view that 26⁻ and 51⁻ extracts can furnish sufficient folate compounds to support in vitro T4D phage formation.

DISCUSSION

Although the addition of synthetic pteroyl hexaglutamate to extracts of *E. coli* B infected with T4D amber mutant 452 (28⁻) results in only a small increase in the rate of phage formation in vitro and a small increase in the absolute amount of phage formed, the specificity of the reaction indicates that the gene 28 product plays a role in formation of this phage component. These extracts must reduce the pteroyl hexaglutamyl compound to the dihydro compound, and this might limit the ability of the unreduced compound to complement extracts. One other factor undoubtedly limiting the amount of phage formed upon addition of pteroyl hexaglutamate to 28⁻ extracts is the presence of the high-molecular-weight pteroyl compounds containing 7 to 12 glutamate residues (6). Experiments with 28⁻ extracts to complement folate-depleted 7⁻ or 10⁻ extracts

showed significant phage formation (of the order of 2×10^{10} to 3×10^{10} new particles) could be stimulated by the addition of pteroyl hexaglutamate. This result shows that 28^- extracts are deficient in pteroyl hexaglutamate and supports the conclusion that T4D gene 28 product is involved in pteroyl hexaglutamate formation.

From these in vitro complementation experiments, it is apparent that the primary product of T4D gene 28 is a catalytic protein required for either the formation of pteroyl hexaglutamate or the regulation of its concentration. From the observation (6) that very high-molecular-weight folate-like compounds are produced in T4D 28^--infected cells, it seems likely that the 28 product is a cleavage enzyme which has an important physiological function. This enzyme not only forms the pteroyl hexaglutamate from higher-molecular-weight compounds, but also removes any larger folates which interfere with phage tail assembly.

ACKNOWLEDGMENTS

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