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Bacteriophage Tail Components

III. Use of Synthetic Pteroyl Hexaglutamate for T4D Tail Plate Assembly

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The assembly of T4D tail plates occurring during in vitro complementation reactions was found to be stimulated by pteroyl hexaglutamate. Neither the pteroyl pentaglutamate nor the pteroyl heptaglutamate substituted for the hexaglutamate. A small stimulation of the rate and amount of T4D tail plate assembly was observed in untreated extracts. A greater stimulation occurred when activated charcoal-treated bacterial extracts were used. Charcoal treatment inhibited complementation only when no preformed tail plates were present in the extracts, and the inhibition was reversed by the addition of 9×10^{-6} M chemically synthesized pteroyl hexaglutamate. The stimulation is apparently due to a requirement for the pteroyl hexaglutamate for tail plate assembly.

The complex T-even bacteriophage tail structure was shown recently by Kozloff and coworkers (5-7) to contain an unusual virus-induced folic acid conjugate as a structural component. On the basis of its spectrum and behavior during gel permeation chromatography, this compound appeared to be a dihydropteroyl penta- or hexaglutamate. Recent developments in solid-phase peptide synthesis have led to the synthesis of folate conjugates containing up to seven glutamyl residues (8). With these compounds, it has been possible to examine the role of the folate conjugate during in vitro phage morphogenesis. The need for a specific folate conjugate during phage tail plate assembly has now been demonstrated by use of the in vitro complementation system developed by Edgar and his colleagues (1-4, 11). Only the pteroyl hexaglutamate has been found to restore activity to systems depleted of their endogenous folate conjugates by charcoal treatment. Further, these systems have permitted mapping of the specific reaction in which the folate compound is required.

MATERIALS AND METHODS

Preparation and purification of bacteriophage stocks and extracts of Escherichia coli B infected with T4D amber mutants. Most of the biological materials and methods were identical to those used earlier (6, 7). Various T4D amber mutants were obtained from R. S. Edgar and colleagues (1-3). These mutants were grown on the permissive host *E. coli* CR63 and were purified by standard procedures. The mutants used were as follows. (i) Defective in tail plate formation: gene 6 (N102), gene 7 (B16), gene 8 (N132), gene 10 (B255), gene 26 (N131), gene 27 (N120), gene 28 (A452), and gene 53 (H28). (ii) Defective in tail tube formation: gene 48 (N85) and gene 54 (H21). (iii) Defective in sheath formation: gene 18 (E18). (iv) Defective in head formation: gene 23 (B17). (v) A quadruple mutant defective in tail fiber formation: genes 34/35/37/38 (B25/B252/N52/B262). (vi) A double mutant defective in completing tail formation: genes 11/12 (N93/N69). Extracts of the mutant-infected nonpermissive host *E. coli* B were prepared by the procedure used earlier (7), except that the bacteria, at 4×10^8/ml, were infected with a multiplicity of infection of 4:1 at 30 C. After 30 min of incubation, the infected cells were chilled and centrifuged at $1,400 \times g$ for 5 min; they were then resuspended in cold BUM (3) plus deoxyribonuclease to 1.0% of their original volume and were stored at −70 C. After thawing at 30 C, the T4D titer was usually 10^9 to 2×10^9/ml, and the titer after complementation normally increased 10- to 300-fold.

Charcoal treatment of bacterial extracts. Two types of activated charcoal were used, Norit A, from Pfanstiehl Laboratories, Inc., and Darco, grade G-60, from Atlas Chemical Industries. Norit A was used in most experiments, but the Darco was equally effective. Three different procedures were used for treating the bacterial extracts. (i) Weighed amounts of charcoal were added directly to an empty chilled reaction tube; then the two complementing amber extracts were added and the mixture was kept at 0 C for 5 min. The reaction mixture containing the charcoal was then incubated at 30 C for various times. This procedure is referred to as treatment with free charcoal in Fig. 2. (ii) In some experiments, weighed amounts of Norit A were pressed on strips of cellulose tape coated on both sides with adhesive. These strips were immersed into individual extracts for various times in the cold and then removed. The extracts were then mixed for complementation. This method is called the "bound" charcoal treatment (see Fig. 2), and it

permits the later addition of reagents which might otherwise be adsorbed by charcoal. (iii) Various extracts were also treated separately with charcoal contained in washed dialysis bags. The bags containing the charcoal (usually 100 mg suspended in 0.3 ml of BUM) were immersed in the extracts (0.5 ml) for 4 to 5 hr in the cold; they were then removed, and the treated extracts were mixed for complementation in the usual manner. This procedure prevented any contact between the proteins in the extracts and the charcoal, but it also removed various freely diffusible substances.

Other methods and sources of chemicals. Aminopterin inhibition of complementation was assayed as described previously (7). Aqueous solutions of pteroyl pentaglutamate, pteroyl hexaglutamate, and pteroyl heptaglutamate, prepared by solid-phase chemical synthesis, were the generous gift of Carlos Krumdieck and Charles Baugh (8). Other reagents were those used earlier (7).

RESULTS

Effect of folate conjugates on in vitro formation of T4D particles. Figure 1 shows that *p*-aminobenzoyl glutamate, pteroyl glutamate (folic acid), and pteroyl pentaglutamate, at approximately 10^{-5} M, failed to affect complementation between gene 10 and gene 7 extracts. However, pteroyl hexaglutamate at the same concentration stimulated complementation. The stimulation was largest for short periods of incubation. However, at 2.2×10^{-5} M, the pteroyl hexaglutamate (and the pteroyl pentaglutamate) inhibited complementation. These results are consistent with the view that the pteroyl hexaglutamate is used for virus assembly but that it first must be reduced to the dihydro compound. Any excess unreduced pteroyl compound acts as an inhibitor of dihydrofolate reductase and thus of assembly (7). The relatively low degree of stimulation (at most twofold) is due presumably to the high endogenous level of folate conjugates. This property and the inhibitory effect of higher concentrations of the added conjugates make it difficult to assess the role of various folate conjugates when the usual bacterial extracts are used.

Inhibition of in vitro complementation of T4D gene 10 and gene 7 extracts by activated charcoal. Activated charcoal is well known for its ability to adsorb folic acid conjugates. The effect of adding 16 mg of Norit A per ml to the complementation reaction between gene 7 and gene 10 extracts is shown in Fig. 2. Charcoal addition greatly depressed the initial rate of phage formation but not the total amount of phage formed by 300 min. The decrease in the initial rate of phage formation was found to be proportional to the amount of charcoal added (Fig. 3). "Bound" charcoal (on strips of tapes) was less effective than "free"

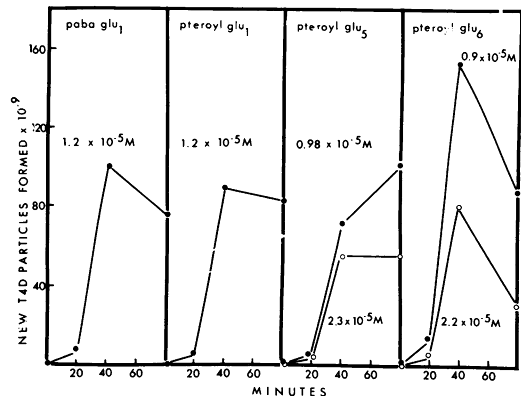

FIG. 1. *Effect of various folate conjugates on in vitro complementation between extracts made with T4D gene 10 and T4D gene 7 amber mutants. The test compounds in either water or BUM amounted to only 5 to 10% of the final volume. The complementation was carried out as described in Materials and Methods. Paba glu$_1$ = p-aminobenzoyl glutamate; pteroyl glu$_1$ = pteroyl glutamate; pteroyl glu$_5$ = pteroyl pentaglutamate; pteroyl glu$_6$ = pteroyl hexaglutamate.*

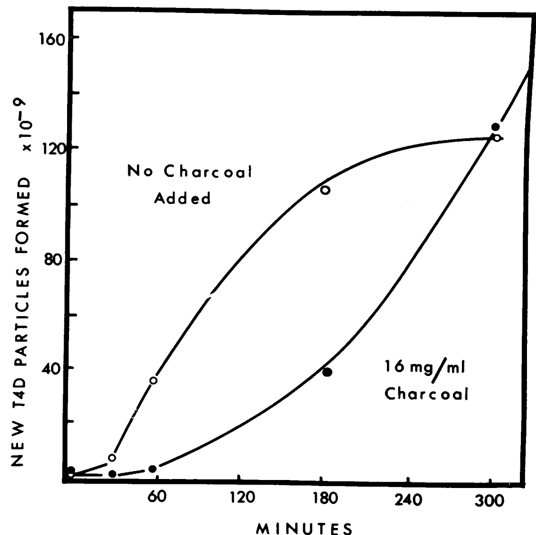

FIG. 2. *Inhibition by activated charcoal of in vitro complementation between extracts of E. coli B made with T4D amber mutant in gene 10 and a similar extract made with T4D amber mutant in gene 7. The complementation was carried out as described in Materials and Methods.*

charcoal, but both experiments (shown in Fig. 2 and 3) suggest that charcoal adsorbs some substance from the extracts which is rate determining.

Several properties of the charcoal-inhibited system were studied to determine the compound or

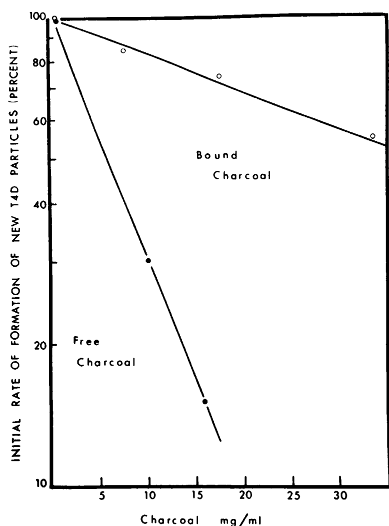

FIG. 3. *Inhibition of the initial rate of formation of new T4D particles in an in vitro complementation system by free charcoal or bound charcoal. The initial rate was calculated from the increase in phage titer from 30 to 60 minutes after mixing of bacterial extracts made with T4D gene 10 and T4D genp 7 mutants. In the experiment with free charcoal, various amounts of charcoal were added to separate reaction mixtures at 4 C. The tubes were then incubated at 30 C and assayed at various times. In the experiment with "bound" charcoal, weighed amounts of charcoal were pressed on strips of cellulose tape coated on both sides with adhesive. These strips were immersed in separate tubes containing the extracts and kept at 0 C for 1 hr. The strips with the "bound" charcoal were then removed, and the gene 10 and gene 7 extracts were mixed and incubated in the usual manner.*

compounds adsorbed. One possibility considered was that these levels of charcoal might be removing indole, which occurs in these extracts, and which Nashimoto and Uchida (10) have shown to stimulate tail fiber attachment. Results given below clearly indicate that, although indole has an effect on complementation (and might be removed under certain conditions), the removal of indole is not the cause of charcoal inhibition at levels of free charcoal of 8 mg/ml or less.

In view of the stimulation of the initial rate of complementation shown for pteroyl hexaglutamate in Fig. 1, it seemed more likely that these low levels of charcoal were removing some but not all of the endogenous folate compounds. This would cause a decrease in the rate of phage formation, but, since the folates are in considerable excess over that needed for phage assembly, the total amount of phage formed over long periods of incubation should not be decreased. One test of this hypothesis of charcoal inhibition is given in Table 1. Phage assembly in vitro occurring in mixtures of T4D gene 7 and gene 10 extracts was found previously to be sensitive to the addition of 10^{-4} to 10^{-3} M aminopterin, a dihydrofolate reductase inhibitor (7). This compound apparently blocked the incorporation of the phage-induced dihydrofolate reductase into the phage tail plate. In these previous experiments, it was observed that the concentration of aminopterin required to inhibit complementation was higher than that normally needed to inhibit enzymatic activity (9). This was presumed to be due to the high concentration of folates in the extracts. Table 1 shows that charcoal treatment greatly increased the sensitivity of these extracts to aminopterin inhibition and supports the view that charcoal treatment removes endogenous folate compounds necessary for assembly.

Mapping of the site of charcoal inhibition in the pathway of T4D morphogenesis. The effect of various amounts of free charcoal on three in vitro assembly reactions is shown in Fig. 4. The system containing gene 10 and gene 7 extracts was sensitive to as little as 2 mg/ml. The other two systems,

TABLE 1. *Effect of charcoal treatment on the inhibition by aminopterin of complementation with T4D gene 10 and gene 7 extracts*

Expt	Prepn	Initial rate of formation of new T4D particles[a]	Inhibition, %
I	Untreated extracts	2.1×10^9	—
	+7 × 10^{-4} M aminopterin	0.4×10^9	81
	+2.3 × 10^{-4} M aminopterin	1.2×10^9	43
	Treated extracts[b]	1.6×10^9	—
	+7 × 10^{-4} M aminopterin	0	100
	+2.3 × 10^{-4} M aminopterin	0	100
II	Untreated extracts	14.3×10^9	—
	+2.3 × 10^{-4} M aminopterin	10.4×10^9	27
	Treated extracts[c]	4.1×10^9	—
	+2.3 × 10^{-6} M aminopterin	2.1×10^9	49

[a] Net new particles formed at 30 C between 30 and 60 min after mixing extracts.

[b] Each extract was treated separately with bound charcoal at a concentration of 20 mg/ml for 25 min at 0 C.

[c] Each tube of extract was treated separately by adding a dialysis bag containing 100 mg of charcoal to 0.5 ml of extract and keeping the tube at 0 C for 4 hr; the bag containing the charcoal was then removed and the extracts were mixed.

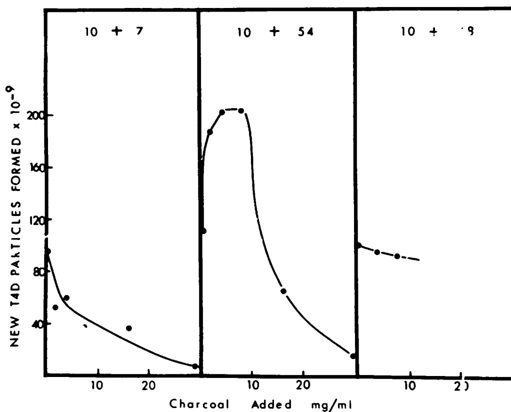

FIG. 4. *Effect of charcoal on in vitro complementation between extracts of E. coli B made with different T4D amber mutants. The complementation was carried out in the presence of the charcoal as described in Materials and Methods.*

gene 10 plus gene 54 and gene 10 plus gene 48, were either stimulated or unaffected by the added charcoal. At concentrations above 20 mg/ml, all complementation mixtures were inhibited, including those shown later in Table 2. This nonspecific inhibition was not further studied.

It should be noted that gene 54 and gene 48 extracts both contain preformed tail plates (11), whereas gene 10 and gene 7 do not contain this substructure. Table 2 shows that the small amounts of charcoal used (8 mg/ml) inhibited a very specific site in viral assembly. All complementation systems in which either one of the bacterial extracts contained a tail plate or more complex tail substructure were either stimulated or unaffected by the charcoal.

These results (Fig. 4 and Table 2) also show that the inhibition due to added free charcoal at 8 mg/ml cannot be due to removal of indole, because the indole-sensitive reaction, the addition of tail fibers to fiberless particles, occurs in every system given in Table 2 except that in experiment 15. The simplest interpretation of the increase in aminopterin sensitivity and the specific site of inhibition of morphogenesis is that charcoal inhibits tail plate formation by removing a folate conjugate.

Effect of synthetic folate conjugates on complementation in charcoal-treated extracts. The highly charcoal-sensitive complementation extracts gene 7 and gene 10 were treated separately with charcoal contained in a dialysis bag. This permitted the later addition of synthetic pteroyl glutamates. The results of adding these compounds to these treated extracts are shown in Fig. 5–7. The charcoal inhibition observed after this type of treatment was different from that shown in Fig. 2. Both the initial rate and the level of complementation at the final point taken (300 or 180 min) were decreased. Further, indole dialyzes into the bag containing the charcoal, and addition of indole at a concentration of 1.4×10^{-4} M partially restored the rate of complementation. Increasing the indole concentration to 5×10^{-4} M did not further stimulate complementation to the level in the untreated extracts. It was also found that indole did not stimulate complementation in the untreated extracts as did pteroyl hexaglutamate (Fig. 1).

Figure 5 shows that 9×10^{-6} M synthetic pteroyl hexaglutamate stimulated complementation. The addition of this compound alone to treated extracts increased the rate of complementation, and the addition of pteroyl hexaglu-

TABLE 2. *Inhibition of T4D tail assembly by activated charcoal*[a]

Expt no.	Substructures present	Extracts	Relative complementation
1–4	None	10 + (7 or 8, or 28 or 53)	0.1–0.3
5–8	None	27 + (6 or 8, or 26 or 53)	0.0–0.2
9–10	Tail plate	10 + (54 or 48)	0.9–1.8
11	Tail plate	27 + 54	0.7
12	Tail plate + tube	27 + 18	1.2
13	Tail plate + tube + sheath	27 + 23	1.9
14	Fiberless particle	10 + 34/35/37/38	0.8
15	Defective particle	10 + 11/12	1.6

[a] In experiments 1–13, 0.8 mg of charcoal was added to a 0.1-ml mixture of equal portions of each of the two extracts. This mixture was incubated for 5 min at 0 C, a sample was removed for assay, and the reaction mixture was assayed again after 3 hr at 29 C. The net fold increase in T4D in the absence of charcoal was: expt 1, 125; expt 2, 8; expt 3, 3; expt 4, 97; expt 5, 8; expt 6, 17; expt 7, 3; expt 8, 8; expt 9, 111; expt 10, 100; expt 11, 50; expt 12, 87; expt 13, 129; expt 14, 325; expt 15, 250. In experiments 14 and 15, 0.8 mg of charcoal was added to a mixture of 0.01 ml of the purified defective particles, suspended in saline, plus 0.09 ml of gene 10 extract. This kept the charcoal to bacterial extract ratio similar to that in other experiments. The relative complementation given is ratio of net complementation in the presence of the charcoal to the net complementation in the absence of charcoal (7).

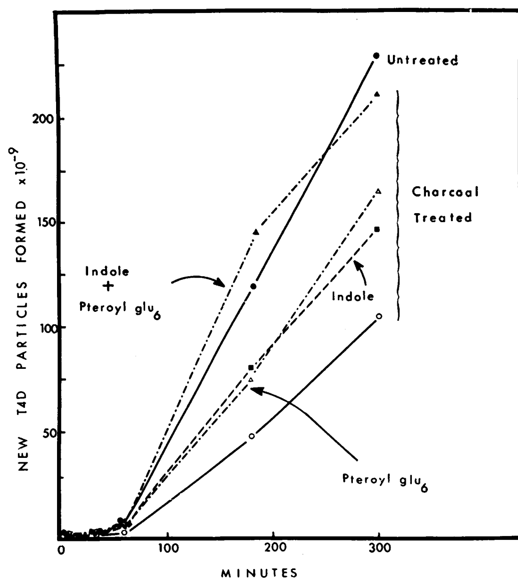

FIG. 5. *Effect of pteroyl hexaglutamate (pteroyl glu₆) and indole on in vitro complementation between gene 7 and gene 10 extracts pretreated with charcoal. The added pteroyl glu₆ was at a final concentration of 9.0 × 10⁻⁶ M, and the indole was at 1.4 × 10⁻⁴ M. These two compounds were added as concentrated solutions and amounted together to only 10% of the final reaction volume. The treatments and other procedures are given in Materials and Methods.*

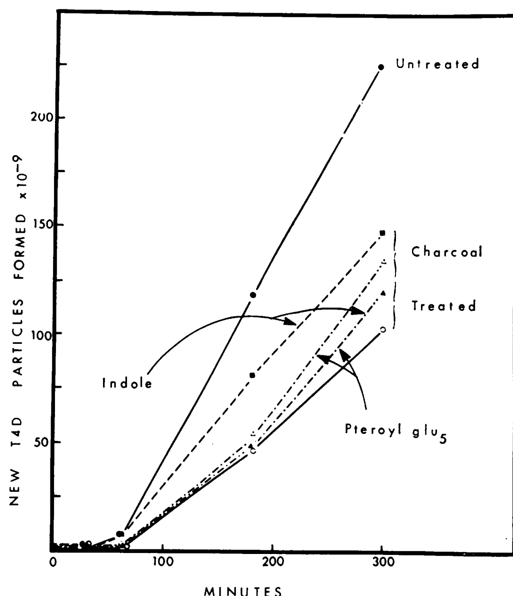

FIG. 6. *Effect of pteroyl pentaglutamate (pteroyl glu₅) and indole on in vitro complementation between gene 7 and gene 10 extracts pretreated with charcoal. The added pteroyl glu₅ was at a final concentration of 9.0 × 10⁻⁶ M, and the indole was at 1.4 × 10⁻⁴ M. These two compounds were added as concentrated solutions and amounted together to only 10% of the final reaction volume. The treatments and other procedures are given in Materials and Methods.*

tamate plus indole completely restored the ability of these extracts to form new T4D particles. On the other hand, both synthetic pteroyl pentaglutamate (Fig. 6) and pteroyl heptaglutamate (Fig. 7) at 9.0×10^{-6} M were either inactive or inhibitory to complementation. Further, in contrast to the results with pteroyl hexaglutamate, both the penta- and hepta- derivatives in the presence of indole inhibited complementation. Higher concentrations of pteroyl hexaglutamate did not stimulate the assembly rate above that shown in Fig. 5 for 9.0×10^{-6} M. Final phage tails contain the dihydro form of this compound, and the added synthetic compound presumably must first be reduced to the "dihydro" form; any excess unreduced compound is inhibitory (see Fig. 1). This feature of the extracts prevented any testing of the possibility that pteroyl hexaglutamate in the absence of indole could completely restore the level of complementation.

DISCUSSION

The use of activated charcoal to remove selectively the folate conjugates from extracts of bacteria infected with T4D amber mutants has permitted the direct demonstration that petroyl hexaglutamate stimulates assembly of the tail plate. Since the pteroyl hexaglutamate is known to be a component of phage substructure consisting of the tail tube and the tail plate, it can be proposed that this effect of the hexaglutamate is due to its incorporation into the phage structure. Further, since dihydrofolate reductase is also directly involved in tail plate assembly, and the inhibition of assembly by aminopterin and by charcoal map at the same site (7), it seems probable that the reductase plus the bound pteroyl hexaglutamate are incorporated together into the tail plate. Based on what is known of the sequence of reactions involved in tail plate assembly (Edgar, *personal communication*), the addition of this enzyme plus cofactor to the other components may be the final step in the formation of the tail plate.

These experiments do not permit a direct determination of the number of folate molecules needed for assembly, but it is obvious that the rate of assembly is directly dependent on the concentration of the folate conjugate. Each infected bacterium contains about 400,000 folate molecules (5), of which a maximum of 10% (6)

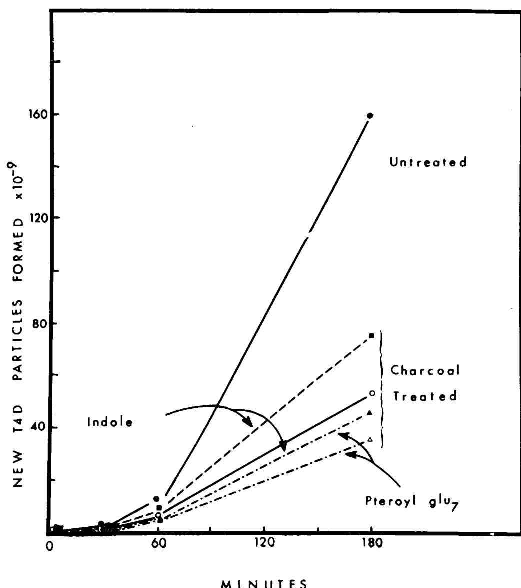

FIG. 7. *Effect of pteroyl heptaglutamate (pteroyl glu$_7$) and indole on in vitro complementation between gene 7 and gene 10 extracts pretreated with charcoal. The added pteroyl glu$_7$ was at a final concentration of 9.0×10^{-6} M, and the added indole was at 1.4×10^{-4} M. These two compounds were added as concentrated solutions and amounted together to only about 10% of the final reaction volume. The treatments and other procedures are given in Materials and Methods.*

or 40,000 are dihydropteroyl hexaglutamates. The formation of 300 phage particles each containing possibly six folates (6) would only require 1,800 molecules out of those present in the cell. This large excess raises the question of whether, in addition to participating in and influencing the rate of phage tail plate assembly, the phage-induced folate compound might play some other role in the formation of other viral components.

Twelve T4D genes, 5, 6, 7, 8, 10, 25, 26, 27, 28, 29, 51, and 53, have been identified so far as being required for tail plate formation (1, 2, 11). One or more of these genes or an as yet unidentified gene must be responsible for inducing the formation of the phage pteroyl hexaglutamate. In view of the chloramphenicol sensitivity of pteroyl hexaglutamate formation (6), it appears that a new enzyme(s) must be formed. The mechanism of the action of such an enzyme is not certain, because the unregulated stepwise addition of glutamate residues to the host dihydropteroyl triglutamate might well result in the formation of significant amounts of pteroyl tetraglutamate, pentaglutamate, and possibly heptaglutamate, all of which would inhibit phage assembly. Other biosynthetic mechanisms for the formation of the pteroyl hexaglutamate deserve consideration. In any case, the induction of an enzyme to make a small critical molecule for viral assembly, such as pteroyl hexaglutamate, represents a unique and unexpected expression of the viral genome.

ACKNOWLEDGMENTS

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