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Bacteriophage Tail Components

II. Dihydrofolate Reductase in T4D Bacteriophage

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The protein component of the T-even bacteriophage coat which binds the phage-specific dihydropteroyl polyglutamate has been identified as the phage-induced dihydrofolate reductase. Dihydrofolate reductase activity has been found in highly purified preparations of T-even phage ghosts and phage substructures after partial denaturation. The highest specific enzymatic activity was found in purified tail plate preparations, and it was concluded that this enzyme was a structural component of the phage tail plate. Phage viability was directly correlated with the enzymological properties of the phage tail plate dihydrofolate reductase. All reactions catalyzed by this enzyme which changed the oxidation state of the phage dihydrofolate also inactivated the phage. Properties of two T4D dihydrofolate reductase-negative mutants, *wh*1 and *wh*11, have been examined. Various lines of evidence support the view that the product of the *wh* locus of the phage genome is normally incorporated into the phage tail structure. The effects of various dihydrofolate reductase inhibitors on phage assembly in in vitro complementation experiments with various extracts of conditional lethal T4D mutants have been examined. These inhibitors were found to specifically block complementation when added to extracts which did not contain preformed tail plates. If tail plates were present, inhibitors such as aminopterin, did not affect further phage assembly. This specific inhibition of tail plate formation in vitro confirms the analytical and genetic evidence that this phage-induced "early" enzyme is a component of the phage coat.

The finding that T-even phage infection induced the formation of an unusual dihydropteroyl polyglutamate which was then incorporated into the phage tail structure (16) leads directly to the problem of identifying the phage protein binding this folate derivative. It has been pointed out by Baker that folate derivatives, and especially those with several glutamyl residues, can form extremely stable complexes with proteins (4). However, the properties and specific location of the phage dihydropteroyl polyglutamates suggest that a specific phage protein may be involved. Among the virus-induced proteins formed after infection which have a high affinity for dihydrofolate is the enzyme dihydrofolate reductase (20, 22). We have reported briefly that dihydrofolate reductase activity is present in preparations of T-even phage ghosts (Verses et al., Bacteriol. Proc., p. 163, 1968), and evidence will be presented now that the enzyme is a component of the phage tail plate.

The conclusion that this enzyme is a phage tail plate structural component is so unexpected that, in addition to its presence and specific location, three additional confirmatory experiments have been carried out. (i) It has been found that the enzymological properties of dihydrofolate reductase of whole phage are correlated with phage viability; changes in the oxidation state of the phage dihydrofolate result in phage inactivation. (ii) The phage gene, *wh*, for dihydrofolate reductase has been found to normally control the structure of the tail plate as judged by changes in the enzymatic, immunological, and physical properties of phage *wh* mutants. (iii) In vitro assembly of T4D, using the technique of in vitro complementation of Edgar and his colleagues (7, 14, 18, 27), has been found to be sensitive to the presence of inhibitors of dihydrofolate reductase. The folate antagonists appear to be the inhibitors of phage tail plate assembly.

MATERIALS AND METHODS

Preparation and purification of bacteriophage stocks, ghosts, and substructures, and extracts of Escherichia coli B infected with T4D amber mutants. Most of the biological materials and methods were identical to those used in the accompanying paper (16). Various T4D amber mutants were obtained from R. S. Edgar and were used in addition to those given earlier.

These mutants, which do not form tail plates under restrictive growth conditions (27), were N102 (gene 6), B16 (gene 7), B255 (gene 10), N67 (gene 25), N120 (gene 27), A452 (gene 28), B7 (gene 29), and S29 (gene 51). The amber phage mutants were grown on the permissive host, *E. coli* CR63, and were purified by standard procedures. Extracts of the nonpermissive host, *E. coli* B, infected with amber mutants were prepared by procedures given by Edgar and Wood (7) and by King (14). They were stored at −70 C and used in in vitro complementation reactions in the usual manner. Net fold increase in phage titer after complementation was calculated as the final titer minus the original titer divided by the original titer. The relative complementation in the presence of inhibitors was the ratio of the net fold increases (D. Shapiro and L. M. Kozloff, J. Mol. Biol., *in press*).

The origin and properties of T4D*wh*1, a point mutant in the *wh* gene, have been described by Hall et al. (10). They backcrossed this mutant twice with wild-type T4D and we backcrossed it two more times against wild-type T4D. It is presumably identical to wild-type T4D in every gene except *wh*. Measurements of dihydrofolate reductase activity after infection with T4D*wh*1 confirmed that *wh*1 does not cause any increase in dihydrofolate reductase activity after infection of either *E. coli* B or *E. coli* CR63.

T4D*wh*11, obtained from D. H. Hall (9), is an amber mutant in the *wh* gene. Stocks of this amber mutant were grown on the permissive strain of *E. coli* CR63 (designated T4*wh*11 (Cr63) or on the nonpermissive strain of *E. coli* B (designated T4D*wh*11 (B). Hall's (9) observations on the increase in dihydrofolate reductase activity after infection of *E. coli* CR63, but not after infection of *E. coli* B, were confirmed. Additional evidence on the nature of this amber *wh* mutant is presented later.

Enzymatic assays. Dihydrofolate reductase was assayed microspectrophotofluorometrically by following the decrease in fluorescence of reduced nicotinamide adenine dinucleotide phosphate (NADPH). The reaction mixture was similar to that used in the standard spectrophotometric assay of Mathews and Sutherland (22) and contained 5 μmoles of phosphate buffer (*p*H 7.5), 1.2 μmoles of NADPH, and various amounts of protein in a final volume of 0.135 ml. After allowing 3 min for temperature equilibration, the NADPH was added and the initial rate of change of the NADPH fluorescence was measured. The exciting light was 340 nm and the emitted light was 456 nm. The Aminco-Bowman spectrophotofluorometer was modified by placing a heating element and a thermister in the cuvette holder support and connecting it to a proportional temperature controller (Athena, model 51). Temperature was readily maintained at 37 C. In some experiments, the change in fluorescence was recorded with a Sargent recorder.

The fluorometric method could readily detect amounts of enzymatic activity 1/10 to 1/15 of that measurable in the standard spectrophotometric assay (22). However, the fluorometric enzyme activity values were not constant from day to day and therefore absolute rate measurements or standard units of enzyme activity were not calculated. All values for enzymatic activity were relative and were compared to each other on a given day.

T4D-induced dihydrofolate reductase was partially purified by using a modification of the procedure of Mathews and Sutherland (22). The sonic extract of T4D-infected *E. coli* B was clarified, treated with streptomycin and ammonium sulfate (0 to 60%), and chromatographed on a calcium phosphate column; the peak fraction was used for the immunological experiments.

Thymidylate synthetase activity was also measured by a microfluorometric modification of the optical method of Mathews and Cohen (21), in which the rate of conversion of tetrahydrofolate to dihydrofolate was followed. The assay mixture contained 5.3 μmoles of tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.4), 6.4 μmoles of 2-mercaptoethanol, 0.13 μmoles of ethylenediaminetetraacetic acid (EDTA), 210 μmoles of formaldehyde, 3.2 μmoles of magnesium chloride, 0.0027 μmoles of tetrahydrofolic acid, 0.0067 μmoles of deoxyuridine-5-phosphate, and various amounts of enzyme in a final volume of 0.157 ml. The temperature was maintained at 37 C, the exciting light was at 317 nm, and the emitted light was measured at 420 nm.

Disruption of phage with urea or formamide to release dihydrofolate reductase activity. Ghost preparations were treated with urea in the following manner. The ghosts (usually a total of 5×10^{12} to 10×10^{12} particles) were initially incubated for 5 min at room temperature in 2.3 M urea in the presence of 4×10^{-5} M dihydrofolate, 1.5×10^{-4} M NADPH, 0.15 M mercaptoethanol, and 0.075 M phosphate at *p*H 7.5. A solution of 8 M urea was then added to bring the final urea concentration to 6 M, and the solution was incubated for 15 min more at room temperature and then for an additional 45 min at 37 C. The solution was then stored at −20 C.

Reagent grade formamide (25.2 M) was added to suspensions of phage ghosts in the presence of 0.05 M phosphate buffer (*p*H 7.5), 0.02 M mercaptoethanol, and 5×10^{-5} M dihydrofolate. The amount of formamide required to liberate the enzymatic activity was found to vary sharply with the concentration of ghost protein. At concentrations above those found to be optimal, enzymatic activity was lost. For solutions containing a final concentration of 2 mg/ml (1.3×10^{13} particles/ml), 1.4 M formamide was necessary; for solutions containing 4 mg/ml, 2.3 M formamide was required; and for solutions containing 6 mg/ml, 3.2 M formamide was required for optimum release. It was also necessary to incubate the formamide-ghost suspensions at 37 C for 5 min to complete the liberation of the activity.

Inactivation of bacteriophages by pyridine nucleotides. It was necessary to incubate solutions containing reduced pyridine nucleotides under anaerobic conditions to prevent air oxidation. In the initial experiments, the reaction was carried out in evacuated 10-ml Thunberg tubes. The solution in the bottom of the tube contained 50 μmoles of phosphate buffer, (*p*H 7.5), 3 mg of gelatin dissolved in 0.5 ml of 0.9% NaCl, and approximately 10^{11} viable phage particles.

Weighted amounts of solid NADPH [or reduced nicotinamide adenine dinucleotide (NADH)] were placed in the side arm. The tubes were evacuated, mixed, and incubated at 37 C in a water bath. The reaction was stopped by the addition of 10 volumes of cold tryptone broth. In later experiments, it was found that incubations could be successfully carried out in 3.0-ml tubes with mineral oil layered over the solution. Incubation of phage with oxidized pyridine nucleotides such as nicotinamide adenine dinucleotide phosphate ($NADP^+$) or nicotinamide adenine dinucleotide (NAD^+) was carried out in 3.0-ml tubes without a mineral oil top layer. In these experiments in which the change in phage titer was not large, the number of dilution steps was kept to a minimum, and at least 400 plaques were counted per assay.

Preparation of antisera to T4D tail plates. Approximately 20 μg of T4D tail plates, taken from the peak tube of the second sucrose gradient of the purification procedure described in the accompanying paper [*see* Fig. 6C (16)], plus 50% Freund's adjuvant in a total volume of 0.2 ml was injected into the popliteal gland of a 2-kg New Zealand white rabbit. Three weeks later, 40 μg of tail plates in 0.4 ml of 10% Freund's adjuvant was injected intramuscularly. Serum was obtained at various times and assayed for the killing titer for T4D by standard procedures; at 6 weeks after the first injection, the K value for inactivation was about 300 and it stayed at this level for several months.

Chemical and other materials. Most of the reagents were similar to those employed in the accompanying paper (16). Chemically and enzymatically reduced NADPH, enzymatically reduced NADH, $NADP^+$ NAD^+, tetrahydrofolate, aminopterin, amethopterin, and dihydrofolate were from Sigma Chemical Co. Deoxyuridine-5-monophosphate was from Calbiochem. The irreversible inhibitor of dihydrofolate reductase, compound 15f (a *p*-phenyl propionyl derivative of 4,6 diamino-1,2 dihydro-2,2-dimethyl-1 phenyl-*S* triazine with a terminal sulfonyl fluoride), was a gift of B. R. Baker (5).

RESULTS

Dihydrofolate reductase activity in disrupted phage and phage substructure preparations. No dihydrofolate reductase activity could be detected in preparations of phage ghosts, even with the sensitive spectrophotofluorometric method, unless the preparations were first treated with a protein denaturant such as urea or formamide (Fig. 1). Maximal dihydrofolate reductase activity was liberated in 30 min in 6 M urea at 37 C and then remained constant. If the urea concentration was only raised to 3 M, a small fraction of the activity found after 6 M treatment was released. In the absence of added dihydrofolate

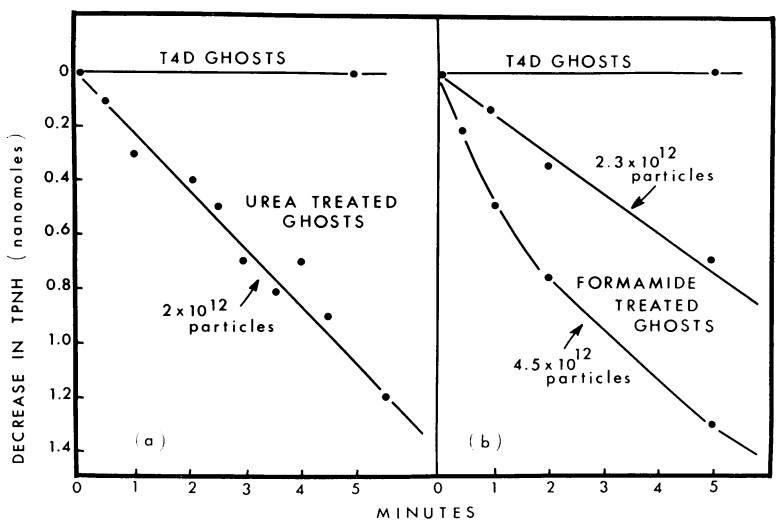

FIG. 1. *Dihydrofolate reductase in T4D ghost preparations. (a) Ghosts were treated with 6 M urea as described. Samples (0.1 ml) equal to 2 × 10^{12} ghost particles were then added to the cuvette and then 5 μmoles of phosphate buffer (pH 7.5), 6.5 nmoles of dihydrofolate, 1 μmole of mercaptoethanol, and finally 22 nmoles of NADPH (TPNH) were added in a final volume of 0.150 ml. The decrease in NADPH fluorescence at 29.5 C was then determined. (b) The different amounts of T4D ghost particles were treated with different concentrations of formamide; 2.3 × 10^{12} particles were treated for 5 min at 37 C with 1.8 M formamide in a volume of 0.12 ml, whereas the sample containing 4.5 × 10^{12} particles required a concentration of 3.15 M formamide (in a volume of 0.125 ml). The incubation with the formamide was carried out in the presence of 6 nmoles of dihydrofolate and 7.5 μmoles of mercaptoethanol, and the reaction was started by the addition of 0.01 ml of a solution containing 30 nmoles of NADPH. The decrease in fluorescence was measured at 37 C.*

during treatment, 6 M (or 3 M) urea destroyed all activity. Formamide treatment was also found to liberate enzymatic activity from ghost preparations. The amount of formamide required to release maximal enzymatic activity depended on the amount of ghost protein present; excess formamide destroyed all activity. The amount of dihydrofolate reductase liberated, as determined from the initial reaction rate, was proportional to the number of ghost particles used in the assay system. Relative activities were always compared on the same day by using identical reagents. The formamide treatment was quicker and somewhat more reproducible than the urea treatment, but appeared to liberate less activity.

Although chicken liver dihydrofolate reductase activity is stimulated by treatment with urea or formamide (13), no similar effect on the phage-induced enzyme has been found (22), and the effect of urea and formamide on phage ghosts is probably due to the disruption of its complex quaternary structure and the release of a buried or concealed enzyme.

Almost identical levels of dihydrofolate reductase activity were also found in urea-disrupted T2L ghost preparations. In view of the similarities of dihydrofolate content of all of the T-even phages (16) and the similarities of the reaction with NADPH of all of the T-even phages (*see below*), it seems likely that all T-even phage preparations contain dihydrofolate reductase. Major attention was paid to T4D because of the availability of both purified T4D tail substructures (16) and of two dihydrofolate-minus mutants of T4D.

Highly purified T4D tail substructures obtained from lysates of amber mutants were also analyzed for their dihydrofolate reductase activity. No activity was found in any substructures until they were disrupted with 6 M urea. The correlation between enzyme activity and sedimentation behavior for one particular tail substructure, tail tube plus sheath plus plate, is shown in Fig. 2. Similar data were obtained for the other tail structures, and it is apparent that the enzyme behaves as a genuine tail component during zonal centrifugation.

The results shown in Table 1 indicate that tail substructures are enriched for dihydrofolate reductase and that the tail is the most probable site of enzyme location on the whole phage ghost. Since the tail plate is 2 to 3% of the total mass of the ghost, essentially all of the phage ghost enzymatic activity can be accounted for by the activity in its tail plate. It should also be emphasized that the phage-specific dihydropteroyl polyglutamate appears to be located at the juncture between the tail plate and the tail tube (16).

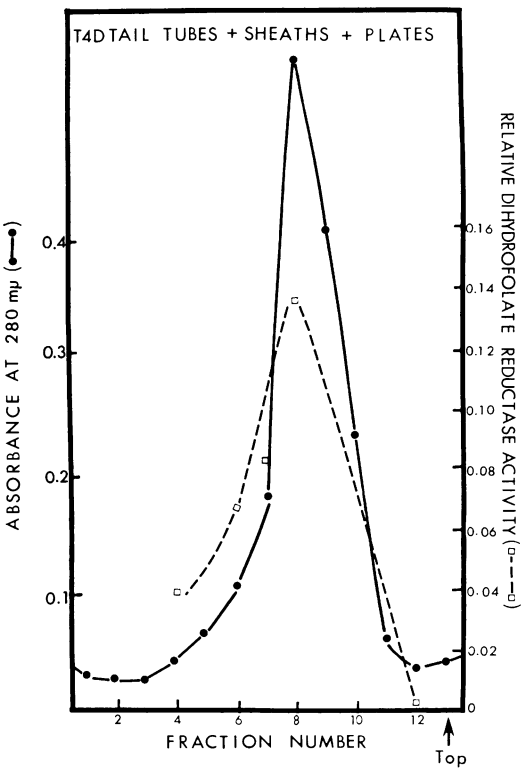

FIG. 2. *Sedimentation properties of the T4D tail substructure consisting of the tail tube plus sheath plus plate and its associated dihydrofolate reductase activity. The purified tail substructure was subjected to zonal centrifugation (16), fractions were collected and dialyzed, and the absorbance was determined for each fraction. A 0.1-ml amount of tail substructure solution of the absorbance given in the figure was treated with 6 M urea (final volume during treatment, 0.18 ml) and then 0.05 ml was taken for the standard fluorometric assay.*

Thymidylate synthetase, for which dihydrofolate is a cofactor, is also induced by phage infection (21), and the possibility was examined that this protein might also participate in dihydrofolate binding. Phage ghosts and purified tail plates (equivalent to 10^{12} particles in the assay cuvette) were assayed for thymidylate synthetase activity by using a highly sensitive spectrophotofluorometric method. No activity was found in undisrupted preparations, and disruption by 3 M or 6 M urea (using deoxyuridine monophosphate as a protective cofactor) did not liberate any activity.

Phage viability and enzymological properties of phage dihydrofolate reductase. Although there is strong evidence that a phage-specific dihydropteroyl polyglutamate is an integral part of the tail of the virus particle (16), it was still possible

TABLE 1. *Dihydrofolate reductase activity*

T4D component	Expt I		Expt II	
	Specific activity[a]	Relative activity	Specific activity[a]	Relative activity
Ghosts	0.014	1	0.005	1
Tail tubes + sheaths + plates	0.023	2	0.011	2
Tail tubes + plates	0.11	8	0.043	9
Tail plates	0.56	40	0.11	22

[a] After 6 M urea treatment, defined as the initial rate of decrease of NADPH fluorescence in arbitrary units per milligram of protein. Usually 50 to 100 μg of protein was used per assay. All dihydrofolate reductase assays for each experiment were carried out on a single day. Separate preparations of T4D ghosts and tail substructures were used for the two experiments.

that the dihydrofolate reductase activity found localized on tail plates might be an artefact. Therefore, properties of the phage enzyme were examined to determine whether its presence and activity were essential to phage viability. Since normally this enzyme catalyzes the reaction NADPH + dihydrofolate $\rightleftarrows$ $NADP^{++}$ tetrahydrofolate, the effects of NADPH, NADH, $NADP^{+}$, and NAD^{+} on various phages were examined.

It was found that all of the T-even phages examined were inactivated upon incubation with NADPH (Table 2). At *p*H 7.5, there was little inactivation of T4D for the first 2 to 3 hr (Fig. 3a, b, and c) and then the rate of inactivation appeared logarithmic. Even when the concentration of NADPH was increased, there was always an initial lag in the inactivation curve. This suggests that inactivation requires reduction by more than one NADPH molecule. This would be in agreement with the fact that T4D contains several molecules of dihydrofolate. Although the initial lag made it impossible to calculate specific rate constants, the reaction rate (as judged from the logarithmic part of the curve) was very low with less than 0.001 M NADPH, proportional between 0.001 and 0.005 M NADPH, and maximal at 0.006 M or higher.

The properties of T4D phage inactivated by NADPH were examined. All of the phage particles appeared morphologically normal in electron micrographs. However, the ability of these phage particles to both adsorb to and kill their host cells at 37 C was greatly reduced. Accurate measurements of adsorption and host cell killing were difficult to make because of the effect of O_2

TABLE 2. *Inactivation of T-even bacteriophages by NADPH*[a]

Phage	Plus 0.006 M NADPH (% inactivated)
T2L	58
T2H	12
T4B	30
T4D	57
T4D*wh*1	50

[a] After 6 hr at 37 C, at *p*H 7.5 in evacuated Thunberg tubes.

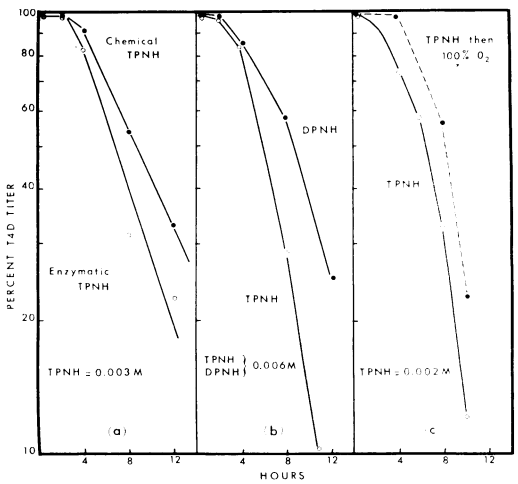

FIG. 3. *Inactivation of T4D by various reduced pyridine nucleotides. (a) T4D was incubated with two kinds of NADPH (TPNH) from P. L. Biochemicals. (One prepared by chemical reduction and one by reduction with isocitric acid dehydrogenase.) The incubation was carried out in phosphate buffer at pH 7.5 in Thunberg tubes in a 37 C water bath. (b) T4D was incubated as in (a) by using enzymatically prepared NADPH and NADH (DPNH). (c) T4D was incubated with NADPH under oil. At various times, 0.1-ml samples were removed, added to 2.0 ml of broth, and then treated with 100% O_2 at 37C for 10 min.*

reversal at 37 C (Fig. 3c). These results do indicate that NADPH reduction is probably affecting tail function.

The requirement for a stereospecific form of the reduced NADPH is shown in Fig. 3a. Enzymatically prepared NADPH, presumably in the β configuration [by analogy to the known form of the NADH which also inactivated phage (Fig. 3b)], is considerably more active than chemically prepared NADPH which is an equimolar mixture of the α and β stereoisomers. This stereospecificity supports the view that viral inactivation is due to an enzyme-catalyzed reduction and not to

some extraneous property of the NADPH preparation.

It has been reported by Mathews and Cohen (20) that the virus-induced dihydrofolate reductase can react with NADH as well as NADPH, although at equivalent concentrations NADH is only ¼ as active. As shown in Fig. 3b, enzymatically prepared NADH (in the β configuration) is about ⅓ as active as enzymatically prepared NADPH in inactivating T4D. This property supports the view that the enzyme in phage tail plates may be the virus-induced enzyme.

The reversibility of NADPH inactivation by both O_2 and, more significantly, by large quantities of one of the presumed products of the enzymatic reaction was also examined. It was found that treatment with 100% O_2 at 37 C revived a significant fraction of the inactivated virus particles (Fig. 3c). At 4 C, oxygen treatment did not reactivate any virus particles. The total phage reactivated was a function of the degree of inactivation. It seems likely that there are multiple sites for reduction and that only after several of them have reacted is the phage permanently inactivated.

It was also found that the initial phage concentration greatly influenced the amount of phage inactivated by NADPH. For example, with 0.004 M NADPH and at an initial phage concentration of 10^{12} particles per ml, there was relatively little inactivation (about 50%), whereas at 2×10^8/ml there was 99.9% inactivation in 24 hr. These results are in agreement with the concept that NADPH/$NADP^+$ determines the degree of inactivation. At higher phage concentrations, there would be much more $NADP^+$ formed which would inhibit further inactivation.

Although Mathews (19) was unable to detect spectrophotometrically the reversibility of the reduction of dihydrofolate by NADPH with purified phage-induced dihydrofolate reductase, the oxidation of tetrahydrofolate by $NADP^+$ back to dihydrofolate has been shown with dihydrofolate from other sources (13). T4D and T4D*wh*1 were first inactivated by NADPH and then diluted into a high concentration of $NADP^+$ so that the ratio of $NADP^+$ to NADPH was 25–50 to 1 (Table 3). Under these circumstances, the phage titer largely returned; for example, in experiment 1, in which there was 60% inactivation by NADPH, all of the inactivated virus was reactivated upon subsequent treatment with $NADP^+$. When a lower initial concentration of T4D was incubated with NADPH (Fig. 4), there was greater phage inactivation (98%). Upon subsequent treatment with $NADP^+$, the phage titer increased 2.5-fold but most of the

TABLE 3. *Reactivation by $NADP^+$ of T4D and T4Dwh1 inactivated by NADPH*[a]

Expt	Phage	Initial NADPH concn	Incubation time	Phage titer after incubation	$NADP^+$ concn	Phage titer after incubation with $NADP^+$
			hr			
I	T4D	None	7	7.0×10^8	None	7.0×10^8
		0.0023 M	7	2.7×10^8	0.011 M (and 0.0002 M NADPH)	7.0×10^8
II	T4D	None	16	4.0×10^{10}	None	4.0×10^{10}
		0.005 M	16	1.7×10^{10}	0.04 M (and 0.001 M NADPH)	2.7×10^{10}
III	T4D	None	10	1.6×10^9	None	1.6×10^9
		0.004 M	10	0.35×10^9	0.1 M (and 0.0004 M NADPH)	0.8×10^9
IV	T4D*wh*1	None	10	1.4×10^9	None	1.4×10^9
		0.004 M	10	0.40×10^9	0.01 M (and 0.0004 M NADPH)	0.66×10^9
V	T4D*wh*1	None	7	1.2×10^9	None	1.2×10^9
		0.0023 M	7	0.5×10^9	0.001 M (and 0.0002 M NADPH)	0.9×10^9

[a] In all of these experiments, the original NADPH inactivation was carried out at 37 C under oil in 0.05 M phosphate buffer (*p*H 7.5). For reactivation, 1 volume of the phage-NADPH solution was added to 5 to 10 volumes of the appropriate $NADP^+$ solution, and this second solution was incubated in air for 16 hr at 37 C.

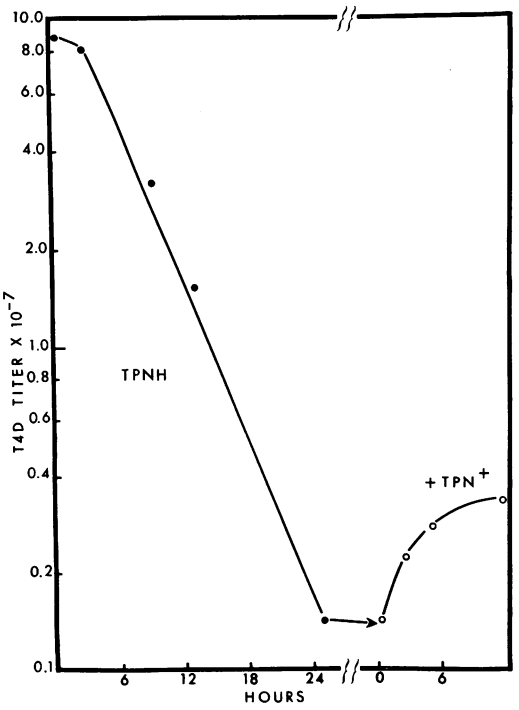

FIG. 4. *T4D phage inactivation by NADPH (TPNH) and reactivation by NADP+ (TPN+). T4D was incubated under oil at 37 C with 0.0036 M NADPH in 0.005 M phosphate buffer at pH 7.5 containing 2 mg of gelatin per ml. Samples were removed at various times, diluted, and assayed. The 25-hr sample was diluted 1 to 4,000 into a similar phosphate buffer containing 0.036 M NADP+ yielding a [NADP+/NADPH] of 4 × 10⁴. This solution was then incubated further in air at 37 C.*

phage particles were apparently irreversibly inactivated. This finding is similar to that observed with reversal of NADPH inactivation by 100% O_2 and supports the view that there are multiple sites for inactivation and that only after several of them have reacted is the phage permanently inactivated.

In control experiments, NAD^+, at concentrations equivalent to the $NADP^+$ concentrations used, was unable to reactivate NADPH-treated T4D (or T4D*wh*1) particles. An attempt was also made to determine whether $NADP^+$ could reactivate phage inactivated by reduction with formamidine sulfinic acid (24). As expected, after chemical reduction, incubation with $NADP^+$ did not revive the inactivated particles.

Mathews and Sutherland (22) reported that they were unable to detect any ability of the phage-induced dihydrofolate reductase to reduce folate to dihydrofolate with NADPH as the reducing agent. However, dihydrofolate reductase from other sources are known to carry out this reaction (11, 12), and it has also been reported that enzymes from yeast and *Streptococcus* (3, 6) can readily catalyze not only the reduction of folate to dihydrofolate, but also the reversal of that reaction which is the oxidation of dihydrofolate by $NADP^+$. Presumably the reaction stops when only a fraction of the phage have been inactivated because of the accumulation of NADPH. Figure 5 shows that T4D is slowly inactivated by $NADP^+$ but not by NAD^+. When T4D inactivated by $NADP^+$ was treated with NADPH (NADPH/$NADP^+$ = 400), all of the phage were rapidly reactivated. Continued incubation of these reactivated particles with NADPH then, as predicted, again caused phage inactivation.

These effects of pyridine nucleotides on phage viability are summarized in Fig. 6. Although it has not been possible to identify either the "folate" or the "H_4-folate" presumably produced in these reactions, the similarity of these reaction patterns to the known reactions cata-

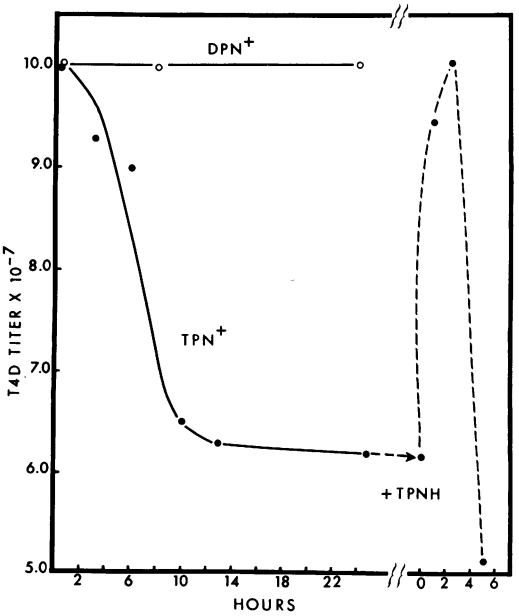

FIG. 5. *T4D phage inactivation by NADP+ (TPN+) and reactivation by NADPH (TPNH). T4D was incubated at 37 C in air with 0.036 M NaDP+ in 0.03 M phosphate buffer at pH 7.3 containing 2 mg of gelatin per ml. Samples were removed at various times, diluted, and assayed. The 25-hr sample was diluted 1 to 4,000 into a similar phosphate buffer containing 0.0036 M NADPH giving a [NADPH/NADP+] of 450. This solution was covered with oil and then incubated at 37 C.*

lyzed by dihydrofolate reductase (or folate reductase) is apparent. The correlation between the enzymological properties of dihydrofolate reductase (plus its bond dihydropteroyl polyglutamate) and phage viability indicates that the phage tail not only contains dihydrofolate reductase but that it can function catalytically to reduce and oxidize the phage-specific dihydrofolate. It should be noted here that previous experiments have demonstrated that phage viability depended critically on the oxidation state of this phage component (16). Further, these experiments with the pyridine nucleotides strongly support the earlier conclusion that dihydrofolate is buried or trapped in the phage tail structure. Since oxidation results in a loss of phage viability which can be reversed, this means that the folate product of this reaction must remain in situ.

Properties of T4Dwh1. The properties of two phage mutants, *wh*1 and *wh*11, neither of which induces production of active dihydrofolate reductase, were examined. No dihydrofolate reductase activity could be detected in intact or disrupted T4D*wh*1 preparations by the spectrophotofluorometric assay procedure. This indicates that the dihydrofolate reductase of normal T4D particles was coded for by the phage *wh* gene and raised the question of how a viable phage particle could be formed in a cell infected with a *wh*1 phage. Various properties of T4D*wh*1 particles suggested that these particles contained a protein very much like the phage-induced dihydrofolate reductase. (i) T4D*wh*1 is a point mutant in the *wh* gene and presumably produces an altered protein. (ii) T4D*wh*1 particles contained dihydropteroyl polyglutamates in normal amounts (16). (iii) T4D*wh*1 was inactivated by NADPH and reactivated by $NADP^+$ (Tables 2 and 3) at rates comparable to the reaction of wild-type T4D. These observations suggest that the *wh*1 phage mutant induced the formation of a protein which resembled the phage-induced dihydrofolate reductase in that it bound dihydropteroyl glutamyl compounds and also transferred electrons to and from pyridine nucleotides but lacked the ability to catalytically reduce *free* dihydrofolate. A protein with these properties could presumably also serve a structural role in the phage tail plate.

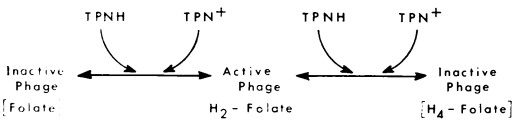

FIG. 6. *Effects of pyridine nucleotides on viability of T4D bacteriophage. TPNH (NADPH); TPN^+ ($NADP^+$).*

To determine whether T4D*wh*1 tail plates contained a protein similar to the T4D dihydrofolate reductase, immunological measurements of the presence of dihydrofolate reductase-like antigens in T4D and T4D*wh*1 were carried out. When antiserum prepared by injecting highly purified wild-type T4D tail plates was added to T4D, the phage were rapidly inactivated. Under standard conditions (2), the K value was 280 ($\pm$20). It was also found that antiserum to T4D plates markedly inhibited the enzymatic activity of partially purified T4D dihydrofolate reductase (Table 4). When the antiserum was preadsorbed with either T4D or T4D*wh*1 ghosts, similar amounts of antibody to dihydrofolate reductase were removed. It can be concluded that both T4D and T4D*wh*1 contain antigenic determinants, presumably on their tail plates, similar if not identical to those of the phage-induced dihydrofolate reductase.

Properties of T4Dwh11. The properties of a *wh* amber mutant, T4D*wh*11, were examined since it can produce viable phage not only after infection of the permissive strains of *E. coli* but also after infection of the nonpermissive strain of *E. coli*. Under these latter circumstances, no phage induction of dihydrofolate can occur. Nevertheless, it was found that T4D*wh*11(CR63) and T4D*wh*11(B) ghost particles *both* exhibited

TABLE 4. *Presence of dihydrofolate reductase-like antigens in T4D and T4Dwh1 bacteriophages*[a]

Substances added	Purified T4D virus-induced dihydrofolate reductase (% activity)	
	Expt I	Expt II
Normal rabbit serum	100	100
Antiserum to T4D-tail plates	46	67
Antiserum preadsorbed with T4D ghosts	61	82
Antiserum preadsorbed with T4D*wh*1 ghosts	67	82

[a] A 0.05-ml amount of antiserum to T4D tail plates, K = 289 (or normal rabbit sera) plus 0.1 ml of 0.05 M phosphate buffer (pH 7.5) was incubated at 25 C for 30 min with 0.05 ml of saline or T4D (or T4D*wh*1) ghosts equivalent to 1.4×10^{12} particles. These solutions were clarified at 20,000 $\times$ g for 2 hr. A 0.1-ml amount of the supernatant solution was incubated with 0.01 ml of purified T4D-induced dihydrofolate reductase for 60 min at 28 C. A 0.02-ml amount was then removed and assayed for dihydrofolate reductase activity spectrophotofluorometrically.

dihydrofolate reductase activity after treatment with formamide. The enzymatic activity per particle for both types of preparations of T4D-*wh*11 was identical to that obtained with preparations of wild-type T4D particles. It was also found that T4D*wh*11(CR63) and T4D*wh*11(B) were inactivated at identical rates by purified hog kidney conjugase (*see below*; Table 5), indicating that both phage types probably contained the phage-specific pteroyl conjugate.

These observations are those expected for T4D*wh*11(CR63), since *E. coli* CR63 possesses a suppressor for the amber mutation and will allow the synthesis of an enzyme very similar to the normal phage-induced dihydrofolate reductase. However, the presence of the same amount of enzymatic activity in preparations of T4D*wh*11(B) suggests that in the absence of phage-induced enzyme another dihydrofolate reductase must be incorporated into the phage structure. The only known source of this enzyme in *E. coli* B infected with T4D*wh*11 is the pre-existing host dihydrofolate reductase. This host enzyme has been partially characterized, especially with regard to its differences from the phage-induced enzyme (D. Shapiro and L. M. Kozloff, J. Mol. Biol., *in press*), and any phage containing this bacterial enzyme as part of its structure might be expected to have different properties from both wild-type T4D and T4D*wh*-11(CR63).

Some chemical and physical properties of these phage strains are shown in Fig. 7 and are summarized in Table 5. All experiments shown in Fig. 7 were repeated two or more times. Although T4D, T4D*wh*1, and T4D*wh*11(CR63)

TABLE 5. *Inactivation of T4D and T4Dwh strains by various treatments*

Strain	Treatment						
	Antisera[a]	Conjugase[a]	Heat[a]	*N*-methyl hydroxylamine	$Cd(CN)_3^-$	NADPH or NADH	$NADP^+$
T4D	280 ± 20	0.11 ± 0.01	0.14 ± 0.03	*See* Fig 6b	*See* Fig. 6c	*See* Fig. 8	*See* Fig. 4
T4D*wh*1	280 ± 20		0.14 ± 0.03	Identical to T4D	Identical to T4D	Identical to T4D	
T4D*wh*11(CR63)	290 ± 20	0.10 ± 0.01	0.14 ± 0.03	*Slower* than T4D	Identical to T4D	Identical to T4D	Identical to T4D
T4D*wh*11(B)	290 ± 20	0.11 ± 0.01	0.07 ± 0.02	Identical to T4D	*Faster* than T4D	*Faster* than T4D	*Not inactivated*

[a] The inactivation with antiserum is given as standard *K* values (2). The inactivation with purified conjugase (15) or heat (1) s given as the first-order reaction rate for the linear portion of the inactivation.

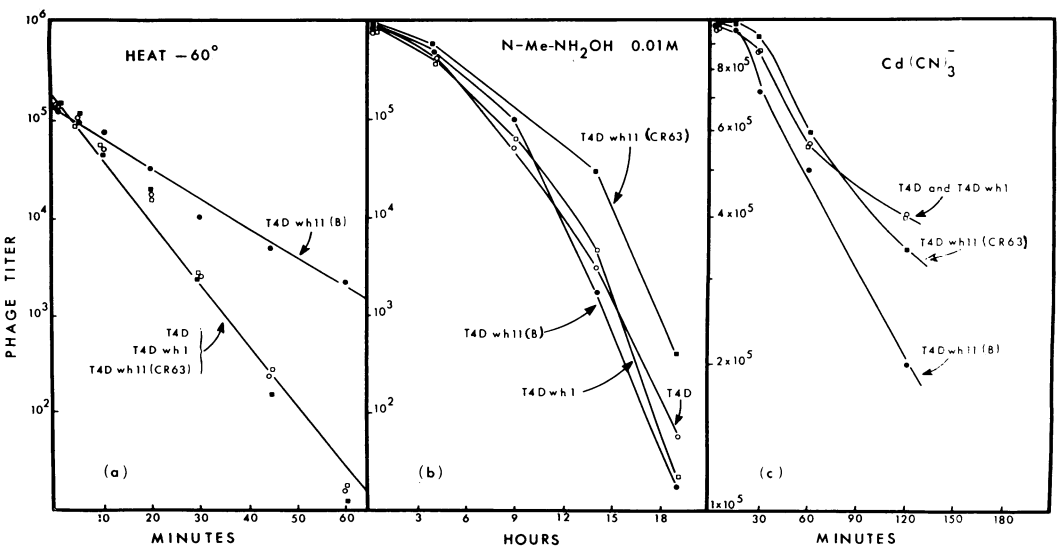

FIG. 7. *Physical and chemical properties of T4D and T4Dwh1 strains. (a) Heat inactivation was carried out as described by Adams (1); (b) inactivation by 0.01 M N-methyl hydroxylamine (8) was carried out in 0.02 M phosphate buffer (pH 6.6) containing 10^{-4} M $MgSO_4$ at 37 C; (c) the procedure was that described earlier (18).*

have identical susceptibilities to heat inactivation, T4D*wh*11(B) was markedly more resistant to heat inactivation. Further, T4D*wh*11(B) was more sensitive than the other three phages to inactivation by $Cd(CN)_3^-$, a reagent known to affect the phage tail tip (18). On the other hand, T4D*wh*11(CR63) was more resistant than the other three types of phages to inactivation by *N*-methyl hydroxylamine, a reagent which also reacts with the tail of the phage (18). This difference in susceptibility to *N*-methyl hydroxylamine indicates that suppression of this amber mutation by substitution of another amino acid (presumably a serine residue) for the original amino acid also changes the properties of the phage coat. It is apparent that the *wh*11 mutation does affect the structure of the phage tail and that the nature of the host in which the phage is assembled determines the physical and chemical properties of the phage produced.

The interaction of pyridine nucleotides with the different *wh*11 preparations was also examined. The reaction of T4D*wh*11(B) as compared to T4D, with NADPH and NADH at *p*H 7.3 and at *p*H 5.0, is shown in Fig. 8. (Both phage preparations were stable at either *p*H in the absence of the reduced nucleotide.) Under all conditions, T4D*wh*11 (B) was more sensitive to inactivation than was T4D. Unexpectedly, it was found that at *p*H 5.0 both phage types were inactivated about four times more rapidly than at *p*H 7.3. The typical initial lag found at *p*H 7.3 was not observed at *p*H 5.0, and the reaction appeared first order with time. Since the optimum *p*H for either the phage-induced or the host dihydrofolate reductase is *p*H 7.0 to 7.5, the rapid inactivation at *p*H 5.0 must represent a property of the quaternary structure of the phage tail plate.

The effect of $NADP^+$ on T4D*wh*11 strains as compared to T4D was also examined. T4D*wh*11-(CR63) was inactivated by $NADP^+$ at a rate equal to that shown for wild-type T4D in Fig. 5. On the other hand, T4D*wh*11(B) was not affected by $NADP^+$. These results clearly indicate that the dihydrofolate reductase in T4D*wh*11(B) particles must be different from that found in T4D*wh*11(CR63) particles (or in T4D particles).

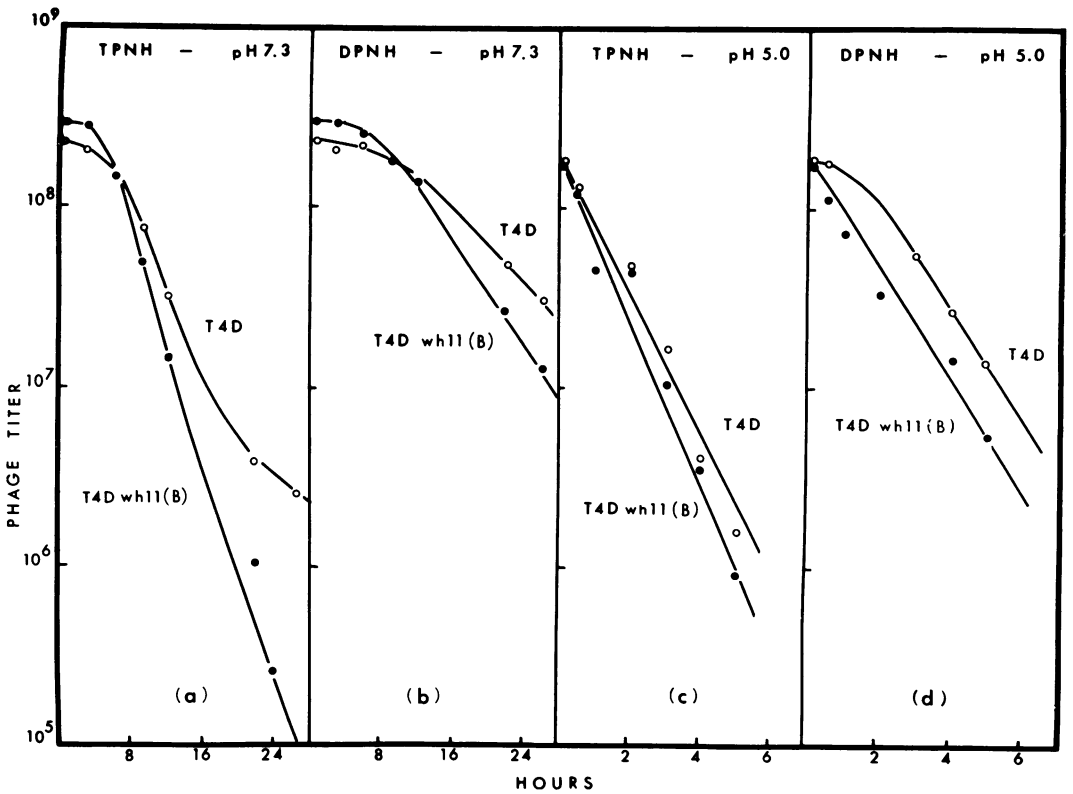

FIG. 8. *Inactivation of T4D and T4wh11 (B) by 0.003 M NADPH (TPNH) and 0.003 M NADH (DPNH). The phage were incubated with the reduced pyridine nucleotide in either 0.05 M phosphate buffer (pH 7.3) or 0.05 M acetate buffer (pH 5.0) under oil.*

Table 5 summarizes the properties of T4D and various T4D*wh* strains. It is apparent that the phage *wh* gene controls not only the production of dihydrofolate reductase but the phage tail structure as well, presumably because this enzyme is a tail component. The properties of T4D*wh*1 and T4D*wh*11(B) indicate that dihydrofolate reductase, or a protein structurally similar to dihydrofolate reductase, must be a component of all the T-even phage particles.

Inhibition of T4D tail plate assembly by dihydrofolate reductase inhibitors. The effects of inhibitors of dihydrofolate reductase on the in vitro assembly of T4D are shown in Fig. 9 and 10 and in Table 6. Extracts were made from *E. coli* B after infection with various amber mutants of T4D defective in different genes required for tail assembly (27). For example, the products of gene 7 and gene 10 are required for tail plate formation, and in vitro complementation between these two extracts allows first tail plate formation and then, after a number of additional reactions, formation of infective phage (27). Figures 9 and 10a show that aminopterin and amethopterin (11) are equally effective in inhibiting in vitro assembly between gene 10 and 7 extracts. As shown in Fig. 9a, the inhibition is dependent on the amount of inhibitor added. An irreversible inhibitor of dihydrofolate reductase, compound 15f described by Baker (5), and folate, which also has been reported to be an inhibitor of the phage-induced dihydrofolate reductase (D. Shapiro and R. M. Kozloff, J. Mol. Biol., *in press*), both inhibited complementation between gene 10 and 7 extracts. But compounds such as glutamate, *p*-amino-benzoyl glutamate, and uracil, all at 10^{-3} M, did not inhibit complementation.

The effect of dihydrofolate reductase inhibitors on complementation between various pairs of other extracts of T4D amber mutants is shown in Fig. 10b and 10c and in Table 6. Complementation between gene 10 and gene 53 was also sensitive to inhibitors (Fig. 10b). But assembly

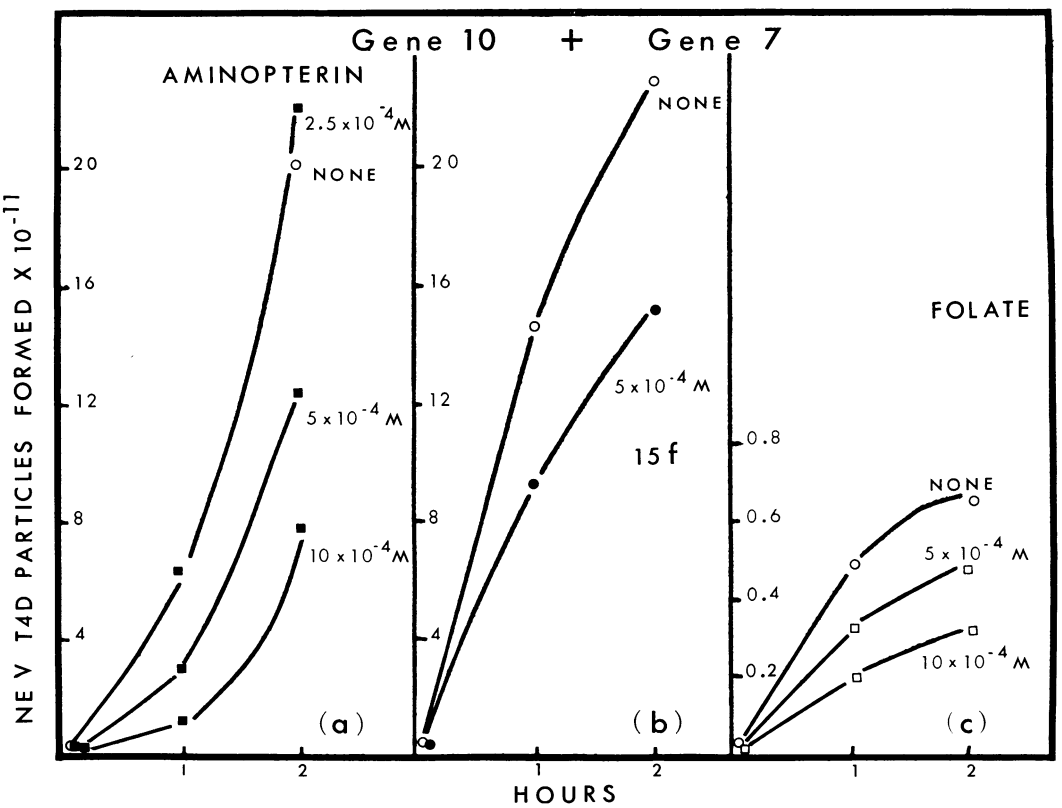

FIG. 9. *Inhibition of in vitro complementation between extracts made from amber T4D gene-infected E. coli B and amber T4D gene 7-infected E. coli B by three inhibitors of dihydrofolate reductase; aminopterin, compound 15f, and folate. The complementation reaction was carried out as described in Materials and Methods. 15f is the irreversible inhibitor of dihydrofolate reductase synthesized by Baker et al. (5).*

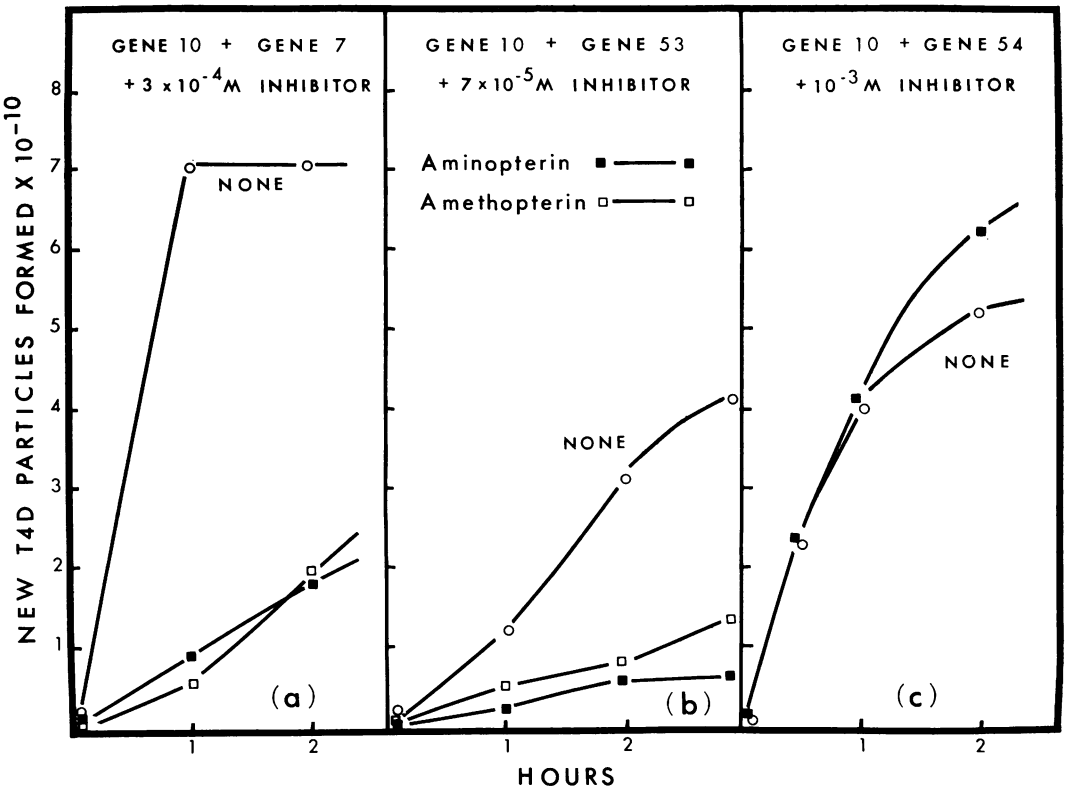

FIG. 10. *Inhibition by aminopterin and amethopterin of in vitro complementation between extracts of E. coli B made with different amber T4D mutants. The complementation was carried out as described in Materials and Methods.*

of T4D in mixtures of extracts of gene 10 and gene 54 was not inhibited even in the presence of 10^{-3} M aminopterin (Fig. 10c). It should be noted that gene 54 extracts contain preformed tail plates. The effect of 10^{-3} M aminopterin on the steps in T4D tail assembly is shown in Table 6. In all mutant pairs tested, aminopterin prevented complementation only if no tail plates were present in either of the extracts. These results indicate that only before dihydrofolate reductase has been incorporated into the complex tail plate is the inhibitor able to bind to this protein and prevent its use for phage tail assembly.

The concentrations of inhibitors necessary to prevent complementations, such as the 10^{-3} to 10^{-4} M shown in Fig. 9 and 10 and in Table 6, are much higher than the 10^{-7} to 10^{-8} M concentrations reported to be effective with the purified phage-induced or bacterial enzymes (19). However, these extracts are known to contain high concentrations of the phage-induced dihydropteroyl polyglutamate, and dialysis to remove these compounds inactivated the extracts. Under these circumstances, it is impossible to predict the concentration of inhibitor needed to compete with phage-induced compound to prevent assembly.

On the basis of the finding that all four inhibitors of dihydrofolate reductase tested, i.e., aminopterin, amethopterin, folate, and compound 15f, inhibited assembly, and on the finding that there is great specificity in the step in tail assembly which is inhibited, it can be concluded that dihydrofolate reductase is involved in the formation of phage tail plates.

DISCUSSION

The results of these studies and those in the accompanying paper (16) can be used to amplify the scheme of T4D tail assembly proposed by Wood et al. (27), as shown in Fig. 11. During normal T4D tail assembly, the product of the phage *wh* gene is incorporated into the tail plate. This product need not be fully active dihydrofolate reductase, as in the case of the assembly of T4D*wh*1, but it must be able to bind the phage-induced dihydropteroyl polyglutamate and probably must be able to react with NADPH. In a

TABLE 6. *Inhibition of T4D tail assembly reactions by aminopterin*

Expt	Most complex tail substructure present	Extracts[a]	Relative complementation with aminopterin
1	No recognizable tail substructure	10 + 7	0.4
2		10 + 7	0.4
3		10 + 7	0.2
4		10 + 26	0.4
5		10 + 6	0.1
6		10 + 53	0.6
7		7 + 26	0.4
8	Tail plate	7 + 48	1.0
9		7 + 54	0.9
10		10 + 54	1.6
11	Tail plate + tail tube	10 + 18	1.2
12	Tail plate + tube + sheath	54 + 23	1.0
13	Tail-fiberless particle	X4E + 23	0.8

[a] The variation in complementation between a given pair of extracts without added inhibitor on any day was ±20%. Equal portions of each extract and the control solution or the inhibitor solution were mixed and incubated at 30 C. In the control experiment (without inhibitor), either 10^{-3} M phosphate buffer at *p*H 7.4 or 10^{-3} M glutamate at *p*H 7.4 was added. Immediately after mixing and after 2 hr at 30 C, samples were taken, diluted in tryptone broth, and assayed on CR63. The net fold increase in phage titer in the absence of the inhibitor for each experiment was: experiment 1, 120; experiment 2, 54; experiment 3, 17; experiment 4, 3; experiment 5, 4; experiment 6, 14; experiment 7, 2; experiment 8, 126; experiment 9, 146; experiment 10, 36; experiment 11, 2; experiment 12, 200; and experiment 13, 35. The final concentration of aminopterin (adjusted to *p*H 7.4) was 10^{-3} M in all experiments, except for experiment 5 with extracts 10 + 6 when it was 3×10^{-5} M, and for experiment 6 with extracts 10 + 53 when it was 10^{-4} M.

most unusual case of an amber mutation, in the *wh* gene where no suitable phage protein is produced, another dihydrofolate reductase (most likely the host enzyme) is incorporated into the structure, producing a phage with altered phenotypic properties (a new type of host-dependent modification). The number of dihydrofolate reductase molecules incorporated per tail plate is very difficult to determine. The amount of activity liberated by urea or formamide is not too helpful, since there is no way to estimate either the efficiency of enzyme liberation or the amount

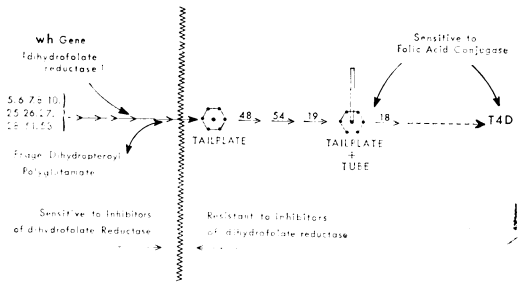

FIG. 11. *Tail assembly of bacteriophage T4D. The roles of the phage wh gene product, dihydrofolate reductase, and the binding of the phage-induced dihydropteroyl polyglutamate are indicated on the scheme put forth by Wood et al. (27).*

of enzyme which might be destroyed by the denaturing treatment. Based on the number of dihydropteroyl polyglutamates found in phage particles, there may be somewhere between two and six enzyme molecules per tail plate.

The phage-induced pteroyl polyglutamate can be added either before or after the tail plate is assembled. None of the products of the 14 genes (other than the *wh* gene) needed for tail assembly has been identified, and it is possible that one of these genes may induce the synthesis of the enzyme needed to form the phage pteroyl polyglutamate. The addition of tail tube to a tail plate containing the dihydropteroyl polyglutamate effectively traps the pteroyl polyglutamate. Further, tail and phage assembly is then no longer sensitive to dihydrofolate reductase inhibitors since these inhbitors cannot react with the appropriate site on the enzyme. It should be noted that the site for pyridine nucleotide binding, however, is available to added reduced or oxidized nucleotides and that the glutamate residues of the folate compound extend from the plate structure and are susceptible to hydrolysis by added folic acid conjugase.

Although dihydrofolate reductase and bound dihydropteroyl polyglutamate are essential elements of the phage tail structure, their role in the attachment and injection process is not certain. Recently Simon and Anderson (25, 26) showed that the tail plate undergoes a morphological transition during interaction with the host cell. One of the unusual properties of dihydrofolate reductase (J. Burchall, Fed. Proc. **25**:277, 1966; J. Burchall and M. Chan, Fed. Proc. **28**:352, 1969) is that its conformation can be radically altered by cofactor attachment (23). It may be this property of the enzyme which is important for phage physiology. It can be supposed that the cell leakage occurring immediately after phage at-

tachment liberates enough NADPH to react with the phage tail plate dihydrofolate reductase, reducing the dihydro-compound to the tetrahydro-compound, and that this change in oxidation state triggers first a change in the conformation of the phage enzyme and then a morphological change in the phage tail plate. Preliminary experiments on morphological changes produced in the phage tail substructure consisting of plate plus tube after treatment with NADPH tend to support the supposition that dihydrofolate reductase plays a role in phage infection.

Finally, it may be emphasized that the use of an "early" enzyme, formed within 1 to 2 min after infection (20), as a component of the phage coat is not in agreement with the widespread view that capsid components are only synthesized as "late" proteins. Since this enzyme amounts to only about 0.02% of the total mass of the phage coat, and indeed under unusual circumstances may possibly be replaced by a host enzyme, it clearly represents a special case.

ACKNOWLEDGMENTS

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