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Bacteriophage Tail Components

I. Pteroyl Polyglutamates in T-Even Bacteriophages

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A pteroylpolyglutamate has been found to be a constituent of all *Escherichia coli* T-even bacteriophages and has been characterized with regard to its oxidation state, molecular weight, origin, and location on the phage particle. The phage compound has been shown to be a dihydropteroyl penta- or hexaglutamate on the basis of its chemical and physical properties. Analyses of extracts of uninfected and T2L-infected *E. coli* have indicated that the phage dihydropteroyl polyglutamate was present only in infected cells. Its synthesis was sensitive to the addition of chloramphenicol before infection, and the compound appeared to be specifically induced by phage infection. Analyses of isolated phage ghosts and tail substructures have shown that each phage particle contains between two and six phage-specific pteroyl derivatives and that the juncture of the phage tail plate with the tail tube is the most likely site of binding of the phage-induced pteroyl compound.

The T-even bacteriophage capsids are composed of discrete morphologically distinct protein substructures firmly bonded to each other (6, 13, 19, 22). Little is known about the nature of the bonds between these substructures, except that these bonds are unlikely to be covalent since the intact T-even phage particle can be disrupted by a variety of methods which normally do not break covalent bonds (8, 34).

In 1965, Kozloff and Lute (20) presented evidence that an unusual folic acid conjugate was an essential constituent of the capsid of *Escherichia coli* T4 bacteriophage. In addition to the presence of the folate compound in highly purified T4Bo1 phage preparations, it was shown that an enzyme purified from hog kidney both hydrolyzed the γ-glutamyl bonds of folic acid conjugates and irreversibly inactivated all T-even phage strains. Phage particles treated with this enzyme appeared morphologically intact but were no longer able to attach to host cells.

The phage folate conjugate has now been found in every strain of T-even bacteriophage analyzed, and, although originally thought (20) to vary chemically in some unknown way among the different phage strains, it has now been identified as a dihydrofolate conjugate in all strains. Any change in oxidation state of the dihydrofolate was correlated with loss of viability. All strains of T2 and T4 phages analyzed had similar amounts of dihydrofolate which in different ghost preparations varied from two to six molecules per phage particle. Measurement of the molecular size of both the phage compound and the new folate compounds found in infected cells by chromatography on gel-filtration columns indicated that the phage compound contained five to six glutamyl residues. This compound was not found in uninfected cells and it represents a unique compound formed after infection.

The availability of a large number of T4D amber mutants through the courtesy of R. S. Edgar and his colleagues (12, 13, 35) has allowed the preparation of a variety of bacterial lysates rich in different phage substructures. Methods have been devised for the isolation and purification of these substructures, and they have been analyzed for their folate content. The probable location of the phage folate on the phage tail has been determined. In the accompanying paper (23), evidence on the nature and location of the protein component of phage and phage substructures which binds the phage folate is presented. It has been concluded that the phage-induced enzyme, dihydrofolate reductase, is a structural component of the phage tail plate and that this enzyme is the site of binding of the folate compound. Preliminary reports on the location of the phage compound (Kozloff, Lute, and Crosby, Fed. Proc. **25**:778, 1966) and the presence of dihydrofolate reductase in phage capsids (Verses et al., Bacteriol. Proc., p. 153, 1968) have appeared.

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MATERIALS AND METHODS

Preparation and purification of bacteriophage stocks, ghosts, and substructures. A variety of *E. coli* bacteriophage stocks were grown, purified, and assayed by standard procedures (2). A list of these phages with their relevant properties and the sources of the original stocks is given in Table 1. Phage ghosts were normally prepared by the osmotic shock method of Herriott and Barlow (16) with Na_2SO_4, but the glycerol osmotic shock method of Cummings (7) was also used for some preparations. T4Bol preparations, which are highly resistant to osmotic shock by Na_2SO_4 and glycerol, were subjected to osmotic shock with sucrose (7) as the osmotic agent. Ghost preparations were freed from any remaining whole-phage particles by centrifugation in a Spinco SW 25 rotor using a density gradient. Usually 10 ml of the concentrated ghost preparation was placed on top of 10 ml of a 90% D_2O–1.2 M CsCl solution (density 1.30), which in turn had been layered over 10 ml of 70% sucrose solution (density of 1.35). After centrifugation at 40,692 × *g*, all whole-phage particles (density of 1.50) had been sedimented to the bottom of the tube and the protein phage ghosts (density about 1.3) were readily removed from the center of the centrifuge tube. The concentration of phage ghosts was determined by their protein content (33).

Samples of purified T4st, T6, and λ were furnished by D. J. Cummings.

Stocks of T4D amber mutants were grown on the permissive *E. coli* host, CR63, and purified by standard procedures. When these strains were used to infect *E. coli* B, the nonpermissive host, various phage substructures were synthesized (Table 1; reference 13). Normally 16 liters of growth medium was inoculated with *E. coli* B, grown to 4 × 10^8/ml, and then infected with the appropriate amber mutant at a multiplicity of four phage per bacterium. After vigorous aeration at 37 C for 90 min, chloroform (1 ml/liter) and deoxyribonuclease (2 μg/ml) were added. The lysate was incubated with slow aeration for 1 hr at 37 C and chilled. Some of the purification procedures were modifications of those outlined by Edgar and Wood (12), King (18), and Edgar and Lielausis (11), which depend upon the sedimentation properties of the substructures and their identification by electron microscopy. The microscopy was carried out as described earlier (21).

Particles free from tail fibers, prepared from the T4D quadruple amber mutant, X4E (12), were purified in a manner identical to standard phage preparations since they sediment as fast or faster than intact phage particles.

The phage tail substructure, consisting of tail plates attached to tail cores, was isolated from lysates of the T4D mutant defective in the two genes, 14 and 15, by a series of differential and zonal centrifugation procedures. The lysate was clarified at 6,000 × *g* for 15 min, and the tail substructure was sedimented by centrifugation in 250-ml bottles for 16 hr at 35,000 × *g* in the A-57 head of an IEC B-35 ultracentrifuge. The pellets were resuspended in 1% ammonium acetate and clarified at low speed. The tail substructure was then concentrated in the

TABLE 1. *Escherichia coli bacteriophage strains*

Strain	Distinctive properties	Source
T2L	Cofactor-independent for adsorption	(17)[a]
T2H	Indole inhibits adsorption	(17)
T2HI^r	Indole-insensitive	S. Champe
T4B	Requires tryptophan for adsorption	(19)
T4BC$^+$	Tryptophan-independent	S. Champe
T4Bol	Requires tryptophan, resists osmotic shock	(20)
T4D	Wild type, cofactor-independent	(13)
T4Dwh 1	Dihydrofolate reductase minus	I. Tessman (15, 17)
T4D *amber mutants*	(*Under restrictive conditions*)	
B25/B252/N52/B262(X4E)	Defective in genes 34, 35, 37, and 38; yields tail-fiberless particles	R. S. Edgar (12)
B20/N133	Defective in genes 14 and 15; yields free tail cores plus tail plates	R. S. Edgar
B17	Defective in gene 23; yields 3 tail substructures; tail plates plus cores, tail plates plus cores plus sheaths, and tail fibers	R. S. Edgar
H21	Defective in gene 54; yields free tail plates and free phage heads	R. S. Edgar
N140	Defective in gene 27; yields tail fibers	R. S. Edgar
T5st	Resistant to head destruction	D. J. Cummings
T6	Wild-type phage	D. J. Cummings
λvir	Virulent strain	D. J. Cummings

[a] Numbers in parentheses indicate reference numbers.

no. 30 head of a Spinco L-2 centrifuge (4 hr at 67,000 × *g*). These pellets were again resuspended and the substructure was subjected to zonal centrifugation on a 5 to 25% sucrose gradient (plus 1% ammonium acetate) in 30-ml tubes of the SW-25 Spinco rotor. After 4 hr at 40,692 × *g*, 2.0-ml fractions were collected and analyzed for protein and peak tubes were examined in the electron microscope. Under these conditions, tail plates plus tail tubes have a sedimentation coefficient of about 70 to 80*S* and move to the center of the gradient. The main peak of tail substructure was pooled, concentrated, and again subjected to zonal centrifugation in a similar gradient. In all cases, only a symmetrical single peak of tail substructure was obtained after this second centrifugation. The evidence for purity and other characteristics of these structures are given later.

Two kinds of phage tail substructures were isolated from the gene 23 amber mutant (B17). Lysates of this mutant grown on *E. coli* B yielded the same tail substructure as did the 14/15 mutant of tail plates plus tail cores and, in addition, the substructure consisting of tail plates plus tubes plus sheaths [King (18)]. Normally the tail sheath is constructed around the tail core, but sheath substructure is not stable and the sheath subunits tend to fall off upon storage. Centrifugation procedures similar to those used with 14/15 lysates above were used to purify these two tail substructures. On sucrose gradients, the tail substructure with sheath had a sedimentation rate approximately 60 to 70% faster than that of tail substructure without sheath. Concentrated tail substructures from gene 18 lysates formed two peaks on sucrose gradients which were readily separated. The fractions from the fast peak representing the tail substructure with sheaths were pooled, concentrated, and again subjected to zonal centrifugation. Similarly, the substructures in the slow peak (tail plate plus tube) were pooled and purified. This procedure yielded highly purified preparations of both types of tail substructures. The yield amounted to a total of 3 to 4 mg of purified tail substructures per liter of lysate.

Two quite different types of phage substructures were isolated from gene 54 (amber mutant H21) lysates. This mutant cannot form tail tubes, and both free empty heads and free tail plates are liberated upon lysis of *E. coli* B. These two substructures were readily sedimented as were the other tail substructures. Separation of phage heads and tail plates was performed by using a gel-filtration procedure. Concentrated substructures were put on an agarose A-50m column (40 by 400 mm), and the material was eluted with 1% ammonium acetate by using an LKB drop counter-fraction collector. Phage heads came out in the void volume (fraction 41 with 64 drop fractions), whereas tail plates came out in a single peak with a maximum in fraction 53. The tail-plate peaks were pooled, rechromatographed, and then subjected to concentrated zonal centrifugation in 5 to 40% sucrose-ammonium acetate gradients. Plates were found in the middle fraction after 4.5 hr at 59,000 × *g* in the SW 25 rotor. Free tail plates sedimented slightly faster than the substructures consisting of tail plates plus tail core and had a sedimentation constant of about 80 to 90*S*.

In later experiments, phage substructures such as tail plates or tail plates plus tubes were concentrated from the lysates by using a modification (S. Ward, *personal communication*) of the three-phase method of Albertsson and his colleagues (3, 27). The following compounds were added slowly with stirring to the chloroform- and deoxyribonuclease-treated lysate: polyethylene glycol, 91.5 g/liter; NaCl, 30.0 g/liter; and sodium dextran sulfate, 2.0 g/liter. This mixture was allowed to stand overnight. Three phases formed and the great bulk of the supernatant solution was siphoned off and discarded, leaving the interface material and bottom solution. The interface material and bottom solution were centrifuged at 2,200 × *g* for 20 min. The interface material was retained, deoxyribonuclease was added (2 μg/ml), and enough 1% sodium dextran sulfate was added to form a homogeneous suspension. Then 0.15 volume of 3.0 M KCl was added with stirring and the solution was allowed to stand in the cold overnight. The precipitated dextran sulfate was centrifuged off at 2,200 × *g* for 10 min. The substructures in the supernatant were concentrated in either a Spinco L-2 (3 hr at 75,000 × *g* in the no. 30 rotor) or IEC B-35 ultracentrifuge (3 hr at 70,000 × *g* in the no. 170 rotor).

A detailed description of the properties of these phage substructures will be given later.

Microbiological assays. Folic acid was assayed microbiologically by using both *Streptococcus faecalis* (ATCC 8043) and *Lactobacillus casei* (ATCC 7469) following the procedures described earlier by Kozloff and Lute (20). Special precautions were necessary to measure dihydrofolate. These included the addition of fresh potassium ascorbate at *p*H 6.0 to a final concentration of 1% during all extractions, digestions, and sterilizations (never above 95 C) and to a concentration of 0.1% during the growth of the test organisms. These precautions, outlined by Bakerman (5), prevent the oxidation and destruction of the highly labile reduced folate. It was found that under these conditions *L. casei* responded equally to folate and to dihydrofolate. The dihydrofolate used for assays was measured optically with $E_{283} = 21{,}000$. Dihydrofolate either was obtained from Sigma Chemical Co. or was prepared by the Zakrewski modification of Futterman's procedure (36) with potassium ascorbate and $Na_2S_2O_4$ as reducing agents.

Purified hog kidney conjugase was used in many experiments to hydrolyze the polyglutamyl derivatives of folic acid conjugates. This enzyme was purified by the previously described method (20), except that the acetone precipitation step was replaced by chromatography on Sephadex G-75. The peak fraction was stored at −20 C and had a specific activity of 500 units/ml (20).

p-Aminobenzoic acid (PABA), a constituent of all of the forms of folic acid (even those which are of varying activity for *L. casei* and *S. faecalis*) was also assayed microbiologically. Some initial assays were carried out with *L. plantarum* (ATCC 8014). This microorganism did not respond to folic acid or to

p-aminobenzoyl glutamic acid. Trial experiments with folic acid indicated that heating a sample at 110 C in a sealed tube in 6 N HCl (under N_2) would release growth factors for this organism. However, yields from known amounts of folate were usually between 20 and 40%. Although corrections for phage samples were made with folate controls in the hydrolytic procedure, there was still some uncertainty in these corrections since the phage compound is not a pteroyl monoglutamic acid. However, the results obtained were in general agreement with those by other methods and are included later.

The most reliable microbiological test organism for measuring PABA was the *E. coli* mutant used by Lampen et al. (25). This organism was obtained from the American Type Culture Collection (9723a), and a single PABA-requiring colony was isolated and carried on tryptone slants. The final assay medium contained, per liter: 2 g of glucose; 12 mg each of adenine, guanine, uracil, and xanthine; 100 mg each of the 20 common amino acids; and 0.025 M KH_2SO_4, 0.001 M $MgSO_4$, 0.05 M NH_4Cl, 0.05 M NaCl, and 5×10^{-5} M CaCl, all at *p*H 6.8. Normally the bacterial inoculum was prepared by adding a loopful of bacteria to 4.0 ml of basal medium containing a total of 25×10^{-12} moles of PABA. This was shaken for 6 hr at 37 C until the bacterial concentration reached 5×10^7 per ml, and 0.04 ml was used to inoculate 4.0-ml assay tubes. The tubes were shaken at 28 C for 20 hr and the turbidity was read in an Aminco-Bowman fluorometer at 400 nm. PABA standard solutions were prepared and standardized optically ($E_{266} = 1.47 \times 10^9$). Proportional growth was obtained from 0.5×10^{-12} to 40×10^{-12} moles of PABA per 4.0-ml assay tube.

Hydrolysis of phage samples preparatory to microbiological analysis for PABA was carried out by several different methods. Acid hydrolysis always resulted in significant losses of PABA. The alkaline hydrolysis procedure adopted was based on the observation of Kuiken et al. (24) that cysteine protected PABA from air oxidation. Samples (0.2 ml) were put in alkali-resistant Pyrex tubes (Corning 9455); 0.02 ml of 17% mercaptoethanol and 0.5 ml of 10 N NaOH were added; the tubes were then gassed with N_2 and sealed under N_2. These tubes were autoclaved for various times and then chilled and opened. Solid Dowex-50 resin in the acid form was added to neutralize the base to the phenolpthalein end point and to remove sodium ions, the solution was made just pink again with dilute NaOH, and the resin was centrifuged down. The supernatant solution plus three water washes of the resin were combined and evaporated to dryness to remove the mercaptoethanol. The remaining solids were taken up in 4.0 ml of basal assay medium and three 1.0-ml samples were taken for analysis. Under these conditions, added PABA was quantitatively recovered and folate samples gave the theoretical yield of PABA.

E. coli strain 9723a also responded to unhydrolyzed glutamyl derivatives of PABA. *p*-Aminobenzoyl glutamic acid was equally as active as PABA. Although pure samples of higher glutamyl conjugates of PABA were not available for testing, various observations indicated that compounds containing PABA plus three to six glutamyl residues could also serve as efficient growth factors. For example, samples from molecular sieve chromatography of bacterial extracts, both phage-infected and uninfected, contained PABA compounds having molecular weights corresponding to PABA(glu)$_3$ and PABA(glu)$_5$ and these compounds acted as growth factors. Further, phage ghost extracts (*see below*) known to contain only dihydrofolate as a higher conjugate gave similar results for PABA content when the sample was assayed after prolonged hydrolysis with 5 N NaOH (plus mercaptoethanol), or was assayed after treating the virus preparation with urea at room temperature. These observations indicated that this bacterial *E. coli* mutant probably can use, with similar efficiencies, PABA, PABA(glu)$_1$, PABA(glu)$_3$, and very probably PABA(glu)$_{5-6}$.

Other analytical methods, chemicals, and other materials. Protein was measured by the modified biuret method of Westley and Lambeth (33) with crystalline bovine serum albumin as a standard. Serum-blocking power was measured by the method of De Mars (10). All common chemicals were analytical grade and purchased from commercial sources. *p*-Aminobenzoyl glutamic acid was obtained from Sigma Chemical Co. Formamidine sulfinic acid was a product of Aldrich Chemical Co. This compound was normally stored in a desiccator at −20 C, since after storage at room temperature it lost most of its reducing power. The anti T4-serum had a *K* value over 2,000. D_2O (90%) was obtained from Bio-Rad Laboratories and CsCl from Gallard Schlesinger. The gel filtration resin Sephadex G-75 was a product of Pharmacia Corp., and P-2 gel (100 to 200 mesh) and agarose A-50m were from Bio-Rad Laboratories.

RESULTS

Phage ghosts were examined for their content of reduced folate compounds by using extraction procedures known to minimize breakdown (26). Extracts of purified ghosts were prepared by heating (95 C for 5 min in the dark) usually 10^{13} to 2×10^{13} phage ghost particles in a total volume of 0.5 ml containing 2% purified mercaptoethanol as a protective agent. These samples were cooled and then ultrafiltered in the cold and dark. The ultrafiltrates, which were protein-free, were adjusted to various *p*H values and then examined in the Aminco-Bowman spectrophotofluorometer.

Uyeda and Rabinowitz (32) have reported the fluorescence characteristics of a variety of folic acids. Both dihydrofolate and tetrahydrofolate have characteristic spectra; dihydrofolate fluoresces maximally at 420 nm when excited at 325 nm at *p*H 9.0, whereas tetrahydrofolate fluoresces maximally at 360 nm at *p*H 3.0 when excited at 310 nm. Folic acid has little or no fluorescence. The fluorescence spectra of three different phage extracts and dihydrofolate are

shown in Fig. 1. All T-even ghost preparations, including T4Bo1, gave extracts having spectra essentially identical to that of dihydrofolate. No tetrahydrofolate could be detected even though it is at least 10-fold more fluorescent than dihydrofolate compound.

Molecular weight of T2L phage dihydropteroyl polyglutamate. The folate conjugate extracted from T4Bo1 phage previously (20) was estimated to be most likely a penta- or hexaglutamyl conjugate on the basis of its mobility upon high-voltage paper electrophoresis. It migrated twice as fast as pteroyl triglutamate, but samples of the higher conjugates were not available for comparison. Column gel-filtration chromatography was used to determine the molecular size of the phage compound. In initial experiments, it was found that both folate and dihydrofolate interacted with P-2 polyacrylamide gel material and eluted much later than would be expected on the basis of their molecular weights. However, PABA and *p*-aminobenzoyl glutamate did not react with the column material and behaved as expected.

It proved feasible to convert the highly labile phage dihydro compound quantitatively into a *p*-aminobenzoyl polyglutamate and then to determine the apparent molecular size of this portion of the phage compound. A 0.5-ml amount of D_2O-sucrose gradient-purified T2L ghosts (containing 2.5×10^{13} particles) was vigorously mixed with 8 volumes of 9 M urea at room temperature and in the light. After 15 min, the solution was passed through an ultrafiltration membrane, the protein-free filtrate (plus molecular weight markers) was placed on a Bio-Rad P-2 poly gel filtration column, and the material was eluted with 0.01 M potassium phosphate buffer (*p*H 7.0). Fractions (about 1.2 ml) were collected and the eluting position of the markers was determined by their ultraviolet light absorption. The position of the phage compound was determined by microbiological assay with *E. coli* 9723a as the test organism. Over 70% of the phage compound was recovered as a single substance which behaved as if it had a molecular weight of about 1,200 daltons (Fig. 3). Since this procedure caused breakdown of the original phage compound, it appeared likely that T2L phage capsids contained a single species of a relatively high-molecular-weight glutamyl derivative of PABA.

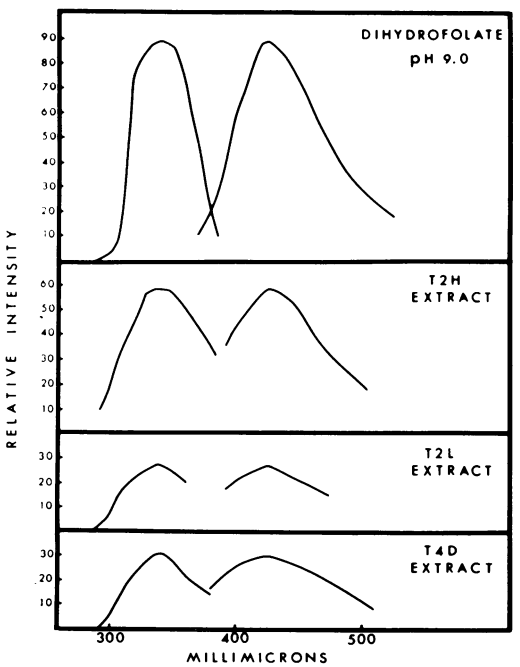

FIG. 1. *Fluorescence spectra at pH 9.0 of dihydrofolate and extracts of T2L, T2H, and T4D. The curve at the lower wavelengths represents the spectra for excitation, whereas the curve at the higher wavelengths represents the spectra for the emitted light. The preparation of the extracts is described in the text.*

The *p*-aminobenzoyl hexaglutamate (with 6 K^+) has a weight of 1,157 daltons and would agree with that of the phage compound. However, molecular weight estimation from gel filtration elution volumes is subject to error since the correlation is with molecular diameter rather than weight (1). A compound containing a negatively charged polyglutamyl chain would be expected to be asymmetric and to behave on these gel columns as if it were somewhat larger than it actually was. The behavior of the degraded phage compound upon gel filtration, in agreement with the earlier work (20), suggests that the phage compound is a penta- or hexaglutamyl derivative of dihydropteroic acid.

Inactivation of various T-even phages by folic acid conjugase. Various strains of T-even phages were treated with purified hog kidney conjugase under conditions described previously (20). This enzyme hydrolyzes specifically γ-glutamyl bonds in folic acid conjugates. All of the T-even phage preparations were rapidly and irreversibly inactivated (over 90% in 30 min). Two other coliphages with quite different tail structures, T5st and λ_{vir}, were unaffected by incubation with this enzyme (Table 2).

Inactivation of various T-even phages by reducing agents. The requirement that the pteroyl polyglutamyl compound be at the dihydro stage of reduction was examined. Since phage which contain "dihydrofolate" were grown with vig-

TABLE 2. *Phage inactivation by conjugase and formamidine sulfinic acid*

Phage	Conjugase-sensitive	Formamidine sulfinic acid-sensitive
T2L	Yes	Yes
T2H	Yes	Yes
T4Bol	Yes	Yes
T4B	Yes	Yes
T4D	Yes	Yes
T6	Yes	
T5st	No	No
λv	No	No

orous aeration, a condition which normally oxidizes free dihydrofolate, it appears that the phage dihydrofolate is not only tightly bound to the phage capsid but also is protected against air oxidation. The chemical reduction of free dihydrofolate to tetrahydrofolate is usually carried out either with hydrosulfite plus ascorbate at 70 C (31) or with H_2 gas in acid with a metal catalyst, conditions which cannot be used with viable phage. At 37 C and *p*H 8.0, neither hydrosulfite nor borohydride, another active reducing agent, had any affect on the titer of T-even phage preparations.

A powerful reducing agent, formamidine sulfinic acid, which at neutral *p*H values has a redox potential of −1.5 v (as contrasted with −0.5 v for hydrosulfite), has been described by Shashoua (30). Solid formamidine sulfinic acid (4 mg) was added to the side arm of a Thunberg tube containing 3.0 ml of folic acid at 1.7×10^{-8} M in 0.1 M phosphate buffer (*p*H 6.6) in the main body of the tube. The tube was evacuated and mixed and then incubated in the dark at 37 C for 18 hr.

The solution containing the formamidine sulfinic acid-treated folate was examined in the spectrophotofluorometer. Characteristic spectra (32) for both dihydrofolate (at *p*H 9.0) and tetrahydrofolate (at *p*H 3.0) were found. Based on the relative fluorescences (32), it was estimated that 70% of the original folate had been reduced to dihydrofolate and 7% had been reduced to tetrahydrofolate.

Formamidine sulfinic acid (0.01 M) was then incubated with various phages under anaerobic conditions at *p*H 6.6 in 0.2 M phosphate buffer containing 1 mg of gelatin per ml. The results are given in Table 2 and Fig. 2. All of the T-even phages were slowly inactivated by this treatment (usually 50% in 18 hr), whereas neither T5 nor

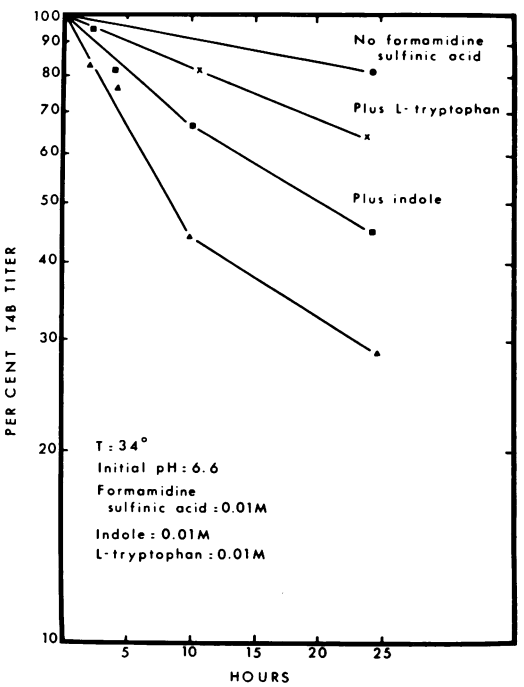

FIG. 2. *Inactivation of T4B bacteriophage by 0.01 M formamidine sulfinic acid. The reaction was carried out anaerobically using Thunberg tubes and adding the solid reducing agent to the side arm. The 0.2 M phosphate buffer contained 1 mg of gelatin per ml. The reaction was stopped by chilling the evacuated tube in the dark and then opening it and adding 10 volumes of cold broth. The pH usually fell in 24 hr to approximately 6.0 as the reaction proceeded.*

λ was affected. The slow rate of inactivation is that expected from the slow rate of reduction of dihydrofolate to tetrahydrofolate. Additional experiments on the inactivation of various phages by another reducing agent, reduced nicotinamide adenine dinucleotide phosphate, are given in the accompanying paper (23).

Evidence was also obtained that this reducing agent was acting on the phage tail. Figure 2 shows that the rate of T4B inactivation is inhibited markedly by the addition of either L-tryptophan or by indole. T2H phage, whose absorption is indole-sensitive, was also completely protected by indole from inactivation by formamidine sulfinic acid. Since L-tryptophan and indole are known to interact with the phage tail, it seems likely that the dihydropteroyl polyglutamate of the T-even phage tail is the site acted upon by this reducing agent.

Virus-induced production of a high-molecular-weight dihydrofolate. In an earlier study (20), no measurable change was found in the kinds of

folic acids in infected cells as compared to uninfected cells. Both infected and uninfected *E. coli* had the same folate content (approximately 200,000 molecules/cell), and practically all of the folic acid extracted by heating was present as pteroyl triglutamates. However, when bacterial extracts were chromatographed on a P-2 gel filtration column, it was found that phage infection did cause the production of a small amount of a new high-molecular-weight labile pteroyl polyglutamic acid. Although a molecular weight could not be estimated accurately because of interaction with supporting gel, the new compound, representing only 0.5% of the total folate, behaved as if it were larger than a pteroyl triglutamate.

The lability of phage dihydropteroyl glutamate made it possible to examine whether phage infection influenced the molecular size distribution of the various *p*-aminobenzoate compounds in bacterial extracts. A 500-ml amount of both T2L-infected and uninfected bacteria was concentrated 100-fold by centrifugation and then disrupted for 10 min in the cold with a Bronson Sonifier. The insoluble material was sedimented by centrifuging at 100,000 × *g* for 15 min, and the supernatant solution was lyophilized. The dried powder was dissolved in 1 to 2 ml of 8 M urea at room temperature in the light, and this solution was then ultrafiltered and chromatographed on a P-2 gel-filtration column (Fig. 4). Infected cells contained a unique compound, eluting in tubes 38 to 39, active as *p*-aminobenzoate in a biological assay and with a molecular weight of approximately 1,200 daltons (*see* Fig. 3 for the relationship between the added markers and their molecular weights). This compound amounted to 8% of the total *p*-aminobenzoate and corresponded to the PABA compound that was isolated from the T2L ghosts. Extracts from uninfected cells did not contain a similar compound. The limit of detection of the compound in uninfected cells was of the order of 0.1% of the total *p*-aminobenzoate of 1/80 of the amount of the new compound found in infected cells.

The effect of an inhibitor of protein synthesis, chloramphenicol, on the appearance of the new high-molecular-weight PABA compound was examined by methods identical to those shown in Fig. 4. Three identical cultures of *E. coli* B were infected with T2L at a multiplicity of four T2L per bacterium; in one culture no chloramphenicol was added, in the second culture chloramphenicol was added to a final concentration of 150 μg/ml at the time of infection, and in the last culture chloramphenicol was added 10 min after infection. Chloramphenicol added

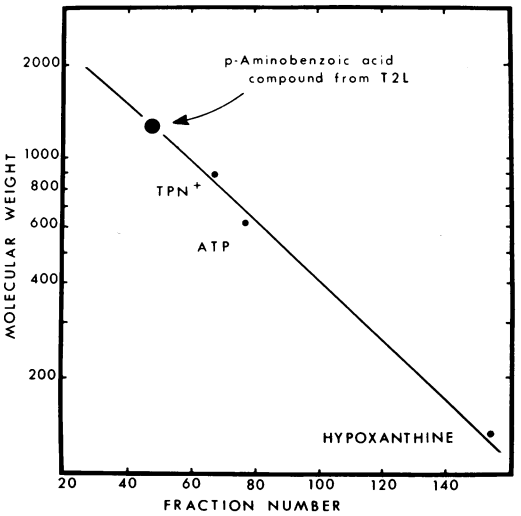

FIG. 3. *Gel-filtration chromatography of the material released from T2L ghosts by 8 M urea. Material from 2.5 × 10^{13} ghosts was placed on a P-2 (100 to 200 mesh) polyacrylamide gel column 58 cm in length and 2.0 cm in diameter; the volume was 190 ml. About 0.6 mg of nicotinamide adenine dinucleotide phosphate (TPN+), 0.9 mg of adenosine triphosphate (ATP), and 0.2 mg of hypoxanthine were used as markers. The phage compound was measured microbiologically by its ability to supply p-aminobenzoic acid for E. coli 9723a without additional hydrolysis.*

at the time of infection completely prevented the appearance of the high-molecular-weight PABA compound. When added 10 min after infection, chloramphenicol had no effect on the appearance or amount of the high-molecular-weight PABA compound and results identical to those in Fig. 4 were obtained.

These observations on the appearance in T2L-infected cells of a new dihydropteroyl pentaglutamate indicate that phage infection specifically induces the synthesis of this compound. The chloramphenicol sensitivity not only supports the view that these compounds are virus-induced but also suggests that virus-induced protein synthesis is required for its formation.

Dihydropteroyl polyglutamate and p-aminobenzoate content of various T-even phages. Samples were taken from three specific phage ghost preparations and analyzed for both dihydropteroyl glutamate and PABA. The results are given as molecules per phage particle in Table 3. The agreement found for the amount of dihydrofolate and for PABA supports the conclusion that dihydrofolate is the only form of pteroyl glutamate present in phage preparations. Further, the results of separate analyses for dihydrofolate and PABA on various preparations of different

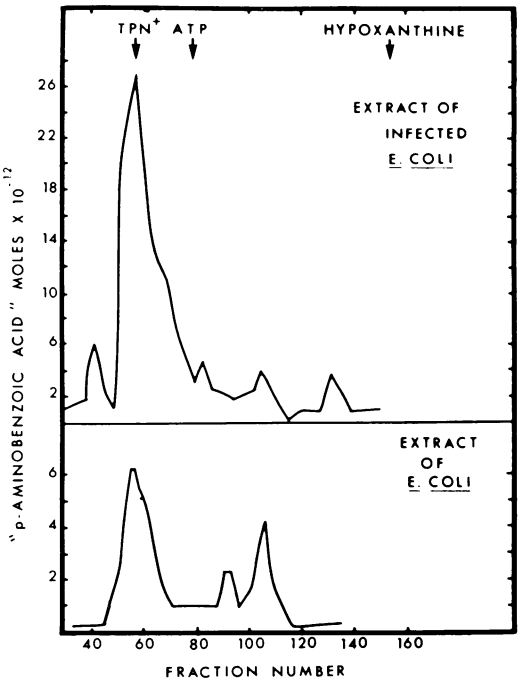

FIG. 4. *Gel filtration chromatography of p-aminobenzoate compounds extracted from uninfected E. coli and T2L-infected E. coli. The uninfected bacteria were grown to 3 × 10⁸ cells/ml in a glucose-NH_4Cl medium (2) and then concentrated in the cold and extracted. The infected cells were grown to the same concentration, infected with four T2L per bacterium, incubated with aeration for 10 min, and then chilled, concentrated, and extracted. The extraction procedure described in the text was designed to cause the breakdown of all dihydropteroyl polyglutamates into p-aminobenzoyl glutamate compounds. The analytical procedure was similar to that described for Fig. 3.*

TABLE 3. *Dihydrofolate and p-aminobenzoate content of specific phage ghost preparations*[a]

Phage ghost prepn	Molecules per phage particle	
	Dihydrofolate	*p*-Aminobenzoate
T2L	1.3	0.8
T4D	1.6	1.6
T4B	1.9	1.0

[a] Both analyses were carried out microbiologically as described in Materials and Methods. Dihydrofolate activity for *L. casei* activity required prior conjugase treatment. The *p*-aminobenzoic acid values were obtained after alkaline hydrolysis.

TABLE 4. *Dihydrofolate and p-aminobenzoate content of various T-even phage ghost preparations*[a]

Different prepn of phage	Molecules/phage particle	
	Dihydrofolate	*p*-Aminobenzoate
T2L	1.1, 1.3, 1.4	0.8, 1.1, 1.8, 2.0, 5.0
T2H		1.1, 2.1, 2.8, 6.0
T2HI^r		4.1, 5.9
T4B	1.9	1.0
T4Bo1		1.9, 2.5, 3.0, 4.0
T4BC$^+$		1.0
T4D	1.6	1.0, 1.1, 1.4, 1.8, 2.0, 4.5

[a] Each value represents a separate analysis of a different preparation. For instance, five different T2L and four different T2H preparations were analyzed for *p*-aminobenzoic acid. Average values are not given since the meaning of the average would not reflect the biological situation.

strains of T-even phages given in Table 4 clearly show that all of the T-even strains examined contained about the same amount of dihydrofolate (and PABA).

The exact number of pteroyl glutamates per phage particle is of considerable interest. The analytical results indicate that phage ghost preparations usually have on the order of one to two molecules per particle, and, since there must be a whole number of folates per ghost particle, two is probably the lower limit. However, since the phage folate compound is not bound by covalent bonds to the phage capsid, it seems likely that two molecules per ghost particle also must represent the minimum number per phage particle. The preparation of ghosts involves a variety of environmental conditions which damage the phage particle, and electron microscope pictures of ghosts invariably show some with contracted tails and missing tail plates. It therefore seems likely that some folate originally present in the phage particle will be lost. It is important to note that occasional preparations of phage ghosts are obtained (T2L, T2H, T2HI^r, T4Bo1, and T4D in Table 4) which have four to six folate molecules per ghost particle, and it is possible that intact phage particles may contain as many as six molecules per particle.

Location of dihydropteroyl glutamate on a substructure of the phage tail. Earlier experiments (20) showed that conjugase treatment of T4 phage specifically inhibited adsorption and killing ability. This observation implied that the pteroyl polyglutamate influenced phage tail function. The experiments reported in Fig. 2 and Table 2 on the inactivation of the T-even phages by formamidine sulfinic acid and the protection

of phage particles by such adsorption cofactors as L-tryptophan and indole support this conclusion. Since phage tail fibers are the part of the phage structure making the initial contact with the bacterial host cell, the possibility was examined that the phage pteroyl glutamate was a component of the rather complicated phage tail fiber. Tail-fiberless T4D particles were prepared by using the quadruple T4D mutant, X4E (7). These particles were purified and osmotically shocked, and the concentrated ghosts were then analyzed for their pteroyl glutamate content by the fluorometric method of Allfrey et al. (4). These particles were found to have amounts of pteroyl glutamate equivalent to those found in whole phage particles (Table 5), and it is apparent that the phage compound is not a component of the tail fibers themselves.

A variety of substructures formed by T4D amber mutants under restrictive growth conditions were prepared and purified as described above. The substructure preparations all behaved as a single component upon sucrose gradient centrifugation or gel-filtration chromatography, or with both techniques. The substructures isolated are given in Table 5.

The T4D head preparation isolated from gel filtration columns came out in the elution front along with a few phage ghosts. No effort was made to characterize further this substructure since it was not enriched for dihydropteroyl glutamate (Table 5).

Some features of the three other substructures are shown in the electron micrographs of Fig. 5 and the spectra are given in Fig. 6. The tail tubes plus sheaths plus plate preparations shown in Fig. 5A always contained small amounts of material without the sheath portion. As pointed out by King (18), sheaths on tail substructures tend to be unstable. The substructure consisting of the tail tube plus plate was purified from both B17 lysates (gene 23) and from lysates of the double amber mutant B20/N133 [genes 14 and 15 (Fig. 5B)]. Tail plates were obtained from H21 lysates (gene 54) and stored in the cold. Most of the tail plates are regular hexagons, but after storage the preparation appeared to contain more "starlike" structures than it had initially (Fig. 5C).

In agreement with the data of King (18) and Edgar and Lielausis (11), the sedimentation coefficient of sheaths plus tail tube plus plates was approximately 130*S*. Substructures consisting of tail tubes plus plates had an *S* value of about 90. The spectra given in Fig. 7 show that the preparations are free from nucleic acid and any detectable amounts of other chromogens.

TABLE 5. *Dihydrofolate and p-aminobenzoate content of phage components*[a]

Phage component	Amt (pmoles) per mg of protein	
	Dihydrofolate	*p*-Aminobenzoate
Whole T4D	?	?
T4D ghosts	15.7	15.9
Tail-fiberless T4D ghosts	~16.0	
T4D heads	1.4	
Tail tubes plus sheaths plus plates	97.0	119.0
Tail tubes plus plates	127.0	125.0
Tail plates	1.5	

[a] Tail-fiberless T4 were prepared with T4D amber mutant 4XE (Table 1); T4D heads and plates with T4D amber mutant H21; and the remaining tail portions with T4D amber mutant B17. Tail tubes plus plates were also prepared with the double amber mutant B20 and N133. The dihydrofolate and *p*-aminobenzoate were assayed as described in Table 3, except for the tail-fiberless particles which were assayed by a fluorometric method (4).

A comparison of the absorbancy with amounts of protein as measured by the biuret reaction (35) showed that these substructures differed with respect to their relative content of aromatic amino acids. For example, the amount (milligrams) of protein per optical density unit at 280 nm (corrected for light scattering) was 0.69 for T4 ghosts, 1.1 for T4D tail sheaths plus tail tubes plus plates, 1.0 for tail tubes plus tail plates, and 1.7 for tail plates. Tail plates are apparently quite low in tyrosine and tryptophan as compared to the rest of the phage.

Based on their sedimentation properties, their spectra, and their appearance in the electron microscope, these substructure preparations appeared suitable for analysis to determine the location of the phage dihydropteroyl glutamate compound. The results of analyses for dihydrofolate and *p*-aminobenzoate are given in Table 5. These results show that the phage folate compound is specifically located on phage tail structures and that the tail substructure having the highest concentration is that consisting of the tail tube plus tail plate.

It should be noted that the tail plates, although the site of attachment of the tail fibers, as isolated contain little or no folate. Evidence presented in the accompanying paper (23) indicates that the tail plate is involved in the folate binding. It seems that the free tail plate, without the additional structure of the tail tube (and tail

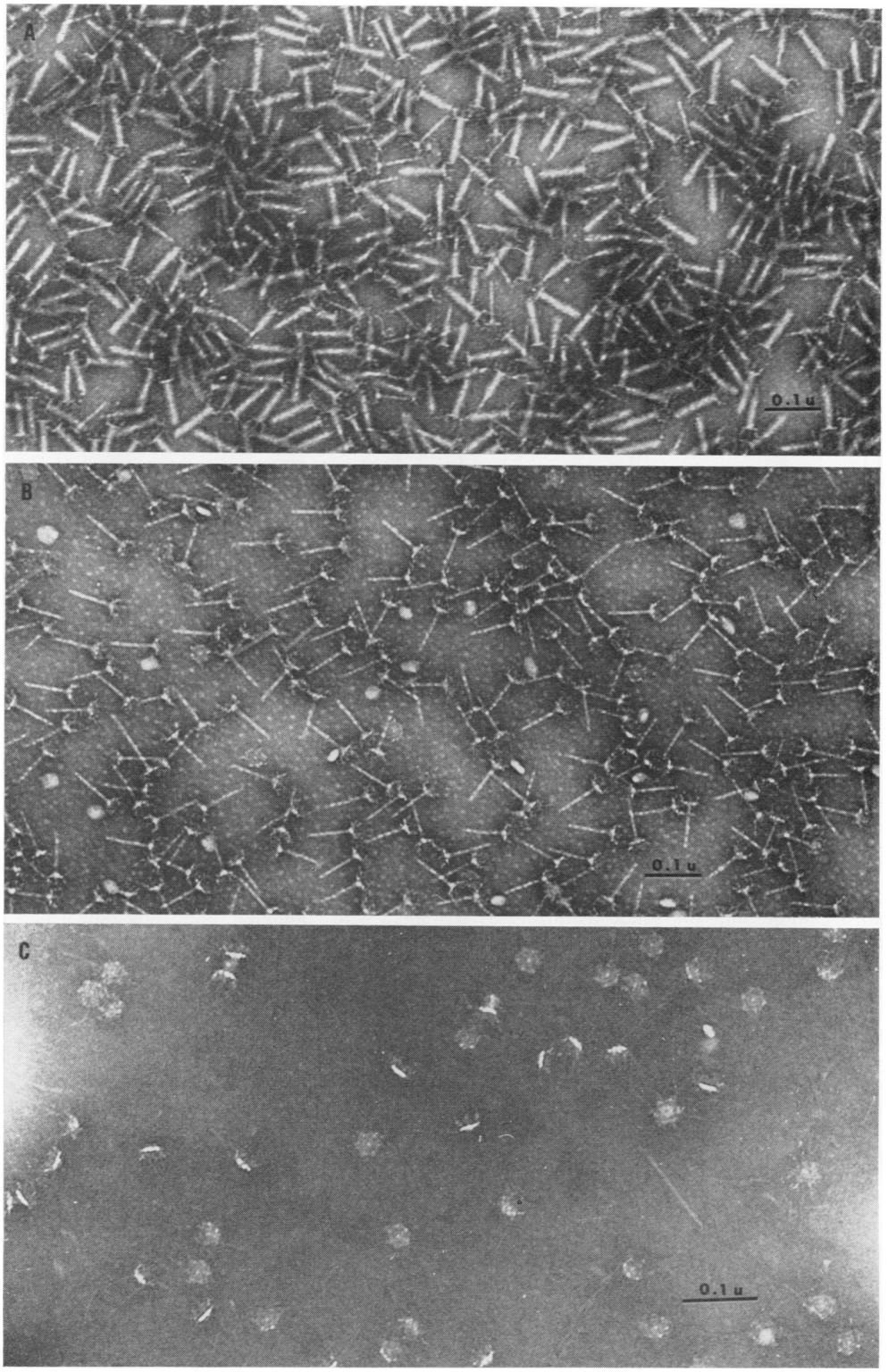

FIG. 5. *Electron micrograph of three different T4D phage tail substructures. (A) Tail tubes plus plates plus sheaths; note the appearance of a few structures free from sheaths. (B) Tail tubes plus plates. (C) Tail plates; most appear hexagonal with solid centers although an occasional "starlike" structure is visible; on edge plates appear to have at least three projections.*

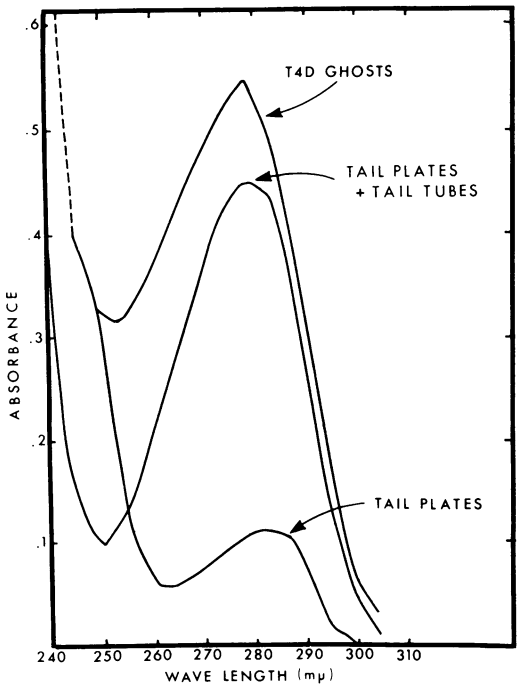

FIG. 6. *Spectra of T4D phage ghosts and T4D tail substructures. The absorbancies are corrected for light scattering, assuring that the absorbancy above 310 nm was entirely due to scattering. The substructure consisting of the tail plate plus tail tube was purified from a lysate made from a T4D amber mutant in genes 14 and 15; the tail plates were purified from a T4D gene 54 lysate.*

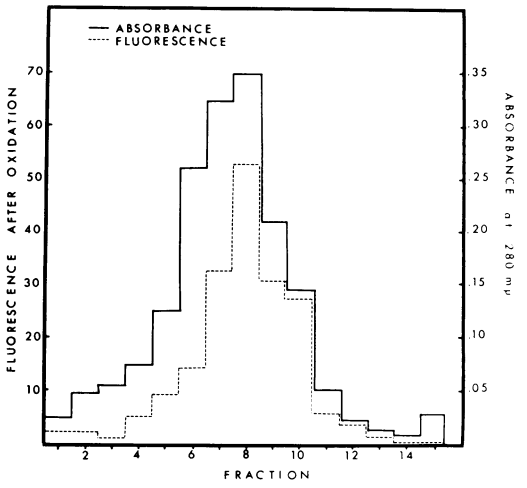

FIG. 7. *Sucrose gradient centrifugation of T4D tail substructures consisting of tail tubes plus tail plates purified from lysates of a T4D amber mutant in genes 14 and 15. About 4 mg of tail substructures in 2.0 ml of 1% ammonium acetate was placed on top of 28 ml of a 5 to 40% sucrose gradient (plus 1% ammonium acetate), and the tube was centrifuged for 4.5 hr at 59,000 × g. Fractions (2 ml) were collected (fraction no. 1 is from the bottom of the tube) and dialyzed against 1% ammonium acetate; then the absorbancy was read at 280 nm. Samples of each fraction were also taken and oxidized, and the fluorescence due to the 6-pteroic acid was measured (4).*

sheath), cannot bind the folate as firmly as it does in the whole phage particle.

Nature of the dihydrofolate found in tail sheath plus tail plate substructure. The substructure containing the highest amount of folate per milligram was examined to determine whether it was the same compound found in phage preparations. Figure 7 shows the relationship between the sedimentation behavior of the tail tube plus plate substructure (from a B20/N133 preparation) in terms of absorbance at 280 nm and the sedimentation behavior of the folate compound [measured as fluorescence after permanganate oxidation (4)]. The correspondence between folate content and protein is good and supports the conclusion that the folate is a constituent of this phage substructure. Extraction of purified substructures with 2% mercaptoethanol gave a fluorescent substance with a spectrum identical to those given in Fig. 1 for the various phage extracts and for that of dihydrofolate. No other material was detectable. Another feature of phage compound is that it contains five to six glutamyl residues (20); the compound extracted from this tail substructure was active microbiologically for *L. casei* only after treatment with conjugase, indicating that it had four or more glutamyl residues. Based on these observations, it seems highly likely that the dihydropteroyl glutamate found in the isolated tail substructures is identical to that found in phage ghost preparations.

The nature of the binding of the pteroyl glutamate to the tail tube plus tail plate substructure was also examined. Since whole-phage particles are readily inactivated by hog kidney conjugase, it can be assumed that the terminal glutamyl residues of the phage dihydrofolate are exposed on the final phage structure. The experiment shown in Fig. 8 is consistent with the conclusion that the tail substructure compound also contains exposed substrate for this enzyme. The addition of this tail substructure to a test system containing viable T4B protected the T4B phage from conjugase inactivation. In this experiment, the tail substructure contained 30 to 40 times the folate content of the test T4B phage.

Role of the phage dihydropteroyl polyglutamate in maintaining tail structure. The importance of

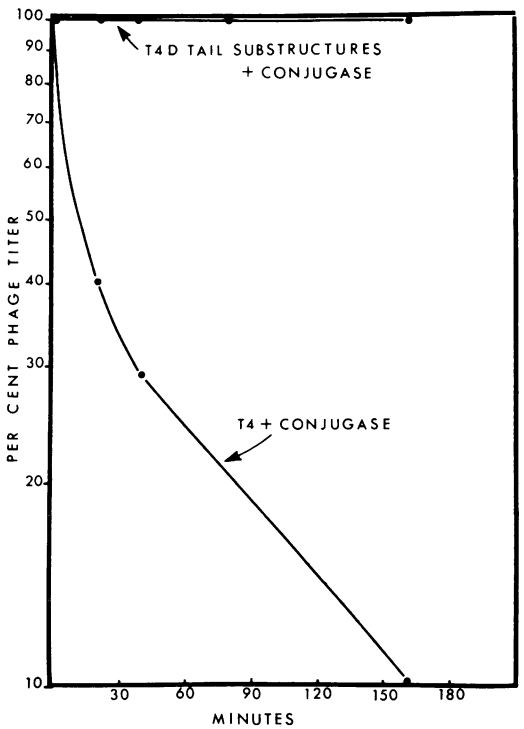

FIG. 8. *Protection of T4D phage against inactivation by hog kidney conjugase by a preparation of T4D tail substructures. The system consisted of T4D phage at 10^{12} per ml, 50 units of conjugase (20), and about 80 μg of highly purified T4D tail substructures from a gene 14/15 lysate which consists of tail tubes plus tail plates.*

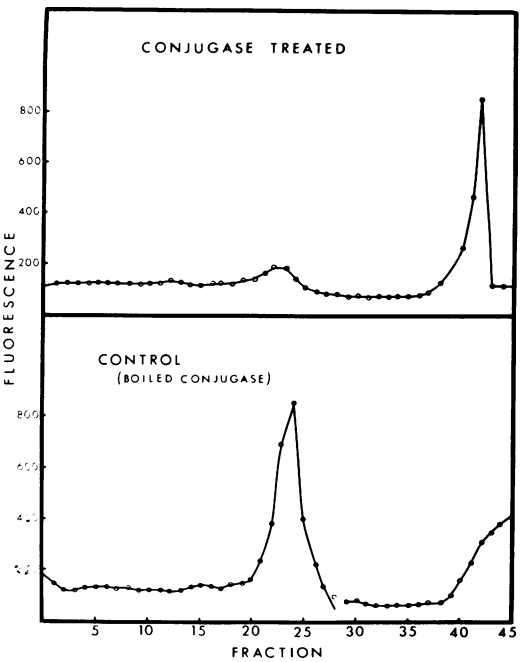

FIG. 9. *Sedimentation behavior of the T4D tail substructure consisting of tail plates plus tail tubes after conjugase treatment. After the enzyme treatment and centrifugation described in the text, 2.0-ml fractions were collected. (Fraction no. 1 is from the bottom of the tube.) The relative protein content of each fraction was measured by its fluorescence at 340 nm when excited at 280 nm.*

the glutamate residues of the phage compound for the maintenance of the structure of the tail components is illustrated in Fig. 9. Purified tail tubes plus plates (about 50 μg of protein) were incubated with 50 units of conjugase (or boiled conjugase) in 0.2 M acetate buffer (*p*H 4.5) containing 0.002 M cysteine. After 2 hr at 30 C, the reaction mixtures were placed on 12 ml of a 5 to 40% sucrose gradient containing 1% ammonium acetate. These tubes were centrifuged for 3.5 hr at 130,000 × *g* in a B-35 ultracentrifuge. Relatively low recovery (30%) of the total substructures added was found in the boiled enzyme control, but nonetheless it can be seen that enzyme treatment broke down the tail tube plus plate substructure into material which remained near the top of the gradient. (It was not possible to examine the morphological changes because the protein in the conjugase preparation obscured the phage structures.)

It should be noted that no material having the sedimentation property of free tail plates (~90*S*) remained after enzyme treatment. Since isolated tail plates contain no folate, hydrolytic removal of a glutamyl residue should not necessarily cause tail plate destruction. The most likely interpretation of these results is that the tail plate plus tube substructure and, especially, the rather fragile tail plate are stable under these mildly acid circumstances (*p*H 4.5) only when they contain the pteroyl polyglutamate. This would explain the poor recovery in the boiled conjugase control since those substructures without dihydrofolate would also be destroyed. Recently, preliminary results have been obtained showing that tail plates free from folate are indeed unstable at these *p*H values (Male et al., Bacteriol. Proc., p. 183, 1970).

Properties of conjugase-treated particles, with regard to tail fiber attachment and sedimentation rate. As would be expected, the phage particle lacking just tail fibers is quite stable to conjugase treatment and does not break down when incubated at *p*H 4.5. The ability of tail fibers (12) to attach to conjugase-treated tail-fiberless particles was determined immunologi-

cally as serum-blocking power (10) after gradient purification to remove excess tail fibers. It was found that conjugase treatment did not prevent tail fiber attachment. This result suggests that the folate conjugate does not participate in the structures at the apexes of hexagonal base plates to which the fibers are attached.

Another feature of the attachment of tail fibers is their mobility about their attachment at the apexes of the base plates, especially for those phages which interact with adsorption cofactors such as L-tryptophan (9). T4Bol was 95% inactivated by conjugase. The conjugase was removed by sedimenting the phage, and the phage was resuspended in phosphate buffer. When examined in a Spinco model E ultracentrifuge, the treated T4B particles all had a sedimentation coefficient of 1,000*S* in the absence of tryptophan and a rate of 900*S* in the presence of 20 μg of L-tryptophan per ml. These properties are identical to those of untreated phage and reflect the change in position of the tail fibers. These observations also support the conclusion that the phage folate conjugate does not participate in the structure at the apexes of the base plate.

DISCUSSION

These investigations show that all of the T-even *E. coli* bacteriophages possess a unique type of folic acid, dihydropteroyl penta- or hexaglutamate, as a structural component of their complex tail. However, several experimental problems make it possible to answer only tentatively several important questions. First, how many molecules of this compound are there in viable virus particles? It is impossible to obtain reliable values by directly analyzing viable virus preparations since their head capsids trap a variety of molecules of the size of the phage folate. Values on ghosted phage preparations (with ruptured heads) varied from about two to occasionally six. Since ghost preparations always contain some particles with damaged tails, the analytical values support the view that viable phage, which has sixfold symmetry including a hexagon-shaped base plate and six tail fibers, may have six molecules of this particular compound. However, two or three folates per ghost would also be compatible with a structure with sixfold symmetry.

For the substructure consisting of tail sheath plus tail tube plus tail plate, which is also enriched for folate, a reasonable estimate can be made of the molecular size and number of folates per substructure unit. The mass of this substructure (28, 29) may be estimated to be about 13×10^6 daltons. Since ghosts have a mass of about 85×10^6 to 90×10^6 daltons (19), the phage substructure containing sheath plus tube plus plate represents about $\frac{1}{4}$ of the total phage weight. Since this substructure contains seven times the folate content of phage ghosts, all of the phage folate can be accounted for by that found in this tail structure. As isolated, and with the reservations discussed above in mind, these tail structures therefore contain about two dihydrofolates per tail substructure. The structure richest in folate per milligram of protein is that consisting of the tail plate plus tail tube. This tail substructure has an estimated particle mass of possibly 5×10^6 to 6×10^6 daltons, which is $\frac{1}{12}$ of the total mass. This substructure was found to contain only eight times the folate content of phage ghosts. On the average then, each substructure contains $\frac{8}{18}$ of that found in the phage particle or about one dihydrofolate molecule. Nonetheless, it must be concluded that this portion of the viral tail is the most probable locus for the dihydrofolate binding.

Although folate compounds are notorious for the avidity they display toward proteins, the ability of the phage structure to retain this compound "permanently" by noncovalent bonds is unexpected. The analyses of the tail substructures and some of their properties offer clues to the probable mechanism of the firm binding. It can be visualized that during tail assembly the dihydrofolate binds reversibly to the dihydrofolate reductase of the tail plate (23), probably near the center junction where the tail tube is to be attached. Attachment of the tail tube to the tail plate (35), either directly or by an allosteric effect, improves the binding and blocks the release of the dihydrofolate. The later addition of the sheath components (18) to this structure further stabilizes the binding of the dihydrofolate, essentially trapping it at this critical juncture. The structure now has almost the stability of the final phage structure with regard to its dihydrofolate content. The experiments showing that attachment of tail fibers to the apexes of the hexagonal base plate is essentially normal after conjugase treatment also support the view that the dihydrofolate is probably at the juncture between the base plate and tail tube.

There is no question that the dihydropteroyl glutamate found in phage, in phage tail substructures, and in phage-infected host cells is virus-induced. Its formation is chloramphenicol-sensitive, but the exact mechanism of its induction is not apparent. It is possible that a new enzyme(s) is formed which adds glutamates to the host cell dihydropteroyl triglutamate. This would be a unique type of an early virus-

induced enzyme, but in view of the role this compound plays in viral assembly it deserves consideration.

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LITERATURE CITED

1. Ackers, G. K. 1967. A new calibration procedure for gel filtration columns. J. Biol. Chem. 242:3237–3238.
2. Adams, M. H. 1959. Bacteriophages. Interscience Publishers, Inc., New York.
3. Albertsson, P.-A., and G. Frick. 1959. Partition of virus particles in a liquid two-phase system. Biochim. Biophys. Acta 37:230–237.
4. Allfrey, V., L. J. Teply, C. Geffen, and C. G. King. 1948. A fluorometric method for the determination of pteroylglutamic acid. J. Biol. Chem. 178:465–480.
5. Bakerman, H. A. 1961. A method for measuring the microbiological activity of tetrahydrofolic acid and other labile reduced folic acid derivatives. Anal. Biochem. 2:558–567.
6. Brenner, S., G. Streisinger, R. W. Horne, S. P. Champe, S. Benzer, and M. W. Rees. 1958. Structural components of bacteriophage. J. Mol. Biol. 1:281–292.
7. Cummings, D. J. 1964. Sedimentation and biological properties of T-phages of *Escherichia coli*. Virology 23:408–418.
8. Cummings, D. J., V. A. Chapman, and S. S. DeLong. 1968. Disruption of T-even bacteriophages by dimethyl sulfoxide. J. Virol. 2:610–620.
9. Cummings, D. J., V. A. Chapman, and S. S. DeLong. 1969. The sedimentation and conformational variance among T-even bacteriophages. Virology 37:94–108.
10. De Mars, R. I. 1955. The production of phage-related materials when bacteriophage development is interrupted by proflavine. Virology 1:83–99.
11. Edgar, R. S., and I. Lielausis. 1968. Some steps in the assembly of bacteriophage T4. J. Mol. Biol. 32:263–276.
12. Edgar, R. S., and W. B. Wood. 1966. Morphogenesis of bacteriophage T4 in extracts of mutant-infected cells. Proc. Nat. Acad. Sci. U.S.A. 55:498–505.
13. Epstein, R. H., A. Bolle, C. M. Steinberg, E. Kellenberger, E. Boy de la Tour, R. S. Edgar, M. Susman, G. H. Denhardt, and A. Lielausis. 1963. Physiological studies of conditional lethal mutants of bacteriophage T4D. Cold Spring Harbor Symp. Quant. Biol. 28:375–394.
14. Hall, D. H. 1967. Mutants of bacteriophage T4 unable to induce dihydrofolate reductase activity. Proc. Nat. Acad. Sci. U.S.A. 58:584–591.
15. Hall, D., I. Tessman, and O. Karlström. 1967. Linkage of T4 genes controlling a series of steps in pyrimidine biosynthesis. Virology 31:442–448.
16. Herriott, R. M., and J. L. Barlow. 1957. The protein coats or ghosts of coliphage T2. J. Gen. Physiol. 40:809–825.
17. Kanner, L. C., and L. M. Kozloff. 1964. The reaction of indole and T2 bacteriophage. Biochemistry 3:215–223.
18. King, J. 1968. Assembly of the tail of bacteriophage T4. J. Mol. Biol. 32:231–262.
19. Kozloff, L. M. 1968. Biochemistry of the T-even bacteriophages of *Escherichia coli*, p. 435–496. *In* H. Fraenkel-Conrat (ed.), Molecular basis of virology. Reinhold Book Corp., New York.
20. Kozloff, L. M., and M. Lute. 1965. Folic acid, a structural component of T4 bacteriophage. J. Mol. Biol. 12:780–792.
21. Kozloff, L. M., M. Lute, L. K. Crosby, R. Wong, and B. Stern. 1969. Critical arginine residue for maintaining the bacteriophage tail structure. J. Virol. 3:217–227.
22. Kozloff, L. M., M. Lute, and K. Henderson. 1957. Viral invasion. I. Rupture of thiol ester bonds in the bacteriophage tail. J. Biol. Chem. 228:511–528.
23. Kozloff, L. M., C. Verses, M. Lute, and L. K. Crosby. 1970. Bacteriophage tail components. II. Dihydrofolate reductase in T4D bacteriophage. J. Virol. 5:740–753.
24. Kuiken, K. A., C. M. Lyman, and F. Hale. 1947. Factors which influence the stability of tryptophan during the hydrolysis of proteins in alkaline solution. J. Biol. Chem. 171:551–560.
25. Lampen, J. O., R. R. Roepke, and M. J. Jones. 1947. The replacement of *p*-aminobenzoic acid in the growth of a mutant strain of *Escherichia coli*. J. Biol. Chem. 164:789–790.
26. Lowry, H., A. Bessey, and J. Crawford. 1949. Photolytic and enzymatic transformations of pteroylglutamic acid. J. Biol. Chem. 26:389–398.
27. Philipson, L. P., A. Albertsson, and G. Frick. 1960. The purification and concentration of viruses by aqueous polymer phase systems. Virology 11:553–571.
28. Sarkar, N., S. Sarkar, and L. M. Kozloff. 1964. Tail components of T2 bacteriophage. I. Properties of the isolated contractile tail sheath. Biochemistry 3:511–517.
29. Sarkar, S., N. Sarkar, and L. M. Kozloff. 1964. Tail components of T2 bacteriophage. II. Properties of the isolated tail cores. Biochemistry 3:517–521.
30. Shashoua, V. E. 1964. Formamidine sulfinic acid as a biochemical reducing agent. Biochemistry 3:1719–1720.
31. Silverman, M., and J. M. Noronha. 1961. A method for the preparation of tetrahydrofolic acid. Biochem. Biophys. Res. Commun. 4:180–182.
32. Uyeda, K., and J. C. Rabinowitz. 1963. Fluorescence properties of tetrahydrofolate and related compounds. Anal. Biochem. 6:100–108.
33. Westley, J., and J. Lambeth. 1960. Protein determination on the basis of copper-binding capacity. Biochim. Biophys. Acta 40:364–366.
34. Williams, R. C., and D. Fraser. 1956. Structural and functional differentiation in T2 bacteriophages. Virology 2:289–307.
35. Wood, W. B., R. S. Edgar, J. King, I. Lielausis, and M. Henninger. 1968. Bacteriophage assembly. Fed. Proc. 27:1160–1166.
36. Zakrzewski, S. F. 1960. Studies on the substrate specificity of folic acid reductase. J. Biol. Chem. 235:1780–1784.