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Bacteriophage T4 Baseplate Components

I. Binding and Location of the Folic Acid

LLOYD M. KOZLOFF,* MURL LUTE, AND LOUISE K. CROSBY
Department of Microbiology, University of Colorado Medical School, Denver, Colorado 80220

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Two different proteins with high affinities for the pteridine ring of folic acid have been used to determine the location of this portion of the folate molecule in the tail plate of T4D and other T-even bacteriophage particles. The two proteins used were (i) antibody specific for folic acid and (ii) the folate-binding protein from bovine milk. Both proteins were examined for their effect on various intact and incomplete phage particles. Intact T2H was weakly inactivated by the antiserum but not by the milk protein. No other intact T-even phage, including T4D, was affected by these two proteins. When incomplete T4D particles were exposed in an in vitro morphogenesis system, it was found that neither of the two proteins affected either the addition of the long tail fibers to fiberless particles or the addition of tail cores to tail plates. On the other hand, these two proteins specifically blocked the addition of T4D gene 11 product to the bottom of T4D baseplates. After the addition of the gene 11 protein, these two reagents did not inhibit the further addition of the gene 12 protein to the baseplate. It can be concluded that the phage folic acid is a tightly bound baseplate constituent and that the pteridine portion of the folic acid is largely covered by the gene 11 protein.

The baseplate of the T-even bacteriophages is one of the more complex substructures of these virus particles. At least 14 gene products (31) are known to be involved in assembling this structure. Most of the viral gene products are protein, and they have been partially characterized chemically and the morphogenetic pathway has been outlined by King and his colleagues (12; Y. Kikuchi and J. King, personal communication). This complex structure has the physiological role of controlling the attachment of a virus particle to its bacterial host and in aiding and regulating the entrance of the viral DNA into the host cell (1, 2, 20, 32). One of the unusual features of the baseplate is its ability to undergo a conformational change from a hexagon-like structure containing an apparent solid center plug to a six-pointed star configuration with an apparent center opening (25, 26). Although this change is required for DNA injection, little is known of either of the factors influencing this change or the components involved.

An unusual form of folic acid, dihydropteroyl hexaglutamate, is induced by phage infection and is known to be a component of the phage tail and to function during DNA injection (16). Its exact location, however, has not been determined. In this connection it is interesting to note that two phage-induced proteins, dihydrofolate reductase (22) and thymidylate synthetase (6), probably also play a role in the initial stage of infection (14, 15, 19) and are either known or suspected to be tail components.

This paper presents evidence on the location and binding of the phage folic acid to the phage structure. This compound was first reported as a capsid component in 1965 (16) and was shown to be involved in tail function. An attack on the polyglutamate portion of the folate by conjugase prevented phage adsorption. In 1970, it was found that the T4D phage substructure, consisting of the tail core plus the baseplate, contained all the phage folic acid (18). However, preparations of isolated baseplates did not contain folic acid. At the same time, an enzyme involved in folic acid metabolism, dihydrofolate reductase, was also shown to be a baseplate component (19).

Specific reagents have now been used to examine the binding of the folate compound in the phage capsid, and the evidence indicates that the folic acid is also located in the baseplate. These reagents include antibodies specifically made against the pteridine ring of the folic acid (24) and the folate-binding protein, which is a normal constituent of bovine milk (7, 8, 23, 28, 30). The reaction of these compounds with both whole phage particles and incomplete phage particles lacking a variety of protein components suggests that the pteridine ring is buried within the complex baseplate largely under

the gene 11 protein, while a portion of polyglutamate extends free of the baseplate. The accompanying papers (14, 15) indicate that phage-induced dihydrofolate reductase (*dfr*) and thymidylate synthetase (*td*) also are partially covered by the gene 11 protein and are probably closely associated with the phage folic acid in the baseplate.

MATERIALS AND METHODS

Preparation and purification of bacteriophage stocks and substructures. *Escherichia coli* bacteriophage stocks were grown, purified, and assayed by standard procedures (18). A list of these phages with their relevant properties and the sources of the original stocks is given in Table 1.

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 and gene products were synthesized (Table 1). Particles free from long tail fibers, prepared from the T4D quadruple amber mutant X4E (4), defective in genes 34, 35, 37, and 38, were purified in a manner identical to standard phage preparations since they sediment as fast or faster than intact phage particles. Similarly, preparations of intact phage particles (T4D 12^-) lacking the gene 12 protein (P12) were prepared and purified. These particles can be activated upon incubation with *E. coli* B extracts containing the gene 12 protein (11). In addition, preparations of intact but incomplete phage particles were purified lacking both the gene 12 protein (P12) and the gene 11 protein (P11) (29). These particles were prepared after infection with T4D 11^-. Both T4D 11^- and T4D 12^- particles do have the long tail fibers. During baseplate morphogenesis the 11 protein is bound to the bottom of the tail plate before the 12 protein (32). Incomplete particles made with T4D 11^- first add the 11 protein, and then the 12 protein is added (10, 21). The 12 protein cannot bind to 11^- particles. The 11^- incomplete particles can be activated upon incubation with bacterial extracts containing both the 11 and 12 proteins. These two tail proteins are known to be involved in the first steps of infection (27, 29).

The bacterial extract used as the source for both the gene 11 and gene 12 proteins was prepared in the following manner. One liter of *E. coli* B (the nonpermissive host for phage amber mutants) was grown in broth to a concentration of 4×10^8 cells/ml at 30 C. The bacteria were infected with five particles of the T4D double amber mutant $23^-/27^-$ per cell, incubated for 7 min, and then superinfected with five more phage per cell. This T4D mutant forms neither the 23P nor the 27P and cannot form either phage heads or tailplates. After an additional 40 min of incubation at 30 C, the suspension was cooled in an ice bath, and the infected cells were centrifuged down and then resuspended in about 4 to 5 ml of 0.06 M phosphate buffer, pH 7.0, containing 0.01 M EDTA at pH 7.0. The suspension was sonicated with a Branson sonifier in the cold until the viscosity was greatly decreased. The suspension was clarified at $8,000 \times g$ for 90 min and then dialyzed overnight against BUM (10, 21). A small precipitate was centrifuged off; the solution was incubated at room temperature for 30 min and clarified once again, and then 0.1 ml of 0.02 M EDTA, pH 7.0, was added. The volume was usually about 5.5 ml at this point, and the solution was distributed in small capped tubes and stored at −20 C. The extract was reasonably stable for several months, and 1.0 ml usually contained enough 11P and 12P to complement 2×10^{11} incomplete phage particles in vitro in 5 h at 30 C.

Folate-binding compounds. Serum from rabbits immunized against folic acid linked covalently through the glutamic acid carboxyl group to bovine serum albumin was kindly furnished by J. Russell Little. This serum has been called FA-1 by Rubenstein and Little (24) and has been shown to have affinity exclusively toward the pteridine (*pte*) ring. For example, the affinity of an altered pteridine ring

TABLE 1. *E. coli bacteriophage strains*

Strain	Distinctive properties	Source
T2L	Cofactor-independent for adsorption	(9)
T2H	Indole inhibits adsorption	(9)
T4B	Requires tryptophan for adsorption	(13)
T4D	Wild type, cofactor-independent	(4)
T4D amber mutants		
N93	Defective in gene 11; yields particles lacking both the 11 and 12 proteins	R. S. Edgar (5)
N69	Defective in gene 12; yields particles containing the 11 protein but not the 12 protein	R. S. Edgar (5)
E1137	Defective in gene 19; yields tail plates	R. S. Edgar (5)
N85	Defective in gene 48; unable to make tail cores	R. S. Edgar (5)
H21	Defective in gene 54; yields free tail plates and free phage heads	R. S. Edgar (5)
B17/N140	Defective in genes 23 and 27; source of gene 11 and gene 12 proteins	R. S. Edgar (5)
B25/B252/N52/B262 (X4E)	Defective in genes 34, 35, 37, and 38; yields tail fiberless particles	R. S. Edgar (5)

such as that of methotrexate is about 2% that of the folate pteridine ring; the affinity toward the *p*-aminobenzoyl glutamic residues in the folic acid was less than 0.01% of that to the whole folic acid.

Carnation Instant nonfat dry milk (obtained locally) was used as a source of folate-binding protein. Based on its ready availability and its content of the milk folate-binding protein (8, 23), this milk powder has been used to develop a radioassay method for folic acid (28, 30). The binding protein has been shown to specifically bind to the pteridine ring (30) rather than to other portions of the folate molecule. Five grams of milk powder was dissolved in 50 ml of BUM buffer (4) and dialyzed against 100 volumes of BUM in the cold. After dialysis the milk powder suspension was stored at 4 C. The preparation slowly lost the ability to bind folate over a period of 2 weeks. For some experiments the albumin fraction of dry milk was prepared by two different methods as described by Ford et al. (7). The first method involved a sodium sulfate step to remove the casein, whereas the second method used a rennin treatment to remove the casein, leaving the binding protein in the whey fraction. In the second method, to obtain active binding protein it was necessary to remove the folate from the binding protein by treatment with charcoal at low pH. The whey proteins in BUM containing 50 mg of dithiothreitol per ml were titrated to pH 3.6 with 1 N acetic acid. Eighty milligrams of Norit A was added per ml of solution. After stirring for 10 min in the cold, the charcoal was removed by centrifugation and the pH was raised to 7.0 with 2 N NaOH. The solution was again dialyzed against BUM containing dithiothreitol, and it is referred to later as the whey fraction.

The ability of the milk albumin fractions and whey fractions to bind [^{3}H]folate was measured by the methods of Waxman et al. (30) and Tajuddin and Gardyna (28). Both the albumin and whey fractions bound folate equally well (per milligram of protein), but the yield of folate-binding protein was threefold higher using the whey procedure. Both fractions bound folate 30-fold better than unfractionated milk. However, neither preparation was stable, and most of the folate-binding ability was lost upon storage of lyophilization.

RESULTS

Interaction of whole phage particles with proteins that react with folic acid. It has been shown that hog kidney conjugase (16), a peptidase for the gamma glutamyl bonds of folic acid polyglutamates, inactivated all the T-even phages by damaging their tail structure. Further, this enzyme reacted with the T4D phage substructure, consisting of the tail core and baseplate (18). However, whereas these observations show that the polyglutamate portion is partially exposed, they do not indicate the precise location of the pteridine group of the folic acid. All the T-even phage were treated with the two proteins known to bind to pteridine groups, the antiserum to folate prepared by Rubenstein and Little (24), and the folate-binding protein from milk (7, 8, 23, 28, 30).

All the T-even phages examined, T2H, T2L, T4B, and T4D, were unaffected by the presence of the folate-binding protein from milk. Phage at a final concentration of 10^7/ml, in the presence or absence of adsorption cofactors, were incubated with 3.5% milk solids or purified albumin fractions at 30 C from 3 to 20 h. None of the phage strains was inactivated. This amount of folate-binding proteins will be shown below to react with incomplete phage particles. It can be concluded that the pteridine group on the tail of whole phage particles is not exposed sufficiently to react with the folate-binding protein.

When T2L, T4D, and T4B phages were treated with antiserum specific for folic acid, they were also completely resistant. However, one strain of the T-even phages, T2H, was partially inactivated by the antiserum. The inactivation of this phage strain is shown in Fig. 1. Over 50% of the particles were inactivated by a 1:6 dilution of the antiserum within 1 h. The reason for the failure to inactivate more than 50% of the phage particles is unknown. The addition of folic acid as a competitor initially protected the phage, and it can be concluded that the antifolate serum is probably reacting with the pteridine group of the T2H phage folic acid. It is also apparent that the exposure of the pteridine group is different in T2H as compared to the other phage strains.

T2H is known to have unique features of its tail structure since its adsorption to its host cell is sensitive to the presence of indole (9), whereas the other T-even phages are not sensitive to indole. It was found that 1 mM indole blocked the inactivation of this phage particle by the antiserum, presumably by altering the conformation of the baseplate and tail fibers. Adenosine diphosphoribose, another reagent known to alter the configuration of the tail plate of T-even phages by binding to the phage baseplate dihydrofolate reductase (20), at 5 mM also blocked the inactivation of the T2H by the antiserum. These results suggest that the pteridine portion of the phage particle folate is contained within the tail baseplate and that alterations of the baseplate may block the ability of relatively large molecules such as gamma globulins or milk albumin to bind to it. The relative inaccessibility of the pteridine group, especially as compared to the polyglutamate end of the molecule, is in accord with the observation that phage folic acid is so tightly bound that it cannot be removed by prolonged wash-

ing, dialysis, or prolonged exposure to activated charcoal.

Effect of antifolate serum on the joining of tail fibers and tail cores to the baseplate. The antiserum to folic acid was tested on specific steps involved in the assembly of the tail of T4D. It was possible to use antiserum in in vitro morphogenesis systems (3, 29) since complete T4D particles are insensitive to this serum. The effect of this serum on the addition of tail fibers to fiberless particles is shown in Fig. 2. The rate of in vitro complementation was identical in the presence of the antiserum and normal serum. It appears that neither the tail fibers themselves nor phage particles without tail fibers contained an exposed phage pteridine residue.

Similarly, the exposure of the pteridine group during the reactions involving the addition of the T4D tail core proteins to the tail plate was examined in three in vitro systems (11). The three systems were: (i) extracts of gene 54^--infected cells mixed with extracts of gene 19^--infected cells; (ii) gene 54^- extracts mixed with gene 48^- extracts; and (iii) gene 48^- extracts mixed with gene 19^- extracts. In all three systems the antiserum did not affect the rate (or amount) of phage assembly. From these experiments it seems likely that the pteridine group is neither exposed on the top of the baseplate nor is the pteridine likely to be a component of the tail core.

Effect of hog kidney conjugase and antiserum specific to folic acid on T4D particles lacking gene 11 and 12 proteins. It has been known for some time that the gene 11 and gene 12 proteins are baseplate components which can either be used to form the baseplate early in morphogenesis or be added at the end of the morphogenetic pathway to particles which are otherwise complete (3). T4D particles lacking the gene 11 and gene 12 proteins were prepared, purified, and complemented in vitro with gene 11 and gene 12 proteins as described in Materials and Methods.

The presence and exposure of the folic acid on particles lacking the gene 11 and gene 12 proteins were examined in a variety of ways. These incomplete particles were treated with hog kidney conjugase at pH 4.5 as described earlier (16). Samples were removed at various times, the pH was raised back to 7.4 with pH 8.0

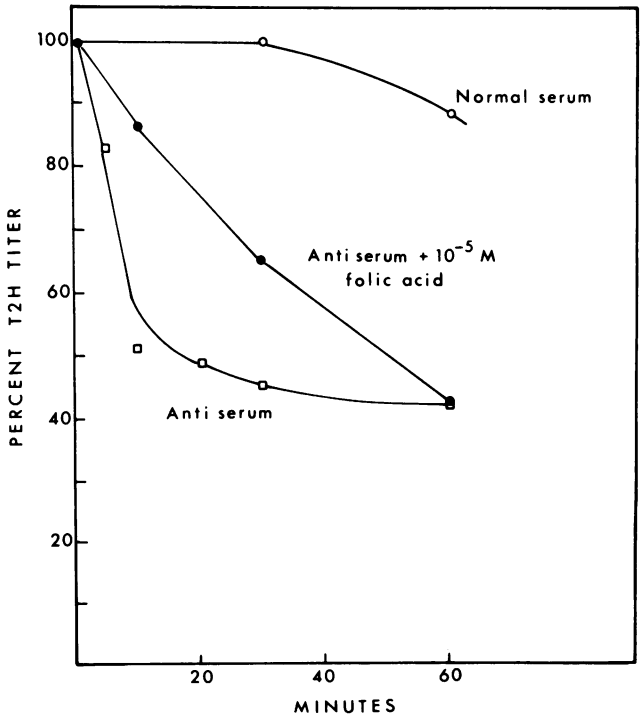

FIG. 1. *Effect of antibodies specific for folic acid on T2H phage. Purified T2H was diluted in 0.1 M phosphate buffer, pH 7.0, so that its initial concentration was 6.6 × 10^6 in the reaction mixture; the antiserum to folic acid (or normal rabbit serum) was diluted 1:6 in the reaction mixture. The incubation temperature was 24 C.*

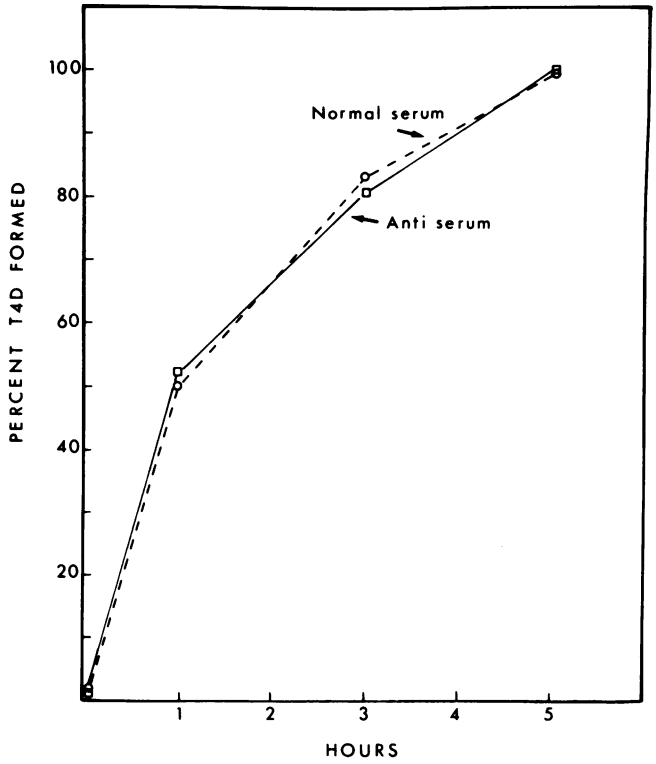

FIG. 2. *Effect of antibodies specific for folic acid on addition of tail fibers to fiberless T4D particles. Purified T4D amber mutant X4E particles lacking tail fibers (initial titer of 3.0 × 10^{10} in the reaction mixture) were incubated with extract made with T4D23⁻ at 30 C. In 5 h the titer had increased to 6 × 10^{11} (equals 100% T4D formed). The serum was used at a 1:10 dilution.*

phosphate buffer (a pH at which conjugase is largely inactive), and then the extract containing the gene 11 and gene 12 proteins was added. Incomplete T4D 11⁻ particles were sensitive to the action of conjugase, but their rate of inactivation was approximately the same as that of intact T4D gene 11⁻ particles, which had been grown on the permissive host cell and had both the gene 11 and gene 12 proteins on their baseplates. It can be concluded that, as expected, T4D particles lacking the 11 and 12 proteins do contain a folate polyglutamate but that the exposure of the polyglutamate portion is not very different in these incomplete particles from what it is in completed particles.

On the other hand, the pteridine ring of the folate is exposed in particles lacking the 11 protein, whereas it is not exposed in intact T4D particles. Figure 3 shows that antiserum to folic acid inhibited the steps in morphogenesis involving the addition of the gene 11 (and gene 12) proteins to T4D particles. The action of the antiserum was partially reversed by the addition of 2 × 10^{-6} M folic acid. This inhibition by the antiserum was observed in five separate experiments, and the inhibition was always relieved by folic acid. The addition of gene 12 protein to particles lacking this protein was not effected by the presence of antiserum to folic acid (Fig. 4). Further, T4D phage particles lacking the 12 protein were completely resistant to inactivation by the antiserum. These observations demonstrated that the folate polyglutamate is a baseplate component and that the pteridine portion of the folate must be shielded by the gene 11 protein.

These observations suggested that the folate polyglutamate was incorporated into the baseplate structure quite early during its morphogenesis and was bound irreversibly. The possibility that some 11⁻ particles might have lost all or some of their dihydropteroyl hexaglutamate and that folate could be incorporated later was examined by adding the phage folate compound (17) to 11⁻ particles and then later adding the extract containing the 11 and 12 proteins. In this experiment the phage titer increased from 4 × 10^{7} to 1.74 × 10^{10}/ml in the absence of phage folate and from 4 × 10^{7} to 1.67 × 10^{10} in the presence of 9 × 10^{-5} M folate

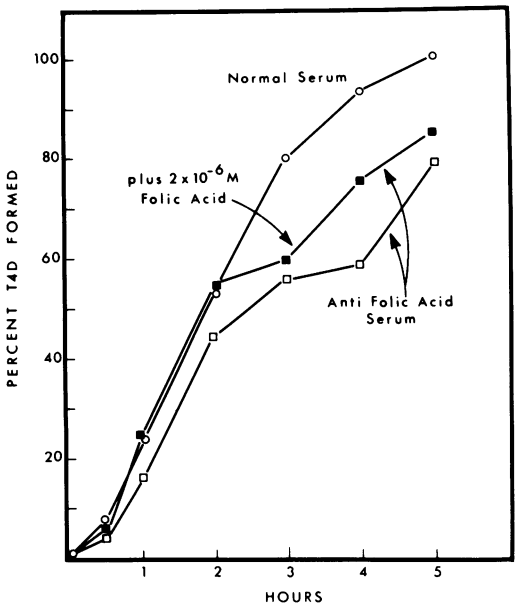

FIG. 3. *Effect of antibodies specific for folic acid on addition of P11 and P12 to T4D 11⁻ particles. The initial titer of live T4D 11⁻ particles was 6 × 10⁶/ml, and in the control tube with normal rabbit serum the final titer (100% T4D formed) at 5 h was 9.9 × 10⁹/ml. The normal rabbit serum or antifolic acid serum was incubated at a 1:3 dilution overnight in the cold with the phage particles with or without the folic acid. Then the P11 and P12 contained in the 23/27 extract as described in Materials and Methods was added and the reaction was incubated at 30 C. The final serum dilution was 1:10.*

hexaglutamate. Further, in separate experiments it was also found that the presence of charcoal did not affect the addition of the 11 protein. These results confirm the earlier observations that inhibition of baseplate formation (17, 19) by charcoal or aminopterin is due to the inhibition of incorporation of the folate compound into the baseplate structure, but that once assembled the folate can neither be removed nor reincorporated later.

Effect of folate-binding proteins from milk on T4D morphogenesis. The exposure of the pteridine group on phage particles lacking the gene 11 and gene 12 proteins was confirmed by examining the effect of folate-binding proteins from milk on the addition of P11 (and P12) to T4D 11⁻ particles. Figure 5 shows that milk solids inhibit the addition of the gene 11 (and gene 12) proteins and that the degree of inhibition was proportional to the amount of milk solids added. The inhibition of complementation by milk solids was destroyed by heating the milk (Fig. 6). This is in agreement with the known heat lability of the folate-binding protein in milk. Further, the addition of folic acid (3.5×10^{-4} M) also blocked the effect of the milk solids on gene 11 protein addition (Fig. 6).

The effect of milk folate-binding proteins solely on the addition of the gene 12 protein to T4D 12⁻ particles was examined. In an experiment identical to that shown in Fig. 4 on the effect of antiserum to folic acid on 12P addition, it was found that the presence of milk solids has no effect on the addition of the 12 protein.

The correlation of the ability of milk solids to bind folic acid and to inhibit complementation in this system is shown in Table 2. The albumin and rennet whey fractions of milk are known to contain the folate-binding protein. It can be seen that the ability of these fractions to bind free folate is correlated with their ability to inhibit P11 addition in the complementation mixture and thus prevent formation of viable T4D.

Although both the antiserum to folate and the milk folate-binding protein are known to bind free folate largely irreversibly, the data from Fig. 3 and 5 suggest that these large proteins react reversibly with the pteridine ring bound to proteins on the phage tail structure. Since the addition of the 11 protein is irreversible, the reagents then may only decrease the rate of 11 protein addition but have a lesser effect on the amount of total new particles formed.

Although the evidence indicated that antiserum for folic acid and milk folate-binding protein were reacting with phage pteridine, the possibility that these two proteins were reacting with some component in the extract used as the source for the gene 11P and gene 12P was examined (Fig. 7). The maximum inhibition of complementation was found when the milk folate-binding protein was preincubated with the incomplete 11⁻ phage particles for 18 h at room temperature prior to the addition of the extract. There was no inhibition of complementation when the same amount of folate-binding protein was preincubated with the extract containing the P11 and P12 and then the incomplete phage particles added. Further, there was less inhibition if the milk solids were added to the complete complementation mixture without preincubation.

DISCUSSION

The results reported above offer insight into the role of phage-induced dihydropteroyl hexaglutamate in the morphogenesis of the phage tail plate. Even though isolated and purified free tail plates do not contain the folate com-

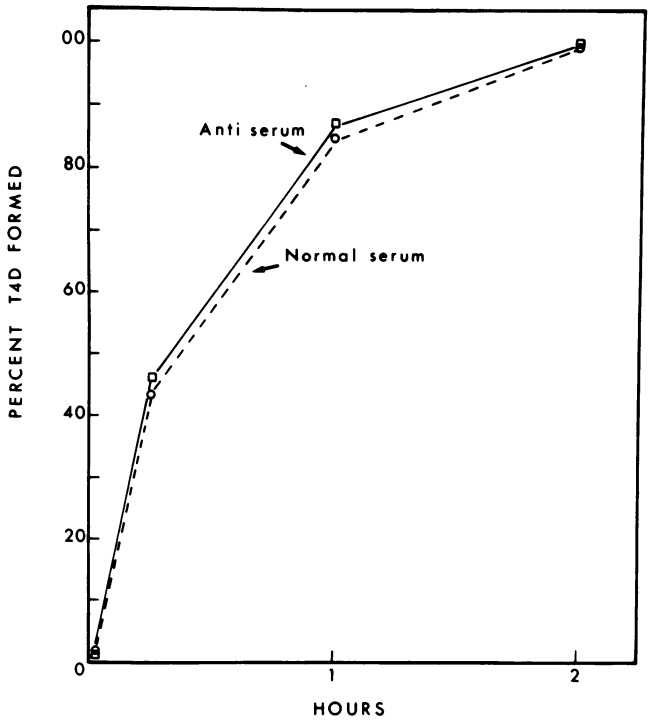

FIG. 4. *Effect of antibodies specific for folic acid on addition of P12 to T4D 12⁻ particles. T4D 12⁻ particles were diluted in BUM so that the initial titer of live particles was 7 × 10⁹/ml. Antifolate serum (or normal serum) was used at a final dilution of 1:2. The source of P12 was the 23/27 extract described in Materials and Methods, and it was added last to the reaction mixture. The mixture was incubated at 37 C, and after 2 h the phage titer had increased to 2.3 × 10¹⁰.*

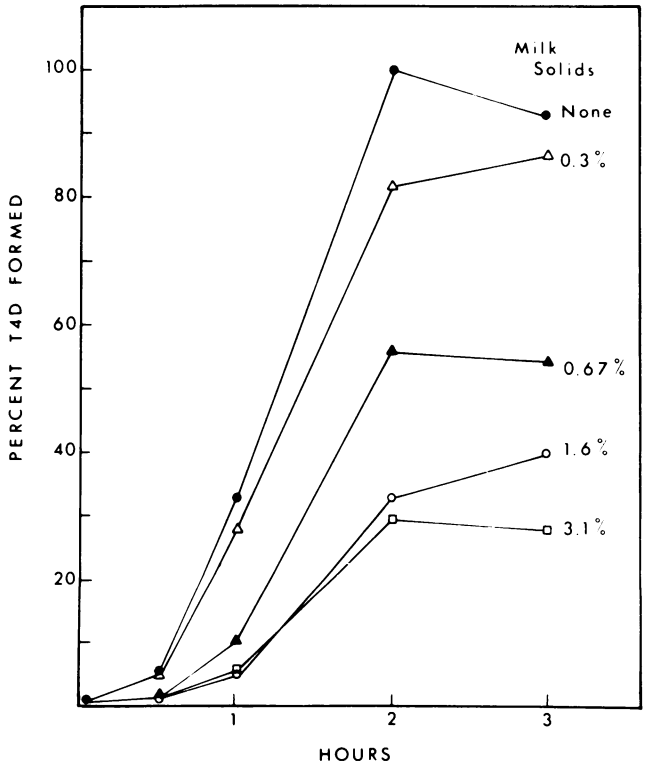

FIG. 5. *Effect of milk solids on addition of P11 and P12 to T4D 11⁻ particles. Various amounts of a dialyzed 5% suspension of dry nonfat milk solids were incubated with T4D 11⁻ particles for 30 min at 30 C, and the complementation was initiated by the addition of the T4D 23/27 extract. The amounts of milk solids listed were the final concentrations in the reaction mixture. The initial titer was 3 × 10⁷, and the maximum titer in the absence of milk solids was 1.2 × 10¹⁰ (equals 100% T4D formed).*

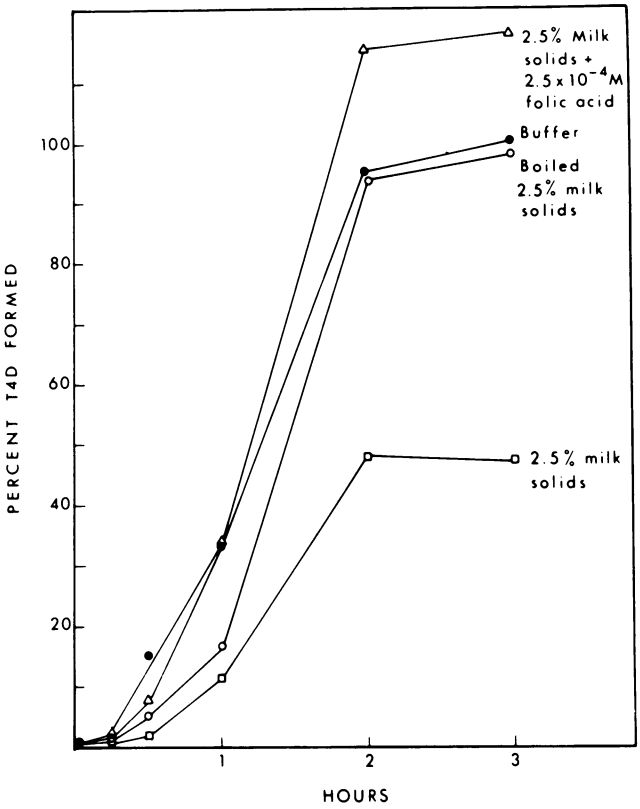

FIG. 6. *Effect of heat and folic acid on the inhibition of P11 and P12 addition to T4D 11⁻ particles by milk solids. This experiment was carried out similar to that in Fig. 5, except that one portion of the milk solids was boiled for 5 min before being added to the reaction mixture. The initial titer was 3 × 10⁷, and in the absence of milk solids the maximum titer was 9.5 × 10⁹ (equals 100% T4D formed).*

TABLE 2. *Ability of milk protein fractions to bind folate and to inhibit the addition of P11 and P12 to T4D 11⁻ particles*

Milk fraction	Folate binding (pmol/ml)[a]	T4D (%) formed at various times (min)[b]		
		15	30	120
None	0	100	100	100
Albumin fraction	0.06	5	50	54
Rennet whey fraction (charcoal treated)	0.17	0	1.5	44

[a] Measured by the method of Waxman et al. (30) and Metz et al. (23).

[b] Measured by methods used in Fig. 3, 5, and 6. Identical volumes were used of the albumin and rennet whey fractions. The percentage given reflects the amount of phage formed at a given time with the milk protein as compared to that of the control reaction without milk protein.

pounds, it can be concluded that during normal morphogenesis the folate does bind to tail plate proteins. Most likely the folate binds to the phage-induced dihydrofolate reductase and thymidylate synthetase, as described in the companion papers (14, 15), to form a critical element possibly at the center of the tail plate. Folate is not irreversibly bound to the tail plate until the tail core has been constructed on the top of the plate. Presumably the addition of the tail core causes conformational changes in adjacent proteins which irreversibly lock in place the pteridine portion of the folate. Later, the addition of the gene 11 protein on the bottom of the plate (at least for T4D and probably for T2L and T4B, but not for T2H) covers the pteridine portion so that large molecules such as rabbit serum globulin or milk albumin cannot react with this structure.

The role played by this unusual viral component is still not defined, but it must be involved both in the morphogenesis of the tail plate and

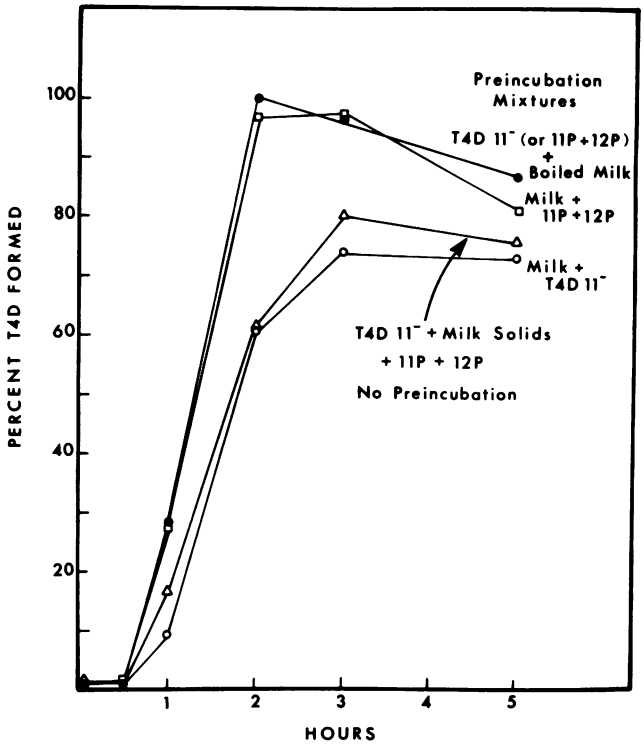

FIG. 7. *Effect of preincubation of T4D 11⁻ particles with milk solids on the subsequent addition of P11 and P12. T4D 11⁻ particles (or 23/27 extract, the source of P11 and P12) were incubated with 6% dialyzed milk solids for 18 h at 24 C. Then the complementation was carried out as described for similar experiments. Boiled milk was preincubated with the two components to serve as the controls. Similarly in another control the milk solids, T4D 11⁻ particles, and the 23/27 extract were all added simultaneously without preincubation.*

in the conformational changes the tail plate undergoes upon infection of the host cell. A probable, and quite tentative, suggestion for the location of the folate is shown diagramatically in an accompanying paper (14). It is known that the binding of this small molecule to viral tail plate proteins influences their quaternary structure (15), and it seems likely that changes in its binding are involved in the "hexagon" to "star" transition necessary for DNA injection.

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