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Bacteriophage T4 Virion Dihydrofolate Reductase: Approaches to Quantitation and Assessment of Function

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This paper is concerned with the physiological role(s) of T4 phage-coded dihydrofolate reductase, which functions both in DNA precursor metabolism and as a virion protein. (i) We have detected enzyme activity in noninfectious particles produced under restrictive conditions by gene 11 mutants. This supports the conclusion of Kozloff et al. (J. Virol. 16:1401-1408, 1975) that the protein lies in the baseplate, covered by the gene 11 protein. (ii) We have obtained further evidence for virion dihydrofolate reductase as the target for neutralizing activity of T4 dihydrofolate reductase antiserum and as a determinant of the heat lability of the virion. This derives from our observation that the reductases specified by T4B and T4D differ in several properties. (iii) We have investigated several anomalous properties of T4 mutants bearing deletions that reportedly extend into or through the *frd* gene, which codes for dihydrofolate reductase. Evidence is presented that the deletions in fact do not extend through *frd*. These strains direct the synthesis of material that cross-reacts with antiserum to homogeneous dihydrofolate reductase. Moreover, they are all quite sensitive to the phage-neutralizing effects of this antiserum. In addition, they are restricted by several of the hospital strains, wild-type strains of *Escherichia coli* supplied by the California Institute of Technology group. (iv) We have attempted to detect dihydrofolate reductase among early-synthesized proteins present in T4 tails. Two such proteins are seen, one of which is evidently the gene 25 product and one that is a bacterial protein. Quantitation of our electrophoretic technique has allowed determination of the number of molecules of some T4 tail components present per virion. (v) Finally, we have compared the T4 dihydrofolate reductase with the corresponding enzyme specified by two plasmids conferring resistance to trimethoprim (Skold and Widh, J. Biol. Chem. 249:4324-4325, 1974). Although the enzymes are similar in some properties, they differ in several important respects, including immunological activity.

For several years this laboratory has investigated the molecular properties and biological roles of T4 phage-coded dihydrofolate reductase. Although the enzyme duplicates a preexisting host cell activity, the viral enzyme does participate in the greatly increased rates of DNA precursor synthesis seen in infected cells, for T4 *frd* gene mutants, unable to induce dihydrofolate reductase (23), show reduced rates of DNA synthesis, due to partial thymine nucleotide deficiency (22). Subsequent to this demonstration, Kozloff et al. (20) detected dihydrofolate reductase activity in the baseplates of disrupted phage ghosts, suggesting an additional role for this enzyme as a structural protein. Mathews (25) presented genetic evidence that the structural reductase is the same as the soluble enzyme, and Mathews et al. (28) showed that antiserum to homogeneous T4 dihydrofolate reductase has T4 phage-neutralizing activity, presumably because it reacts with virion dihydrofolate reductase. Dawes and Goldberg (7) reported similar findings.

In this paper we describe several approaches to directly demonstrating and quantitating virion dihydrofolate reductase activity and to evaluating the role of this enzyme as a virion protein. The accompanying paper (18) from Kozloff's laboratory presents complementary data, both for dihydrofolate reductase and for thymidylate synthetase, another early enzyme that has been implicated as a virion protein (6, 16).

MATERIALS AND METHODS

Cells and bacteriophage strains. Phage strains used included T4D, T4BO$_1$, and T6, which have been maintained in this laboratory for some time, as have *Escherichia coli* strains B (wild type), B3 (low-thy-

mine-requiring strain), and CR63 (permissive host for propagation of amber mutants). Phage bearing deletions between genes 63 and 32 were obtained from T. Homyk, Jr., and J. Weil, of Vanderbilt University (11). These included T4 *del* (63-32) −1, −7, and −9. All three strains also contained the *r*1589 deletion in the *r*II gene. They are referred to herein as *del*1, *del*7, and *del*9, respectively. The *r*1589 phage was provided from the same source. T4 *am*N93 (gene 11) was obtained from L. Kozloff, University of Colorado, and other gene 11 amber mutants were obtained from the California Institute of Technology (Cal. Tech.) collection, through the courtesies of I. Lielausis, H. Revel, and W. Wood. T4 *am*B272 (gene 23) was obtained from H. Bernstein of this institution. T4 *frd*11, an amber *frd* mutant (10), was obtained from D. Hall, Duke University; T4 *frd*2 was isolated in this laboratory (23); T4 *td*8 (thymidylate synthetase negative) was originally provided by I. Tessman, Purdue University; and T4 *td*N54, an amber *td* mutant isolated by D. Hall, was provided by L. Kozloff.

The hospital strains, a set of 26 wild-type *E. coli* strains (38), were also provided through the courtesy of the Cal. Tech. group. *E. coli* J53 strains bearing R factor R388 or R483 were provided by R. Hedges, Royal Postgraduate Medical School, London. Both R factors, which specify resistance to trimethoprim, have been shown to code for a drug-resistant form of dihydrofolate reductase (33).

Growth media and procedures for growth and infection of cells, preparation of cell-free extracts, purification of phage, assay for enzymes, and immunodiffusion have been described in previous publications from this laboratory (5, 6, 21, 24–27).

The isolation of phage tails was accomplished essentially as described by King and Laemmli (14). *E. coli* B was grown at 37°C in 40 ml of M9 medium with vigorous aeration to a density of about 2×10^8/ml. The culture was infected with T4 *am*B272 (gene 23) at a multiplicity of about five phage per bacterium, and superinfection at the same multiplicity was carried out at 7 min, to promote lysis inhibition. Labeling was carried out at 0.8 μCi/ml with a ^{14}C-labeled amino acid mixture (New England Nuclear; specific activities of individual amino acids, from 100 to 400 mCi/mmol) for the periods indicated in the individual figure legends. When labeling was to be terminated before 45 min, this was accomplished by addition of a 1,000-fold molar excess of Casamino Acids, and incubation was continued until 45 min after infection. Cells were harvested by centrifugation and suspended in 1 ml of M9 salts. Cells were lysed by two cycles of freeze-thawing, and then pancreatic DNase and $MgSO_4$ were added to give concentrations of 20 μg/ml and 0.01 M, respectively. After a 30-min incubation in an ice bath, 1 ml of 0.1 M EDTA and 40 μg of pancreatic RNase were added. After an additional 30-min incubation at room temperature, debris was removed by centrifugation at $6,000 \times g$ for 10 min.

A 0.5-ml amount of the above extract was layered onto a 5-ml 5 to 20% (wt/vol) linear sucrose gradient, which was then centrifuged for 40 min at 43,000 rpm in a Beckman SW65 rotor at 20°C. Fractions of 0.2 ml each were collected by pumping from the bottom of the tube. The distribution of radioactivity through the gradient was determined by liquid scintillation counting of 50-μl samples of each fraction. Each gradient showed a single peak of radioactivity. Electron microscopic examination of these fractions showed them to consist largely of "naked" (unsheathed) phage tails. Sheathed tails are, apparently, unstable under these centrifugation conditions.

Labeled proteins in these preparations were analyzed by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis on 10% gels, as described by Studier (34). Electrophoresis was carried out at 150 V for approximately 2 h, until the tracking dye was about 2 mm from the bottom of the gel. The labeled proteins were visualized by fluorography, as described by Bonner and Laskey (3).

Stoichiometry of T4 tail proteins. Radioactively labeled T4D and phage tails were prepared by infecting *E. coli* B with T4D or *am*B272, respectively. The cells were grown at 37°C to a density of 2×10^8 ml in 40 ml of M9 supplemented with 50 μg of leucine per ml. Cultures were infected with five phage per bacterium, and, at 4 min postinfection, the surviving bacteria were measured. One minute later, the infected cells were pelleted by centrifugation and suspended in 40 ml of fresh M9, prewarmed to 37°C and containing 5 μg of leucine per ml plus 300 μCi of [^{3}H]leucine (New England Nuclear; specific activity, 84 Ci/mmol). The *am*B272 culture was superinfected at the same multiplicity 2 min later. One-milliliter samples of each culture were removed at 20-min intervals for T4D infection and at 10-min intervals for the mutant-infected cells, measured from the time the cells were suspended with [^{3}H]leucine. These samples were rapidly membrane filtered (Millipore Corp.), placed on ice, and treated as follows. A 500-μl amount of each filtrate was mixed with 200 μg of bovine serum albumin (20 μl of a 1% [wt/vol] solution), and 500 μl of 10% trichloroacetic acid was added. This mixture was allowed to stand at room temperature for 15 min, after which the precipitated protein was pelleted by centrifugation. The amount of radioactivity in the supernatant was taken as a measure of the amount of labeled leucine remaining in the growth medium. This was done to ensure that the specific activity of the pool of labeled leucine available for protein synthesis did not change significantly throughout the course of infection.

At 2 h after infection, chloroform was added to complete lysis of the T4D-infected cells, and cellular debris was removed by centrifugation at $3,000 \times g$ for 10 min. The *am*B272-infected cells were harvested 45 min after infection and treated as described in Results. The supernatant containing T4D was returned to 37°C, and 40 μl of a saturated $MgSO_4$ solution, 40 μg of bovine pancreatic DNase I (Sigma Chemical Co.), and 40 μg of RNase A (bovine pancreas; Sigma type II-A) were added. After 30 min, the phage were pelleted by centrifugation in a Beckman type 35 rotor at 35,000 rpm for 45 min at 5°C. The pellet was suspended gently in 0.4 ml of M9 salts, and the large pieces of insoluble debris were

removed manually by holding the centrifuge tube horizontally and rotating it. The insoluble material sticks to the wall of the centrifuge tube and the rotational motion carries the debris out of the phage suspension. This method was preferred to a second centrifugation because it was necessary to minimize breakage of the fragile tail fibers, as the stoichiometric calculations were to be based on the known number of tail fibers per phage particle. The labeled tails were pelleted by centrifugation in a Beckman SW65 rotor at 65,000 rpm for 2 h at 5°C. There was essentially no visible debris in this preparation, and often the pellet was invisible. The tails were resuspended in 0.4 ml of M9 salts and were further purified by sedimentation through a sucrose gradient as described previously. The intact phage were also further purified by sedimentation through a 5-ml 5 to 20% (wt/vol) sucrose gradient. The centrifugation was carried out in a Beckman SW65 rotor at 25,000 rpm for 20 min at 20°C. The gradients were fractionated, and the distribution of radioactivity was determined as previously described.

Electrophoretic analysis of the contents of the peak fractions of the sucrose gradients was accomplished on 10% sodium dodecyl sulfate-polyacrylamide slab gels. The gels were impregnated with 2,5-diphenyloxazole, dried, and exposed to X-ray film as described earlier. Each developed film was realigned with the gel that exposed it, and regions of the gel containing certain proteins that are products of known phage genes were identified and cut out of the gel. These gel fragments were solubilized by treatment with 0.5 ml of either a Protosol-water solution (9:1, vol/vol) at 55°C overnight or 0.88 M NH_4OH in 30% H_2O_2 at 37°C for 36 h. The latter treatment is slightly more efficient for releasing counts from the gel. A 0.5-ml amount of 0.1 M HCl and 7.5 ml of a Triton X-100-based cocktail were added, and the amount of radioactivity present was determined by scintillation counting. Counting efficiency was about 20%.

RESULTS

Enzyme activity in gene 11-defective viral particles. To demonstrate dihydrofolate reductase activity in phage particles, Kozloff et al. (20) had to partially disrupt T4D ghosts with urea or dimethyl-formamide. More recently, the same group (17) presented indirect evidence supporting the idea that the enzyme protein is partially buried within the baseplate, shielded by the gene 11 protein. Infection with gene 11 mutants under restrictive conditions is known to generate noninfectious particles lacking gp11 and gp12, the products of genes 11 and 12, respectively (32). On the expectation that such particles might contain partially exposed baseplate dihydrofolate reductase, we assayed purified defective phage from a T4 *am*N93 infection of *E. coli* B. Such phage showed enzyme activity even without prior disruption of the particles (Table 1). The activity per particle, although severalfold higher than that noted by Kozloff et al. in disrupted ghosts, still represents but a small percentage of the activity in a single molecule of purified T4 dihydrofolate reductase, as calculated from the turnover number of the purified enzyme (8). Activity was below detectable limits with T4D particles, supporting the idea that the enzyme is buried within the baseplate. Moreover, 11^- particles containing an additional mutation in gene *frd* also showed activity below the limits of detection, indicating that the observed enzyme activity does represent the *frd* gene product.

TABLE 1. *Dihydrofolate reductase activity in phage particles*[a]

Particle	Activity (nmol/min per 10^{13} particles)
Urea-treated T4D ghosts[b]	1.1
*am*N93 (11^-) defective phage	9.1
Intact T4D phage	<0.4
*am*N93-*am frd*11 defective phage	<0.4
Purified T4 dihydrofolate reductase (1 molecule)[c]	69.8

[a] Enzyme activity was assayed by the spectrophotometric method (23) at 37°C. Particle concentrations, for both intact phage and defective particles, were determined by ultraviolet light absorption measurements (36).

[b] Reported by Kozloff et al. (20).

[c] Determined from the turnover number of purified dihydrofolate reductase (8).

In principle, the availability of an enzymatic assay for virion dihydrofolate reductase presents us with an opportunity for quantitating the particle enzyme, namely, titration with aminopterin or some other stoichiometric inhibitor (23). However, the 11^- particle activity is relatively resistant to these folate analogs, possibly because the enzyme still binds pteroylhexaglutamate, another known tail constituent (19). In addition, we have no way to tell what proportion of the virion enzyme molecules are accessible to added substrate and, hence, detectable in our assay.

Differences between dihydrofolate reductases specified by T4D and T4B. Infectivity of T4D phage is neutralized by antiserum prepared against purified dihydrofolate reductase specified by T4D (28). In an earlier publication we presented evidence that the target site for the phage-neutralizing activity was, in fact, the virion dihydrofolate reductase (28). This was based partly upon our finding that T6 phage infectivity is neutralized quite slowly by the antiserum, linked with our knowledge that T6 dihydrofolate reductase differs from the T4D enzyme in several respects (23). During one

neutralization experiment we included T4BO$_1$, a T4B strain, and were surprised to see that it, like T6, was slowly neutralized by the antiserum. Figure 1 presents averaged data from three such experiments. We also found that the soluble dihydrofolate reductases specified by T4D and T4B can be differentiated on the basis of heat stability. The T4B enzyme was readily inactivated by incubation at 40°C (like the T6 enzyme [23]), whereas the T4D enzyme was relatively stable (Fig. 2). Since T4B and T4D are much more closely related than are T4D and T6, these observations strengthen our conclusion that virion dihydrofolate reductase is the target for neutralizing action of the antiserum.

In an earlier study (25) we presented evidence that the *frd* gene, known to code for soluble dihydrofolate reductase (10), also is a determinant of the heat lability of the T4 virion. In view of the difference between the soluble reductases of T4D and T4B, it seemed appropriate to ask whether the virions of these phages differ in heat lability as well. As shown in Fig. 3, they do. One cannot definitely ascribe this difference to the *frd* gene, but, in view of the close relatedness of these two phages plus previous data on the *frd* gene as a determinant of heat lability, this seems to be a reasonable interpretation of these experiments.

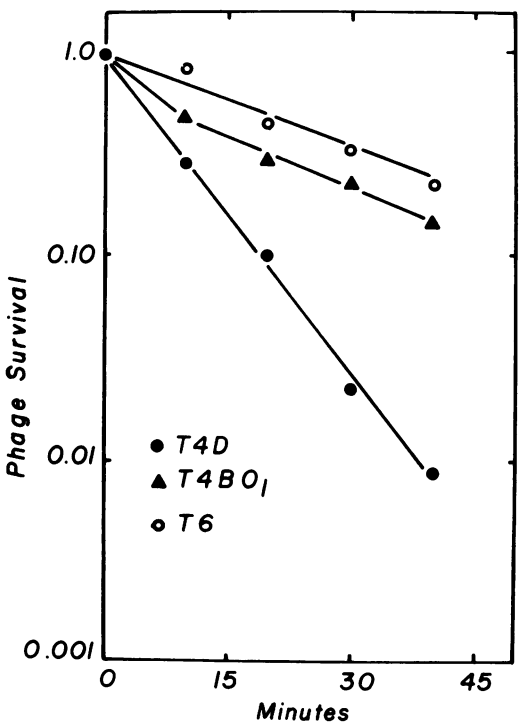


FIG. 1. *Neutralization of phage infectivity by antiserum against T4D dihydrofolate reductase. Procedures are as described by Capco and Mathews (6). Each data point represents an average value obtained from three separate experiments.*

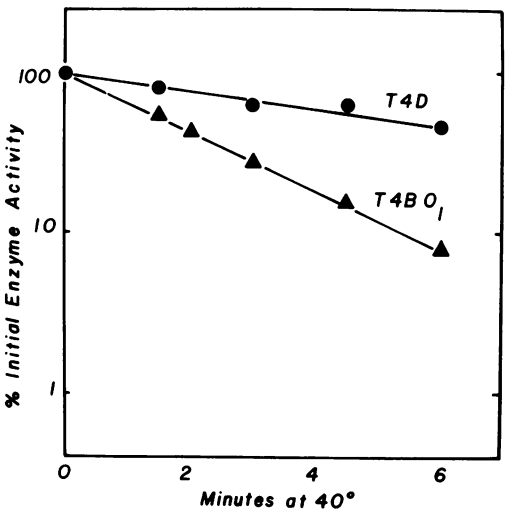


FIG. 2. *Heat lability of T4D and T4BO$_1$ dihydrofolate reductases, determined as described by Mathews (25). Each data point represents an average value obtained from three separate experiments.*

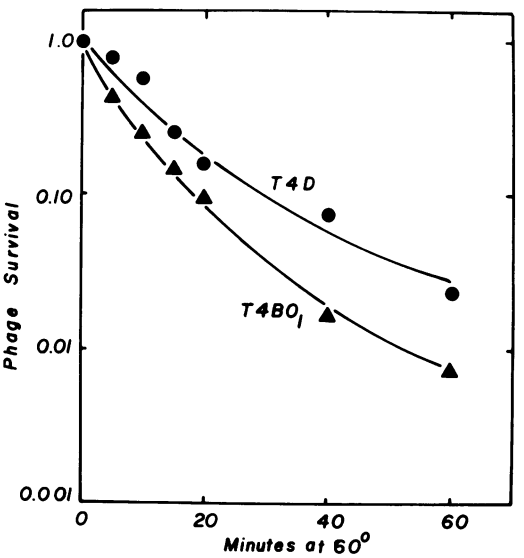


FIG. 3. *Heat inactivation of phage infectivity, determined as described by Capco and Mathews (6). Each data point represents an average value obtained from three separate experiments.*

Properties of phage bearing deletions between genes 63 and 32. Homyk and Weil (11) have reported extensively upon the isolation

and properties of T4 strains bearing long deletions in the region between genes 63 and 32 on the T4 linkage map. This area includes genes coding for enzymes of deoxynucleotide metabolism, enzymes that serve to augment preexisting host cell activities. In general agreement with their heteroduplex mapping data, we found (6) that three strains provided to us by T. Homyk, Jr., namely, *del*1, *del*7, and *del*9, are all unable to induce thymidylate synthetase activity after infection, whereas *del*7 and *del*9 are also unable to induce dihydrofolate reductase activity. The viability of these mutants in standard *E. coli* strains is not surprising in terms of our knowledge of DNA precursor metabolism; the corresponding host cell enzymes could provide precursors, albeit at a reduced rate. However, in terms of virion structure and morphogenesis, the viability of these strains suggests either that the missing enzymes are not essential structural proteins or that the deletions do not extend completely through the *td* and/or *frd* genes, such that partial proteins are still made.

If the *td* and/or *frd* genes are completely deleted in these strains, as suggested by the data of Homyk and Weil (see Discussion), and if the *td* and *frd* gene products in virions represent the neutralizing targets of the respective antisera, then these phage should be resistant to neutralization by the antisera. However, as we previously reported (6), all three strains are more readily inactivated by T4 thymidylate synthetase antiserum than is the wild-type parent. Moreover, extracts of cells infected by any of these strains contain material that cross-reacts with the thymidylate synthetase antiserum, as shown by immunodiffusion. The same experiments were repeated with the T4 dihydrofolate reductase antiserum. All three strains were inactivated by this serum more rapidly than was T4D (Fig. 4). The high sensitivity of *del*1 is not surprising, since this strain is known to induce dihydrofolate reductase (6). However, *del*7 and *del*9, which do not, were even more sensitive to this antiserum than was *del*1. Moreover, all three deletion mutants synthesized material in infected cells that cross-reacted with T4 dihydrofolate reductase antiserum, as shown by immunodiffusion (Fig. 5). Note also that two point mutants in the *frd* gene also form this cross-reacting material. One of these, *frd*11, is an amber mutant, but, evidently, it synthesizes an incomplete polypeptide nearly as long as the wild-type protein (17). These observations involve us in the same apparent paradox as do our similar experiments with thymidylate synthetase antiserum: mapping data indicate the presence of long

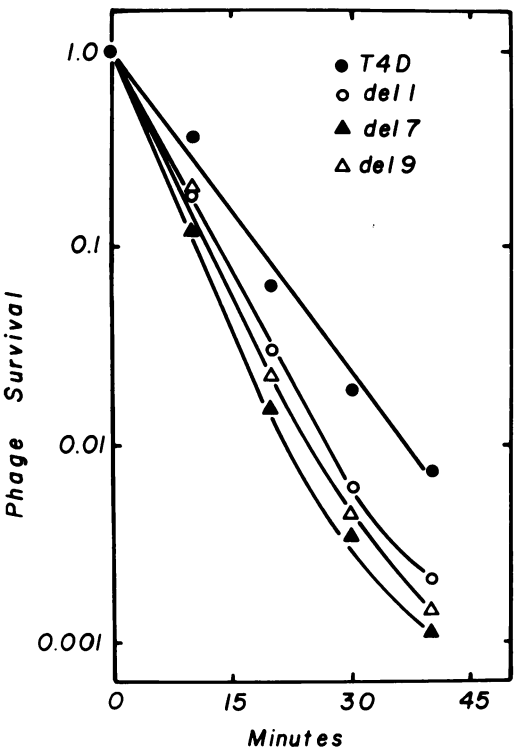


FIG. 4. *Neutralization of infectivity of T4D and 63-32 deletion mutants by antiserum against T4D dihydrofolate reductase. Procedures are as described by Capco and Mathews (6). Each data point represents an average value obtained from three separate experiments.*

deletions in this area of the genome, yet immunological data suggest that the deletions extend only partway into the relevant genes.

More recent experiments indicate that the dihydrofolate reductase-negative phenotype of *del*7 and *del*9 is not due to deletion of the *frd* gene, either wholly or in part. We wished to identify portions of the T4 genome corresponding to the 63-32 region by comparing restriction endonuclease digests of T4D DNA with those of DNA from the mutants. Since *del*7 and *del*9 each contains an *r*II deletion, we wished to cross this out to avoid misidentification of a fragment that actually corresponded to the *r*II region. Each strain was crossed to T4D, and we selected clones that plated on a lambda lysogen but not on *E. coli* CT526 (see below). Three independently derived clones from crosses involving *del*9 were selected, and two were selected from *del*7. All five clones retained the original thymidylate synthetase-negative phenotype (Table 2). However, all five synthesized near normal levels of dihydrofolate reductase. To show that the *r*II gene was not regulating

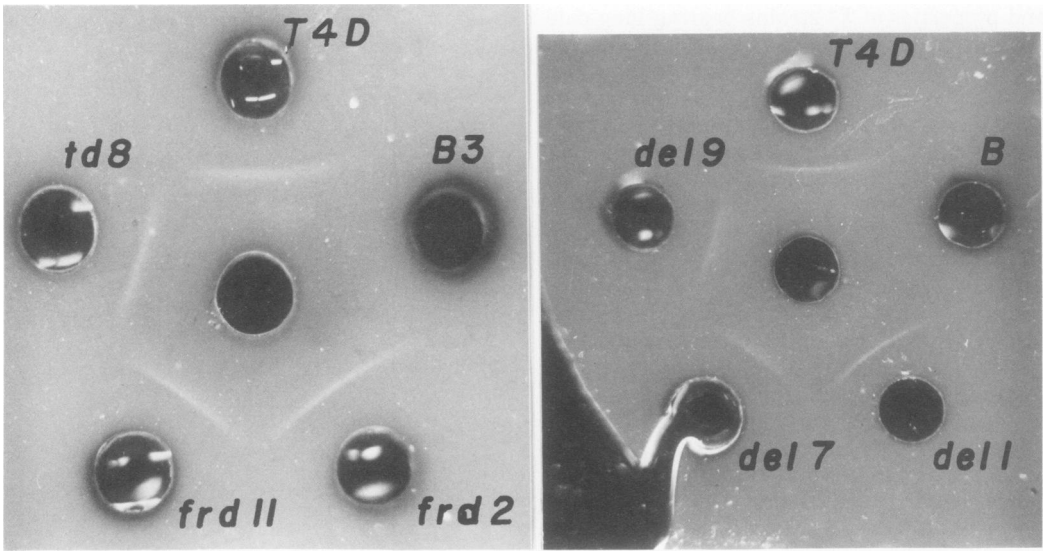


FIG. 5. *Immunodiffusion involving anti-dihydrofolate reductase and crude extracts of uninfected E. coli (B and B3) or cells infected with T4D or various T4 mutants. An immunoglobulin G fraction was prepared from the T4 dihydrofolate reductase antiserum, and any antibodies to E. coli proteins were absorbed out; immunodiffusions were run as described by Capco and Mathews (6).*

TABLE 2. *Properties of recombinants in crosses of del7 or del9 to T4D*[a]

Group	Phage	Plating efficiency relative to CR63		Enzyme sp act (nmol/min per mg of protein)	
		594 (λ)	CT526	Dihydrofolate reductase	Thymidylate synthetase
A	T4D	1.0	1.0	22.9	4.1
	del1	2×10^{-5}	10^{-6}	36.4	<0.1
	del7	3×10^{-6}	4×10^{-7}	ND[b]	<0.1
	del9	5×10^{-6}	10^{-6}	1.8	<0.1
B	*del7* rII$^+$	1.0	10^{-5}	25.0	<0.1
	del7 rII$^+$	1.0	2×10^{-5}	34.5	<0.1
	del9 rII$^+$	1.0	5×10^{-6}	18.1	ND
	del9 rII$^+$	1.0	2×10^{-5}	20.3	ND
	del9 rII$^+$	1.0	8×10^{-6}	25.7	ND
C	*del7* r1589	$<10^{-7}$	$<10^{-7}$	24.8	0.4
	del7 rUV363	10^{-6}	$<10^{-7}$	23.3	0.4
	del9 r1589	$<10^{-7}$	5×10^{-5}	23.7	<0.1
	del9 rUV363	2×10^{-7}	$<10^{-7}$	24.2	<0.1

[a] The results in group A represent the properties of our original stocks. *del1*, *del7*, and *del9* all contain the r1589 deletion. In each case the rII mutation is recognized as an inability to plate on a lambda lysogen, 594(λ), and the 63-32 deletion is recognized as an inability to plate on *E. coli* CT526. Group B represents five independently derived recombinants from crosses to T4D (input ratio, 10:1 T4D over *del7* or *del9*; crosses carried out in *E. coli* CR63). In the experiment of group C, rII mutations, either the original r1589 deletion or a point mutation, rUV363, were crossed back into *del7* or *del9* to show that the state of the rII gene does not affect the expression of the *frd* gene. All enzyme assays were carried out after infection of *E. coli* 201, a thymidylate synthetase-negative host.

[b] ND, Not determined.

the expression of *frd* in some unknown way, we crossed rII mutations back into our rII$^+$ *del*63-32 strains, either the original r1589 deletion or rUV363, a point mutation. In all cases the reductase-plus, synthetase-minus phenotype was retained (Table 2). Thus, it seems clear that *frd* could not have been deleted in the original *del7* and *del9* strains. It seems likely that each

strain contained an additional mutation, which had the effect of turning off dihydrofolate reductase synthesis even in the presence of an intact *frd* gene, and that this mutation was bred out when the strain was crossed to T4D.

Restriction of deletion mutants in hospital strains of *E. coli*. The accumulated information on mutants in genes *td* and *frd*, whether point mutants or deletions, indicates that enzymatically active products of these genes are not absolutely required for phage growth in common laboratory strains of *E. coli*. Inactivation of either enzyme by mutation reduces the rate of DNA synthesis some two- to threefold, because of a reduced rate of thymine nucleotide production (22), but the corresponding host cell activities provide sufficient synthetic capacity for significant phage yields to be maintained. To date, little information is available regarding the role of either enzyme as a virion protein. One way to seek such information would be to identify a phage-host system in which infection is restricted due to absence of either or both proteins. One could then identify the specific step in the infective process that was aberrant, such as adsorption, DNA injection, a specific step in morphogenesis, or some other process. The hospital strains, a set of wild-type *E. coli* strains isolated by the Cal. Tech. group, provide a suitable opportunity to seek such a system. Included in this set of *E. coli* strains are restrictive hosts for T4 strains bearing mutations in several other "nonessential" genes. By means of semiquantitative spot testing, we examined 26 of the hospital strains with respect to their ability to support infection by the three deletions as well as by point mutants in the *td* and *frd* genes. None of the point mutants was restricted by any of the hospital strains. However, *del*1 was restricted by six of the strains, and *del*7 and *del*9 were restricted by seven strains each. Quantitative determinations of relative plating efficiency were determined for each system, as summarized in Table 3. In each case we established that the *r*II deletion borne by each phage strain was not responsible for the restriction, since T4 *r*1589 could plate on each host restrictive for the 63–32 deletions. It seems clear that the basis of restriction does not relate to DNA precursor deficiencies, first, because all experiments were carried out in nutritionally complete medium, and, second, because the relative plating efficiencies seen in abortive infections seem far too low to be accounted for in terms of precursor deficiencies, unless the deficiencies involved the unique T-even phage deoxynucleotide, 5-hydroxymethyldeoxycytidine monophosphate. However, none of the gene products encoded by the 63–32 region is known to play a direct role in hydroxymethylcytosine metabolism. In any event, the large number of restrictive hosts found for these deletions suggests that even though the missing gene products largely duplicate preexisting enzyme activities, they are essential to growth in certain host cells.

Electrophoretic analysis of early-labeled proteins in T4 tails. One possible explanation for the rapid inactivation of the 63–32 deletion mutants by antisera against purified dihydrofolate reductase or thymidylate synthetase is that these proteins are not elements of the virion but that the antisera react with different targets, possibly the products of homologous genes elsewhere on the T4 chromosome. Although this seems quite unlikely, for reasons covered in Discussion, it seemed desirable to verify the existence of these proteins in baseplates by a direct approach in addition to the already described approach of detecting enzyme activity in purified virions. Since most structural proteins are synthesized late in infection, whereas the two enzymes are early proteins, a suitable approach seemed to be electrophoretic analysis of phage proteins synthesized early in infection and incorporated into tails late in infection. Labeling was carried out in infections with a gene 23 mutant that makes no heads, such that labeled tails could be isolated and analyzed independently.

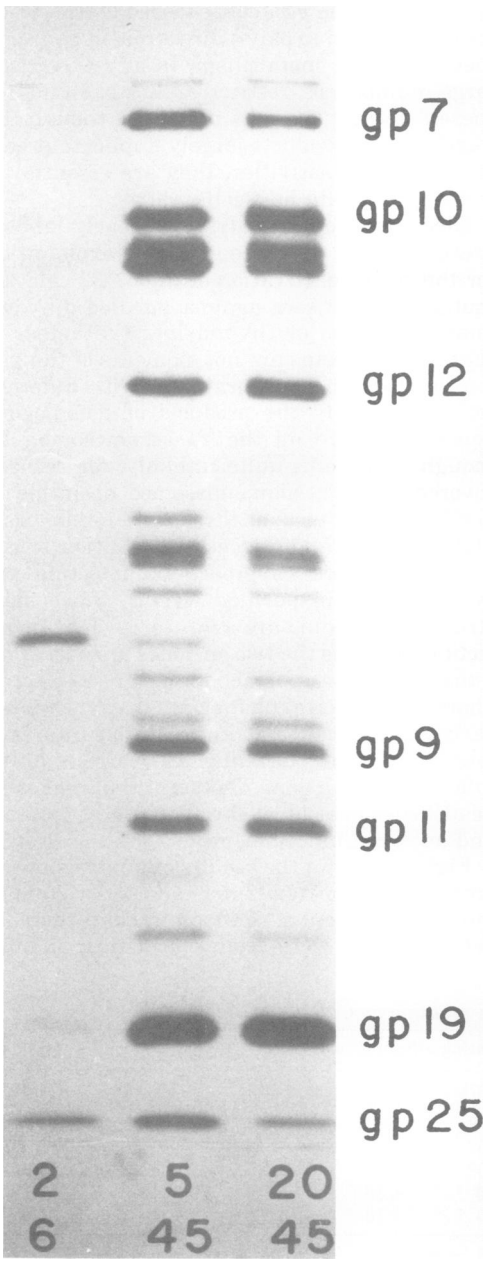
Figure 6 shows the results of analysis of tail proteins labeled from 2 to 6, 5 to 45, or 20 to 45 min after infection. Two prominent early-labeled proteins were seen, one with a subunit

TABLE 3. *Restriction of 32–63 deletion mutants by Cal. Tech. strains*[a]

Phage strain	Relative plating efficiency in *E. coli*							
	B	CT89	CT196	CT271	CT312	CT511	CT526	CT569
T4D	1.00	0.62	0.59	0.69	0.24	0.11	0.46	0.07
T4*r*1589	1.00	0.84	0.25	0.52	0.05	0.21	0.20	0.31
*del*1	1.00	1.09	$<10^{-7}$	0.09	$<10^{-7}$	$<10^{-6}$	$<10^{-7}$	$<10^{-7}$
*del*7	1.00	$<10^{-6}$	$<10^{-7}$	$<10^{-7}$	$<10^{-7}$	$<10^{-7}$	$<10^{-7}$	$<10^{-7}$
*del*9	1.00	$<10^{-7}$	$<10^{-7}$	$<10^{-6}$	$<10^{-7}$	$<10^{-7}$	$<10^{-7}$	$<10^{-7}$

[a] Data from three separate experiments are averaged in this table.

molecular weight of about 40,000 and a smaller one with a molecular weight of 15,000 or less. Dihydrofolate reductase has a molecular weight of 29,000 (8), and thymidylate synthetase is a dimer of 28,000-dalton subunits (5). No labeled bands were detected in the corresponding region of the gel, which is in the vicinity of gp9 and gp11 (molecular weights, 34,000 and 24,000, respectively [12]; recently, we have found that the purified enzyme actually migrates slightly faster than gp11, which itself migrates at an anomalously slow rate in our system [R. A. Mosher, unpublished results]). In an effort to relate the 40,000-dalton band or the smaller polypeptide to precursor or cleaved forms of the *td* or *frd* gene product, we crossed the gene 23 mutation into *del*1, *del*7, *del*9, *frd*11, and *am*N54. All of these strains gave a pattern identical to that shown in Fig. 6. Thus, we conclude that neither band is related to either dihydrofolate reductase or thymidylate synthetase. By comparing our electrophoretic patterns with those of Kikuchi and King (12), we have tentatively identified the low-molecular-weight polypeptide as gp25. Confirmation of this will be of interest, inasmuch as it seems to represent another exception to the generalization that structural proteins are synthesized late in infection. The 40,000-molecular-weight protein has not been identified, but it may be an *E. coli* outer membrane protein, as suggested by King and Laemmli (14). The slow turnoff of bacterial envelope protein synthesis after T4 infection (2, 30) would be consistent with our observation of heavy labeling of this material within the first few minutes after infection.



If the 40,000-dalton polypeptide is a bacterial protein, is it specifically associated with T4 tails, or is it present in material that happens to cosediment with our labeled tails? If it is a bacterial protein, it should be detectable in corresponding gradient fractions from cells labeled only before infection. If it is specifically associated with T4 tails, then it should be absent from sucrose gradient fractions prepared from cells infected with a DO mutant, which expresses no late functions. To answer both questions we labeled *E. coli* for one generation of growth and then infected it in nonradioactive medium with either T4 *am*B272 (gene 23) or T4 *am*E10 (gene 45). Tail-containing fractions from the 23^- infection and corresponding fractions from the 45^- infection were analyzed electrophoretically (Fig. 7). Substantial radioactivity was detected in the 40,000-dalton polypeptide from the 23^- infection, indicating that it is a host-specific protein. Although radioactivity was detected in the corresponding band

FIG. 6. *Slab gel electrophoretic analysis of T4 tail proteins. Cells were grown, infected, and labeled as described in the text. Labeling intervals were 2 to 6, 5 to 45, and 20 to 45 min after infection, as indicated on the figure. Gene product identifications were obtained by comparison of this figure with a comparable illustration in Kikuchi and King (12), except that the preparation of these investigators did not contain gp19, the major tail core protein.*

FIG. 7. *Slab gel electrophoretic analysis of proteins labeled before infection. Growth, infection, and preparation of material for electrophoresis were as described in the text, except that labeling was carried out for one generation during bacterial growth (for the left and center tracks). The left track shows the pattern obtained in infection by T4 amB272 (gene 23), and the center track depicts the pattern obtained in infection by T4 amE10 (gene 45). Infections were terminated at 45 min. The right track represents, for comparison, the pattern obtained from fully labeled T4 tails, labeled from 10 to 45 min after infection by T4 amB272.*

from the 45⁻ infection, it was greatly reduced in amount, suggesting that most of the protein is specifically associated with the tail. Regarding the nature of this association, Brown and Anderson (4) showed some years ago that T4 particles newly released from infected cells contain electron microscopically visible "debris" attached to their baseplates. This debris, which was identified as cell wall material, is gradually lost from the baseplate as the phage ages. Consistent with the idea of a reversible association between T4 tails and host cell wall material, we find that the radioactivity of the 40,000-dalton band varies depending upon the method used to lyse the cells. When chloroform is used to aid lysis, the intensity of this band is much lower than when lysis is carried out simply by freeze-thawing (data not shown).

Stoichiometry of tail proteins. To interpret the significance of our inability to find an early-synthesized protein with a molecular weight of 30,000 in the T4 tail, it was necessary to know the sensitivity of our technique. Specifically, would a protein in this size range be detected on our gels if it were present in only one copy per tail? Answering this question required knowledge of the number of copies of some of the proteins in the baseplate. To determine these values, we prepared intact phage and phage tails, all of whose protein components had been labeled with [^{3}H]leucine drawn from a pool whose specific activity had not changed during the entire labeling period.

The protocol was designed such that all of the leucine utilized for protein synthesis was supplied exogenously. This was accomplished by growing the cells, before infection, in medium supplemented with 50 μg of leucine per ml, which represses the leucine-biosynthetic enzymes (9). The infection was carried out such that the cells had no opportunity to induce any enzymes in response to the lowered levels of leucine present during the phage growth cycle (see Materials and Methods). When the [^{3}H]-leucine was added after infection, sufficient nonradioactive leucine was present to insure that the amount of isotope utilized for protein synthesis was a small percentage (less than 10%) of the total (data not shown). Thus, all of the leucine utilized in the manufacture of proteins in the infected cells after the addition of label and up to the termination of infection was drawn from a pool with a constant specific activity. Therefore, the amount of isotope present in a specific gene product in the purified phage or tail preparation is proportional to the number of copies of that gene product, with appropriate corrections for molecular weight and leucine content. Both the phage tails and

intact phage were purified by a cycle of differential centrifugation and, finally, on sucrose gradients (Fig. 8) as described in Materials and Methods. The protein components of the peak fractions from the sucrose gradients were separated by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis and visualized by fluorography. The products of genes 37, 7, 34, 10, and 12 have been identified by other laboratories on gels of complete phage (13, 14, 15, 37) and are well separated from their nearest neighbors in the gel (Fig. 9). These bands were then cut from the gel, and their radioactivity was determined. The numbers of copies of gp's 7, 10, and 12 were calculated by asssuming that there are 12 copies of gp37 in one phage particle (37). The numbers calculated with p34 as standard are quite low because of its high leucine content (7.99%) (35). The values obtained are presented in Table 4. Calculations for the purified tails were based on six being the number of copies of gp7 in one tail. This is the number postulated by Kikuchi and King (12) and is in good agreement with our experimentally determined value for whole phage. gp's 7, 10, 12, 9,

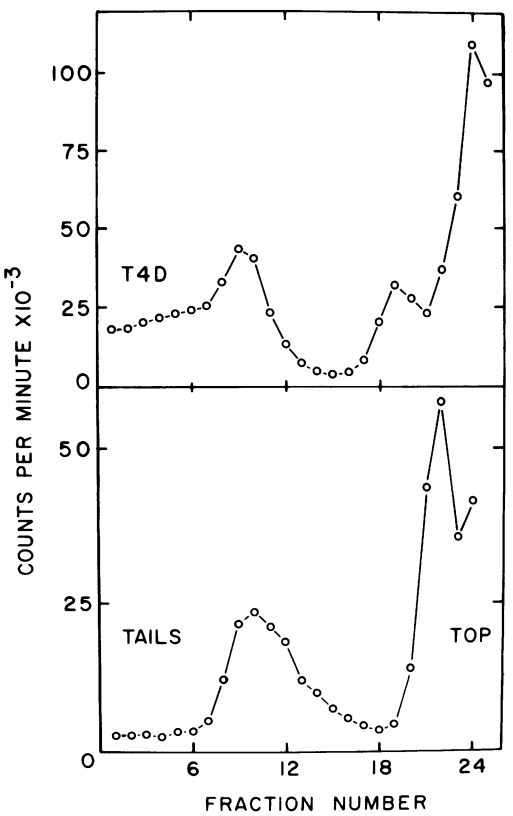


FIG. 8. *Distribution of radioactivity on 5 to 20% sucrose gradients used to purify intact T4D (upper panel) or phage tails (lower panel). Centrifugation conditions were as in the text. Fifty-microliter portions of each fraction were counted.*

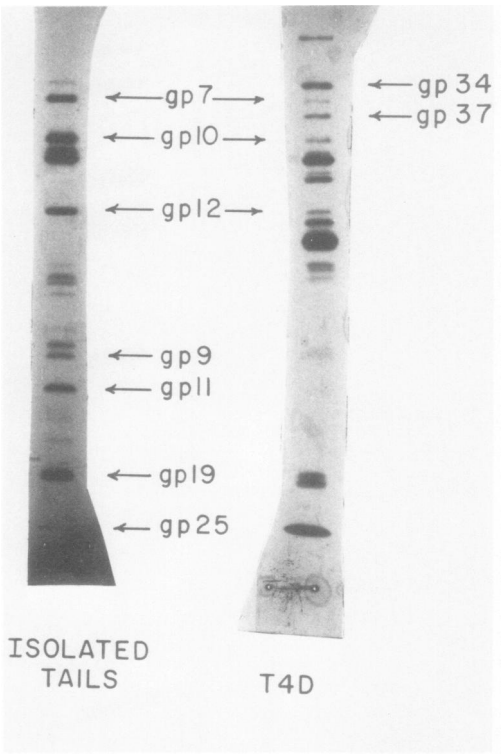


FIG. 9. *Slab gel electrophoretic analysis of the stoichiometry of T4 proteins. Gene product identifications were made by comparison with published sodium dodecyl sulfate-gel electropherograms (13, 14, 15, 37). The gene products identified were cut out of the corresponding gels, and their radioactivity was determined.*

TABLE 4. *Stoichiometry of T4 tail proteins*[a]

gp	Molecules per particle	
	T4D phage	T4 *am*B272 tails
7	5.2	S (6)
9	ND	8.9
10	17.0	15.5
11	ND	21.4
12	20.6	19.6
19	ND	87.2
25	ND	11.9
34	15.8	NP
37	S (12)	NP

[a] Abbreviations: S, Gene product used as standard for quantitating other proteins; ND, not determined; NP, not present.

11, 19, and 25 are well separated from adjacent proteins in the gels of purified tails and are easily identified (12; Fig. 9). The calculated values for these proteins are presented in Table 3. It should be emphasized that these values are not corrected for leucine content. Such a correction could easily change some of the values by 50%.

The implications of these data with respect to detection of the *frd* and *td* gene products in isolated tails are reviewed in the Discussion.

Comparison of T4 dihydrofolate reductase with a corresponding enzyme encoded by plasmids. We felt that further light could be shed on the biological functions of phage-coded dihydrofolate reductases if we could compare these enzymes with other microbial dihydrofolate reductases. Specifically, we were intrigued with the report of Skold and Widh (33) that two R factors conferring resistance to trimethoprim coded for dihydrofolate reductases that appear to be similar to the phage reductases in two interesting respects: (i) relative resistance to trimethoprim (23) and (ii) high sensitivity to heat. This raised the possibility of a common evolutionary origin for the plasmid and phage enzymes. This possibility was tested by further comparison of the T4 enzyme with partially purified enzymes encoded by R factors R388 and R483. Table 5 compares the *E. coli* chromosomal dihydrofolate reductase with the T4 enzyme and the R483 plasmid enzyme with respect to precipitability with ammonium sulfate and ability to use reduced nicotinamide adenine dinucleotide instead of reduced nicotinamide adenine dinucleotide phosphate. In both these regards the plasmid enzyme is distinct from the phage enzyme. Similar results were seen with the R388 enzyme (data not shown). Moreover, the phage and plasmid enzymes are immunologically distinct. Neither plasmid reductase formed a precipitin band in immunodiffusion against antiserum to T4 dihydrofolate reductase (data not shown). In addition, when we examined binding of the T4 reductase antibody to the R483 enzyme by sucrose gradient centrifugation, we similarly found no evidence either for inactivation of the enzyme by antibody or for binding in the absence of inactivation (Fig. 10). Figure 10 also shows that the R483 enzyme has an approximate molecular weight of 26,000, somewhat lower than that of the T4 enzyme.

TABLE 5. *Properties of dihydrofolate reductases specified by phage T4, by the E. coli chromosome, and by R factor 483*[a]

Enzyme source	% of total activity[b]		
	In 0–50% ammonium sulfate fraction	In 50–85% ammonium sulfate fraction	With NADH as compared with NADPH
E. coli B	10	90	<1
T4-infected B	92	8	19
R483	6	94	7

[a] Cultures (600 ml) of *E. coli* B and J53 (containing R483) were grown at 37°C in glycerol-Casamino Acids medium to about 3×10^8 cells/ml, and one *E. coli* B culture was infected with T4 phage (five phage per bacterium; cells harvested at 20 min). Crude cell-free extracts were prepared and treated with streptomycin sulfate as described previously by Mathews and Sutherland (29). Ammonium sulfate fractions were prepared, and the most active fraction from each culture was assayed with reduced nicotinamide adenine dinucleotide (NADH) as well as with reduced nicotinamide adenine dinucleotide phosphate (NADPH).

[b] The total activity in each streptomycin supernatant is taken as 100%.

DISCUSSION

The data presented in this paper provide further support for the proposal that dihydrofolate reductase is a functional protein in the T4 virion. First, enzyme activity, controlled by the *frd* gene, can be detected in defective virions in which parts of the baseplate are exposed by the absence of the gene 11 product. Second, the difference between dihydrofolate reductases specified by T4D and T4B supports the concept that this protein is both a determinant of the heat lability of the virion and a target for the phage-neutralizing activity of the T4 dihydrofolate reductase antiserum. Third, the fact that deletions affecting the expression of the *frd* gene restrict growth in 7 out of 26 wild-type *E. coli* strains tested indicates that the gene product is essential in some natural environments. Regarding this latter point, we emphasize that none of these 7 restrictions has yet been shown to be a specific effect of inactivation of the *frd* gene. All of the 63–32 deletions tested extend over several genes (11; Fig. 11). Until point mutants restricted by one or more of the hospital strains have been shown to map in *frd*, we cannot conclude that dihydrofolate reductase deficiency is responsible for restriction. However, since all of the known genes between 63 and 32 control steps in DNA precursor synthesis that were previously thought to be nonessential for growth, clarification of the mechanism of restriction should be of interest whether or not *frd* is directly involved.

The accompanying paper by Kozloff et al. (18)

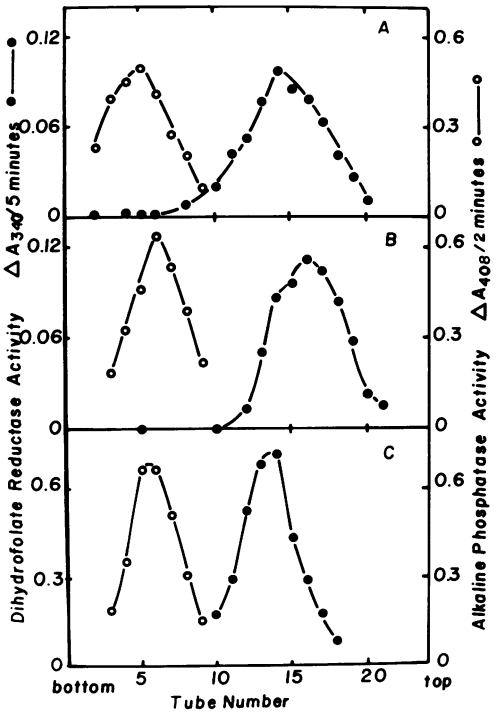


FIG. 10. *Failure of T4D dihydrofolate reductase antiserum to react with dihydrofolate reductase specified by R factor 483. The 0 to 50% ammonium sulfate fraction described in the footnote to Table 4 was used as the source of the T4 enzyme, and the 50 to 85% fraction described in the same footnote was used as the source of the plasmid-specified enzyme. A portion of the 483 enzyme was incubated with an equal volume of T4D dihydrofolate reductase antiserum for 18 h at 4°C. A 0.2-ml amount of this mixture was centrifuged through a 5 to 20% neutral sucrose gradient along with an equal amount of R483 enzyme that had not been treated with antiserum and a sample of T4 phage-coded enzyme. Centrifugation was carried out for 24 h at 40,000 rpm in a Beckman SW65 rotor, in the presence of E. coli alkaline phosphatase as a sedimentation marker (29). (A) Antiserum-treated R483 enzyme; (B) untreated R483 enzyme; (C) untreated T4D enzyme.*

presents data comparable to ours, in support of the idea that thymidylate synthetase is a functional protein of the T4 virion. The existence of a *ts* mutation simultaneously affecting temperature sensitivity of thymidylate synthetase and heat lability of the virion is especially convincing in this regard. These workers have presented similar evidence with respect to dihydrofolate reductase (18).

However, two aspects of our work are disturbing: (i) our failure to detect early-labeled proteins of the T4 tail that correspond either to thymidylate synthetase or to dihydrofolate reductase; and (ii) the synthesis by all three of the 63–32 deletions tested of material that is immunologically related to both enzymes.

With respect to the first point, it is clear that dihydrofolate reductase is present as a virion protein in extremely small amounts. The activity in a single virion is less than that of a single molecule of purified reductase (Table 1). However, it seems likely that the activity of the particle enzyme is lower than that of the soluble enzyme because of factors like tight binding of the endogenous folate (19) or partial shielding of the enzyme by other proteins, even in 11⁻ defective phage particles. In any event, it seems likely that any rare protein (five copies or less per virion) would probably not be detected. Silverstein and Goldberg (31) have recently discussed this point with regard to the gene 2 protein, which may be present at only two copies per virion. In an attempt to quantitate the particle-bound enzyme molecules, we developed a technique for determining the stoichiometry of T4 tail components. The values we have determined for well-characterized gene products are in good agreement with numbers predicted by Kikuchi and King (12). For example, these authors described a wedge-shaped baseplate precursor, which is present at six copies per virion. Since this substructure contains gp's 7, 10, and 11, one would expect these proteins to be present in multiples of six. The values listed in Table 3 suggest that the numbers of protein molecules per wedge structure are 1, 3, and 3 or 4 for gp's 7, 10, and 11, respectively.

The radioactivity in each well-characterized band, plus knowledge of the molecular weight and leucine content of each protein, should make it possible to predict the radioactivity associated with a band containing dihydrofol-

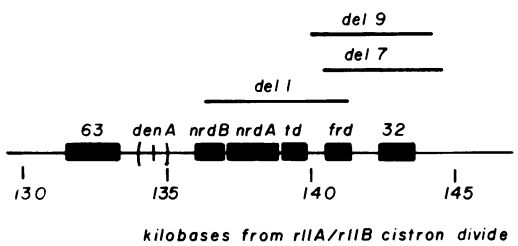


FIG. 11. *63–32 region of the T4 genetic map. The end points of each deletion are taken from the data of Homyk and Weil (11). The positions of the genes were taken from Wood's map (39). Genes 63 and 32 have been physically mapped, but the genes in between have not. The length of each gene has been estimated from the molecular weight of the respective gene product, where known.*

ate reductase (this, of course, assumes that the virion-bound enzyme has the same molecular weight as the soluble enzyme). Our failure to detect a 30,000-dalton band in the experiment of Fig. 9 sets a probable upper limit of six early-synthesized polypeptides per particle. This value is quite approximate, however, because in the experiment of Fig. 9 the labeling interval began at 5 min after infection, by which time considerable early enzyme synthesis has occurred (1, 26). Additional experiments involving increased specific activities, isotopes of higher energy, and extended labeling intervals are under way.

A priori, the data on the 63-32 deletion mutants could be rationalized by assuming that the deletions extend only partway into a gene, such that an incomplete protein, containing the antigenic specificity of the native protein, is synthesized. However, all three mutants tested are reported to contain long deletions, extending some 4,000 to 5,000 nucleotide pairs, at least five times the length of the *frd* gene (8). On the other hand, there is some uncertainty concerning the end points of the deletions. Figure 11 shows the end points as reported from the heteroduplex mapping data of Homyk and Weil (11). This is juxtaposed with the physical map of the 63-32 region, as taken from Wood's T4 linkage map (39). This juxtaposition shows both *del*7 and *del*9 to extend through gene 32, a situation clearly incompatible with viability of the phage. Moreover, *del*1 is shown to cover *frd*, even though this mutant does induce normal levels of dihydrofolate reductase (6). We conclude that the end points of the deletions must lie at least 2,000 nucleotide pairs to the left of the points indicated on Fig. 11. This would give *del*1 a *den*A$^-$ phenotype, as has been reported (11). Even so, it is difficult to visualize all three of the mutants making immunologically active proteins.

Additional uncertainty regarding the positions of *frd* and *td* relative to the deletions comes from our finding that the capacity to synthesize dihydrofolate reductase is regained in recombinants between *del*7 or *del*9 and T4D, even though the recombinants still contain the original deletion, as shown by failure to induce thymidylate synthetase activity and restriction by *E. coli* CT526 (Table 2). Since the same results were seen with five independent recombinants, it seems impossible to explain this in terms of a rare illegitimate recombination event occurring within the deleted segment. It seems far more likely that *del*7 and *del*9 each contains an additional mutation affecting the expression of the *frd* gene, and that this mutation maps at a site distinct from that of the 63-32 deletion, such that it is not recovered in the recombinant. That such a mutation would be present in both strains, in addition to the 63-32 and *r*II deletions, is not implausible a priori, for two reasons. (i) Construction of these strains involved heavy UV irradiation before the deletion-bearing strains were crossed into a T4D-*r*1589 background (11); this could have introduced new point mutations that were not removed in subsequent backcrossing. (ii) There is ample reason for believing that *del*7 and *del*9 are identical (11); thus, the idea that they contain the same additional mutation is not surprising. Further attempts to characterize this presumed mutation and the gene in which it lies are under way in our laboratory.

The above considerations make it seem more probable that the T4 dihydrofolate reductase cross-reacting material formed in infection by *del*7 or *del*9 is, in fact, a product of the *frd* gene, even though it is enzymatically inactive. This, in turn, supports the idea that the target site for phage inactivation by the reductase antiserum is indeed the *frd* gene product, present in the virion. Whether it plays an essential role in the phage life cycle as a structural element must remain, to some extent, an open question. However, the results of Table 2 relieve us of the obligation to explain how T4 can reproduce in the total absence of a structural *frd* gene product.

In considering an alternate possibility, namely, that the target site for the phage-neutralizing effect of the reductase antiserum is not the *frd* gene product, we should emphasize the following. (i) The enzyme used in preparing the antiserum was homogeneous by several criteria (8). (ii) Even if the enzyme preparation had contained minor impurities, they could not have been structural proteins, for the enzyme was prepared from cells infected with a DO mutant, which makes no late proteins. (iii) The serum does inactivate T4 dihydrofolate reductase activity (28). (iv) The antiserum displays only one band in immunodiffusion (Fig. 5). It seems virtually certain, from the above, that this band represents an antigen-antibody complex involving the *frd* gene product.

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