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Genetic and Immunological Studies of Bacteriophage T4 Thymidylate Synthetase

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Thymidylate synthetase, which appears after infection of *Escherichia coli* with bacteriophage T4, has been partially purified. The phage enzyme is immunologically distinct from the host enzyme and has a molecular weight of 50,000 in comparison to 68,000 for the host enzyme. A system has been developed to characterize T4 *td* mutants previously known to have impaired expression of phage thymidylate synthetase. For this system, an *E. coli* host lacking thymidylate synthetase was isolated. Known genetic suppressors were transduced into this host. The resulting isogenic hosts were infected with phage T4 *td* mutants. The specific activities and amounts of cross-reacting material induced by several different types of phage mutants under conditions of suppression or non-suppression have been examined. The results show that the phage carries the structural gene specifying the thymidylate synthetase which appears after phage infection, and that the combination of plaque morphology, enzyme activity assays, and an assay for immunologically cross-reacting material provides a means for identifying true amber mutants of the phage gene.

Infection of *Escherichia coli* by T-even and T5 phage markedly stimulates the biosynthesis of the enzyme thymidylate synthetase both in thymine-requiring and thymine-independent bacteria (2, 4). The new enzyme, which appears after phage infection, can be separated from the *E. coli* host enzyme (6). In the case of T4 infections, the phage locus responsible for the appearance of the second enzyme, the "*td*" region, has been identified (17, 18) and maps in a cluster of at least five genes controlling sequential metabolic reactions in the synthesis of thymidylate (20).

Interestingly, the phage enzyme appears to be non-essential; phage *td* mutants are able to multiply well in the presence of either the host enzyme or exogenous thymine. However, under certain conditions, viable phage yields from phage *td* mutants are decreased in comparison to wild type, suggesting that the ability to induce thymidylate synthetase formation confers a selective advantage on wild-type phage (13). In order to better understand the nature of the phage-induced enzyme and its relationship to the host enzyme, we studied a group of phage mutants presumably involving the thymidylate synthetase gene locus. We were especially interested in characterizing nonsense mutations in the phage enzyme gene, since with such mutants we hoped to observe the effect of amino acid substitution and protein chain termination on the function and tertiary structure of phage thymidylate synthetase.

To achieve the above goals we employed an experimental system consisting of an isogenic set of thymidylate synthetase *E. coli* mutants of *su*$^-$, *suI* (serine), *suII* (glutamine), and *suIII* (tyrosine) genotypes infected with T4 *td* nonsense mutants. For this purpose, a thymine-requiring strain of *E. coli* was selected which was shown to be unable to synthesize its own thymidylate synthetase. Since the *E. coli* strain also contained two known amber mutations for tryptophan and lactose, the successful transduction of suppressor genes could be easily monitored. With this system, it was predicted that prematurely terminated phage thymidylate synthetase would result from infection of the *su*$^-$ host with a phage nonsense mutant and that phage thymidylate synthetase with a known amino acid substitution would be obtained from the infection of a *su*$^+$ strain. The effect of these alternatives at the same site and at different sites within the phage *td* gene could then be evaluated. This article reports the characterization of the system in which these substitutions can be studied.

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MATERIALS AND METHODS

Bacterial media. L broth contained, per liter: 10 g of tryptone (Difco), 5 g of yeast extract (Difco), 5 g of NaCl, and 1 ml of 1 *N* NaOH. Medium M9 (1) was supplemented with 25 μg of thymidine per ml, 2 μg of vitamin B_1 per ml, 50 μg of tryptophan per ml, and 5 mg of glucose per ml. For P1 transduction, R buffer was composed of 0.01 M Tris (pH 7.5), 0.15 g of $MgSO_4 \cdot 7H_2O$ per liter, and 0.01 g of gelatin per liter. R agar was prepared according to Gussin (7). Tris-glucose medium and plates for T4B *td* plaque morphology were made as described by Simon and Tessman (18). A-N medium contained, per liter: 7 g of K_2HPO_4, 3 g of KH_2PO_4, 0.5 g of Na-citrate $\cdot 2H_2O$ and 0.1 g of $MgSO_4 \cdot 7H_2O$.

Buffers. Buffer A: fifty mM Tris-10 mM mercaptoethanol-1 mM disodium ethylenediamine adjusted to pH 7.4 with HCl. Buffer B: same components as Buffer A but adjusted to pH 8.4 with HCl.

Bacterial strains and their sources are listed in Table 1.

Two amber mutant phage, *T4am6* and *T4N82*, and the transducing phage P1 (S. Brenner) were obtained from M. Malamy. Phage T4D (td^+) was provided by E. Goldberg. Wild-type Bol and T4 *td* mutants were obtained from Irwin Tessman. The *T4BN* series of phage mutants were originally isolated, after nitrous acid mutagenesis, by Dwight Hall in 1965.

Chemicals. Deoxyuridine-5'-monophosphate (dUMP) was obtained from Calbiochem, Los Angeles, Calif., and thymidine, folic acid, cytochrome c (horse heart), and Tris were from Sigma Chemical Company, St. Louis, Mo. Trimethoprim was obtained from Burroughs Wellcome and Co. (U.S.A.). Tetrahydrofolate was prepared from folic acid by catalytic reduction with platinum oxide in the presence of hydrogen (8). It was stored at −20 C in 1 M mercaptoethanol. Egg albumin, 5× crystallized, was purchased from Nutritional Biochemical Corp., Cleveland, Ohio, and bovine gamma globulin was from Armour Pharmaceutical Co., Kankakee, Ill. Diethylaminoethyl (DEAE)-cellulose, type 40, with 0.9 meq of binding sites per g, was purchased from Brown Company, Berlin, N.H. It was suspended in distilled water, fines were removed, and the resin was treated with 5% ammonium hydroxide, and then washed with distilled water to pH 7. Finally, it was equilibrated with Buffer B at 4 C.

Preparation of T4B lysates. The 200B strain was grown in L broth to a cell density of 10^8 cells/ml. L-tryptophan (25 μg/ml) and thymidine (100 μg/ml) were added, and the culture was inoculated with a freshly picked phage plaque. Five minutes were allowed for absorption, the infected culture was shaken for 3 h at 37 C, and the cells were then lysed with chloroform. Lysates of 10^{10} to 10^{11} phage/ml were obtained. The phage were assayed by the overlay agar method on L plates with SW-2 as the indicator host. The L soft agar contained 25 μg of thymidine per ml and was added at the time of plating.

Preparation of crude extracts for assay of thymidylate synthetase. Bacterial cells were grown in 200 ml of L broth, in a 2-liter flask, to a density of 1.5×10^8 cells/ml. One minute before infection, tryptophan and thymidine were added as above. At zero time, phage were added at a multiplicity of infection of six. Five minutes were allowed for absorption, and the culture was then placed on a rotary shaker at high speed. At 15 min the cultures were poured over half their volume of ice and centrifuged immediately at 5,000 rpm for 10 min to pellet the cells. Control cultures were handled in the same way except for omission of the infection.

Pelleted cells, infected or uninfected, were suspended in chilled Buffer B at about 2×10^9 cells/ml and subjected sonic treatment in a Branson sonifier model LS-75 (Branson Corporation, Stamford, Conn.). During sonic treatment, the samples were immersed in an ice-water bath. The bacterial suspension was sonically treated twice at 20 kc for 30 s with a 1-min interval to allow for cooling. The resulting sonicate was centrifuged for 15 min at 16,000 rpm. The decanted supernatant fluid served as the crude extract for thymidylate synthetase assay.

Protein determination. Protein was determined by a turbidimetric assay (9).

Test for plaque morphology of the T4B td mutants. The methods of Simon and Tessman (18) were used for plating the phage and doing spot tests. The bacterial strains were grown in M9 supplemented with thymidine, B_1, tryptophan, and glucose. The Tris-glucose plates had no thymidine in the bottom agar; the 2.5-ml overlay generally contained 20 μg of thymidine. For spot testing, the plate with its overlay was incubated for 1 h at 37 C before it was spotted with the phage.

Transduction. The basic procedures of Adams (1) were followed for P1 propagation and titration. The procedure of Lennox (10) was followed for the transduction.

Thymidylate synthetase assays. The major assay for thymidylate synthetase was the spectrophotometric assay of Wahba and Friedkin (20). The radioactiv-

TABLE 1. *Bacterial strains*

Strain	Genotype	Source
200B (D. Zipser)	F^-, *thi, strR, trpam, lacam*	M. Malamy
SW-2	F^-, *thi, strR, trpam, lacam, thy, su*$^-$	Isolation described in this paper
SK-I	F^-, *thi, strR, trpam, lacam, thy, suI*	Isolation described in this paper
SK-II	F^-, *thi, strR, trpam, lacam, thy, suII*	Isolation described in this paper
CA5013 (E. Signer)	*HfrC, thi, lac, strS, suI*	J. Beckwith
CA161 (S. Brenner)	*Hfr, i*$^+$*z*$^-$*y14, suII*	J. Beckwith
X7035	F^-, *thi, his, mal, strR, suIII*	P. Strigini

ity assay, involving the labilization of tritium attached to C-5 of dUMP, was a modification of the methods of Lomax and Greenberg (11) and of Roberts (16) as described by Reid (Ph.D. thesis, Tufts University, 1969).

Wild-type E. coli thymidylate synthetase. Thymidylate synthetase purified through fraction VI (5) was used for preparation of antiserum 240 against wild-type *E. coli* B enzyme.

RESULTS

Genetics. In order to characterize the thymidylate synthetases of various phage mutants it was necessary to select host strains which themselves did not synthesize thymidylate synthetase; thus any of the enzyme present would be due to infection by the phage. From *E. coli* 200 B a spontaneous thymine-dependent strain was selected. The use of trimethoprim, an inhibitor of dihydrofolate reductase, proved to be very effective as a means of selecting thymine-dependent mutants without the use of a mutagenic agent (3, 19). About 10^6 cells/ml were introduced into tubes containing supplemented M9 medium, but with 50 μg of thymidine per ml and trimethoprim at levels of 5, 7.5, or 10 μg/ml. A turbid culture (5×10^8 cells/ml) was obtained after incubation for 65 h at 37 C in the tubes containing 5 μg of trimethoprim per ml; cultures grew more slowly in the presence of the higher concentrations of trimethoprim. Almost 50% of the colonies were able to grow on plates supplemented with thymine (10 μg/ml) but were unable to grow on plates without thymine. Any of these thymine-dependent strains which could grow on plates lacking thymine but supplemented with deoxyuridine were discarded. Finally, a strain called "SW-2" was selected and was found to contain no detectable thymidylate synthetase activity in either the spectrophotometric or radioactive assays. The thymidylate synthetase mutation in the SW-2 strain was stable (reversion rate less than 1 in 10^8 cells) and was not suppressed by amber suppressors. This was an important requirement so that during suppression of amber phage mutants a concurrent suppression of the host enzyme mutation would not occur.

Generalized transduction with bacteriophage P1 was employed to introduce known suppressor genes into the SW-2 strain. *E. coli* strains CA5013, CA161, and X7035, known to carry *suI*, *suII*, and *suIII* respectively, served as the donors. This procedure generated a set of strains which were isogenic except for the specific suppressor gene present. Transductants were tested for concomitant expression of suppression of their *lac am* and *trp am* markers, retention of thymine dependence, and ability to suppress known T4 amber phage (Table 2). The SK-I strain (*suI*) was studied in detail.

Extraction and purification of phage thymidylate synthetase from T4D phage grown on E. coli 200 B. Preliminary experiments indicated that infection at a cell density of 1.5×10^8 cells/ml with a multiplicity of infection of 6 resulted in infection of more than 90% of the cells. Infective center assays detected progeny phage at 15 min; lysis followed this within 5 min. Thus experimental infections were terminated routinely at 15 min. Infected cell pellets from 39 liters of medium (see Materials and Methods) were sonically treated, centrifuged, and then fractionated as follows. Nucleic acids were removed by the addition of streptomycin (3.75 mg per ml of extract) and this step was followed by two additions of $(NH_4)_2SO_4$, to 40 and 80% saturation. The enzyme present in the second $(NH_4)_2SO_4$ fraction was redissolved and then concentrated by dialysis against solid sucrose.

Thymidylate synthetase was further purified by DEAE-cellulose chromatography either by gradient elution (0–0.34 M NaCl in Buffer A) or directly in a one-step elution with 0.08 M NaCl in Buffer A. As has been previously observed (6), phage enzyme consistently appeared in earlier elution fractions than the host *E. coli* enzyme. When the isolated *E. coli* and phage enzymes were mixed and rechromatographed, two distinct peaks were again found, corresponding to the respective positions of the phage and host enzymes. Each of these peaks showed its original elution pattern when rechromatographed again. The final specific activities of seven purified phage T4D thymidyl-

TABLE 2. *Characterization of su⁺ transductants of E. coli strain SW-2 (thymidylate synthetase minus)*[a]

Expression	Recipient SW-2	Transductants	
		SK-I (*suI*)	SK-II (*suII*)
Growth on Lac B[b]	–	–	–
Growth on Lac BT[c]	–	+	+
Plaque formation by *T4am6*[d]	–	+	+
Plaque formation by *T4N82*[d]	–	+	+

[a] *E. coli* strains CA5013 and CA161 served as donors of *suI* and *suII*. Cells were washed twice and suspended in A-N before being streaked on Lac B and Lac BT plates.

[b] M9 medium, lactose, thiamine.

[c] M9 medium, lactose, thiamine, thymine.

[d] *Amber* mutants.

ate synthetase preparations ranged between 2.4 to 3.4 units of protein per mg.

Preliminary experiments indicated that direct DEAE-cellulose chromatography of crude extracts, by-passing the customary streptomycin and ammonium sulfate fractionations, may aid in obtaining higher yields of the phage enzyme. This simplified procedure had the advantage of a 30% recovery in comparison to the total of 15% recovery with the alternate method.

Different molecular weights of host and phage enzymes. The host and phage enzymes previously chromatographed on DEAE-cellulose emerged at different positions from a Sephadex G-100 column. In order to estimate their molecular weights, a Sephadex G-100 column (1 by 100 cm) was calibrated with proteins of known molecular weights: bovine gamma globulin, 160,000 (15); ovalbumin, 45,000 (21); and cytochrome c, 12,700 (12). Phage thymidylate synthetase was eluted at a point corresponding to a molecular weight of 50,000; the host enzyme emerged in the region of 68,000. The results were reproducible in independent experiments. The average recovery of active phage enzyme was 5% as compared to a recovery of 70% for the *E. coli* enzyme.

Different stabilities of host and phage thymidylate synthetases. The two enzymes exhibited markedly different stabilities and recoveries throughout the purification procedures. The recoveries of phage enzyme from ammonium sulfate fractionation, DEAE-cellulose chromatography, and Sephadex gel filtration were consistently lower than the recoveries of the host enzyme. The host enzyme, with specific activities up to 37 units of protein per mg, could be stored in sucrose at −20 C for a period of years with little loss of activity. Under any conditions employed, the phage enzyme decreased from a maximum specific activity of 10 units of protein per mg to 2 to 3 units/mg over a period of 18 h; the activity then remained at that level for several months during storage at −20 C.

Wild-type host and phage T4 thymidylate synthetases are immunologically distinct. Rabbits were immunized with preparations of wild-type host and phage T4 thymidylate synthetases partially purified by ammonium sulfate fractionation, DEAE-cellulose chromatography, and Sephadex gel filtration. As a control, antiserum was obtained from a rabbit immunized with an extract from the uninfected SW-2 strain which lacked thymidylate synthetase activity.

Antisera were prepared by immunization of New Zealand white rabbits with the following antigens: phage T4D thymidylate synthetase (rabbits #239 and 243); *E. coli* B thymidylate synthetase (rabbit #240); and a crude extract of *E. coli* SW-2 (rabbit #242). Except for the immunization of rabbit 239, the antigen was emulsified in an equal volume of complete Freund adjuvant. The first dose was injected intradermally in multiple sites. Two weeks later another course of multi-site intradermal injections was administered and a week later the final dose of antigen was given through the marginal ear vein. After 7 days the animals were bled. The rabbits were boosted intradermally or intravenously at intervals and bled a week after such injection.

To characterize an antiserum, measurements were made of its ability to inhibit the enzymatic activity of thymidylate synthetase from *E. coli* or phage T4. It was found that antiserum to phage T4 enzyme inhibited only phage T4 enzyme (Fig. 1). Similarly, antiserum to host enzyme inhibited only host and not phage thymidylate synthetase (Fig. 1). Neither normal serum nor the control antiserum to the protein in an *E. coli* strain lacking thymidylate synthetase (SW-2) inhibited either enzyme. Thus, the host and phage enzymes are immunologically distinct proteins.

Denatured enzyme cross-reacts with antiserum to homologous active thymidylate synthetase. Both the host and phage enzymes were inactivated gently. The host enzyme was heated to 45 C for 1 h; the phage enzyme was allowed to stand for 12 h at room temperature. Both of these inactive preparations were tested

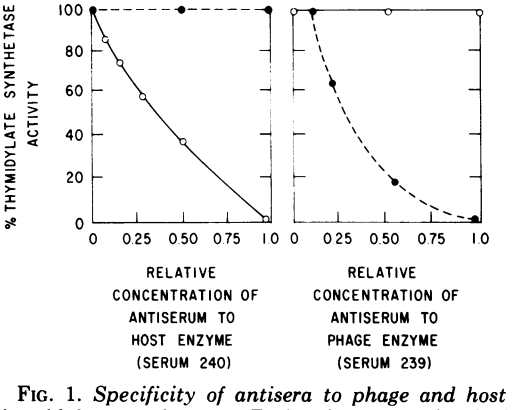

FIG. 1. *Specificity of antisera to phage and host thymidylate synthetases. Each mixture consisted of 0.4 ml of diluted serum as indicated in the figure and 50 μliters of enzyme (activity equivalent to an absorbance change of 0.035 per min at 340 nm for E. coli and 0.015 per min for T4D enzyme). After 18 h at 4 C each mixture was centrifuged and the supernatant fluid was assayed spectrophotometrically for thymidylate synthetase activity.* (○), *E. coli enzyme;* (●), *phage T4D enzyme.*

in their homologous and non-homologous enzyme-serum systems for their ability to compete with the active enzyme for inhibiting antibody. The net effect of a cross-reaction was to reduce the inhibitory effect of the antiserum on native enzyme.

Inactivated phage enzyme cross-reacted only with antiserum to phage thymidylate synthetase and not with antiserum to host enzyme, while inactivated host thymidylate synthetase cross-reacted only with anti-host enzyme serum and not with anti-phage enzyme serum (Fig. 2 and 3).

The fact that inactivated preparations could cross-react in their homologous systems indicated that loss of enzyme activity, possibly due to a local unfolding in the region of the catalytic site, could occur without a gross change in the tertiary structure of the protein. The reciprocal lack of cross-reactivity with the heterologous serum showed that inactivation did not reveal any antigenically similar regions common to both the host and phage enzymes.

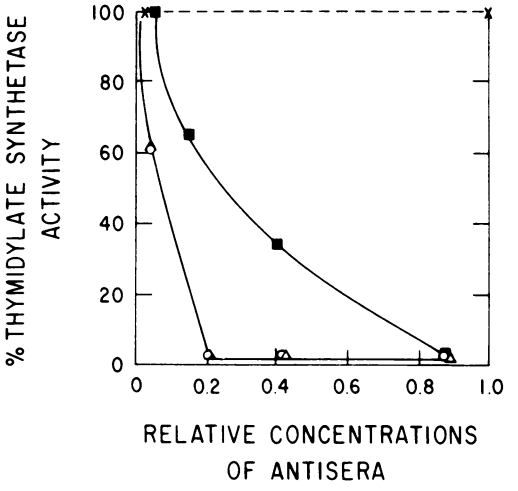

FIG. 2. *Both active and inactivated phage thymidylate synthetases react with antiserum to phage enzyme. Each mixture consisted of 0.2 ml of diluted serum and various enzyme preparations as labeled. The amounts of enzyme added were as follows: active T4D, 0.02 (as absorbance change per min at 340 nm); T4D inactivated by standing at room temperature for 12 h, 0.033 (before inactivation); E. coli inactivated by heating at 45 C for 1 h, 0.030 before inactivation. After 4 h at 4 C each mixture was assayed spectrophotometrically for thymidylate synthetase activity. (×), anti-SW-2 antiserum 242 + active T4D enzyme; (○), anti-phage enzyme serum 239 plus active T4D enzyme; (■), anti-phage enzyme serum 239 + active T4D enzyme in the presence of inactivated T4D enzyme; (△), anti-phage enzyme serum 239 + active T4D enzyme in the presence of inactivated E. coli enzyme.*

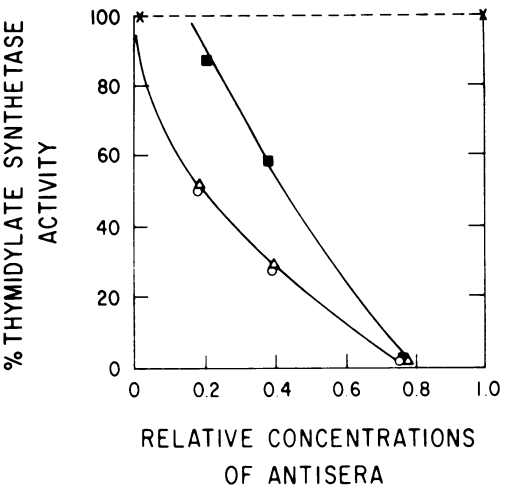

FIG. 3. *Both active and inactivated E. coli thymidylate synthetases react with antiserum to E. coli enzyme. Same experimental procedure as in Fig. 2, with the same enzyme preparations. (×), anti-SW-2 serum 242 + active E. coli enzyme; (○), anti-E. coli enzyme serum 240 + active E. coli enzyme; (■), anti-E. coli enzyme serum 240 + active E. coli enzyme in the presence of inactivated E. coli enzyme; (△), anti-E. coli enzyme serum 240 + active E. coli enzyme in the presence of inactivated T4D enzyme.*

Identification of phage with amber mutations in the thymidylate synthetase gene. Phage that are mutant in thymidylate synthetase (*td* mutants) can be recognized by their plaque morphology under the plating conditions of Simon and Tessman (18). The *td* mutants produce a smaller plaque on a "thymidylate synthetase minus" (Thy^-) host than do the wild-type phage; both wild-type and *td* mutant plaques are large on a Thy^+ host.

The availability of the newly constructed isogenic su^- and su^+ host mutants in thymidylate synthetase enabled us to identify *td* phage possessing an amber mutation. Such an amber *td* would be expected to produce a small plaque on a su^- host but a large plaque on a su^+ host in which the amber mutation was suppressed and the phage gene could be expressed (see Fig. 4). Strains SW-2 (su^-), SK-I (*suI*), and SK-II (*suII*) were sent to Irwin Tessman at Purdue University, who screened his collection of *td* mutants for the predicted phenotype and returned 5 presumptive *td* nonsense mutants (Table 2). In all cases in which the small plaque morphology was produced (*N33* and *N37* infections of SW-2 and SK-I; *N43* and *N54* infections of SW-2) no enzyme activity could be detected in the crude extracts (Table 3). In the case of *N57*, which had an intermediate size plaque on both hosts, a low level of enzymatic activity, bordering on the limit of detection, was found in the crude

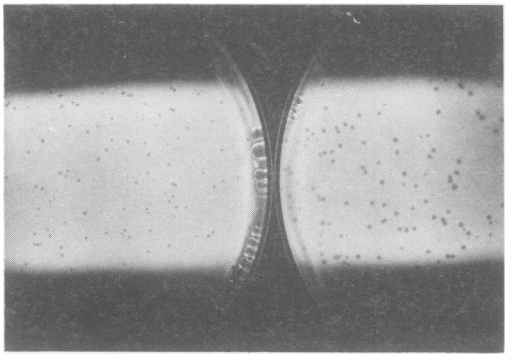

FIG. 4. *Plaque morphology of T4td mutants. Left plate: phage tdN43 on SW-2 (su⁻) host. Right plate: phage tdN43 on SK-I (su⁺) host.*

extracts. Appreciable enzymatic activity was detected in only one of the cases where a large plaque morphology occurred (*N43* infections of SK-I). The most puzzling case was the *N54* infection of SK-I, where no enzymatic activity could be detected even though large plaques were produced.

Measurement of cross-reacting material produced by phage T4 td mutants on su⁻ and su⁺ hosts. The immunoassay technique described above was used to measure the production of cross-reacting material in phage T4 *td* mutants grown on *su⁻* and *su⁺* hosts. The presence of denatured thymidylate synthetase could be detected by reversal of the primary inhibition of active enzyme by antiserum. The results of such an experiment, depicted in Fig. 5, show that there was a linear relationship between the reversal of inhibition and the amount of cross-reacting material present at any of the serum concentrations used.

Crude extracts from infections of the SW-2 (*su⁻*) and SK-I (*su⁺*) hosts with each of the phage *td* mutants were assayed in order to determine their content of cross-reacting material (Fig. 6). Crude extracts of *N33* infections of either SW-2 or SK-I contained no detectable cross-reacting material. Crude extracts of *N37* infections of SW-2 and SK-I both contained one-third of the amount of wild-type cross-reacting material. Thus none of these phage had the properties expected for simple amber mutants. No cross-reacting material was detected in crude extracts from *N43* infections of the *su⁻* host SW-2. However, cross-reacting material was produced in *N43* infections of the *su⁺* host SK-I, so that this phage did behave as an amber mutant. The amount of this suppressed cross-reacting material was less than that resulting from *N37* infections. Similarly, although no cross-reacting material could be detected in crude extracts of *N54* infections of SW-2, there was an appreciable amount in crude extracts of *N54*-infected SK-I cells, even though the latter extracts had no detectable enzyme activity. The *N57* infections of strain SW-2 produced cross-reacting material which increased slightly in *N57* infections of SK-I.

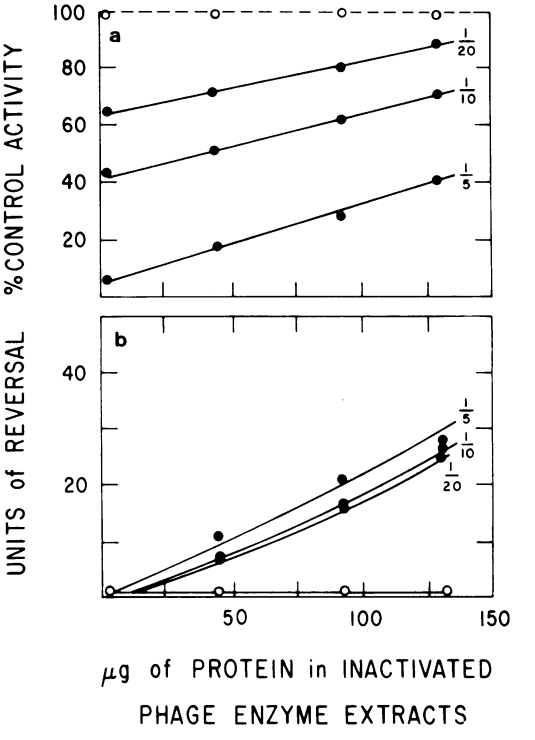

FIG. 5. *The assay of cross-reacting material by reversal of immunological inhibition of phage thymidylate synthetase activity. a, Antiserum 239 to wild-type phage T4D (on 200 B host) thymidylate synthetase was diluted with Buffer A. Cross-reacting material in inactivated extracts of wild-type T4Bol (on SW-2 host) was brought to a total volume of 0.1 ml with Buffer A. Then 0.2 ml of diluted serum 239 and active phage enzyme in crude extracts of T4Bol (on SW-2 host) was added (enzyme activity giving an absorbance change of 0.015 per min). The mixtures were incubated for 4 h at 4 C and centrifuged. Samples of the supernatant fluid, 75 μliters, were assayed for thymidylate synthetase activity by the spectrophotometric method. The fractions in the figure indicate the dilutions of serum 239 used. (●), assays with serum 239; (○), control assays with antiserum 242 to crude extracts of uninfected SW-2. b, The same data as in part a, plotted as arbitrary units of reversal of inhibition of phage thymidylate synthetase as a function of the amount of cross-reacting material present where: units of reversal equals percentage of control activity with cross-reacting material minus percent of control activity without cross-reacting material. The fractions indicate the dilution of serum present.*

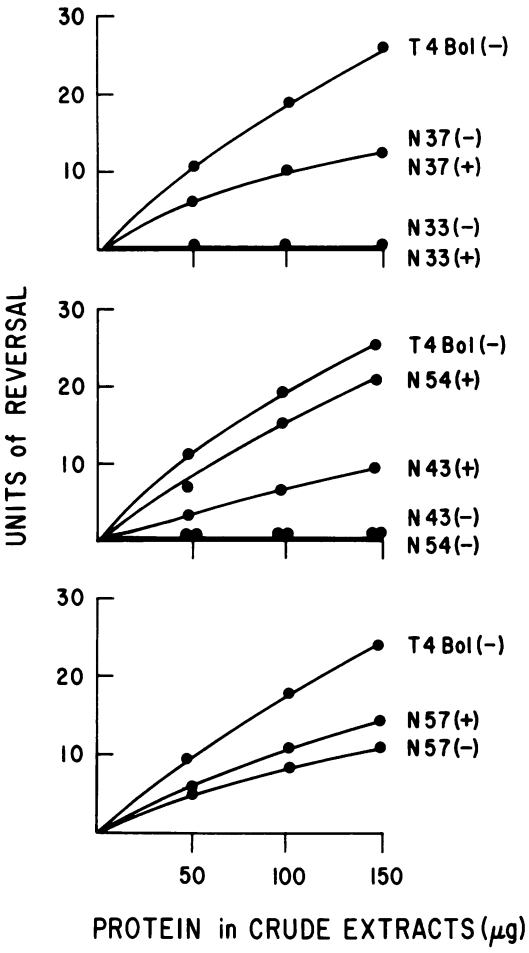

FIG. 6. *Reversal of immunological inhibition of phage thymidylate synthetase by crude extracts of td mutants after infection of su⁻ and su⁺ hosts. Anti-phage T4D serum 239 was diluted 1/6.6 with Buffer A. The crude extract was brought to a total volume of 0.2 ml with the same buffer before its addition to 0.2 ml of the antiserum. Then 30 μliters of T4Bol-infected crude extract (enzyme activity equivalent to an absorbance change of 0.015 per min at 340 nm) was added. After 4 h at 4 C, the mixture was centrifuged and 150 μliters of the supernatant fluid were taken for spectrophotometric assay of thymidylate synthetase. See legend of Fig. 4 for definition of units of reversal. (−), extract from su⁻ host SW-2; (+), extract from su⁺ host SK-I. T4Bol (−) was an inactivated crude extract.*

DISCUSSION

These data confirm and extend the observations of Greenberg, Somerville, and De Wolf (6) indicating that the phage and host enzymes are distinct proteins. In addition to different elution properties on DEAE-cellulose, the enzymes have different molecular weights as judged by Sephadex gel filtration and, in agreement with previous findings (6), also differ markedly in stability. Further, reactions with antibodies directed against either protein showed complete and reciprocal lack of immunological cross-reactivity between the phage and host enzymes.

Previous work strongly suggested that phage T4 DNA contains the structural gene specifying phage thymidylate synthetase. This was inferred from the differential inhibition with F-dUMP (14), from the separation of the two enzymes on DEAE-cellulose (6) and from the characterization of phage *td* mutants. The demonstrated immunological distinctness of the host and phage enzymes in this study further supports the belief that phage thymidylate synthetase is a phage gene product and is not an altered host enzyme.

The data obtained on the specific activity of the enzyme from phage mutants, their plaque size, and the amount of cross-reacting material they produce under conditions of genetic suppression and nonsuppression provide added evidence that the T4 phage *td* locus' protein product is the phage thymidylate synthetase. The results correlate well with a preliminary map of the *td* mutants deduced from both two- and three-factor crosses between pairs of the *td* mutants. Proceeding from the *frd* gene (dihydrofolate reductase) toward the *nrd* gene (nucleotide reductase), the apparent map order was: *tdN33*, *tdN43*, and *tdN54*, *tdN57*, *tdN37* (D. Hall, personal communication). All of the phage *td* mutants employed in the present studies were known to be single site mutants (D. Hall, M.S. thesis, Purdue University, 1965).

As summarized in Table 3, phage mutants *tdN33* and *N37* produced small plaques on both su⁻ and su⁺ hosts. Crude extracts from infections of both hosts had no enzymatic activity. No cross-reacting material was detected in crude extracts from *tdN33* infections of either host; this mutant mapped adjacent to the T4 phage gene for dihydrofolate reductase. In the case of *tdN37*, however, crude infections of either host produced cross-reacting material in an amount equal to about 30% of the enzyme protein present with wild-type phage infection. Two possibilities can be proposed for the nature of their lesions. First, the *tdN33* and *tdN37* mutations may cause chain terminations that are not suppressible by amber suppressors due to the nature of the mutant codon or to its inaccessibility to an incoming suppressor tRNA. For example, chain-terminating codons of the UGA or UAA type are not translated by amber suppressors I, II, and III. If this is indeed the case, the results of the immunological

TABLE 3. *Plaque size, thymidylate synthetase activity and cross-reacting material in phage-infected su⁻ and su⁺ E. coli cells*

Phage *td* strain	SW-2 (*su⁻*)			SK-I (*suI*)			SK-II (*suII*)
	Plaque size[a]	Enzyme activity[b]	Cross-reacting material[c]	Plaque size[a]	Enzyme activity[b]	Cross-reacting material[c]	Plaque size[a]
N33	Small	<0.01	<0.01	Small	<0.01	<0.01	Small
N37	Small	<0.01	0.10	Small	<0.01	0.10	Small
N43	Small	<0.01	<0.01	Large	0.12	0.052	Large
N54	Small	<0.01	<0.01	Large	<0.01	0.13	Large
N57	Intermediate	0.03	0.09	Intermediate	0.04	0.11	Intermediate
T4Bol		0.72	0.17		0.70	0.17	

[a] The phage sample was incubated with two drops of indicator bacteria at 5×10^8 cells/ml for 15 min at 37 C before plating. The Tris-glucose plates contained no thymidine. The 2.5-ml agar overlay contained 20 μg of thymidine and two drops of indicator bacteria (method of Simon and Tessman [18]).

[b] Specific activity in units per mg of protein (average values for three separate infections).

[c] Units of reversal per mg of phage protein in immunological assay (fig. 4).

assays are compatible with the tentative order of mapping presented above. That is, up to the point of termination, *N33* produced no cross-reacting material, while *N37* produced a much more extended chain which could cross-react immunologically. A second possibility is that *N33* and *N37* are missense mutants. In the case of *N33*, the amino acid substitution in the protein would have to not only alter the nature of the catalytic site, rendering the enzyme inactive, but would also have to alter the conformation of the protein beyond what is achieved by mild denaturation, so that it is no longer cross-reactive. The *N37* substitution may yield a cross-reactive protein with an altered and inactive catalytic site.

The *tdN43* and *tdN54* mutants are the only two studied that can be properly classified as amber mutants (Table 3). First, they showed the expected phenotype: small plaques were produced on the SW-2 (*su⁻*) strain and large plaques appeared on the SK-I (*su⁺*) strain. Second, both produced more cross-reacting material during infection of the *su⁺* host than during infection of the *su⁻* host. As expected, the crude extracts from infections of SW-2 contained no detectable enzymatic activity. Under conditions of suppression, thymidylate synthetase was produced upon infection with *tdN43* giving one-sixth the specific activity of the wild-type phage infection. The amount of cross-reacting material closely paralleled the level of enzyme activity. In the *tdN43* infections of SK-I, it is not possible to distinguish between two possibilities: the production of a small amount of active enzyme due to inefficient suppression or, second, the production of a large amount of partially active enzyme. If the latter were true, the substitution of serine in the protein chain resulted in an alteration of both the enzymatic activity and the protein conformation.

Surprisingly, no thymidylate synthetase activity was found in crude extracts from four independent infections of SK-I with *tdN54* although the phage produced large plaques on SK-I. The substantial amount of cross-reacting material present in the crude extracts indicated that chain propagation had occurred and that the conformation of the resulting protein was not drastically altered. The absence of detectable enzymatic activity suggested two major possibilities. First, because of the particular site of the amber mutation, suppression may lead to an altered inactive catalytic site. Second, and more likely since the morphological tests indicated that thymidylate was being synthesized in vivo, the substitution of serine for the wild-type amino acid at the site of the amber mutation may have rendered the enzyme very labile to the conditions encountered during the preparation of the extracts.

Hall found that the map positions of *tdN43* and *tdN54* were indistinguishable; no wild-type recombinants were observed when a host was mixedly infected with these two mutants (D. Hall, personal communication). If *tdN43* and *tdN54* are indeed *am* mutations, then since the same suppressor mutation gave rise to different suppressed phenotypes, it is likely that the two mutations are in different codons. They may be so close to each other that recombination is a very rare event. The *N54* site may be more critical for the formation of a stable catalytic site.

The *tdN57* mutant represented a third type. This phage produced an intermediate size plaque on both SW-2 and SK-I strains. Correlated with this was a very low level of enzymatic activity. It was difficult to conclude

that the *tdN57* was an amber since the cross-reacting material produced on infection of a su^+ strain was only slightly higher than that produced on infection of a su^- strain (Table 3). Since no cross-reacting material was detected in crude extracts from *tdN43* or *tdN54* of SW-2 but was present with *tdN57* infection, it appears that the *tdN57* permitted the production of a protein chain which extended beyond the *N43* or *N54* sites and which had a conformation similar enough to the native enzyme to be immunologically active.

In summary, the ability to demonstrate differences in the properties of the enzyme thymidylate synthetase from cultures infected with phage *td* mutants of both amber and non-amber types allows one to conclude, in agreement with previous investigators, that the infecting T4 phage codes for the thymidylate synthetase which appears after infection. In the identification of true amber mutants, the immunological measurement of cross-reacting material was an effective indicator even when enzyme activity measurements alone were not sufficient, as with *tdN54*. As such mutants are identified, their thymidylate synthetase proteins should allow more detailed genetic mapping and further study of structure-function relationships as the effects of single specific amino acid replacements are defined.

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