*INABILITY OF THYMINE-DEPENDENT MUTANTS OF BACTERIOPHAGE T4 TO INDUCE THYMIDYLATE SYNTHETASE**

By David M. Shapiro,† Joseph Eigner,‡ and G. Robert Greenberg

DEPARTMENT OF BIOLOGICAL CHEMISTRY, UNIVERSITY OF MICHIGAN

Communicated by J. L. Oncley, November 20, 1964

In 1954 Barner and Cohen[1] discovered that infection of a thymine-dependent mutant of *Escherichia coli* B by bacteriophage T2 or T5 brought about the net synthesis of thymine derivatives and subsequent multiplication of the phage. Later work showed that infection of *E. coli* B or of thymine-dependent mutants of *E. coli* by T2 or T5 induced the formation of thymidylate synthetase activity.[2,3] The concept that this enzyme is, at least in part, phage-determined is supported by the physical separation in this laboratory of the phage-induced and bacterial thymidylate synthetases[4] and the kinetic evidence of Mathews and Cohen[5] that the dTMP[6] synthetases induced by T4 and T6 are different. The reaction catalyzed by bacterial thymidylate synthetase,[7] and apparently by the "early" enzyme induced by phage infection, proceeds with the following stoichiometry:

$$5,10\text{-Methylenetetrahydrofolate} + \text{dUMP} \xrightarrow{Mg^{++}} \text{dTMP} + \text{dihydrofolate}.$$

In a recent communication, Simon and Tessman[8] reported the isolation of a new class of mutants of T4 bacteriophage which require thymine in order to replicate in a thymine auxotroph of the host. These mutants, designated as *td*,[8] did not complement each other and were considered to have suffered lesions in a region of their DNA involved with the initiation of thymidylate synthetase activity.

The present study provides direct evidence that the level of thymidylate synthetase activity induced by infection of *E. coli* B3 with these thymine-dependent mutants of T4 phage is markedly lower than after infection by the wild-type phage.

Materials and Methods.—*E. coli* B3, a thymine-requiring mutant of *E. coli* B originally isolated by S. Brenner, University of Cambridge, England, was obtained from N. Melechen, St. Louis University. Bacteriophage T4B and thymine-requiring mutants *td*8, *td*9, *td*10, and *td*15, derived from T4BO$_1$ were generously provided by Edward Simon and Irwin Tessman, Purdue University. Bacterial stocks were maintained on nutrient agar slants. Wild-type and mutant bacteriophage were propagated on either *E. coli* R2 or on *E. coli* B3, using a glycerol-salts-casamino acids medium[9] supplemented with 10 μg/ml thymine.

Bacteriophage were assayed by the agar-layer technique[10] using *E. coli* S/6 as the indicator bacteria. Viable cells were determined by plating on tryptone Bacto-agar plates. Infected bacteria were measured as described by Adams.[10]

dl-Tetrahydrofolate was obtained from Nutritional Biochemicals Corp., Cleveland, and was approximately 50% pure, based on the calculated concentration of the *l*,L-isomer and assayed with *E. coli* dTMP synthetase purified by chromatography on DEAE-cellulose.[4] DEAE-cellulose, dUMP, dCMP, and dCTP were obtained from Calbiochem, Los Angeles; chemically reduced TPN was a product of the Sigma Chemical Co., St. Louis. C^{14}-labeled formaldehyde was purchased from Research Specialities Co., Richmond, Calif. Dihydrofolate was prepared from folic acid (Nutritional Biochemicals Corp., Cleveland) according to the procedure of Futterman.[11] *E. coli* alkaline phosphatase, chromatographically purified, was obtained from Worthington Biochemical Corp., Freehold, N. J.

Assay of dTMP synthetase: Method I was the spectrophotometric procedure of Wahba and Friedkin[7] as previously employed.[4] Cuvette temperatures were maintained at 25°. *Method II* measured the incorporation of C^{14}-formaldehyde into dTMP. The reaction mixture contained:

enzyme; Tris-formate solution, pH 7.4, 50 μmoles as Tris; 2-mercaptoethanol, 50 μmoles; ethylenediamine-tetraacetate, pH 7.4, 0.5 μmoles; magnesium acetate, 10 μmoles; *dl*,L-tetrahydrofolate, 0.5 mg; C^{14}-formaldehyde, 1 μmole; dUMP, 1 μmole, in a final volume of 0.5 ml. The reaction mixture was incubated at 37° for 40 min and the reaction terminated by chilling in an ice bath and adding 100 μmoles of formaldehyde or hydroxylamine. dTMP was isolated on a Dowex-1-formate column.[12] The ratio of the rates by this method at 25° and 37° was 0.4. In *method III* the reaction mixture was scaled up 10-fold and the radioactive products isolated by carrier techniques. After incubation at 37° for 60 min, 4.80 μmoles of carrier dTMP and 4.35 μmoles of carrier thymine were added, and the reaction was stopped by heating for 3 min in a boiling water bath. Precipitated protein was removed by centrifugation. The supernatant solution was adjusted to pH 8.2 and incubated with *E. coli* alkaline phosphatase until 90% or more of the phosphate ester of dTMP (and dUMP) was hydrolyzed. Trichloroacetic acid was added to a final concentration of 5%, the suspension centrifuged, and the supernate applied to a 0.5 × 8-cm Dowex-50W-X8 column (H^+ form, 200–400 mesh). The fraction which was eluted with the water wash and which contained the nucleosides and free bases was adjusted to pH 4.4. Unreacted C^{14}-formaldehyde was removed after addition of 100 μmoles of carrier formaldehyde by precipitation as the dimedon (5,5-dimethylresorcinol) derivative. The addition of formaldehyde, followed by an excess of dimedon and the removal of precipitate by centrifugation, was repeated three times, and finally 100 more μmoles of formaldehyde than remaining dimedon were added. The sample was adjusted to pH 11 and placed on a 0.5 × 5-cm Dowex-1-X8 column (formate form, 200–400 mesh). Nucleosides and free bases were eluted with 0.05 *M* ammonium formate, pH 4.4, and then concentrated. Descending chromatography on Whatman no. 3 MM paper with n-butanol saturated with water separated thymine and thymidine from deoxyuridine,[13] and chromatography with 0.25 *M* formic acid (Whatman no. 3 MM, ascending) separated thymidine from thymine. The isolated thymidine showed the expected spectral characteristics at pH 2 and 12. Less than 10% of the radioactivity recovered at this point appeared as thymine. The incorporation of C^{14} label into dTMP was calculated from the specific activity of the isolated thymine and thymidine fractions. For each extract examined by this method, a control lacking dUMP was carried through all of the above steps.

Other assays: dCMP hydroxymethylase was measured as previously described,[14] except that the product was isolated on small Dowex-1-formate columns.[15] Deoxycytidinetriphosphatase was assayed by the method of Wiberg *et al.*[12] Dihydrofolate reductase was measured spectrophotometrically.[16] All enzyme activities are expressed in units or in milliunits; a unit is the activity catalyzing the formation of 1 mμmole of product per minute.

Protein concentration was determined by a modification of the microbiuret method[17] or, where indicated, by the method of Lowry *et al.*[18] Inorganic phosphate was estimated by the molybdate method.[19] Formaldehyde was assayed colorimetrically[20] using a standard which was measured gravimetrically as the dimedon derivative. C^{14}-formaldehyde was determined by adding carrier and counting the dimedon derivative. Radioactivity was measured by an end window gas-flow counter.

Infection and preparation of extracts: *E. coli* B3 was grown aerobically at 37° to a concentration of 5×10^8 to 1×10^9 cells per ml, either on the glycerol-salts-casamino acids medium plus 10 μg thymine per ml, or on a medium containing 0.8% Difco nutrient broth and 0.5% sodium chloride plus 10 μg thymine per ml, as indicated in the legends. Immediately before infection L-tryptophan was added to a concentration of 10 μg per ml. The culture was infected with a multiplicity of 5 phage per bacterium and aeration continued. The process of infection was terminated by pouring the culture over approximately one half its volume of crushed ice. The cells were collected by centrifugation at 6000 × *g* for 15 min and stored at −15°. Extracts were prepared by one of two procedures. An amount of cell paste equivalent to 8–30 × 10^{10} cells was suspended in 10 ml of 0.05 *M* Tris-hydrochloride buffer, pH 7.4, and twice subjected to ultrasonic treatment at 20 kc for 30-sec periods using a one-half inch probe (model LS-75 Sonifier, Branson Co., Stamford, Conn.). During sonication the temperature was maintained at 0–15° by immersion in a −10° bath. Cell debris was removed by centrifuging the suspension at 30,000 × *g* for 15 min. In the second procedure the cell paste was ground with twice its weight of alumina (Aluminum Co. of America, A305), the mixture extracted with 4 vol of 0.01 *M* Tris-hydrochloride buffer, pH 7.4, containing 0.005 *M* 2-mercaptoethanol and 0.01 *M* magnesium acetate, and the resulting suspen-

sion centrifuged at 30,000 × *g* for 30 min. The precipitate was extracted again with 2 vol of the buffer solution, the mixture centrifuged as before, and the supernates were combined. In some cases this fraction was centrifuged for 4 hr at 100,000 × *g* and the supernatant solution saved. All centrifugations and extractions were carried out at 0–4°, and extracts were stored at −20°.

Chromatographic separation of phage-induced dTMP synthetase: *E. coli* R2 was grown aerobically on the glycerol-salts-casamino acids medium at 37° in the absence of added thymine. The infection process was the same as described for *E. coli* B3. Extracts from alumina ground cells were incubated with 1 μg/ml each of pancreatic DNase and RNase for 45 min at 30° and centrifuged at 100,000 × *g*. The resulting supernate was dialyzed in alkali-treated[7] Visking tubing against 0.005 *M* potassium phosphate buffer, pH 6.5, and applied to a 1.2 × 15-cm column of DEAE-cellulose previously washed with NaOH solution and equilibrated with 0.005 *M* potassium phosphate buffer. Phage-induced dTMP synthetase was eluted with 0.10 *M* potassium phosphate, pH 6.5, and host dTMP synthetase with 0.25 *M* potassium phosphate, pH 6.5, by the procedure previously described for their separation after infection of *E. coli* by T2 phage.[4] Additional details are given in the legend to Figure 1.

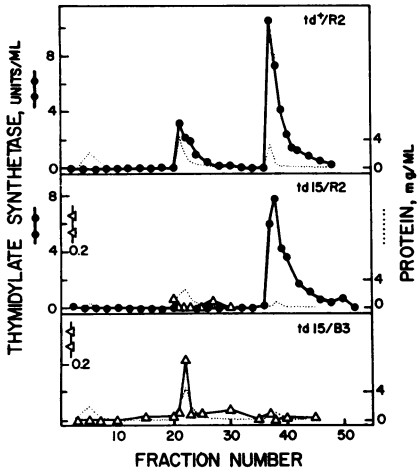

FIG. 1.—DEAE-cellulose chromatography of extracts of *E. coli* infected by *td* bacteriophage. The values are uncorrected. Filled circles indicate dTMP synthetase assays made spectrophotometrically, while triangles indicate assays by method II; note the 20-fold difference in ordinates. Dotted lines indicate protein[18] concentration. Elution was accomplished with potassium phosphate buffers at pH 6.5 according to the following schedule: 0.005 *M* to tube 17, 0.10 *M* to tube 33, 0.25 *M* to tube 55. Fraction volumes were 3.3 ml. In the case of the *td*⁺/R2 extract 77 mg of protein containing 160 units of dTMP synthetase were chromatographed and 88% of the unit were recovered; for *td*15/R2, the corresponding figures were 60 mg protein, 140 units, and 81% recovery; for *td*15/B3, 88 mg protein, 1 unit, and approximately 75% recovery. The 30,000 *g* supernatant fractions of *td*⁺/R2, *td*15/R2, and *td*15/B3, respectively, contained 1.46, 1.4 (estimated from the value for 100,000 *g* supernate), and 0.023 units of thymidylate synthetase/mg protein. Recoveries of total units of dTMP synthetase activities through the manipulations preceding these column steps were rather variable: for *td*⁺/R2 62%, *td*15/R2 62% (estimated), and *td*15/B3 22%. The major losses were on dialysis. dCMP hydroxymethylase levels per mg protein in the crude extracts were: *td*⁺/R2 1.36 units, *td*15/R2 2.25 units, and *td*15/B3 1.37 units.

Results.—Induction of dTMP synthetase and other enzymes by td and td⁺ phage: An indispensable requirement in comparing enzyme levels in cell cultures infected by bacteriophage is that equivalent degrees of infection prevail. Neither the per cent of cells killed nor the number of cells which eventually produced progeny phage ("infected bacteria") were reliable indices of early enzyme induction (see also refs. 15 and 21). We have therefore taken the activity of dCMP hydroxymethylase,[22] which is found only after phage infection, as a criterion of successful infection by the *td*-phage. However, such variables as differences in the effective duration of infection and in the rate of increase of the "early" enzymes are involved so that correction for differences in levels of dCMP hydroxymethylase are presented with some reserve. In Table 1 are summarized three experiments in which comparable levels of this enzyme activity appeared in *E. coli* B3 after infection with either the wild-type phage or the thymine-requiring mutants. In the last column the data are presented as the ratios of the specific activities of dTMP

TABLE 1

PHAGE-INDUCED ENZYMES FOLLOWING INFECTION OF *E. coli* B3 BY *td* MUTANTS OF T4

Extract	Period of infection (min)	dTMP Synthetase Method I	dTMP Synthetase Method II	dCMP Hydroxymethylase	dCTPase	TSase/HMase*
		(Units per mg protein)				
Expt. 1						
B3		<0.05		<0.003	1.2	
*td*8/B3†	15	<0.05		1.2	23	<0.04
*td*15/B3	15	<0.05		1.2	22	<0.04
td$^+$/B3	15	1.6		1.3	25	1.2
Expt. 2						
*td*8/B3	5		0.053	1.7		0.031
*td*8/B3	10		0.062	3.7		0.017
*td*8/B3	20	<0.04	0.038	3.3		0.012
td$^+$/B3	0		0.033	0.012		
td$^+$/B3	5	0.13	0.24	1.5		0.16
td$^+$/B3	10	0.52	0.64	2.6		0.25
td$^+$/B3	20	0.33	0.59	1.4		0.42
Expt. 3					Folate-H_2 reductase‡	
B3			<0.001	<0.001	3	
*td*8/B3	15	<0.06	<0.002	0.93	13	<0.002
*td*9/B3	15	<0.04	<0.002	1.72	19	<0.001
*td*10/B3	15	<0.04	<0.002	3.44	15	<0.0006
*td*15/B3	15	<0.06	0.074	0.89	14	0.079
td$^+$/B3	15	0.56‡	0.58‡	1.21§ 1.38‡	19	0.42

* Ratio of specific activities of thymidylate synthetase to dCMP hydroxymethylase. In expts. 2 and 3 these ratios were based on the determination of dTMP synthetase by method II.

† See abbreviations.[6]

‡ These values were obtained from the 100,000 *g* supernate. In expt. 3 the 100,000 *g* supernate fractions from cells infected by any of the mutant phage showed less than 0.05 units of dTMP synthetase per mg protein by method I.

§ This is the calculated sum from the separate analyses for activities of the 100,000 *g* supernate and the ribosomal fractions.

Except as indicated, the analyses were on the supernates after centrifugation of the crude extract at 30,000 *g*. *Experiment 1:* The bacteria were grown in glycerol-salts-casamino acid medium supplemented with 10 μg thymine per ml under forced aeration and extracted by the sonication method. *Experiment 2:* The bacteria were grown and infected in nutrient broth plus 10 μg thymine per ml under forced aeration and extracted by the sonication method. The control for the assay of dTMP synthetase by method II was omission of the enzyme. C^{14}-formaldehyde had an activity of 185,000 cpm/μmole. *Experiment 3:* Growth and infection were as in expt. 1, aerating by rotary shaking. The cells were extracted by the grinding method. The control for the dTMP synthetase assay by method II was omission of dUMP. The activity of the C^{14}-formaldehyde was 342,000 cpm/μmole. Within a given experiment the recovery of total protein in each extract was approximately constant.

synthetase to dCMP hydroxymethylase. It is evident that other phage-initiated enzyme activities, namely, dCTPase[23, 24] and dihydrofolate reductase,[25] also appeared at approximately the same levels whether the cells were infected by the *td* mutants or by *td*$^+$ phage. On the other hand, dTMP synthetase activity was much lower in extracts of cells infected by any of the *td* mutant phage than in those infected by *td*$^+$ phage. The results were essentially the same whether dTMP synthesis was assayed by *method II* or spectrophotometrically. Since the two assay methods differ in the reaction conditions and the products measured, the values obtained are not quantitatively comparable in crude extracts. Infection of *E. coli* B3 by *td*15 led to the induction of about 19 per cent of the dTMP synthetase activity of the wild-type control, corrected on the basis of the dCMP hydroxymethylase levels. In spite of this "leakiness" *td*15 produces the same low burst sizes on *E. coli* B3 in the absence of thymine as the other *td* mutants.[8] The variation in the results with *td*8 in experiments 2 and 3 probably is related to the different controls used (see legend to Table 1).

At the low levels of enzymatic activity induced by the *td* mutants and the necessarily high concentrations of protein employed, the resulting high blank values

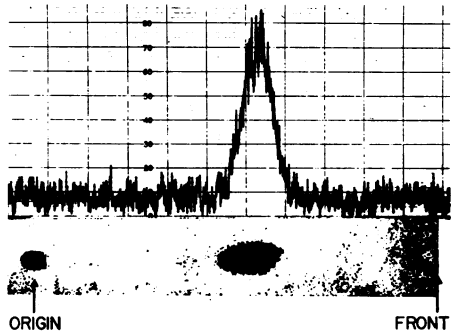

FIG. 2.—Correspondence of the radioactivity and the ultraviolet absorption of thymidine (from dTMP formed by the extract from *td*15/B3). C^{14}-thymidine was isolated from the reaction mixture by method III and chromatographed in water-saturated n-butanol. The distance from the starting line (spot at left) to the peak of radioactivity was 10.8 cm (R_F = 0.55). The same results were obtained with the system, td^+/B3.

in the spectrophotometric assay lead to a decrease in precision. The isotopic assay (*method II*) is subject to an error caused by the conversion of a fraction of the dTMP to thymidine and thymine by action of phosphatase(s) and phosphorylase present in the crude extracts. Since thymidine and thymine are eluted from the Dowex-1 column in the same position as radioactive by-products of the reaction, they cannot easily be assayed by this method. Therefore, in order to obtain a confirming value for dTMP synthetase activity of cells infected by *td*15 phage and to establish the nature of the nucleotide product specifically, *method III* was employed. The radioactive thymidine isolated by this method was rechromatographed in n-butanol saturated with water (Whatman no. 1, ascending). The radioactive product isolated from either reaction mixture, from *td*15/B3 or td^+/B3, cochromatographed with carrier thymidine as measured by scanning for radioactivity and by photography under a filtered mercury lamp (Fig. 2). The results given in Table 2 for *td*15/B3 are in essential agreement with the level obtained by *method II* (Table 1, expt. 3).

Mixing experiments: These studies were designed to test for the presence of a substance produced by cells infected by mutant phage which would specifically inhibit phage-induced dTMP synthetase. An example of such a substance is described by Friedkin and co-workers[26] who concentrated a natural inhibitor for dTMP synthetase from *E. coli* B. The results of several experiments in which extracts of cells infected by wild-type phage and of cells infected by mutant phage were mixed are given in Table 3. The variations observed approached the experimental errors of the methods at the high protein concentrations employed. The important result of these studies was that substantially no inhibition of the dTMP

TABLE 2

ASSAY OF dTMP SYNTHETASE BY ISOLATION OF C^{14}-THYMIDINE*

Extract	Plus dUMP	Minus dUMP	Difference	Per cent of wild-type phage
	(Milliunits per mg protein)			
td^+/B3	337	4.0	333	100
*td*15/B3	47.3	1.1	46.2	14 (27)†
B3	0.98	0.88	0.10	—

* Method III.

† The figure in parentheses represents a corrected value, based on the deviation from enzyme linearity at the high protein concentration employed, as determined by method II (× 1.41) and the ratio of dCMP hydroxymethylase activities in the two extracts (× 1.36). The linearity correction is a maximal value and neglects loss of C^{14}-dTMP in method II by phosphatase.

The extracts were those used in expt. 3, Table 1. The activity of the formaldehyde was 342,000 cpm/μmole. The 30,000 *g* supernates of B3 and *td*15/B3 and the 100,000 *g* supernate of td^+/B3 were used, and the latter was corrected to the 30,000 *g* value (× 0.66). 27.5 mg of protein of the extract of B3, 27.9 mg of extract *td*15/B3, and 4.1 mg of extract td^+/B3 were used. Actual values for the isolated thymidine fraction in terms of cpm, with and without dUMP as substrate, were, respectively: for td^+/B3, 13,300 and 214, for *td*15/B3, 7,970 and 232, and for B3, 203 and 225. The recoveries of dTMP as thymidine ranged from 30 to 45%.

TABLE 3

EFFECT OF EXTRACTS OF *td*-INFECTED *E. coli* B3 ON dTMP SYNTHETASE ACTIVITY INDUCED BY WILD-TYPE PHAGE

	Extract	Protein (mg)	Single extract (cpm)	Combined Extracts (cpm) Calculated	Found
(*A*)	*td*$^+$/B3	0.33	1820		
	*td*8/B3	0.36	160	1980	1570
	*td*8/B3	0.72	290	2110	1830
(*B*)	*td*$^+$/B3	0.8	7350		
	*td*15/B3	0.7	810	8160	7750
	*td*15/B3	1.4	1360	8710	7580
(*C*)	*td*$^+$/B3	0.8	2860		
	*td*8/B3	1.4	25	2885	2740
	*td*9/B3	1.3	28	2878	2830
(*D*)	*td*$^+$/B3	0.8	2550		
	*td*10/B3	1.3	29	2579	2690
	*td*15/B3	1.4	755	3305	3820

Each extract first was assayed for dTMP synthetase and then tested for its effect on the wild-type enzyme present in the extract of *td*$^+$/B3 by assaying an extract of *td*$^+$/B3 in the presence of each of the other extracts. The calculated values are based on the addition of the individual activities.

In expts. *A–D*, dTMP synthetase activity was measured by method II, and the results are given as the total incorporated into dTMP. The extracts of *td*$^+$/B3 and *td*8/B3 under (*A*) were those used in expt. 2, Table 1 (10-min samples). The extracts under (*B*) were those of expt. 3, Table 1; *td*$^+$/B3 was a 100,000 *g* supernate, *td*15/B3, a 30,000 *g* supernate. In (*C*) and (*D*), 30,000 *g* supernates were used.

synthetase activity took place in extracts of *E. coli* B3 infected by *td*$^+$ phage on addition of extracts prepared from *E. coli* B3 infected with the *td* mutant phage.

Chromatographic studies: Extracts of *E. coli* R2, infected either by phage *td*15 or *td*$^+$, were passed through a DEAE-cellulose column to separate the host and the phage-induced activities. In Figure 1 (*td*$^+$/R2) the first peak eluted from the column is known to be the phage-initiated enzyme, and the second, the host enzyme.[4] No phage-induced enzyme was detected in an extract of *E. coli* R2 infected with *td*15 (*td*15/R2). As expected, the *td*15/B3 control possessed low activity in the region of the column corresponding to the phage-induced dTMP synthetase. Had *td*15 induced the same level of dTMP synthetase activity on *E. coli* R2 as on *E. coli* B3 (Tables 1 and 2), it should have been detected. In any event, it is clear that activity comparable to that induced by *td*$^+$ was not detected. In that case 33 units of phage-induced enzyme were recovered from the column. However, the possibility remains that *td*15 induces a very unstable enzyme which may have been lost in the steps prior to the chromatography. Only 17 per cent of the "leaky" activity of the *td*15/B3 control extract survived these steps.

Discussion.—These thymine-dependent mutants of T4 phage are not able to induce levels of thymidylate synthetase activity in *E. coli* B3 comparable to those induced by wild-type phage. The finding of early phage-induced enzymes other than dTMP synthetase after infection by these mutants is taken as evidence that (*a*) good infection took place, and (*b*) the mutation, whatever its nature, appears to be related to the dTMP synthetase system.

The mutant phage, *td*15, clearly induced the formation of the enzyme in *E. coli* B3 to a level somewhere between 14 and 27 per cent of the value induced by the wild-type phage. Nevertheless, little or no phage-induced dTMP synthetase was found on chromatography of extracts of *E. coli* B3 or *E. coli* R2 infected by mutant *td*15 (Fig. 1), perhaps because of an instability of an altered enzyme.

The results obtained by mixing of extracts (Table 3) appear to exclude the presence of an inhibitor as an explanation of the low levels of phage-induced enzyme. A similar conclusion can be drawn from *in vivo* experiments[8] in which

normal yields of td^+ were obtained when *E. coli* B3 was simultaneously infected by td^+ and each of the *td* mutants.

The experiments reported in this paper do not distinguish between a lesion of a structural gene carried by the phage genome and a number of other possible mutations. Studies on the temperature-sensitive mutants *ts* L13 and *ts* G25W[27] and on the corresponding noncomplementing amber mutant *Am* N122[28] have given evidence that these mutations lead to structural changes in dCMP hydroxymethylase. In addition, Streisinger and co-workers[29] have provided evidence that structural changes in lysozyme, a "late" phage-induced enzyme, occur in mutations of the endolysin gene.

Epstein *et al.*[30] and Sarabhai *et al.*[31] have reported a great number of mutations resulting in structural alterations of the phage such as coat protein and tail structure. On the other hand, thus far in the group of "early" enzymes, phage mutations affecting only dCMP hydroxymethylase[12, 27, 28] and dTMP synthetase have been reported. Yet a considerable number of genes in the T4 genome are known to be necessary for the synthesis of DNA.[30] It is obvious that a further search for the enzymes related to these genes is important to our understanding of the infection process.

Summary.—Infection of *Escherichia coli* by three thymine-requiring mutants of T4 phage induced little or no dTMP synthetase compared to the wild-type controls. A fourth mutant, *td*15, was clearly "leaky." At the same time a number of other phage-induced enzymes reached normal levels. These findings suggest that the mutations are related to thymidylate synthetase but do not indicate the nature of the mutations.

* Supported, in part, by grant A1669 from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, and by a grant from the National Science Foundation.

† Postdoctoral fellow of the Institute of Science and Technology of the University of Michigan and the National Institutes of Health. Present address: Department of Biochemistry, Woman's Medical College of Pennsylvania, Philadelphia.

‡ Postdoctoral fellow of the National Science Foundation and postdoctoral trainee of the National Institutes of Health, Genetics training grant to the University of Michigan. Present address: Department of Microbiology, Washington University, St. Louis.

[1] Barner, H. D., and S. S. Cohen, *J. Bacteriol.*, **68**, 80 (1954).

[2] Barner, H. D., and S. S. Cohen, *J. Biol. Chem.*, **234**, 2987 (1959).

[3] Flaks, J. G., and S. S. Cohen, *J. Biol. Chem.*, **234**, 2981 (1959).

[4] Greenberg, G. R., R. L. Somerville, and S. DeWolf, these PROCEEDINGS, **48**, 242 (1962).

[5] Mathews, C. K., and S. S. Cohen, *J. Biol. Chem.*, **238**, 367 (1963).

[6] Abbreviations employed are those defined by *J. Biol. Chem.* except as follows: DEAE-cellulose, diethylaminoethyl-cellulose; *td*8/B3, *E. coli* B3 infected by the phage mutant, *td*8.

[7] Wahba, A. J., and M. Friedkin, *J. Biol. Chem.*, **237**, 3794 (1962).

[8] Simon, E. H. and I. Tessman, these PROCEEDINGS, **50**, 526 (1963). We have followed the designation *td* suggested by these authors, though this terminology might be confused with the well-studied tryptophan synthetase mutants. Perhaps thy^- would be more accurate since the mutants are thymine-requiring. Another thymine-requiring mutant of T4 has been independently isolated by D. L. Wulff and K. Metzger [*Virology*, **21**, 499 (1963)].

[9] Fraser, D., and E. A. Jerrel, *J. Biol. Chem.*, **205**, 291 (1953).

[10] Adams, M. H., *Bacteriophages* (New York: Interscience Publishers, 1959), pp. 449–451.

[11] Futterman, S., *J. Biol. Chem.*, **228**, 1031 (1957).

[12] Wiberg, J. S., M.-L. Dirksen, R. H. Epstein, S. E. Luria, and J. M. Buchanan, these PROCEEDINGS, **48**, 293 (1962).

[13] 5-HydroxymethyldUMP could be a hydrolytic side product of the hypothetical intermediate having a methylene bridge between the 5 position of dUMP and the no. 5 atom of tetrahydrofolate.[7] In all solvents examined by R. E. Cline, R. M. Fink, and K. Fink [*J. Am. Chem. Soc.*, **81**, 2521 (1959)], 5-hydroxymethyldeoxyuridine migrated to the same position as 5-hydroxymethyluracil or was slightly slower, and thymidine and thymine always migrated far ahead of both. We could not detect radioactivity on our chromatograms in the region between the starting line and the position of thymidine and thymine (R_F, 0.64). 5-Hydroxymethyluracil showed an R_F of 0.49. From the background level radioactivity on the chromatogram, 5-hydroxymethyldUMP could not account for more than 5% of the synthesis. 5-HydroxymethyldUMP does not appear to be an intermediate in the *E. coli* dTMP synthetase reaction.[3]

[14] Somerville, R. L., K. Ebisuzaki, and G. R. Greenberg, these PROCEEDINGS, **45**, 1240 (1959).

[15] Dirksen, M. L., J. S. Wiberg, J. F. Koerner, and J. M. Buchanan, these PROCEEDINGS, **46**, 1425 (1960).

[16] Osborn, M. J., and R. M. Huennekens, *J. Biol. Chem.*, **233**, 969 (1958).

[17] Zamenhof, S., in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1957), vol. 3, p. 702.

[18] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

[19] Fiske, C. H., and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925).

[20] Nash, T., *Biochem. J.*, **55**, 416 (1953).

[21] Cohen, S. S., *Federation Proc.*, **20**, 641 (1961).

[22] Flaks, J. G., and S. S. Cohen, *J. Biol. Chem.*, **234**, 1501 (1959).

[23] Koerner, J. F., M. S. Smith, and J. M. Buchanan, *J. Biol. Chem.*, **235**, 2691 (1960).

[24] Kornberg, A., S. B. Zimmerman, S. R. Kornberg, and J. Josse, these PROCEEDINGS, **45**, 772 (1959).

[25] Mathews, C. K., and S. S. Cohen, *J. Biol. Chem.*, **238**, PC853 (1963).

[26] Friedkin, M., E. J. Crawford, E. Donovan, and E. J. Pastore, *J. Biol. Chem.*, **237**, 3811 (1962).

[27] Wiberg, J. S., and J. M. Buchanan, these PROCEEDINGS, **51**, 421 (1964).

[28] Dirksen, M. L., J. C. Hutson, and J. M. Buchanan, these PROCEEDINGS, **50**, 507 (1963).

[29] Streisinger, G., F. Mukai, W. J. Dreyer, B. Miller, and S. Horiuchi, in *Cellular Regulatory Mechanisms*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 26 (1961), p. 25. See also Anfinsen, C., *Federation Proc.*, **20**, 634 (1961).

[30] Epstein, R. H., A. Bolle, C. M. Steinberg, E. Kellenberger, E. Boy de la Tour, R. Chevalley, R. S. Edgar, M. Susman, G. H. Denhardt, and A. Lielausis, in *Synthesis and Structure of Macromolecules*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 28 (1963), p. 375.

[31] Sarabhai, A. S., A. O. W. Stretton, S. Brenner, and A. Bolle, *Nature*, **201**, 13 (1964).

---

*LIPID-PHOSPHOACETYLMURAMYL-PENTAPEPTIDE AND LIPID-PHOSPHODISACCHARIDE-PENTAPEPTIDE: PRESUMED MEMBRANE TRANSPORT INTERMEDIATES IN CELL WALL SYNTHESIS**

BY JOHN S. ANDERSON,† MICHIO MATSUHASHI, MARY A. HASKIN, AND JACK L. STROMINGER

DEPARTMENT OF PHARMACOLOGY, UNIVERSITY OF WISCONSIN MEDICAL SCHOOL, MADISON

Communicated by Henry Lardy, February 15, 1965

The uridine nucleotides, uridine diphospho-acetylmuramyl·L-ala·D-glu·L-lys·D-ala·D-ala (UDP-MurNAc-pentapeptide) and uridine diphospho-acetylglucosamine (UDP-GlcNAc), are substrates for a reaction catalyzed by a particulate enzyme prepared from *Staphylococcus aureus* in which a linear glycopeptide com-