EVIDENCE FOR A DUAL ROLE FOR THE BACTERIOPHAGE T4-INDUCED DEOXYCYTIDINE TRIPHOSPHATE NUCLEOTIDOHYDROLASE*

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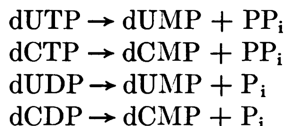
The induction of dCTP nucleotidohydrolase (E.C. 3.6.1.12), hereafter referred to as dCTPase, in *E. coli* infected by T-even bacteriophages has been previously reported.[1,2] Wiberg[3] has recently found that *amber* mutations[4] in gene 56 of bacteriophage T4 block dCTPase induction in *E. coli* B infected with these mutants. When we learned that Greenberg[5] had demonstrated increased dUTPase activity in *E. coli* infected with bacteriophage T2, it ocurred to us that one enzyme might catalyze both reactions, a situation analogous to the phage-induced polyfunctional deoxynucleoside monophosphate kinase.[6] Zimmerman and Kornberg[7] purified the dCTPase induced by phage T2 in *E. coli* B, and tested at least ten deoxynucleoside triphosphate substrates other than dCTP and found the enzyme to be active only on dCTP. They did not report trying dUTP. This paper presents evidence to support the hypothesis that a single T4 phage-induced enzyme catalyzes the following four reactions:[8]



$$\text{dUTP} \rightarrow \text{dUMP} + \text{PP}_i$$
$$\text{dCTP} \rightarrow \text{dCMP} + \text{PP}_i$$
$$\text{dUDP} \rightarrow \text{dUMP} + \text{P}_i$$
$$\text{dCDP} \rightarrow \text{dCMP} + \text{P}_i$$

Extracts of uninfected *E. coli* have been shown to contain significant amounts of dUTPase activity,[9,10] and only trace amounts of dCDPase and dCTPase activities,[7] but the number of enzymes involved has not been elucidated.

Methods and Materials.—Materials: The *amber*[4] (*am*)[11] mutants described in this work were generously supplied by R. S. Edgar. The dCTP and dUTP were purchased both from Sigma Chemical Co. and Calbiochem, and the dUDP was purchased from Sigma Chemical Co. The substrate solutions were analyzed spectrally from 230 to 300 mμ before use. Streptomycin sulfate was purchased from Calbiochem. The DEAE cellulose was purchased from Schleicher and Schuell, and Hypatite C, a hydroxylapatite preparation, from Clarkson Chemical Co.

Methods: Preparation of cell extracts: All cultures were grown with forced aeration in media similar to that described by Fraser and Jerrel,[12] except that 5 gm casamino acids were used per liter rather than 15 gm. Phage stocks of the amber mutants were grown in *E, coli* CR63 at 37°, and stocks of T4 am^+ were grown in *E. coli* B at 37°. The stocks were prepared and titered as described by Wiberg *et al.*[13] All *amber* mutant stocks were checked for mutant phenotype before use. Cultures (10-liter) of uninfected *E. coli* B cells and cells infected with T4 *am* N82 were grown and used for the preparation of large quantities of cell extracts. Smaller, 500-ml cultures were grown for scanning mutants for failure to induce dUTPase. For preparation of these cell extracts, cultures were grown at 37° until the absorbance at 660 mμ reached 0.7 (about 5×10^8 cells/ml) on the Beckman DB spectrophotometer. Phage stocks were added to give a m.o.i. of 3–5, and aeration was continued until time to harvest. Ice was then added to the cultures to cool them quickly to 0–5°, and the infected cells were harvested in the Sharples continuous-flow centrifuge (10-liter cultures), or by centrifuging for 15 min at 5,000 × *g* (500-ml cultures). Cell extracts were prepared either by grinding with alumina[14] or by sonication for 1–2 min using a Bronwill Biosonik sonicator. The method of preparation of extracts did not appear to affect the observed

enzyme activities. For simplicity, these extracts will henceforth be referred to as uninfected extracts, am^+ extracts, *am* E56 extracts, etc. The protein concentrations in these extracts were measured by the procedure of Lowry *et al.*[15] with trypsin as a standard.

Enzyme assay: The assays for dCTPase and dUTPase were a modification of the procedure described by Wiberg *et al.*[13] Unless indicated otherwise, incubation mixtures containing 25 μmoles $MgCl_2$, 40 μmoles Tris-Cl buffer, pH 8.5, 10 μmoles mercaptoethanol, 1 μmole EDTA, 0.16 μmole each of dUTP and dCTP, and enzyme in a total volume of 1.0 ml, were incubated for 15 min at 30°. The following controls were always included: no enzyme, no substrate, neither enzyme nor substrate. Then 0.1 ml 0.1 *M* EDTA was added and the solutions were placed in a boiling water bath for 1 min. These assay mixtures were then placed on 3.3 × 50-mm columns of Biorad, AG1-x8, 200–400 mesh, previously washed with 2 *M* $NH_4^+HCOO^-$, pH 2.9, to reduce the background UV-absorbing material in the assay eluates. These columns were then washed successively with 2 ml water and the following ammonium formate solutions: 3 ml 0.01 *M*, pH 4.3; 3 ml 0.06 *M*, pH 2.9; 2 ml 0.05 *M*, pH 4.3; 2 ml 0.25 *M*, pH 4.3; and 3 ml 0.25 *M* pH 4.3. The 0.06 *M* fraction was collected and contained all the dCMP eluted from the column; the two 0.25 *M* fractions were both collected and the dUMP occurred primarily in the second fraction, but occasionally some appeared in the first fraction. The absorbance of these fractions was determined at 280 mμ(dCMP) and 260 mμ(dUMP), and the amount of dCMP or dUMP present was determined using the extinction coefficients 13.0×10^3 and 9.9×10^3, respectively.

Enzyme purification: Streptomycin sulfate fractions were prepared by adding slowly with stirring 0.3 vol of a 5% solution of streptomycin sulfate in deionized water to the crude extracts, and then centrifuging for 10 min at 10,000 × *g*. The precipitates were resuspended in 0.2 *M* potassium phosphate buffer, pH 6.9, containing 0.002 *M* mercaptoethanol and then recentrifuged for 10 min at 10,000 × *g*. The precipitate from *am* N82 extracts was readily solubilized by this procedure, whereas the precipitate from uninfected extracts was very slowly solubilized. The phosphate concentration of these extracts was then adjusted to 0.05 *M* by diluting with 0.01 *M* potassium phosphate buffer, pH 6.9, containing 0.002 *M* mercaptoethanol (buffer A). A 1 × 15-cm column of DEAE cellulose was equilibrated with buffer A. The enzyme solution to be fractionated was applied to the column, followed by 25 ml of buffer A. The remaining proteins were then eluted with a linear NaCl gradient. The mixing chamber contained 100 ml of buffer A, whereas the reservoir contained 100 ml of buffer A containing 0.4 *M* NaCl for elution of the streptomycin supernatant fractions, or 100 ml of buffer A containing 0.7 *M* NaCl for elution of the streptomycin precipitate fractions. A 1.7 × 5-cm column of Hypatite C was equilibrated with buffer A. A portion of the combined enzyme fractions from the DEAE column chromatography of the streptomycin precipitate from the *am* N82 extract was applied directly to the column and then eluted with a linear potassium phosphate gradient. The mixing chamber contained 100 ml of buffer A, and the reservoir 100 ml of 0.4 *M* potassium phosphate buffer, pH 6.9, containing 0.002 *M* mercaptoethanol.

Results and Discussion.—The results of dUTPase assays with extracts of *E. coli* infected by various *amber* mutants of bacteriophage T4 are shown in Table 1. The mutants are listed in order of their positions on the genetic map.[16] Mutations in 19 different genes were studied, and it is clear that (1) the *amber* mutant, E56, containing a mutation in gene 56 failed to induce dUTPase, whereas all other mutants studied induced this enzyme, and (2) extended synthesis of dUTPase occurs if phage DNA synthesis is interfered with as has been previously observed with many other early enzymes.[13] Data shown later in Table 4 indicate that two other gene 56 mutants, *am* E51 and *am* E114, also fail to induce dUTPase.

To confirm that *am* E56 is also unable to induce dCTPase as reported by Wiberg,[3] we assayed dCTPase and dUTPase activity in am^+ and *am* E56 extracts as a function of infection time. These results are shown in Figure 1, and indicate that neither dUTPase nor dCTPase is induced by *am* E56 in *E. coli* B whereas T4 am^+ induces both of these enzymes. The dUTPase assays reported in Table 1 and Figure 1 were performed at pH = 8.1. Analysis at this pH accounts for the lower specific

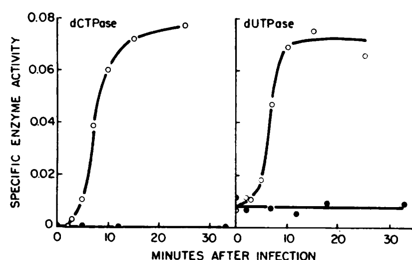


FIG. 1.—Increase in dCTPase and dUTPase activities in *E. coli* B infected with T4 am^+ (○) and T4 *am* E 56 (●). The dCTPase activity was assayed at pH = 8.5 and the dUTPase was assayed at pH 8.1. Only dCTP or dUTP was present in each assay mixture. The specific activity is expressed as μmoles product formed/min/mg protein.

activities observed in these experiments than those obtained in later experiments performed at pH = 8.5. No dCTPase was detected in uninfected cells in these assays. To check the success of the *am* E56 infection, these extracts were also assayed for dCMP deaminase,[17] dihydrofolate reductase,[17] dCMP hydroxymethylase, and thymidylate synthetase, all of which were induced. Hence, it appears that gene 56 is specifically involved in the induction of both dCTPase and dUTPase in these phage-infected cells, but is not involved in the synthesis of at least four other early enzymes. Whether gene 56 is a structural gene or a regulator gene has not yet been unequivocally demonstrated, however.

To investigate further the relationship between the host dUTPase and the phage-induced dUTPase and dCTPase, uninfected and *am* N82 extracts were fractionated with streptomycin sulfate as shown in Table 2. The dUTPase in the uninfected extracts is not precipitated by streptomycin sulfate, whereas streptomycin sulfate precipitates more than 97 per cent of the dCTPase and 93 per cent of the dUTPase in the *am* N82 extract. Zimmerman and Kornberg[7] found that the dCTPase induced by phage T2r+ in *E. coli* B was recovered in the streptomycin sulfate soluble fraction in 76 per cent yield, in contrast to our results with T4 *am* N82. To test whether this discrepancy could be accounted for by species differences, we treated

TABLE 1

dUTPASE ACTIVITY IN EXTRACTS OF *E. coli* B INFECTED WITH VARIOUS AMBER MUTANTS OF BACTERIOPHAGE T4

Gene no.	Mutant phenotype	Extract	Infection time (min)	dUTPase
—	—	Uninfected	—	0.012
—	—	T4 am^+	15	0.062
30	DO[11]	*am* H 39	31	0.144
63	DO	*am* E1072	20	0.112
32	DO	*am* A453	20	0.179
59	DO	*am* HL628	20	0.093
52	DD	*am* H17	19	0.062
60	DD	*am* HL626	20	0.085
39	DD	*am* N116	20	0.115
56	DO	*am* E56	33	0.015
58	DD	*am* HL627	20	0.116
61	DD	*am* E219	20	0.133
41	DO	*am* N81	30	0.137
42	DO	*am* N122	20	0.127
43	DO	*am* B22	20	0.138
62	DO	*am* E1140	32	0.217
44	DO	*am* N82	35	0.236
45	DO	*am* E10	30	0.187
46	DA	*am* N130	30	0.167
47	DA	*am* A456	15	0.095
1	DO	*am* B24	20	0.135

The assay conditions were those described in *Methods and Materials*, except that pH was 8.1 and dUTP was the only substrate included in these assay mixtures. All extracts were dialyzed against 0.01 *M* potassium phosphate buffer, pH 6.9, containing 0.001 *M* β-mercaptoethanol before assay. The dUTPase activity is expressed as μmole dUMP formed/min/mg protein.

TABLE 2

SEPARATION OF HOST AND PHAGE-INDUCED dUTPASE WITH STREPTOMYCIN SULFATE

Enzyme fraction	Total Enzyme Units dCTPase	Total Enzyme Units dUTPase	Specific Activity dCTPase	Specific Activity dUTPase
Uninfected *E. coli* B				
1. Crude extract	0	0.43	0	0.018
2. Streptomycin supernatant	0	0.59	0	0.018
3. Streptomycin precipitate	0	<0.01	0	<0.001
am N82 infected *E. coli* B				
1. Crude extract	8.56	12.4	0.212	0.308
2. Streptomycin supernatant	0.21	0.82	0.006	0.020
3. Streptomycin precipitate	7.78	10.4	0.553	0.733

Extracts (5 ml) of uninfected cells and cells infected with *am* N82 were prepared by grinding the frozen cells with alumina, and were then treated with streptomycin sulfate. The resulting fractions were assayed simultaneously for dCTPase and dUTPase as described in *Methods and Materials*. Enzyme units are expressed as μmoles dUMP or dCMP formed per minute.

T2 and T4 am^+ extracts with streptomycin sulfate under conditions identical to those described in *Methods and Materials*. The results indicated that whereas the T4 enzyme is precipitated by streptomycin sulfate from both T4 am^+ and *am* N82 extracts, the T2 enzyme remains in the supernatant after treatment of the T2 extract with streptomycin sulfate. This suggests that there are significant structural differences between the T2 and T4-induced enzymes. The host dUTPase activity appears to remain in the supernatant fraction after treatment of the *am* N82 extract with streptomycin sulfate, and about a $2^1/_2$-fold purification of the phage-induced enzyme was obtained by this streptomycin precipitation.

The streptomycin supernatant fractions from the uninfected and *am* N82 extracts and the streptomycin precipitate from the *am* N82 extract were each chromatographed on DEAE and the fractions assayed for dUTPase and dCTPase activities simultaneously. The results are shown in Figure 2. No dCTPase activity was detectable in the fractions containing the *E. coli* dUTPase activity (graph *A*). The dUTPase activity in the streptomycin supernatant from the *am* N82 extract (graph *B*) has chromatographic and catalytic properties similar to those of the

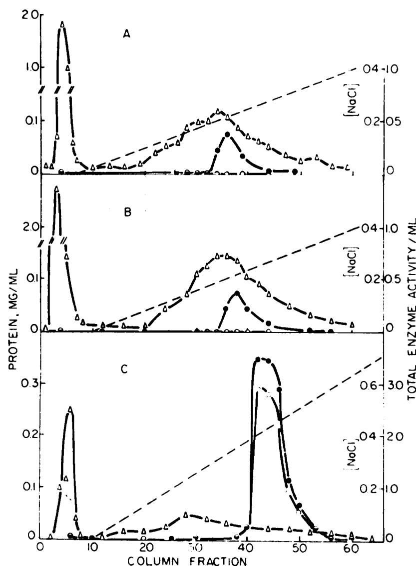


FIG. 2.—Chromatography of crude extracts on DEAE-cellulose. Graph *A*—5 ml (about 25 mg protein) of streptomycin supernatant from uninfected *E. coli* B extract. Graph *B*—5 ml (about 30 mg protein) of the streptomycin supernatant from extract of *E. coli* B infected with T4 *am* N82. Graph *C*—4 ml (about 9 mg protein) of the streptomycin precipitate from the *am* N82 extract were diluted with 12 ml of 0.01 *M* potassium phosphate, pH = 6.9, containing 0.002 *M* β-mercaptoethanol before application to the column. Protein concentrations (Δ), dUTPase (●), dCTPase (○), and NaCl concentration (—) are plotted as a function of the column fraction. Enzyme activities are expressed as μmoles product formed per 15-min assay per ml enzyme solution.

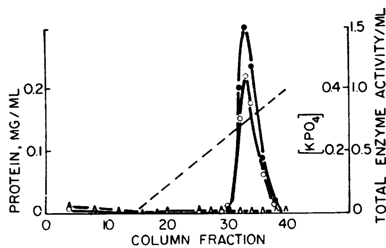


FIG. 3.—Chromatography of enzyme on Hypatite C. About 1.5 mg of protein from the combined active fractions from the DEAE-cellulose column, Fig. 2C, were directly applied and chromatographed on Hypatite C. The data are plotted in a similar fashion to that in Fig. 2, except that a potassium phosphate gradient was used instead of a sodium chloride gradient.

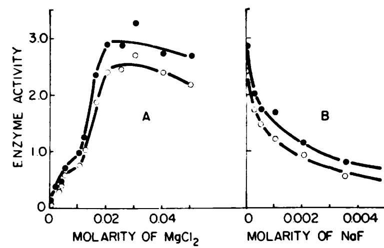


FIG. 4.—Effect of magnesium (graph *A*) and fluoride (graph *B*) ions on enzyme activity. Incubation mixtures contained 0.03 ml of DEAE-cellulose fraction 46, Fig. 2C, in addition to the usual assay components. The $MgCl_2$ concentration in the NaF-containing assays was 0.025 *M*. The data are expressed as the μmoles dUMP (●) or dCMP (○) formed per 15-min assay per ml enzyme solution.

E. coli dUTPase and presumably is the *E. coli* enzyme. In contrast, the dUTPase activity in the streptomycin precipitate from the *am* N82 extract is eluted from DEAE-cellulose at a much higher NaCl concentration than the *E. coli* enzyme and coincides exactly with the dCTPase activity in the column fractions (graph *C*). The ratio of the two activities is constant throughout the peak.

A portion of the enzyme peak shown in Figure 2*C* was rechromatographed on Hypatite C as shown in Figure 3. Once again, dCTPase and dUTPase activities correspond exactly, and the ratio of the two activities is constant throughout the enzyme peak. Protein concentrations in these fractions were too low to measure accurately.

The ratio of dCTPase to dUTPase activity and the over-all per cent recovery and highest specific activity of the dCTPase (assayed in presence of dUTP) in the *am* N82 crude extract, streptomycin precipitate, DEAE-cellulose column fractions, and hydroxylapatite column fractions are, respectively: 0.69, 0.75, 0.84, 0.81; 100, 91, 99, 101 per cent; and 0.21, 0.55, 8.1, > 21.

The results of experiments to test the effect of magnesium ion and fluoride ion concentrations on the phage-induced dCTPase and dUTPase activities are shown in Figure 4. Both enzymes respond identically to both ions. An interesting feature of the magnesium dependence of these two activities is the discontinuity observed at low magnesium concentrations. The dUTPase activity possesses marked sensitivity to fluoride ion, as previously reported for dCTPase.[7]

The similar chromatographic and biochemical properties of the phage-induced dUTPase and dCTPase activities suggest that the same enzyme molecule catalyzes the hydrolysis of dUTP and dCTP to the corresponding deoxynucleoside monophosphates. Bello and Bessman[6] have already shown that a single enzyme probably catalyzes the phosphorylation of dTMP, dGMP, and dHMP. The discovery of polyfunctional phage-induced enzymes in *E. coli* after infection with T-even phages is not surprising. Phage-induced metabolism need not be as versatile as *E. coli* metabolism, and the phage chromosome is much smaller than the *E. coli* chromosome. Hence, the "genetic potential" of a specific amount of phage DNA

TABLE 3

EFFECT OF DEOXYNUCLEOSIDE TRIPHOSPHATE CONCENTRATIONS ON dCTPASE AND dUTPASE ACTIVITIES

Enzyme	mμMoles Substrate			mμMoles Product Formed		
	dCTP	dUTP	dTTP	dCMP	dUMP	dTMP
Experiment 1						
0.03 ml T4 am^+	0	160	...	<1	50	...
Crude extract (dialyzed)	80	160	...	16	37	...
	160	160	...	27	33	...
	160	80	...	31	22	...
	160	0	...	43	<1	...
Experiment 2						
0.30 ml Fraction 38 (Fig. 2*B*—	0	160	...	<1	93	...
Presumed to be *E. coli* enzyme)	80	160	...	<1	100	...
	160	160	...	<2	99	...
Experiment 3						
0.02 ml Fraction 46 (Fig. 2*C*—	0	160	0	<1	97	—
Presumed to be phage-induced	80	160	0	30	75	—
enzyme)	160	160	0	45	58	—
	160	80	0	67	42	—
	160	0	0	66	<1	<1
	160	0	160	74	—	4
	0	0	160	<1	—	7

The assay mixtures containing the indicated amount of each deoxynucleoside triphosphate were incubated for 15 min and assayed as described in *Methods and Materials*.

could be increased by the induction of polyfunctional enzymes, whose presence is necessary for phage replication throughout the latent period.

Although it is difficult to prove that the two activities reside in the same protein, all of our results support this idea. The dUTPase and dCTPase have been purified more than 100-fold with very good recovery, and exhibit identical physical and chemical properties all through the purification. Furthermore, gene 56 mutants of bacteriophage T4 fail to induce either enzyme, an indication that gene 56 is either a regulator gene for both enzymes, or that the gene product of gene 56 is found in both enzymes. No unequivocal proof that gene 56 is a structural gene for either enzyme has yet been obtained, but the genetic evidence combined with the purification data strongly suggest that gene 56 is a structural gene for a single enzyme catalyzing the hydrolysis of dUTP and dCTP.

If indeed the same molecule catalyzes both reactions, it might be expected that the catalytic site is the same for each substrate. If so, each substrate should inhibit the hydrolysis of the other. Data to support this hypothesis are shown in Table 3. The *E. coli* dUTPase activity is not inhibited by dCTP (experiment 2), whereas the phage-induced dUTPase activity is inhibited by dCTP and the dCTPase activity is inhibited by dUTP (experiments 1 and 3). In contrast, dTTP is not an inhibitor of the phage-induced dCTPase, and is a poor substrate, at best. Another experiment with CTP and UTP indicated that neither of these compounds is either a substrate or an inhibitor of the phage-induced enzymes. It was consistently observed that under the assay conditions used, dUTP is a better substrate for the enzyme than was dCTP. Little or no dUMP formation was detected when only dCTP was incubated with either the am^+ extract or the partially purified *am* N82 enzyme, an indication that this preference for dUTP is not an artifact resulting from the phage-induced dCMP deaminase activity. The data shown in Table 3 suggest that the catalytic site may be the same for dUTP and dCTP hydrolysis, but more rigorous studies are necessary to determine whether the inhibition is truly competitive and whether $Km = Ki$. Common catalytic sites have

TABLE 4

RELATIONSHIP OF PHAGE-INDUCED dUDPASE ACTIVITY TO dUTPASE AND dCTPASE ACTIVITIES

Experiment 1

Crude extract	Specific Activity dUDPase	Specific Activity dUTPase
T4 *am*+	0.103	0.184
Uninfected	0.007	0.029
am E51 (gene 56 mutant)	0.006	0.017
am E114 (gene 56 mutant)	0.008	0.021
am N103 (gene 43 mutant)	0.407	0.583

Experiment 2

mμmole Substrate dCTP	dUDP	dUTP	mμmoles Product Formed dCMP	dUMP
160	—	—	77	<2
160	160	—	19	24
—	160	—	<1	40
160	—	160	31	45
—	—	160	<1	87

In expt. 1, undialyzed crude extracts were incubated with assay mixtures containing either dUDP or dUTP, and no dCTP. In expt. 2, 0.03 ml of fraction 46, Fig. 2*C* (presumed to be the phage-induced enzyme), stored more than 2 months at 4°, was incubated with assay mixtures containing the indicated substrates.

previously been implicated for hydrolysis of dCTP and dCDP[7] and phosphorylation of dCMP, dHMP, and dTMP[6] by T-even phage-induced enzymes.

Infection of *E. coli* with T-even phage also results in induction of dCDPase[7] and dUDPase[5] activities. Wiberg[18] found that no increases in dCDPase occurred in *E. coli* B infected with gene 56 mutants of phage T4, so it was of interest to see whether gene 56 also controls dUDPase induction as well as dCTPase, dCDPase, and dUTPase. Results not recorded in Figures 2 and 3 indicated that the phage-induced dUDPase possesses chromatographic properties similar to those of the dUTPase on DEAE-cellulose and Hypatite C. The data in Table 4 indicate that two gene 56 mutants, *am* E51 and *am* E114, induce neither dUDPase nor dUTPase activity, although T4 *am*+, *am* N103, and *am* N82 induce both of these activities. Furthermore, dUDP inhibits the hydrolysis of dCTP by the partially purified enzyme, and dCTP inhibits the hydrolysis of dUDP, suggesting that a common catalytic site may be involved in the hydrolysis of all three substrates tested: dCTP, dUDP, and dUTP. By inference,[7] dCDP may also be hydrolyzed at this site.

Since no net DNA synthesis occurs in *E. coli* B infected with gene 56 *amber* mutants, it appears that the host dCTPase and dUTPase cannot "rescue" the infection, and that either cytosine or uracil or both must be excluded from T4 phage DNA if phage replication is to proceed normally. Wiberg[19] has suggested that the DNA made in *E. coli* B infected with gene 56 *amber* mutants is nuclease-sensitive as a result of its presumed cytosine content. This view must now be modified to include the possibility that uracil may also occur in this phage DNA and affect its nuclease sensitivity. A more accurate evaluation of the lethality of gene 56 mutations will be possible when base analyses on this "mutant DNA" have been performed, and the role and specificity of *E. coli* and phage-induced nucleases have been elucidated.

Summary.—Data have been presented indicating that gene 56 of bacteriophage T4 controls the synthesis of both the phage-induced dCTPase and dUTPase activities. Fractionation of extracts of phage-infected cells with streptomycin sulfate, DEAE-cellulose chromatography, and hydroxylapatite chromatography indicated that these two activities probably reside in the same protein. Mutual inhibition studies indicated that the same site may catalyze both reactions.

Furthermore, the purified enzyme also hydrolyzes dUDP, and this dUDPase activity is not induced in *E. coli* B by gene 56 mutants. The dUDP inhibits dCTP hydrolysis, suggesting that there is a common catalytic site for dUTP, dCTP, and dUDP hydrolysis on the phage-induced enzyme. The results of others [7, 18] would imply that dCDP is also hydrolyzed at this site.

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[11] The abbreviations used in this report are: *am*, *amber* mutants of phage T4 which replicate in *E. coli* CR63 but not in *E. coli* B; DO, *amber* mutants unable to induce DNA synthesis in *E. coli* B; DD, *amber* mutants inducing delayed DNA synthesis in *E. coli* B; DA, *amber* mutants inducing arrested DNA synthesis in *E. coli* B; dHMP, deoxyhydroxymethyl cytidine 5′-monophosphate; DEAE-cellulose, diethylaminoethyl cellulose; and m.o.i. multiplicity of infection, in addition to standard abbreviations.

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