*NEW dUTPASE AND dUDPASE ACTIVITIES AFTER INFECTION OF ESCHERICHIA COLI BY T2 BACTERIOPHAGE**

By G. Robert Greenberg

DEPARTMENT OF BIOLOGICAL CHEMISTRY, UNIVERSITY OF MICHIGAN, ANN ARBOR

Communicated by S. E. Luria, August 3, 1966

After infection of *E. coli* by the T-even bacteriophage, new enzymes are found which do not appear to be present in the uninfected host.[1, 2] Several of these "early" phage-induced[3] enzymes apparently duplicate the function of host cell enzymes which have been regarded as being on the pathway from UDP to dTTP.[4–7] Deoxyuridinetriphosphatase, an enzyme which catalyzes the conversion of dUTP to dUMP and inorganic pyrophosphate in the host organism and which has been considered to be a step in the pathway to the biosynthesis of thymidylate,[8–10] has not been reported to be a phage-induced activity.

After infection by T2 phage, the dUTPase activity rises between five- and tenfold by 15 min, and a new dUTPase activity can be identified. This paper presents evidence that the phage-induced dUTPase activity is distinct from the host enzyme previously described.

In an accompanying paper, Warner and Barnes[11] have provided convincing evidence that a comparable dUTPase activity arising after infection by T4 is, in fact, identical to the phage-induced enzyme, dCTPase.[12–14]

Methods.—dUTPase activity was measured in two ways: spectrophotometrically by coupling to thymidylate synthetase by the method of Wahba and Friedkin,[15] as described previously,[10] or by release of phosphate in the presence of excess pyrophosphatase. The spectrophotometric analysis employed a Beckman DU monochromator with an automatic cuvette changer and a recorder system with an expanded scale (Gilford Instruments Laboratories, Oberlin, Ohio). The assay was run at 25°. In the coupled system, with thymidylate synthetase present at a level of 5 units, the assay was linear to about 2.5 units of dUTPase with either the host or phage enzyme.

A unit of dUTPase activity catalyzes the hydrolysis of 1 mμmole of dUTP per minute. The same unit is used for thymidylate synthetase. Unless stated otherwise, dUTPase units are the values measured at pH 7.4 by coupling to the thymidylate synthetase system.

Thymidylate synthetase was isolated from *E. coli* by a modification of the procedure of Wahba and Friedkin.[16] The enzyme was also purified from a DEAE-cellulose column fraction (Fig. 1, *Uninfected*). It could be freed of dUTPase on a hydroxylapatite column. Seventy-four ml of a host thymidylate synthetase fraction, containing 281 units of activity and 36 mg protein from the DEAE-cellulose column, were dialyzed against 0.01 *M* potassium phosphate buffer, pH 6.8, and adsorbed on a 2 × 10.5-cm hydoxyl apatite column previously washed with the same buffer. Thymidylate synthetase was eluted with 0.06 *M* phosphate, pH 6.8, in about 68% yield and purified about sixfold. dUTPase was eluted at 0.08 *M* phosphate. After concentration by dialysis against silicic acid, the thymidylate synthetase fraction was frozen in small aliquots.

dUTPase was assayed by the release of inorganic orthophosphate from dUTP in the presence of contaminating bacterial pyrophosphatase and added crystalline yeast pyrophosphatase. Enzyme fractions were dialyzed against 0.05 *M* Tris-chloride, pH 7.4, and 0.001 *M* ethylenediaminetetraacetate. The reaction mixture contained 4 μmoles of magnesium acetate, 20–50 μmoles of buffer (imidazole at pH 6.0–6.5, Tris-chloride from pH 7.0 to 8.9 or sodium glycinate buffer, pH 9 and above), 0.1 μmole of dUTP and about 0.5–4 units of dUTPase in a volume of 0.5 ml. In some experiments about 5–10 units of yeast inorganic pyrophosphatase were included to supplement the endogenous pyrophosphatase which might have been limiting. The reaction was run 10 min at 37° and stopped by trichloroacetic acid. Controls using dUTPase fractions heated 10 min in a boiling water bath were always included. In order to determine that pyrophosphatase activity was not limiting, particularly as the pH values were varied, in critical experi-

ments additional reaction mixtures were incubated with all the reactants of the dUTPase assay plus 0.05 μmole of sodium pyrophosphate. This addition always increased the rate of release of inorganic phosphate three- to tenfold, or the added PP_i was completely degraded.

dCTPase[12-14] activity at pH 8.5 and 37° in 0.008 *M* Mg^{++} was measured by separation of dCMP on Dowex-1-formate columns[12] or by release of P_i in the presence of excess inorganic pyrophosphatase. Activity on other nucleoside di- and triphosphates was determined at pH 8.5 and 37° in 0.008 *M* Mg^{++} by P_i release in the presence of excess pyrophosphatase. In each determination appropriate controls were run for P_i and PP_i in the nucleotide preparations and included incubations with boiled enzyme.

Phage-induced dUTPase has been prepared by infection of either *E. coli* R2[17] or a thymine-dependent mutant, *E. coli* B3. For large-scale preparations *E. coli* R2 was grown at 37° in a Biogen using the procedure and medium described previously[5, 10] and infected at a multiplicity of 2 with $T2r^+$ phage which had been irradiated with a mercury lamp until 5×10^{-5} of the plaque-forming particles survived. After 3 hr of infection the culture was harvested, and the paste was stored at −20°. The cell paste (100 gm) was ground with alumina and extracted with 400 ml of 0.05 *M* potassium phosphate buffer, pH 6.5. The mixture was centrifuged for 45 min at 25,000 $\times$ *g*, re-extracted with 200 ml of buffer, recentrifuged, and the combined supernates were fractionated on DEAE-cellulose columns by a modification[19] of the methods described earlier[5, 10] (Fig. 1). The uninfected control consisted of *E. coli* R2 cells grown in continuous culture in the Biogen in the same culture medium as used for the infected cells, collected, extracted, and fractionated as described above and in the legend to Figure 1, using the same DEAE-cellulose column.

In the isolation of phage-induced dUTPase from *E. coli* B3 infected with five irradiated phage per cell, the ground paste from 76 gm of cells was extracted with 5 vol of a solution containing 0.05 *M* Tris buffer, pH 7.4, 0.01 *M* magnesium acetate, and 0.01 *M* 2-mercaptoethanol, centrifuged 20 min at 20,000 $\times$ *g*, and treated with 3 μg of 1$\times$ recrystallized pancreatic DNase/ml. This solution was again centrifuged at 30,000 $\times$ *g* for 20 min, and the supernate was centrifuged 4 hr at 78,000 $\times$ *g*. The supernate was treated dropwise with 0.3 vol of 5% streptomycin sulfate (calculated as the free base) and centrifuged at 20,000 $\times$ *g* for 20 min. The supernate was fractionated by slow addition of 0.49 vol of acetone, decreasing the temperature from 0° to just above the freezing point of the acetone-water mixture during the addition. The precipitate was redissolved in the Tris-mercaptoethanol-Mg^{++} solution overnight and carried through a separation on a DEAE-cellulose column.

When the fractions corresponding to the phage-induced and the host dUTPases were rechromatographed on small columns as in Figures 1 and 2, dUTPases I and II were found at positions corresponding almost exactly to those observed when unfractionated extract was chromatographed and rechromatographed, and they also showed the same enzymatic behavior.

Materials.—dUTP was prepared by deamination of dCTP with nitrous acid as described previously[10] and also was purchased from Sigma Chemical Co. dUDP was obtained by action of a preparation of hydroxymethyl dCMP kinase on dUMP[10] and by deamination of dCDP (Sigma Chemical Co.). dUDP was characterized by paper chromatography, by ultraviolet absorption, by labile and total phosphate, and by conversion to dUMP on acid hydrolysis.[10] Hydroxylapatite[20] was kindly supplied by Dr. W. Robinson. Crystalline yeast inorganic pyrophosphatase was a gift from Dr. M. J. Coon.

Results.—Separation of phage-induced and host dUTPases: Figure 1 shows the elution patterns of dUTPase and thymidylate synthetase activities on chromatography of extracts of infected and uninfected *E. coli* R2 cells on DEAE-cellulose. It is clear that after infection far more dUTPase activity is eluted from the columns, even accounting for the 31 per cent more protein employed in the chromatography of the infected cell extract and even with the very large loss in activity suffered on chromatography. In addition, a new dUTPase fraction appears at a considerably lower concentration of the phosphate eluant.

In order to establish that this fraction from the chromatography of the infected cell extract was distinct from the host dUTPase, fractions were rechromatographed. When the fractions from 2.89 to 3.42 liters (Fig. 1, *Infected*) were combined and

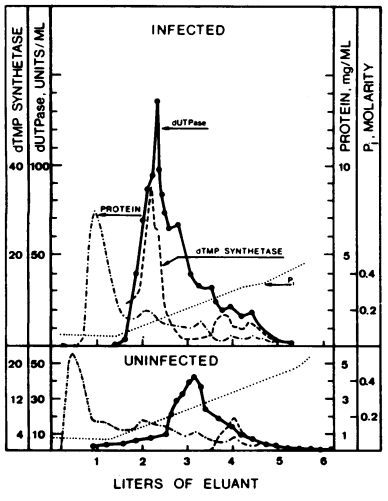

FIG. 1.—Separation on DEAE-cellulose columns of dUTPase activities from *E. coli* before and after infection with T2 bacteriophage. The combined 25,000 × *g* supernates of the extracts of infected cells (*Methods*), containing 8.0 gm of protein and having 738,000 units of dUTPase and 36,000 units of dTMP synthetase, were passed, over a period of 15 hr, through a 4.8 × 25-cm DEAE-cellulose column, prepared and washed as described previously,[5, 10, 19] and then equilibrated with 0.05 *M* potassium phosphate buffer, pH 6.5. After washing with 70 ml of the same buffer, the adsorbed material was eluted by a series of linear gradients of phosphate buffer at pH 6.5. The dotted line is the phosphate analysis of the eluates. Fractions (25-ml) were collected, and all operations were carried out at 0–4°. The recovery of dUTPase was only about 17%.

Proteins (6.1 gm), extracted in the same manner from 76 gm of the paste of uninfected cells and containing 111,000 units of dUTPase activity and 8,000 units of dTMP synthetase, were subjected to chromatography on the same DEAE-cellulose column after regeneration. The recovery of dUTPase was about 42%.

rechromatographed on a smaller DEAE-cellulose column, two major peaks (Fig. 2*A*) were found, one with a maximum at 1.03 liters (*I*) and the other at about 1.62 liters (*II*). Rechromatography of the fractions from 1.72 to 2.43 liters (Fig. 1, *Infected*) produced only one band with a maximum at 1.0 liter of eluant and eluted between 0.09 and about 0.15 *M* phosphate (Fig. 2*B*). Rechromatography of the dUTPase from host cells on the small column (Fig. 2*C*) also gave only one peak, but with a maximum at 1.59 liters between phosphate concentrations of 0.15 *M* and about 0.22 *M*, corresponding closely to the second peak (dUTPase II) from infected cell extracts.

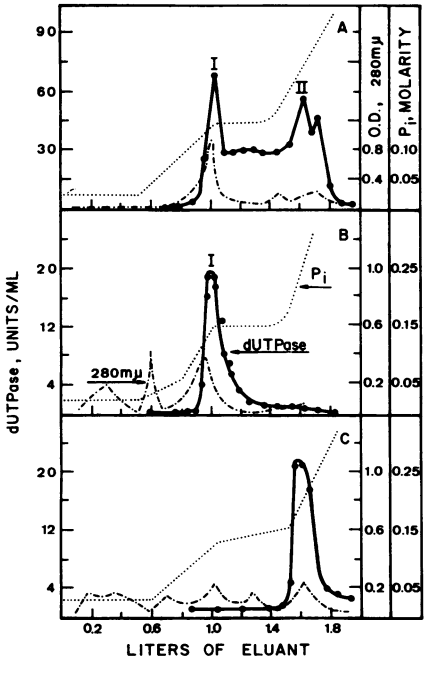

FIG. 2.—Rechromatography of dUTPase fractions on smaller DEAE-cellulose columns. Fractions from the large DEAE-cellulose columns (Fig. 1), concentrated and dialyzed against 0.02 *M* potassium phosphate buffer, pH 6.5, were rechromatographed on 2.8 × 18-cm columns. In (*A*), fractions from 2.89 to 3.42 liters from the column in Fig. 1 (*Infected*) containing 324 mg of protein and 51,000 units of dUTPase were placed on the column. In (*B*), fractions between 1.72 and 2.48 liters from the column in Fig. 1 (*Infected*) and containing 197 mg protein and 5,000 units were chromatographed through the small column. (*C*) shows the rechromatography of the dUTPase from the host (Fig. 1, *Uninfected*) using 281 mg and 3,100 units. The columns were washed with an equal volume of 0.02 *M* phosphate (total volume of sample and wash was about 500 ml). Elution of the protein was effected by the schedule: 500 ml of a linear gradient of buffer, pH 6.5, 0.02–0.15 *M*, 450 ml of 0.15 *M* phosphate solution, 250 ml of a linear gradient of phosphate buffer 0.15–0.35 *M*, and finally 0.35 *M* phosphate solution to the end. The dotted lines are the measured concentrations of phosphate in the eluates and indicate the slight variations in the elution schedules. A considerable part of the activity of the phage-induced dUTPase fractions from the large column was lost during the process of concentration by ultrafiltration prior to rechromatography. Large losses also occurred during rechromatography.

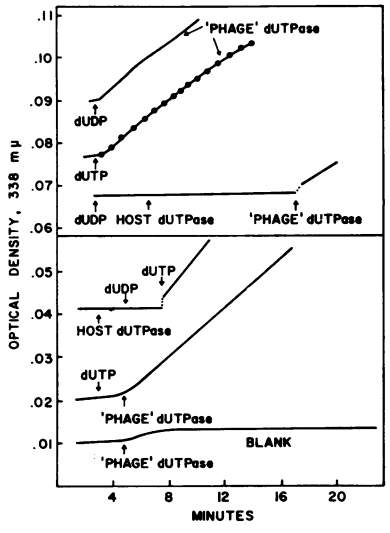

FIG. 3.—Comparison of the activities of phage-induced dUTPase and host dUTPase on dUDP and dUTP. In this figure the optical density values are presented to show relative change. The apparatus was balanced against a blank containing tetrahydrofolate, Mg^{++}, mercaptoethanol, buffer, and formaldehyde. Full-scale readings with the Gilford-Beckman apparatus usually were adjusted to an absorbance of 0.100. At the beginning of each indicated run, the test cuvette contained thymidylate synthetase free of dUTPase and in nonlimiting amounts and all of the components of the assay system. At the indicated times, dUTP or dUDP and host or phage-induced dUTPase were added. In the upper section of the figure in those curves labeled as *'phage' dUTPase* the enzymes were added at the beginning of the runs. The curve with black dots at approximately 1-min intervals is presented to show the range of variation of the individual points. Actual measurements were taken at 15-sec intervals. The results show also that the dUTP and dUDP preparations contained little or no dUMP. Comparable activities of rechromatographed host and phage-induced dUTPases were used in each experiment.

Comparison of the phage-induced dUTPase and the host enzyme: Figure 3 provides the evidence that the phage-induced dUTPase fraction (dUTPase I, Fig. 2*B*) converted both dUTP and dUDP[21] to dUMP. The reaction was assayed spectrophotometrically by coupling to the thymidylate synthetase system. Several sets of experiments are presented in Figure 3. Both the host dUTPase (Fig. 2*C*) and the DEAE-cellulose column fraction from infected cells corresponding in position to the host dUTPase (dUTPase II, Fig. 2*A*) acted only on dUTP and not on dUDP.[21] Actually, even crude 30,000 × *g* supernatant fractions of extracts from uninfected cells showed only slight activity on dUDP. The ratio of dUDP to dUTP activities varied between 0.73 and 0.95 in the various fractions corresponding to the phage-induced dUTPase.

Because of contamination by inorganic pyrophosphatase, the exact nature of the primary phosphate products formed from dUTP has not been established; 2 moles of inorganic phosphate were liberated per mole of dUMP formed. The host enzyme forms PPi and dUMP as the primary products.[8–10]

At 56° the host dUTPase lost about 10 per cent of its activity in 20 min; at about the same protein concentration the phage-induced dUTPase lost 50 per cent in this time and was not stabilized by mixing with host enzyme. Host enzyme and dUTPase fraction II (Fig. 2*A*) showed very similar heat stabilities.

The pH optimum of the phage-induced enzyme was about 9.5 with a shoulder or peak at 8.5 showing an activity varying between 80 and 90 per cent of the maximum. At pH 10.6 the activity was about 30 per cent, and at pH 7.4 the activity was about 35 per cent. The host enzyme showed a maximum at about 9.5 also, approximately 75 per cent of the maximum activity at pH 8.5, 55 per cent at 10.6, and 45 per cent at 7.4.

The K_m for the dUTPase induced by T2 infection was approximately 3.3×10^{-6} moles/liter at pH 7.4. The host enzyme showed a K_m of approximately 9.3×10^{-6} moles/liter.

At a concentration of 10 μmoles of potassium fluoride per ml of reaction mixture, the host dUTPase showed no inhibition when assayed by coupling to thymidylate

synthetase. At the same fluoride concentration, rechromatographed phage-induced dUTPase activity was reduced to 18–34 per cent of the control depending on the enzyme source. With dUDP as the substrate the activity was reduced to a negligible value by 10 μmoles of F^-. Thymidylate synthetase was not affected by this concentration of fluoride.

On the basis of P_i liberation in the presence of inorganic pyrophosphatase the partially purified phage-induced dUTPase (fraction I, Fig. 2*B*) showed no activity on dATP, dTTP, dGTP, UTP, CTP, and UDP. ATP showed a rate which was about 6 per cent of that of dUTP. The activity on dCTP relative to dUTPase activity varied widely from fraction to fraction. dCTP at a ratio of 200 mμmoles to 60 mμmoles of dUTP reduced the rate of phage-induced dUTPase activity to 61 per cent of the control value assayed by coupling to thymidylate synthetase. At a ratio of 200 mμmoles of dCTP to 20 mμmoles dUTP the rate was reduced to 34 per cent.

The increase in dUTPase approached completion 15 min after infection by unirradiated T2 phage. Addition of 50 μg of chloramphenicol per ml of culture just prior to infection prevented the appearance of the new dUTPase activity.

Discussion.—After infection of *E. coli* by T2 bacteriophage, a dUTPase activity is found which is distinguished from that in the uninfected host by the following properties: (1) it is separated from the normal host enzyme by DEAE-cellulose chromatography; (2) it acts on both dUTP and dUDP whereas the host enzyme will not degrade dUDP;[21] (3) it is inhibited by fluoride whereas the host enzyme is not; (4) it is considerably more unstable to heat inactivation than the host enzyme; (5) its K_m for dUTP is about 3.3×10^{-6} *M*, while that of the host enzyme is about 9.3×10^{-6} *M;* and (6) while the two enzymes appear to have about the same pH optima, the shapes of the pH-activity curves are different. While the dUDP to dUTP activity was always found in approximately the same ratio and while fluoride inhibited dUDPase activity, it has not been rigorously established that the two activities are identical.

The finding that a phage-induced protein catalyzes a new dUTPase activity distinct from that of the host suggests some rather interesting considerations. Firstly, taken together with previous observations,[2, 4–7] of the seven enzyme systems considered to be on the pathway to dTTP in *E. coli* (Fig. 4, *a–h*) four new enzymes are formed after infection by T-even phages, two have not been studied to date, and in one case (*h*) a new enzyme is apparently not formed.[22] In addition, a new bypass not found in the host, dUDP → dUMP, has now been described.

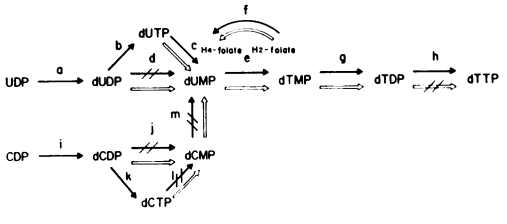

FIG. 4.—Phage-induced enzymes on the postulated pathways leading to dTTP. The broad arrows represent enzymes which are known to be phage-induced by isolation, the thin arrows those of the host. ⇢ indicates either that the enzyme is not found in the host or that only trace activities corresponding to the step have been reported. In the step CDP → dCDP an increase has been reported after phage infection,[23] but the enzyme activities have not been separated or characterized. When a broad or narrow arrow is omitted, the system has not been reported.

Secondly, the recent demonstration by Fleming and Bessman[24] that phage-induced dCMP deaminase[25] is activated by dCTP provides the sys-

tem (*i*) through (*m*) as an additional pathway to dUMP and thence to dTTP. Accordingly, the two systems appear to represent a very efficient mechanism for guaranteeing an adequate supply of dUMP.[26] At the same time the system (*i*) to (*l*) provides dCMP for 5-hydroxymethyl dCMP formation and dCTP degradation. Since dCTP is, in fact, degraded and since dTTP inhibits the deaminase,[24] it might be considered that a controlled balance between the dUTPase and the dCTPase-dCMP deaminase pathways could exist during infection. The finding by Warner and Barnes[11] that dCTPase and dUTPase activities induced by T4 infection are catalyzed by the same enzyme and the fact that conversion of hydroxymethyl dCMP to its diphosphate and of dTMP to dTDP is catalyzed by the same kinase[7] appear to increase the possibility of interplay between these systems.

A number of properties of the dUTPase induced by T2 phage infection fit the hypothesis that it may be dCTPase. These include its activity on dUDP, its inhibition by fluoride and by dCTP, its pH optimum, and its position on a DEAE-cellulose column.[14] In addition, the level of dUTPase activity in crude extracts (about 70 units/mg protein at pH 7.4) is comparable to the levels reported for dCTPase.[12, 13] It should be stated that dUTP previously had not been tested as a substrate for the dCTPase[12, 14] induced by infection with T2 phage. The findings of Warner and Barnes[11] that the two activities are the same after T4 phage infection strongly suggests that the two activities are the same after infection by T2 phage. However, the dCTPase/dUTPase ratios in the phage-induced dUTPase column fractions (Fig. 2*B*) varied widely from fraction to fraction. Therefore, while it is likely that the dCTPase and dUTPase activities after T2 infection are identical, more extensive purification is necessary to clarify this ambiguity.

Finally, mention should be made of the recent report that dUTPase activity increases after infection of *B. subtilis* by phage SP8.[28]

Summary.—After infection of *Escherichia coli* by bacteriophage T2, the dUTPase activity increases by 5–10 times in 15 min. This activity has been shown to be separable from the dUTPase of the host. It differs from the enzyme of the host primarily in its sensitivity to fluoride, its stability, and its chromatographic behavior. In addition, unlike host dUTPase which acts only on dUTP, the phage-induced fraction acts both on dUTP and dUDP. The possible role of the new dUTPase activity and its relationship to phage-induced dCMP deaminase in the biosynthesis of thymidylic acid have been discussed.

The author is greatly indebted to Dr. Huber Warner for volunteering, before publication, the findings in his laboratory on the nature of dUTPase in T4-infected cells and for his cooperation in publishing our findings together. He also wishes to thank Mrs. Norine Morrison for her careful and faithful help, and Dr. Nancy Nossal for kindly providing preparations from DEAE-cellulose column fractionations of *E. coli* extracts. M. Max Zollinger, Laboratoire de Biophysique, Université de Genève, kindly prepared the figures.

* Supported in part by grant AM-01669 from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health and by a grant from the National Science Foundation.

The abbreviations employed are those suggested in the *Journal of Biological Chemistry*.

[1] Flaks, J. G., and S. S. Cohen, *Biochim. Biophys. Acta*, **25**, 667 (1957).

[2] Cohen, S. S., *Ann. Rev. Biochem.*, **32**, 83 (1963).

[3] The term "phage-induced" is employed here because of convention. It does not denote classical enzyme induction, the nature of the mechanism, or the origin of the enzyme.

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

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

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

[7] Bello, L. J., and M. J. Bessman, *J. Biol. Chem.*, **238**, 1777 (1963).

[8] Bertani, L. E., A. Häggmark, and P. Reichard, *J. Biol. Chem.*, **236**, PC67 (1961).

[9] *Ibid.*, **238**, 3407 (1963).

[10] Greenberg, G. R., and R. L. Somerville, these PROCEEDINGS, **48**, 247 (1962).

[11] Warner, H. R., and J. E. Barnes, these PROCEEDINGS, **56**, 1233 (1966).

[12] Koerner, J. F., M. S. Smith, and J. M. Buchanan, *J. Am. Chem. Soc.*, **81**, 2594 (1959), and *J. Biol. Chem.*, **235**, 2691 (1960).

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

[14] Zimmerman, S. B., and A. Kornberg, *J. Biol. Chem.*, **236**, 1480 (1960).

[15] Wahba, A. J. and M. Friedkin, *J. Biol. Chem.*, **236**, PC11 (1961).

[16] *Ibid.*, **237**, 3794 (1962).

[17] A variant of *E. coli* B originally selected for its relative resistance to premature lysis by T2 bacteriophage.[18]

[18] Hershey, A. D., J. Dixon, and M. Chase, *J. Gen. Physiol.*, **36**, 777 (1953).

[19] Nossal, N., doctoral dissertation, University of Michigan, June, 1963.

[20] Levin, O., in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1962), vol. 5, p. 27.

[21] Before the discovery of the phage-induced dUTPase, we erroneously reported that the normal *E. coli* dUTPase attacked dUDP.[10] This was based on the present author's inadvertent use for the assay of dUDP (ref. 10, Table 2) of the dUTPase fraction obtained from a DEAE-cellulose column separation of the extract of T2-infected cells.

[22] Bello, L. J., and M. J. Bessman, *Biochim. Biophys. Acta*, **72**, 647 (1963).

[23] Barner, H. D., and S. S. Cohen, *J. Biol. Chem.*, **237**, PC1376 (1962).

[24] Fleming, W. H., and M. J. Bessman, *J. Biol. Chem.*, **240**, PC4108 (1965).

[25] Keck, K., H. R. Mahler, and D. Fraser, *Arch. Biochem. Biophys.*, **86**, 85 (1960).

[26] Hall and Tessman[27] have reported that mutants of T4 phage unable to induce the formation of dCMP deaminase yield burst sizes of about one half those of the wild-type phage. Their finding provides evidence for a pathway to dUMP other than dCMP deaminase. Both the host dUTPase and the phage-induced dUTPase could give rise to dUMP.

[27] Hall, D. H., and I. Tessman, *Federation Proc.*, **25**, 776 (1966).

[28] Kahan, F., E. Kahan, and B. Riddle, *Federation Proc.*, **23**, 318 (1964).