Early Biochemical Events Occurring After Infection of *Bacillus cereus* 569-SP1 with Bacteriophage GSW

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After infection of *Bacillus cereus* 569-SP1 with the 5-hydroxymethyluracil-containing phage GSW, new dTTPase, dUTPase, and dUMP-hydroxymethylase activities appear. No significant changes in activities of other pyrimidine ribonucleoside or 2'-deoxyribonucleoside triphosphate nucleotidohydrolases were detected. dUTP and dUMP inhibit the dTTPase activity, whereas dTTP failed to inhibit dUTPase activity. The K_m value for the substrate dUTP is 10^{-4} M and for dTTP is 4.85×10^{-4} M. Thymidylate synthetase activity is inhibited only when cells are infected during the late lag or very early log phases of growth; when cells are infected with phage during mid-log, thymidylate synthetase activity is unaffected. The data support the suggestion that, although phage GSW may inhibit an otherwise expected increase in activity of thymidylate synthetase, it fails to affect the already existing activity. The data presented do not allow discrimination as to whether the phage specifies inhibition of de novo synthesis of thymidylate synthetase or the increase in activity of already existing but not fully expressed enzyme.

Upon infection of *Bacillus subtilis* with hydroxymethyluracil (HMU)-containing phages, at least three phage-specified proteins are known to take part in the exclusion of the thymine from the HMU-containing phage genome: the enzyme dTTPase (10, 11; H. Ben-Horin and A. K. Saz, Abstr. Annu. Meet. Amer. Soc. Microbiol., 73rd, p. 235, 1973; F. Kahan, E. Kahan, and B. Riddle, Fed. Proc. 23:318, 1964), catalyzing the hydrolytic conversion of dTTP to dTMP and PP_i , the enzyme thymidylate 5'-nucleotidase (9; Ben-Horin and Saz, Abstr. Annu. Meet. Amer. Soc. Microbiol.), catalyzing the conversion of dTMP to deoxythymidine and orthophosphate, and a protein that acts as an inhibitor of the enzyme thymidylate synthetase (5).

A common property of all HMU-containing phages so far studied is their ability, upon infection, to specify dUMP-hydroxymethylase, an enzyme that catalyzes the formation of 5-hydroxymethyl deoxyuridine monophosphate ($[5\text{-CH}_2\text{OH}]$ dUMP) from dUMP (13; Kahan et al., Fed. Proc.). dUMP ($5\text{-CH}_2\text{OH}$) is a precursor for dUTP ($5\text{-CH}_2\text{OH}$), which is utilized, probably by the phage-specific DNA polymerase, for phage DNA synthesis.

In this communication, we report studies on the course of dTTPase, dUTPase, dUMP-hydroxymethylase, and thymidylate synthetase activities after infection of *B. cereus* 569-SP1 with the HMU-containing phage GSW (M. A. Walz, Ph.D. thesis, Georgetown Univ., Washington, D.C., 1973; M. A. Walz and A. K. Saz, Bacteriol. Proc., p. 210, 1971). A preliminary report of this study was presented elsewhere (Ben-Horin and Saz, Abstr. Annu. Meet. Amer. Soc. Microbiol.).

MATERIALS AND METHODS

Bacteriophage and bacterial strain. *B. cereus* 569-SP1 (kindly supplied by J. Duerksen, Univ. of Kansas) was used in all experiments. The phage GSW was originally isolated by Garon (Ph.D. thesis, Georgetown Univ., Washington, D.C., 1969) and further characterized as an HMU-containing phage by Walz and Saz (Walz, Ph.D. thesis; Walz and Saz, Bacteriol. Proc.).

Infection and preparation of bacterial extracts. *B. cereus* 569-SP1 was grown overnight in PA media (16) at 37°C with aeration by shaking. From the overnight culture, 40 ml was inoculated into 1 liter of fresh PA media, and the cells were allowed to grow, under the same conditions until the optical density at 525 nm (OD_{525}) approached 0.3 to 0.4, a point at which there are 1.5×10^7 to 2.5×10^7 viable colony-forming units/ml. Infection was initiated by adding the phage GSW to the bacterial culture at a multiplicity of 10 to 15, and the cultures were incu-

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bated as above. Approximately 95% of the cells lost their viability and became infective centers within 15 to 20 min. Complete lysis of cells occurred 90 to 100 min after infection.

The infectious process and the growth of noninfected cells were stopped by chilling the cells in dry ice and acetone. All subsequent manipulations were performed at 0 to 4 C. Cells were removed by centrifugation for 10 min at $16,000 \times g$, resuspended in 20 ml of buffer A, and disrupted in the Branson Sonifier (20 kc) for 5 min at 0 C. The debris was removed by centrifugation for 30 min at $35,000 \times g$, and the supernatant fluid, if not further purified, was dialyzed overnight against 1,000 volumes of buffer A. This preparation is termed "crude extract."

Preparation of partially purified enzymes. Partially purified preparations were prepared by stepwise fractionation with solid ammonium sulfate (3). To isolate dUMP-hydroxymethylase activity, the enzyme was precipitated in 35 to 40% saturated ammonium sulfate after 0 to 35% saturation with the salt. The precipitate obtained after 35% saturation was removed by centrifugation for 30 min at $35,000 \times g$. The precipitate obtained at 35 to 40% saturation was resuspended in 1 to 2 ml of Tris buffer B and dialyzed overnight against 1,000 volumes of the same buffer. To isolate thymidylate synthetase activity, the enzyme was precipitated in 55 to 90% saturated ammonium sulfate, after 0 to 55% saturation with the salt. The precipitate obtained in 0 to 55% saturation was removed as described above for purification of dUMP-hydroxymethylase. The precipitate obtained at 55 to 90% saturation was resuspended in 3 to 4 ml of Tris buffer B and dialyzed overnight against 1,000 volumes of the same buffer.

Determination of enzyme activities. (i) dTTPase activity was determined with some modifications by the method of Roscoe (11).

The reaction mixture contained, in a final volume of 0.4 ml, 2.5 μCi of [*methyl*- ^3H]dTTP in 0.9 μmol dTTP, 4 μmol of MgCl_2 , 5 μmol of β -mercaptoethanol, 24 μmol of Tris buffer (pH 8.9), and extract equivalent to 0.03 to 0.09 mg of protein. The reaction was run at 37 C for 10 min and stopped by the addition of 0.04 ml of 60% trichloroacetic acid. The resultant precipitate was removed by centrifugation for 10 min at $3,000 \times g$. Control incubations in which buffer A replaced the extract were treated similarly and run routinely. Reaction products were separated and identified by thin-layer chromatography (TLC) on Eastman Kodak nonfluorescent cellulose sheets or Whatman no. 1 paper chromatography by using the ascending technique with deoxythymidine, dTMP, dTDP, and dTTP as markers. The chromatograms were developed in a solvent system consisting of 66 parts by volume of isobutyric acid, 33 parts water, and 1 part ammonia (28 to 30%). By using the TLC technique, it was possible to separate deoxythymidine, dTMP, and dTDP and dTTP. The R_f values were 0.71, 0.52, and 0.35, respectively. By means of paper chromatography, it was also possible to separate dTDP from dTTP; the R_f values for deoxythymidine, dTMP, dTDP, and dTTP, when separated on paper chromatography, were 0.71, 0.41, 0.27, and 0.17, respectively. The separated spots were

viewed in UV light, cut out, immersed in scintillation fluid [toluene-2,5-diphenyloxazole [PPO]-1,4-bis-(5-phenyloxazolyl)-benzene [POPOP]], and counted in an ambient temperature Beckman liquid scintillation counter.

(ii) The dUTPase activity was determined as described above for dTTPase activity, with the following changes: 2.5 μCi of [*5*- ^3H]dUTP in 0.9 μmol of dUTP replaced [*methyl*- ^3H]dTTP in the reaction mixture. Only paper chromatography was used for separation of the reaction products. The R_f values for deoxyuridine, dUMP, dUDP, and dUTP were 0.57, 0.31, 0.20, and 0.10, respectively. In the control incubations, less than 2% of the total [^3H]dTTP and 1.5% of the total [^3H]dUTP counts were found in the corresponding nucleoside and nucleoside monophosphate spots. These amounts were subtracted from all values presented below.

(iii) dUMP-hydroxymethylase activity was determined by the method of Haslam et al. (5), with some modifications. The reaction mixture contained, in a final volume of 0.3 ml, 10 μmol of β -mercaptoethanol, 0.1 μmol of tetrahydrofolic acid (THF), 20 μmol of NaF, 0.5 μmol of dUMP, 25 μmol of Tris buffer (pH 7.4), 1 μCi of H^{14}CHO per 0.1665 μmol HCHO, and partially purified enzyme containing 0.15 to 0.45 mg of protein. The reaction was run at 37 C for 20 min and stopped by adding 0.03 ml of 60% trichloroacetic acid. The precipitate was removed by centrifugation for 10 min at $3,000 \times g$. The product of the reaction, dUMP (5- CH_2OH), was separated on paper chromatography by the method of Haslam et al. (5) and observed with a UV lamp, and the spot was cut out, immersed in scintillation fluid (Triton X-toluene-PPO-POPOP), and counted as above.

Control incubations from which dUMP was omitted were carried out for each sample assayed. In the control incubations, less than 1.5% of the total H^{14}CHO counts was found in dUMP (5- CH_2OH) spots. These small amounts were subtracted from all values reported.

(iv) Thymidylate synthetase activity was determined by following the oxidation of THF to dihydrofolic acid (DHF) during the conversion of dUMP to dTMP. The spectrophotometric assay method of Wahba and Friedkin (17), which was modified according to Dunlap et al. (1), was used here with some further modifications: two cuvettes each contained 100 μmol of β -mercaptoethanol, 0.2 μmol of THF, 7 μmol of HCHO, 5 μmol of NaHCO_3 , 25 μmol of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 ml of 0.5 M potassium phosphate buffer (pH 6.8), partially purified enzyme (equivalent to 0.5 to 1 mg of protein), and water, to make the total volume 1.05 ml. To start the reaction, 0.05 ml of 10 mM dUMP was added to one cuvette and 0.05 ml of water was added to the other (used as blank). The increase in absorbance at 340 nm was followed spectrophotometrically for 15 min at room temperature. The net absorbance change was used to calculate the enzymatic activity. According to Wahba and Friedkin (17), a change of +6.4 OD_{340} occurs when 1 μmol of THF is oxidized to DHF.

Proteins were determined by the method of Lowry et al. (8) by using crystalline bovine serum albumin as a standard.

Buffers. Tris buffer A contained 10^{-3} M EDTA, 10^{-2} β mercaptoethanol, and 5×10^{-2} M Tris, adjusted to pH 8.9 by HCl. Tris buffer B was the same as buffer A, but contained 5×10^{-2} M KCl with the pH of 7.4.

Chemicals. Unlabeled nucleosides and nucleoside phosphates were purchased from Sigma Chemical Co., and $[5\text{-}^3\text{H}]\text{dUTP}$ was purchased from Amer-sham/Searle. All other radioactive materials were purchased from Schwarz/Mann.

RESULTS

(i) dTTPase and dUTPase activities. The activity of dTTPase is measured by determination of the tritiated dTMP formed from tritiated dTTP (Fig. 1). In the course of the reaction about 10% of the formed dTMP is further hydrolyzed by dTMPase into deoxythymidine. Although this relatively small loss of dTMP might for most purposes be ignored, we prefer to express the dTTPase activity as dTMP plus deoxythymidine produced in the reaction. Similarly, dUTPase activity is expressed as tritiated dUMP plus tritiated deoxyuridine produced from tritiated dUTP, since in the course of the reaction about 7% of the formed dUMP is further hydrolyzed to deoxyuridine (Fig. 2). Whereas the conversion of dTTP into dTMP is linear for at least 10 min (Fig. 1), there is no ready explanation for the observation that the conversion of dUTP into dUMP is not a strictly linear process (Fig. 2). Both dTTPase and dUTPase activities are linear when 0.03 to 0.095 mg of protein is present in the assay reaction mixture (Fig. 3). The conversion of dTTP into dTMP and dUTP into dUMP proceeded until

FIG. 1. Conversion of dTTP into dTMP by dTTPase in extracts of infected cells as a function of time. Extract capable of hydrolyzing $0.43 \mu\text{mol}$ of dTTP per mg of protein per min was used in the assay.

FIG. 2. Conversion of dUTP into dUMP by dUTPase in extract of infected cells as a function of time. Extract capable of hydrolyzing $0.38 \mu\text{mol}$ of dUTP per mg of protein per min was used in the assay.

FIG. 3. Conversion of dTTP into dTMP and of dUTP into dUMP in infected cells in the presence of different amounts of extract from infected cells. Extract capable of hydrolyzing $0.68 \mu\text{mol}$ of dTTP and $0.65 \mu\text{mol}$ of dUTP per mg of protein per min was used in the assay. About 6% of dTMP and 4% of dUMP were further hydrolyzed to deoxythymidine and deoxyuridine, respectively, in the course of the reaction.

at least 75% of dTTP and 90% of dUTP were hydrolyzed.

Upon infection of *B. cereus* 569-SP1 cells with phage GSW, the activities of dTTPase and dUTPase increased approximately 34- and 20-fold, respectively (Fig. 4). Activity increases for a period of 45 min after infection. No increase in

FIG. 4. Appearance of new dTTPase and dUTPase activities after infection of *B. cereus* 569-SP1 with the phage GSW. Infectious process and growth of noninfected cells were stopped at different times. Extracts were prepared and determined for dTTPase and dUTPase activities, and cells were infected as described. Infected cells are represented by solid lines and noninfected cells are represented by broken lines.

activity is seen in noninfected cells. Infected cells show specific increase only in dTTPase and dUTPase activities, whereas other pyrimidine nucleoside triphosphate nucleotidohydrolases show no change in activity (Table 1) except for a barely significant rise in dCTPase activity. In contrast to the dCTPase specified by T4 phage which can also hydrolyze dUTP, dCDP, and dUDP (18, 19), we could not find any significant differences in dTDPase activity between infected and noninfected cells. This is in agreement with previous work (11) showing that the dTTPase specified by the phage ϕ does not utilize dTDP as a substrate.

Zimmerman and Kornberg (19) have shown inhibitory effects on T2-specified dCTPase by dTTP (50%), UTP (40%), and CTP (30%). In their report, dCMP, the product of dCTPase and dCDPase activities, behaved as a competitive inhibitor for the hydrolysis of both dCTP and dCDP by the dCTPase.

We studied the effect of some nucleosides and nucleoside phosphates on the dTTPase and dUTPase activity in infected cells. The results are summarized in Table 2.

UMP, UTP, dCTP, and dCMP are shown to have a slight inhibitory effect (in the range of 15 to 25%) on dUTPase activity, and dCTP and dTMP exhibit similar low percent of inhibition (15 to 22%) on the dTTPase activity. At present, it is impossible to decide, based upon these

slight effects, whether the inhibition is competitive or allosteric.

dUTP, when added to the reaction mixture in equimolar concentration to dTTP, causes as

TABLE 1. Activity of various pyrimidine nucleotidohydrolases before and after infection of *B. cereus* 569-SP1 with the phage GSW^a

Substrate	Nucleotidohydrolase activity (μ mol of nucleoside monophosphate formed/mg of protein/min ^b)	
	Noninfected cells	Infected cells
dTTP	0.02	0.65
dUTP	0.032	0.68
dCTP	0.014	0.023
CTP	0.017	0.022
UTP	0.033	0.038
dTDP	0.042	0.051

^a Infectious process and growth of noninfected cells were stopped 45 min after infection, and extracts were prepared as described. The assay conditions are the same as described for dTTPase and dUTPase, except that the assay for determination of dCTPase, CTPase, UTPase, and dTDPase activities contained 2.5 μ Ci of [3 H]dCTP in 0.9 μ mol of dCTP, 2.5 μ Ci of [3 H]UTP in 0.9 μ mol of UTP, and 2.5 μ Ci of [3 H]dTDP in 0.9 μ mol of dTDP. The reaction products were separated and identified by cellulose TLC or paper chromatography as described.

^b About 10% of the nucleoside monophosphates produced in the reaction were further hydrolyzed to the corresponding nucleosides. These nucleosides were considered as part of the formed nucleoside monophosphates.

TABLE 2. Effect of some nucleoside and nucleoside phosphates on dTTPase and dUTPase activity in extracts derived from *B. cereus* 569-SP1 infected with the phage GSW

Inhibitor added (1 μ M)	Relative dUTPase activity (%) ^a	Relative dTTPase activity (%) ^b
None	100	100
UTP	80	93
CTP	101	94
dCTP	74	86
dUTP		2.7
dTTP	97	
UMP	84.5	89.5
CMP	90	101
dUMP	90	41.5
dCMP	86	99
dTMP	90	78.5
Deoxyuridine	100	78.5

^a 2.5 μ Ci of [3 H]dUTP per 0.9 μ mol was used as substrate in the assay.

^b 2.5 μ Ci of [3 H]dTMP per 0.9 μ mol was used as substrate in the assay.

much as 90% inhibition of the dTTPase activity (Fig. 5; Table 2). dUMP and deoxyuridine also inhibit the dTTPase activity 60 and 22%, respectively (Table 2). Similarly, by using highly concentrated enzyme, dUTP and dUMP exert strong inhibitory effects on dTTPase in noninfected cells. The effects of nucleoside diphosphates on the dTTPase and dUTPase activity could not be studied in our crude preparation because of the existence of ATP-independent phosphoryl transferase reactions according to the following scheme: (i) $[^3\text{H}]\text{dTTP} + \text{XDR} \rightarrow [^3\text{H}]\text{dTDP} + \text{XTP}$; (ii) $[^3\text{H}]\text{dUTP} + \text{XDP} \rightarrow [^3\text{H}]\text{dUDP} + \text{XTP}$, where X indicates pyrimidine ribonucleosides or pyrimidine 2'-deoxyribonucleosides. Phosphoryl transferase reactions do not occur between nucleoside triphosphates and nucleoside monophosphates or between nucleoside diphosphates and nucleoside monophosphates. Nucleoside diphosphokinase, which might be responsible for such phosphoryl transfer reactions, was isolated by Sedmak and Ramaley from *B. subtilis* (14).

The K_m for the substrates dUTP and dTTP were determined according to Lineweaver and Burk (7) to be 10^{-4} M and 4.85×10^{-4} M, respectively (Fig. 6).

In contrast to the very high sensitivity to fluoride ion of dCTPase specified by the phages T2 (4, 19) and T4 (18), we could not detect any significant effect on dTTPase and dUTPase activities when NaF was added to reaction mixtures in concentrations up to 30 mM.

FIG. 5. Inhibition of dUTP of dTTPase activity in extract from infected cells. The reaction mixture contained 2.5 μCi of [methyl- ^3H]dTTP per 0.9 μmol and extract capable of hydrolyzing 0.38 μmol of dTTP per mg of protein per min.

FIG. 6. Lineweaver-Burk plot of the rate of dTTPase and dUTPase activities as a function of substrate concentrations. Protein (0.01 mg) of extract from infected cells capable of hydrolyzing 0.43 μmol of dTTP and 0.4 μmol of dUTP per mg of protein per min was used in the assay. V is expressed in μM dUMP (x) or dTTP (•) formed per min.

(ii) **dUMP-hydroxymethylase activity.** dUMP-hydroxymethylase is precipitated in 35 to 40% saturated ammonium sulfate, and the precipitated fraction contains about 3 to 5% of the total proteins in the extract. For reasons that are not clear, we were not able to measure activity of the enzyme in the crude extract. On the basis of protein content of the crude and purified extracts, we estimate that, by this one-step purification procedure, the enzyme is purified approximately 20- to 30-fold.

In the assay for dUMP-hydroxymethylase activity, the incorporation of H^{14}CHO into dUMP, to form dUMP ($5\text{-}^{14}\text{CH}_2\text{OH}$), was determined. Such incorporation is linear for about 20 min when 0.15 to 0.45 mg of protein is present in the reaction mixture.

The dUMP-hydroxymethylase appears within 20 min after infection of *B. cereus* 569-SP1 by the phage GSW (Fig. 7). The enzymatic activity increases for about 40 min

after infection and then declines. No significant activity of this enzyme could be detected in noninfected cells.

(iii) Thymidylate synthetase activity. Thymidylate synthetase is precipitated in 55 to 90% saturated ammonium sulfate, and the precipitated fraction contains about 16 to 21% of the total protein in the extract. As in the case of the hydroxymethylase, we were not able to measure the activity of the enzyme in the crude extract. As above, we estimate that by this one-step purification procedure, the enzyme is purified approximately five- to sixfold.

Extracts from cells of *B. cereus* 569-SP1 show an increase in activity of thymidylate synthetase during the late lag and early exponential phases of growth (Fig. 8). The activity starts to decrease at the middle of the exponential phase when there are 4×10^7 to 5×10^7 colony forming units/ml (cells entering the stationary phase have 10^8 to 2×10^8 colony forming units/ml). Extracts obtained from *B. cereus* 569-SP1 cells in the stationary phase for about 10 h contain thymidylate synthetase activity in amounts capable of oxidizing 0.6 nmol of THF per mg of protein per min. Thus, extracts of *B. cereus* 569-SP1, when prepared from cells at the middle of the exponential growth phase, contain threefold higher thymidylate synthetase activity compared to extracts prepared from cells in the stationary phase.

FIG. 7. Appearance of new dUMP-hydroxymethylase activity after infection of *B. cereus* 569-SP1 with the phage GSW. Infection and growth of noninfected cells were stopped at different times, and partially purified enzyme preparations were prepared as described. Infected cells (x); noninfected cells (o).

FIG. 8. Thymidylate synthetase activity in extracts of *B. cereus* SP1 in various stages of cell cycle. Growth of cells was stopped at different times, and partially purified enzyme preparations were prepared as described.

The increase in thymidylate synthetase activity is markedly inhibited when cells are infected during the late lag or the very early exponential growth periods (Fig. 9). However, in distinction to the results obtained in infection during late lag or very early log phases, when cells are infected during the mid-exponential growth phase, no change in the thymidylate synthetase activity is seen, compared to noninfected cells (Fig. 10). This has been shown in two different ways. (i) From an overnight culture, cells were inoculated into fresh PA medium and allowed to grow until the OD_{525} approached 0.4 to 0.5, when there are about 2×10^7 to 3×10^7 colony forming units/ml. At that time, phage was added to part of the culture to initiate infection, and both infected and noninfected cells continued to grow under the same conditions. When the thymidylate synthetase activity of such infected cells was compared to that of noninfected cells, no difference could be observed (Fig. 10A). The decrease in thymidylate synthetase activity in these cells is expected, as cells grown under such conditions maintain the peak level of thymidylate synthetase activity for a very short time. A marked decrease in the enzyme activity starts shortly after the cells are in mid-log phase (Fig. 10A). (ii) By using overnight cultures as inoculum, cells were inoculated into fresh PA medium and allowed to grow until the OD_{525} approached 0.5 to 0.6, when there are about 3×10^7 to 4×10^7 viable colony forming units/ml, i.e., at the middle of the exponential phase. To prevent decrease in thymidylate synthetase activity, the culture was maintained in the exponential growth phase by diluting the cells 25-fold with fresh PA media. Cells continued to grow until the OD_{525}

FIG. 9. Thymidylate synthetase activity in *B. cereus* 569-SP1 infected with the phage GSW during the late lag or the very early exponential growth. Cells were grown in PA medium until OD_{525} approached 0.220 (when there are about 10^7 to 1.5×10^7 viable colony forming units/ml), and phage was added to initiate infection, as described. Infectious process and growth of noninfected cells were stopped at different times, and partially purified enzyme preparations were prepared as described. Symbols: (○) noninfected cells; (×) infected cells.

approached 0.25. At this point, one-half of the culture was infected with phage, the other one-half remained as a control, and both infected and noninfected cells were incubated under the same conditions. Here again, no differences in thymidylate synthetase activity between infected and noninfected cells were observed (Fig. 10B). The decrease in thymidylate synthetase activity was prevented by this procedure. Under the conditions of infection as described in Materials and Methods, 90 to 95% of all cells exposed gave rise to infectious centers whether cells were infected in late lag to early log or mid- to late log.

DISCUSSION

Like other HMU-containing phages, the phage GSW specified dTTPase and dUTPase activities upon infection of *B. cereus* 569-SP1. However, the fact that, of all nucleoside phos-

phates studied (Table 2), only dUTP and dUMP show strong inhibition of dTTPase activity in our system (but not in other phage-*Bacillus* interrelationships), whereas dTTP and dTMP have only marginal effects on dUTPase activity, may indicate that the system described herein is unique. Two lines of explanations might be used to interpret this phenomenon.

(i) Assuming that the phage GSW specifies for one bifunctional enzyme that can hydrolyze both dTTP and dUTP (as is probably the case in ϕ e-specified dTTPase) (11), we might explain such inhibition on the basis of differences in the K_m values towards the substrates dUTP and dTTP. It is recognized that K_m values obtained in cell-free systems may not be appli-

FIG. 10. Thymidylate synthetase activity in *B. cereus* 569-SP1 cells infected with the phage GSW. Infection was initiated when cells contained high or maximal activity of the enzyme. The experimental conditions for (A) and (B) are described in the text. (A) Infected cells (×); noninfected cells (○). (B) Infected cells (●); noninfected cells (×).

cable to the in vivo situation, particularly when relatively small differences in values are being discussed. The affinity of the enzyme towards dUTP is fivefold higher than towards dTTP (Fig. 6), a fact that can explain the inhibition of dTTPase by dUTP in the presence of dTTP (Fig. 5).

On the basis of differences in affinity towards substrate and inhibitor, the inhibition of dTTPase but not of dUTPase by dUMP may be similarly explained. dUMP cannot be considered as a substrate for dUTPase or dTTPase, and it is assumed that it acts as competitive inhibitor (as is the case with T2-specified dCTPase, which is competitively inhibited by dCMP) (19). Although we did not determine the K_i for dUMP, we might expect the following order of affinities towards the active site of the enzyme: $dTTP \leq dUMP < dUTP$. This may explain inhibition of dTTPase activity by dUTP and dUMP and the failure of dTTP or dUMP to inhibit the dUTPase activity.

(ii) The other possibility is that dTTPase and dUTPase are two separate enzymes specified by the phage GSW. In such a case, the enzyme dUTPase will act to remove dUTP which may both inhibit the dTTPase activity and be incorporated into the phage genome. If this is the case, a new and very interesting control mechanism specified by the phage is possible. Here, the phage would specify for a particular enzyme aimed at eliminating inhibitor material that might interfere with other phage-specified enzymes. Further studies, particularly further enzyme purifications, are needed to elucidate which one, if either, of these two proposed possibilities is operative.

Like other HMU-containing phages, the phage GSW, upon infecting *B. cereus* 569-SP1, specifies dUMP-hydroxymethylase. Thus, the synthesis of the phage DNA is dependent on the availability of dUMP. dUMP also serves as a substrate for thymidylate synthetase, catalyzing the production of dTMP. Haslam et al. (5) have shown that, when the phage ϕ e infects *B. subtilis*, it specifies a protein that acts as an inhibitor for thymidylate synthetase. The role thymidylate synthetase inactivation plays in the replication of HMU-containing phage is still not completely clear. Haslam et al. (5) suggested that phage SP8 and possibly also phage ϕ e do not depend on the inhibition of thymidylate synthetase activity to prevent the incorporation of thymine into the phage genome. Roscoe (12) has shown that inhibition of thymidylate synthetase is not responsible for the arrest of host DNA synthesis. An almost general property for all HMU-containing phages infecting *B.*

subtilis is the capacity to specify dCMP deaminase which catalyzes the conversion of dCMP into dUMP. This is considered as probably the major method used by the phage to provide dUMP for its own genome synthesis. However, the phage GSW does not specify dCMP deaminase activity (Ben-Horin and Saz, Abstr. Annu. Meet. Amer. Soc. Microbiol.; and manuscript in preparation) and thus may face a shortage in available levels of dUMP. Under such conditions, one might expect that upon infection the phage would cause a complete inhibition of thymidylate synthetase activity, resulting in the raising of levels of dUMP available as a substrate for dUMP-hydroxymethylase activity. However, the data we present support the suggestion that, although the phage may cause inhibition of the increase in activity of thymidylate synthetase, it fails to affect the already existing activity, and this might be interpreted as an indication of a possible correlation between thymidylate synthetase activity and host DNA synthesis. An increase in activity of thymidylate synthetase is seen during the late lag and early exponential phases of growth when the rate of DNA synthesis in the cells is high. When the number of cells in the culture approached 4×10^7 viable colony forming units/ml (approximately 1 to $\frac{1}{2}$ generations before the culture entered stationary phase), the activity of thymidylate synthetase started to decrease markedly. The data shown in Fig. 8 are unexpected in prokaryotic cells (but not eukaryotic cells) in that we have been unable to find in the literature reports indicating that thymidylate synthetase activity increases during the growth cycle of prokaryotic cells. Thus, it seems as if there is some control and/or regulation relationship between thymidylate synthetase production (or activity) and DNA synthesis. Such a relationship, if it should occur in *Escherichia coli*, might explain the need for T-even phages to specify for thymidylate synthetase when infecting *E. coli* (2, 6). A similar relationship was shown to occur between dCMP deaminase activity and DNA synthesis during synchronous growth of *Chlorella pyrenoidosa* (15). When infection with phage GSW is initiated during the late lag or the very early log phases, the phage either inhibits the synthesis of new thymidylate synthetase or inhibits the increase in activity of already existing enzyme (Fig. 9). The data do not allow us to discriminate between these two possibilities. However, when the phage infects cells containing high or maximal thymidylate synthetase activity, it fails to affect the activity of the enzyme (Fig. 10). It is not clear yet whether there is any difference in

phage replication or yield under conditions in which increase in activity of thymidylate synthetase is inhibited (infection during the late lag or early log phases) as compared to conditions in which the enzyme is not affected by the phage (infection during the middle log phase). Preliminary observations indicate that, in the latter case, lysis of the cells was sometimes delayed for as long as 180 min after infection (compared to the 90 to 100 min in the first case), and the yield of phage progeny was lower. However, the data are still inconsistent, and more studies are needed to clarify this point. Our data ruled out the possibility that the phage GSW specifies a protein that acts as an inhibitor of thymidylate synthetase activity. Such an inhibitor, if it precipitated in the 55 to 90% saturated ammonium sulfate, should be present in the partially purified enzyme preparation and should affect the activity of the enzyme. Figure 10 indicates that this is not the case. If an inhibitor were to precipitate in 0 to 55% saturated ammonium sulfate, it would be removed from the partially purified enzyme preparation and therefore could not account for the inhibition presented in Fig. 9.

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