A dTMPASE FOUND AFTER INFECTION OF BACILLUS SUBTILIS WITH PHAGE SP5C

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Bacillus subtilis phage SP5C is derived from the group of bacteriophages reported by Romig and Brodetsky (1961). A number of these phages (SP5, SP6, SP7, SP8, SP9, and SP13) have been found to be serologically related (Brodetsky and Romig, 1964). The buoyant densities of their DNA's in cesium chloride are similar and their DNA's when denatured yield bimodal bands in CsCl gradients (Marmur and Cordes, 1963). The DNA of one of these phages, SP8, contains hydroxymethyluracil in place of thymine (Kallen, Simon and Marmur, 1962). Another B. subtilis phage, Øe, also has been reported to contain hydroxymethyluracil in it's DNA (Roscoe and Tucker, 1962).

This communication [1] describes the increase, after infection of B. subtilis SB19 with phage SP5C, of an enzyme activity that cleaves deoxythymidylic acid (dTMP) to deoxythymidine (dT) and inorganic phosphate (P_i) and also describes some properties of the partially purified enzyme fraction.

METHODS

Phage SP5C was obtained as a single plaque isolate of SP5 and is distinguished from the latter by its requirement for calcium. A calcium concentration of 0.2%, in broth and on agar plates is necessary in order for it to propagate in the host strain SB19. Hydrolysis

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of purified SP5C DNA by pancreatic DNase and E. coli diesterase (Exonuclease 1 - Lehman, 1960) and separation of the deoxynucleoside monophosphates on a Dowex-1-acetate column yields deoxyhydroxymethyluridylate (dHMUMP) as one of the nucleotides.

The medium used for growing B. subtilis SB19 and for phage propagation is the TY broth described by Romig and Brodetsky (1961) except that the NaCl was omitted and the $MnCl_2$ concentration was increased ten fold. The bacteria were grown at 37° C with vigorous aeration to a cell titer of about 2 x 10^8/ml. Three SP5C phages per cell were then added. At appropriate times, 100 ml of the culture were poured onto crushed ice and the infected cells collected by centrifugation. The cells were resuspended in 2 ml of buffer containing: 0.05 M glycylglycine, pH 7.0 - 0.002 M GSH - 0.002 M EDTA. They were then disrupted for 1 minute with a Mullard Ultrasonic Generator. After centrifugation at 8,000 xg for 30 minutes, the extracts were immediately assayed for enzyme activity.

Infected cells used for enzyme purification were harvested 30 minutes after infection from 20 to 60 liters of culture.

P^{32} -Labeled deoxynucleoside 5' -monophosphates were prepared by the method of Lehman et al. (1958). Protein concentrations were determined, after precipitation with cold 10% trichloracetic acid, by the method of Lowry et al. (1951).

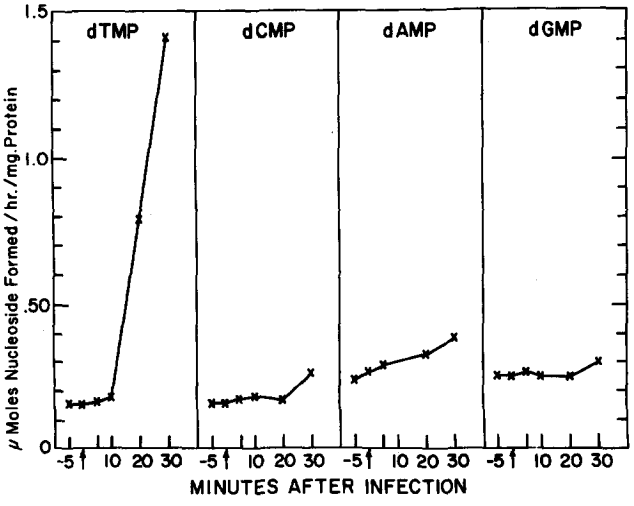
Assay of dTMPase: This assay measures the formation of Norit nonadsorbable P^{32}. The reaction mixture (0.25 ml) consisted of : 10 µmoles glycylglycine buffer, pH 7.0; 2 µmoles $MgCl_2$; 60 mµmoles Na^+K^+EDTA, pH 7.0; 150 mµmoles $dTMP^{32}$ (2.76 x 10^5 cpm/µmole); and an amount of enzyme liberating about 5 to 40 mµmoles P^{32} per 30 minutes. After incubation for 30 minutes at 37° C, the reaction was chilled and 0.4 ml of 0.1 N HCl, 0.2 ml of a norit adsorbing solution (containing 0.50 mg bovine plasma albumin per ml and 25 µmoles potassium phosphate per ml), and 0.15 ml of acid washed

Norit (20% packed volume) were added. After standing at 0° C for 2 minutes with intermittent stirring, the reaction mixture was centrifuged for 3 minutes at 4,000 xg. An aliquot of the supernatant was plated, dried, and counted in a Nuclear Chicago thin window counter. The C^{14} assay for dTMPase is described in the legend of Table 1.

RESULTS AND DISCUSSION

dTMPase activity of phage infected B. subtilis: After infection of B. subtilis SB19 with phage SP5C, there is an approximately ten fold increase in an activity that splits dTMP to dT and P_i (Figure 1). No such increase in dTMPase activity has been found after continued incubation of the uninfected host cells in the absence of SP5C. Although only the results obtained using the Norit nonadsorbable P^{32} assay are presented, similar results have been found using C^{14} -dTMP as the substrate and measuring the amount of C^{14} -deoxythymidine formed. After infection, there is little or no increase in the rate of degradation of either dCMP, dAMP or dGMP (Figure 1). The small changes observed in the rates of dCMP, dAMP and dGMP breakdown have not been consistent.

dTMPase Activity of B. Subtilis After Infection with Phage SP5C



The induction of this enzyme does not appear to be due to small molecular weight inducers or derepressors present in the phage preparations. The addition to the bacterial culture of the supernatant obtained after sedimentation of the phage by high speed centrifugation does not induce the enzyme. Nor does the enzyme seem to be related to the defective phages present in B. subtilis, (Romig, personal communication; and Seaman, Tarmy, and Marmur, 1964) since the treatment of B. subtilis SB19 cells with mitomycin, an inducer of these prophages, does not produce dTMPase.

Properties of partially purified dTMPase: The dTMPase of phage SP5C-infected B. subtilis has been purified approximately 15 fold. The enzyme is extremely labile. After nucleic acids have been removed from the preparation, 50% of the enzymatic activity is lost after 3 hours at 0° C. (Even for this amount of stability, dTMP, $MgCl_2$, EDTA, and GSH must be present as stabilizers). The enzyme requires Mg^{++} (8×10^{-3}M). Absence of Mg^{++} in the reaction mixture decreases the activity to less than 1%.

The substrate specificity experiments (Table 1) indicate that with the 15 fold-purified enzyme, under these conditions, the enzymic breakdown of dTMP is greater than the other deoxy- or ribonucleoside monophosphates and dTTP. The activity of the enzyme fraction on dAMP as well as dUMP and dHMUMP (Table 1) may be due to another enzyme present in the preparation. This will be resolved with further purification. Kahan et al. (1964) have reported a dTTPase activity in crude extracts of B. subtilis infected with phage SP8. The 15 fold-purified dTMPase fraction does not have dTTPase activity (Table 1). (The assay using C^{14} dTTP would detect dTDP, dTMP as well as dT production). The question of whether dTMPase will specifically release the 5' phosphate connected to the terminal end of deoxythymidine in a polynucleotide chain must await further purification of the enzyme.

After incubation of $dTMP^{32}$ with the enzyme one of the reaction products has been shown to be P^{32} orthophosphate by its cochromatography

TABLE 1. Substrate Specificity - SP5C dTMPase

Assay	Relative Activity										
	dTMP	dCMP	dGMP	dAMP	dUMP	dHMUMP	dTTP	CMP	GMP	AMP	UMP
P^{32}	100	7	7	-	-	-	-	-	-	-	-
C^{14}	100	7	-	12	-	17	<1	-	-	-	-
P_i	100	-	-	11	20	-	-	<1	<1	<1	<1

All nucleotides were 5'PO_4.
When $dTMP^{32}$ was used as the substrate 35.8 mμmoles of phosphate were released. This value was set at 100 and the other values are expressed relative to it when another nucleotide was used in the place of $dTMP^{32}$. Three micrograms of SP5C dTMPase fraction (15 fold purified) was used. The P^{32} assay is described in the text. The C^{14} assay measures the conversion of C^{14} nucleotide to C^{14} nucleoside which is separated by paper chromatography on Whatman 3 MM paper using ammonium acetate 1M, pH 7.5 (3 parts): 95% ethanol (7 parts). In addition to separating the nucleoside formed it also separates any dTMP if it were formed from dTDP + dTTP when the latter is used as the substrate.
P_i was measured by the method of Chen et al. (1956).

with carrier orthophosphate on Dowex-1-chloride and its reactions in the Chen et al. (1956) method for inorganic phosphate. When C^{14} dTMP is used as the substrate, the C^{14} product travels as thymidine in a variety of chromatographic systems.

Role of dTMPase in the economy of a phage-infected cell: By comparing the relative breakdown of $dTMP^{32}$ and $dCMP^{32}$, a similar dTMPase activity that increases to the same magnitude has been found after infection of B. subtilis SB19 with other subtilis phages whose DNA's contain hydroxymethyluracil, uracil or thymine (Nishihara, Trilling, and Aposhian, in preparation). There has also been a preliminary report of a dTMPase activity in a crude

extract of B. subtilis infected with a uracil containing phage (Kahan, 1963). The dTMPase of phage SP5C infected B. subtilis may serve to prevent incorporation of thymidylate into the DNA of the infected host and of the bacteriophage, thus, allowing replication of the phage DNA which contains hydroxymethyluracil in place of thymine. However, the finding of a similar dTMPase activity of similar magnitude in B. subtilis cells infected with a thymine-containing phage is unexpected and suggests a new pathway for the formation of dTTP which may not involve the phosphorylation of dTMP.

References

Brodetsky, A.M., and Romig, W.R., Bacteriol. Proc., 118 (1964).
Chen, P.S., Toribara, T.Y., and Warner, H., Anal. Chem., 28, 1756 (1956).
Kahan, F.M., Fed. Proc., 22, 406 (1963).
Kahan, F., Kahan, E. and Riddle, B., Fed. Proc., 23, 318 (1964).
Kallen, R.G., Simon, M., and Marmur, J., J. Mol. Biol., 5, 248 (1962).
Lehman, I.R., J. Biol. Chem., 235, 1479 (1960).
Lehman, I.R., Bessman, M.J., Simms, E.S., and Kornberg, A., J. Biol. Chem., 233, 163 (1958).
Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. Biol. Chem., 193, 265 (1951).
Marmur, J., and Cordes, S., Informational Macromolecules (1963).
Romig, W.R., and Brodetsky, A.M., J. Bacteriol., 82, 135 (1961).
Roscoe, D.H., and Tucker, R.G., Biochem. Biophys. Res. Commun., 16, 106 (1964).
Seaman, E., Tarmy, E., and Marmur, J., Biochemistry 3, 607 (1964).