THE BIOSYNTHESIS OF A PYRIMIDINE REPLACING THYMINE IN BACTERIOPHAGE DNA

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A phage, øe, which attacks a Marburg strain of Bacillus subtilis, NCTC 3610, has been isolated from soil and its DNA composition determined. Adenine, guanine and cytosine are present (G+C = 40.3%) but thymine is completely replaced by 5-hydroxymethyl uracil. Measurement of the thermal transition of øe DNA gives a Tm of 76.5° in 1.5×10^{-1} M-NaCl + 1.5×10^{-2} M-sodium citrate. The phage is thus similar to another subtilis phage, SP-8, analysed by Kallen, Simon and Marmur (1962). There are however some differences between the two phages: øe has a head diameter of 50 mμ and a tail length of 70 mμ while the corresponding values for SP-8 are 100 mμ and 170 mμ respectively (Davison, 1963); also no glucose has been detected bound to øe DNA in contrast to SP-8 (Takahashi and Marmur, 1963). Some of the enzymic changes occurring on infection by øe have been studied and the results indicate that 5-hydroxymethyldeoxyuridylic acid (dHMUMP) is formed from deoxyuridylic acid (dUMP) by a hydroxymethylation reaction analogous to the hydroxymethyldeoxycytidylate-synthesising mechanism found in phage-infected Escherichia coli (Flaks and Cohen, 1959).

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Methods. (i) Crude extracts were prepared from cultures grown with vigorous aeration at 37° in double strength minimal medium (Davis and Mingioli, 1950) containing 4×10^{-2} M-glucose. Organisms in the log phase of growth (5×10^8 cells/ml.) were infected with a five-fold excess of ø*e* (which has a latent period of 60 min. under these conditions) and aeration continued for 20 min. The cultures were rapidly chilled to 2° and the organisms collected by centrifugation. After washing and resuspension in 10^{-1} M-Tris buffer, pH 7.5, the cells were disrupted using a Mullard Ultrasonic Generator, Type E 7590B. Unbroken cells and other particles were removed by centrifugation at 10,000 g for 5 min. and 100,000 g for 60 min. Extracts from uninfected bacteria were prepared by the same method from parallel cultures.

(ii) Deoxycytidylic acid (dCMP) deaminase was measured spectrophotometrically (Wang, 1955). Each cuvette contained in 3.5 ml: 175 µmoles Tris buffer, pH 7.5, and 1 µmole dCMP; freshly prepared extract was added to one cuvette and the rate of decrease in optical density at 285 mµ measured at 25°.

(iii) Thymidylate (TMP) synthetase was estimated according to Wahba and Friedkin (1962). Cuvettes contained: 25 µmoles Tris buffer, pH 7.5, 50 µmoles mercaptoethanol, 12.5 µmoles $MgCl_2$, 0.5 µmoles ethylenediaminetetraacetate, 10 µmoles formaldehyde, 0.1 µmoles tetrahydrofolic acid (THFA) and extract in a total volume of 0.45 ml. To start the reaction 0.05 µmoles dUMP were added to one cuvette and the increase in optical density at 340 mµ followed at 25°.

Results and Discussion. The changes in the specific activities of dCMP deaminase and TMP synthetase on infection are given in Table 1. An increase in dCMP deaminase also occurs during growth of SP-8 (Marmur and Greenspan, 1963) and

TABLE 1

Comparison of dCMP deaminase and TMP synthetase activities in extracts of B. subtilis, uninfected and infected with ϕe.

Extract	*dCMP deaminase	*TMP synthetase
Uninfected	< 0.1	0.015
Infected	2.0	< 0.001

*Units of activity: dCMP deaminase, μmoles dCMP utilised/mg. protein/hr.; TMP synthetase, μmoles THFA oxidised/mg. protein /hr.

this change suggests that the product of the enzymic reaction, dUMP, is an intermediate in the biosynthesis of dHMUMP. Extracts of Neurospora convert thymine and thymidine to HMU derivatives (Abbott, Kadner and Fink, 1964) but with the decrease of TMP synthetase activity on infection it seems unlikely that a similar step occurs in the viral system. A more probable pathway is direct hydroxymethylation of dUMP and an enzyme bringing about this reaction has been detected.

Enzymic extracts were incubated for 45 min. at 37° with 50 μmoles Tris buffer, pH 7.5, 10 μmoles mercaptoethanol, 1 μmole $H^{14}CHO$ (1.1×10^6 cpm/μmole), 0.2 μmole THFA and 1 μmole dUMP in a total volume of 1 ml. The reaction was stopped by heating in boiling water for 5 min. and precipitated protein removed. The supernatant fluid was applied to Whatman No. 1 chromatography paper and the spots washed overnight with n-butanol/ammonia (Markham and Smith, 1949) to remove unwanted radioactive material. The nucleotides, which remain at the origin, were eluted with water, treated with alkaline phosphatase and the resulting nucleosides paper-chromatographed in 86% aq. n-butanol (Markham and Smith, 1949). A peak of radioactivity was detected and had the chromatographic properties of synthetic

HMU deoxyriboside in 3 solvents: 86% aq. n-butanol and n-butanol /ammonia (Markham and Smith, 1949) and isopropanol/HCl (Wyatt, 1951). The radioactive area was eluted with water and counted. Control incubations were treated in the same manner and the corresponding regions of the chromatogram eluted. The results are shown in Table 2. The dUMP hydroxymethylase activity detected by this means (0.01 µmoles dHMUMP formed/mg. protein /hr.) is not sufficient to account for the rate of DNA synthesis in infected bacteria. This may be due to the method of measurement but the possibility of a second pathway cannot be eliminated.

TABLE 2

Fixation of $H^{14}CHO$ into dHMUMP by extracts of øe-infected B. subtilis.

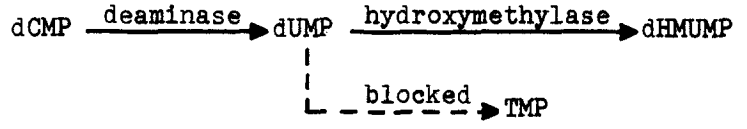
Incubation mixture	cpm/ml. mixture
*Complete system	5600
" " minus dUMP	125
" " " THFA	65
" " " mercaptoethanol	4450
" " " extract	95
**Reagent mixture + boiled extract	105
" " + extract of uninfected cells	115

*Complete system as described in text
**Extract from infected bacteria was heated for 5 min. in boiling water

It has been suggested that TMP synthetase limits the rate of synthesis of DNA containing thymine (Flaks and Cohen, 1959 a); it is not known at this stage whether the hydroxymethylase could exert a similar control. Preliminary results suggest that the dCMP deaminase is subject to inhibition by

dUMP and TMP, and in this respect the enzyme is like that found in rapidly growing animal tissue (Scarano, Bonaduce and de Petrocellis, 1960; Maley and Maley, 1962). The reduction of TMP synthetase activity may be caused by the increased production of an inhibitor similar to that demonstrated by Friedkin, Crawford, Donovan and Pastore (1962); if extract from infected bacteria is mixed with that from normal bacteria the synthetase activity of the latter is decreased.

In conclusion it appears that dHMUMP can be synthesised in ø-infected B. subtilis by the following pathway:



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