The enzymic synthesis of 5-hydroxymethyldeoxycytidylic acid*

A unique viral pyrimidine, 5-hydroxymethylcytosine(HMC), is present in the deoxyribose nucleic acid (DNA) of the T-even bacteriophages[1], and is absent from the nucleic acids of the host bacterium, *E. coli*, which contain cytosine. Work in this laboratory has shown that the pyrimidine ring of viral HMC can be derived from the cytosine of *E. coli* DNA[2]. The hydroxymethyl substituent of HMC, as well as the methyl group of thymine, can be derived from the β-carbon of serine[2], but not from the methyl group of methionine[3]. It has been shown that the formation of HMC does not occur in infected cells at the level of the free pyrimidines or their nucleosides[4,5].

The formation of HMC has now been found to take place at the level of the nucleotide, with deoxycytidine-5'-phosphate (deoxy-CMP) and formaldehyde as substrates in the presence of 5,6,7,8-tetrahydrofolic acid (THFA) and an enzyme preparation derived from *E. coli* infected with T6 bacteriophage. The reaction has been followed by measuring the amount of formaldehyde-^{14}C rendered non-volatile and acid-stable. Two bacterial extracts were studied; one obtained from exponentially growing cultures of *E. coli*, and the second from similar cells infected with T6r$^+$. The hydroxymethylation of deoxy-CMP was far greater (at least six-fold) with extracts of infected cells than with extracts of uninfected cells. Indeed, it is not certain that the very slight uptake of $H^{14}CHO$ with deoxy-CMP in extracts of uninfected cells led to the formation of hydroxymethyldeoxy-CMP as it did with extracts of infected cells. The hydroxymethylation of deoxyuridine-5'-phosphate (deoxy-UMP) proceeded with extracts of both uninfected and infected cells, the activity of the latter being 2- to 3-fold greater. With extracts of infected cells the product of this reaction was thymidylic acid[6].

Infected cells were centrifuged 15 min after adsorption of the virus in an aerated synthetic medium. Cell-free extracts were prepared by grinding with alumina, followed by extraction with 0.1 *M* phosphate buffer, pH 7.0. The extracts were dialyzed against 0.01 *M* phosphate buffer, pH 7.0, and treated with Dowex-1-chloride resin. Table I shows the fixation of formaldehyde-^{14}C obtained with the extract from infected cells and deoxy-CMP and deoxy-UMP.

TABLE I

INCORPORATION OF $H^{14}CHO$ INTO ACID-STABLE, NON-VOLATILE FORM

Additions to system	*Moles $H^{14}CHO$ incorporated*
None	0.003
THFA	0.056
Deoxycytidylate	0.021
Deoxyuridylate	0.013
THFA + deoxycytidylate	0.202
THFA + deoxyuridylate	0.099

The system (0.5 ml) contained: potassium phosphate, pH 7.0 (25 μmoles), $MgSO_4$ (5 μmoles), $H^{14}CHO$ (1 μmole; specific activity, 19,100 CPM/μmole), enzyme from T6r$^+$ infected cells of *E. coli* (0.63 mg protein). Where added: THFA (0.48 μmole), deoxy-CMP or deoxy-UMP (2.5 μmoles). After incubation for 30 min at 37°, the reaction was stopped with 0.5 ml. 10% trichloroacetic acid. After centrifugation, aliquots were placed on planchets with 0.05 ml 2 *N* HCl and dried. The planchets were assayed for fixed ^{14}C.

5-hydroxymethyldeoxy-CMP has been isolated as a product of the reaction with deoxy-CMP and formaldehyde-^{14}C in the presence of THFA and an enzyme prepared from T6r$^+$ infected cells under conditions described in Table I, with quantities increased 60-fold. Formaldehyde uptake was maximal after incubation for 45 min (11.4 μmoles $H^{14}CHO$). The reaction was terminated with cold $HClO_4$. After centrifugation, the excess perchlorate was removed as $KClO_4$. Ion-exchange chromatography on Dowex-1-acetate revealed the pattern for the cytosine nucleotide region shown in Fig. 1. Deoxy-CMP, free of isotope, was eluted first. 5-methyldeoxy-CMP could not be detected. The peak containing 5-hydroxymethyldeoxy-CMP followed deoxy-CMP, and accounted for 58% of the isotope incorporated. The ratio ^{14}C: HMC: organic phosphate was 0.97: 1.0: 1.0. The nucleotide showed an ultraviolet absorption maximum at 283–284 mμ, at pH 1.0, with an ε_{max} of $11.7 \cdot 10^3$ based on the phosphate content. The ultraviolet-absorption spectra at pH 1, 7, and 13, were similiar to those reported for the deoxyriboside[5].

Enzymic dephosphorylation of the nucleotide yielded a compound with properties identical to 5-hydroxymethyldeoxycytidine isolated from T6r$^+$ bacteriophage DNA. The R_F of the nucleoside

in *n*-butanol-5 % NH_4OH was 0.18, identical with the nucleoside from viral DNA. In this system it separated from deoxycytidine (R_F 0.24) and 5-methyldeoxycytidine (R_F 0.30).

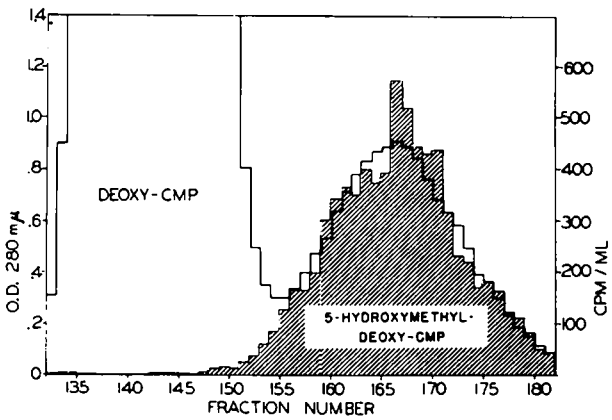

Fig. 1. Ion-exchange chromatography on Dowex-1-acetate, of the cytosine nucleotide area. The column was eluted at this point with 0.06 *M* ammonium acetate, pH 4.2. The shaded area represents the fixed radioactivity.

Acid hydrolysis of the nucleotide in 6N HCl yielded a base whose R_F was identical with synthetic HMC and which differed from cytosine or 5-methylcytosine. With both the nucleoside and the free base, the ratio of ultraviolet absorption to radioactivity was the same as that in the nucleotide.

Two different hydroxymethylation reactions of pyrimidine deoxyribotides are presented below:

$$\text{Deoxyuridylate} + \text{HCHO} + 2\text{H} \xrightarrow{\text{THFA}} \text{thymidylate} \qquad (1)$$

$$\text{Deoxycytidylate} + \text{HCHO} \xrightarrow{\text{THFA}} \text{5-hydroxymethyldeoxycytidylate} \qquad (2)$$

The reaction with deoxy-UMP proceeds past the hydroxymethyl level and involves a reduction, leading to the formation of the methyl pyrimidine, thymine. In extracts of infected cells, the reaction with deoxy-CMP appears to stop with the production of the hydroxymethylcytosine deoxyribotide. The mechanisms of these reactions and the reasons for this difference in the nature of the products have not yet been clarified.

The problem of the origin of the marked increase of activity in the hydroxymethylation of deoxy-CMP by extracts of infected cells is now under investigation.

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