Biological Activity of 5-Hydroxymethyluracil and Its Deoxynucleoside in Noninfected and Phage-infected *Bacillus subtilis*

MUTSUKO NISHIHARA,¹ NORMAN FRIEDMAN, AND H. VASKEN APOSHIAN

Department of Cell Biology and Pharmacology, University of Maryland School of Medicine,
Baltimore, Maryland 21201

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¹⁴C-hydroxymethyldeoxyuridine (dHMU) is specifically incorporated into the deoxyribonucleic acid (DNA) of bacteriophage SP8. Incorporation experiments demonstrate that the initiation of phage SP8 DNA synthesis occurs between 12.5 to 15 min after infection. Incorporation into host DNA does not occur. ¹⁴C-dHMU can be used as an analytical tool for screening conditionally lethal phage mutants containing hydroxymethyluracil in their DNA to select those that are defective in DNA synthesis under restrictive conditions. The pyrimidine, ¹⁴C-hydroxymethyluracil (HMU), is not incorporated into bacterial or phage DNA. Neither HMU nor dHMU can replace thymine as a growth requirement for *Bacillus subtilis* 168 Ind⁻Thy⁻. HMU does not inhibit the utilization of thymine. Although dHMU inhibits deoxythymidine utilization, the inhibition is not competitive.

The pyrimidine 5-hydroxymethyluracil (HMU) is found in place of thymine in the deoxyribonucleic acid (DNA) of a number of bacteriophages that infect *Bacillus subtilis* (1, 4, 6, 10, 11). The structure of HMU differs from thymine by the substitution of an hydroxymethyl group for a methyl group (Fig. 1). Some analogues of thymine, for example, the 5-bromo-, the 5-chloro- and 5-iodo- derivatives are incorporated into bacterial DNA in place of thymine (2, 15, 16). Others have been useful metabolic inhibitors, for example 5-fluorodeoxyuridine. In the present report, the following questions have been asked about HMU and its deoxynucleoside. (i) Will either compound support the growth, in the absence of thymine or deoxythymidine, of bacterial mutants requiring thymine? (ii) Will either compound inhibit the utilization of thymine or deoxythymidine in such mutants? (iii) Are these compounds incorporated into *B. subtilis* or phage DNA? (iv) Can they be used as analytical tools for screening conditionally lethal phage mutants containing HMU in their DNA, to select those that are defective in DNA synthesis under restrictive conditions?

MATERIALS AND METHODS

Phages and bacteria. Stocks of phage SP3 and phage SP8 were produced by propagation on *B. subtilis* SB19 as described previously (9, 14). Phage

SP82 and its mutants were obtained from Eunice Kahan. Lysates, after low-speed centrifugation to remove bacterial debris, were centrifuged for 60 min at $39,100 \times g$. The phage pellet was then resuspended in a buffer, described by Spizizen (13), composed of 14 g of K₂HPO₄, 6 g of KH₂PO₄, 1 g of sodium citrate·2H₂O, 2 g of (NH₄)₂SO₄, 0.2 g of MgSO₄·7H₂O per liter of distilled water. Glucose was omitted. Purified, resuspended phage were used in all experiments. *B. subtilis* 168 Ind⁻Thy⁻ (3) was obtained from Frank Rothman. *Escherichia coli* 15T-H⁻U⁻, which requires thymine, histidine, and uracil for growth, was obtained from P. C. Huang.

Media. The synthetic medium used for *B. subtilis* was that of Spizizen (13) supplemented with 0.02% casein hydrolysate, 50 μg of L-tryptophan per ml, and 5 μg of deoxythymidine (dT) per ml. This medium will be referred to as S1 medium. *E. coli* 15T-H⁻U⁻ was grown in the medium described by Maaløe and Hanawalt (7) except that 25 μg of L-histidine was substituted for arginine. This medium will be referred to as S2 medium. TY broth, as modified by Trilling and Aposhian (14), was used where indicated.

Materials. ¹⁴C-5-hydroxymethyldeoxyuridine (dHMU), labeled in the hydroxymethyl carbon, was prepared by treating ¹⁴C-5-hydroxymethyldeoxyuridylylate (dHMUMP) with *E. coli* alkaline phosphatase. The nucleoside was separated from unreacted substrate by applying the reaction mixture to diethylaminoethyl (DEAE) paper sheets (Whatman DE-81). Chromatography was then carried out with water as the solvent. Under these conditions, only the non-phosphorylated compound migrates from the origin. The separated ¹⁴C-dHMU was eluted from the paper

¹ Postdoctoral fellow of the American Cancer Society.

FIG. 1. Structural formulae for 5-hydroxymethyluracil and thymine.

with water, and the ratio of its absorption at 250:260 nm and 280:260 nm were found to be 0.72 and 0.60, respectively, at pH 7.

¹⁴C-dHUMP was prepared as previously described (1). Deoxythymidine-2-¹⁴C and deoxyuridine-2-¹⁴C were purchased from Schwarz Bio Research Inc., Orangeburg, N.Y. Hydroxymethyluracil-2-¹⁴C was a product of New England Nuclear Corp., Boston, Mass. Nonradioactive pyrimidines and their deoxynucleosides were purchased from Calbiochem, Los Angeles, Calif.

Bacterial growth studies. *B. subtilis* 168 Ind⁻Thy⁻ cultures were started by adding spores to S1 medium. The cells were grown with shaking at 37 C to an absorbancy at 600 nm of 0.5 to 1.0, as measured in a Zeiss PMQII spectrophotometer. They were centrifuged, washed once with S1 medium lacking dT, and then resuspended in one-tenth the original volume of S1 medium lacking dT. This suspension of cells was used to inoculate a series of tubes containing various concentrations of pyrimidine or pyrimidine deoxynucleoside. The inoculum added was 1% of the volume in such tubes. The tubes were then placed on an Eberbach reciprocal shaker and incubated at 37 C for 3.5 hr. Growth was measured as an increase in absorbancy at 600 nm.

E. coli T⁻H⁻U⁻ cultures were started with cells grown in S2 medium. Cells were grown and washed as described above for *B. subtilis*, except that S2 medium minus dT or uridine was used for the wash and final suspension.

Uptake and incorporation of deoxynucleosides. For incorporation studies, a standard inoculum of spores of *B. subtilis* 168 Ind⁻Thy⁻ was added to S1 medium. The culture was shaken at 37 C until an absorbancy at 600 nm of about 1.0 was obtained. The cells were centrifuged and washed once with S1 medium lacking dT. They were then resuspended in the same medium and incubated with shaking at 37 C for 15 min. To portions of this culture were added the labeled compound and, where appropriate, 10 phage per cell. At various times, samples of 0.5 or 1.0 ml were removed and added to cold 5% trichloroacetic acid containing 1.25 mg of dT per ml. The precipitates were collected on filters (Millipore Corp., Bedford, Mass.) and washed once with 5 ml of cold 5% trichloroacetic acid

and twice with 5 ml of cold water. They were dried and counted in a Mark I scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.) using a toluene solution containing 4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene per liter.

RESULTS

Bacterial growth studies. The results of adding HMU or its deoxynucleoside to a culture of *B. subtilis* Ind⁻Thy⁻ in medium lacking thymine or deoxythymidine are shown in Fig. 2. As can be seen, neither HMU nor its deoxynucleoside can be utilized in place of thymine or deoxythymidine for growth of these cells.

The inhibitory activity of HMU and dHMU was then examined. As seen in Fig. 3, the presence of HMU does not inhibit the utilization of thymine as measured by the growth of this organism. If dHMU (2 μmoles/ml) is added to the culture in the presence of varying amounts of deoxythymidine, a consistent inhibition of growth occurs (Fig. 3B). This inhibition, however, does not appear to be competitive since increasing concentrations of deoxythymidine do not reverse the inhibition. In the presence of dHMU and low levels of dT, a consistent but small stimulation of growth has always been observed (Fig. 3B). Whether this is due to the utilization of dHMU or its sparing the utilization of dT in some metabolic reactions is not known. The amounts of ¹⁴C-dHMU used in the incorporation studies reported in this paper are much less than these inhibitory concentrations.

Neither HMU nor its deoxynucleoside will

FIG. 2. Growth of *B. subtilis* 168 Ind⁻Thy⁻ in the presence of T, dT, HMU, or dHMU.

FIG. 3. Influence of HMU or dHMU on the growth of *B. subtilis* 168 Ind⁻ Thy⁻ in the presence of T or dT. The concentration of HMU or dHMU, where present, was 2 μmoles/ml.

FIG. 4. Growth of *E. coli* T⁻H⁻U⁻ in the presence of uridine, dT, HMU, or dHMU. Readings of absorbancy were made 6 hr after inoculation into medium containing the pyrimidine base or nucleoside. (A) All tubes contained dT; (B) all tubes contained uridine.

substitute as a growth factor for either uridine or dT in another organism, *E. coli* T⁻H⁻U⁻ (Fig. 4). The growth requirements of *E. coli* and *B. subtilis*, therefore, are not satisfied by these 5-hydroxymethyl analogues of thymine and deoxythymidine under these conditions.

Uptake and incorporation of ¹⁴C-HMU and its deoxynucleosides. Since neither HMU nor its deoxynucleoside was utilized as a growth factor under these conditions, their possible incorporation into bacterial and phage DNA was next studied. ¹⁴C-HMU or ¹⁴C-dHMU was added to

cultures of *B. subtilis* Ind⁻ Thy⁻ in the presence or absence of phage SP8. S1 medium lacking dT was used. The amount of ¹⁴C-HMU incorporated into the acid-precipitable fraction was neither significant nor different for noninfected or SP8-infected cells. However, the addition of ¹⁴C-dHMU to the culture at the time of infection of the host by phage SP8 results in the incorporation of the radioactivity into trichloroacetic acid-precipitable material (Fig. 5). ¹⁴C-dHMU is not incorporated after infection of the host by SP3, a phage whose DNA contains thymine instead of

FIG. 5. Incorporation of ^{14}C -dHMU and ^{14}C -dU by uninfected and SP8-infected *B. subtilis* 168 Ind⁻ Thy⁻ into trichloroacetic acid-insoluble material. (A) ^{14}C -dHMU; the specific activity was 1.4×10^7 counts per min per μmole ; the amount in the medium was 1.6×10^4 counts per min per ml. (B) ^{14}C -dU; the specific activity was 1.4×10^6 counts per min per μmole ; 2.1×10^4 counts/min were present per ml of medium. Results are expressed as trichloroacetic acid-insoluble material, counts per min per ml of culture.

FIG. 6. CsCl gradient centrifugation of ^{14}C -dHMU-labeled SP8 DNA. Cells were grown and infected with SP8 in the presence of ^{14}C -dHMU. At 80 min after the addition of phage, the lysate (80 ml) was centrifuged at $12,100 \times g$ for 10 min. To the supernatant fluid was added 160 μg of pancreatic ribonuclease, 16 μg of pancreatic deoxyribonuclease, and MgSO_4 to a concentration of 0.003 M. Incubation for 60 min at 37 C was followed by centrifugation for 60 min at $39,100 \times g$. The phage pellet was covered with 2 ml of SSC (0.15 M NaCl, 0.015 M sodium citrate) and left overnight at 4 C. The resuspended phage were centrifuged at $39,100 \times g$ for 60 min, and the pellet was resuspended in 2 ml of SSC. The phage DNA was extracted at 4 C with SSC-saturated phenol. An equal amount, based on absorbancy at 260 nm of unlabeled phage SP8 DNA was added to the ^{14}C -labeled DNA obtained from phages made in the presence of ^{14}C -dHMU. CsCl was added to a density of 1.74 g/cm³. Centrifugation was performed in an SW65 rotor of a Spinco L2-65B ultracentrifuge at 35,000 rev/min for 3 days at 25 C. Drops were collected from the bottom of the tube and samples from the fractions were taken for measurements of absorbancy at 260 nm. For radioactivity measurements, 0.05 ml was placed on glass filters (Whatman GF/C), dried, and counted. Densities were determined from the refractive index at 25 C.

FIG. 7. Incorporation of ^{14}C -dT by uninfected and SP8-infected *B. subtilis* 168 Ind⁻ Thy⁻ into trichloroacetic acid-insoluble material. ^{14}C -deoxythymidine (1.7×10^6 counts per min per μmole), 2.0×10^4 counts per min per ml of medium, was present. Results are expressed as trichloroacetic acid-insoluble material, counts per min per ml of culture.

HMU (D. Trilling, Ph.D. Thesis, Tufts Univ. Boston, 1967), again demonstrating that the radioactivity is not incorporated unless the host is infected by a phage containing HMU. Alkali digestion of samples prior to acid precipitation showed that the radioactivity is incorporated into DNA and not into RNA.

As judged by the incorporation of radioactivity, the first indication of SP8 DNA synthesis occurs between 12.5 and 15 min after infection. The latent period before lysis is approximately 45 min in this medium.

To be certain that the ^{14}C -dHMU was actually incorporated into phage DNA, the DNA was extracted with phenol and centrifuged in CsCl . As seen in Fig. 6, the radioactivity appears in the same position as added unlabeled SP8 DNA. The density of this peak was 1.748 g/cm^3 , similar to that reported previously (8). This again indicates the usefulness and specificity of ^{14}C -dHMU as a label for following HMU phage DNA synthesis.

If ^{14}C -deoxyuridine (dU) is used as the label, incorporation into acid-precipitable material occurs in both uninfected and infected cells (Fig. 5B) at the same rate. Thus, the time at which host DNA synthesis ceases and phage DNA synthesis begins cannot be distinguished when deoxyuridine is used (Fig. 5B). In contrast to the results observed with ^{14}C -dHMU and ^{14}C -dU, the cessation of host DNA synthesis after SP8 infection can be observed if ^{14}C -dT is used (Fig. 7). Thus, the use of ^{14}C -dHMU and ^3H -dT should be useful in double-labeling experiments in which the synthesis of SP8 DNA and the cessation of host DNA synthesis require simultaneous study.

These experiments on the uptake and incorporation of ^{14}C -deoxynucleosides used synthetic medium. Since many reports dealing with phage infection of *B. subtilis* have used enriched medium, the incorporation of ^{14}C -dHMU was repeated using TY broth. Under identical conditions except for the growth medium, the amount of ^{14}C -dHMU incorporated into the acid-precipitable fraction after SP8 infection was found to be much lower (less than 10%) in TY broth than in a synthetic medium.

Screening of conditional phage mutants blocked in DNA synthesis. Since the incorporation of ^{14}C -dHMU can be used as a specific tool for detecting and measuring the synthesis of HMU-containing phage DNA, this labeled deoxynucleoside should be useful for rapidly determining the extent of DNA synthesis in cells infected with conditional phage mutants whose DNA contain HMU. Mutants which are blocked or abnormal in DNA synthesis under restrictive conditions should be easily detected by this method.

Figure 8 summarizes the results with two such temperature-sensitive conditional phage mutants. SP82-H26 and SP82-H23 have been reported to be blocked in phage DNA synthesis at higher temperatures (5). The temperature-sensitive enzyme involved in DNA synthesis in the case of SP82-H26 is unknown and, in the case of SP82-H23, it is believed to be the hydroxymethylase which catalyzes the conversion of deoxyuridylylate (dUMP) to dHMUMP (E. Kahan, *personal communication*). Phage SP82-wild, when grown in its host at either 37 or 46 C, rapidly incorporates ^{14}C -dHMU. At 46 C, the incorporation of ^{14}C -dHMU by cells infected with SP82-H26 is approximately 8% of that found at the permissive temperature of 37 C, showing a definite block in DNA synthesis at the restrictive temperature (Fig. 8B).

Incorporation of ^{14}C -dHMU by SP82-H23-infected cells at 46 C would be expected, since this would be due to a bypass of the temperature-

FIG. 8. Use of ¹⁴C-dHMU to screen phage mutants defective in DNA synthesis. Results are expressed as trichloroacetic acid-insoluble material, counts per min per ml of culture. Specific activity of ¹⁴C-dHMU is given in legend of Fig. 5. *B. subtilis* 168 Ind⁻ Thy⁻ was the host.

sensitive hydroxymethylase. This was found to be so (Fig. 8C). However, the rate of incorporation does not equal that at the lower temperature.

These results demonstrate the usefulness of ¹⁴C-dHMU for screening mutants of HMU-containing phages for defects in phage DNA synthesis.

DISCUSSION

Although the structure of hydroxymethyluracil appears to be closely related to thymine, the substitution of a hydroxymethyl group for a methyl group on the number 5 carbon atom of the ring prevents its utilization for the growth of thymine-requiring mutants of *B. subtilis* and *E. coli*. Neither do these compounds have strong inhibitory properties in the *B. subtilis* system studied. Any explanation as to the different activity of thymine and HMU requires a more basic understanding of the subtle physical and chemical interactions between substrate and enzyme. It is of interest to note that space-filling atomic models of thymine (T) and HMU show the presence of a steric interference present in HMU but not T. There is a nonbonded interaction between the 6-keto group and the 5-hydroxymethyl group that limits free rotation of the bond between the hydroxymethyl carbon atom and the number 5 ring carbon atom. This steric interference is not present in thymine. Direct observations of restricted rotation in HMU using nuclear magnetic resonance techniques might be of aid in measuring these rotational differences.

The deoxynucleoside of HMU can, however, be incorporated into phage SP8 DNA (Fig. 5 and 6). In studying the mechanisms involved in phage infection of *B. subtilis*, it is often essential to know at what time and under what conditions the synthesis of phage DNA occurs. The DNA of many of these phages (SP8, SP5C, ϕ e, SP01 and SP82) contain the unusual pyrimidine, HMU, in place of thymine (1, 4, 6, 10, 11). In cells infected with phage SP8, host DNA is not rapidly degraded (8) as it is in the *E. coli* T-even phage systems. Furthermore, *B. subtilis* DNA synthesis does not immediately cease (8). This complicates many methods used to measure the time at which phage DNA synthesis occurs and the amount of DNA synthesized after infection. For example, the incorporation of the radioactive deoxynucleosides of uracil, cytosine, guanine, or adenine into acid-precipitable material is not a specific indication of the synthesis of the HMU-containing phage DNA (Nishihara and Aposhian *unpublished data*). Moreover, although the synthesis of HMU-containing DNA can be detected by equilibrium centrifugation of lysed infected cells in cesium chloride, such a method is not only time consuming but is also limited by the number of samples it is possible to deal with in the ultracentrifuge at one time. The centrifugation procedure, also, is too burdensome and slow to be used if one needs a rapid screening method for detecting phage DNA synthesis. The incorporation of ¹⁴C-dHMU can be used as a rapid and specific measure of the synthesis of phage DNA contain-

ing HMU, as shown by the evidence presented in this paper.

After infection of *B. subtilis* by phage SP8 or ϕ e, a new enzyme appears which catalyzes the synthesis of dHMUMP by the hydroxymethylation of dUMP (11, 12). The incorporation of ^{14}C -dHMU into SP8 DNA, as shown by the present work, suggests that still another pathway, perhaps analogous to the dT kinase reaction, is possible for dHMUMP synthesis. These results suggest either a host- or phage-induced deoxynucleoside kinase is converting ^{14}C -dHMU to ^{14}C -dHMUMP.

The observation that the uninfected host does not incorporate ^{14}C -dHMU into acid-precipitable material may be due to one of two reasons. The host deoxynucleoside kinase, e.g., dT kinase, may have such a high degree of specificity that the conversion of dHMU to dHMUMP does not occur. An alternative explanation is that enzymes which are necessary to raise dHMUMP to the level of phosphorylation required for DNA synthesis are lacking in the uninfected cells. Preliminary evidence in support of the latter has been reported (F. Kahan, E. Kahan, and B. Riddle, Federation Proc., p. 23, 1964).

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