Biosynthesis of 5-(4′,5′-Dihydroxypentyl)Uracil as a Nucleoside Triphosphate in Bacteriophage SP15-Infected *Bacillus subtilis*

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The nucleoside triphosphate of 5-(4′,5′-dihydroxypentyl)uracil (DHPU) was detected in the acid-soluble extract from bacteriophage SP15-infected *Bacillus subtilis* W23. No uracil was found in the DNA of either replicating or mature phage. Labeled thymidine added during phage DNA synthesis was incorporated into phage DNA. The presence of DHPU as a nucleoside triphosphate in the acid-soluble pool and the incorporation of thymidine into phage DNA suggest that both DHPU and thymine are incorporated into SP15 DNA via their nucleoside triphosphates. 5-Fluorodeoxyuridine inhibited biosynthesis of SP15 DNA, and this inhibition was reversed by thymidine, resulting in the synthesis of a DNA containing reduced amounts of fully modified DHPU. It is proposed that 5-fluorodeoxyuridine, or its metabolic product, inhibits a step in the biosynthetic pathway to the nucleoside triphosphate of DHPU.

The DNA of *Bacillus subtilis* bacteriophage SP15 contains the unusual base 5-(4′,5′-dihydroxypentyl)uracil (DHPU), replacing approximately half the thymine (3, 18). The base is further modified with three glucose substituents, one of which is phosphodiester linked (C. Brandon, Ph.D. thesis, Yeshiva University, New York, N.Y., 1974), and this hypermodification appears responsible for the following unusual properties of the DNA: (i) an unusually high buoyant density in CsCl of 1.761 g/cm³; (ii) a very low thermal denaturation temperature in standard saline citrate of 61.5°C; and (iii) degradation of the DNA under alkaline conditions to fragments that sediment at 5.5 S (18).

It has been proposed that synthesis of the unusual base DHPU occurs at the macromolecular level after replication of phage DNA. It was suggested that uracil, incorporated into light DNA presumably via dUTP, is modified to DHPU and to thymine and that the heavy component is then added (20). However, bacteriophage DNA containing 5-hydroxymethyl- cytosine in place of cytosine is synthesized by utilizing the deoxynucleoside triphosphate of the unusual base (10), and the same is true for bacteriophage DNA containing 5-hydroxymethyluracil (23; F. Kahan, E. Kahan, and B. Riddle, Fed. Proc. 23:318, 1964). Furthermore, the nucleoside monophosphate of α-putrescinythymine, which replaces approximately half the thymine in *Pseudomonas acidovorans* phage φW14 DNA, has been detected in the acid-soluble pool of infected cells before the onset of phage DNA synthesis (13). Its presence suggests that α-pu- trescinythymine may also be synthesized as a nucleotide before its incorporation into phage DNA, although the nucleoside triphosphate has not been detected in extracts from phage-infected cells (R. A. J. Warren, personal communication).

The purpose of this paper is to report evidence suggesting that both DHPU and thymine are synthesized as deoxynucleoside triphosphates before their incorporation into SP15 DNA.

**MATERIALS AND METHODS**

Phage, bacterium, and medium. Bacteriophage SP15 was propagated on *B. subtilis* W23 in the NLM medium of Neubert and Marmur (20). Infection was at a multiplicity of 5 to 6 phage per bacterium when the culture was at an optical density of 0.6 at 550 nm (Bausch & Lomb Spectronic 20).

Materials. Bases, nucleosides, and nucleotides were obtained from Calbiochem. S1 nuclease was purchased from Miles Laboratories, Inc., and snake venom phosphodiesterase came from Worthington Biochemicals Corp. DNase I and T1 RNase were from Calbiochem, and pancreatic RNase A was from Sigma Chemical Co. 6-(p-Hydroxyphenylazo)uracil was a gift from B. W. Langley of Imperial Chemical Industries, Ltd. CsCl was obtained from Metallgesellschaft A.G., Frankfurt, West Germany. [6-3H]uracil, 32P, [5,6-3H]-uridine, and [methyl-3H]thymidine were supplied by Schwarz/Mann. [6-3H]uridine was obtained from New England Nuclear Corp.
Preparation of phage DNA. When DNA was to be prepared from infected cells, growth was rapidly stopped by adding an equal volume of frozen 0.15 M NaCl-0.1 M EDTA, pH 8.0, containing 2 × 10⁻⁴ M sodium azide. In certain instances, 6-(p-hydroxyphenylazo)uracil, an inhibitor of DNA synthesis for B. subtilis but not for SP15 (4, 22), was added after infection at a concentration of 200 μM. DNA was isolated by the Marmur procedure (17). When removal of RNA was desired, the solution was incubated with pancreatic RNase, 100 μg/ml, and T1 RNase, 10 U/ml, at 37°C for 1 h. DNA was isolated from purified phage by the phenol method (14). The phage was purified by density centrifugation essentially as described by Bøvre and Szymbalski (2).

Intermediate-density phage DNA was prepared from either infected cells or lysates to which 5-fluorodeoxyuridine (25 μg/ml)-thymidine (250 μg/ml)-deoxyadenosine (to inhibit phosphorylation of the deoxynucleosides [5, 26]) (125 μg/ml) had been added 15 min after phage infection. The large batch of intermediate-density DNA used in the renaturation experiment was prepared from 2 liters of an infected culture. Shaking was continued for 3 h after infection, and the culture was left at 37°C overnight. After clarification by centrifugation at 5,000 × g for 10 min, the lysate was incubated with DNase I, 10 μg/ml, for 1 h. Phage was concentrated by the method of Yamamoto and Alberts (29) and banded by CsCl centrifugation. The upper phage band was collected, and DNA extraction was as above. The intermediate-density DNA was further purified by preparative CsCl density gradient centrifugation.

Preparative density gradient centrifugation. Solutions of DNA in 0.02 M Tris-hydrochloride, pH 8.0, were brought to a density of 1.74 g/cm³ with CsCl. Centrifugation was in a type 50 Ti rotor at 35,000 rpm for at least 36 h at 10°C. Fractions were collected from the top with a peristaltic pump connected to a Buchler Auto Densi-Flow instrument and monitored at 254 nm with an ISCO model UA-5 absorbance monitor. If the DNA was to be processed further, CsCl was removed by dialysis against 0.015 M NaCl-0.0015 M sodium citrate, pH 7.0. Cerenkov radiation of 32P-labeled DNA was detected in a Beckman liquid scintillation spectrometer after dilution of a portion of each fraction with 2.0 ml of water.

Analytical density gradient centrifugation. The density profile of the DNA was determined by centrifugation at 42,040 rpm for 20 to 23 h at 25°C in a Beckman model E ultracentrifuge equipped with a UV scanning system. The buoyant density was determined (16) relative to a marker of Escherichia coli DNA (buoyant density in CsCl taken as 1.710 g/cm³).

Alkaline hydrolysis and trichloroacetic acid precipitation of intermediate-density DNA. Pooled fractions of intermediate-density DNA from a preparative density gradient centrifugation, after dialysis, were divided into equal portions. One portion was incubated in 0.3 N NaOH at 55°C for 46 h and then neutralized with 0.1 N HCl. To both portions, 150 μg of calf thymus DNA and an equal volume of 10% trichloroacetic acid were added. After 10 min in an ice bath, the precipitates were filtered on 0.45-μm membrane filters (Millipore Corp.) and washed with 5% trichloroacetic acid-95% ethanol. The filters were dried and counted in toluene-2,5-diphenyloxazole-1,4-bis-(5-phenyloxazolyl)benzene.

Digestion of DNA to nucleoside monophosphates. The DNA was precipitated with 2 volumes of ethanol, washed several times with 70% (vol/vol) ethanol, washed once with 95% (vol/vol) ethanol, and dried in vacuo. After solution in a minimal volume of water, the DNA was denatured by heating for 5 min at 100°C followed by quick cooling in an ice bath. Solutions of sodium acetate (pH 4.5), ZnSO₄, and NaCl were added to final concentrations of 0.03, 10⁻⁴, and 0.01 M, respectively, the S1 nuclease was added at a concentration of 1 U/μg of DNA. The solution was incubated at 55°C for 2 h, after which the pH was adjusted to 9 with 2 N NaOH. Glycine buffer, pH 9.2, was added to a concentration of 0.1 M, MgCl₂ was added to a concentration of 0.013 M, and the digest was incubated with snake venom phosphodiesterase, 0.2 U/ml of DNA, for 2 h at 37°C. The reaction was terminated by heating at 100°C for 3 min.

Thin-layer chromatography. Thin-layer sheets were washed with methanol by ascending chromatography. The solvent was brought up to a filter paper strip stapled across the top of the chromatogram. Deoxyribonucleotides were separated by two-dimensional chromatography according to Dawid et al. (8). The desalted enzymatic digest was spotted on an unmodified cellulose sheet (20 by 20 cm; EM Laboratories, Inc.) with 20 μg each of the marker deoxyribonucleotides dAMP, dCMP, dGMP, dTMP, and dUMP. Development was for 15 cm from the origin in each direction with isobutyrac acid-water-concentrated ammonium hydroxide (66:20:1) and with saturated ammonium sulfate–1 M sodium acetate–isopropanol (80:18:2). The UV-absorbing spots were excised and counted in toluene-2,5-diphenyloxazole-1,4-bis-(5-phenyloxazolyl)benzene. The migration position of the hypermodified nucleotide of DHPU was determined by autoradiography of a 32P-labeled digest of SP15 DNA, and that region of the chromatogram was also excised and counted.

The one-dimensional chromatograms were developed 15 cm from the origin with 1 M LiCl brought to pH 4.2 with boric acid for the polyethyleneimine-cellulose sheet (20 by 20 cm; Brinkmann Instruments, Inc.) or with the upper layer of ethyl acetate-water-formic acid (90:35:5) (20) for the unmodified cellulose sheet. The migration of the radioactive compounds was detected by cutting 0.25-inch (ca. 0.64 cm) strips and counting them directly in toluene-2,5-diphenyloxazole-1,4-bis-(5-phenyloxazolyl)benzene.

High-pressure liquid chromatography. A Glenco HPLC System I instrument with a 254-nm detector was used with prepackaged columns of either Partisol-10 SAX or Partisol-10 SCX (Reeve Angel) for high-pressure liquid chromatography. The system was operated at room temperature, and effluent was collected in an ISCO model 1200 fraction collector. All buffers were filtered through 0.45-μm nitrocellulose membranes (Millipore Corp.) and degassed in vacuo.

Deoxynucleoside monophosphates were separated on the Partisol SAX column operating at a flow rate of 0.84 ml/min. Up to 100 μl of sample was added after the column was equilibrated with 0.01 M potas-
sium phosphate, pH 3.15. The following elution program was followed: 0.01 M potassium phosphate, pH 3.15, 15 min; 0.02, 0.05, and 0.10 M potassium phosphate, pH 4.15, 5 min each; and 0.25 M potassium phosphate, pH 4.15, 12 min. Fractions were collected for 0.6 min, and radioactivity was determined by counting a portion of each fraction in 2.0 to 3.0 ml of PCS solubilizer (Amersham/Searle) or ScintiVerse (Fisher Scientific Co.).

Bases and/or nucleosides were separated and identified by using the Partisol SCX column; authentic compounds were located in separate trials. The column, operating at a flow rate of 0.4 ml/min, was equilibrated and eluted with 0.2 M ammonium formate-formic acid at pH 3.2. Ten microliters of a solution containing the bases uracil, thymine, guanine, cytosine, and adenine, each 100 μg/ml, was added to the sample to be injected, and elution was monitored at 254 nm. Fractions were collected for 0.4 min, and radioactivity was determined as above. Formate esters of DHPU were hydrolyzed by heating at 90°C for 15 min in 0.1 N HCl. The sample was taken to dryness in vacuo, and the residue was dissolved in 0.1 ml of water.

Charcoal desalting. Enzymatic DNA digests or column fractions were desalted on charcoal columns in Pasteur pipettes. Darco G-60 activated charcoal (Fisher Scientific Co.), which had been heated for several minutes at 100°C in 2 N HCl and washed free of acid, was added to a glass wool-plugged pipette to a height of from 0.5 to 1.0 cm. The sample was added, and the column was washed with several milliliters of water and then eluted with 50% ethanol containing 1% concentrated ammonium hydroxide (vol/vol) until elution was complete, as determined by monitoring the radioactivity of the eluate.

Formic acid hydrolysis. Desalted column fractions to be hydrolyzed were brought to dryness in vacuo in thick-walled Pyrex centrifuge tubes (17 by 100 mm). One-half milliliter of 91.4% formic acid was added, and hydrolysis was carried out as described elsewhere (20).

Extraction of acid-soluble material from infected cells. The radioactive labeling compounds were added 45 min after phage infection, and 30 min later the cultures were chilled and the cells were collected by centrifugation. An equal volume of 2 M formic acid was added to the cells (6), and the suspension was kept in an ice bath for 15 min, after which it was clarified by centrifugation at 12,000 × g for 10 min at 5°C. The supernatants labeled with [6-3H]uridine were lyophilized to dryness. The 32P, [5-6-3H]uridine doubly labeled supernatant was added to a Pasteur pipette charcoal column prepared as described above. The column was washed with 1 to 2 ml of water and eluted with 65% ethanol-0.3% concentrated ammonium hydroxide. The eluate was collected in an ice bath, maintained at a neutral pH by addition of dilute formic acid, and then lyophilized to dryness. The residues were dissolved in 0.1 ml of water for chromatography on Partisol SAX.

RESULTS

Inhibition of synthesis of bacteriophage DNA by 5-fluorodeoxyuridine. The proposal

that thymine in SP15 DNA is synthesized from uracil present in the polymerized DNA and not by the action of thymidylate synthetase suggests that 5-fluorodeoxyuridine, a potent inhibitor of thymidylate synthetase upon its conversion to 5-fluorodeoxyuridine monophosphate (7), would not affect bacteriophage replication. However, addition of 5-fluorodeoxyuridine to SP15-infected cultures of B. subtilis W23 prevented lysis of these cultures. To determine if this inhibition of lysis was due to inhibition of DNA synthesis, 32P, was added to infected cultures both with and without added 5-fluorodeoxyuridine, and the DNA was isolated and banded in CsCl. The incorporation of label into phage DNA is shown in Fig. 1A. There was no incorporation of label into DNA in the presence of 5-fluorodeoxyuridine (Fig. 1B). The addition of thymidine to a 5-fluorodeoxyuridine-inhibited culture resulted in incorporation of 32P into DNA (Fig. 1C), but, surprisingly, the newly synthesized DNA appeared to have a density intermediate between those of B. subtilis and SP15 DNAs.

Renaturation studies of intermediate-density DNA with normal phage DNA. Lysis

![Fig. 1. Inhibition of synthesis of bacteriophage DNA by 5-fluorodeoxyuridine. Deoxyadenosine, 200 μg/ml, was added 15 min after infection. At 50 min after infection, 32P, 10 μCi/ml, was added, and 20 min later growth was rapidly stopped and the cells were harvested. The DNA was extracted with no incubation with RNase and banded by density gradient centrifugation in CsCl. Density increases from left to right. (A) No further additions; (B) 5-fluorodeoxyuridine, 50 μg/ml, added 15 min after infection; (C) 5-fluorodeoxyuridine, 50 μg/ml, and thymidine, 200 μg/ml, added 15 min after infection.](http://jvi.asm.org/DownloadedFrom/502-WALKER-AND-MANDEL-J-VIROL)
of SP15-infected bacteria in the presence of 5-fluorodeoxyuridine and thymidine was delayed, and the burst size was reduced. Bacteriophage isolated from such a lysate was banded in CsCl, and phage containing the intermediate-density DNA appeared as a wide, opalescent band above the smaller, narrow band of phage with normal DNA. Phage containing the intermediate-density DNA appeared as infective as normal phage. Intermediate-density DNA was prepared from the upper phage band, and it was separated from any residual heavy DNA by CsCl centrifugation. The resulting DNA preparation, which was essentially free of heavy DNA, had a characteristic broad band in the analytical ultracentrifuge, with a peak at a density of 1.745 g/cm³.

The intermediate-density DNA and SP15 DNA were denatured both separately and together and allowed to renature. A portion of the denatured mixture was added to CsCl and chilled before renaturation. The density profiles of the renatured DNAs and the denatured mixture are given in Fig. 2. Denatured intermediate-density DNA and normal phage DNA annealed with each other to give a new band of DNA with a density midway between their two densities (Fig. 2D); hence, the same sequences are present in both DNAs.

Analysis of nucleotides in intermediate-density DNA. Alkaline hydrolysis of 32P-labeled intermediate-density DNA suggested that the DNA contained less alkali-labile phosphodiester-linked glucose (Table 1). Fractions from a CsCl gradient centrifugation of 32P-labeled intermediate-density DNA were hydrolyzed, and fractions 15 to 17, which contained chiefly heavy DNA, released a greater percentage of counts to the acid-soluble material than did fractions 9 to 11 and 12 to 14, which contained intermediate-density DNA. The alkali-labile phosphate of fractions 15 to 17 compares well with amounts previously reported in SP15 DNA (18).

To determine whether the reduced density of the intermediate-density DNA was due to a decrease only in the phosphodiester-linked glucose of the DNA, labeled intermediate-density DNA was enzymatically digested to its deoxy-nucleoside monophosphates. Two-dimensional thin-layer chromatography of the digest did not indicate the presence of a new deoxyribonucleoside monophosphate, such as dDHPUMP or the mono- or diguanylated derivatives of dDHPUMP.

To compare the nucleotide composition of intermediate and normal phage DNAs, both DNAs were labeled with [6-3H]uracil and enzymatically digested to their deoxyribonucleoside monophosphates. Figure 3 shows the high-pres-

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**FIG. 2. Analytical CsCl density gradient centrifugation of renatured SP15 and intermediate-density DNAs.** The DNAs in 0.3 M NaCl-0.03 M sodium citrate, pH 7.0, were heated at 100°C for 5 min and, with the exception of (C), slowly cooled to 37°C and allowed to renature for 4 h. E. coli DNA, 0.5 μg/ml, with a density of 1.710 g/cm³, was added as a marker. (A) 2.8 μg of intermediate-density DNA; (B) 2.0 μg of SP15 DNA; (C) 1.75 μg each of SP15 DNA and intermediate-density DNA added to CsCl and chilled immediately after denaturing; (D) 1.75 μg each of SP15 and intermediate-density DNAs.

**TABLE 1. Release of 32P from intermediate-density SP15 DNA on alkaline hydrolysis**

<table>
<thead>
<tr>
<th>Fraction*</th>
<th>Precipitated by acid</th>
<th>Released to solubles (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Alkaline hydrolyzed</td>
</tr>
<tr>
<td>6–8</td>
<td>43,374</td>
<td>39,712</td>
</tr>
<tr>
<td>9–11</td>
<td>121,153</td>
<td>113,751</td>
</tr>
<tr>
<td>12–14</td>
<td>91,650</td>
<td>87,310</td>
</tr>
<tr>
<td>15–17</td>
<td>45,665</td>
<td>37,878</td>
</tr>
</tbody>
</table>

* Numbers represent the pooled fractions from a preparative CsCl gradient of 32P-labeled intermediate-density DNA prepared as in the text. 6-(p-Hydroxyphenylazo)uracil (200 μM) was added at the time of infection.

sure liquid chromatographic profile of the two digests, and the decreased quantity of the hypermodified nucleotide of DHPU (dDHPUMPglc3P) in the intermediate-density DNA digest is readily apparent. The absorption profiles of the two digests also indicate relatively increased dTMP in the intermediate-density
DNA digest. It can be concluded that the intermediate-density DNA contains less hypermodified DHPU and increased thymine.

**Incorporation of thymidine into SP15 DNA.** The inhibition of synthesis of SP15 DNA by 5-fluorodeoxyuridine suggests that synthesis of dTMP is indeed necessary for bacteriophage DNA replication. The fact that this inhibition is reversed by thymidine and the results in Fig. 3, which show no label in dTMP of phage DNA replicated in the presence of labeled uracil, unlabeled thymidine, and 5-fluorodeoxyuridine, suggest that thymidine is incorporated into phage DNA. To test this possibility, [methyl-3H]thymidine was added to a culture 50 min after phage infection, and 20 min later the cells were harvested and the DNA was prepared. This is the time period during which phage DNA synthesis occurs most rapidly (20). The phage DNA, which was well labeled, was separated from bacterial DNA, which had only a trace of counts, by CsCl centrifugation, and, after enzymatic digestion of the DNA, the deoxynucleotides were chromatographed on a thin-layer sheet and the labeling of each was determined (Table 2). Label from [methyl-3H]thymidine was incorporated almost exclusively into the dTMP of bacteriophage DNA.

Confirmation of the incorporation of thymidine into SP15 DNA was obtained by preparing phage DNA containing 5-bromouracil. An infected culture with 5-fluorodeoxyuridine, 5-bromodeoxyuridine, and deoxyadenosine added to the medium was allowed to lyse, and the DNA was prepared from the purified phage. Phage DNA prepared in the same way, but with thymidine added to the culture medium instead of 5-bromodeoxyuridine, has a buoyant density in CsCl of 1.745 g/cm³. The DNA labeled with 5-bromodeoxyuridine had a broad band in the analytical ultracentrifuge with peaks at 1.791 and 1.800 g/cm³.

**Search for uracil in SP15 DNA.** The presence of uracil in SP15 DNA would be compatible with the suggestion that DHPU is synthesized at the macromolecular level from uracil in the DNA. Therefore, DNA from both mature and replicating phage was assayed for uracil. [6-3H]uracil-labeled DNA from mature phage was enzymatically digested, and the deoxynucleotides were separated by thin-layer chromatography and their labeling was determined (Table 2). As expected, dCMP, dTMP, and dDHPUMPglc3P were all labeled by [6-3H]uracil, but there were negligible counts at the migration position of dUMP.

DNA from replicating phage was prepared from an infected culture pulse-labeled for 2 min with [6-3H]uracil at the time of maximal DNA synthesis. In none of our pulse-labeling experiments were we able to detect any light SP15 DNA. The deoxynucleotides from the enzymatic digest of the DNA were separated by high-pressure liquid chromatography on Partisil SAX. Since dTMP and dUMP are not well resolved by this method, tubes containing the radioactive peaks in the area of their elution were pooled in two portions, one containing “early peak”

TABLE 2. Incorporation of label from [methyl-3H]thymidine and from [6-3H]uracil into nucleoside monophosphates of SP15 DNA

<table>
<thead>
<tr>
<th>Nucleotide*</th>
<th>3H cpm recovered</th>
<th>from [methyl-3H]thymidine</th>
<th>from [6-3H]uracil</th>
</tr>
</thead>
<tbody>
<tr>
<td>dAMP</td>
<td>357</td>
<td>335</td>
<td></td>
</tr>
<tr>
<td>dCMP</td>
<td>42</td>
<td>6,253</td>
<td></td>
</tr>
<tr>
<td>dGMP</td>
<td>493</td>
<td>144</td>
<td></td>
</tr>
<tr>
<td>dTMP</td>
<td>29,027</td>
<td>4,428</td>
<td></td>
</tr>
<tr>
<td>dDHPUMPglc3P</td>
<td>404</td>
<td>7,962</td>
<td></td>
</tr>
<tr>
<td>dUMP</td>
<td>ND</td>
<td>89</td>
<td></td>
</tr>
</tbody>
</table>

* Purified SP15 DNA enzymatically digested to mononucleotides was chromatographed in two dimensions as described in the text. Spots were excised and counted in toluene-based scintillation fluid.

** Fifty minutes after infection, [methyl-3H]thymine, 6.7 μCi/ml, was added. Infected cells were harvested 20 min later, and DNA was isolated and centrifuged to equilibrium in CsCl; the DNA at a density of 1.76 g/cm³ was collected, dialyzed, and digested.

* [6-3H]uracil, 2.5 μCi/ml, was added 40 min after phage infection. After lysis, phage particles were purified, and the DNA was isolated and enzymatically digested.

* ND, Not detected.
fractions and the other containing “late peak” fractions. Tubes containing dCMP were also pooled, and all three samples were subjected to formic acid hydrolysis. The resulting bases were chromatographed on Partisil SCX, and their positions of elution were compared with those of added standard bases (Fig. 4). In neither the early-peak fractions (Fig. 4B) nor the late-peak fractions (Fig. 4C) was there evidence of any uracil; however, uracil was detected in the hydrolysate of dCMP, due to its deamination during acid hydrolysis. These results indicate that uracil is not present in the DNA of either replicating or mature SP15.

**Attempt to incorporate label from the deoxynucleoside of DHPU into SP15 DNA.** Neubort and Marmur had reported that DHPU is not incorporated into SP15 DNA (20). However, SP8-infected cells can incorporate the nucleoside 5-hydroxymethyldeoxyuridine, but not the base 5-hydroxymethyluracil, into phage DNA (21). Therefore, the deoxynucleoside of DHPU was prepared to determine whether it can be incorporated into phage DNA. The fully modified nucleotide of DHPU was isolated by high-pressure liquid chromatography of an enzymatic digest of [6-3H]uracil, D-[U-14C]glucose-labeled phage DNA. The phosphodiester-linked glucose was removed by alkaline hydrolysis, giving the diglucosylated derivative, and the nucleoside was prepared by incubation with E. coli alkaline phosphatase. After purification of the diglucosylated deoxynucleoside by high-pressure liquid chromatography, the remaining two glucoses were removed by incubation with α-glucosidase. The resulting deoxynucleoside of DHPU was added to a phage-infected culture in two separate experiments, and there was no detectable incorporation of radioactivity into bacteriophage DNA in either case.

**Presence of the nucleoside triphosphate of DHPU in the acid-soluble pool of SP15-infected bacteria.** Although the deoxynucleoside of DHPU was not incorporated into SP15 DNA, the presence of DHPU, especially at the triphosphate level, in the acid-soluble pool of infected cells would indicate its availability for incorporation into phage DNA. [6-3H]Uridine-labeled pools were extracted from both infected and uninfected bacteria and chromatographed on Partisil SAX (Fig. 5). Tube 9 of the infected-cell pool contained a sharp peak that was not present in the pool from uninfected cells. The formic acid hydrolysate of the contents of tube 9 was chromatographed on Partisil SCX both before and after heating in 0.1 N HCl (Fig. 6).

**Fig. 4.** High-pressure liquid chromatography of bases from formic acid hydrolysis of deoxynucleoside monophosphates from [6-3H]uracil-pulse-labeled phage DNA. An SP15-infected culture, containing 200 μM 6-(p-hydroxyphenylazo)uracil, was pulse-labeled with [6-3H]uracil, 100 μCi/ml, for 2 min. The DNA was enzymatically digested to its deoxynucleoside monophosphates, which were separated on Partisil SAX, and the specified peaks, after formic acid hydrolysis, were chromatographed on Partisil SCX. (A) Formic acid hydrolysates of dCMP peak; (B) formic acid hydrolysate of dTMP-dUMP early-peak fractions; (C) formic acid hydrolysate of dTMP-dUMP late-peak fractions.

**Fig. 5.** High-pressure liquid chromatography of acid-soluble extracts from [6-3H]uridine-labeled bacterial and phage-infected cultures. The cultures were labeled for 30 min with [6-3H]uridine, 5 μCi/ml. Extracts were prepared, and chromatography on Partisil SAX was performed as described in the text for deoxynucleoside monophosphates with the following modification: the 12-min elution with 0.25 M potassium phosphate, pH 4.15, was followed by 20 min of elution with 1.0 M potassium phosphate, pH 4.15. (A) Bacterial extract; (B) phage-infected bacterial extract.
Formic acid hydrolysis of nucleosides or nucleotides of hydroxalkylated pyrimidines yields their formate ester, which can be hydrolyzed by heating in 0.1 N acid (M. Mandel and M. S. Walker, unpublished data). Since DHPU has two hydroxyls, both mono- and diformate esters are formed, and unequivocal identification of DHPU can be readily achieved by chromatography of formic acid hydrolysates before and after acid hydrolysis. The formic acid hydrolysate of tube 9 shows the typical profile of DHPU. The monoformate esters elute between the positions of thymine and guanine, whereas the diformate ester elutes between guanine and cytosine. After 0.1 N acid hydrolysis, there is only one peak, which elutes at the position of DHPU. As a further confirmation of the identification, the formic acid hydrolysate of tube 9 was chromatographed on a thin-layer sheet before and after 0.1 N HCl hydrolysis and compared with labeled DHPU prepared from the purified hypermodified DHPU nucleotide from SP15 DNA (Fig. 7). The two preparations exhibited identical profiles.

The identification of the peak in tube 9 as DHPU demonstrated the presence of DHPU in the acid-soluble pool of infected cells. However, nucleoside triphosphates elute quite late on Partisil SAX. To facilitate detection of DHPU as a nucleoside triphosphate, the phage-infected pool was doubly labeled with $^{32}$P$_{i}$ and [6-3H]joridine, and more precautions were taken to minimize conditions under which triphosphates would be hydrolyzed. The elution profile of the pool on Partisil SAX is given by Fig. 8. Portions of the contents of the tubes containing the tritium-labeled peak in the triphosphate region of elu-
tion were hydrolyzed by formic acid. Tubes 90 and 91 exhibited the typical elution profile of DHPU (Fig. 9); an unidentified compound that eluted early was also present in tube 90.

To show that the DHPU was present as a nucleoside triphosphate, portions of the contents of tubes 90 and 91 were hydrolyzed in 1 N HCl at 100°C for 7 min. Thin-layer chromatography of tube 90 on polyethyleneimine-cellulose both before and after 7 min of hydrolysis is given in Fig. 10. The release of Pi on 7 min of acid hydrolysis is seen in the lower part of the figure, and the decrease in 32P labeling of the tritium-labeled compound is consistent with its being a monophosphate after hydrolysis. Also, its migration on the ion-exchange sheet after 7 min of acid hydrolysis is as would be expected for a less charged compound. Similar results were obtained for tube 91, chromatographed on Partisil SAX before and after hydrolysis (Fig. 11). A small quantity of base or nucleoside can be seen eluting early in the 7-min acid-hydrolyzed preparation, with dDHPU eluting near where dTMP and dUMP elute. From the above results, it can be concluded that DHPU is present as a nucleoside triphosphate in SP15-infected bacteria.

**DISCUSSION**

The presence of the nucleoside triphosphate of DHPU in the acid-soluble extract of SP15-

![Fig. 9. High-pressure liquid chromatography of the formic acid hydrolysate of 3H- and 32P-labeled fractions from Partisil SAX chromatography of the acid-soluble pool from infected cells. Fractions are those indicated as dDHPUTP in Fig. 8. □, Formic acid hydrolysate; ○, after 0.1 N HCl hydrolysis.](image)

![Fig. 10. Polyethyleneimine (PEI)-cellulose thin-layer chromatography of 3H- and 32P-labeled fraction 90 before and after acid hydrolysis. Desalted fraction 90 from Partisil SAX chromatography (Fig. 8) was spotted both before and after hydrolysis in 1 N HCl at 100°C for 7 min. The acid hydrolysate was taken to dryness in vacuo and dissolved in water before spotting.](image)

![Fig. 11. Partisil SAX chromatography of 3H- and 32P-labeled fraction 91 from Partisil SAX chromatography (Fig. 8) was applied both before and after hydrolysis in 1 N HCl at 100°C for 7 min. The acid hydrolysate was taken to dryness in vacuo and dissolved in water before being added to the column. High-pressure liquid chromatography was as for Fig. 8.](image)
label from the nucleoside of DHPU into phage DNA is not necessarily inconsistent with this conclusion, since phage T4, which incorporates 5-hydroxymethylcytosine into its DNA from 5-hydroxymethyldeoxycytidine triphosphate, is unable to utilize 5-hydroxymethyldeoxycytidine (19). H. Witmer and M. Dosmar (personal communication) have not been able to detect an enzyme activity in extracts of SP15-infected cells capable of phosphorylating the deoxynucleoside of DHPU.

The incorporation of thymidine into SP15 DNA and our inability to demonstrate the presence of uracil in phage DNA further decrease the likelihood that uracil in the phage DNA is modified to either DHPU or thymine. The incorporation of thymidine into phage DNA was readily demonstrated when thymidine was added during phage DNA synthesis. In earlier experiments (20), thymidine was added at the time of phage infection, and it is possible that, during the long latent period before phage DNA synthesis began, all the thymidine was phosphorylated to thymidine and deoxyribose-1-phosphate. Previous reports of the presence of uracil in SP15 DNA (20) could have been due to the detection of formate esters of DHPU, which occur in formic acid hydrolysates of DHPU-containing DNA and which migrate near uracil in the thin-layer systems used. Deamination of cytosine during hydrolysis also results in the formation of a small quantity of uracil.

The inhibition of SP15 DNA synthesis by 5-fluorodeoxyuridine and its reversal by thymidine are also consistent with the synthesis of thymine before its incorporation into phage DNA. It was interesting that the DNA synthesized in the presence of 5-fluorodeoxyuridine and thymidine differed from normal phage DNA in density and base composition. The renaturation studies of this intermediate-density DNA with normal-density phage DNA indicated the presence of completely complementary sequences in the two DNAs and that, therefore, the intermediate-density DNA was not the result of the induction of a defective phage. The discovery that the intermediate-density DNA contained less hypermodified DHPU and increased thymine suggests that 5-fluorodeoxyuridine, or, more likely, 5-fluorodeoxyuridine monophosphate, inhibits a step in the biosynthetic pathway of the nucleoside triphosphate of DHPU, although other explanations, such as an effect on the DNA polymerase, are not ruled out. A determination of the relative pool sizes of the nucleoside triphosphate of DHPU in cells infected both with and without added 5-fluorodeoxyuridine and thymidine should help to resolve this question.

The mechanism that determines which base, thymine or DHPU, is chosen to pair with adenine during replication of SP15 DNA is unknown, and the possibility exists that the mechanism is dependent on pool size. It has been shown that the synthesis of pyrimidine nucleotides by T4 bacteriophage is regulated at the same ratio as their occurrence in DNA (11). If such controls operate for nucleotide synthesis in SP15, it is possible that the quantities of DHPU and thymine in SP15 DNA are determined by the relative synthesis of the nucleoside triphosphates of these two bases. Whether the incorporation of DHPU and thymine into the DNA occurs at specific sites in the DNA is also unknown.

The function of the hypermodified base in SP15 DNA remains unclear. In recent years, there have been reports of restriction enzymes in some strains of B. subtilis (1, 24, 25), and it is possible that glucosylation of SP15 DNA protects the DNA from such enzymes; preliminary results have demonstrated that SP15 DNA is resistant to the action of the restriction endonuclease EcoRI, BamHI, Sal I, Hpa II, and HindIII (M. Mandel, S. Lloyd, and N. Lapeyre, unpublished data). It has been shown that glucosylation of T-even phage DNA protects that DNA from degradation by host-cell nucleases (22). Also, the presence of 5-hydroxymethylcytosine in T-even phage DNA protects the DNA from phage-induced nucleases (27). However, since SP15 is a generalized transducing phage that transfers a larger fragment of the Bacillus genome than does any other known phage (26), degradation of bacterial DNA in SP15-infected cells cannot be very extensive, and protection against phage-induced nucleases specific for DNA with no base substitutions appears unnecessary. It can be speculated that the modified base may also function in controlling transcription; recent work indicates two discrete waves of RNA synthesis in SP15-infected cells (9).

Transcription of late protein messengers does not occur from T-even phage DNA containing cytosine (12, 15). Another possibility is that there exists an interaction between the protein coat molecules and the hypermodified DNA that determines that phage DNA is packaged preferentially in the presence of the degraded bacterial DNA. The ability to grow phage in the presence of 5-fluorodeoxyuridine and thymidine with DNA having an altered ratio of hypermodified DHPU to thymine should provide an experimental approach that could aid in answering some of the above questions.

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LITERATURE CITED


