5-[(Hydroxymethyl)-*O*-pyrophosphoryl]uracil, an Intermediate in the Biosynthesis of α-Putrescinylthymine in Deoxyribonucleic Acid of Bacteriophage φW-14†

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ABSTRACT: In a nonpermissive host, an amber mutant, *am* 37, of bacteriophage φW-14 synthesizes deoxyribonucleic acid (DNA) of considerably greater buoyant density than the DNA synthesized by wild-type phage. The *am* 37 DNA lacks the hypermodified pyrimidine, α-putrescinylthymine (putThy). Instead, it contains a new modified base, 5-[(hydroxymethyl)-*O*-pyrophosphoryl]uracil (hmPPUra). Extracts of cells infected with wild-type φW-14 convert the hmPPUra in *am* 37 DNA to putThy when incubated with putrescine.

In the deoxyribonucleic acid (DNA)[1] of bacteriophage φW-14, half of the thymine residues are replaced with α-putrescinylthymine (putThy)[1] (Kropinski et al., 1973). Thymine and putThy are formed from (hydroxymethyl)uracil (hmUra) at the polynucleotide level (Neuhard et al., 1980). These modifications of hmUra are coupled tightly to DNA replication, and intermediates between hmUra and thymine or putThy have not been detected in replicating DNA of cells infected with wild-type φW-14 (Neuhard et al., 1980).

The isolation of amber suppressor mutants of the host, *Pseudomonas acidovorans* (Warren, 1979), has allowed the isolation of amber mutants of φW-14 (P. Miller, unpublished results). In this paper we describe the isolation and identification of a derivative of hmUra that accumulates in the replicating DNA of one of these mutants and the conversion of this compound to putThy by extracts of cells infected with wild-type φW-14.

Materials and Methods

Organisms. Wild-type φW-14 (Kropinski & Warren, 1970) was propagated on *P. acidovorans* strain 29 (ATCC 9355; Stanier et al., 1966). The amber mutant, *am* 37, was propagated on strain *sup*2, a *sup* derivative of strain 29 (Warren, 1979). Strain 3L, a thymidine auxotroph of strain 29 (Kelln & Warren, 1973a), was used for the tritium-release experiments. All biochemical experiments were performed at a multiplicity of infection of 20 and an exponential culture density of 3×10^8 cells mL^{-1}.

Medium. Tris–casamino acids–succinate medium (Lewis et al., 1975) was used throughout. For labeling infected cells with [6-^{3}H]uracil, the phosphorus content was ~20 μg mL^{-1}, and for labeling with ^{32}P, the content was ~5 μg mL^{-1}.

Labeling and Isolation of DNA. The procedures for the labeling, extraction, and purification of replicating phage DNA were described previously (Maltman et al., 1980).

Release of Tritium from [5-^{3}H]Uracil. The release of tritium from [5-^{3}H]uracil was measured by using charcoal adsorption (Tomich et al., 1974).

Analysis of DNA. Conditions for the isopycnic density gradient centrifugation of DNA and for its hydrolysis with acid were described previously (Maltman et al., 1980).

Enzymatic Digestion of DNA. The amount of DNA was determined spectrophotometrically, and then it was precipitated with 2 volumes of 95% ethanol, washed several times with 70% ethanol, once with 95% ethanol, and several times with ether, and then air-dried. It was redissolved in the minimal volume of sterile distilled, deionized water and then boiled for 5 min to inactivate residual nucleases and to ensure denaturation of the DNA. Ammonium acetate, pH 5.0, was added to give a final concentration of 50 mM; $ZnSO_4$ was added to a final concentration of 0.1 mM, and 10 units of S_1 nuclease was added/μg of DNA. The solution was incubated for 4 h at 55 °C and then lyophilized. The residue was suspended in deionized distilled water and lyophilized again. The residue was suspended in the minimal volume of deionized distilled water, and NH_4HCO_3 (pH 8.4) and $MgCl_2$ were added to final concentrations of 100 and 15 mM, respectively. Snake venom phosphodiesterase was added (final concentration 20 μg mL^{-1}) and the sample incubated at 37 °C for 2 h.

Limit digestion of DNA with DNase I and snake venom phosphodiesterase was performed as follows. The DNA was treated as described above for S1–snake venom phosphodiesterase digestion. The dried sample was dissolved in sterile distilled water. Tris-HCl, pH 8.2, and $MgCl_2$ were added to give final concentrations of 50 and 15 mM, respectively. Then 20 μg of each enzyme was added/mL of DNA solution, and the mixture was incubated at 37 °C for 4 h.

The nucleotides released were separated by thin-layer chromatography and detected by fluorography (Randerath, 1969) for ^{3}H-labeled samples or by radioautography for ^{32}P-labeled samples.

Thin-Layer Chromatography. Enzymatic digests were spotted directly on sheets of unmodified cellulose (Eastman Chromogram 6064, without fluorescent indicator). The sheets were washed twice by ascending development with 95% ethanol to remove inorganic salts. Mononucleotides were separated by development with solvent E in the first dimension followed by solvent A in the second dimension. The procedures for the

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[1] Abbreviations used: DNA, deoxyribonucleic acid; putThy, α-putrescinylthymine; putdTMP, α-putrescinyldeoxythymidine 5′-monophosphate; hmUra, (hydroxymethyl)uracil; hmdUrd, (hydroxymethyl)-deoxyuridine; hmPPUra, 5-[(hydroxymethyl)-*O*-pyrophosphoryl]uracil; hmPPdUMP, 5-[(hydroxymethyl)-*O*-pyrophosphoryl]deoxyuridine 5′-monophosphate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; Cl_3CCOOH, trichloroacetic acid; RNA, ribonucleic acid.

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separation of the bases released from DNA by acid hydrolysis were described previously (Maltman et al., 1980).

Solvents for Thin-Layer Chromatography. The solvents were as follows: (A) saturated $(NH_4)_2SO_4$–1 M sodium acetate–2-propanol (80:12:2 v/v); (B) *tert*-butyl alcohol–2-butanone–concentrated HCl–water (40:30:10:20 v/v); (C) 1-butanol–water (86:14 v/v); (D) 1-butanol–water–concentrated NH_4OH (86:9:5 v/v); (E) isobutyric acid–water–concentrated NH_4OH (66:20:1 v/v).

Column Chromatography. ^{32}P-Labeled *am* 37 DNA was digested to mononucleotides with S1 nuclease and snake venom phosphodiesterase. The digest was diluted to 3 mL with 20 mM Tris-HCl buffer, pH 7.5. Sufficient dTDP and dTTP were added to give final concentrations of ~75 μg mL^{-1}. The digest was applied to a DEAE-Sephadex A-25 column (1 × 40 cm). The resin was washed with 1 bed volume of 20 mM Tris-HCl, and then the nucleotides were eluted with a linear gradient of NaCl (0–0.4 M, total volume 500 mL) in 20 mM Tris-HCl–7 M urea, pH 7.5. Fractions of 5 mL were collected and assayed for Cerenkov radiation and for absorbance at 267 nm.

Purification of Unknown Nucleotide. Large-scale S1–snake venom phosphodiesterase digests of *am* 37 replicating DNA were applied as a band 9 cm from the top of a sheet of Whatman SFC-40 filter paper (40 × 20 cm). The sheet was washed twice by descending development with 95% ethanol. It was developed for 24 h by descending chromatography with solvent E. After radioautography to locate the nucleotides, strips containing them were cut from the sheets and washed with 95% ethanol by descending chromatography. The nucleotides were recovered by descending elution of the strips with distilled water.

Preparation of Cell-Free Extracts. Strain 29 was grown to a cell density of 3 × 10^8 mL^{-1}, and then infected with phage. The cells were collected by centrifugation (3000*g* × 5 min at ambient temperature) 35 min after infection, washed once by centrifugation with the growth volume of buffer (50 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, 10 mM $MgCl_2$, and 50 mM KCl), and then resuspended at 5 × 10^9 mL^{-1} in the same buffer. Lysozyme was added to a final concentration of 1 mg mL^{-1}. The suspension was incubated at 30 °C for 30 min and then frozen and thawed rapidly 3 times. The resulting extract was passed several times through a 26-gauge needle to reduce the viscosity.

In Vitro Synthesis of putThy. Reaction mixtures contained 30 μL of putrescine (10 mM), 30 μL of crude extract, 30 μL of labeled *am* 37 DNA (400 μg mL^{-1}), and 210 μL of buffer (see above). At intervals during incubation at 30 °C, 50-μL samples were removed to 0.5 mL of ice-cold 0.01 M Tris-HCl, 0.15 M NaCl, and 0.01 M EDTA, pH 7.5, containing 100 μg of unlabeled φW-14 DNA. Cold 95% ethanol (2 volumes) was added to the samples. After the solution stood on ice for 30 min, the DNA was collected by centrifugation, and 500 μL of the supernatant was removed for the determination of released radioactivity. The DNA was digested and the radioactivity in the mononucleotides was determined as described above.

Chemicals and Enzymes. All chemicals were of reagent grade. Reference bases, nucleosides, nucleotides, and DNase 1 were from Calbiochem-Behring Corp., La Jolla, CA; nuclease S1 was from Miles Laboratories, Elkhart, IN; snake venom phosphodiesterase and bacterial alkaline phosphatase were from Millipore, Montreal, Canada.

Results

Isolation of am 37. The proportions of thymine and putThy in φW-14 DNA appear to be rigidly controlled because they do not vary (K. L. Maltman, unpublished observations). Therefore, we assumed that phage mutants which were unable to modify replicating DNA normally would be unable to form viable progeny under nonpermissive conditions. Accordingly, a number of temperature-sensitive (K. L. Maltman, unpublished observations) and amber (P. Miller, unpublished observations) mutants of φW-14 were screened for defects in DNA modification. Wild-type φW-14 DNA is of unusually low buoyant density (1.666 g cm^{-3}) because of the putThy residues (Kropinski et al., 1973), so mutant-infected cultures of strain 29 were screened for the accumulation of DNA of buoyant density >1.666 g cm^{-3}. Of the mutants examined, *am* 37 was of particular interest.

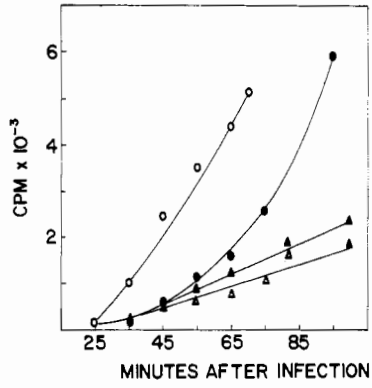

FIGURE 1: DNA synthesis by phage-infected cells. The incorporation of [6-^{3}H]uracil into alkali-resistant, Cl_3CCOOH-insoluble material was determined for wild-type phage in strain 29 (○) and in strain *sup*2 (●) and for *am* 37 in strain 29 (△) and in *sup*2 (▲).

Conditionally Lethal Nature of am 37. Although *am* 37 infected strain 29 cells synthesized DNA (Figure 1), this mutant was conditionally lethal. The burst size in strain 29 was <1; in strain *sup*2 it was ~50. The normal burst size is ~300 (Kropinski & Warren, 1970). This difference in burst size was greater than the difference in DNA synthesis shown by *am* 37 and wild-type phage in strain *sup*2 (Figure 1). The density of the DNA formed by *am* 37 in strain 29 by 45 min after infection was much greater than 1.666 g cm^{-3} (Figure 2). The multiple bands probably represented molecules containing two progeny strands, one parental and one progeny strand, and sometimes host DNA synthesized by cells surviving the infection. The density of the DNA became more homogeneous as the infection progressed: by 75 min after infection it was all of the greatest density shown in Figure 2C. The exact nature of this DNA is being determined.

[5-^{3}H]Uracil can be used to measure both the synthesis of DNA and the synthesis of the 5-substituted bases derived from uracil (Tomich et al., 1974). Strain 3L is a thymidine auxotroph of strain 29 (Kelln & Warren, 1973a), so it does not release tritium from [5-^{3}H]uracil. φW-14 shuts off host DNA synthesis by ~10 min and phage DNA synthesis starts ~20 min after infection [Figure 3 and Kelln & Warren (1973b), Maltman et al., 1980]. The release of tritium from [5-^{3}H]-uracil starts ~20 min after infection of strain 3L with wild-type φW-14.

In strain 3L cells infected with wild-type φW-14, the rates of tritium release and of the incorporation of uracil into DNA were the same throughout the infective cycle (Figure 3). In 3L cells infected with *am* 37, however, the rate of incorporation slowed relative to the rate of tritium release as the infection progressed (Figure 3).

Base Composition of am 37 DNA. The DNA synthesized by *am* 37 infected *sup*2 cells contained a small amount of hmUra and almost normal levels of thymine and putThy

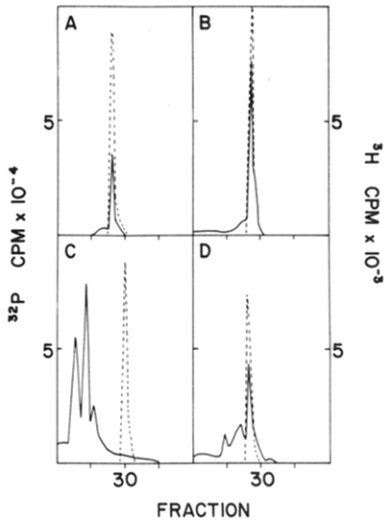

FIGURE 2: Buoyant density of DNA synthesized by phage-infected cells. Infected cells were labeled with [6-^{3}H]uracil, and the DNA was extracted from the cells 35 min after infection. Strain 29 infected with wild-type phage (A) and with *am* 37 (C); strain *sup*2 infected with wild-type phage (B) and with *am* 37 (D). (—) [6-^{3}H]Uracil-labeled DNA; (---) ^{32}P reference DNA from phage particles. The bottom of the gradient is on the left.

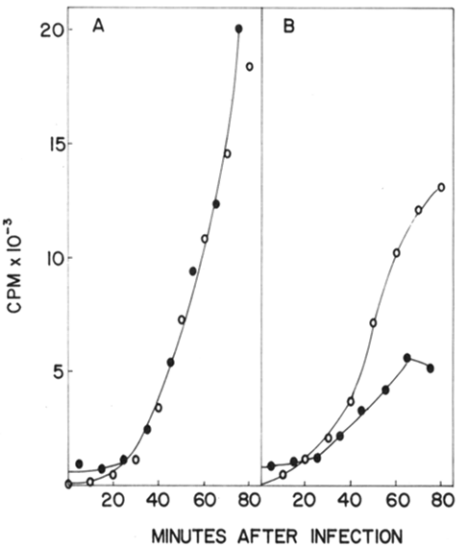

FIGURE 3: DNA synthesis and tritium release by infected cultures. Infected cells of strain 3L were labeled with [5-^{3}H]uracil. Samples were assayed for the incorporation of label into alkali-resistant, Cl_3CCOOH-insoluble material (●) and for the release of tritium (○). (A) Wild-type phage; (B) *am* 37.

Table I: Base Compositions of ϕW-14 and *am* 37 DNAs[a]

base	source of DNA: *am* 37/ strain 29	*am* 37/ strain *sup*2	ϕW-14/ strain 29
putThy	1 074[b] (2.7)[c]	5 062 (19.9)	14 430 (18.2)
cytosine	18 016 (44.5)	10 539 (41.5)	34 585 (43.6)
hmUra	5 903 (14.6)	1 028 (4.0)	157 (0.2)
uracil	1 209 (3.0)	176 (0.7)	180 (0.2)
thymine	9 353 (23.1)	6 173 (24.3)	21 976 (27.7)
unhydrolyzed nucleotides	4 964 (12.3)	2 441 (9.6)	7 987 (10.1)

[a] Replicating DNA was labeled with [6-^{3}H]uracil. After hydrolysis, the bases were separated by thin-layer chromatography, using solvent B in the first and solvent D in the second dimension. [b] cpm in the area cut from the chromatogram. [c] The figures in parentheses are the percentages of the total radioactivity recovered from the chromatogram. Recoveries were routinely >90%.

(Table I). The presence of hmUra, usually barely detectable in replicating ϕW-14 DNA (Neuhard et al., 1980), presumably reflected the efficiency of the amber suppressor mutation employed. The DNA synthesized in strain 29 cells contained the normal amount of thymine, only a small amount of putThy and a considerable amount of hmUra (Table I).

Nucleotide Composition of am 37 DNA. The DNA synthesized by *am* 37 infected *sup*2 cells contained dTMP, α-putrescinyl dTMP (putdTMP), and traces of hmdUMP and

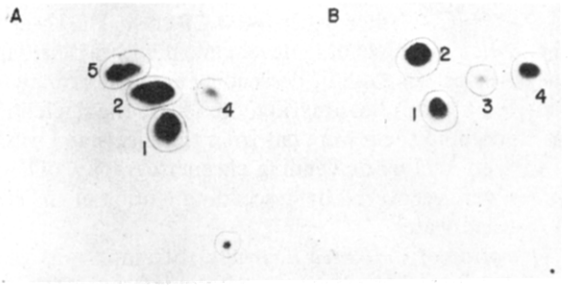

FIGURE 4: Nucleotides in *am* 37 DNA. Cells were labeled with [6-^{3}H]uracil after infection with *am* 37. The DNA was extracted from the cells at 45 min after infection and digested to mononucleotides which were separated by thin-layer chromatography. (A) Strain *sup*2 as host; (B) strain 29 as host. (1) dTMP; (2) dCMP; (3) hmdUMP; (4) unknown nucleotide; (5) putdTMP.

of an unknown nucleotide (Figure 4A and Table II). The DNA synthesized by infected strain 29 cells contained dTMP but only traces of putdTMP; the content of hmdUMP was increased, and that of the unknown nucleotide was increased markedly (Figure 4B and Table II). The unknown nucleotide represented a greater proportion of the ^{32}P label than of the ^{3}H label, suggesting that it contained extra phosphates. The hmdUMP and the unknown nucleotide were purified by paper chromatography. The only pyrimidine released from both compounds by acid hydrolysis was hmUra. This confirmed the presence of hmdUMP in *am* 37 DNA and showed that

Table II: Nucleotide Composition of ϕW-14 and *am* 37 DNAs[a]

nucleotide	source of DNA: *am* 37/strain 29 ([6-^{3}H]uracil)	*am* 37/strain 29 ([^{32}P]orthophosphate)	*am* 37/strain *sup*2 ([6-^{3}H]uracil)	ϕW-14/strain 29 ([^{32}P]orthophosphate)
dGMP	40[b] (0.1)[c]	3 944 (16.1)	0	13 355 (24.1)
dAMP	72 (0.2)	3 012 (12.3)	0	13 921 (25.1)
dTMP	8 080 (27.6)	2 972 (12.1)	41 218 (27.0)	7 219 (13.0)
dCMP	13 252 (45.2)	5 943 (24.2)	81 876 (53.7)	14 007 (25.2)
putdTMP	385 (1.3)	87 (0.4)	27 835 (18.3)	6 904 (12.4)
hmdUMP	2 043 (7.0)	865 (3.5)	256 (0.2)	29 (<0.1)
unknown	5 430 (18.5)	7 750 (31.5)	1 264 (0.8)	47 (<0.1)

[a] Replicating DNA was labeled with [6-^{3}H]uracil or [^{32}P]orthophosphate. After digestion with nuclease S1 and snake venom phosphodiesterase, the mononucleotides were separated by thin-layer chromatography. [b] cpm in the area cut from the chromatogram. [c] The figures in parentheses are the percentages of the total radioactivity recovered as nucleotides.

Table III: Properties of Unknown Nucleotide[a]

treatment[b]	unknown nucleotide			hmdUMP				
	^3H	^{32}P	$^{32}P/^3H$	^3H	^{32}P	$^{32}P/^3H$	P_i[c]	hmdUra
none	2298	6759	2.9					
1 N HCl, 37 °C, 30 min				1903	2040	1.1	4626	
1 N HCl, 98 °C, 7 min				1978	2334	1.2	4550	
alkaline phosphatase							6292	2018

[a] The DNA from which the nucleotides were purified was labeled with [6-^{3}H]uracil and [^{32}P]orthophosphate. [b] The products were identified by thin-layer chromatography with known standards. [c] P_i = inorganic phosphate.

the unknown nucleotide was probably a derivative of hmdUMP. A sample of [2-^{14}C]adenine-labeled *am* 37 DNA was prepared from infected strain 29 cells to ensure that the unknown nucleotide was not an oligonucleotide resulting from incomplete digestion of the DNA. Digestion of this preparation with nuclease S1 and snake venom phosphodiesterase released dAMP and dGMP as the only radioactive nucleotides.

Digestion of *am* 37 DNA from strain 29 cells with DNase I and snake venom phosphodiesterase was incomplete. However, digestion of [2-^{14}C]adenine-labeled DNA yielded dGMP and dAMP as the only labeled products. Therefore, the oligomers obtained from the DNA with these enzymes contained only pyrimidines. Significantly, some of the unknown nucleotide but very little hmdUMP was released from the DNA by DNase I and the phosphodiesterase. Digestion with these enzymes was performed at pH 8.2 and that with nuclease S1 at pH 5.0. Either the unknown nucleotide was labile at pH 5.0 and partially converted to hmdUMP during the digestion with nuclease S1 or the hmdUMP in *am* 37 DNA was present in sequences resistant to digestion by DNase I and phosphodiesterase.

Characterization of Unknown Nucleotide. A culture of strain 29 was infected with *am* 37. Half of the infected culture was labeled with [6-^{3}H]uracil and the other half with [^{32}P]-orthophosphate. DNA was extracted from the cells 50 min after infection. The two preparations of DNA were mixed to give approximately equal amounts of each label. After digestion to mononucleotides with nuclease S1–phosphodiesterase, the unknown nucleotide was purified by descending paper chromatography. The properties of the purified compound are summarized in Table III.

Treatment of the nucleotide with bacterial alkaline phosphatase converted it to (hydroxymethyl)deoxyuridine (hmdUrd). The hmdUrd was identified on the basis of its R_f's by using all of the solvents given under Materials and Methods. Treatment with 1 N HCl for 7 min at 98 °C or 30 min at 37 °C converted the nucleotide to a compound with the chromatographic properties of hmdUMP. The ratio of acid-labile to acid-stable phosphate was ~2. These properties showed the nucleotide to be a derivative of hmdUMP with extra phosphate(s) most likely attached to the 5-hydroxymethyl.

A sample of ^{32}P-labeled *am* 37 DNA from strain 29 cells was digested to mononucleotides. The digest was mixed with unlabeled dTDP and dTTP as markers. The mixture was chromatographed on a DEAE-Sephadex A-25 column at pH 7.5. A ^{32}P-labeled nucleotide with a net charge of about −5 relative to a net charge of −4 for dTTP was present in the digest (Figure 5). This would be expected if the unknown nucleotide was hmdUMP with an additional pyrophosphate group on the 5-hydroxymethyl. This structure was consistent with the alkaline phosphatase and acid-lability data. Thus the nucleotide appeared to be 5-[(hydroxymethyl)-*O*-pyrophosphoryl]dUMP (abbreviated to hmPPdUMP).

Conversion of hmPPdUMP to putdTMP. A cell-free extract was prepared from strain 29 cells infected with wild-type

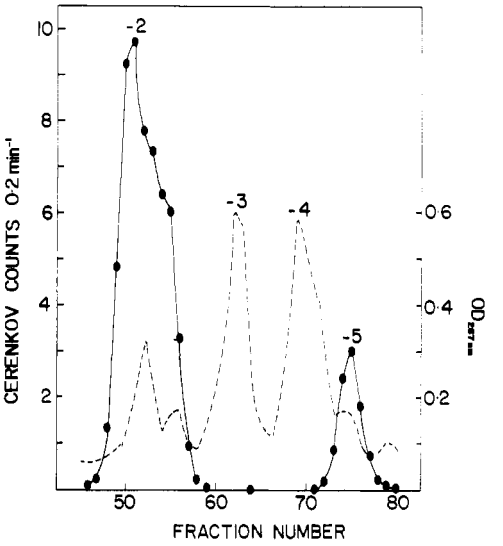

FIGURE 5: Column chromatography of nucleotide mixture from *am* 37 DNA. A sample of ^{32}P-labeled *am* 37 DNA was prepared from infected cells of strain 29. The DNA was digested to mononucleotides which were chromatographed on a DEAE-Sephadex A-25 (1 × 40 cm) column with unlabeled dTDP and dTTP as reference compounds. Fractions of 5 mL were collected and assayed for Cerenkov radiation (●) and absorbance at 260 nm (---). The relative charges are given for each peak.

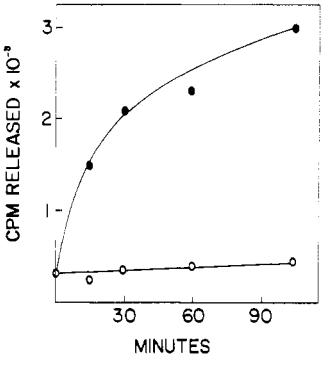

FIGURE 6: Release of radioactivity from ^{32}P-labeled *am* 37 DNA. A sample of ^{32}P-labeled *am* 37 DNA was prepared from infected cells of strain 29. The DNA was incubated with putrescine and a cell-free extract from infected cells of strain 29. Samples were removed at intervals and assayed for alcohol-soluble radioactivity (because of the acid lability of hmPPUra). Extracts were from wild-type infected cells (●) and *am* 37 infected cells (○).

φW-14. When ^{32}P-labeled *am* 37 DNA from strain 29 cells was incubated with the extract and putrescine, alcohol-soluble radioactivity was released (Figure 6). Radioactivity was not released when the DNA was incubated with an extract prepared from *am* 37 infected strain 29 cells (Figure 6). The release of radioactivity was accompanied by a decrease in the amount of hmPPdUMP and an increase in the amount of putdTMP in the DNA (Figure 7). However, the amounts of dTMP and of hmdUMP did not change (Figure 7). The nucleotides were obtained from the DNA by digestion with

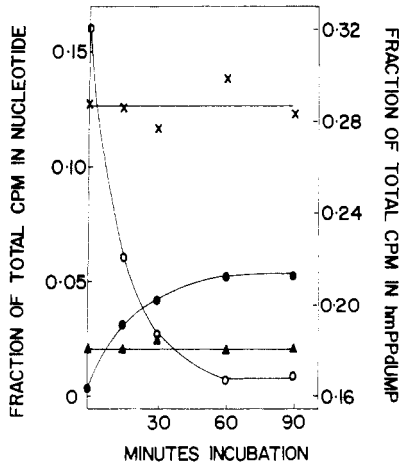

FIGURE 7: Conversion of hmPPUra in *am* 37 DNA into putThy. The procedure was essentially as described in the legend to Figure 6. The DNA in samples was digested to mononucleotides which were separated by thin-layer chromatography. The nucleotides were detected by radioautography and excised from the sheets, and their radioactivity was determined. dTMP (×); hmdUMP (▲); putdTMP (●); hmPPdUMP (○).

nuclease S1 and venom phosphodiesterase. Since the hmdUMP released remained constant while the level of hmPPdUMP decreased, hmdUra was probably present in *am* 37 DNA and was not an artifact resulting from the acid lability of the hmPPdUra residues.

The fraction of the total radioactivity lost as hmPPdUMP was ~3 times the amount appearing as putdTMP (Figure 7). This was consistent with the unknown nucleotide being hmPPdUMP.

Discussion

The DNA accumulated by *am* 37 infected strain 29 cells contains a novel base, hmPPUra. The acid lability of both phosphates in the pyrophosphoryl group is not surprising. The hmUra group has benzylic character (Brown et al., 1968; Santi, 1967), and the rate of hydrolysis in strong acid is ~100-fold greater for monobenzyl phosphate than for simple aliphatic phosphates (Kumamoto & Westheimer, 1955). Furthermore, allyl pyrophosphates are quite unstable below pH 5, cleaving into allyl alcohols and inorganic pyrophosphate (Goodman & Popjak, 1960).

The synthesis of putThy in ϕW-14 DNA appears to be at least a two-step process: the conversion of hmUra to hmPPUra and the conversion of hmPPUra to putThy. At present nothing is known about the synthesis of hmPPura. Attempts to remove the pyrophosphoryl groups from *am* 37 DNA with alkaline phosphatase, which would generate a substrate for probing the pyrophosphorylation reaction, have been unsuccessful. This DNA may represent a poor substrate for alkaline phosphatase, since the pyrophosphoryl groups lie to one side of the major groove, adjacent to the phosphates of the backbone. Attempts to pyrophosphorylate the hmUra residues of ϕe DNA by using extracts from ϕW-14-infected *P. acidovorans* have also been unsuccessful. Normally, the synthesis of putThy is coupled tightly to DNA replication (Neuhard et al., 1980), so the pyrophosphorylation of a fully hydrogen-bonded double-helical DNA may be hindered. Furthermore, if the thymine and putThy residues in ϕW-14 DNA are not randomly distributed, then ϕe DNA may contain few of the sequences normally pyrophosphorylated in ϕW-14 DNA.

The formation of putThy from hmPPUra involves the displacement of a pyrophosphoryl group by an incoming amino nitrogen with the formation of a carbon–nitrogen bond. This type of reaction occurs, for example, in the formation of phosphoribosyl anthranilate from anthranilate and phosphoribosyl pyrophosphate and in the formation of dihydropteroate from (hydroxymethyl)pteridine pyrophosphate and *p*-aminobenzoate (Walsh, 1979). The situation here is more complex. HmUra will alkylate poorly nucleophilic aromatic amines in aqueous alkaline medium (Santi, 1967), so the hydroxymethyl group is susceptible to nucleophilic displacement. Such displacement could be enhanced by the presence of a pyrophosphoryl leaving group. Some esters of hmUra are also sensitive to nucleophilic displacement (Santi, 1971). Such reactions may proceed via an initial attack at the 6 position of the pyrimidine ring (Pogolotti & Santi, 1977). It remains to be seen whether or not the conversion of hmPPUra to putThy occurs by direct displacement at the 5-methylene group or via an initial nucleophilic attack of the enzyme or putrescine at the 6 position.

In the mechanism proposed for the thymidylate synthetase reaction, the enzyme makes a nucleophilic attack on the 6 position of the pyrimidine ring in dUMP, followed by attachment of CH_2-H_4folate to the 5 position. The complex 5,6-dihydropyrimidine intermediate than undergoes β elimination to yield a reactive exocyclic methylene intermediate that is reduced to dTMP by using H_4folate as the reductant [for a review see Pogolotti & Santi (1977)]. It is possible that the hmPPUra found in *am* 37 DNA is also a precursor of Thy. Nucleophilic attack at the 6 position of the pyrimidine ring could lead to the formation of an exocyclic methylene intermediate by displacement of the pyrophosphoryl group. As in the thymidylate synthetase reaction, the intermediate could be reduced to Thy. If this is true, then the accumulation of hmPPUra in *am* 37 DNA argues that the formation of putThy and Thy is sequence specific. However, the development of ϕW-14 is not inhibited by concentrations of trimethoprim up to 10-fold greater than the minimal inhibitory concentration for the host (K. L. Maltman, unpublished observations). This argues against the involvement of H_4folate in ϕW-14 DNA synthesis.

ϕW-14 DNA is of extremely low buoyant density, a consequence of the methylene groups in the putrescinyl groups and probably of the exclusion of cesium ions by the positively charged amino groups (Kropinski et al., 1973). The DNA synthesized by *am* 37 infected cells is considerably denser, and this is probably a consequence of the pyrophosphoryl groups binding extra cesium ions as well as of the presence of hydroxymethyl groups. In SP15 DNA, glucose 1-phosphates are attached to the dihydroxypentyl side chains (Brandon, 1974), and the buoyant density of this DNA is unusually high (Marmur et al., 1972). Conversely, the thermal transition temperature of ϕW-14 DNA is unusually high (Kropinski et al., 1973) while that of SP15 DNA is unusually low (Marmur et al., 1972), reflecting the stabilizing effect of the positively charged amino groups and the destabilizing effect of negatively charged phosphates. It is of considerable interest that the modification of ϕW-14 DNA involves steps which first increase and then decrease the net negative charge on the double helix. Less DNA is synthesized by *am* 37 infected strain 29 cells than by cells infected with wild-type phage. In the latter case, modification is coupled tightly to replication (Neuhard, et al., 1980), so that the DNA does not accumulate significant amounts of extra negative charges. Only some 50% of the hmPPdUMP residues in *am* 37 were converted to putThy. This might be increased by determining the reaction optimums. The DNA accumulated by the *am* 37 infected cells may be a poor template for the replication enzymes, so that DNA synthesis eventually stops. The slowing of the rate of [5-

[3H]uracil incorporation relative to the rate of tritium release suggested that under nonpermissive conditions DNA synthesis in *am* 37 infected cells was affected more by the nature of the DNA being synthesized than by a decline in the rate of synthesis of nucleotide precursors. Furthermore, this DNA may also be a poor template for RNA polymerase, so that late gene expression would be impaired. Finally, even if there was some expression of late genes, the DNA might prove difficult to package. The pyrophosphorylated DNA is being purified so that its physical–chemical properties may be examined.

The results obtained with *am* 37 confirm that putThy and thymine arise from hmUra by separate routes. The modification of ϕW-14 DNA is thus a complex process, involving at least two steps for the formation of putThy and an unknown number for the formation of thymine. The relationship of this modification system to the replication machinery for ϕW-14 DNA poses some intriguing questions. For example, are the modification reactions base-sequence specific? If ϕW-14 DNA synthesis involves a multienzyme complex, are the modification enzymes found in the complex? Such an association could couple modification to replication.

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Primary Structure of the Phage P22 Repressor and Its Gene *c2*†

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ABSTRACT: The amino acid sequence of the Salmonella phage P22 repressor and the DNA sequence of its gene *c2* have been determined. Sequential Edman degradations on intact P22 repressor and repressor peptides generated by proteolytic and chemical cleavages have been overlapped to give approximately 97% of the complete protein sequence. Additionally, the nucleotide sequence of the P22 *c2* repressor gene has been determined by DNA sequencing techniques. The DNA sequence and partial protein sequence are collinear and together define the complete amino acid sequence of P22 repressor. The repressor is a single-chain 216 amino acid polypeptide. Basic residues in the sequence tend to be clustered, and residues 9–20 are highly basic, containing five arginyl and three lysyl residues. The carboxyl-terminal 133 amino acids of the *c2* repressor are homologous to the carboxyl-terminal sequence of the coliphage λ *cI* repressor. The amino-terminal sequences of these two repressors show little similarity.

Upon infection of their hosts, members of the lambdoid family of temperate bacteriophages can either form lysogens or undergo lytic growth. In a lysogen, the phage DNA is integrated into the host chromosome, and all of the phage genes necessary for lytic growth are turned off. This negative regulation of phage transcription is mediated by a repressor protein. In the case of Salmonella phage P22, the repressor protein encoded by the phage *c2* gene is necessary for maintenance of lysogeny (Levine, 1957; Levine & Smith, 1964). Phage with mutant *c2* polypeptides, or phage in which the synthesis of the *c2* repressor is reduced, cannot form lysogens and grow only lytically.

The P22 *c2* repressor controls gene expression by binding to the phage DNA at two operator regions (Bronson & Levine, 1972; Ballivet & Eisen, 1978). The nucleotide sequence of these operator regions has been determined, and the interaction

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