DNA SYNTHESIS AND MODIFICATION
IN \( \phi W-14 \)-INFECTED \textit{PSEUDOMONAS ACIDOVORANS}

by

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We accept this thesis as conforming to the
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Date 13/10/91
Experiments with $\Phi W$-14-infected, thymidine-requiring mutants of \textit{P. acidovorans} strain 29 demonstrated that deoxyuridine but not thymidine was a precursor of thymine in $\Phi W$-14 DNA. Deoxyuridine was also a precursor of the $\alpha$-putresciny1thymine found in $\Phi W$-14 DNA. The biosynthesis of $\alpha$-putresciny1thymine and thymine was mediated by enzyme activities appearing after infection. $\Phi W$-14 DNA synthesis and DNA modification was resistant to the antibiotics trimethoprim and 5-fluorodeoxyuridine. This indicated that endogenous thymidine biosynthesis was unlike that observed in the uninfected host or in other biological systems. These observations helped demonstrate that hydroxymethyluracil-containing nucleotides were precursors of thymine and $\alpha$-putresciny1thymine-containing nucleotides (Neuhard et al., 1980). The absence of $\alpha$-putresciny1 thymine and thymine nucleotides in $\Phi W$-14-infected cell nucleotide pools suggested that these nucleotides might be synthesized from hydroxymethyluracil at the polynucleotide level. Degradative analysis of nascent $\Phi W$-14 DNA demonstrated the presence of hydroxymethyluracil. Enzymatic degradation of pulse-labelled, nascent $\Phi W$-14 DNA followed by TLC suggested the presence of three or more novel nucleotides not found in uniformly labelled DNA samples. These observations were consistent with neutral CsCl analysis of pulse-labelled $\Phi W$-14 DNA. This DNA contained unusual heavy density components.

$\Phi W$-14 \textit{ts} and amber mutants were screened for defects in DNA
replication or DNA modification by CsCl gradient and/or degradative analysis. Some DO mutants were identified. In addition, two DNA modification mutants were found. Am 42 made φW-14 DNA containing lower-than-normal levels of α-putrescylthymine and increased levels of thymine. Am 37 accumulated intermediates in α-putrescylthymine biosynthesis. The conditionally lethal nature of the DNA modification lesion was demonstrated. DNA synthesis was adversely affected by this mutation but DNA precursor supplies were not impaired.

Two atypical mononucleotides were purified from am 37 DNA. One was identified as hydroxymethyldeoxyuridylate. The second nucleotide was an acid-labile derivative of hydroxymethyldeoxyuridylate. Analysis of [6-3H]-uracil and $^{32}$P$_4$ labelling ratios, chemical and enzymatic degradation and chromatographic analysis of this nucleotide demonstrated that it was the novel compound 5-(hydroxymethyl-0-pyrophosphoryl)-deoxyuridylate (abbreviated to hmPPdUMP).

5-(hydroxymethyl-0-pyrophosphoryl)-uracil was shown to be a precursor of α-putrescylthymine by in vitro modification of am 37 DNA with φW-14 wild-type infected P. acidovorans cell-free extracts. In vitro modification confirmed that α-putrescylthymine was formed at the polynucleotide level. φW-14 DNA modification was not necessarily coupled to replication. The presence of hydroxymethyluracil in am 37 DNA agreed with the suggestion that hmPPura was formed by pyrophosphorylation of hydroxymethyluracil in nascent DNA. HmPPdUMP had chromatographic properties similar to one of the compounds detected in pulse-labelled φW-14 wild-type DNA.
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INTRODUCTION

\( \Phi W-14 \) is a lytic bacteriophage of some *Pseudomonas acidovorans* strains (Kropinski and Warren, 1970). The bacteriophage contains double-stranded DNA with a mol % G + C content of 51.4 (M. Mandel, personal communication). The native virion DNA has a lower-than-expected buoyant density in neutral CsCl density gradients, and a higher-than-expected melting temperature (Tm) (Kropinski et al., 1973). These aberrant properties are due to a hypermodified pyrimidine which replaces approximately 50 percent of the total expected thymine fraction in the bacteriophage DNA. The base is a C-5 modified pyrimidine, 5-(4-aminobutylaminomethyl)uracil, also called \( \alpha \)-putrescinyllthymine or putThy. The aminomethyl group of the putThy molecule is at the same oxidation level as the hydroxymethyl function of hydroxymethyluracil (hmUra). The structure of the acid-hydrolyzed base is assumed to accurately reflect the structure of the putThy nucleotide. This assumption is consistent with the physical and chemical properties of \( \Phi W-14 \) DNA. The positive charges carried by the putrescinyll function could theoretically neutralize one quarter of the total negative charges on the DNA molecule at physiological pH (Kropinski and Warren, 1973). These positive charges stabilize the DNA helix since \( \Phi W-14 \) DNA has a very high melting temperature (Tm = 99.3° in 1xSSC) (Table 1). The low buoyant density (\( \rho \)) of \( \Phi W-14 \) DNA is also a reflection of the presence of the putrescinyll side chains. Positively charged amines exclude cesium from the cesium-DNA
TABLE 1.—Properties of phage DNAs containing modified bases

<table>
<thead>
<tr>
<th>Phage Host</th>
<th>DNA molecular weight (x 10^{-6})</th>
<th>Moles % G+C</th>
<th>Base change</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4 Escherichia coli</td>
<td>110</td>
<td>34</td>
<td>50</td>
</tr>
<tr>
<td>5-hmCyt for Cyt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Øe Bacillus subtilis</td>
<td>100</td>
<td>39</td>
<td>43</td>
</tr>
<tr>
<td>5-hmUra for Thy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS2 B. subtilis</td>
<td>150</td>
<td>28</td>
<td>43</td>
</tr>
<tr>
<td>Ura for Thy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>xP12 xanthomonas oryzae</td>
<td>30</td>
<td>67</td>
<td>64</td>
</tr>
<tr>
<td>5-mCyt for Cyt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-2L Synechococcus elongatus</td>
<td>28</td>
<td>69</td>
<td>70</td>
</tr>
<tr>
<td>2-nAde for Ade</td>
<td></td>
<td></td>
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<tr>
<td>SP15 B. subtilis</td>
<td>250</td>
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<td>43</td>
</tr>
<tr>
<td>5-dhpUra for Thy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP10 B. subtilis</td>
<td>59^f</td>
<td>43</td>
<td>43</td>
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<tr>
<td>α-gluThy for Thy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0W-14 Pseudomonas acidovorans</td>
<td>92</td>
<td>51^e</td>
<td>67</td>
</tr>
<tr>
<td>α-putThy for Thy</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a The extent to which the modified base replaces the normal base.
b Thermal transition temperatures are extrapolated to the value in 0.15 M NaCl.
c Buoyant densities in neutral CsCl, assuming a value of 1,710 g ml^{-1} for E. coli DNA.
d These are the values expected for a DNA of the same moles % G+C and of normal composition.
e M. Mandel, personal communication.
f Not reported, but may be the value given (K. Bott, personal communication in ref. 6).

<table>
<thead>
<tr>
<th>Extent of change&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>T&lt;sub&gt;b&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt; observed</th>
<th>T&lt;sub&gt;b&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt; expected&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Buoyant density&lt;sup&gt;c&lt;/sup&gt; observed</th>
<th>Buoyant density&lt;sup&gt;c&lt;/sup&gt; expected&lt;sup&gt;d&lt;/sup&gt;</th>
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<td>84</td>
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<td>85.3</td>
<td>1.742</td>
<td>1.703</td>
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<tr>
<td>100</td>
<td>76.5</td>
<td>81.5</td>
<td>1.722</td>
<td>1.690</td>
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<tr>
<td>100</td>
<td>101.5</td>
<td>95.4</td>
<td>1.710</td>
<td>1.726</td>
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<tr>
<td>100</td>
<td>101.9</td>
<td>98.3</td>
<td>1.731</td>
<td>1.728</td>
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<td>61.7</td>
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<tr>
<td>15-20</td>
<td>81.5</td>
<td>86.9</td>
<td>1.723</td>
<td>1.703</td>
</tr>
<tr>
<td>50</td>
<td>99.3</td>
<td>90.3</td>
<td>1.666</td>
<td>1.716</td>
</tr>
</tbody>
</table>

<sup>a</sup> Extent of change

<sup>b</sup> T (temperature)

<sup>c</sup> Buoyant density

<sup>d</sup> Expected values
complex \((\rho = 1.666 \text{ g cm}^{-1})\). The low buoyant density of the \(\Phi W-14\) DNA can also be partially attributed to the presence of the four methylene functions in the amino-butyl group (Warren, 1980).

The biosynthetic origins of the structural components of the putThy base are known. Uracil is a precursor of the pyrimidine ring portion of the molecule (Kelln, Ph.D. Thesis, 1973). Serine labels putThy and Thy residues in \(\Phi W-14\) DNA. The labelling of this methylene function with \([2^{-14}\text{C}]-\text{serine}\) or \([2,3^{-3}\text{H}]-\text{serine}\) is consistent with the transfer of a one carbon fragment from \(N_5-N_{10}\) methylene-tetrahydrofolic acid (THFA) (Karrer, M.Sc. Thesis, 1973) and with thymine not being a precursor of putThy. Ornithine is a precursor of the putThy side chain, since ornithine labels putThy residues in \(\Phi W-14\) DNA (Quail et al., 1976). \(P.\ acidovorans\) is impermeable to putrescine under normal conditions (Karrer and Warren, 1974).

Many other organisms and bacteriophages with modified bases in their DNA have been identified, and many of the bases have been characterized. Most procaryotes and eucaryotes contain variable amounts of the base 5-methylcytosine. In mammalian cells, between 2 and 8 percent of the total deoxycytidylate residues are found in this form (Hall, 1966). Procaryotic DNA also contains small amounts of 5-methyldeoxycytidylate and \(N_6\)-methyldeoxyadenylate. Methylated bases in specific DNA sequences can protect DNA from restriction endonucleases recognizing those sequences. The mechanism of synthesis of methylated bases is well understood but is not relevant to this discussion.

Bacteriophages are the richest sources of modified bases in DNA (Table 1) (Figure 1). The \(T\)-even bacteriophage group provided the first
FIGURE 1.—The structure of modified bases found in bacteriophage DNA.

Figure 1 Structures of modified bases in phage DNAs: 1. 5-hydroxymethylcytosine (hmCyt); 2. 5-methylcytosine (mCyt); 3. uracil (Ura); 4. 5-hydroxymethyluracil (hmUra); 5. α-putrescylthymine (putThy); 6. 5-dihydroxypentyluracil (dhpUra); 7. α-glutamylthymine (gluThy); 8. 2-aminoadenine (nAde).
examples of a modified base in bacteriophage DNA (Wyatt and Cohen, 1953). T-even phage included T2, T4 and T6 which are similar but not identical in many respects. They all contain the base 5-hydroxymethylcytosine (hmCyt) which completely replaces cytosine in the bacteriophage DNA. 5-hydroxymethylcytosine base pairs with guanine in the same manner as cytosine. 5-hydroxymethylcytosine residues are further modified by the covalent and stereospecific addition of d-glucose molecules. The glucosylation patterns are specific for each bacteriophage type (Revel and Luria, 1970).

The cases of the T-even bacteriophage and other bacteriophage containing modified bases in their DNA will be considered in more detail. The presence of modified bases in DNA raises many intriguing possibilities. The biosynthetic origins and functions of modified bases will be examined.

The structures of modified bases include a variety of permutations upon the four normal bases found in DNA. The best studied examples are bases which are modified pyrimidines. Modification takes place in a manner which does not alter the base-pairing properties of the base. Modification occurs after the formation of the complete pyrimidine ring structure. Therefore, uridine monophosphate (UMP), is a precursor of all modified pyrimidine bases. The C-5 hydrogen bond is a favoured site for the substitution reactions generating modified bases. This is probably due to the high intrinsic reactivity of this site. Modifications occurring here can also extend out and away from the base-pairing regions of the DNA helix. For example, the methyl group of
thymine lies in the major groove of the DNA double helix (Mahler and Cordes, 1971).

Although base modification occurs in a way which does not alter base-pairing properties, modification often alters appreciably the physical properties of the DNA. A catalogue of effects is shown in Table 1 (Warren, 1980). The equations used to predict mol % G + C content of DNA are derived from studies of normal DNA molecules. Properties of DNA such as melting temperature or buoyant density are dependent upon the GC content of the DNA. Alterations in the basic components of the DNA have effects upon melting temperature and buoyant density. Differences in mol % G + C contents predicted by melting temperature analysis or from buoyant density determinations are usually indicative of the presence of a modified base in DNA. Degradative analysis of DNA suspected of containing a modified base is used to confirm suspicions; in addition, the normal base substituted is identified by its absence or partial replacement. DNA may be degraded by acid hydrolysis to bases or by enzymatic digestion to nucleotides or nucleosides. If acid hydrolysis of DNA is used to demonstrate the presence of a modified base it is also necessary to prove that the hydrolysis procedure does not alter the base, e.g. hmCyt residues in T4 DNA are glucosylated but acid hydrolysis of DNA removes the glucose. Unusual bases which are stable under conditions of acid hydrolysis are called hypermodified bases. Enzymatic digestion of DNA-containing acid-labile structures generally releases bases in their unaltered form. Glucosylated T4 DNA is resistant to restriction endonucleases (Revel and Luria, 1970). SP 15 DNA is not
completely degraded to mononucleotides by sequential DNase I and snake venom phosphodiesterase (SVPD) treatments (Brandon, Ph.D. Thesis, 1973).

The presence of a modified base in bacteriophage DNA provides opportunities for the dynamic analysis of phage nucleotide metabolism and biochemistry. If infection increases the rate of DNA synthesis over that seen in an uninfected cell then the rate of synthesis or supply for the precursors of DNA must also increase. If the mol % G + C content of the infecting phage DNA is different from that of the uninfected host, then the phage must encode functions to reallocate precursors to reflect the changes in DNA base composition. These changes may appear as alterations in the size of the nucleotide pool. However, the size of the nucleotide pool is not likely to be as important as the rate of flow of nucleotides through the pool. If the phage DNA contains a modified nucleotide which is not found in uninfected cells then the phage must encode functions that allow the de novo synthesis of the modified nucleotide. In addition, the phage must also inhibit the synthesis and/or prevent the incorporation of the normal nucleotide into phage DNA. Bacteriophages which partially replace a normal base must regulate the levels of substitution of each base. Modified bases or normal bases may also be altered after polymerization and these modification functions must also be regulated. The manner by which various phages accomplish the synthesis of DNA containing unusual components will be considered with respect to the preceding points. The biological consequences of base substitution will also be considered.
T-even bacteriophage (T2, T4, T6)

In *E. coli* infected with T-even bacteriophage, many of the points made above are illustrated. T-even phage make hydroxymethylcytosine at the mononucleotide level and use it to completely replace cytosine in their DNA (Wyatt and Cohen, 1953). Glucosylation of T-even phage DNA provides a model for post-replicational modification of DNA.

The reprogramming of the cellular biosynthetic machinery in T4-infected cells begins with the inhibition of host DNA synthesis and other cellular biosynthetic processes. Cytosine-containing host DNA is degraded. The deoxyribonucleases which mediate these processes are encoded in the T4 genome (Mathews, 1977). The initial steps in the degradation sequence are due to nucleases which specifically recognize and cleave cytosine-containing DNA. Hydroxymethylcytosine-containing DNA is not a substrate for these enzymes (Kutter and Wiberg, 1969). Degradation of host DNA not only destroys all host-encoded information but also generates a pool of precursors which can be used for T-even phage DNA synthesis (Price and Warner, 1969).

dCMP, formed by host DNA degradation and by de novo synthesis, is a precursor of hmdCMP (Cohen, 1968). The synthesis of hmdCMP involves the $\text{N}_5\text{-N}_{10}$-methylene-tetrahydrofolate-mediated reaction catalyzed by the T4 enzyme deoxycytidylate hydroxymethylase. The reaction is analogous to the $\text{N}_5\text{-N}_{10}$-methylene-THFA-mediated C-1 transfer occurring in thymine biosynthesis; however, transfer at the oxidation level of the hydroxymethyl group does not result in the oxidation of THFA to DHFA (Flaks and Cohen, 1957; Cohen, 1968). In addition to converting dCMP to hmdCMP, T4-infected cells induce a kinase capable of making hmdCTP (Mathews, 1977).
The phage must ensure that dCTP is eliminated from the nucleotide pools since incorporation of cytosine into T4 DNA would result in the destruction of the DNA by cytosine-recognizing nucleases. dCTP is eliminated from infected-cell nucleotide pools by the synthesis of a T4-coded dCTPase (Price and Warner, 1969). dCTP is converted to dCMP and pyrophosphate. dCMP is a substrate for dCMP hydroxymethylase (Cohen, 1968). dCMP is also a substrate for the T4 enzyme dCMP deaminase. The product of dCMP deaminase is dUMP, which, in turn, is a substrate for another T4 enzyme, thymidylate synthase. Thirty-five percent of the dCMP arising from mononucleotides released from host DNA is ultimately reincorporated into T4 DNA. The rate of DNA synthesis in T4-infected cells increases ten fold after infection but the deoxyribonucleoside pools remain at a constant size (Mathews, 1972). This reflects the increased rate of flow of precursors through the active pool. Pool sizes in T4-infected cells reflect the GC content of the DNA synthesized. dCMP deaminase is a T4 enzyme which helps reroute pyrimidines from the cytidylate pathways in the uninfected host (mol % G + C = 50) to the thymidylate pathway in the infected host (mol % G + C = 34). In T4-infected E. coli the ratio of Thy and hmCyt deoxyribonucleotides in the pools is 2.0 to 1.0 (Flanegan and Greenberg, 1977; Chiu et al., 1977). This is the same ratio that is found for these nucleotides in T4 DNA. The maintenance of a precise ratio linking the supply of precursors to DNA polymerization suggests a complex regulatory mechanism (Mathews et al., 1979). The regulation of pyrimidine deoxyribonucleotides involves a multienzyme complex. There is some controversy over the components of this complex. Mathews (1979) has proposed that the complex contains the T4 enzymes dCMP hydromethylase,
thymidylate synthase, dCTP/dUTPase, ribonucleotide diphosphate reductase, deoxynucleoside monophosphate kinase, dCMP deaminase and the host-coded enzyme nucleoside diphosphate kinase. Experiments performed in Greenberg's lab have suggested the complex contains dCMP hydroxymethylase, thymidylate synthase, dCTP/dUTPase, ribonucleotide diphosphate reductase, T4 DNA polymerase, β-glucosyltransferase and gene products 32 (unwinding protein) or 44 (DO) and 45 (DO) (Tomich and Greenberg, 1973; Wovcha et al., 1973; Wovcha et al., 1977; Tomich et al., 1974; Chiu et al., 1977; Flanagan et al., 1977; Greenberg and Chiu, 1978).

He believes the complex channels the deoxynucleoside precursors directly to the replicating forks of T4 DNA. The T4 complex proposed by Mathews contains enzymes involved in the generation of pyrimidine precursors but not any proteins involved directly in DNA replication (Reddy et al., 1977). The evidence upon which these conclusions are based is derived partially from the analysis of carefully fractionated cell-free extracts, and plasmolyzed mutant-infected cells. Cells infected with gene 1 (T4 deoxyribonucleotide kinase) or gene 42 (dCMP hydroxymethylase) mutants cannot synthesize DNA in vitro, even when the metabolic block is thwarted by the addition of deoxynucleoside 5'-triphosphates. These data were interpreted as evidence for protein complexes. Assembly of enzymes into multienzyme complexes channeled DNA precursors into DNA and activated enzymes involved in precursor synthesis. Evidence supporting these conclusions was obtained in vivo. dCMP hydroxymethylase and thymidylate synthase do not function in vivo until they are assembled into multienzyme complexes (Tomich et al., 1974). These activities sediment quickly in vitro as one peak of enzyme activity (Reddy et al., 1977).
It should be noted that T4 DNA polymerase mutants accumulate DNA precursors when DNA synthesis is blocked (Chiu et al., 1976). However, gene 42 mutants have been found which can accumulate hmdCMP in vitro but which will not make T4 DNA (Tomich et al., 1974). These are probably enzyme complex assembly mutants. dCMP hydroxymethylase and dTMP synthase can be assayed in vivo using Greenberg's tritium release assay. [5-\(^3\)H]-uracil enters nucleotide pools and some is converted to [5-\(^3\)H]-dCMP or [5-\(^3\)H]-dUMP. These nucleotides are substrates for dCMP hydroxymethylase and thymidylate synthase. Enzymatic transfer of one carbon fragments from THFA to the C-5 position of the pyrimidine ring results in the formation of hmdCMP or dTMP. Tritium is released as \(^3\)H\(_2\)O. Radioactivity in \(^3\)H\(_2\)O can be recovered by filtering samples through a PCA and charcoal slurry. Labelled nucleotides are retained in the charcoal but the tritiated water is not. The amount of tritium detected in water is proportional to the level of tritium-releasing enzyme activity.

Although the data are incomplete, it can be concluded that T4 DNA synthesis is strictly regulated. DNA precursors are made in the correct ratios and fed to the polymerizing complex at a precise rate which determines the rate of DNA synthesis. It is useful for an organism to ensure delivery of an adequate supply of a precursor to its intracellular site of utilization. Compartmentalization of related functions by formation of enzyme complexes performs this function in T4-infected cells.

T-even DNA glucosylation

Glucosylation of hmCyt residues in T4 DNA is a model system for post replicational modification of DNA (Revel and Luria, 1970). T2, T4
and T6 all have different DNA glucosylation patterns which are distinct and reproducible. T4 has two kinds of glucosylating enzyme: \( \alpha \)-glucosyl transferase and \( \beta \)-glucosyl transferase. T2 and T6 have only \( \alpha \)-glucosyl transferases. In T4 70 percent of the glucosylated \( \text{hmCyt} \) molecules are \( \alpha \)-glucosylated, while 30 percent of the linkages are \( \beta \)-glucosylated (Lehman and Pratt, 1960). Uridine diphosphoglucose (UDPG), which is synthesized by host enzymes, transfers glucose to \( \text{hmCyt} \) in T4 and to \( \text{hmCyt} \) and the 6 position of glucose in T2 and T6 (Kornberg et al., 1961; Josse and Kornberg, 1963). T6 has 72 percent of its \( \text{hmCyt} \) residues diglucosylated while only 5 percent of T2 \( \text{hmCyt} \) is diglucosylated. T4 \( \alpha \)-glucosyl transferase mutants produce DNA with all the hydroxymethylcytosine residues \( \beta \)-glucosylated. T4 \( \beta \)-glucosyl transferase mutants produce DNA with all the \( \text{hmCyt} \) \( \alpha \)-glucosylated. Twenty-five percent of T2 and T6 \( \text{hmCyt} \) is not glucosylated \textit{in vivo}. Non glucosylated T-even DNA may be produced in UDPG\(^{-}\) hosts. However, phage with non glucosylated DNA is subject to restriction in some bacterial strains (Georgopoulos, 1967). Restriction of non glucosylated T-even DNA is governed by two host genes which can destroy \( \text{hmCyt} \)-containing DNA (Fleischman and Richardson, 1971). In T4-infected \textit{E. coli} the DNA-restricting ability is lost. T4 must code for a product which inhibits T4 restriction. The function of T4 DNA glucosylation appears to be protection from restriction (Hewlett and Mathews, 1975).

All the T-even \( \alpha \)-glucosyl transferase show some sequence specificity. T2 DNA glucosylated \textit{in vivo} cannot serve as a substrate for \textit{in vitro} T2-mediated glucosylation, but will allow glucosylation by T6 \( \alpha \)-glucosyl transferase. In T4 DNA purine residues linked to the 3'
position of a hmCyt residue appear to hinder glucosylation (Lunt et al., 1964). T4 α-glucosyl transferase incubated with non glucosylated T6 DNA could glucosylate all hmCyt residues except those adjacent to other hmCyt residues (de Waard et al., 1967). They did not find any β-glucosyl transferase specificity. However, α (or β)-glucosyl transferase will glucosylate sites \textit{in vitro} that are normally β (or α)-glucosylated (Josse and Kornberg, 1962). The structure of the DNA helix is important for modification; native hmCyt-containing DNA is a better substrate for glucosylation than heat-denatured hmCyt-containing DNA (Josse and Kornberg, 1962). The reasons for non random distribution of glucosylated hmCyt residues are not understood. Glucosylation is not required for phage viability. Wild-type mutants of T4 grow poorly on a rifampicin-resistant mutant of \textit{E. coli}. They form "indistinct plaques". Indistinct plaque formers can be converted to distinct plaque formers by a mutation mapping in the β-glucosyl transferase gene. Mathews (1977) has interpreted this experiment as evidence for the involvement of glucosylated hmCyt in RNA polymerase template interactions. Transcription in T4-infected cells is carried out by host RNA polymerase. Specific phage-encoded modifications of RNA polymerase occur at specified times in T4 infection. These modifications allow transcription of previously proscribed domains on the bacteriophage genome. In normal T4 DNA all hmCyt residues are glucosylated. However, non glucosylated T4 phage replicates normally. The substitution of cytosine for hmCyt in T4 DNA blocks late gene expression. There seems to be a requirement for hmCyt-containing DNA for late gene expression (Kutter et al., 1975; Kutter and Wiberg, 1968). There is no corresponding requirement for
glucosylated hmCyt residues. Mathews' conclusions regarding β-glucosyltransferase do not bear upon the situation pertaining to wild-type RNA polymerase interaction with glucosylated hmCyt-containing template.

T4 DNA glucosylation is coupled to replication (McNichol and Goldberg, 1973). Glucosylated T-even DNA is immunogenic. Antisera with specificity for α or β or α and β-glucosylated T4 DNA can be prepared. Non-glucosylated T4 DNA is not immunologically detectable in T4-infected E. coli. β-glucosyl transferase is supposed to be a component of a multienzyme DNA precursor synthesizing complex (Mathews, 1979).

In T6-infected E. coli newly synthesized non glucosylated DNA was demonstrable (Erikson and Szybalski, 1964). In vivo, T6 DNA synthesis begins 2 minutes before the onset of DNA glucosylation. After the synthesis of glucosylating enzyme begins, all the T6 DNA formed was glucosylated.

**Bacillus subtilis bacteriophage**

*Bacillus subtilis* bacteriophages with hmUra-containing DNA are common. They include Øe, SP8, SP01, SP82, SP5 and 2C. The presence of hmUra in DNA causes the DNA to band at a heavier density than expected in neutral CsCl density gradients. Øe-infected *B. subtilis* reprogram cellular processes to stop host biosynthesis of Thy-containing nucleotides and to eliminate Thy-containing nucleotides. HmdUTP completely replaces dTTP in the nucleotide pools of infected cells (Roscoe, 1969). Øe accomplishes this in a manner which is understood. dTMP cannot be converted to HmdUMP. dTTP is destroyed by the action of a nucleotidase which converts dTTP to dTMP and pyrophosphate (Price et al., 1972). A multiple gene phage mutant with a defective dTTPase and a temperature-sensitive hydroxymethylase has thymine in its DNA replacing up to 20
percent of the hmUra. It can grow and transfer Thy-containing DNA to progeny phage (Marcus and Newlon, 1971).

HmUra in Φe DNA comes from the induction of a phage-specific dUMP hydroxymethylase enzyme (Price et al., 1972). All the hmUra in the Φe DNA is synthesized from de novo nucleotide precursors. Φe inhibits host DNA synthesis but the host DNA is not degraded to mononucleotides (Roscoe, 1969). Φe-infected cells induce an inhibitor of thymidylate synthase. This leaves dUMP available as a substrate for the phage coded hydroxymethylase (Roscoe and Tucker, 1969; Haslam et al., 1967). In the Φe-related phage SP5, a new enzyme, thymidine phosphatase, is induced. It cleaves dTMP to thymidine and orthophosphate (Aposhian and Tremblay, 1966). A phage-encoded dCMP deaminase is induced. This pivotal enzyme routes the correct amount of dCMP to dCTP or hmUdUTP pathways. Such rerouting of precursors is required whenever the host GC content is different from the phage GC content. This enzyme would be a good target for allosteric regulation, but is the only known dCMP deaminase not subject to feedback regulation by dNTPs (Nishihara et al., 1967).

Phage with hmUra-containing DNA are susceptible to host restriction. Φe DNA is susceptible to several restriction endonucleases (Berkner and Folk, 1979). The hydroxymethyluracil groups in Φe DNA are not modified by the addition of sugars or any other moieties (Allegria and Kahan, 1968).

The hmUra in SP01 or Φe DNA is not required for replication or transcription. SP01 induces a phage-specific DNA polymerase. This enzyme will use either hmUdUTP or dTTP as a substrate in vivo or in vitro (Yehle and Ganesan, 1973). Φe DNA requires host DNA polymerase III
since a $ts$ DNA pol III $B. subtilis$-$\phi$ infected cell shuts off $\phi e$ DNA synthesis when it is shifted from the permissive to the non permissive temperature (Lavi et al., 1974). It is believed that a phage-encoded pol III modifying protein is synthesized which increases the affinity of the polymerase for the $\phi e$ template (Lavi et al., 1974). This cannot be an absolute requirement for $\text{hmUra}$ in the $\phi e$ DNA since $\phi e$ DNA containing thymine replicates normally.

DNA replication in $B. subtilis$ is inhibited by the antifolate drugs trimethoprim and aminopterin. DNA replication in $\phi e$-infected $B. subtilis$ is resistant to these drugs. Thymidine biosynthesis is inhibited in cells exposed to antifolate drugs. Dihydrofolate reductase is the target enzyme for trimethoprim and aminopterin inhibition. Thymidine biosynthesis is inhibited because, during the formation of $\text{dTMP}$ from $\text{dUMP}$ and $N_5 N\_10^-\text{methylene-tetrahydrofolic acid}$, a C-1 transfer is catalyzed by thymidylate synthase. This transfer results in the oxidation of $\text{THFA}$ to $\text{DHFA}$. Normally, $\text{THFA}$ is regenerated from $\text{DHFA}$ and NADPH by the action of the enzyme dihydrofolate reductase (Mahler and Cordes, 1971). When dihydrofolate reductase is inhibited, the stoichiometric requirement for $\text{THFA}$ in thymidylate biosynthesis eventually depletes the cellular supply of $\text{THFA}$. $\text{THFA}$ is required for many cellular biosynthetic processes such as the formation of purines, pyrimidines and the amino acids methionine, serine and glycine. This requirement for $\text{THFA}$ is catalytic but not stoichiometric. The action of antifolate drugs is antagonized by the addition of thymidine since this reduces the endogenous requirement for the regeneration of $\text{THFA}$. Cells infected by bacteriophage $\phi e$ synthesize $\text{hmDUMP}$ instead of $\text{dTMP}$. $\text{hmDUMP}$ biosynthesis
requires the cofactor $N_5^--N_{10}^-$ methylene tetrahydrofolic acid, the dUMP hydroxymethylase reaction does not result in the oxidation of THFA. Therefore, dihydrofolate reductase activity is not required to regenerate large quantities of DHFA. In all cells C-1 transfer to THFA is catalyzed by the enzyme serine transhydroxymethylase (Mahler and Cordes, 1971). Caution should be exercised in interpreting trimethoprim resistance as demonstrating a requirement for dTMP biosynthesis. Drug resistance factors, encoding resistance to antifolate drugs, act by increasing the number of dihydrofolate reductase molecules. Plasmid or phage-coded dihydrofolate reductase may be intrinsically more resistant to antifolate drugs (Meynell, 1973).

PBS1 is a pseudolysogenic bacteriophage of $B. subtilis$. The DNA of PBS1 contains uracil which completely replaces thymine in bacteriophage DNA. PBS2 is a clear plaque derivative of PBS1 (Takahashi and Marmur, 1963). As expected, PBS2 DNA has a higher-than-predicted buoyant density in CsCl and a lower-than-predicted Tm (Table 1). Infection of $B. subtilis$ by PBS2 requires the exclusion of dTTP and its replacement by dUTP in nucleotide pools. The phage must also inhibit host-encoded enzymes which destroy uracil-containing DNA. Misincorporation of uracil or the deamination of cytosine residues in DNA occurs at a low frequency in all cells (Tye et al., 1977). Mechanisms to reduce the frequency of transition mutations that deamination of cytosine would cause have evolved. Cells possess the enzyme uracil-DNA glycosylase which cleaves the N-glycosidic bond between uracil and deoxyribose moieties in DNA. This is the initial step in a process which then excises the deoxyribose sugar and repairs the molecule using the complimentary DNA strand.
(Friedberg et al., 1975; Duncan et al., 1976). PBS2 codes for a small, heat stable inhibitor of the enzyme uracil-DNA glycosylase (Tomita and Takahashi, 1976; Friedberg et al., 1975). PBS2 infection also induces thymidine phosphorohydrolase and dUMP kinase activities (Kahan, 1963). Both enzymes serve to limit the supply of dTMP. dUMP kinase also increases the supply of dUTP. Thymidylate synthase is not inhibited in PBS2 infected cells (Hitzeman et al., 1978). PBS2-infected cells also contain a dCTP deaminase activity (Tomita and Takahashi, 1969). Coupled with host dCMP deaminase these enzymes increase the availability of dUTP or its precursors. These enzymes route precursors to dUTP precursor pathways (Price and Frato, 1975; Rima and Takahashi, 1973). Thirty to 50 percent of the dUTP used for PBS2 DNA synthesis is generated by the action of dCTP deaminase. The rest comes from de novo sources via dCMP deaminase and ribonucleotide reductase (Rima and Takahashi, 1979; Price, 1980). Host DNA synthesis is inhibited by PBS2 but host DNA is not degraded, therefore, all deoxynucleotides come from de novo synthesis.

PBS2 is a transducing phage (Takahashi, 1963). It is obvious that Thy-containing DNA is not discriminated against during packaging. The biological consequences which are due to the presence of uracil in PBS DNA are clear. Uracil-containing DNA is susceptible to nuclease or restriction endonuclease cleavage although rates may differ from normal substrates (Berkner and Folk, 1979). PBS2-infected cells have a RNA polymerase activity which is resistant to rifampicin or streptolydigin. Transcription occurs even if the antibiotics are added prior to infection. Uracil DNA has little or no template activity with host RNA
polymerase while the viral RNA polymerase shows appreciable template activity only with PBS2 DNA or poly (dA-dT) (Clark et al., 1974; Price and Frabotta, 1972; Price et al., 1974; Rima and Takahashi, 1973). The nature of early PBS2 transcription is obscure. PBS2-specified RNA polymerase does not appear until the middle of the lytic cycle, but early phage transcription is rifampicin resistant (Clark, 1978). This suggests that PBS2 virions carry a RNA polymerase-modifying enzyme which could render transcription drug resistant.

DNA replication is active with purified PBS2 polymerase using either dTTP or dUTP as a substrate (Price, 1980). Small amounts of dTTP-containing DNA can be made in vivo by growing cells at a high pH. At this pH the inhibition of host dUTPase is inactivated and enough dUMP to allow the formation of dTMP accumulates. dTMP phosphorohydrolase must also be inhibited at this high pH (Price and Fogt, 1973; Price and Frato, 1975). The PBS2-induced DNA polymerase does not require dUTP in vivo for polymerization.

Lindahl has pointed out that the low mol % G + C content of PBS2 DNA could be a biological consequence of the presence of uracil in PBS2 DNA (Lindahl, 1979). He proposes that PBS2 DNA has undergone all the allowable GC to AU transitions and that there would be a strong selective pressure against further transition mutations. This hypothesis should be testable. It predicts that PBS2 phage would be extremely susceptible to killing by a transition-inducing mutagen such as nitrous acid and that the third base in DNA codons should be some other base than uracil in all or most allowable cases.

A question about PBS1, the pseudolysogenic parent of PBS2, also
arises. Does lysogenic PBS1 DNA contain Thy instead of Ura? If not, how does PBS1 protect its DNA against host uracil N-glycosidase?

Uracil in PBS2 is not modified after incorporation into DNA. 5-FU is incorporated into PBS2 DNA (Lozeron and Szybalski, 1967). *B. subtilis* infected in the presence of \( [2^{-14}C] \)-FUdR make DNA in which uracil is replaced by FUdR. The buoyant density of the DNA in CsCl density gradients increases from 1.722 to 1.737-1.745 gcc\(^{-1}\). Label is quantitatively recovered from the heavy DNA fraction and acid hydrolysis and TLC of the DNA samples shows that the label remains associated with 5-FU. A replacement of one mol % of uracil by 5-FU is accompanied by an increase in buoyant density of 0.34 mg cc\(^{-1}\). This paper proves that 5-FdUTP must be a substrate for PBS2 DNA polymerase. PBS2 phage formed with 5-FU in DNA replicate but are more UV-sensitive than normal PBS2 phage. In other phage systems incorporation of 5-FU into DNA would indicate that the phage used dUTP as a polymerization substrate.

XP 12 is a bacteriophage of *Xanthomonas oryzae* (Kuo et al., 1968). It has a mol % G + C content of 67 compared to 64 for its host. The observed melting temperature value for the phage DNA is 101.5\(^\circ\), higher than expected. The buoyant density of the DNA is 1.710 gcc\(^{-1}\), lower than expected. 5-methylcytosine completely replaces cytosine in the DNA of the phage. 5-methylcytosine is synthesized from dCMP by a \( N_5-N_10^-\)methylene-THFA-dependant enzyme. XP 12 also encodes its own thymidylate synthase. Host DNA replication is inhibited after infection but DNA is not degraded (Erlich et al., 1977).

SP 10 and SP 15 are examples of bacteriophages in which thymine DNA residues are partially replaced by another hypermodified pyrimidine.
α-glutamylthymine (gluThy) replaces thymine in SP 10 DNA and 5-dihydroxy- 
pentyluracil (dhpUra) replaces thymine in SP 15 DNA. Both DNA types are 
similar to φW-14 DNA in that they contain five major DNA bases. The 
biosynthetic origins of these bases have been investigated. 

SP 15 DNA contains dhpUra, and the nucleotide dhpUTP has been 
found in infected cell nucleotide pools (Walker and Mandel, 1978a). 
dhpUra is derived from uracil and ribose (Walker and Mandel, 1978b). 
SP 15 DNA synthesis is inhibited by FUdR. FUdR inhibition of DNA syn­ 
thesis can be reversed by the addition of TdR to cultures. SP 15 phage 
DNA which is made in the presence of FUdR and TdR has lower-than-normal 
levels of dhpUra. SP 15 phage with lower levels of dhpUra and increased 
levels of thymine are viable. Exogenously supplied TdR and BUDr are 
incorporated into phage DNA. dTTP is present in the nucleotide pools of 
infected cells. The formation of dhpUra nucleotides is also inhibited 
by the presence of FUdR. This is obvious since reversal of FUdR inhibi­ 
tion of DNA synthesis results in abnormally high levels of Thy in DNA. 
However, inhibition of DNA synthesis with aminopterin, followed by rever­ 
sal of the inhibition by TdR results in the synthesis of SP 15 DNA with 
normal amounts of dhpUra and Thy. dUMP but not dTMP is a dhpUra precur­ 
sor (Walker and Mandel, 1978a; Walker and Mandel, 1978b; Walker, Ph.D. 

Host DNA is not degraded by SP 15 infection. Since SP 15 is a 
generalized transducing phage of B. subtilis (Taylor and Thorne, 1963), 
dhpUra-containing DNA is not required for DNA packaging. 

dhpUra in SP 15 DNA is triglucosylated with one sugar linked in 
a phosphodiester bond. The sugars are added to DNA after replication in
UDPG-mediated reactions which may be coupled to replication. A structure for the sugar-phosphate modifying functions has been proposed (Brandon, Ph.D. Thesis, 1973). Only dhpUra is isolated from acid hydrolysates of SP 15 DNA. There is only one chromatographically resolvable dhpUra-glucosylated and phosphorylated nucleotide in S1-snake venom phosphodiesterase digests of SP 15 DNA.

The most remarkable feature about SP 15 is that DNA replication naturally proceeds using five nucleotide triphosphate precursors. Under normal conditions the ratio of dhpUra to Thy is controlled. Forty-two percent of the potential DNA thymine content is actually dhpUra (Marmur et al., 1972). The mechanism used to regulate the level of dhpUra substitution has not been investigated. It is also not known if the distribution of dhpUra and Thy in SP 15 is random.

SP 15 replication is sensitive to inhibitors of RNA polymerase (Dosmar et al., 1977). Therefore, it is likely that SP 15-infected cells use host RNA polymerase or phage-modified forms of host-modified polymerase. The presence of glucosylated dhpUra residues has no effect upon transcription of SP 15 DNA.

Bacteriophage SP 10 is another B. subtilis generalized transducing phage (Taylor and Thorne, 1963). α-glutamylthymine replaces 15 to 20 percent of the thymine residues in SP 10 DNA. The gamma carboxyls of the glutamyl sidechain carry an unidentified hydrophilic substituent carrying a primary amine (Warren, 1980). Like other DNA species with modified bases, SP 10 DNA displays unusual behaviour in neutral cesium chloride density gradients (ρ = 1.723). The melting temperature of SP 10 DNA is 81.5°, lower than predicted from its mol % G + C content.
The biosynthesis of SP 10 DNA is not inhibited by FUdR. Thymidine incorporation stops permanently after infection of a Thy− host (Markewych et al., 1977). Thymidylate synthase activity declines and a dTTPase activity appears in infected cells. dTTP levels fall to less than 5 percent of preinfection levels (Markewych et al., 1979). Although host DNA is degraded in SP 10-infected cells, none of the Thy released is reincorporated into phage DNA (Markewych et al., 1977). These results suggest that dTTP is not an in vivo substrate for SP 10 DNA polymerase.

Infected cells induce a dUMP hydroxymethylase activity and a novel kinase activity capable of generating hmddUTP from hmddUDP (Witmer and Dosmar, 1978). SP 10-infected cells contain hmddUDP but not dUTP. hmddUTP is likely a precursor of both gluThy and Thy in SP 10 DNA. Soluble gluThy nucleotides are not found in infected cells. hmUra-containing SP 10 DNA does not accumulate under normal conditions during DNA replication. Modification of hmUra to Thy and gluThy is probably coupled to DNA replication. It is not known if DNA modification in SP 10-infected cells is sequence specific.

SP 10 dUMP hydroxymethylase must be resistant to FUdR. Susceptibility of the enzyme to 5FdUMP, in vitro, has not been tested.

The partial substitution of hmUra for thymine has been reported in Gyrodinium cohnii, a dinoflagellate (Rae, 1973). Rae has used density and melting temperature profiles to screen dinoflagellate DNA for the presence of other unusual bases. Cryptothecum (Gyrodinium) cohnii contains 11 percent of its DNA nucleotides in hydroxymethyldeoxyuridylylate. C. cohnii also replaces 3 percent of its cytosine residues.
with 5-methylcytosine. The simultaneous presence of hmUra, Thy, Cyt and 5-MeCyt is common in dinoflagellates. *Exuviaella cassebia* contains hmUra, Thy, Cyt and 5-MeCyt in its DNA. The counteracting effects due to the presence of hmUra and 5-MeCyt in DNA average out the effects upon buoyant density and melting temperature. Buoyant density and melting temperature values accurately predicted the Tm and ρ values for *E. cassebia* DNA. This misleading result suggested that *E. cassebia* DNA contained only the four normal DNA bases and demonstrated the wisdom of coupling CsCl gradient and Tm analysis with base or nucleotide analysis of unknown DNA samples when screening for unusual nucleotides.

The distribution of hmUra and Thy in the DNA of *C. cohnii* is not random. CsSO₄-AgCl density gradients demonstrate the presence of three distinct density populations of DNA (Rae, 1973). hmUra is preferentially located in the dinucleotides 5'-hmUra-Ade-3' and hmUra-Cyt. hmUra is also enriched in trinucleotide sequences 5'-purine-hmUra-purine-3'. Methylcytosine occurs predominantly in the sequence 5'-methylCyt-Gua-3' (Steele and Rae, 1980).

The biosynthetic pathways for the dinoflagellate DNA nucleotides have not been investigated.

To a certain extent the properties of cells infected by phage carrying modified bases can be generalized. In all cases but one, a major substitution of a modified base leads to the complete replacement of the substituted base in the nucleotide pools. For all the phage, except SP 15, this ensures that DNA replication proceeds using only four nucleotides. Replacement of a base involves steps to remove any residual base. The flow of precursors is rerouted to reflect differences.
between the GC content of the phage and that of the host. Post-replicative modification occurs rapidly after the synthesis of unmodified DNA and unmodified DNA does not accumulate.

The question of the biological significance of modified significance of modified bases in DNA remains largely unanswered. In some phage DNA modification prevents restriction of DNA. Generally speaking, the modified base is not required for phage viability. Modified bases may aid phage enzymes in recognizing templates for transcription or replication. The absence of a modified base is sometimes enough to ensure the degradation of host DNA. This thesis presents results which show that most of these generalizations apply to the replication and modification of ΦW-14 DNA.
MATERIALS AND METHODS

Organisms

The bacterial strains employed were all *Pseudomonas acidovorans* strains (Table 2). Working cell stocks were stored at -20° in 40 percent glycerol and Casamino acids-mannitol (CAA-M) medium. Working stocks were prepared from permanent stocks in the following manner: 25 ml of cells were grown overnight at 30° in a water bath shaking at 200 rpm. The cells were spun down and resuspended in 4 ml of sterile CAA-M. One ml of the cell suspension was added to 4 ml of 50 percent glycerol-CAA-M. The suspension was stored at -70°, until needed. Working stocks were stored at -20°, and were stable at this temperature for three to six months.

Media

CAA-M was used as a complete, undefined medium. It consists of 10 g CAA (Difco), technical grade; 5 g yeast extract; 5 g mannitol and 0.05 g tryptophan per litre of medium. Medium was adjusted to pH 7.0 with concentrated NaOH.

TCS 1 x P₁ was used for experimental purposes requiring defined medium. TCS 1/5 x P₁ is the same as TCS 1 x P₁ except that it has one-fifth the amount of orthophosphate. It was used in ³²PO₄⁻-labelling experiments. TCS (g/l) is tris(hydroxymethyl)aminomethane (Tris) 12.1; KCl, 5.0; Na₂SO₄, 0.0227; FeCl₃·6H₂O, 0.0008; CaCl₂, 0.017; KH₂PO₄, 0.0174. Medium is adjusted to pH 7.0.
**TABLE 2.—Bacterial strains used in this study.**

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas acidovorans</td>
<td>29 prototrophic</td>
<td>R.Y. Stanier</td>
</tr>
<tr>
<td></td>
<td>3L derived from strain 29; requires 250 μg TdR ml⁻¹ for growth.</td>
<td>R.A. Kelln</td>
</tr>
<tr>
<td></td>
<td>3L/FU² sup 1 derived from strain 29</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>sup 2 derived from strain 29</td>
<td>R.A.J. Warren</td>
</tr>
<tr>
<td></td>
<td>sup 3 derived from strain 29</td>
<td>R.A.J. Warren</td>
</tr>
<tr>
<td></td>
<td>sup 4 derived from strain 29</td>
<td>R.A.J. Warren</td>
</tr>
<tr>
<td></td>
<td>sup 5 derived from strain 29</td>
<td>R.A.J. Warren</td>
</tr>
<tr>
<td></td>
<td>JE 1 prototrophic; soil isolate.</td>
<td>J. Ethier</td>
</tr>
<tr>
<td></td>
<td>JE 1 sup 1 derived from JE 1</td>
<td>P. Miller</td>
</tr>
<tr>
<td></td>
<td>JE 1 sup 2 derived from JE 1</td>
<td>P. Miller</td>
</tr>
<tr>
<td></td>
<td>JE 1 sup 3 derived from JE 1</td>
<td>P. Miller</td>
</tr>
</tbody>
</table>
TCS was supplemented with 2 ml 10 percent succinate and 0.5 ml 10 percent charcoal-filtered CAA per 100 ml of medium.

Solid media were prepared by the addition of 15 g agar ml$^{-1}$. Soft agar overlays were prepared by adding 7.5 g agar-1 to CAA-M.

Phage were maintained in a one-to-four mixture of Luria Broth (LB) and TCS, or in 3XD buffer at 4°.

Other Buffers and Media

- **TN**: 10 mM Tris-HCl pH 7.4, 0.15 M NaCl.
- **TNE**: 10 mM Tris-HCl pH 7.4, 0.15 M NaCl, 0.01 M EDTA.
- **SSC**: 0.15 M NaCl, 0.015 M Na$_3$ citrate.
- **LB**: 2.5 percent nutrient broth, 0.5 percent yeast extract.

Growth of Bacteria

Cultures were usually grown at 30°. Liquid cultures in TCS were grown overnight in one-tenth the normal amount of succinate and CAA in a gyrotory water bath shaking at 200 rpm. In the morning the normal concentrations of succinate (0.2 percent) and CAA (0.05 percent) were added and the shaking rate of the water bath was increased to 250 rpm. Cell density was determined with a Klett-Summerson colorimeter, equipped with a No. 54 filter. Klett numbers were converted to cell numbers by reference to a standard curve.

Plating cultures for plaque assays were prepared by growing cells in CAA-M medium or in a four-to-one mixture of CAA-M and TCS.

Bacteriophage

Bacteriophage ϕW-14 $^+$ was prepared from stocks derived from Kropinski and Warren's (1970) original isolate. Working stocks were always prepared from the original lysates.
ϕW-14 ts mutants were isolated as described in Results section; am mutants were isolated by P. Miller.

Preparation of high-titre phage lysates

ϕW-14 w+ - 200 ml of P. acidovorans were grown to a cell density of 3.0 x 10^8 cells per ml. ϕW-14, freshly prepared from a small volume of lysate, was added (approximate moi = 0.1). The culture was incubated until lysis was complete. A few drops of chloroform were added to the lysate and incubation was continued for 30 minutes. At this point lysates were generally stored overnight at 4°. In the morning, lysates were warmed to 30°. DNAse I (~5 μg ml⁻¹) was added and the lysate was gently agitated for 60 minutes. The lysate was digested with 10 μg ml⁻¹ of pronase (self-digested 30 minutes at 37°) for two hours or until most of the visible cell debris were digested. The lysate was centrifuged at 4° for 5 minutes and 5,000 rpm; the supernatant was transferred to polycarbonate centrifuge tubes and spun at 13,000 rpm for 40 minutes. The pellet was gently resuspended in a four-to-one mixture of TCS and LB (TCS/LB). After resuspension, the lysate was subjected to another cycle of differential centrifugation. The high-speed pellet was resuspended in 20 ml of TCS/LB and stored at 4° over CHCl₃.

The purification procedures for am and ts lysates were similar; however, high-titre lysates could only be obtained with cells grown on TCS/CAA-M at four-to-one v/v (TCS/CAA-M). Ts lysates were prepared at 20-22°. Twenty-five ml of P. acidovorans strain 29 cells were grown to saturation on TCS/CAA-M. Two ml of this culture were diluted into 25 ml of fresh medium and incubated at 20-22° until cell density reached 2-3 x 10^8
per ml. Several individual plaques were picked with a Pasteur pipette and added to the cultures. Cultures were incubated until lysis then treated in the same manner as \( w^+ \) lysates. The lysates were titered at 20° and 30°.

Am \( \phi W-14 \) lysates were prepared in the same way on strain 29 sup 2 cells at 30°. The lysates were titered on strain 29 and strain sup 2.

**Phage titration**

\( \phi W-14 \) was titered on CAA-M using the soft agar overlay technique (Adams, 1959).

**Conditioned medium**

Conditioned medium was prepared using strain 29 or 3L cells grown on TCS to a cell density of \( 3 \times 10^8 \) ml\(^{-1} \). Cells were centrifuged down and the supernatant was used immediately or filtered through 0.45 μ Millipore membranes into sterile glass bottles and stored at 4°. Media conditioned with 3L originally contained 500 μg ml\(^{-1} \) thymidine.

**Testing for antibiotic sensitivity**

Antibiotics were added at the appropriate concentration to exponentially growing cultures of \( P. \ acidovorans \). Growth was monitored with the Klötz.

The effect of various antibiotics on phage growth was monitored by plating for pfu throughout infection in the presence or absence of antibiotics. For antibiotics which did not affect the growth of the host cell, antibiotic sensitivity was also measured by testing for pfu on plates.
Phage mutagenesis

$\Phi W^{-14} w^+$ stocks were mutagenized to 0.1 percent survival with nitrous acid. They were prepared according to the methods of Roth.

The isolation of $\Phi W^{-14}$ ts mutants

Mutagenized $\Phi W^{-14}$ were diluted and plated so that each plate contained 100 to 200 plaques at 20°. Plaques were picked with sterile toothpicks and replicated onto lawns of $P. \text{acidovorans}$ strain 29. Replicas were incubated at 30° and 20°. Phage which lysed lawns of strain 29 at 20° but not at 30° were plaque purified for further study.

Isolation of $\Phi W^{-14}$ am mutants

$\Phi W^{-14}$ am mutants were isolated from mutagenized stocks of $\Phi W^{-14}$ by P. Miller. $P. \text{acidovorans}$ strain 29 (sup 2) was the permissive host and $P. \text{acidovorans}$ strains 29 or 3L were the non permissive hosts.

Complementation of ts $\Phi W^{-14}$

Ts stock lysates were diluted to approximately $10^6$ to $10^7$ pfu ml$^{-1}$. Samples were spotted onto a plate backed by a numbered grid and cross-tested against other $\Phi W^{-14}$ ts mutants. Plates were incubated at 30°. Lawn clearing at 30° by two separate ts mutants was scored as +. The method was not satisfactory for leaky ts mutants.

Screening ts and am mutants

Ten ml cultures of $P. \text{acidovorans}$ strain 29, growing at 30°, were infected at multiplicities of infection of 20. At 20 minutes after infection, samples for survivors were plated on CAA-M agar. At 25 minutes after infection, 1.0 $\mu$Ci ml$^{-1}$ of [6-3H]-uracil without carrier was
added and incorporation was continued until 45 minutes post-infection for am mutants and later for ts mutants. DNA was purified from the phage-infected cells as described below.

**DNA purification**

Samples were poured into an equal volume of ice-cold TNE and centrifuged at 8,000 rpm for 5 minutes at 4°C. The supernatant was discarded. (Infected cells stick to the side of the centrifuge tube.) Ice-cold TNE was added to the tube and the cells scraped from the side. Two mg ml⁻¹ of self-digested pronase was added. Then an equal volume 1xSSC and SDS to a final concentration of 0.5 percent was added. The viscous lysate was incubated overnight at 37°C. In the morning, SSC-washed, redistilled phenol was added and the DNA was extracted by rolling at low speed for 2 hours. The mixture was spun at 5,000 rpm for 5 minutes and the phenol layer and the interface was removed. The aqueous phase was reextracted once with phenol as described above. The aqueous layer was washed three times with water-saturated ether. The DNA was precipitated with two volumes of 95 percent ethanol. The precipitated DNA was resuspended overnight in TNE. The next day 50 μg ml⁻¹ of heat-treated pancreatic RNase was added. Digestion with RNase was carried our for 1 hour at 37°C. The DNA was reextracted once with phenol and then ether washed. The DNA was precipitated with 95 percent ethanol and resuspended in TNE. DNA solutions were kept frozen at -20°C until ready for use.

**Acid hydrolysis of DNA samples**

A DNA sample was ethanol-precipitated, washed three times with 95 percent ethanol and twice with ether. The DNA was air-dried and resuspended
in 0.2 ml of 6N HCl. The sample was incubated until it had dissolved and was transferred to a hydrolysis vial. The hydrolysis vials were sealed under reduced pressure and then placed at 100° for 2 hours. This treatment converted 80 to 90 percent of the pyrimidine nucleotides to free bases. Although conversion was greater than 90 percent for a longer hydrolysis time, the deamination of cytosine was increased also. The other pyrimidine bases were stable. After hydrolysis, vials were opened and the HCl was removed from samples by evaporation in a vacuum dessicator over NaOH.

**TLC of acid-hydrolyzed DNA**

The samples were resuspended in 25 µl of 0.2 N HCl and 25 µl of a standard base mixture containing 1 mg ml⁻¹ each of putThy, Cyt, Ura, hmUra and Thy. The total hydrolysates were spotted as small (<0.5 cm) diameter spots on cellulose thin-layer sheets. Bases were resolved by two-dimensional development with solvents B and D. The thin-layers were thoroughly air-dried after chromatography in each solvent.

**Pulse-labelling procedures**

The pulse-labelling protocol was similar for all the isotopes. Cultures were grown to a density of 3 x 10⁸ ml⁻¹ and infected at a multiplicity of infection (moi) of 10. At 35 to 40 minutes after infection, the cells were labelled with ³²P₀₄, [6-³H]-uracil or [5-³H]-uracil for 10 seconds. Cells for ³²P₀₄ pulse-labelling were grown in TCS 1/5 x P₁ and the pulse was stopped by pouring the culture over one volume of partially frozen 0.1 M sodium phosphate buffer pH 7.0, containing EDTA (0.01 M) and 0.02M KCN.
Cells for tritiated uracil pulse-labelling were grown in TCS 1 x $P_\text{i}$ and the pulse was stopped by pouring the culture over one volume of partially frozen TNE buffer containing 1 mg ml$^{-1}$ uracil and 0.02 M KCN. The cells were spun down at 5,000 rpm for 5 minutes, resuspended in ice-cold TNE and repelleted. Once again the cells were resuspended in TNE and processed, as described previously, for the extraction of intracellular DNA. All pulse experiments were carried out at 30°.

**Nucleotide pool analysis**

$[2\,{}^{14}\text{C}]$-uracil and $^{32}\text{P}_4$-labelled nucleotide pools were prepared according to published procedures.

$^{32}\text{P}_4$ was added one to two cell doublings prior to infection in order to allow equilibrium labelling of the nucleotide pools. At a density of $3 \times 10^8$ cells ml$^{-1}$ the culture was infected at a multiplicity of infection of 10. Five ml samples were taken at the indicated times and passed through 0.45 µ Millipore HA membranes. The filters were washed with 2.5 ml each of ice-cold TCS 1 x $P_\text{i}$ and transferred to a beaker containing 2.0 ml of 0.3 N HCOOH. The formic acid-extracted cells on the filter were kept on ice. After 30 minutes the cells were resuspended in the formic acid by scraping them off the filters. The filters were re-extracted with 0.5 ml of 0.3 N HCOOH. The two fractions were combined and centrifuged at 8,000 rpm for 10 minutes at 4°. The supernatant was removed taking care not to disturb the cell pellet, which was discarded. The formic acid extract was lyophilized, and the residue resuspended in a measured volume of $\text{dH}_2\text{O}$. The nucleotide pools were stored at -20° until chromatography.
Chromatography of nucleotide pool preparations of PEI-cellulose

Chromatography procedures were described by Randerath and Randerath (1966).

The PEI sheets used were prepared according to the procedures of Randerath (1966).

CsCl density gradient analysis of DNA

Five g of a saturated solution of CsCl in dH₂O was added to a polyallomer or nitrocellulose ultracentrifuge tube. DNA samples and TNE were added until the total gradient weight was 6.0 g. The gradients were mixed, overlayed with paraffin oil and centrifuged for 72 hours at 30,000 rpm in a SW 50.1 or SW 39 rotor. The bottoms of the tubes were punctured and the gradients dripped onto glass fiber filters or Whatman 3MM paper squares. The glass fibre filters were dried at 80°, overlayed with scintillant and counted.

Gradients containing ³H and ¹⁴C label were dripped onto numbered squares of paper. The squares were washed three times in ice-cold 5 percent TCA, three times in 95 percent ethanol and twice in ether. The DNA samples were eluted from the paper in vials overnight with 0.5 ml of 0.1 N HCl. In the morning, 0.5 ml of 0.1 N NaOH was added to neutralize the acid, 10 ml of Triton:Toluene or Bray's scintillant (1960) was added and the samples were mixed, allowed to stabilize, and counted. Cross channel overlap was estimated by preparing standards containing known ratios of isotopes and treating them in an identical manner to authentic samples. Quenching was assumed to be identical for all similarly processed samples and standards.
Sonication of DNA

DNA samples were diluted to the same concentration before sonication. Sonication was for a total of 1 minute (4 x 15 second bursts at a setting of 50 on a Bronwill sonic oscillator). Samples were kept on ice prior to and during sonication.

Heat treatment of DNA

DNA samples in the TNE or 1 x SSC were boiled for 5 minutes in a capped tube and then quick-cooled by placing the tube in an ice bucket.

Measurement of DNA accumulation

$\left[^{3}\text{H}\right]$-uracil - Cultures contained $1.0 \mu\text{Ci ml}^{-1}$ of label and $10 \mu\text{g ml}^{-1}$ of cold uracil. $0.1\text{ ml}$ samples were taken directly into $1.0\text{ ml}$ of $0.3\text{ NaOH}$ and incubated overnight at $37^\circ$. In the morning $1.0\text{ ml}$ of $0.3\text{ N HCl}$ and $0.25\text{ ml}$ of 50 percent TCA were added to the samples. After thorough mixing the samples were chilled on ice for at least 60 minutes. The precipitated material was trapped on $0.45\mu\text{ Millipore membranes}$. The tubes were washed three times with ice-cold 5 percent TCA and then three times with 95 percent ethanol, each washing being passed through the membrane. The filters were placed in scintillation vials, dried at $80^\circ$, overlayed with Toluene-based scintillant and counted.

$\left[^{3}\text{H}\right]$-ornithine - Cultures contained $1.0 \mu\text{Ci ml}^{-1}$ of label and $10 \mu\text{g ml}^{-1}$ of ornithine and $100 \mu\text{g ml}^{-1}$ of arginine. $0.1\text{ ml}$ samples were taken into $1.0\text{ ml}$ of TNE containing 0.5 percent SDS and $100 \mu\text{g ml}^{-1}$ each of arginine and ornithine. Pronase was added to a final concentration of $2 \mu\text{g ml}^{-1}$ and digestion was carried out overnight at $37^\circ$. In the
morning, TCA was added to a final concentration of 5 percent. The
samples were collected, washed, dried and counted as described above.

\[^{3}H\]-TdR - Cultures contained 10 \( \mu \)Ci \([^{3}H]-\text{TdR}\) per ml of cul-
ture and 250 \( \mu \)g TdR per ml. 0.1 ml samples were taken into 1 ml of 5
percent TCA. Samples were chilled and processed as described above.

**Measurement of tritium release from \([5-^{3}H]\)-uracil**

\([5-{^{3}H}]\)-uracil was added to the cultures containing 10 \( \mu \)g cold
uracil ml\(^{-1}\). 0.5 ml samples were taken at intervals into 1.0 ml of a
PCA-Norit slurry (20 g charcoal per 100 ml of 4 percent PCA). The sam-
ples were filtered through a GF/F filter and the filtrate was retained.
An aliquot of the filtrate was counted in Bray's solution or in Scinti-
verse.

**Enzymatic digestion of DNA**

The concentration of the DNA sample was determined spectrophoto-
metrically and the required amount of DNA was precipitated with two volumes
of 95 percent ethanol, washed several times with 70 percent ethanol, sev-
eral times with 95 percent ethanol, then with ether and air-dried. The
sample was redissolved in the minimum volume of sterile distilled, deion-
ized water, then boiled for 5 minutes to inactivate residual nucleases and
to ensure denaturation of DNA. Ammonium acetate, pH 5.0 was added to give
a final concentration of 50 mM, \( \text{ZnSO}_{4} \) was added to give a final concentra-
tion of 0.1 mM and 10 units of \( S_{1} \) nuclease were added per \( \mu \)g of DNA. The
sample was incubated for 4 hours at 55°, then lyophilized. The residue
was resuspended in deionized, distilled water and lyophilized again. The
residue was suspended in the minimum volume of deionized, distilled water
and \( \text{NH}_{4}\text{CO}_{3} \), pH 8.4, and \( \text{MgCl}_{2} \) were added to final concentrations
of 100 mM and 15 mM, respectively. Snake venom phosphodiesterase was added to a final concentration of 20 \( \mu \text{g ml}^{-1} \) and the sample was incubated at 37° for 2 hours.

Limit digestion of DNA with DNase I and snake venom phosphodiesterase was performed as follows. The DNA was treated as described for \( S_1 \) digestion. The dried sample was dissolved in distilled, deionized water. Tris-HCl, pH 8.2 and MgCl\(_2\) were added to give final concentrations of 50 mM and 15 mM, respectively. Then 20 \( \mu \text{g} \) of each enzyme were added per ml of DNA solution and the mixture incubated at 37° for 4 hours.

The nucleotides released were separated by thin-layer chromatography and detected by fluorography for tritium-labelled samples or by autoradiography for \( ^{32}\text{P} \)-labelled samples, (see below).

**Thin-layer chromatography of enzymatic DNA digests**

The 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 percent ethanol to remove inorganic salts. Mononucleotides remained at the origin. Mononucleotides were separated by development with solvent E and then with solvent A. The direction of separation in the first dimension was perpendicular to the direction of washing. Chromatography sheets were dried in between first dimension and second dimension chromatography.

**Solvents for TLC on unmodified cellulose**

**Solvent A** - saturated (NH\(_4\))\(_2\)SO\(_4\):1 M Na acetate:isopropanol

\[(80:12:2 \text{ v/v}).\]
Solvent C - n-butanol:H₂O (86:14 v/v).

Detection of radioactivity on thin-layer sheets

Tritium-labelled nucleotides were detected by impregnating sheets with a 7 percent solution of PPO in ether. The ether was evaporated and the chromatogram was exposed to film at -70° for an appropriate length of time prior to development (Randerath, 1969).

Chromatograms containing ³²P-labelled nucleotides were exposed to film and the film was developed after an appropriate length of time.

Bacterial alkaline phosphate digestion

0.03 units of BAP-F (Millipore Corp.) was added to the sample in 50 mM Tris-HCl, pH 8.4 containing 15 mM MgCl₂. Digestion was carried out at room temperature for one hour. The enzymatic digests were spotted directly on thin-layer sheets.

Column chromatography

Biogel P₂ chromatography of S₁-snake venom phosphodiesterase digest of am 37 DNA. The column dimensions were 45 x 1 cm. The elution buffer was 0.1 M ammonium acetate, pH 7.0. The sample was loaded in a volume of 0.8 ml of elution buffer, and 2.5 ml fractions were collected.

DEAE - Sephadex chromatography. The column was 8 x 1 cm. The sample was applied in 20 mM Tris-HCl, pH 8.0. The column was
eluted with a linear gradient of NaCl from 0 to 0.4 M, total volume 160 ml, and 3 ml fractions were collected.

**DEAE-Sephadex-7 M urea chromatography.** Twenty mM Tris-HCl pH 7.5 $^{32}$P-labelled am 37 DNA was digested to mononucleotides with S₁ nuclease and snake venom phosphodiesterase. The digest was diluted to 3.0 ml with 20 mM Tris-HCl pH 7.5, 7 M urea. dTTP and dTDP were added to give final concentrations of 75 μg ml$^{-1}$. The sample was applied to a DEAE-Sephadex A-25 column (1 x 40 cm). The resin was washed with one bed volume of 20 mM Tris-HCl, 7 M urea and the nucleotides were eluted with a linear gradient on NaCl (0 to 0.4M) in this buffer. The total volume of eluate was 500 ml. It was pumped through the column at a constant rate of 1 ml min$^{-1}$. Fraction volume was 5 ml. Samples were assayed for Cerenkov radiation and for absorbance at 267 nm. DEAE-Sephadex columns were in the chloride form.

**Purification of the unknown nucleotide**

$^{32}$P-labelled 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 x 20 cm). The sheet was washed by descending development in 95 percent ethanol. It was then dried and developed in one dimension by descending development with Solvent E. The bottom of the sheet had been serrated to ensure the uniform flow of solvent as it dripped off the paper. After autoradiography, to locate the nucleotides, strips containing them were cut from the sheets and washed free of isobutyrate with 95 percent ethanol by descending chromatography. The salt-free nucleotides were recovered from paper strips by descending development with distilled water.
Preparation of cell-free extracts

Strain 29 was grown to a density of $3 \times 10^8$ cells ml$^{-1}$ then infected with phage at a multiplicity of infection of 20. The cells were collected by centrifugation at 35 minutes after infection (3,000 g x 5 minutes at ambient temperature). The cell pellet was resuspended in one growth volume of buffer (50 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, 10 mM MgCl$_2$ and 50 mM KCl) and repelleted. The cells were resuspended in the Tris buffer at a density of $5 \times 10^9$ ml$^{-1}$. Lysozyme was added to a final concentration of 1 mg ml$^{-1}$ and the suspension was incubated at 30° for 30 minutes. The suspension was quick-frozen in an alcohol-dry-ice bath and quickly thawed at 30°. The freeze-thaw step was repeated three times. The resulting extract was passed through a 26 gauge needle to reduce the viscosity.

In vitro synthesis of putThy

Reaction mixtures contained 30 µl putrescine (10 mM), 30 µl crude cell extract, 30 µl of $^{32}$P-labelled am 37 DNA (400 µg ml$^{-1}$) and 210 µl of Reaction buffer (see above). The am 37 DNA substrate contained an unknown proportion of strain 29 DNA and parental am 37 DNA. The effect of these substrates on the reaction is not known. At intervals during incubation at 30°, 50 µl samples were removed to 0.5 ml of ice-cold Tris-HCl (10 mM), 0.15 M NaCl, 0.01 M EDTA, pH 7.5, containing 100 µg ml$^{-1}$ of unlabelled øW-14 DNA. Two volumes of ice-cold 95 percent ethanol were then added to the samples. After standing on ice for 30 minutes the DNA was collected by centrifugation and 500 µl of the ethanol-buffer supernatant was removed for the determination of released radioactivity.
The DNA was repurified and digested with S1 and snake venom phosphodiesterase as described earlier. The mononucleotides were separated by 2-dimensional-thin-layer-chromatography on cellulose thin-layer sheets.

**Chemicals**

Chemicals were of analytical grade and were used without further purification.

**Enzymes**

Pancreatic RNase was purchased from Calbiochem. It was dissolved in TNE at a concentration of 1 mg ml$^{-1}$ and boiled at 100° for 5 minutes prior to use. DNase I and snake venom phosphodiesterase were purchased from Calbiochem and were used without further treatment or purification. Nuclease S1 was obtained from Miles Laboratories Inc. Bacterial alkaline phosphatase F (BAP-F) was purchased from the Millipore Corporation.

**Radiochemicals**

All radiochemicals were purchased from New England Nuclear and were used without further purification.
RESULTS AND DISCUSSION

Nucleotide pools

Kelln (1973) detected putThy nucleotides in acid-soluble extracts of \( \Phi W-14 \)-infected \( P. \) acidovorans strain 29. This was presumptive evidence that putdTTP was a precursor of putThy in \( \Phi W-14 \) DNA. Several attempts to repeat this experiment were unsuccessful. Specific activities of \([^{3}H]\)-ornithine ten times greater than used in normal DNA accumulation protocols did not label any nucleotides resolvable by 2D-PEI cellulose thin-layer chromatography.

\([^{14}C]\)-uracil labelling of nucleotide pools in infected and uninfected \( P. \) acidovorans strain 29 was also undertaken. All uracil-labelled compounds separable using the total pool separation protocol appeared in both infected and uninfected cell extracts (Figure 2). Micromole quantities of labelled nucleotides could have been detected and resolved. Thy, \( hUra \) or Ura nucleotides containing the same number of phosphate groups were not resolved from each other. Within the limits of detectability there were no putThy nucleotides in the soluble pools of infected cells. Subsequently Neuhard and Warren (1980) were able to demonstrate the presence of \( hmdUTP \) in the nucleotide pools of \( \Phi W-14 \)-infected cells. dTTP is excluded from the nucleotide pools of infected cells. dTTP, dUTP and \( hmdUTP \) were not resolved in the normal PEI-cellulose system used for the resolution of nucleotide triphosphates. A
FIGURE 2.—Resolution of [2-$^{14}$C]-uracil-labelled nucleotides in uninfected and infected cells.

Samples were collected from A) Uninfected *P. acidovorans* strain 29, and B) *P. acidovorans* strain 29 at 35 minutes after infection with ØW-14. The labelling, extraction and chromatography of the nucleotide pools are as described in the Materials and Methods section.
three-dimensional system employed by Neuhard resolves dTTP and hmUTP from each other (Neuhard, personal communication).

**In vitro**, the sole detectable product of THFA-mediated $^{14}$C-formaldehyde labelling of dUMP was $^{14}$C-hmdUMP (Neuhard et al., 1980). Kinases capable of generating hmdUTP from hmdUMP were also detected. dTTP is excluded from infected cell nucleotide pools by 30 minutes after infection. ØW-14 infection induces an activity capable of inhibiting host thymidylate synthase as well as a potent dUTP/dTTPase (Neuhard et al., 1980).

Measurements of nucleotide levels in pools extracted from cells at various times after infection were performed (Table 3). Infected cell pools uniformly labelled with $^{32}$P$_{4}$ were separated by 2D-TLC on PEI-cellulose. In ØW-14-infected cells the levels of all measured nucleotides dropped despite the fact that DNA synthesis rates after infection are greater than rates in uninfected cells (Kelln and Warren, 1973). This means that the flow of precursors through nucleotide pools was in equilibrium with the demands of DNA synthesis for precursors. More meaningful determinations of nucleotide pool activities could probably be obtained using procedures to measure the differential synthesis rate of each pool nucleotide. The dCTP and hmdUTP pools declined in size after infection until they reached an equilibrium level (Table 3). This contrasts with the data of Neuhard (personal communication), who observed increases in pool sizes for hmdUTP and dCTP after an initial decline. The differences between
TABLE 3.—The deoxynucleoside triphosphate pools of \( \Phi W-14 \)-infected

\( P.\ acidovorans \) strain 29.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>0 min</th>
<th>10 min</th>
<th>20 min</th>
<th>30 min</th>
<th>40 min</th>
<th>50 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>dGTP</td>
<td>1318</td>
<td>601</td>
<td>366</td>
<td>274</td>
<td>266</td>
<td>218</td>
</tr>
<tr>
<td>dATP</td>
<td>429</td>
<td>338</td>
<td>362</td>
<td>-</td>
<td>169</td>
<td>271</td>
</tr>
<tr>
<td>dCTP</td>
<td>5555</td>
<td>1281</td>
<td>1376</td>
<td>1961</td>
<td>1225</td>
<td>1181</td>
</tr>
<tr>
<td>dUTP/dTTP/hmdUTP</td>
<td>1704</td>
<td>750</td>
<td>414</td>
<td>421</td>
<td>408</td>
<td>236</td>
</tr>
</tbody>
</table>

Nucleotide pools were labelled, prepared and separated as described in
the Materials and Methods. The values given are cpm in the area cut
from the chromatogram. dUTP, dTTP and hmdUTP were not separated from
one and other in this experiment.
results can probably be explained by the use of different media, growth temperature and aeration conditions. In our laboratory, accumulation of nucleotides in infected cells is seen only in DO amber mutants grown under nonpermissive conditions (P. Miller, unpublished observations). These mutants accumulate nucleotides after infection because they are unable to make phage DNA. Unlike T4, in \( \Phi W-14 \) DNA hmdUTP and dCTP are not found in the soluble nucleotide pools at the same ratios that they are found in phage DNA. The size of the dCTP pool in infected cells was approximately four times greater than the size of the hmdUTP pool. This difference did not appear to have any significance. The size of the infected cells' dCTP and hmdUTP pools was less important than the rate of flow of precursors through the pool. The flow of dCTP, hmdUTP, dGTP and dATP flow through the nucleotide pools was balanced.

In vitro assays and pool studies by Neuhard and Warren suggested that hmdUTP and not dTTP is the precursor of putThy and Thy in \( \Phi W-14 \) DNA.

Deoxyuridine but not deoxythymidine was a \( \Phi W-14 \) DNA precursor

Before radioactive deoxyuridine could be used to label \( \Phi W-14 \) DNA, it was necessary to isolate a derivative of strain 3L which would not incorporate exogenous uracil into DNA. This was necessary because commercial preparations of \( [6-^3H]d\text{UdR} \) and \( [2-^{14}C]d\text{UdR} \) contain appreciable quantities of radioactive
uracil. Uracil phosphorylase mutants are resistant to 5-fluorouracil because they cannot make 5-fluoro UMP (O'Donovan and Neuhard, 1970). Therefore host mutants without uracil phosphorylase activity are readily isolated by growing cells in the presence of 5-fluorouracil.

5-fluorouracil derivatives of 3L were isolated from plates spread with 3L and seeded with crystals of 5-FU. Colonies appearing inside zones of growth inhibition after 2 to 3 days were picked, restreaked and reisolated as described above. Five 3L/FU^R strains were obtained this way. They required TdR for growth and were sensitive to ϕW-14 infection. The isolates were tested for the ability to incorporate [6-^3H]-uracil (Figure 3). Of the five clones, only one, 3L/FU^R_2, was found which did not incorporate [^3H]-uracil into TCA insoluble counts in uninfected or infected cells (Figure 3). 3L/FU^R_2 was tested for the ability to incorporate [6-^3H]-dUdR into uninfected ϕW-14 infected cell DNA. Only infected cells accumulated [6-^3H]-dUdR in alkali-resistant, TCA-precipitable material (Figure 4). Acid hydrolysis and thin-layer chromatography of ϕW-14 DNA labelled with [6-^3H]-dUdR showed that it contained labelled Thy and putThy (Table 4). Cytosine was not labelled. As a control, [^3H]-ornithine-labelled ϕW-14 DNA grown in 3L/FU^R_2 was prepared and subjected to acid hydrolysis and thin-layer chromatography. Ornithine labels this DNA, and it was assumed that the compound detected in hydrolysates was putThy. In ϕW-14 infected 3L/FU^R_2 labelled with [6-^3H]-dUdR, putThy and Thy accumulated in the DNA at equal rates throughout infection (Figure 5).
FIGURE 3.—The incorporation of uracil by 3L/FU$^{R_2}$.

At 0 minutes [6-$^3$H]-uracil (1.0 µCi ml$^{-1}$, 10 µg ml$^{-1}$) was added to cultures of 3L, 0; 3L/FU$^{R_2}$, •; and φW-14 infected 3L/FU$^{R_2}$, △. Incorporation of the label into TCA precipitable material was followed.
FIGURE 4.—The incorporation of deoxyuridine in $\phi W$-14 infected $3L/FU^R_2$.

A growing culture of $3L/FU^R_2$ was split into two halves. One half was infected with $\phi W$-14. [6-$^3$H]-UdR (4.0 $\mu$Ci ml$^{-1}$, without carrier) was added at 10 minutes after infection. The incorporation of radioactivity into alkali resistant, TCA precipitable material was followed. $\phi W$-14 infected $3L/FU^R_2$, ★; $3L/FU^R_2$, ○. The arrow indicates the beginning of cell lysis in the infected culture.
TABLE 4.—Deoxyuridine labelling of bases in ΦW-14 infected 3L/FU^R_2.

<table>
<thead>
<tr>
<th>Base</th>
<th>[³H]-orn (cpm)</th>
<th>[6-³H]-Ura (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>putThy</td>
<td>1593 (0.95)^a</td>
<td>1853 (0.48)</td>
</tr>
<tr>
<td>Cyt</td>
<td>37 (0.02)</td>
<td>88 (0.02)</td>
</tr>
<tr>
<td>Thy</td>
<td>55 (0.03)</td>
<td>1903 (0.50)</td>
</tr>
</tbody>
</table>

ΦW-14 infected cultures of 3L/FU^R_2 were labelled with [³H]-orn (0.1 μCi μg⁻¹) or [6-³H]-dUdR (4.0 μCi ml⁻¹). The cultures were allowed to lyse and the DNA was purified from the phage particles. The DNA was acid-hydrolyzed and the bases were separated by one dimensional thin-layer chromatography in solvent E.

^a The values shown are cpm in areas cut from the chromatogram. The values in parentheses are the fractions of the radioactivity recovered in labelled bases.
FIGURE 5.—The accumulation of Thy and putThy in $\Phi W$-14 DNA.

$3L/FU^R_2$ was infected with $\Phi W$-14 and labelled with [6-$^3$H]-UdR as described in Figure 4. Aliquots of the infected culture were withdrawn at intervals after infection and DNA was purified from the cells. The DNA was hydrolyzed in 6N HCl and Thy and putThy were separated by 1D-TLC. $^3$H-label in Thy, $^3$H-label in putThy,
Kelln had reported that $[\text{methyl-}^{3}\text{H}]$-TdR would label $\Phi W$-14 DNA in $P$. acidovorans 3L. This result could not be reproduced. The incorporation of thymidine in uninfected and infected 3L was measured. Only uninfected 3L accumulated alkali-resistant, TCA-precipitated label (Figure 6). Warren has obtained similar results, and he has also shown that host DNA prelabelled with $[^3\text{H}]$-TdR is not degraded and reincorporated into $\Phi W$-14 DNA (Maltman et al., 1980).

Although the data strongly suggested that $\Phi W$-14-infected cells could not incorporate deoxythymidine, it was possible that this was due to an overwhelming preference for endogenously supplied deoxythymidine nucleotides. $\Phi W$-14-infected strain 3L produced viable phage bursts after exogenous thymidine was removed by centrifugation and washing (Figure 7). $\Phi W$-14-infected 3L was spun down, washed and resuspended in conditioned TCS medium without cold thymidine but containing 25 $\mu$Ci ml$^{-1}$ $[\text{methyl-}^{3}\text{H}]$-TdR. The infected cells were allowed to lyse and DNA was extracted from the purified phage. A small amount of tritium-labelled $\Phi W$-14 DNA was obtained which banded with reference $\Phi W$-14 DNA in neutral CsCl gradients (Figure 8). Acid-hydrolysis and TLC of this DNA showed that none of the label was in thymine; most of the label in bases ran in the guanine, adenine region of the chromatogram. Only 6 percent of the label was recovered in bases. Therefore, even specific activities of label great enough to cause "garbage labelling" of $\Phi W$-14 DNA, failed to label the thymine bases of $\Phi W$-14 DNA.
FIGURE 6.—Thymidine incorporation in strain 3L and in ØW-14-infected strain 3L.

[Methyl-³H]-thymidine (0.04 μCi μg⁻¹, 250 μg ml⁻¹) was added to a culture of ØW-14 infected 3L; and to an uninfected culture of 3L, O. The incorporation of radioactivity into TCA precipitable radioactivity was determined.
FIGURE 7.—Thymidine was not required for plaque production in \( \phi W-14 \) infected strain 3L.

At 10 minutes after infection of strain 3L with \( \phi W-14 \), the infected culture was spun down and resuspended in one volume of thymidine free conditioned TCS medium. Aliquots of the infected culture were plated through CHCL\(_3\) at intervals.
MINUTES AFTER
RESUSPENSION
FIGURE 8.--Nonspecific labelling of φW-14 DNA by high specific activity thymidine.

P. acidovorans 3L was infected with φW-14 and at 10 minutes after infection the cells were spun down and resuspended in conditioned medium containing 25.0 μCi ml⁻¹ of [methyl-³H]-TdR (without carrier). The cultures were allowed to lyse and the DNA was purified from phage particles. Part of the DNA was acid hydrolyzed and the bases were separated by 1D-TLC in solvent B. The remaining DNA was analyzed on a neutral CsCl density gradient. [³H]-TdR, O; ³²P-labelled φW-14 reference DNA, •.
Attempts were also made to density label \( \Phi W-14 \) DNA with the thymidine analogue, BUdR. 3L grown in TdR was spun down and resuspended in 500 \( \mu g \, ml^{-1} \) of BUdR. The \( \Phi W-14 \) DNA, also labelled with \(^3\text{H}\) ornithine, was extracted from phage and analyzed on neutral CsCl density gradients (Figure 9). Reference DNA and \( \Phi W-14 \) DNA from phage grown in the presence of BUdR banded at the same density, showing that BUdR was not incorporated into DNA in \( \Phi W-14 \)-infected cells (Figure 9).

It was concluded that exogenously supplied TdR was not a precursor of thymine in \( \Phi W-14 \) DNA. The ability of [6-\(^3\text{H}\)]-UdR to label putThy and Thy bases demonstrated that the enzyme thymidine kinase was active in \( \Phi W-14 \)-infected \textit{P. acidovorans} (Table 4).

**Endogenous thymidine utilization in \( \Phi W-14 \)-infected \textit{P. acidovorans} 3L**

Exogenously supplied TdR was not a precursor of thymine in \( \Phi W-14 \) DNA. This did not exclude the possibility that endogenously supplied TdR was the source of the thymine residues in DNA. The methods for demonstrating endogenous requirements for TdR are well established but indirect, and rely upon the use of inhibitors with known mechanisms of action.

FUdR was found to inhibit the growth of \textit{P. acidovorans} strain 29 at concentrations greater than 250 \( \mu g \, ml^{-1} \) (Kelln and Warren, 1973). \textit{P. acidovorans} 3L, a derivative of strain 29, is resistant to high concentrations of FUdR. 3L is a thymidylate synthase mutant which requires at least 250 \( \mu g \, ml^{-1} \) TdR for cell growth (Kelln, Ph.D. Thesis, 1973). Cell growth was measured in these experiments by plating on solid CAA-M medium in the presence of the drug. 500 \( \mu g \, ml^{-1} \) FUdR did not prevent
FIGURE 9.—Buoyant density of φW-14 DNA synthesized in the presence of B UdR.

φW-14-infected *P. acidovorans* strain 3L was spun down and resuspended in conditioned TCS medium without thymidine. B UdR (500 μg ml⁻¹), adenosine (100 μg ml⁻¹) and ³H-ornithine (1.0 μCi ml⁻¹, 10 μg ml⁻¹) were added and the cells were allowed to lyse. DNA was purified from phage particles and analyzed on neutral CsCl buoyant density gradients. ³H-orn label, O; ³²P-labelled φW-14 φ⁺ phage reference DNA, ⋆.
the normal accumulation of plaque-forming units in \( \Phi W-14 \)-infected strain 3L (Figure 10). \( \Phi W-14 \) also formed plaques on 3L growing on CAA-M plates containing TdR and FUdR.

The failure of FUdR to inhibit phage formation in \( \Phi W-14 \)-infected cells did not exclude the possibility that FUdR could affect DNA replication or modification. FUdR had little or no effect upon the accumulation of DNA measured using \([2-^{14}C]\)-uracil or \([3^H]\)-ornithine (Figure 11). The differences in label accumulated by infected cultures with and without added FUdR were not significant. When the data points were replotted as a percentage of the final incorporation value, the relative ratios of accumulation for all cell cultures were identical (Figure 12). Identical accumulation rates for \([2-^{14}C]\)-Ura and \([3^H]\)-Orn label suggested that DNA replication and DNA modification proceeded normally in the presence of FUdR.

Permeability of \( P. \ acidovorans \) to FUdR was evident. Growth of strain 29 was inhibited by FUdR and radioactive deoxyuridine labelled \( \Phi W-14 \) DNA in 3L/FU \( ^R_2 \).

FUdR could exert a non lethal effect upon DNA modification in \( \Phi W-14 \)-infected cells. FUdR might be incorporated into phage DNA or FUdR might inhibit the accumulation of putThy or Thy residues. \([6-^{3}H]\)-Ura-labelled \( \Phi W-14 \) DNA prepared in the presence of FUdR was examined. FUdR added to cells 30 minutes prior to, or at the onset of phage infection had no effect upon the buoyant density of the DNA synthesized (Table 5). After acid hydrolysis and TLC, phage DNA prepared in the presence of FUdR showed normal labelling ratios for putThy, Cyt and Thy bases (Table 6). Gradient and hydrolysis data were similar whether strains 29 or 3L were used (Table 7).
FIGURE 10.—The effect of trimethoprim and FUdR upon plaque production in ØW-14-infected strain 3L.

Cultures of strain 3L growing in TCS medium with 250 μg ml\(^{-1}\) TdR were supplemented with 500 μg ml\(^{-1}\) FUdR, △; or with 100 μg ml\(^{-1}\) Tp,○; no addition,●. The cultures were then grown to a density of 3.0 x 10\(^8\) cells ml\(^{-1}\) and infected with ØW-14. The number of plaque forming units in each culture was determined at intervals after infection.
FIGURE 11.—The effect of fluorodeoxyuridine upon DNA synthesis in \( \Phi W-14 \)-infected \emph{P. acidovorans} 3L.

Strain 3L was infected with \( \Phi W-14 \) and label was added to cultures 20 minutes later. Ornithine labelling of \( \Phi W-14 \) DNA was followed by measuring the incorporation of \(^3\text{H}\)-orn into pronase resistant, TCA precipitable material. The incorporation of \([2-^{14}\text{C}]\text{-uracil}\) was followed by measuring the accumulation of alkali resistant, TCA precipitable material. Infected cells labelled with \(^3\text{H}\)-orn, ●; infected cells grown in FUdR (500 \( \mu \text{g ml}^{-1} \)) and labelled with \(^3\text{H}\)-orn, ○; infected cells labelled with \([2-^{14}\text{C}]\text{-uracil}\), ▲; infected cells grown in FUdR (500 \( \mu \text{g ml}^{-1} \)) and labelled with \([2-^{14}\text{C}]\text{-uracil}\), △.
FIGURE 12.—The accumulation of \( \phi W-14 \) DNA in the presence of FUdR.

The data was taken from the previous figure and replotted as a percentage of the final incorporation value. The symbols are defined in the legend of Figure 11.
PERCENT OF MAXIMUM ACCUMULATION

MINUTES AFTER INFECTION
TABLE 5.—The effect of trimethoprim or FUdR upon the buoyant density of \(\Phi_{W-14}\) DNA synthesized in P. acidovorans

<table>
<thead>
<tr>
<th>Time of addition of FUdR</th>
<th>(\Phi_{W-14})-infected strain 29</th>
<th>(\Phi_{W-14})-infected strain 3L</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min.</td>
<td>+(^{a})</td>
<td>+</td>
</tr>
<tr>
<td>30 min. before infection</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Time of addition of Tp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>90 min. before infection</td>
<td>-(^{b})</td>
<td>+</td>
</tr>
</tbody>
</table>

The DNA used in these experiments was labelled with [6-\(^{3}\)H]-uracil and extracted from the infected cells at 45 minutes after infection. These procedures are described in the Materials and Methods.

\(^{a}\) + indicates that the DNA made in infected cells had the same buoyant density as authentic \(\Phi_{W-14}\) reference DNA in neutral CsCl gradients.

\(^{b}\) Strain 29 cells with an established inhibition by trimethoprim did not support \(\Phi_{W-14}\) plaque production or DNA synthesis.
TABLE 6.—The base composition of \( \Phi W \)-14 DNA prepared in the presence of Trimethoprim or FUdR.

<table>
<thead>
<tr>
<th>Phage/Host/Inhibitor</th>
<th>putThy</th>
<th>Cyt</th>
<th>Thy</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Phi W )-14 ( \text{w}^+ )/29/none</td>
<td>2931 (0.24)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6027 (0.49)</td>
<td>3454 (0.28)</td>
</tr>
<tr>
<td>( \Phi W )-14 ( \text{w}^+ )/3L/none</td>
<td>4113 (0.20)</td>
<td>10912 (0.52)</td>
<td>5798 (0.28)</td>
</tr>
<tr>
<td>( \Phi W )-14 ( \text{w}^+ )/29/Tp (100 ( \mu g \text{ ml}^{-1} ))</td>
<td>1787 (0.21)</td>
<td>4027 (0.51)</td>
<td>2255 (0.28)</td>
</tr>
<tr>
<td>( \Phi W )-14 ( \text{w}^+ )/3L/Tp (100 ( \mu g \text{ ml}^{-1} ))</td>
<td>10980 (0.21)</td>
<td>27315 (0.54)</td>
<td>12543 (0.25)</td>
</tr>
<tr>
<td>( \Phi W )-14 ( \text{w}^+ )/29/FUdR (500 ( \mu g \text{ ml}^{-1} ))</td>
<td>27982 (0.22)</td>
<td>65100 (0.52)</td>
<td>31417 (0.25)</td>
</tr>
<tr>
<td>( \Phi W )-14 ( \text{w}^+ )/3L/FUdR (500 ( \mu g \text{ ml}^{-1} ))</td>
<td>34670 (0.22)</td>
<td>81435 (0.53)</td>
<td>38126 (0.25)</td>
</tr>
</tbody>
</table>

The infected cells were labelled from 25 minutes after infection with \([6-^3\text{H}]-\text{uracil}\). The antibiotics were added just before infection. The DNA was extracted from the infected cells at 45 minutes after infection. DNA samples were acid hydrolyzed and the bases were resolved by 1D-TLC in solvent B.

<sup>a</sup> The values represent cpm in the area cut from the chromatogram. The values in parentheses represent the fraction of the label found in putThy, Thy and Cyt bases.
TABLE 7.--\(\Phi W-14\) DNA modification was independent of the host strain.

<table>
<thead>
<tr>
<th>Base</th>
<th>host strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>29</td>
</tr>
<tr>
<td>putThy</td>
<td>4972 (0.24)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cyt</td>
<td>10858 (0.51)</td>
</tr>
<tr>
<td>Thy</td>
<td>5285 (0.25)</td>
</tr>
</tbody>
</table>

DNA was isolated from \(\Phi W-14\) particles after infection and \([6-^3H]-\)uracil labelling of growing cultures. The DNA samples were processed as described in the Materials and Methods.

<sup>a</sup> The values are cpm in the area cut from the chromatogram. The values in parenthesis are the fraction of the label in the pyrimidine bases.
Incorporation of 5-FUdR into PBS2 DNA has been reported (Lozeron and Szybalski, 1967). Accumulation of FUdR in the DNA made it heavier. It was unlikely ψW-14-infected cells used dUTP as a precursor of putThy or Thy. FUdR is an analogue of UdR. [6-3H]-UdR labelled putThy and Thy residues in ψW-14 DNA, but as shown earlier, DNA prepared in the presence of FUdR had the same buoyant density as ψW-14 reference DNA.

Therefore, the resistance of ψW-14 DNA replication and modification to FUdR also effectively excluded the possibility that uracil might be a precursor of putThy in ψW-14 DNA.

Evidence of a cell's ability to synthesize dTMP or hmdUMP from UdR can be obtained by in vivo measurement of tritium release from [5-3H]-uracil-labelled cells.

**[5-3H]-uracil release in ψW-14-infected P. acidovorans**

Thymidylate synthase or any enzyme catalyzing the release of tritium from [5-3H]-uracil can be detected in vivo in cells capable of converting uracil to dUMP. In vivo, tritium releasing activity was present in strain 29 but not in strain 3L uninfected cells (Figure 13). Both strains retained the ability to accumulate [5-3H]-uracil-labelled DNA. [5-3H]-uracil labels cytosine bases in DNA. 3L is a thymidylate synthase-deficient derivative of strain 29 (Kelln, Ph.D. Thesis, 1973). ψW-14-infected P. acidovorans strains 29 or 3L had almost identical tritium release curves, indicating that a tritium-releasing activity was induced by bacteriophage infection (Figure 13). The in vivo results agree with the in vitro results of Kelln (1973). Similar tritium release curves for strains 29 and 3L infected by ψW-14 also suggested that the
FIGURE 13.—DNA synthesis and tritium release in ΦW-14-infected P. acidovorans.

A) The incorporation of [5-3H]-uracil into alkali resistant, TCA precipitable material. P. acidovorans strain 29, uninfected, O; P. acidovorans strain 3L, uninfected, •; P. acidovorans strain 29, ΦW-14 infected, Δ; P. acidovorans strain 3L, ΦW-14 infected, △.

B) The release of tritium from [5-3H]-uracil. Measurement of the in vivo activity of dUMP hydroxymethylase. P. acidovorans strain 29, uninfected, O; P. acidovorans strain 3L, uninfected, •; P. acidovorans strain 29, ΦW-14 infected Δ; P. acidovorans strain 3L, ΦW-14 infected, △.
host thymidylate synthase activity was suppressed by bacteriophage infection, otherwise tritium release in \( \Phi W-14 \)-infected strain 29 would have been greater than in \( \Phi W-14 \)-infected strain 3L.

These results demonstrated the presence of [5-\(^3\)H] releasing activities in \( \Phi W-14 \)-infected cells but they did not prove that tritium release occurred at the level of the nucleotide pool. Kelln detected enzyme activities capable of releasing tritium from [5-\(^3\)H]-dUMP in vitro. Kelln claimed that the products of these reactions were dTMP and hmdUMP. Neuhard has shown that the sole product of this reaction is hmdUMP (Neuhard et al., 1980). The hmdUMP-synthesizing enzyme is probably resistant to FUDR. Concentrations of 5-FdUMP ten times greater than those inhibiting the host thymidylate synthase do not inhibit the \( \Phi W-14 \) hydroxymethylase activity in extracts (Neuhard and Warren, unpublished observations). The inability of \( \Phi W-14 \)-infected cells to utilize endogenous or exogenous TdR, coupled with the evidence of tritium releasing activity in infected cells supported the theory that hmdUMP was the only precursor of Thy and putThy in \( \Phi W-14 \) DNA.

**Trimethoprim (Tp) and trimethoprim and sulfonamide (sulfa) effects on \( \Phi W-14 \) reproduction**

The minimal inhibitory concentration of Tp for *P. acidovorans* strain 29 is between 2.5 and 5 \( \mu g \) ml\(^{-1} \). 100 \( \mu g \) ml\(^{-1} \) of trimethoprim inhibits the growth of strain 29 but does not inhibit the growth of the thymidylate synthase minus strain, 3L. Tp or Tp and sulfa-induced cultures of strain 29 stop growing by 4 hours after addition of drugs to exponential cultures (Figure 14). Additions of adenosine, thymidine, pantothenic acid and amino acids in attempts to prevent or reverse the
FIGURE 14.—The effects of trimethoprim or trimethoprim and sulfonamide upon the growth of \textit{P. acidovorans} strains 29 and 3L.

Cultures of strains 29 and 3L were grown in TCS medium supplemented with succinate (2 mg ml\(^{-1}\)) or with succinate and Casamino acids (1 mg ml\(^{-1}\)), adenosine (100 \(\mu\)g ml\(^{-1}\)), thymidine (500 \(\mu\)g ml\(^{-1}\)), and pantothenic acid (50 \(\mu\)g ml\(^{-1}\)). At the time indicated by the arrow, Tp, (100 \(\mu\)g ml\(^{-1}\)) or Tp (100 \(\mu\)g ml\(^{-1}\)) and sulfonamide (500 \(\mu\)g ml\(^{-1}\)) were added to the cultures. The increases in turbidity were followed with a Klett colorimeter.

A) Strain 29 grown in succinate; no additions, •; Tp, O; Tp and Sulfa, Δ.

B) Strain 29 grown in succinate, Casamino acids, thymidine, adenosine, and pantothenic acid; no additions, O; Tp, •; Tp and Sulfa, Δ.

C) Strain 3L grown in succinate and thymidine; no additions, O; Tp, •; Tp and Sulfa, Δ.

D) Strain 3L grown in succinate, Casamino acids, thymidine, adenosine, and pantothenic acid; no additions, O; Tp, •; Tp and Sulfa, Δ.
inhibitory effects of the drugs were only partially successful. Overnight incubation of cultures of Tp or Tp and sulfa-treated cultures increased the turbidity by 50 percent if supplements were present. Cultures without supplements did not grow at all. Supplements include all products normally requiring a THFA cofactor for biosynthesis.

Tp had no effect upon plaque production by ΦW-14-infected strain 3L cells grown in TCS medium or plated upon CAA-M plus TdR plus Tp plates (Figure 10). 3L is resistant to Tp because it does not deplete THFA and therefore does not need to regenerate large amounts of THFA from DHFA. Tp inhibits the enzyme dihydrofolate reductase which mediates the reduction of DHFA to THFA (Mahler and Cordes, 1971). DHFA is generated from THFA stoichiometrically during thymidylate biosynthesis. If thymidylate synthesis proceeds while dihydrofolate reductase is inhibited, all cellular activities requiring THFA will halt. Trimethoprim or Tp and sulfa-treated cells synthesized ΦW-14 DNA in strain 29 (Figures 15 and 16) when the inhibitor was added at the onset of phage infection. The buoyant density of ΦW-14 DNA extracted from phage-infected strain 29 or 3L cells was examined. The buoyant density of DNA made in 3L or 29 was the same as the buoyant density of ΦW-14 reference DNA (Table 5). Strain 29 cells, pretreated for long periods of time with Tp, do not support phage growth, presumably because Tp treatment causes the cells to undergo thymine-less death prior to infection. Pretreatment of strain 3L cells with Tp for one doubling prior to infection had no effect upon the buoyant density of ΦW-14 DNA synthesized (Table 5).

[6-3H]-Ura labelling ratios for ΦW-14 DNA prepared in the presence of Tp were also normal (Table 6).
FIGURE 15.—The effect of trimethoprim upon ØW-14 DNA synthesis.

Trimethoprim was added to one-half of an infected culture at 20 minutes after infection. The incorporation of [6-\(^3\)H]-uracil into alkali resistant, TCA precipitable material was followed. _P. acidovorans_ strain 29 infected with ØW-14, no additions, ●; _P. acidovorans_ strain 29 infected with ØW-14, Tp (100 µg ml\(^{-1}\)), ○.
MINUTES AFTER INFECTION

PERCENT INCORPORATION

20 25 30 35 40 45 50
FIGURE 16.—The effect of trimethoprim and sulfonamide upon \( \Phi W-14 \) DNA synthesis.

Tp and Sulfa were added to one-half of a culture at 20 minutes after infection. The incorporation of \([6-^3H]\)-uracil into alkali resistant, TCA precipitable material was determined. \textit{P. acidovorans} strain 29 infected with \( \Phi W-14 \), no additions, •; \textit{P. acidovorans} strain 29 infected with \( \Phi W-14 \), Tp (100 \( \mu g \) ml\(^{-1} \)), Sulfa (500 \( \mu g \) ml\(^{-1} \)), ○.
The gradients contained DNA which was extracted from infected cells at 45 minutes after infection or from phage particles after lysis. The absence of heavy density DNA excluded the possibility that infected cells made unmodified DNA which was not packaged.

Collectively, the Tp, Tp and sulfa, and FUDR results suggested that endogenous thymidine was not a precursor of thymine in \( \Phi W-14 \) DNA. Exogenous TdR is also not a precursor. putThy nucleotides were not found in infected cell pools, but infected cells induced enzymes which catalyzed the release of tritium from \([5-^3H]\)-uracil. This situation is similar to systems where hmDUMP is the product of tritium releasing enzymes. hmDUMP biosynthesis requires a THFA cofactor but the THFA requirement is catalytic. THFA is not oxidized, therefore, hmDUMP synthesis is not sensitive to the presence of trimethoprim. These conclusions are supported by the discovery of hmDUMP and hmUTP in the soluble nucleotide pools of infected cells (Neuhard et al., 1980). Warren has also shown the induction of a dUTP/dTTPase activity in \( \Phi W-14 \)-infected cells. It was concluded that \( \Phi W-14 \) synthesis required the four deoxy-nucleoside triphosphates; dATP, dGTP, dCTP and hmUTP, that dTTP was excluded, and that both Thy and putThy in \( \Phi W-14 \) DNA were generated by post-replication modification of hmUra. These conclusions prompted a detailed evaluation of the nature of replicating \( \Phi W-14 \) DNA. The goals of this work were the demonstration of hmURA in replicating \( \Phi W-14 \) DNA and the elucidation of the biosynthetic pathways for putThy and Thy.

**Tritium is not lost from \([6-^3H]\)-uracil during \( \Phi W-14 \) DNA synthesis**

\( \Phi W-14 \) DNA labelled with \([2-^{14}C]\)-uracil or \([6-^3H]\)-uracil was prepared. After acid-hydrolysis and TLC of the bases, the distribution of
both labels in putThy, Cyt and Thy was the same (Table 8). Base-labelling ratios for both labels reflected the mol % G + C values determined for \( \Phi W-14 \) DNA. The biosynthesis of putThy and Thy in \( \Phi W-14 \) DNA preserved the \([6-^3H]-uracil\) label. Equilibrium labelling of bases with \([6-^3H]-Ura\) accurately reflected their relative levels in \( \Phi W-14 \) DNA.

**Replicating DNA**

hmdUTP is the nucleotide pool precursor of Thy and putThy in \( \Phi W-14 \) DNA (Neuhard et al., 1980). Acid hydrolysis of \( \Phi W-14 \) virion DNA followed by two-dimensional thin-layer chromatography showed that hydroxymethyluracil was not present in the DNA (Figure 17).

\([6-^3H]-uracil\)-labelled \( \Phi W-14 \) grown in strain 29 or 3L had the same base composition (Table 7). Changes in growth temperature, growth medium or infection of cells at varying cell densities had no effect upon the thymine and a putrescinyllthymine levels in \( \Phi W-14 \) DNA (data not presented).

Replicating \( \Phi W-14 \) DNA was analyzed on neutral CsCl density gradients. \( \Phi W-14 \) DNA isolated from infected cells had the same buoyant density as reference marker \( \Phi W-14 \) prepared from purified virion DNA (Table 5). Buoyant density is an accurate reflection of modification because the low density of \( \Phi W-14 \) DNA is a consequence of the putThy in \( \Phi W-14 \) DNA (Warren, 1980). Relatively small changes in putThy levels will result in detectable changes in \( \Phi W-14 \) DNA buoyant density.

Replicating \( \Phi W-14 \) DNA may be examined indirectly using parentally labelled \( \Phi W-14 \) DNA and following it through infection with neutral CsCl gradient analysis (Figure 18). Obviously, the putThy in \( \Phi W-14 \) DNA was
TABLE 8.—Tritium is not lost from $[6^-\text{H}]-\text{uracil}$ during putThy or Thy biosynthesis.

<table>
<thead>
<tr>
<th>Base</th>
<th>$[6^-\text{H}]-\text{uracil}$</th>
<th>[2-$\text{C}^\text{14}$]-uracil</th>
</tr>
</thead>
<tbody>
<tr>
<td>putThy</td>
<td>25840 (0.22)</td>
<td>19089 (0.22)</td>
</tr>
<tr>
<td>Thy</td>
<td>31561 (0.26)</td>
<td>23717 (0.28)</td>
</tr>
<tr>
<td>Cyt</td>
<td>62229 (0.52)</td>
<td>42260 (0.50)</td>
</tr>
</tbody>
</table>

The DNA was labelled with radioactive uracil and the DNA was extracted from the infected cells at 35 minutes after infection. The bases in acid hydrolyzed DNA samples were separated by 1D-TLC on cellulose sheets.

a The values represent cpm in the area cut from the chromatogram. The values in parentheses represent the fraction of the label found in putThy, Thy and Cyt bases.
FIGURE 17.—TLC of the pyrimidine products released from ØW-14 DNA by acid hydrolysis.

[2-$^{14}$C]-uracil-labelled ØW-14 virion DNA was prepared and hydrolyzed at 100° for 90 minutes. The sample was mixed with a standard base mixture and bases were resolved by 2D-TLC on cellulose sheets. The procedures employed are described in greater detail in the Materials and Methods.
FIGURE 18.—CsCl density gradient analysis of $^{32}$P-labelled $\phi W$-14 parental DNA.

$^{32}$P-labelled $\phi W$-14 phage was prepared in P. acidovorans strain 29. The radioactive phage preparation was used to infect a growing culture. At intervals after infection, aliquots of the infected culture were removed and DNA was purified. The DNA was banded in neutral CsCl density gradients. $^{32}$P-label, O; [6-$^3$H]-uracil labelled phage reference and host reference DNA were added to the experimental samples, ●.
not removed or altered after infection. There was no transfer of replicating parental DNA to heavier densities. Hybrid unmodified or hmUra-containing DNA did not accumulate in appreciable quantities in infected cells.

Acid-hydrolysis and TLC of bases from parentally labelled \( \Phi W-14 \) DNA extracted from infected cells allowed similar conclusions regarding the fate of Thy and putThy. Both were stable components of \( \Phi W-14 \) DNA (Table 9).

\( \Phi W-14 \) intracellular parentally labelled DNA heated to just below the Tm of \( \Phi W-14 \) DNA banded with a broader density profile than virion DNA (data not shown). Only short fragments of DNA would be denatured by such heat treatments and they would band at slightly heavier densities than duplex \( \Phi W-14 \) DNA. The density shift and peak broadening probably was a consequence of heat denaturation of nicked regions in replicating DNA. Nicks are a consequence of the replication, recombination and the dispersal of the parental DNA into progeny DNA. This was not a surprising result but it showed that the majority of parental label DNA recovered and banded in these experiments was active in these processes.

Virion DNA must have been less extensively nicked than intracellular DNA since peak splitting or broadening in heat-treated virion DNA samples was not observed (Figure 19). CsCl gradients of virion DNA labelled with \( [5-^3H]\)-uracil and \( [5-^{14}C] \)-ornithine formed a single, homogenous peak before and after heat treatment. \( \Phi W-14 \) intracellular DNA prepared from infected cells labelled with \( [5-^3H]\)-uracil and \( [5-^{14}C] \)-ornithine also formed a single, homogenous peak when banded in neutral CsCl density gradients (Figure 19). Heat treatment of these samples prior
TABLE 9.—The fate of putThy and Thy in parentally labelled \( \Phi W-14 \) DNA.

<table>
<thead>
<tr>
<th>Base</th>
<th>Sample taken (min. after infection)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 min.</td>
</tr>
<tr>
<td>putThy</td>
<td>3657 (0.23)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cyt</td>
<td>8972 (0.55)</td>
</tr>
<tr>
<td>Thy</td>
<td>3578 (0.22)</td>
</tr>
</tbody>
</table>

[6-\(^3\)H]-uracil labelled phage was prepared and was used to infect a growing culture of \( P. \) acidovorans strain 29. At various times after infection aliquots of the culture were removed and the intracellular DNA was purified. The DNA was acid hydrolyzed and the bases were resolved by 1D-TLC.

<sup>a</sup> The values are cpm in the area cut from the chromatogram. The values in parentheses are the fraction of the label recovered in putThy, Thy and Cyt bases.
FIGURE 19.--The effect of heat treatment upon the buoyant density profiles of ϕW-14 intracellular and virion DNAs.

[5-³H]-uracil and [5-¹⁴C]-ornithine-labelled DNA was purified from virions and from infected cells. Labelling, extraction and heat treatment procedures are described in the Materials and Methods.

A) native ϕW-14 intracellular DNA
B) heat treated ϕW-14 intracellular DNA
C) native ϕW-14 virion DNA
D) heat treated ϕW-14 virion DNA

ϕW-14 intracellular DNA was purified from infected cells at 35 minutes after infection. [5-³H]-uracil, ●; [5-¹⁴C]-ornithine, ○.
to loading on the gradient split the sample into two peaks containing approximately equal ratios of label. PutThy or Thy-rich regions of the DNA (if there are any) are not preferentially heat-denatured since ornithine or uracil-labelled DNA contributed equally to the slightly denser single-stranded peak. The original purpose of this experiment was to determine whether or not nascent, unmodified DNA could be freed from non covalent attachment to a larger mass of fully modified DNA. This might have aided in the detection and isolation of bases which were intermediates in the Thy or putThy biosynthetic pathways. These expectations were not realized because such heavy DNA components do not exist in amounts large enough to be detected. The absence of peak splitting in virion DNA after partial denaturation indicates that it is not extensively nicked. Peak splitting in the replicating DNA samples probably was a consequence of the presence of nicks in the DNA of infected cells. Short segments of duplex DNA bounded by single-strand nicks on the same chain of the DNA duplex are completely heat-denatured from DNA. Short duplex DNA fragments have a lower Tm than longer duplex DNA fragments of the same mol % G + C content (Mahler and Cordes, 1971). Reconstruction experiments using purified strain 29 DNA and φW-14 DNA indicated that peak splitting was not due to φW-14 DNA being carried down the gradients by host DNA (Figure 20). DNA isolated from infected cells contained both host and phage DNA. Virion DNA preparations contained only phage DNA. Completely heat denatured φW-14 DNA banded as a single peak of uniform density.

The effect of shearing upon the buoyant density profile of φW-14 DNA was studied. Peak broadness in neutral CsCl density gradients was a
FIGURE 20.—CsCl buoyant density analysis of $\phi W$-14 virion and 
P. acidovorans DNA.

A) $^{32}$P-labelled $\phi W$-14 DNA mixed with 50 $\mu$g unlabelled host DNA.

B) $^{32}$P-labelled $\phi W$-14 DNA mixed with 50 $\mu$g unlabelled host DNA and then heat denatured.

C) $^{32}$P-labelled strain 29 DNA mixed with 50 $\mu$g unlabelled $\phi W$-14 DNA.

D) $^{32}$P-labelled strain 29 DNA mixed with 50 $\mu$g unlabelled $\phi W$-14 DNA and then heat denatured.

The samples were loaded on CsCl, centrifuged and fractionated as described in the Materials and Methods.
reflection of the size of the DNA molecules, small molecules having higher diffusion coefficients than larger DNA molecules. Shearing DNA reduces the average length of the DNA molecules. [2-\(^{14}\)C]-uracil and [5-\(^{3}\)H]-ornithine-labelled virion and intracellular phage DNA were examined after shearing (Figure 21). The distribution of both labels was uniform for both virion and intracellular DNA. Shearing intracellular DNA did not shift a significant proportion of the DNA to a heavier density. The effect of sequential shearing and heat treatment on [6-\(^{3}\)H]-uracil labelled \(\phi W-14\) intracellular DNA was also examined (Figure 22). DNA formed a single homogenous peak in CsCl before shearing or heat treatment. Shearing caused peak broadening while heating caused peak splitting. Shearing and then heating a DNA sample resulted in a broad uniform peak. Heavy density components were not released from \(\phi W-14\) intracellular DNA even through the combined action of shearing and heating. Therefore, there were no extensive putThy, GC nor unmodified base-rich domains in \(\phi W-14\) DNA. If there was any unmodified DNA in infected cells it was present in very small amounts and it did not accumulate during the course of the normal infectious cycle.

In the experiments described above, DNA was extracted from cells at 35 minutes after infection. Normally, at this time, phage DNA synthesis was well-established. It was assumed that intracellular DNA content at this time would reflect the nature of \(\phi W-14\) intracellular DNA extracted at any point during DNA replication.

**Thin-layer chromatography of DNA components**

The degradative analysis of newly formed replicating \(\phi W-14\) DNA was preceded by the evaluation of procedures used for the hydrolysis or
FIGURE 21.—The uniform distribution of putThy in $\Phi W$-14 DNA.

$[^3]H$-ornithine-labelled $\Phi W$-14 DNA was prepared from infected cells and from purified virions. $[2-^{14}C]$-uracil labelled $\Phi W$-14 DNA was prepared from infected cells and from purified virions. The DNA was sheared as described in the Materials and Methods.

A) sheared virion DNA; B) sheared intracellular DNA.

FIGURE 22.—The effect of heat treatment and shearing upon the distribution of label in \( \phi W-14 \) DNA extracted from infected cells, and banded on CsCl gradients.

\([6^-3H]\)-uracil labelled DNA was extracted from \( \phi W-14 \) infected \( P. \) acidovorans at 35 minutes after infection.

A) \( \phi W-14 \) DNA.

B) \( \phi W-14 \) DNA, sheared.

C) \( \phi W-14 \) DNA, heat treated.

D) \( \phi W-14 \) DNA, sheared and heat treated.

The procedure employed is described in the Materials and Methods.
A

B

C

D

\[ \text{Fraction Number} \]

\[ \text{Fraction Number} \]
digestion and separation of φW-14 DNA components. This was necessary because techniques previously employed did not give complete digestion and separation of the DNA components most likely to be precursors of Thy and putThy (Kropinski, Ph.D. Thesis, 1971; Kelln and Warren, 1973).

With solvent B, one-dimensional separation of 6 N HCl hydrolysates of φW-14 DNA did not result in the complete resolution of all possible pyrimidine products. Two-dimensional thin-layer separation of hydrolysates in solvents B and D gave complete resolution of all pyrimidine bases from nucleosides and nucleotides. A sample of [2-14C]-uracil labelled P. acidovorans strain 29 DNA was hydrolyzed and separated by two-dimensional thin-layer chromatography in solvents B and D (Figure 23). The hydrolysis procedure did not result in the complete conversion of nucleotides to bases. However, all nucleotides had Rf values of zero in solvent D. Cytosine was not extensively converted to uracil during hydrolysis.

Background counts for the areas of the chromatogram outside the authentic standard spots were very low and independent of the amount of radioactivity loaded. The procedure was sensitive enough to detect the presence of 5-methylcytosine in the DNA of strain 29. 5-methylcytosine is present as approximately 0.5 percent of the pyrimidine bases labelled (Table 10).

The hydrolysis and TLC of [2-14C]-uracil-labelled φW-14 virion DNA is also shown (Figure 17). Although hydrolysis was not complete, the only major pyrimidine bases released by acid hydrolysis were those reported by Kropinski and Warren (1973). hmUra and uracil are not detectable hydrolysis products of φW-14 virion DNA (Figure 17).
FIGURE 23.—Two-dimensional thin-layer chromatography of acid-hydrolyzed, [2-14C]-uracil-labelled *P. acidovorans* DNA.
TABLE 10.—The base composition of *P. acidovorans* strain 29 DNA.

<table>
<thead>
<tr>
<th>compound</th>
<th>cpm</th>
<th>fraction of cpm in compound</th>
<th>spot #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ade</td>
<td>110</td>
<td>&lt;0.001</td>
<td>5</td>
</tr>
<tr>
<td>meAde</td>
<td>95</td>
<td>&lt;0.001</td>
<td>6</td>
</tr>
<tr>
<td>CdR</td>
<td>700</td>
<td>0.004</td>
<td>7</td>
</tr>
<tr>
<td>Cyt</td>
<td>119126</td>
<td>0.617</td>
<td>8</td>
</tr>
<tr>
<td>meCyt</td>
<td>948</td>
<td>0.005</td>
<td>9</td>
</tr>
<tr>
<td>-</td>
<td>31</td>
<td>&lt;0.001</td>
<td>10</td>
</tr>
<tr>
<td>hmUra</td>
<td>325</td>
<td>0.002</td>
<td>11</td>
</tr>
<tr>
<td>UdR</td>
<td>846</td>
<td>0.005</td>
<td>12</td>
</tr>
<tr>
<td>Ura</td>
<td>1087</td>
<td>0.006</td>
<td>13</td>
</tr>
<tr>
<td>Thy</td>
<td>51057</td>
<td>0.260</td>
<td>14</td>
</tr>
<tr>
<td>putThy</td>
<td>37</td>
<td>-</td>
<td>16</td>
</tr>
<tr>
<td>origin</td>
<td>307</td>
<td>0.002</td>
<td>15</td>
</tr>
<tr>
<td>Gua</td>
<td>301</td>
<td>0.002</td>
<td>17</td>
</tr>
</tbody>
</table>

[2-\(^{14}\text{C}\)]-uracil labelled *P. acidovorans* strain 29 DNA was acid hydrolyzed in 6N HCl. The samples were mixed with a standard base reference mixture and separated by 2D-TLC on cellulose sheets. Spot number refers to the spots which were detected under UV light on the chromatographic sheet. Compounds 1 to 4 are unhydrolyzed nucleotide products. There were 23718 cpm in unhydrolyzed nucleotides. See Figure 23.
This combination of solvent systems had the ideal characteristics of resolving all the tested pyrimidine bases over five to 80 percent of the run length in the first dimension. The second dimension completely freed bases from other hydrolysis products liberated by this procedure. Doubly-labelled DNA (³H and ³²P) samples could be acid hydrolyzed and separated. ³H-labelled bases were completely free of contaminating ³²P background radiation. This two-dimensional system is similar in separation characteristics to isopropanol:HCl:H₂O (70:10:20)/n-butanol:H₂O:NH₃ vapour (86:5:saturated NH₃) (Hall, 1971) but the resolution of hmUra and uracil was better when solvent B was used for the first dimension.

Acid hydrolysis of DNA, especially DNA containing unusual bases was not a completely satisfactory procedure since it could have resulted in the destruction of acid-labile bases. ØW-14 DNA was digested to mononucleotides by sequential S1 and snake venom phosphodiesterase treatment. ØW-14 DNA was digested to five components, one of these, putdTMP had the highest Rf in both solvents A and E (Table 11). putdTMP runs at or near the solvent front in most common PEI-cellulose TLC systems. This is a result of its net positive charge at the pH of chromatography. The putdTMP spot is labelled by ornithine. On two-dimensional thin-layer chromatograms of ØW-14 DNA digests the presence of only one extra spot with the chromatographic properties which would be expected of a putThy-containing nucleotide suggested that the putresciny1 functions of ØW-14 DNA were not modified by acid hydrolyzable groups (Figure 24).

ØW-14 DNA cannot be completely degraded to mononucleotides by
TABLE 11A.—The nucleotide composition of pulse labelled φW-14 DNA digests resolved on PEI-cellulose.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>CPM</th>
<th>(fraction of total label)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>unknown</td>
<td>611</td>
</tr>
<tr>
<td>2</td>
<td>unknown</td>
<td>59</td>
</tr>
<tr>
<td>3</td>
<td>unknown</td>
<td>158</td>
</tr>
<tr>
<td>4</td>
<td>unknown</td>
<td>63</td>
</tr>
<tr>
<td>5</td>
<td>unknown</td>
<td>28</td>
</tr>
<tr>
<td>6</td>
<td>unknown</td>
<td>27</td>
</tr>
<tr>
<td>7</td>
<td>orthophosphate</td>
<td>1305</td>
</tr>
<tr>
<td>8</td>
<td>AMP and dAMP</td>
<td>977</td>
</tr>
<tr>
<td>9</td>
<td>UMP and dTMP</td>
<td>257</td>
</tr>
<tr>
<td>10</td>
<td>CMP and dCMP</td>
<td>309</td>
</tr>
<tr>
<td>11</td>
<td>GMP and dGMP</td>
<td>413</td>
</tr>
</tbody>
</table>

The numbers correspond to the number of the nucleotide or spot on the chromatographic sheet. Radioactivity in each compound was quantitated by cutting the area containing the compound from the sheet and counting it in a toluene-based scintillant. DNA was digested with nuclease S1 and snake venom phosphodiesterase according to procedures described in the Materials and Methods. The nucleotides were separated on PEI-cellulose sheets according to the procedures of Randerath and Randerath.
TABLE 11B.--The nucleotide composition of pulse-labelled $\Phi W$-14 DNA digests resolved on cellulose.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>CPM</th>
<th>(fraction of total label)</th>
<th>Rf E</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 dAMP</td>
<td>1040</td>
<td>(0.12)</td>
<td>56</td>
<td>27</td>
</tr>
<tr>
<td>2 dGMP</td>
<td>489</td>
<td>(0.06)</td>
<td>26</td>
<td>39</td>
</tr>
<tr>
<td>3 dTMP</td>
<td>464</td>
<td>(0.05)</td>
<td>36</td>
<td>52</td>
</tr>
<tr>
<td>4 dCMP</td>
<td>714</td>
<td>(0.08)</td>
<td>43</td>
<td>70</td>
</tr>
<tr>
<td>5 putdTMP</td>
<td>360</td>
<td>(0.04)</td>
<td>55</td>
<td>79</td>
</tr>
<tr>
<td>6 hmdUMP</td>
<td>58</td>
<td>(&lt;0.01)</td>
<td>23</td>
<td>62</td>
</tr>
<tr>
<td>7 unknown</td>
<td>266</td>
<td>(0.03)</td>
<td>9</td>
<td>65</td>
</tr>
<tr>
<td>8 rAMP</td>
<td>981</td>
<td>(0.11)</td>
<td>49</td>
<td>34</td>
</tr>
<tr>
<td>9 rGMP</td>
<td>292</td>
<td>(0.03)</td>
<td>15</td>
<td>51</td>
</tr>
<tr>
<td>10 rCMP</td>
<td>72</td>
<td>(&lt;0.01)</td>
<td>30</td>
<td>77</td>
</tr>
<tr>
<td>11 rUMP</td>
<td>173</td>
<td>(0.02)</td>
<td>17</td>
<td>71</td>
</tr>
<tr>
<td>12 unknown</td>
<td>497</td>
<td>(0.06)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13 orthophosphate</td>
<td>3282</td>
<td>(0.38)</td>
<td>23</td>
<td>100</td>
</tr>
</tbody>
</table>

The numbers correspond to the number of the nucleotide or spot on the chromatographic sheet. The procedures employed are described in part A of this Table except that the nucleotides were separated according to the procedures described by Dawid et al., 1970.
FIGURE 24.—Two-dimensional thin-layer chromatography of $^{32}$P-labelled $\phi$W-14 DNA digested with nuclease S1 and snake venom phosphodiesterase (SVPD).
simultaneous or sequential treatments with DNase I and snake venom phosphodiesterase (Figure 25). Limit digests appeared to accumulate dimers containing putThy and one other base; dAMP, dCMP, dGMP and dTMP, but not putdTMP, were liberated in large quantities. The deficiency in putdTMP and the appearance of only three other $^{32}$P-labelled spots running as dimers suggested that the distribution of putThy in $\varnothing W$-14 DNA was not random since completely random distribution of putThy in dimers would generate five dimers. Lewis (unpublished observations) has subsequently pursued the problem of putThy distribution in $\varnothing W$-14 DNA, showing that the ratio of putThy to Thy for single pyrimidine nucleotides bounded by purine nucleotides is 2.4 to 1.0, indicative of an ordered distribution of putThy in $\varnothing W$-14 DNA. The CsCl gradient results presented earlier merely suggested the absence of gross heterogeneity in the distribution of putThy and Thy in $\varnothing W$-14 DNA.

**Pulse-labelling of DNA in $\varnothing W$-14-infected P. acidovorans**

Of necessity, the development and analysis of digestion and separation procedures preceded the detailed analysis of nascent $\varnothing W$-14 DNA.

The tritiated uracil and $^{32}$PO$_4$ pulses were performed as described in the Materials and Methods. Acid hydrolysis and two-dimensional thin-layer chromatography of [6-$^3$H]-uracil-pulsed $\varnothing W$-14 DNA indicated that putThy and Thy were not recovered in normal ratios (Table 12). Labelled hmUra and uracil were found in the hydrolysates. The uracil could arise from Cyt by deamination during acid hydrolysis, from RNA present in the purified DNA or from the incorporation of
FIGURE 25.—Two-dimensional thin-layer chromatography of $^{32}$P-labelled nucleotides present in $\phi W$-14 DNA digested with DNase I and SVPD.
TABLE 12.—Base composition of replicating \(\Phi W\)-14 DNA

<table>
<thead>
<tr>
<th>Base</th>
<th>6-(^3)H-uracil (10 sec pulse)</th>
<th>5-(^3)H-uracil (10 sec pulse)</th>
<th>6-(^3)H-uracil (20 min labelling)</th>
</tr>
</thead>
<tbody>
<tr>
<td>putThy</td>
<td>2200(^a) (7.7)</td>
<td>531 (1.7)</td>
<td>14430 (18.2)</td>
</tr>
<tr>
<td>cytosine</td>
<td>15230 (53.2)</td>
<td>27024 (86.5)</td>
<td>34585 (43.6)</td>
</tr>
<tr>
<td>hydroxymethyl-uracil</td>
<td>740 (2.6)</td>
<td>26 (&gt;0.1)</td>
<td>157 (0.2)</td>
</tr>
<tr>
<td>uracil</td>
<td>1830 (6.4)</td>
<td>28 (&gt;0.1)</td>
<td>180 (0.2)</td>
</tr>
<tr>
<td>thymine</td>
<td>5205 (18.2)</td>
<td>980 (3.1)</td>
<td>21976 (27.7)</td>
</tr>
<tr>
<td>unhydrolyzed nucleotides</td>
<td>3410 (11.9)</td>
<td>2653 (8.5)</td>
<td>7987 (10.0)</td>
</tr>
</tbody>
</table>

DNA was labelled, extracted and hydrolyzed as described in Materials and Methods. The bases were separated by two-dimensional thin-layer chromatography on cellulose sheets.

\(^a\) Cpm in the area cut from the chromatogram. The figures in parentheses represent the percentage of recovered radioactivity. Total recovery of applied radioactivity was >90 percent.
uracil into DNA. The [5-\(^3\)H]-uracil pulse served as a control. Acid hydrolysis of [5-\(^3\)H]-uracil pulse-labelled DNA did not release labelled uracil, therefore, pancreatic RNase treatment of DNA effectively removed susceptible RNA and acid hydrolysis did not cause the deamination of cytosine to uracil under the conditions employed. Uracil was not incorporated into nascent \(\phi W\)-14 DNA. It is possible that the uracil observed arises from the conversion of some acid-labile C\(^5\) modified base during hydrolysis. The presence of label in putThy and Thy in the [5-\(^3\)H]-uracil pulse-labelled DNA sample was probably a consequence of the contamination of commercial [5-\(^3\)H]-uracil with [6-\(^3\)H]-uracil. 2.6 percent of the total pyrimidine label in the [6-\(^3\)H]-uracil pulse was found in hmUra. This compared to 0.2 percent of the total label in a uniformly labelled \(\phi W\)-14 intracellular DNA hydrolysate. This result demonstrated that the hmUTP found in the acid-soluble pools of infected cells was incorporated into \(\phi W\)-14 DNA. The small amount of hmUra relative to Thy and putThy demonstrated the rapid nature of hmUra conversion. Caution should be exercised in evaluating the relative recovery of label in Thy and putThy. If both put-Thy and Thy were derived by post-replication modification of hmUra then the preponderance of Thy suggested either that the Thy-forming reaction(s) were faster or that the Thy-forming reaction(s) occurred before the putThy-forming reaction(s). It should be remembered that the recovery of nascent DNA sequences rich in putThy or Thy, or their precursors, are not necessarily equal. The possibility that putThy-containing sequences were preferentially lost during purification of DNA cannot be excluded.
The nascent DNA fraction of DNA in T7-infected E. coli is denatured during the phenol extraction step of DNA purification. Sonication of lysates prior to phenol purification stabilizes the nascent DNA fraction (Petkau et al., 1975). Extraction of pulse-labelled DNA in our laboratory is performed using the SDS-pronase-phenol method (Lewis et al., 1976). This method, coupled with ethanol precipitation and winding out of DNA during purification, could have resulted in the preferential loss of single-stranded DNA components. On CsCl gradients \(\Phi W-14\) pulse-labelled DNA often had a heavy shoulder. This could have been due to the presence of short, single-stranded pieces of DNA, which were denatured during DNA purification.

\(^{32}\)PO\(_4\) pulses were also performed. Using \(^{32}\)PO\(_4\) to pulse-label DNA facilitated the detection of novel nucleotides and lowered the costs incurred in the pulse experiments. \(\Phi W-14\) DNA pulse-labelled for 10 seconds with \(^{32}\)PO\(_4\) was purified and digested to mononucleotides with S1 nuclease and snake venom phosphodiesterase. Digests were separated by two-dimensional thin-layer chromatography on unmodified cellulose or on PEI-cellulose sheets. After autoradiography, radioactive areas of the chromatograms were cut out and counted. Part of the DNA sample was retained for analysis of the sensitivity of the nascent DNA to S1 nuclease and for CsCl density gradient analysis.

Uniformly labelled intracellular DNA had the same buoyant density as virion DNA. Pulse-labelled DNA was examined to see if it showed a different density profile than uniformly labelled DNA. DNA labelled with [6-\(^3\)H]-uracil until 35 minutes after infection and pulsed for 10 seconds with \(^{32}\)PO\(_4\) was prepared as described in the Materials
and Methods. The native gradients showed that although almost 100 percent of the tritium label was found at $\phi_{W}-14$ or host density, only 48 percent of the pulse label banded at the light phage density (Figure 26). Thirty-eight and 14 percent of the $^{32}$P label banded at heavy and intermediate densities, respectively. Heat treatment of the pulse-labelled replicating DNA sample caused the splitting of the uniformly and pulse-labelled light peak into a three-peak pattern (Figure 26). The pulse-labelled DNA was shifted toward the heavy end of the gradient. Fifty-two percent of the total pulse label banded in the light peaks, while 11 and 37 percent of the material banded at intermediate and heavy densities, respectively. Heat treatment of $\phi_{W}-14$ pulsed, replicating DNA did slightly shift the light peak DNA to a heavier density. This could be due to the presence of unmodified bases in nascent DNA or it could be due to the hyperdensity shift of easily-denatured nascent DNA fragments. The latter possibility seemed unlikely since the uniformly-labelled peaks on the gradient marked the buoyant density of single-stranded and double-stranded $\phi_{W}-14$ DNA.

The DNA preparations were used to compare the chemical and physical stability of the pulse-labelled and uniformly-labelled DNA samples (Table 13). $^{32}$P-labelled $\phi_{W}-14$ DNA served as a control. Both $^{32}$P and $^3$H label were resistant to the action of pancreatic RNase which was employed during the preparation of the DNA samples. The stability of the DNA samples to heating in distilled water and 0.3 N NaOH was examined. The pulse-labelled material appeared slightly more labile than the uniformly-labelled DNA samples. In addition, 30 percent of the pulse-labelled material was solublized when incubated in S1 buffer,
FIGURE 26.—CsCl density analysis of $^{32}$P-pulse-labelled $\Phi W$-14 DNA.

A) Native DNA. B) Heat treated DNA.

The DNA sample was purified from cells 35 minutes after infection with $\Phi W$-14. The length of the $^{32}$P-pulse was approximately 10 seconds. Cells were labelled with [6-$^3$H]-uracil (0.1 $\mu$Ci $\mu$g$^{-1}$, 10 $\mu$g ml$^{-1}$) from 20 minutes after infection.

[6-$^3$H]-uracil labelled DNA, ●; $^{32}$PO$_4$ pulse labelled DNA, ○.
TABLE 13.—The relative chemical lability of pulse-labelled DNA.

[6-\(^3\)H]-Ura-labelled \(\phi W-14\) DNA was extracted from cells 35 minutes after infection. \(^{32}\)P\(_4\) was used to pulse label \(\phi W-14\) infected \textit{P. acidovorans} for 10 seconds at 35 minutes after infection. \(^{32}\)P-labelled \(\phi W-14\) DNA was extracted from virions as described in the Materials and Methods. After treatment DNA samples were spotted on filter papers and subjected to TCA precipitation as described in the Materials and Methods. Each value in the table represents the average of six determinations.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>CPM ($^3$H/$^{32}$P) % recovery ($^3$H/$^{32}$P)</th>
<th>CPM ($^{32}$P) % recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>[6-$^3$H]-Ura uniformly labelled φW-14 DNA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{32}$PO$_4$ pulse-labelled φW-14 DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>CPM ($^3$H/$^{32}$P) % recovery ($^3$H/$^{32}$P)</td>
<td>CPM ($^{32}$P) % recovery</td>
</tr>
<tr>
<td><strong>dH$_2$O</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0 min.)</td>
<td>4008/478</td>
<td>90/81</td>
</tr>
<tr>
<td>(30 min.)</td>
<td>3581/387</td>
<td></td>
</tr>
<tr>
<td><strong>0.3 N NaOH</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0 min.)</td>
<td>3733/409</td>
<td>95/91</td>
</tr>
<tr>
<td>(30 min.)</td>
<td>3535/370</td>
<td></td>
</tr>
<tr>
<td><strong>0.05 M Na acetate, 0.3 M NaCl, pH 4.5</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0 min.)</td>
<td>5548/923</td>
<td>100/70</td>
</tr>
<tr>
<td>(120 min.)</td>
<td>5567/642</td>
<td></td>
</tr>
</tbody>
</table>
without enzyme, for four hours. Uniform label in intracellular DNA was no more labile to the various treatments than the $^{32}$P-labelled phage virion DNA. There was no preferential solubilization of pyrimidine-containing sequences under the conditions employed (Lewis et al., 1976). The differences in chemical stability for pulse and uniform labels suggested that nascent $\Phi W$-14 DNA might contain novel, unstable nucleotides.

As was noted, 30 percent of $^{32}$P pulse label was released from DNA in the absence of S1. Of the remaining DNA approximately 30 percent of the pulse label and 4 percent of the uniform label remained TCA insoluble after limit digestion with S1 in high salt (Figure 27). S1 digestion in high salt (0.3 M NaCl) of $^{32}$P-labelled DNA was less extensive than S1 degradation of the same DNA sample performed at low salt concentrations (0.05 M ammonium acetate). This could indicate incomplete denaturation of the nascent DNA fraction or the presence of S1-resistant sequences. A control mixture of $\Phi W$-14 DNA uniformly labelled with $^{32}$PO$_4$ and [6-$^3$H]-uracil was almost completely converted to TCA-soluble material (Figure 27). Two-dimensional thin-layer chromatography of a low salt S1 digest on cellulose thin layers resolved a complex nucleotide mixture which contained ribose as well as deoxyribose-containing nucleotides and oligonucleotides (Figure 28). Subsequent snake venom phosphodiesterase treatment of the S1-treated, pulse-labelled material converted it almost completely to mononucleotides (Figure 28). Between 5 and 6 percent of the total counts in the digests were retained at the origin during chromatography. This material was assumed to be contaminating, labelled materials. The
FIGURE 27.—Nuclease S1 degradation of pulse-labelled φW-14 DNA.

A) The DNA was labelled uniformly with [6-^3H]-uracil, ●; for 20 minutes and then pulse labelled with $^{32}$P$_{O_4}$, ○; for 10 seconds.

B) The virion DNA was uniformly labelled with [6-^3H]-uracil, ●; and with $^{32}$P$_{O_4}$, ○.

Digestions were performed in 0.05 M Na acetate pH 4.5, 0.3 M NaCl; $10^{-4}$ M ZnCl$_2$. The samples were then TCA precipitated, washed, dried and counted as described in the Materials and Methods. The values are expressed as a percentage of the original amount of TCA precipitable material.
FIGURE 28.—Two dimensional thin-layer chromatography of $^{32}$P-pulse-labelled $\Phi W$-14 DNA digests.

A) PEI-cellulose thin-layer chromatography of $^{32}$P-pulse-labelled $\Phi W$-14 DNA digested sequentially with nuclease S1 and snake venom phosphodiesterase.

B) Cellulose thin-layer chromatography of $^{32}$P-pulse-labelled $\Phi W$-14 DNA digested sequentially with nuclease S1 and SVPD.

C) Cellulose thin-layer chromatography of $^{32}$P-pulse-labelled $\Phi W$-14 DNA digested with nuclease S1.

D) Cellulose thin-layer chromatography of $^{32}$P-uniformly-labelled $\Phi W$-14 and strain 29 DNA digested with nuclease S1 for 2 hours at 55°. The amount of DNA per sample was 50 µg ml$^{-1}$ in 0.05 M NH$_4$-acetate pH 5.0, 10$^{-4}$ M ZnCl$_2$ and with 10 units of S1 per µg of DNA. The digestion and chromatography procedures are described in detail in the Materials and Methods. The numbers on autoradiograms A and B refer to Table 12.
susceptibility of the remaining material to solubilization by nuclease treatment indicated that it was nucleic acid. Since all digestion steps were performed in a single tube, there was no possibility of preferential loss of any nucleotide.

Partial S1 digestion of \(^{32}\)P-labelled strain 29 and \(\Phi W-14\) DNA was performed (Figure 28). The products were separated by two-dimensional thin-layer chromatography and compared to chromatograms of limit S1 or S1 and snake venom phosphodiesterase digests of \(^{32}\)P pulse-labelled \(\Phi W-14\) DNA. Comparison of the unmodified cellulose chromatograms helped mark migration positions of oligonucleotide digestion products. Migration positions of nucleotides on the commercial cellulose layers were very reproducible. Rf values on PEI-cellulose sheets were not reproducible. This was important since the samples were being analyzed for novel nucleotides with unknown Rf values. These digests were analyzed on cellulose and PEI-cellulose thin layers. Equal amounts of DNA incubated for 2 hours at 55° were analyzed. The yield of smaller oligonucleotides was greater for strain 29 DNA, i.e. the rate of cleavage of \(\Phi W-14\) DNA to small oligonucleotides was slower than that for strain 29 DNA. A comparison of S1 limit digests of \(^{32}\)P uniformly-labelled and \(^{32}\)P pulse-labelled DNA identified at least three compounds which were not found in strain 29 or \(\Phi W-14\) partial DNA digests. These products were still present in digests which were treated with snake venom phosphodiesterase and were probably novel nucleotides found only in pulse-labelled \(\Phi W-14\) DNA preparations.

All but three of the components found on the cellulose thin layer and six of the components on the PEI-cellulose thin layer were
identifiable. In the PEI system, ribose and deoxyribose-containing nucleotides ran together while in the cellulose system they were resolved. Ribonucleotides made up about 15 percent of the total radioactivity on the chromatograms. Free $^{32}$P$\text{O}_4^-$ was also released from DNA digests in much larger amounts than from control S1 and snake venom phosphodiesterase digests of $\phi$W-14 DNA. The release of $^{32}$P$\text{O}_4^-$ was a consequence of the S1 hydrolysis conditions since release of $^{32}$P$\text{O}_4^-$ also occurred from pulsed material treated only with S1 nuclease. One of the extra components found in the cellulose system had the chromatographic properties of hmdUMP. The low Rf values for the extra compounds on PEI-cellulose suggested that they had net negative charges greater than two. Some of the extra compounds were likely oligonucleotides resulting from incomplete digestion of $\phi$W-14 DNA. Approximately 22 percent of the total label on the PEI-cellulose thin-layer was found in six unidentifiable compounds while approximately 10 percent of the label found on the cellulose thin-layer sheets were in the three unidentified spots (four minor compounds ran in areas on cellulose thin-layer sheets which corresponded to the migration positions for oligonucleotides; these compounds were not counted)(Table 11). The presence of 10 to 20 percent of the pulse label in novel compounds agreed well with the analysis of pulse labelled DNA on CsCl gradients. A maximum of 15 percent of the label was RNA, therefore only 15 percent of the label in the heavy peak on the CsCl gradient was RNA. The remaining 35 to 40 percent of the pulse-labelled material banding at heavy and intermediate densities on CsCl gradients must have contained some unmodified or partially modified DNA. The yield of hmdUMP from
the $^{32}\text{P}$-pulse labelled DNA was lower than that predicted from the [6-$^3\text{H}$]-uracil pulse. The ratio of recovery of putdTMP to dTMP for enzymatic digests was approximately equal and was greater than the recovery ratio noted previously for the free bases of these compounds in a [6-$^3\text{H}$]-uracil pulse labelled $\text{\Phi W-14}$ DNA sample. Uniform labelling of nucleotides in the pulse experiments was unlikely. $^{32}\text{P}\text{O}_4$-labelled purine nucleotides more rapidly than pyrimidine nucleotides. putdTMP and dTMP levels were comparable because they were both derived post-replicationally from hmdUMP.

Several conclusions can be made from the analysis of pulse labelled DNA. hmUra was found only in nascent DNA in infected cells. Heavy density DNA did accumulate during DNA synthesis but DNA modification occurs rapidly after DNA synthesis. There may be three or more precursors of putThy and Thy and these compounds probably have unusually low net negative charges. The small amount of nascent DNA available from pulse-labelled infected cells necessitated the development of different approaches to the putThy and Thy biosynthesis problem. Two approaches were considered, screening for inhibitors of DNA modification and the isolation of DNA modification mutants.

**Tests for inhibitors of $\text{\Phi W-14}$ DNA synthesis and modification**

The major purpose of the inhibitor studies described so far in this thesis was the attempt to demonstrate that $\text{\Phi W-14}$ does not utilize thymidine (dTTP) for DNA synthesis. Several other inhibitors were tested in the hope that they might have specific effects upon $\text{\Phi W-14}$ DNA replication or modification.
Two inhibitors of ornithine decarboxylase were tested. Ornithine is a precursor of the putresciny1 sidechain of putThy (Karrer and Warren, 1973). Depriving cells of ornithine and consequently of putrescine might lead to the accumulation of unmodified or partially modified \( \Phi W-14 \) DNA. Neither \( \alpha \)-methylornithine nor difluoromethylornithine inhibited the growth of \( P. \) acidovorans or the ability of the treated cells to support \( \Phi W-14 \) DNA modification or replication or phage production (data not shown).

Chloramphenicol is an inhibitor of procaryotic protein synthesis. The minimal inhibitory concentration of chloramphenicol for \( P. \) acidovorans strain 29 grown in TCS medium was 5 to 10 \( \mu g \) ml\(^{-1} \). The effect of addition of chloramphenicol upon \( \Phi W-14 \) reproduction was examined. Plaque production and DNA synthesis were inhibited when chloramphenicol was added to cultures 20 minutes after infection (Figure 29). This demonstrated that in \( \Phi W-14 \)-infected cells protein synthesis was required before phage DNA could be produced. These observations were not pursued.

Netropsin, a DNA-binding antibiotic, was the only inhibitor found which appeared to have a specific effect upon phage reproduction. Netropsin is known to bind to DNA and to sterically block the movement of polymerases along a DNA duplex (Wartell et al., 1974). Netropsin, at a concentration of 100 \( \mu g \) ml\(^{-1} \), had no effect upon the growth rate of \( P. \) acidovorans strain 29 (Figure 30). Netropsin delayed lysis of infected cells (Figure 30) and plaque production was inhibited (Figure 31). Netropsin-treated cells made DNA which had a heavier peak overlapping a normal phage density peak in CsCl buoyant density gradients
FIGURE 29.—The effect of chloramphenicol upon the incorporation of uracil into $\phi W$-14.

Chloramphenicol was added to one-half of a culture at 20 minutes after infection. The incorporation of [6-$^3$H]-uracil into alkali resistant, TCA precipitable material was followed. *P. acidovorans* strain 29 infected with $\phi W$-14, $\bullet$; *P. acidovorans* strain 29 infected with $\phi W$-14 and treated with chloramphenicol (100 $\mu$g ml$^{-1}$), $\circ$. The incorporation of radioactivity is expressed as the percentage of input label which becomes alkali resistant and TCA precipitable.
MINUTES AFTER INFECTION
FIGURE 30.—The effect of Netropsin upon the growth of *P. acidovorans* strain 29.

The cells were grown in TCS medium supplemented with succinate and Casamino acids. Netropsin (100 μg ml⁻¹) was added at 125 minutes to an aliquot of the growing cells. The turbidity of the infected and the uninfected cultures was followed with a Klett colorimeter. *P. acidovorans* strain 29, no additions, ●; *P. acidovorans* strain 29, netropsin treated, ○; *P. acidovorans* strain 29 infected with ΦW-14 at 240 minutes, netropsin treated, Δ.
FIGURE 31.—Plaque production in netropsin-treated \textit{P. acidovorans} strain 29 infected with $\Phi W$-14.

A culture of growing cells was infected with $\Phi W$-14 and split into two halves. One culture was treated with netropsin. Aliquots of both cultures were plated through chloroform in order to determine the number of plaque forming units. $\Phi W$-14 infected \textit{P. acidovorans} strain 29, no additions, $\bullet$; $\Phi W$-14 infected \textit{P. acidovorans} strain 29, with netropsin (100 $\mu$g ml$^{-1}$), $O$. 
(Figure 32). The aberrant density profile was not due to the presence of unmodified DNA. Complete acid-hydrolysis and two-dimensional thin-layer chromatography of the DNA liberated only normal amounts of putThy, Thy and Cyt. hmUra did not accumulate (Table 14). The nature of netropsin-induced inhibition of phage with respect to reproduction was not established. The heavy density DNA seen on CsCl could have been single-stranded. This possibility was not tested.

**ΦW-14 ts and am mutants**

In attempts to facilitate the investigation of DNA replication and modification, a search for conditionally lethal DNA modification and replication mutants was undertaken in the belief that mutants failing to make putThy or Thy would be unable to reproduce. This assumption was based upon the observation that putThy and Thy levels in ΦW-14 DNA were constant and could not be manipulated (K.L. Maltman, unpublished observations).

Seventy temperature-sensitive mutants of ΦW-14 were isolated from nitrous acid mutagenized phage stocks. These phage were impaired in the ability to form plaques on *P. acidovorans* strain 29 at 30° but not at 20° to 22°. Thirty-six of the mutants were leaky and were set aside. Thirty-four of the mutants were cross-tested against each other and the ability to form plaques during mixed infections on bacterial lawns was determined. Five of the thirty-four mutants gave poor or variable complementation results and could not be segregated into complementation groups. The remaining twenty-nine mutants were grouped into twenty-two separate complementation groups which define twenty-two
FIGURE 32.—CsCl buoyant density gradient analysis of $\phi W$-14 DNA extracted from $P$. *acidovorans* strain 29 cells treated with netropsin.

Netropsin (100 $\mu$g ml$^{-1}$) was added to a growing culture of cells which were then infected with $\phi W$-14. At 25 minutes after infection [6-$^3$H]-uracil (1.0 $\mu$Ci ml$^{-1}$, 10.0 $\mu$g ml$^{-1}$) was added and the cells were incubated until 45 minutes after infection. DNA was extracted from infected cells and analyzed on a neutral CsCl buoyant density gradient. $\phi W$-14 DNA synthesized in the presence of netropsin, $\bullet$; $^{32}$P-labelled $\phi W$-14 reference DNA, $\bigcirc$. 
TABLE 14.—The base composition of \( \Phi W-14 \) DNA made in netropsin-treated cells.

<table>
<thead>
<tr>
<th>Base</th>
<th>cpm in base</th>
<th>fraction of total cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>putThy</td>
<td>25408</td>
<td>(0.22)</td>
</tr>
<tr>
<td>Cyt</td>
<td>55302</td>
<td>(0.49)</td>
</tr>
<tr>
<td>HmUra</td>
<td>233</td>
<td>(&lt;0.01)</td>
</tr>
<tr>
<td>Ura</td>
<td>1947</td>
<td>(0.02)</td>
</tr>
<tr>
<td>Thy</td>
<td>30672</td>
<td>(0.27)</td>
</tr>
<tr>
<td>unhydrolyzed nucleotides</td>
<td>86</td>
<td>(&lt;0.01)</td>
</tr>
</tbody>
</table>

DNA hydrolysis in 6N HCl took place for 3 hours at 100°C. The bases were separated by two-dimensional TLC on cellulose. The labelling and isolation of netropsin treated \( \Phi W-14 \) DNA was described in Figure 32.
different $\phi W$-14 genes. The complementation analysis and grouping is shown in Table 15.

P. Miller has isolated forty-two amber mutants from mutagenized $\phi W$-14 phage stocks. These mutants will form plaques on $P$. acidovorans strain 29 (sup 2) but not on $P$. acidovorans strain 29 wild-type cells. They have also been assigned to complementation groups. The relationship between the ts and amber complementation groups has not been established.

Attempts were made to screen for phage replication and modification mutants using $[^3H]$-ornithine incorporation at 30° and 20°. Phage-infected cells which did not make putThy-containing DNA would not accumulate label in DNA. In wild-type phage-infected cells at 30°, DNA synthesis begins at 20 to 25 minutes after infection and continues until 60 to 70 minutes after infection. At 20°, DNA synthesis begins at 60 to 70 minutes after infection and continues until 140 to 150 minutes after infection (Figure 33).

$\phi W$-14 ts lysates were used to infect strain 29 at 20° or 30° in TCS medium. $[^3H]$-ornithine was added and the accumulation of label in bacteriophage DNA measured. At 30 minutes, many mutants appear to exhibit altered time courses for initiation of DNA synthesis. The amount of DNA made also varied greatly. It was not possible to use ornithine incorporation to screen potential DNA modification mutants in the ts system because little DNA was being synthesized at the non permissive temperature in all mutants tested. The ornithine labelling protocol allowed labelling of DNA at the permissive temperature of 20°.

Instead, both am and ts mutants were tested by preparing
TABLE 15.—Complementation analysis and screening of \( \phi W-14 \) \( ts \) mutants.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Group size</th>
<th>Complementation group</th>
<th>( ts ) index</th>
<th>Bases in DNA</th>
<th>( % ) survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 1a )</td>
<td>1</td>
<td>1</td>
<td>( 1.5 \times 10^5 )</td>
<td>nt nt nt nt</td>
<td>33.0</td>
</tr>
<tr>
<td>( 1b )</td>
<td>1</td>
<td>2</td>
<td>( 6.0 \times 10^3 )</td>
<td>+ + - -</td>
<td>21.0</td>
</tr>
<tr>
<td>( 2a )</td>
<td>-</td>
<td>-</td>
<td>( 1.3 \times 10^6 )</td>
<td>+ + - ±</td>
<td>14.6</td>
</tr>
<tr>
<td>( 2b )</td>
<td>1</td>
<td>3</td>
<td>( 2.5 \times 10^6 )</td>
<td>+ + ± -</td>
<td>33.0</td>
</tr>
<tr>
<td>( 2c )</td>
<td>4</td>
<td>4</td>
<td>( 8.5 \times 10^4 )</td>
<td>+ + - -</td>
<td>27.3</td>
</tr>
<tr>
<td>( 2e )</td>
<td>1</td>
<td>5</td>
<td>( 6.4 \times 10^4 )</td>
<td>+ + - -</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>( 6a )</td>
<td>2</td>
<td>6</td>
<td>&gt;( 10^5 )</td>
<td>+ + - -</td>
<td>14.0</td>
</tr>
<tr>
<td>( 6b )</td>
<td>1</td>
<td>7</td>
<td>( 8.5 \times 10^3 )</td>
<td>+ + - -</td>
<td>12.3</td>
</tr>
<tr>
<td>( 9d )</td>
<td>1</td>
<td>8</td>
<td>( 8.4 \times 10^4 )</td>
<td>+ + - -</td>
<td>10.7</td>
</tr>
<tr>
<td>( 11a )</td>
<td>1</td>
<td>9</td>
<td>( 6.3 \times 10^5 )</td>
<td>+ + - -</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>( 12a )</td>
<td>1</td>
<td>10</td>
<td>( 4.5 \times 10^4 )</td>
<td>+ + - -</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>( 19a )</td>
<td>4</td>
<td>4</td>
<td>&gt;( 10^5 )</td>
<td>+ + - -</td>
<td>10.0</td>
</tr>
<tr>
<td>( 21b )</td>
<td>1</td>
<td>11</td>
<td>( 4.1 \times 10^5 )</td>
<td>+ + - -</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>( 24a )</td>
<td>1</td>
<td>12</td>
<td>( 7.2 \times 10^4 )</td>
<td>+ + - -</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>( 27a )</td>
<td>1</td>
<td>13</td>
<td>leaky</td>
<td>+ + - -</td>
<td>1.3</td>
</tr>
<tr>
<td>( 29a )</td>
<td>-</td>
<td>-</td>
<td>leaky</td>
<td>+ + - -</td>
<td>5.9</td>
</tr>
<tr>
<td>( 30a )</td>
<td>-</td>
<td>-</td>
<td>leaky</td>
<td>+ + - -</td>
<td>16.6</td>
</tr>
<tr>
<td>( 34a )</td>
<td>1</td>
<td>14</td>
<td>leaky</td>
<td>+ + - -</td>
<td>50.0</td>
</tr>
<tr>
<td>( A1b )</td>
<td>1</td>
<td>15</td>
<td>&gt;( 10^4 )</td>
<td>+ + - -</td>
<td>50.0</td>
</tr>
<tr>
<td>( A4a )</td>
<td>2</td>
<td>16</td>
<td>( 3.8 \times 10^4 )</td>
<td>+ + - -</td>
<td>2.8</td>
</tr>
<tr>
<td>( A4c )</td>
<td>2</td>
<td>16</td>
<td>( 5.1 \times 10^6 )</td>
<td>+ + - -</td>
<td>1.1</td>
</tr>
<tr>
<td>( A4d )</td>
<td>1</td>
<td>17</td>
<td>&gt;( 10^5 )</td>
<td>nt nt nt nt</td>
<td>nt</td>
</tr>
<tr>
<td>( A4f )</td>
<td>1</td>
<td>18</td>
<td>( 9.5 \times 10^5 )</td>
<td>+ + - -</td>
<td>5.7</td>
</tr>
<tr>
<td>( A5a )</td>
<td>1</td>
<td>19</td>
<td>leaky</td>
<td>+ + - -</td>
<td>0.8</td>
</tr>
<tr>
<td>( A11a )</td>
<td>2</td>
<td>6</td>
<td>&gt;( 10^3 )</td>
<td>nt nt nt nt</td>
<td>nt</td>
</tr>
<tr>
<td>( A12a )</td>
<td>4</td>
<td>4</td>
<td>&gt;( 10^3 )</td>
<td>nt nt nt nt</td>
<td>nt</td>
</tr>
<tr>
<td>( A12c )</td>
<td>1</td>
<td>20</td>
<td>nt</td>
<td>nt nt nt nt</td>
<td>nt</td>
</tr>
<tr>
<td>( A17b )</td>
<td>4</td>
<td>4</td>
<td>( 5.0 \times 10^5 )</td>
<td>+ + - -</td>
<td>6.0</td>
</tr>
<tr>
<td>( A18c )</td>
<td>2</td>
<td>21</td>
<td>&gt;( 10^5 )</td>
<td>+ + - -</td>
<td>2.3</td>
</tr>
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</table>
TABLE 15.—Continued

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Group size</th>
<th>Complementation group</th>
<th>ts index</th>
<th>Bases in DNA</th>
<th>% survivors</th>
</tr>
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<tr>
<td>A18d</td>
<td>1</td>
<td>22</td>
<td>&gt;10⁴</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>A20c</td>
<td>2</td>
<td>21</td>
<td>&gt;10⁵</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>A21b</td>
<td>1</td>
<td>23</td>
<td>&gt;10⁵</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>A22a</td>
<td>-</td>
<td>-</td>
<td>&gt;10⁵</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>B5a</td>
<td>-</td>
<td>-</td>
<td>&gt;10⁴</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

nt (not tested)

High titre lysates of phage mutants were prepared as described in the Materials and Methods. *P. acidovorans* strain 29 was infected at a multiplicity of 20; at 25 minutes after infection [6⁻H]-uracil was added and the cultures were incubated until the cell suspension began to clump. DNA was extracted and processed as described in the Materials and Methods.

Ts index is the ratio of plaque forming units in a lysate at 20° compared to the number of plaque forming units in the same lysate at 30°. [6⁻H]-uracil-labelled bases detected in acid hydrolysates of DNA were scored (+), bases not labelled by [6⁻H]-uracil were scored (−), bases present in marginal amounts were scored as (±).

Percent survivors is the measure of colony forming units surviving the screening infection.

Complementation analysis was performed as described in the Materials and Methods.
FIGURE 33.—DNA synthesis in ΦW-14-infected *P. acidovorans* strain 29 at 20°C.

The incorporation of [6-³H]-uracil into alkali resistant, TCA precipitable material was followed. The procedures used are described in the Materials and Methods.
ALKALI RESISTANT TCA PRECIPITABLE CPM \times 10^{-3}

MINUTES POST INFECTION
[6-3H]-uracil-labelled DNA from cells infected under non permissive conditions. DNA from am-infected strain 29 cells was harvested 45 to 50 minutes after infection. DNA from ts-infected strain 29 cells grown at 30° was harvested from cells when cultures took on a granular appearance. (Generally, infected cells became sticky, started to clump and had a grainy appearance 10 to 15 minutes prior to lysis. In wild-type cells at this stage, DNA synthesis was well-established.) Survivors of the screening infections were measured (Table 15).

DNA extracted from \( \phi W-14 \) am-infected cells was analyzed on neutral CsCl density gradients. This screening procedure detected phage which did not shut off host DNA synthesis effectively (ambers 36, 38), mutants which did not make \( \phi W-14 \) wild-type density DNA (ambers 6, 35, 45), and mutants making DNA of unusual density (ambers 37, 42). Representative examples of screening gradients are shown in Figure 34 (see Table 16).

The [6-3H]-uracil-labelled amber DNA preparations were acid-hydrolyzed and the bases were separated by two-dimensional thin-layer chromatography in solvents B and D. Authentic base standards were run with hydrolysates. The authentic base mixture contained putThy, Cyt, hmUra, Ura and Thy. The base spots were located under ultraviolet light and cut out and counted. Base spots containing significant amounts of a base were scored as positive (+) (Table 16). Using this procedure, of all the amber mutants screened, only am 37 accumulated hmUra in its DNA. Am 37 DNA contained tritium-labelled Thy, Cyt and hmUra. Little [6-3H]-uracil-labelled putThy was recovered from am 37 DNA. Am 42 DNA was also analyzed by acid hydrolysis. The only labelled products released were putThy, Thy and Cyt (Table 18).
FIGURE 34.—CsCl buoyant density gradients of DNA from *P. acidovorans* strain 29 infected with øW-14 *am* mutants.

The labelling and isolation procedures are described in the Materials and Methods. A) *am* 37 DNA extracted 45 minutes after infection; B) *am* 42 DNA extracted 45 minutes after infection; C) *am* 35 DNA extracted 60 minutes after infection. [6-^3^H]-uracil labelled DNA, ●; ^32^P labelled øW-14 ^+^ phage reference DNA, ○.
A

$^{3}\text{H} \text{ CPM} \times 10^{-3}$

FRACTION NUMBER

B

$^{32}\text{P} \text{ CPM} \times 10^{-4}$

FRACTION NUMBER

C

$^{3}\text{H} \text{ CPM} \times 10^{-3}$

FRACTION NUMBER

$^{32}\text{P} \text{ CPM} \times 10^{-4}$
TABLE 16.—The properties of DNA extracted from ϕW-14 am-infected *P. acidovorans*.

*P. acidovorans* strain 29 was infected with ϕW-14 am mutants at a multiplicity of infection of twenty. The cells were labelled with [6-³H]-uracil from 25 minutes to 45 minutes after infection. DNA was extracted and processed as described in the Materials and Methods.

Bases detected after acid hydrolysis and two-dimensional thin-layer chromatography of DNA were scored (+) when the base was detected in significant quantities. The absence of the base or its marginal presence was scored as (-) or (±), respectively.

The DNA was also analyzed on neutral CsCl density gradients. DNA peaks banding at host, phage or other buoyant density were scored (+). The absence of a peak banding at host, phage or other densities was recorded as (-).

The suppressor index is the ratio of plaque forming units in a lysate plated on *P. acidovorans* sup 2 compared to the number of plaque forming units in the same lysate plated on *P. acidovorans* strain 29. Percent survivors is the measure of colony forming units surviving the screening infection.
<table>
<thead>
<tr>
<th>am mutant</th>
<th>Bases detected in</th>
<th>DNA</th>
<th>DNA detected in CsCl</th>
<th>sup index</th>
<th>% survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>putThy Thy Hmu Ura</td>
<td>host ØW-14 other</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>+ + - - - + -</td>
<td>&gt;10^6</td>
<td>1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>+ + - - + + -</td>
<td>&gt;10^5</td>
<td>&lt;1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>+ + - - - + -</td>
<td>&gt;10^5</td>
<td>8.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>+ + - - - + -</td>
<td>&gt;10^6</td>
<td>0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>- - - - - - -</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>+ + - - - + -</td>
<td>&gt;10^5</td>
<td>0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>+ + - - - + -</td>
<td>1.6 x 10^3</td>
<td>0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>+ + - - - + -</td>
<td>&gt;10^5</td>
<td>0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>+ + - - - + -</td>
<td>&gt;10^5</td>
<td>&lt;1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>+ + - - - + -</td>
<td>&gt;10^6</td>
<td>4.9</td>
<td></td>
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</tr>
<tr>
<td>14</td>
<td>+ + - - - + -</td>
<td>&gt;10^5</td>
<td>&lt;1.0</td>
<td></td>
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</tr>
<tr>
<td>15</td>
<td>+ + - - - + -</td>
<td>&gt;10^5</td>
<td>&lt;1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>+ + - - - + -</td>
<td>&gt;10^6</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>+ + - - - + -</td>
<td>&gt;10^5</td>
<td>&lt;1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>+ + - - - + -</td>
<td>&gt;10^6</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>+ + - - - ± -</td>
<td>&gt;10^6</td>
<td>0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>+ + - - nt nt nt</td>
<td>&gt;10^6</td>
<td>0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>+ + - - - + -</td>
<td>1.5 x 10^5</td>
<td>19.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>+ + - - - + -</td>
<td>&gt;10^5</td>
<td>7.0</td>
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<td></td>
</tr>
<tr>
<td>23</td>
<td>+ + - - - + -</td>
<td>1.4 x 10^5</td>
<td>9.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>+ + - - - + -</td>
<td>leaky</td>
<td>&gt;50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>+ + - - - + -</td>
<td>leaky</td>
<td>&gt;50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>am</td>
<td>Bases detected in DNA</td>
<td>DNA detected in CsCl</td>
<td>sup index</td>
<td>% survivors</td>
<td></td>
</tr>
<tr>
<td>-----</td>
<td>----------------------</td>
<td>----------------------</td>
<td>-----------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td>mutant</td>
<td>putThy</td>
<td>Thy</td>
<td>Hmu</td>
<td>Ura</td>
<td>host</td>
</tr>
<tr>
<td>26</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>27</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>28</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
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<td>+</td>
<td>+</td>
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<td>31</td>
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<td>32</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>33</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>-</td>
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<td>34</td>
<td>+</td>
<td>+</td>
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<td>35</td>
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<td>36</td>
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</tr>
<tr>
<td>37</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>38</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
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<td>39</td>
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<td>40</td>
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<td>42</td>
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<td>43</td>
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<td>+</td>
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<td>+</td>
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<tr>
<td>44</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>45</td>
<td>-</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Twenty-five ts mutants were analyzed by preparation of [6-\(^3\)H]-uracil-labelled DNA. Ts 19a made only small amounts of putThy-containing DNA (Table 15).

Am 37, am 42 and ts 19a were analyzed in more detail. (N.B. The recovery of uracil from ts 2a DNA and of hmUra from ts 2b DNA have not been confirmed.)

\(\phi W-14\) ts 19a-infection of \(P.\ acidovorans\)

Ts 19a did not form plaques on strain 29 grown on CAA-M plates at 30°. Plaques on CAA-M medium at 20° were small. The burst size of ts 19a in liquid TCS medium at 30° was less than 1.0 pfu per cell. The burst size for ts 19a grown on TCS medium at 20° was 16 pfu per cell. Ts 19a phage stocks are prepared on CAA-M medium where phage yields per infected cell are greater.

The accumulation of \(^3\)H-ornithine label in ts 19a-infected cells is shown (Figure 35). Ornithine-labelled DNA accumulated at the permissive temperature of 20° but not at the non permissive temperature of 30°.

Measurement of DNA accumulation in ts 19a-infected cells with [6-\(^3\)H]-uracil gave a different pattern (Figure 36). Label accumulated in cells infected at 30° until 70 or 80 minutes after infection. The final amount of label accumulating in DNA at 20° was more than four times the final amount of label in DNA at 20°. Neutral CsCl density gradient analysis of DNA extracted from ts 19a-infected cells incubated at 30° until 40 minutes after infection demonstrated that all the label was in host-density DNA (Figure 37). An identical culture incubated until 70 minutes after infection contained a small amount of \(\phi W-14\)
FIGURE 35.—The incorporation of $[^3]H$-ornithine in $\Phi W-14$ ts 19a-infected $P. \text{acidovorans}$ strain 29.

The incorporation of $[^3]H$-ornithine into pronase resistant, TCA precipitable cpm was measured in $\Phi W-14$ ts 19a infected $P. \text{acidovorans}$ strain 29 at 30°, ●; and at 20°, ○. The procedures used are described in the Materials and Methods. Lysis of the 30° culture occurred at 90 to 100 minutes after infection.
FIGURE 36.--DNA synthesis in ϕW-14 ts 19a-infected P. acidovorans strain 3L.

The incorporation of [6-³H]-uracil into alkali resistant, TCA precipitable cpm was determined for ϕW-14 ts 19a infected cells at 30°, O; and at 20°, ●. ([6-³H]-uracil 0.5 μCi ml⁻¹, 10 μg ml⁻¹.) Lysis of the 30° culture occurred 90 to 100 minutes after infection.
FIGURE 37.—The buoyant density of DNA made in $\phi W-14$ ts 19a-infected \textit{P. acidovorans} strain 29.

$[6^{-3}H]$-uracil-labelled DNA was extracted from cells and banded on CsCl density gradients, ●.

A) The cells were infected at 30° and DNA was extracted 40 minutes after infection.

B) The cells were infected at 30° and DNA was extracted 75 minutes after infection.

C) The cells were infected at 30° and shifted to 20° at 40 minutes after infection. The DNA was extracted at 75 minutes after infection.

D) The cells were infected at 30° and shifted to 20° at 40 minutes after infection. The DNA was extracted at 85 minutes after infection.

E) The cells were infected at 20° and were allowed to lyse. The DNA was extracted from purified $\phi W-14$ ts 19a virions.

$[6^{-3}H]$-uracil was added to cultures at 20 minutes after infection.

Gradient A includes $^{32}$P-labelled host reference DNA, O; Gradient E includes $^{32}$P-labelled phage reference DNA, O.
wild-type-density DNA. When 30° cultures are shifted down to 20° at 40 minutes after infection, ts 19a DNA synthesis was rescued (Figure 38). ϕW-14 wild-type-density DNA appears in CsCl gradients after shift down (Figure 37). The shut off of host DNA synthesis was not efficient. Survivors of infections were also high. Ts 19a DNA, which is made at 30°, does contain putThy. hmUra and/or Ura did not accumulate in DNA (Table 17). Ts 19a DNA made at 20° was normal. It banded at the same density as wild-type phage reference DNA (Figure 37) and had normal levels of putThy and Thy in its DNA. Ts 19a could be a DNA delay mutant or a leaky Do mutant. In addition, ts 19a could be impaired in its ability to shut off host DNA synthesis. This analysis was hampered due to the high levels of survivors in ts 19a infections.

Am 42-infected P. acidovorans strain 29 accumulated DNA with a density greater than that of the wild-type phage reference DNA.

[6-3H]-uracil accumulation in am 42-infected strain 29 or sup 2 demonstrated that the same amount of label accumulated in both hosts. The DNA synthesis program began 35 to 45 minutes after infection and proceeded until about 105 minutes after infection (Figure 39).

Enzymatic digestion and two-dimensional thin-layer chromatography of [6-3H]-uracil-labelled DNA prepared in the non permissive host showed that DNA was deficient in putThy (Table 18). The deficiency in putThy was compensated for by an increase in levels of Thy. HmUra was not detected in am 42 DNA.

DNase I treatment of chloroform-treated am 42 phage lysates demonstrated that am 42 DNA was packaged after infection of permissive and non permissive hosts (Figure 39).
FIGURE 38.—$\Phi W-14\; ts\; 19a$ DNA synthesis can be rescued by a temperature shift from the nonpermissive to the permissive temperature.

A growing culture of $P.\; acidovorans$ strain 29 was infected with $\Phi W-14\; ts\; 19a$. The infected culture was incubated at $30^\circ$. At 10 minutes after infection $[6-^3H]-$uracil (0.25 μCi ml$^{-1}$, 10 μg ml$^{-1}$) was added to the culture. At 35 minutes after infection one-half of the culture was shifted to $20^\circ$. DNA synthesis was measured by following the incorporation of radioactivity into alkali resistant, TCA precipitable material. $30^\circ$, ●; $30^\circ$ downshifted to $20^\circ$, ○.
MINUTES AFTER INFECTION

ALKALI RESISTANT TCA PRECIPITABLE CPM x 10^3

10.0

8.0

6.0

4.0

2.0

30 50 70 90 110

MINUTES AFTER INFECTION
TABLE 17.—The Base composition of DNA made in $\Phi W-14$ ts 19a-infected P. acidovorans.

<table>
<thead>
<tr>
<th>Base</th>
<th>35 minutes/30°</th>
<th>90 minutes/20°</th>
<th>35 minutes/30° shifted to 20° for 30 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>putThy</td>
<td>407 (&lt;0.01)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32162 (0.18)</td>
<td>10306 (0.05)</td>
</tr>
<tr>
<td>Cyt</td>
<td>29799 (0.58)</td>
<td>104744 (0.59)</td>
<td>115418 (0.57)</td>
</tr>
<tr>
<td>Thy</td>
<td>21373 (0.41)</td>
<td>39802 (0.23)</td>
<td>77772 (0.38)</td>
</tr>
<tr>
<td>Hmu</td>
<td>310 (&lt;0.01)</td>
<td>-</td>
<td>519 (&lt;0.01)</td>
</tr>
<tr>
<td>Ura</td>
<td>221 (&lt;0.01)</td>
<td>-</td>
<td>715 (&lt;0.01)</td>
</tr>
</tbody>
</table>

The DNA was labelled, extracted and hydrolyzed as described in the Materials and Methods. The bases were separated by two-dimensional thin-layer chromatography on cellulose sheets.

<sup>a</sup> The values are cpm in the area cut from the chromatogram. The figures in parentheses represent the fraction of the radioactivity recovered in bases. The total recovery of radioactivity was greater than 90 percent.
FIGURE 39.—DNA synthesis in φN-14 am 42-infected P. acidovorans.

The incorporation of [6-3H]-uracil (1.0 μCi ml⁻¹, 10.0 μg ml⁻¹) into alkali resistant, TCA precipitable material was measured for am 42 infected strain 29, O; and am 42 infected strain sup 2, ●. The two points not connected to the curves represent DNase I resistant, alkali resistant, TCA precipitable material in CHCl₃ treated lysates.
ALKALI RESISTANT
TCA
PRECIPITABLE
CPM x 10^{-3}

MINUTES AFTER INFECTION
TABLE 18.—The nucleotide composition of $\Phi W-14$ am 42 DNA.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>$\Phi W-14$ w$^+$ DNA</th>
<th>$\Phi W-14$ am 42 DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>putdTMP</td>
<td>6904 (0.25)</td>
<td>4001 (0.07)</td>
</tr>
<tr>
<td>dCMP</td>
<td>14007 (0.50)</td>
<td>29812 (0.52)</td>
</tr>
<tr>
<td>dTMP</td>
<td>7219 (0.25)</td>
<td>23639 (0.41)</td>
</tr>
<tr>
<td>hmdUMP</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>putdTMP/dTMP</td>
<td>0.96</td>
<td>0.17</td>
</tr>
</tbody>
</table>

DNA was labelled with [6-3H]-uracil and purified and processed as described in the Materials and Methods. Nucleotides were separated by two-dimensional thin-layer chromatography on cellulose sheets.

a The values are cpm in the area cut from the chromatogram. The figures in parentheses represent the fraction of the radioactivity recovered in the nucleotides. The recovery of radioactivity was greater than 90 percent.
S1 and snake venom phosphodiesterase digests of a sample of [6-3H]-uracil-labelled am 42 DNA prepared in strain 29 were separated by two-dimensional thin-layer chromatography on PEI-cellulose and cellulose thin-layers (Figure 40). Am 42 DNA contained depressed levels of putdTMP and elevated levels of dTMP. The single peak on the CsCl gradient ruled out the possibility that the increase in dTMP levels detected was due to the presence of labelled host DNA (Figure 32). The mol % G + C content suggested by the nucleotide labelling was 52.

The conditionally lethal nature of this mutant has not been confirmed. Potentially, am 42 could provide evidence that precise levels of putThy and Thy in ϕW-14 DNA are required for ϕW-14 viability.

ϕW-14 am 37

A [6-3H]-uracil-labelled sample of DNA prepared in P. acidovorans strain 29 was analyzed on a neutral CsCl density gradient (Figure 34). The label in the DNA was found in three distinct peaks. The lightest of these peaks is believed to be labelled host DNA (ρ = 1.72 g/cc⁻¹). 32PO₄ phage reference DNA (ρ = 1.666 g/cc⁻¹) marks the position where normally-modified ϕW-14 should have banded. Two peaks (ρ = 1.73 to 1.74 and 1.77 to 1.78) mark positions of two new bands of heavy density DNA. Acid hydrolysis and thin-layer chromatography of the DNA sample revealed that Cyt, Thy, and hmUra but little putThy were present in the DNA (Table 20).

The ability of am 37 and wild type phage to plate on P. acidovorans strains 29 and JE1 and several of their sup derivatives was determined (Table 19). ϕW-14 w⁰ plates with high efficiency on
FIGURE 40.—Two-dimensional thin-layer chromatography of [6-\(^{3}H\)]-uracil-labelled nucleotides present in am 42 DNA prepared in *P. acidovorans* strain 29.
TABLE 19.—The plating efficiency of $\Phi W$-14 am 37 on \textit{P. acidovorans} strains.

<table>
<thead>
<tr>
<th>Host strain</th>
<th>$\Phi W$-14 $^+$</th>
<th>e.o.p.</th>
<th>$\Phi W$-14 am 37</th>
<th>e.o.p.$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>$3.8 \times 10^{11}$</td>
<td>1.0</td>
<td>$1.1 \times 10^5$</td>
<td>$2.3 \times 10^{-7}$</td>
</tr>
<tr>
<td>29 (sup 1)</td>
<td>$4.2 \times 10^{11}$</td>
<td>1.1</td>
<td>$4.7 \times 10^{11}$</td>
<td>1.0</td>
</tr>
<tr>
<td>29 (sup 2)</td>
<td>$4.7 \times 10^{11}$</td>
<td>1.2</td>
<td>$4.8 \times 10^{11}$</td>
<td>1.0</td>
</tr>
<tr>
<td>29 (sup 3)</td>
<td>$5.1 \times 10^{11}$</td>
<td>1.3</td>
<td>$5.0 \times 10^{11}$</td>
<td>1.1</td>
</tr>
<tr>
<td>29 (sup 4)</td>
<td>$4.5 \times 10^{11}$</td>
<td>1.2</td>
<td>$4.9 \times 10^{11}$</td>
<td>1.0</td>
</tr>
<tr>
<td>29 (sup 5)</td>
<td>$4.5 \times 10^{11}$</td>
<td>1.2</td>
<td>$5.0 \times 10^{11}$</td>
<td>1.1</td>
</tr>
<tr>
<td>JE1</td>
<td>$3.8 \times 10^{11}$</td>
<td>1.0</td>
<td>$3.0 \times 10^5$</td>
<td>$6.4 \times 10^{-7}$</td>
</tr>
<tr>
<td>JE1 (sup 1)</td>
<td>$5.2 \times 10^{11}$</td>
<td>1.4</td>
<td>$3.5 \times 10^5$</td>
<td>$7.4 \times 10^{-7}$</td>
</tr>
<tr>
<td>JE1 (sup 2)</td>
<td>$4.9 \times 10^{11}$</td>
<td>1.3</td>
<td>$2.6 \times 10^5$</td>
<td>$5.5 \times 10^{-7}$</td>
</tr>
<tr>
<td>JE1 (sup 3)</td>
<td>$5.4 \times 10^{11}$</td>
<td>1.4</td>
<td>$3.1 \times 10^5$</td>
<td>$6.6 \times 10^{-7}$</td>
</tr>
</tbody>
</table>

Lysates of $\Phi W$-14 $^+$ and am 37 phages were plated on various \textit{P. acidovorans} strains. The values are the numbers of plaque forming units in one milliliter of a lysate. Wild type $\Phi W$-14 lysates were prepared on $\textit{P. acidovorans}$ strain 29. $\Phi W$-14 am 37 lysates were prepared on $\textit{P. acidovorans}$ strain sup 2.

$^a$ e.o.p. is the efficiency of plating. It is the ratio of plaques formed by a lysate on the permissive host relative to the number of plaques formed on another host. \textit{P. acidovorans} sup 2 was the permissive host for $\Phi W$-14 am 37.
TABLE 20.—Base compositions of $\phi W$-14 and am 37 DNAs.$^a$

<table>
<thead>
<tr>
<th>Base</th>
<th>am 37/strain 29</th>
<th>Source of DNA</th>
<th>$\phi W$-14/strain 29</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>am 37/strain $^{\text{sup}}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>putThy</td>
<td>1074$^b$ (2.7)$^c$</td>
<td>5062 (19.9)</td>
<td>14430 (18.2)</td>
</tr>
<tr>
<td>cytosine</td>
<td>18016 (44.5)</td>
<td>10539 (41.5)</td>
<td>34585 (43.6)</td>
</tr>
<tr>
<td>hmUra</td>
<td>5903 (14.6)</td>
<td>1028 (4.0)</td>
<td>157 (0.2)</td>
</tr>
<tr>
<td>uracil</td>
<td>1209 (3.0)</td>
<td>176 (0.7)</td>
<td>180 (0.2)</td>
</tr>
<tr>
<td>thymine</td>
<td>9353 (23.1)</td>
<td>6173 (24.3)</td>
<td>21976 (27.7)</td>
</tr>
<tr>
<td>unhydrolyzed</td>
<td>4964 (12.3)</td>
<td>2441 (9.6)</td>
<td>7987 (10.1)</td>
</tr>
<tr>
<td>nucleotides</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Replicating DNA was labelled with [6-$^3$H]-uracil. After hydrolysis, the bases were separated by thin-layer chromatography, using solvent B in the 1st and solvent D in the 2nd 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 greater than 90 percent.
all the tested strains. \( \Phi W-14 \text{ am} 37 \) plated only on the five sup derivatives of \( P. \text{acidovorans} \) strain 29. The \( \Phi W-14 \text{ am} 37 \) reversion frequency is less than \( 10^{-6} \) wild-type plaques formed per mutant phenotype plaque.

The burst size of \( \text{am} 37 \) grown on strains 29 and sup 2 was determined (Figure 41). Aliquots of infected cultures were plated through chloroform at time points after infection. After an eclipse period of 60 to 70 minutes phage began to appear in sup 2 cultures. The lysis time was variable but usually occurred 140 to 180 minutes after infection. There was not any release of infectious virus from \( \text{am} 37 \) infected strain 29 cells. The average burst size for \( \text{am} 37 \) phage in strain sup 2 grown on TCS medium was 50 plaque forming units per cell.

The conditional nature of the DNA modification lesion was demonstrated. In the sup 2 host \( \text{am} 37 \) infected cells accumulated some DNA with the same density as \( \Phi W-14 \text{ w}^+ \) reference DNA. Strain 29 cells infected with \( \text{am} 37 \) accumulated heavy density DNA but not wild type, light density DNA. Suppression of the modification lesion in the suppressor host was incomplete. The \( \text{am} 37 \)-sup 2 infected cells contained DNA with a density greater than normally seen in sup 2 or strain 29 cells infected with wild-type \( \Phi W-14 \) (Figure 42). These experiments suggested that a gene responsible for some aspect of \( \Phi W-14 \) DNA modification was mutated in \( \text{am} 37 \). Furthermore, this mutation was responsible for the conditionally lethal phenotype of \( \text{am} 37 \) phage reproduction.

\textbf{Am 37 DNA contains a novel nucleotide}

Quantitative acid hydrolysis and thin-layer chromatography of [6-\(^3\text{H}\)]-uracil labelled DNA samples was performed (Table 20). DNA
FIGURE 41.—The reproduction of \( \phi \)W-14 \textit{am} 37 in \textit{P. acidovorans} strains 29 or sup 2.

At intervals after infection of strains 29 or sup 2 aliquots of the cultures were removed and diluted through CAA-M over CHCl$_3$. Plaque forming units were assayed by plating on \textit{P. acidovorans} strain sup 2. \textit{P. acidovorans} was grown in TCS medium supplemented with succinate and Casamino acids. \textit{Am} 37 infected strain sup 2, O; \textit{am} 37 infected strain 29, O.
FIGURE 42.—Buoyant density of DNA synthesized by phage-infected cells. Infected cells were labelled with [6-$^3$H]-uracil and the DNA extracted from the cells 35 minutes 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-labelled DNA; ---- $^{32}$P reference DNA from phage particles. The bottom of the gradient is on the left.
isolated from strain 29 cells infected with am 37 had extremely low levels of putThy when compared to am 37 DNA prepared in the sup 2 host or to wild-type DNA isolated from infection of strain 29. Thymine levels in am 37 DNA prepared in either the permissive or the non permissive host were normal. DNA samples from am 37-infected strain 29 contained 14.6 percent of the total pyrimidine label in hmUra residues. DNA samples from am 37 infected strain sup 2 contained 4.0 percent of the total pyrimidine label in hmUra. In \( \phi W-14 \) w\( ^+ \) infected cells 0.2 percent or less of the total pyrimidine label was found in hmUra residues.

\( ^{32}P_4 \) and [6-\( ^3H \)]-uracil labelled am 37 DNA and \( \phi W-14 \) w\( ^+ \) DNA were prepared and analyzed by two-dimensional thin-layer chromatography after nuclease S1 and snake venom phosphodiesterase digestion (Figure 43) (Table 21). Am 37-infected strain 29 cells contained DNA with two extra pyrimidine nucleotides not found in uniformly labelled preparations of wild-type \( \phi W-14 \) DNA digests. The two nucleotides shared chromatographic properties with two of the three extra nucleotides detected in \( ^{32}P \)-pulse labelled \( \phi W-14 \) DNA digests. The Rf value for each nucleotide was measured in solvents E and A on cellulose thin-layer sheets (Table 21). One of the extra components had chromatographic properties similar to hmdUMP. The other unidentified compound was more heavily labelled with \( ^{32}P_4 \) than with [6-\( ^3H \)]-uracil. This suggested that this compound contained more than one phosphate residue per pyrimidine base. It was possible that this compound was an oligonucleotide which resulted from incomplete digestion of am 37 DNA. A relatively large amount of free \( ^{32}P_4 \) was also released from \( ^{32}P \)-labelled am 37 DNA during
FIGURE 43.—Nucleotides in am 37 DNA.

Cells were labelled with [6-\(^3\)H]-uracil after infection with am 37. The DNA was extracted from the cells at 45 minutes 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.
TABLE 21.—Nucleotide composition of φW-14 and am 37 DNAs.¹

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>am 37/strain 29 (6-3H-uracil)</th>
<th>am 37/strain 29 (32P orthophosphate)</th>
<th>am 37/strain sup2 (6-3H-uracil)</th>
<th>φW-14/strain 29 (32P orthophosphate)</th>
<th>Re²</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>dGMP</td>
<td>40 (0.1)³</td>
<td>3944 (16.1)</td>
<td>0</td>
<td>13355 (24.1)</td>
<td>26</td>
<td>39</td>
</tr>
<tr>
<td>dAMP</td>
<td>72 (0.2)³</td>
<td>3012 (12.3)</td>
<td>0</td>
<td>13921 (25.1)</td>
<td>56</td>
<td>27</td>
</tr>
<tr>
<td>dTMP</td>
<td>8080 (27.6)</td>
<td>2972 (12.1)</td>
<td>41218 (27.0)</td>
<td>7219 (13.0)</td>
<td>36</td>
<td>52</td>
</tr>
<tr>
<td>dCMP</td>
<td>13252 (45.2)</td>
<td>5943 (24.2)</td>
<td>81876 (53.7)</td>
<td>14007 (25.2)</td>
<td>43</td>
<td>70</td>
</tr>
<tr>
<td>putdTMP</td>
<td>385 (1.3)³</td>
<td>87 (0.4)</td>
<td>27835 (18.3)</td>
<td>6904 (12.4)</td>
<td>55</td>
<td>79</td>
</tr>
<tr>
<td>hmdUMP</td>
<td>2043 (7.0)³</td>
<td>865 (3.5)</td>
<td>256 (0.2)</td>
<td>29 (&lt;0.1)</td>
<td>23</td>
<td>62</td>
</tr>
<tr>
<td>unknown</td>
<td>5430 (18.5)</td>
<td>7750 (31.5)</td>
<td>1264 (0.8)</td>
<td>47 (&lt;0.1)</td>
<td>9</td>
<td>65</td>
</tr>
</tbody>
</table>

¹ Replicating DNA was labelled with [6-3H]-uracil or 32P-orthophosphate. After digestion with nuclease S1 and snake venom phosphodiesterase, the mononucleotides were separated by thin-layer chromatography.

² Cpm in the area cut from the chromatogram.

³ The figures in parentheses are the percentages of the total radioactivity recovered as nucleotides.

⁴ Rf values are given for nucleotides in solvents E and A.
enzymatic digestion with nuclease S1 and snake venom phosphodiesterase. putdTMP made up 1.3 percent or less of the total pyrimidine label in am 37 DNA extracted from strain 29 but was 18 percent of the total pyrimidine label in am 37 DNA extracted from strain sup 2 cells. dTMP was found in all am 37 DNA samples in normal quantities.

Am 37 was deficient in its ability to make putdTMP in a non permissive host, but was the extra radioactive component a novel nucleotide or an oligonucleotide resulting from the incomplete digestion of hmdUMP containing DNA? It was possible to predict the buoyant density of hmUra containing DNA of known mol % G + C content. The predicted buoyant density of native duplex DNA containing guanine, adenine, cytosine and thymine is defined by the formula $\rho = 0.098 (G + C) + 1.660$. For DNA with a mol % G + C of 52.0 the predicted buoyant density is 1.711 gcc$^{-1}$. For the same mol % G + C value, DNA containing hmUra instead of thymine would have a buoyant density of 1.751 gcc$^{-1}$, while DNA with a mol % G + C value of 52.0 containing equal amounts of Thy and hmUra would have a buoyant density of 1.731 gcc$^{-1}$. These calculations were performed according to formulae described by Rae (1973). The heaviest density am 37 DNA peak has a buoyant density between 1.77 and 1.78 gcc$^{-1}$. This value is much greater than could be predicted, even for complete substitution of putThy and Thy by hmUra. It was likely that am 37 DNA contained a novel nucleotide. This nucleotide was probably an intermediate in the biosynthetic pathway leading to putdTMP.

[6-3H]-uracil labelled hmUra in acid hydrolysates of am 37 DNA prepared in strain 29 formed a greater percentage of the total acid
hydrolyzed label than [6-\(^3\)H]-uracil labelled hmdUMP in enzymatic
digests of aliquots of the same DNA sample (Tables 20 and 21). This
suggested that the novel nucleotide broke down to form hmUra during
acid hydrolysis. Since nuclease S1 digestion employed an acidic buffer
it was possible that the hmdUMP in samples was an acid degradation
product of the novel nucleotide.

The novel pyrimidine nucleotide was also detectable in S1 and
snake venom phosphodiesterase digests of am 37 DNA prepared in strain
sup 2 (Figure 43A) (Table 21). hmdUMP and the novel nucleotide made
up approximately 18.3 percent of the total pyrimidines labelled. As
mentioned earlier, suppression of the am 37 phenotype in sup 2 was
incomplete. It is not known if the presence of the novel nucleotide
in the am 37 DNA from the sup 2 host reflects the mechanism of putThy
formation. If putThy formation is normally coupled to replication
then it is likely that the mutant modification protein could fail to
convert a portion of the novel nucleotide to putdTMP. However, if the
modifying enzyme could convert the novel nucleotide to putdTMP when­
ever it is presented, then the DNA from the sup 2 strain infection
should not contain any intermediate in putThy biosynthesis, unless the
substrate is formed or removed by DNA packaging at a rate greater than
the rate of formation of the product. The analysis of the kinetics of
formation of the novel nucleotide as well as the kinetics of its con­
version to putThy might answer the question: is putThy biosynthesis
absolutely coupled to DNA replication?

Am 37 DNA was labelled with [8-\(^{14}\)C]-adenine in strain 29. The
DNA was digested with S1 and snake venom phosphodiesterase. Two-
dimensional thin-layer chromatography revealed that the only labelled 
products were dAMP and dGMP (Figure 44). Therefore, the novel nucleo-
tide was not a product of the incomplete digestion of am 37 DNA. Sam-
ples of $^{32}$P-labelled $\phi W-14$ w$^+$ DNA were digested with DNase I and snake 
venom phosphodiesterase. Two dimensional thin-layer chromatography 
revealed that little putdTMP was released from $\phi W-14$ w$^+$ DNA (Figure 25). 
Similarly, digestion of $^{32}$P-labelled am 37 DNA prepared in strain 29 
infected cells was not complete (Figure 46). Limit digests of am 37 
DNA contained dGMP, dAMP, dCMP and dTMP as well as some of the novel 
nucleotide. In addition there were three other radioactive compounds 
(Figure 46). These were probably dimers containing the novel nucleo-
tide and another pyrimidine nucleotide. After two-dimensional thin-
layer chromatography of DNase I and snake venom phosphodiesterase 
digests of $[8^{-14}C]$-adenine labelled am 37 DNA prepared in strain 29 
dAMP and dGMP were the only labelled products (Figure 45). Therefore, the radio-
active products accumulating in incomplete DNA digests were digestion 
resistant pyrimidine oligonucleotides. Another feature of the DNase I 
and snake venom phosphodiesterase digests of am 37 DNA was the absence 
of a hmdUMP spot. It is possible that the hmdUMP seen on chromatograms 
of S1 digests of am 37 DNA was an artifact generated by the degradation 
of the novel nucleotide in the acidic S1 digestion buffer. However, 
am 37 DNA labelled with $^{32}$PO$_4$ does not release label when incubated in 
S1 buffer in the absence of enzyme (Table 22). Alternatively, hmdUMP 
residues might have been present in sequences which were resistant to 
DNase I and snake venom phosphodiesterase digestion.

The quantity of $^{32}$PO$_4$ released from am 37 DNA during DNase I
FIGURE 44.—Two-dimensional thin-layer chromatography of [8-^{14}C]-adenine-labelled nucleotides present in S1-SVPD digests of am 37 DNA prepared in P. acidovorans strain 29.

Nucleotides were detected by fluorography after 2D-TLC in solvents E and A. DNA was digested sequentially with nuclease S1 and SVPD.

1) dAMP; 2) dGMP.
FIGURE 45.—Two-dimensional thin-layer chromatography of [8-^{14}C]-adenine-labelled nucleotides present in DNase I-SVPD digests of am 37 DNA prepared in P. acidovorans strain 29.

Nucleotides detected by fluorography after 2D-TLC in solvents E and A. The DNA was digested with DNase I and SVPD.

1) dAMP; 2) dGMP.
$^{14}C$-Adenine, DNA, 0.02M Tris, pH 8.2, 0.015M MgCl$_2$
FIGURE 46.—Two-dimensional thin-layer chromatography of $^{32}$P-labelled nucleotides present in DNase I-SVPD digests of am 37 DNA prepared in P. acidovorans strain 29.

The nucleotides were detected by autoradiography after 2D-TLC in solvents E and A. The DNA sample was digested with DNase I and SVPD.

1) dAMP  6) unknown mononucleotide
2) dGMP  7) unknown
3) dTMP  8) unknown
4) dCMP  9) unknown
5) $PO_4$
32p Am37/29
0.05 M Tris pH 8.2 + 0.1% NaCl
DNase I/SVPD
DAVID
TABLE 22.—\(\Phi W-14\) am 37 DNA was stable in alkali and SI buffer.

<table>
<thead>
<tr>
<th>treatment</th>
<th>cpm</th>
<th>percent recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>16365</td>
<td></td>
</tr>
<tr>
<td>(\text{ddH}_2\text{O}, 37^\circ/16\text{ hours})</td>
<td>15991</td>
<td>98</td>
</tr>
<tr>
<td>none</td>
<td>15993</td>
<td></td>
</tr>
<tr>
<td>(0.3\text{ N NaOH, 37^\circ/16\text{ hours}})</td>
<td>15319</td>
<td>96</td>
</tr>
<tr>
<td>none</td>
<td>9742</td>
<td></td>
</tr>
<tr>
<td>(\text{SI buffer, }0.05\text{ M NH}_4\text{-acetate, pH 5.0, 55^\circ/4\text{ hours}})</td>
<td>9508</td>
<td>98</td>
</tr>
</tbody>
</table>

\(^{32}\text{P}-\text{labelled }\Phi W-14\) am 37 DNA was ethanol precipitated and then resuspended in deionized, distilled water. Aliquots of each sample were spotted onto Whatman 3MM paper squares and washed sequentially with 95 percent ethanol and absolute ether. The remaining portion of the sample was treated as described. Each value represents the average of three determinations. After treatment the samples were subjected to TCA precipitation. The papers were washed three times with 95 percent ethanol and twice with absolute ether and were then dried and counted.
and snake venom phosphodiesterase digestion was less than that released from am 37 DNA during digestion with nuclease S1. The source of the $^{32}$PO$_4$ released is not known.

**DNA synthesis in ØW-14 am 37 infected-cells**

The incorporation of [6-$^3$H]-uracil into alkali resistant, TCA precipitable material was measured for wild-type and am 37-infected strains 29 and sup 2 (Figure 47). DNA accumulation in wild-type infected strain 29 cells began at 25 minutes after infection and proceeded until 60 to 70 minutes after infection. Wild-type infection of sup 2 followed a slower time course. DNA synthesis began around 40 minutes after infection and continued until 100 minutes. The final levels of DNA accumulation for both wild-type infections were similar. Am 37-infected strain 29 or sup 2 cells accumulated approximately equal quantities of DNA and followed similar time courses. Am 37 DNA synthesis began around 40 minutes after infection and proceeded until 100 minutes. The amount of DNA synthesized in am 37-infected cells was less than one-half the amount of DNA made in the same strains during wild-type ØW-14 infection.

Neither am 37 nor wild-type ØW-14 DNA were labelled by [2-$^{14}$C]-hmdU. Am 37 DNA was not labelled by [$^3$H]-ornithine.

**Tritium release from [5-$^3$H]-uracil in amber 37-infected 3L**

Tritium release was used to measure the *in vivo* activity of dUMP hydroxymethylase. Correction of the data for differences in sample volumes and counting efficiencies allowed a direct comparison
FIGURE 47.—DNA synthesis by phage-infected cells.

The incorporation of [6-^3^H]-uracil into alkali-resistant, TCA-insoluble material was determined for wild-type phage in strain 29 (O) and in strain sup 2 (●); and for am 37 in strain 29 (Δ) and in sup 2 (▲). [6-^3^H]-uracil (1.0 μCi ml⁻¹, 10 μg ml⁻¹ uracil).
MINUTES AFTER INFECTION

CPM x 10^{-3}

25 45 65 85

MINUTES AFTER INFECTION
of the accumulation of \([5-{^3}H]\)-uracil label in cytosine bases in DNA and the release of tritium from \([5-{^3}H]\)-dUMP in nucleotide pools. In wild-type \(\Phi W-14\)-infected cells the ratio of tritium release to tritium incorporation was one-to-one (Figure 48). This was not surprising since, in a phage with a DNA mol % G + C of approximately 50 percent, the requirements for cytosine and hmUra nucleotides would be equal. The one-to-one ratio is destroyed in \(\Phi W-14\) DO mutants (P. Miller, unpublished observations) and in am 37-infected strain 3L (Figure 48). In am 37 infection the amount of tritium released was greater than the amount of tritium accumulated in DNA. Tritium release and DNA synthesis began about 20 to 25 minutes after infection. In am 37-infected 3L the rate of DNA incorporation slowed relative to the rate of tritium release as the infection progressed. The rate and absolute amount of tritium release in am 37-infected 3L was less than the rate and the amount of tritium released in wild-type \(\Phi W-14\) infections of 3L. It is possible that the nature of the DNA template had an effect upon tritium release. Since tritium release was greater than DNA synthesis it was unlikely that the lower rate of DNA synthesis was due to a shortage in the supply of DNA precursors.

The stability of am 37 DNA

Measurements of DNA accumulation required exposure of am 37 DNA to extreme conditions of basicity and acidity. The stability of \(^{32}\)P-labelled components of am 37 DNA to treatment with 0.3 N NaOH and 5 percent TCA were investigated (Table 22). Ninety-eight percent of input label was recovered from a TCA precipitated am 37 DNA sample
FIGURE 48.—DNA synthesis and tritium release by infected cultures.

Infected cells of strain 3L were labelled with [5-$^3$H]-uracil. Samples were assayed for the incorporation of label into alkali-resistant, TCA-insoluble material (●) and for the release of tritium (○). A: wild-type phage; B: am 37.
which was preincubated in deionized-distilled water at 37° for 16 hours. Ninety-six percent of the $^{32}$P label was recovered from am 37 DNA samples incubated for 16 hours in 0.3 N NaOH at 37° and then TCA precipitated. It was concluded that the $^{32}$P label in am 37 DNA was stable under alkaline incubation conditions and was not affected by subsequent TCA precipitation. The methods used to measure DNA accumulation in am 37-infected cells were valid.

CsCl gradient analysis at am 37 DNA

$^{32}$P-labelled am 37 DNA was extracted from strain 29 cells at 45, 60 and 75 minutes after infection and analyzed in neutral CsCl density gradients. DNA extracted from cells at 45 minutes after infection contained the two peaks of heavy and intermediate density described earlier (Figure 49A). DNA extracted from cells at 60 or 75 minutes after infection contained only one major peak of heavy density DNA (Figure 49, B and C). Two-dimensional thin-layer chromatography of S1 and snake venom phosphodiesterase digests of $^{32}$P-labelled DNA samples demonstrated that the percentages of novel nucleotide and hmdUMP were constant throughout the 30 minute time interval (Table 23). The intermediate density am 37 DNA peak was probably a hybrid DNA duplex containing "unmodified" DNA in one strand and parental DNA in the other strand. As replication proceeded, parental DNA was shifted to hybrid density by DNA replication. Eventually, parental DNA was dispersed by replication and recombination and the hybrid density peak became a small proportion of the DNA. The constant nature of the am 37 DNA nucleotide composition coupled to the differences in CsCl profiles
FIGURE 49.—CsCl buoyant density analysis of φW-14 am 37 DNA prepared at various times after infection of *P. acidovorans* strain 29.

The DNA was purified from infected cells at 45, 60 and 75 minutes after infection. Equal volumes of DNA extracted from 5 ml of infected cells were loaded on a neutral CsCl gradient. The procedures employed are described in detail in the Materials and Methods.
TABLE 23.—The nucleotide composition of φW-14 am 37 DNA prepared at various times after the infection of *P. acidovorans* strain 29.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>45 min.</th>
<th>60 min.</th>
<th>75 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>dTMP</td>
<td>2972 (0.17)</td>
<td>4195 (0.18)</td>
<td>4507 (0.17)</td>
</tr>
<tr>
<td>dCMP</td>
<td>5943 (0.34)</td>
<td>8457 (0.36)</td>
<td>9333 (0.35)</td>
</tr>
<tr>
<td>putdTMP</td>
<td>87 (&lt;0.01)</td>
<td>117 (&lt;0.01)</td>
<td>105 (&lt;0.01)</td>
</tr>
<tr>
<td>hmdUMP</td>
<td>865 (0.05)</td>
<td>1180 (0.05)</td>
<td>1244 (0.05)</td>
</tr>
<tr>
<td>unknown</td>
<td>7750 (0.44)</td>
<td>9248 (0.40)</td>
<td>11160 (0.42)</td>
</tr>
</tbody>
</table>

The DNA was purified from infected strain 29 cells as described in the Materials and Methods. Nuclease S1 and snake venom phosphodiesterase digestions were performed and the nucleotides were separated by 2D-TLC on cellulose. The DNA used was part of the sample used for CsCl analysis in Figure 47.

a The values are cpm in the area cut from the chromatogram. The values in parentheses are the fraction of the total labelled pyrimidine nucleotides.
proved that the labelled DNA peaks did not differ in their labelled nucleotide composition.

**Parentally labelled am 37 DNA**

The transfer of parental label to hybrid density in am 37-infected strain 29 was demonstrated (Figure 50). Most of the parental am 37 DNA remained at parental density, indicating that it was inactive. This probably was a reflection of the high multiplicities of infection required in am 37 experiments. Parentally-labelled hybrid density DNA was only found after the onset of DNA replication in samples taken 45 minutes after infection. This agreed with the results from the progeny labelled gradients. There was no transfer of parental label to hybrid density in am 37 infected sup 2 cells. The parentally labelled DNA had a broad but uniform density profile.

**Purification of the novel nucleotide found in am 37 DNA**

In order to study the nature of the novel am 37 nucleotide, large amounts of $^{32}$P and [6-$^3$H]-uracil-labelled am 37 DNA were prepared in infected strain 29 cells. S1 and snake venom phosphodiesterase digests of the purified DNA were loaded on Whatman 40 SFC paper and treated as described in the Methods section of this thesis. After chromatography the nucleotides were localized by autoradiography (Figure 51). The novel nucleotide and the nucleotide tentatively identified as hmdUMP were well separated from the origin and from other nucleotides. When the purified nucleotide was eluted from the paper and rechromatographed only one radioactive nucleotide was found on the
FIGURE 50.—CsCl buoyant density analysis of parentally labelled ØW-14 am 37 DNA.

[6-\(^3\)H]-uracil-labelled ØW-14 am 37 phage were prepared in strain sup 2. Radioactive phage was used to infect cultures of strains 29 and sup 2. DNA was extracted from the infected cells at 45 minutes after infection of strain 29, (A); and at 60 minutes after infection of strain sup 2, (B). \(^{32}\)P-labelled ØW-14 w\(^+\) phage reference DNA, ●; [6-\(^3\)H]-uracil labelled sample DNA, O.
A

B

\[ ^3H \text{ CPM} \times 10^{-3} \]

\[ ^{32}P \text{ CPM} \times 10^{-3} \]

\[ \text{FRACTION NUMBER} \]
FIGURE 51.—The purification of the novel nucleotide by paper chromatography.

A $^{32}\text{P}$-labelled $\Phi W-14$ am 37 DNA sample was digested with nuclease S1 and with SVPD. Descending paper chromatography and the recovery of the nucleotides are described in the Materials and Methods.
chromatogram (Figure 52). The purity of the radioactive product was also demonstrated on a short DEAE-Sephadex column (Figure 53). The purified nucleotide and an unlabelled digest of am 37 DNA were loaded on the column. Chromatography of the sample revealed that the novel nucleotide was more strongly retarded on the column when compared to the five other mononucleotides present in the sample. Even on a short 8 cm column the novel nucleotide was almost completely resolved from the other mononucleotides. All of the radioactivity loaded on the column eluted as a single peak indicating that the compound prepared by paper chromatography was radiochemically pure. The chromatographic properties of the nucleotide on DEAE-Sephadex suggested that it had a net negative charge greater than the other mononucleotides. This was not surprising since the chromatographic properties of the nucleotide observed on PEI and unmodified cellulose also suggested the same thing. [6-^3H]-uracil and ^32PO$_4^-$ labelling ratios demonstrated that the novel nucleotide carried extra phosphate residues.

The approximate size of the novel nucleotide was established by gel filtration chromatography on Biogel P$_2$ columns. All the nucleotides eluted as a single peak, although the early peak fractions contained more of the novel nucleotide and dGMP eluted in later peak fractions (data not shown).

Attempts to recover the novel nucleotide from the DEAE-Sephadex and Biogel columns passing the eluate fractions through Norit A were not successful, the novel nucleotide was degraded. Purified salt-free samples of the novel nucleotide and hmdUMP were routinely obtained by paper chromatography.
FIGURE 52.—Two-dimensional thin-layer chromatography of the purified novel nucleotide.

An aliquot of the novel nucleotide purified by descending paper chromatography was spotted on a cellulose sheet and subjected to 2D-TLC with solvents E and A.
FIGURE 53.—DEAE-Sephadex column chromatography of the novel nucleotide.

A $^{32}$P-labelled paper purified sample of the novel nucleotide was loaded on a short (8 cm) column of DEAE-Sephadex, with an unlabelled digest of am 37 DNA. The nucleotides were eluted with a linear gradient of NaCl in 20 mM Tris-HCl, pH 8.0. Three ml fractions were collected. Cerenkov radiation and $A_{260}$ were measured for each fraction. The procedures employed are described in detail in the Materials and Methods. $^{32}$P-label nucleotide, O; $A_{260}$, •.
Samples of a $[6^-\text{H}]$-uracil labelled preparation of hmdUMP and the novel nucleotide were hydrolyzed in 6 N HCl. The free bases were separated by two-dimensional thin-layer chromatography. The only radioactive product released by acid hydrolysis was hmdUra. This result confirmed the identification of hmdUMP and demonstrated that the novel nucleotide was an acid-labile derivative of hmdUMP (Table 24).

The lability of the novel nucleotide under acidic and alkaline incubation conditions was examined. It was fairly stable to boiling in deionized-distilled water or heating in 0.3 N KOH at 37° (data not shown). It decomposed upon treatment with 1 N HCl for 30 minutes at 37° or 7 minutes at 98°. The relevant properties of the novel nucleotide are summarized in Table 25. Acid treatment releases hmdUMP and orthophosphate. The ratio of acid-labile to acid-stable phosphate was two. The apparent ratio of tritium to $^{32}$P label is approximately one to three. The acid lability experiments suggested that the novel nucleotide was a diphosphorylated derivative of hmdUMP.

The sensitivity of the novel nucleotide to the enzyme bacterial alkaline phosphatase (BAP) was determined. Incubation with BAP caused the complete conversion of the nucleotide to $[^3\text{H}]$-hmdUra and PO$_4$ (Table 26). The products were determined chromatographically (Figure 54). hmdUra was identified by comparing its chromatographic behaviour to authentic deoxynucleoside standards in five different solvents (Table 26). All phosphate residues on the novel nucleotide were sensitive to BAP (Table 26). The novel nucleotide was stable in the incubation buffer employed (Figure 54).

The information obtained about the novel nucleotide suggested
TABLE 24.—Acid hydrolysis of nucleotides purified from φW-14 am 37 DNA.

<table>
<thead>
<tr>
<th>Authentic base</th>
<th>unknown nucleotide</th>
<th>hmdUMP&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>putThy</td>
<td>12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>Cyt</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>hmUra</td>
<td>2673</td>
<td>1362</td>
</tr>
<tr>
<td>Ura</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>Thy</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

The labelling, extraction and purification procedures are described in the Materials and Methods. Bases were separated by 2D-TLC in solvents B and D. The recovery of applied label was greater than 90 percent. The nucleotides were labelled with [6-<sup>3</sup>H]-uracil.

<sup>a</sup> The values are cpm in the areas cut from the chromatogram.

<sup>b</sup> Identified as hmdUMP on the basis of its chromatographic properties.
TABLE 25.—Properties of the unknown nucleotide.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>unknown nucleotide</th>
<th>hmdUMP</th>
<th>inorganic phosphate</th>
<th>hmdUra</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^{3}\text{H}$</td>
<td>$^{32}\text{P}$</td>
<td>$^{32}\text{P}/^{3}\text{H}$</td>
<td>$^{3}\text{H}$</td>
</tr>
<tr>
<td>None</td>
<td>2298</td>
<td>6759</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>1 N HCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37°C 30 min</td>
<td></td>
<td>1903</td>
<td>2040</td>
<td>1.1</td>
</tr>
<tr>
<td>1 N HCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>98°C 7 min</td>
<td></td>
<td>1978</td>
<td>2334</td>
<td>1.2</td>
</tr>
<tr>
<td>alkaline phosphatase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a The DNA from which the nucleotides were purified was labelled with $[6-^{3}\text{H}]$-uracil and $^{32}\text{P}$ orthophosphate.

b The products were identified by thin-layer chromatography with known standards.
TABLE 26.—Conversion of the unknown nucleotide to its nucleoside.

<table>
<thead>
<tr>
<th></th>
<th>-BAP</th>
<th>+BAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown nucleotide</td>
<td>3747&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19</td>
</tr>
<tr>
<td>$^{32}\text{PO}_4$</td>
<td>138</td>
<td>3422</td>
</tr>
</tbody>
</table>

Identification of the unknown nucleoside by TLC.

<table>
<thead>
<tr>
<th>Rf in Solvent</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Authentic Standards</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hmUdR</td>
<td>76</td>
<td>59</td>
<td>78</td>
<td>26</td>
<td>20</td>
</tr>
<tr>
<td>TdR</td>
<td>83</td>
<td>46</td>
<td>90</td>
<td>50</td>
<td>49</td>
</tr>
<tr>
<td>UdR</td>
<td>83</td>
<td>61</td>
<td>81</td>
<td>38</td>
<td>27</td>
</tr>
<tr>
<td>CdR</td>
<td>72</td>
<td>56</td>
<td>51</td>
<td>16</td>
<td>26</td>
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</table>

Unknown

<table>
<thead>
<tr>
<th></th>
<th>-BAP</th>
<th>+BAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>front</td>
<td>74</td>
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<td>unstable</td>
<td>0</td>
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<tr>
<td>75</td>
<td>58</td>
<td>78</td>
</tr>
<tr>
<td>27</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> A sample of $^{32}$P-labelled nucleotide was treated with BAP. The efficiency of BAP cleavage was monitored by measuring the formation of $^{32}\text{PO}_4$. The nucleoside was identified by measuring Rf values in 5 different solvents and comparing them to those determined for authentic reference standards. Solvent A, dH$_2$O; solvent B, (NH$_4$)$_2$SO$_4$:Na acetate (1M):isopropanol, 80/12/2 v/v; solvent C, t-butanol:MEK:HCl: H$_2$O, 40/30/10/20 v/v; solvent D, n-butanol:H$_2$O, 86/14 v/v; solvent E, n-butanol:H$_2$O:NH$_4$OH, 86/9/5 v/v.
A sample of $^{32}$P-labelled novel nucleotide was subjected to treatment with Bacterial alkaline phosphatase (BAP). The sample was split into two parts. One part was incubated in 50 mM Tris-HCl pH 8.4, 15 mM MgCl$_2$ at room temperature for one hour. The remaining portion of the sample was dissolved in the same buffer and supplemented with BAP (0.03 units). Digestion was carried out for one hour at room temperature. The products were resolved by 1D-TLC on PEI-cellulose sheets, A; or by 2D-TLC on cellulose sheets, B.
that its base was 5(hydroxymethyl-O-pyrophosphoryl) uracil (Figure 55). This structure was consistent with all the facts:

1) the novel nucleotide is a pyrimidine monophosphate nucleotide derived from S1 and snake venom phosphodiesterase digestion of am 37 DNA;
2) acid hydrolysis of the novel nucleotide generates hmUra;
3) acid treatment of the novel nucleotide liberates hmdUMP and PO\$_4^-$ in a ratio of one to two;
4) BAP treatment of the novel nucleotide generates free hmdUra and PO\$_4^-$.

The structure proposed predicted a molecule with a relative net negative charge of five. This prediction was confirmed. A large batch of crude $^{32}$P-labelled am 37 DNA was prepared and digested to mononucleotides. The sample was loaded on a DEAE-Sephadex-urea column along with dTDP and dTTP as net negative charge markers. The column was washed with starting buffer and then eluted with a linear gradient of NaCl (Figure 56). The radioactive mononucleotides eluted as a large, slightly asymmetrical peak. The mononucleotide peak contained two O.D.\textsuperscript{267} components; however, the second smaller peak is likely protein present in, or added to, the samples during digestion. The other O.D.\textsuperscript{267} peaks mark the elution points of the charge marking standards, dTDP (-3) and dTTP (-4). The second, smaller radioactive peak marks the elution position of the novel nucleotide. Assuming a linear relationship between peak elution position and relative net negative charge, it was determined that the novel nucleotide had a net negative charge of five. This experiment corroborated the prediction made from the previous experiments. The novel nucleotide was assigned the designation of hmPPdUMP (base-hmPPUra).
FIGURE 55.—Proposed structure of the novel pyrimidine base.

\[
\begin{align*}
\text{NH} & - \text{CH}_2 - \text{O-PO}_3^- - \text{O-PO}_3^- \\
\end{align*}
\]

5-(hydroxymethyl-o-pyrophosphoryl) uracil
FIGURE 56.--DEAE-sephadex-urea column chromatography of a nucleotide mixture from am 37 DNA.

A sample of $^{32}$P-labelled am 37 DNA was prepared from infected cells of strain 29. The DNA was digested to mononucleotides which were chromatographed on DEAE-Sephadex A25 (1 x 40 cm) column with unlabelled 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.
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 about 100 times greater for monobenzyl phosphate than for simple aliphatic phosphates (Kumamoto and Westheimer, 1955). Furthermore, allylpyrophosphates are quite unstable below pH 5, cleaving into allyl alcohols and inorganic pyrophosphate (Goodman and Popjak, 1960).

The acid treatments used will selectively hydrolyze other phosphate esters under certain conditions. Heating ATP at 98° in 1 N HCl for 7 minutes removes γ and β phosphate residues. Heating ppGpp at 98° for 7 minutes hydrolyzes all pyrophosphate bonds. Short exposure of ppGpp to 1 N HCl at 37° for 30 minutes converts it to ppGp (Sy and Lippman, 1973).

Amber 37 in vitro DNA modification

The presence of hmPPUra in am 37 DNA suggested that it was an activated precursor of putThy. Cell-free extracts of strain 29 cultures infected with wild-type ϕW-14 or am 37 were prepared. 32P-labelled am 37 DNA was used as a substrate in an in vitro experiment to test for a precursor and product relationship between hmPPUra and putThy.

Cell-free extracts of strain 29 cells infected with wild-type ϕW-14 were capable of releasing 32PO4 from am 37 DNA in the presence of putrescine. Cell-free extracts of am 37-infected strain 29 cells were inactive in this assay (Figure 57).

DNA samples incubated with ϕW-14 wild-type extracts were purified, digested to mononucleotides and separated by two-dimensional
A sample of $^{32}\text{P}$-labelled 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 (○).
thin-layer chromatography on cellulose. Nucleotides were detected by autoradiography. All nucleotides were cut out and counted. The results were corrected for the loss of \(^{32}\text{P}\)O\(_4\) during in vitro modification and for other non specific losses during purification, digestion and chromatography. The radioactivity in each nucleotide was plotted as a percentage of the total radioactivity (Figure 58). As modification proceeded, the level of hmPPdUMP in the DNA declined to about 50 percent of the preincubation level. hmdUMP did not accumulate, therefore, hmPPdUMP was not being converted to hmdUMP by the loss to two phosphates. The uniform level of hmdUMP in the treated DNA also suggested that this nucleotide did not arise from the acid-catalyzed destruction of hmPPdUMP during SI digestion of the DNA samples. DNA samples with 50 percent less hmPPdUMP would yield 50 percent less hmdUMP if the SI digestion conditions were responsible for the formation of hmdUMP. The decline in hmPPdUMP levels in am 37 DNA was accompanied by an increase in putdTMP levels. PutdTMP increased from an original 0.3 percent of the label to almost 6.0 percent of the label. The decline in the amount of hmPPdUMP and the increase in the amount of putdTMP was consistent with the loss of two phosphates or one pyrophosphate from hmPPdUMP during putdTMP formation.

The amount of label detected in dTMP in am 37 DNA did not change. Extracts of \(\phi W-14\) w\(^-\) infected strain 29 cells were not capable of converting hmdUMP or hmPPdUMP to dTMP under the incubation conditions employed.

Attempts to create a substrate for the hmdUMP phosphorylating enzyme by removing the phosphates from am 37 DNA with BAP were not
FIGURE 58:—Conversion of hmPPUra in am 37 DNA into putThy.

Procedure essentially as described in the legend to Figure 57. The DNA in samples was digested to mononucleotides which were separated by thin-layer chromatography. The nucleotides were detected by autoradiography, excised from the sheets and their radioactivity determined. dTMP (X); hmdUMP (Δ); putdTMP (●); hmPPdUMP (O).
successful. Native and denatured Øe DNA were tested as substrates in conjunction with $\gamma^{32}P$ ATP and ØW-14 cell-free extracts. No activity was observed.

The formation of putThy from hmPPUra may involve the displacement of a pyrophosphoryl group by an incoming amino nitrogen with the formation of a carbon-nitrogen bond. This type of reaction is found in the formation of phosphoribosylanthranilate from anthranilate and phosphoribosylpyrophosphate, in the formation of dihydroopterate from hydroxymethylpteridine pyrophosphate and p-aminobenzoate and in the formation of thiamine from a C5-pyrophosphoryl-hydroxymethyl pyrimidine base and thiazole (Walsh, 1979).

hmUra will alkylate poorly nucleophilic aromatic amines in aqueous alkaline medium (Santi, 1967). The hydroxymethyl group is susceptible to nucleophilic attack. Nucleophilic displacement could be enhanced by the presence of a pyrophosphoryl leaving group. Some other esters of hmUra are also sensitive to nucleophilic displacement (Santi, 1971). Such reactions proceed by an initial attack at the 6 position of the pyrimidine ring (Pogolotti and Santi, 1977). Conversion of putThy could conceivably occur by direct displacement at the 5-methylene group or via an initial nucleophilic attack of the enzyme or putrescine at the 6 position. Mechanisms requiring the solubulization of the 6-hydrogen are excluded since [6-$^{3}H$]-uracil labelled putThy and Thy to the same extent as [2-$^{14}C$]-uracil (Table 8).

Trimethoprim did not inhibit ØW-14 DNA synthesis or modification. Therefore THFA is not likely involved in the formation of Thy at the polynucleotide level. This does not rule out the possibility that Thy is derived from hmPPUra.
There are several immediate problems which should be pursued. In vitro formation of putdTMP from hmPPdUMP was only 50 percent effective. Reaction conditions should be optimized and the nature of the am 37 DNA substrate should be investigated. The reaction conditions and necessary substrates for hmPPdUMP biosynthesis should also be investigated. The requirements for dTMP synthesis are also unknown. The search for conditionally lethal DNA modification mutants should continue.
FIGURE 59.—Summary of the pathways of \( \Phi W-14 \) DNA precursor synthesis and modification.

This figure summarizes our knowledge of \( \Phi W-14 \) DNA metabolism. \( \Phi W-14 \) infection of a cell results in the inhibition of host DNA synthesis and the synthesis of products necessary for phage DNA synthesis. The synthesis of dTMP is inhibited and dTTP is destroyed by a phage coded dTTPase. Enzymes required for the synthesis of hmdUMP are made. \( \Phi W-14 \) DNA is synthesized with four nucleoside triphosphates, dGTP, dATP, dCTP and hmdUTP. hmdUMP is modified postreplicationally to dTMP and putdTMP. hmPPdUMP is an intermediate in the biosynthetic pathway leading to putdTMP. Nothing is known about biosynthetic pathway leading from hmdUMP to dTMP. Host pathways (----); \( \Phi W-14 \) pathways (-----).
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