

DNA SYNTHESIS AND MODIFICATION
IN ØW-14-INFECTED PSEUDOMONAS ACIDOVORANS

by

KIRK LEE MALTMAN

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Department of Microbiology

The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

Date 13/10/81

ABSTRACT

Experiments with ϕ W-14-infected, thymidine-requiring mutants of *P. acidovorans* strain 29 demonstrated that deoxyuridine but not thymidine was a precursor of thymine in ϕ W-14 DNA. Deoxyuridine was also a precursor of the α -putrescinylothymine found in ϕ W-14 DNA. The biosynthesis of α -putrescinylothymine and thymine was mediated by enzyme activities appearing after infection. ϕ 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 α -putrescinylothymine-containing nucleotides (Neuhard et al., 1980). The absence of α -putrescinylothymine and thymine nucleotides in ϕ W-14-infected cell nucleotide pools suggested that these nucleotides might be synthesized from hydroxymethyluracil at the polynucleotide level. Degradative analysis of nascent ϕ W-14 DNA demonstrated the presence of hydroxymethyluracil. Enzymatic degradation of pulse-labelled, nascent ϕ 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 ϕ W-14 DNA. This DNA contained unusual heavy density components.

ϕ W-14 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 ØW-14 DNA containing lower-than-normal levels of α -putrescinylothymine and increased levels of thymine. Am 37 accumulated intermediates in α -putrescinylothymine 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 ³²P₄ 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 -putrescinylothymine by in vitro modification of am 37 DNA with ØW-14 wild-type infected P. acidovorans cell-free extracts. In vitro modification confirmed that α -putrescinylothymine was formed at the polynucleotide level. ØW-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 ØW-14 wild-type DNA.

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INTRODUCTION

ØW-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 (T_m) (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-amino-butylaminomethyl)uracil, also called α -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 ØW-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 ØW-14 DNA has a very high melting temperature ($T_m = 99.3^\circ$ in 1xSSC) (Table 1). The low buoyant density (ρ) of ØW-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

Phage Host	DNA molecular weight ($\times 10^{-6}$)	Moles % G+C phage	Host	Base change
T4 <u>Escherichia coli</u>	110	34	50	5-hmCyt for Cyt
ϕ e <u>Bacillus subtilis</u>	100	39	43	5-hmUra for Thy
PBS2 <u>B. subtilis</u>	150	28	43	Ura for Thy
χ P12 <u>Xanthomonas oryzae</u>	30	67	64	5-mCyt for Cyt
S-2L <u>Synechococcus elongatus</u>	28	69	70	2-nAde for Ade
SP15 <u>B. subtilis</u>	250	42	43	5-dhpUra for Thy
SP10 <u>B. subtilis</u>	59 ^f	43	43	α -gluThy for Thy
ϕ W-14 <u>Pseudomonas acidovorans</u>	92	51 ^e	67	α -putThy for Thy

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 $1,710 \text{ g ml}^{-1}$ 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).

SOURCE: R. A. J. Warren, 1980, Modified bases in bacteriophage DNAs, Ann. Rev. Microbiol. 34, pp. 137-56.

TABLE 1.--Continued

Extent of change ^a (%)	T _m ^b		Buoyant density ^c	
	observed	expected ^d	observed	expected ^d
100	84	84	1.700	1.694
100	77.5	85.3	1.742	1.703
100	76.5	81.5	1.722	1.690
100	101.5	95.4	1.710	1.726
100	101.9	98.3	1.731	1.728
41	61.7	86.2	1.761	1.702
15-20	81.5	86.9	1.723	1.703
50	99.3	90.3	1.666	1.716

complex ($\rho = 1.666 \text{ gcc}^{-1}$). The low buoyant density of the ϕ 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 ϕ W-14 DNA. The labelling of this methylene function with $[2\text{-}^{14}\text{C}]$ -serine or $[2,3\text{-}^3\text{H}]$ -serine is consistent with the transfer of a one carbon fragment from $\text{N}_5\text{-N}_{10}$ 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 ϕ 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.

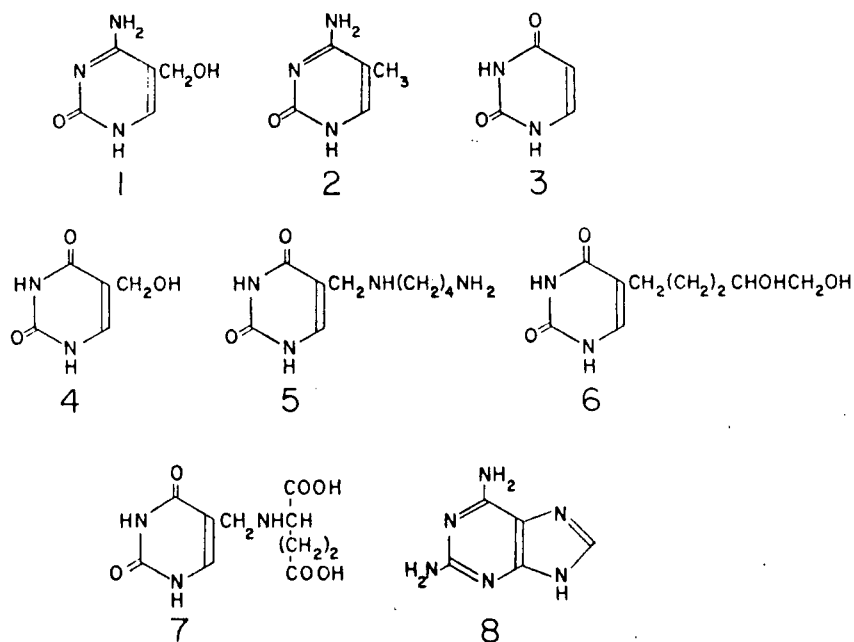


Figure 1 Structures of modified bases in phage DNAs: 1. 5-hydroxymethylcytosine (hmCyt); 2. 5-methylcytosine (mCyt); 3. uracil (Ura); 4. 5-hydroxymethyluracil (hmUra); 5. α-putrescinyllthymine (putThy); 6. 5-dihydroxypentyluracil (dhpUra); 7. α-glutamylthymine (gluThy); 8. 2-aminoadenine (nAde).

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-replicative 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 N_5 - N_{10} -methylene-tetrahydrofolate-mediated reaction catalyzed by the T4 enzyme deoxycytidylate hydroxymethylase. The reaction is analogous to the N_5 - 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 hydroxymethylase,

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. [5-³H]-uracil enters nucleotide pools and some is converted to [5-³H]-dCMP or [5-³H]-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 ³H₂O. Radioactivity in ³H₂O 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: α -glucosyl transferase and β -glucosyl transferase. T2 and T6 have only α -glucosyl transferases. In T4 70 percent of the glucosylated hmCyt molecules are α -glucosylated, while 30 percent of the linkages are β -glucosylated (Lehman and Pratt, 1960). Uridine diphosphoglucose (UDPG), which is synthesized by host enzymes, transfers glucose to hmCyt in T4 and to 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 hmCyt residues diglucosylated while only 5 percent of T2 hmCyt is diglucosylated. T4 α -glucosyl transferase mutants produce DNA with all the hydroxymethylcytosine residues β -glucosylated. T4 β -glucosyl transferase mutants produce DNA with all the hmCyt α -glucosylated. Twenty-five percent of T2 and T6 hmCyt is not glucosylated 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 hmCyt-containing DNA (Fleischman and Richardson, 1971). In T4-infected 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 α -glucosyl transferase show some sequence specificity. T2 DNA glucosylated in vivo cannot serve as a substrate for in vitro T2-mediated glucosylation, but will allow glucosylation by T6 α -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 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 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 ϕ e, 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. ϕ e-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). ϕ e 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 ϕ e DNA comes from the induction of a phage-specific dUMP hydroxymethylase enzyme (Price et al., 1972). All the hmUra in the ϕ e DNA is synthesized from de novo nucleotide precursors. ϕ e inhibits host DNA synthesis but the host DNA is not degraded to mononucleotides (Roscoe, 1969). ϕ e-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 ϕ e-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 hmdUTP 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. ϕ e DNA is susceptible to several restriction endonucleases (Berkner and Folk, 1979). The hydroxymethyluracil groups in ϕ e DNA are not modified by the addition of sugars or any other moieties (Allegría and Kahan, 1968).

The hmUra in SP01 or ϕ e DNA is not required for replication or transcription. SP01 induces a phage-specific DNA polymerase. This enzyme will use either hmdUTP or dTTP as a substrate in vivo or in vitro (Yehle and Ganesan, 1973). ϕ e DNA requires host DNA polymerase III

since a ts DNA pol III B. subtilis- ϕ e infected cell shuts off ϕ 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 ϕ e template (Lavi et al., 1974). This cannot be an absolute requirement for hmdUra in the ϕ e DNA since ϕ e DNA containing thymine replicates normally.

DNA replication in B. subtilis is inhibited by the antifolate drugs trimethoprim and aminopterin. DNA replication in ϕ 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 dTMP from dUMP and N₅-N₁₀-methylene-tetrahydrofolic acid, a C-1 transfer is catalyzed by thymidylate synthase. This transfer results in the oxidation of THFA to DHFA. Normally, THFA is regenerated from DHFA and NADPH by the action of the enzyme dihydrofolate reductase (Mahler and Cordes, 1971). When dihydrofolate reductase is inhibited, the stoichiometric requirement for THFA in thymidylate biosynthesis eventually depletes the cellular supply of THFA. 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 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 THFA. Cells infected by bacteriophage ϕ e synthesize hmdUMP instead of dTMP. hmdUMP biosynthesis

requires the cofactor N₅-N₁₀ 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 T_m (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 $[2-^{14}\text{C}]\text{-FudR}$ 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 g cc^{-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° , higher than expected. The buoyant density of the DNA is 1.710 g cc^{-1} , lower than expected. 5-methylcytosine completely replaces cytosine in the DNA of the phage. 5-methylcytosine is synthesized from dCMP by a $\text{N}_5\text{-N}_{10}$ -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-dihydroxypentyluracil (dhpUra) replaces thymine in SP 15 DNA. Both DNA types are similar to ϕ W-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 synthesis 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 inhibition of DNA synthesis results in abnormally high levels of Thy in DNA. However, inhibition of DNA synthesis with aminopterin, followed by reversal 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 precursor (Walker and Mandel, 1978a; Walker and Mandel, 1978b; Walker, Ph.D. Thesis, 1977).

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 ($\rho = 1.723$). 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 hmdUTP from hmdUDP (Witmer and Dosmar, 1978). SP 10-infected cells contain hmdUTP but not dUTP. hmdUTP 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 hydroxymethyldeoxyuridyate. 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 cassebica 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 T_m and ρ values for E. cassebica DNA. This misleading result suggested that E. cassebica DNA contained only the four normal DNA bases and demonstrated the wisdom of coupling CsCl gradient and T_m 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. CsSO_4 -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 ϕ W-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 $1 \times P_i$ was used for experimental purposes requiring defined medium. TCS $1/5 \times P_i$ is the same as TCS $1 \times P_i$ except that it has one-fifth the amount of orthophosphate. It was used in $^{32}PO_4$ -labelling experiments. TCS (g/l) is tris(hydroxymethyl)aminomethane (Tris) 12.1; KCl, 5.0; Na_2SO_4 , 0.0227; $FeCl_3 \cdot 6H_2O$, 0.0008; $CaCl_2$, 0.017; KH_2PO_4 , 0.0174. Medium is adjusted to pH 7.0.

TABLE 2.--Bacterial strains used in this study.

Bacterium	Strain	Source
<u>Pseudomonas</u> <u>acidovorans</u>	29 prototrophic	R.Y. Stanier
	3L derived from strain 29; requires 250 μg TdR ml^{-1} for growth.	R.A. Kelln
	3L/FU ^R 2 derived from 3L; resistant to 5-FU; requires 250 μg TdR ml^{-1} for growth.	This study
	sup 1 derived from strain 29	R.A.J. Warren
	sup 2 derived from strain 29	R.A.J. Warren
	sup 3 derived from strain 29	R.A.J. Warren
	sup 4 derived from strain 29	R.A.J. Warren
	sup 5 derived from strain 29	R.A.J. Warren
	JE 1 prototrophic; soil isolate.	J. Ethier
	JE 1 sup 1 derived from JE 1	P. Miller
	JE 1 sup 2 derived from JE 1	P. Miller
	JE 1 sup 3 derived from JE 1	P. Miller

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-l 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 Na₃ 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 ØW-14 w⁺ was prepared from stocks derived from Kropinski and Warren's (1970) original isolate. Working stocks were always prepared from the original lysates.

ϕ W-14 ts mutants were isolated as described in Results section; am mutants were isolated by P. Miller.

Preparation of high-titre phage lysates

ϕ W-14 w^+ - 200 ml of P. acidovorans were grown to a cell density of 3.0×10^8 cells per ml. ϕ W-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 ($\sim 5 \mu\text{g ml}^{-1}$) was added and the lysate was gently agitated for 60 minutes. The lysate was digested with $10 \mu\text{g ml}^{-1}$ 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 CHCl_3 .

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 $2-3 \times 10^8$

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 ØW-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

ØW-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 $3 \times 10^8 \text{ ml}^{-1}$. 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 $\mu\text{g ml}^{-1}$ thymidine.

Testing for antibiotic sensitivity

Antibiotics were added at the appropriate concentration to exponentially growing cultures of P. acidovorans. Growth was monitored with the Klëtt.

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

ØW-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 ØW-14 ts mutants

Mutagenized ØW-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. 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 ØW-14 am mutants

ØW-14 am mutants were isolated from mutagenized stocks of ØW-14 by P. Miller. P. acidovorans strain 29 (sup 2) was the permissive host and P. acidovorans strains 29 or 3L were the non permissive hosts.

Complementation of ts ØW-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 ØW-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. 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 µCi 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°. 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°. 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 $\mu\text{g ml}^{-1}$ of heat-treated pancreatic RNase was added. Digestion with RNase was carried out for 1 hour at 37°. 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° 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 μ l of 0.2 N HCl and 25 μ l 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 3×10^8 ml⁻¹ and infected at a multiplicity of infection (moi) of 10. At 35 to 40 minutes after infection, the cells were labelled with $^{32}\text{PO}_4$, [6-³H]-uracil or [5-³H]-uracil for 10 seconds. Cells for $^{32}\text{PO}_4$ pulse-labelling were grown in TCS 1/5 x P_i 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 $1 \times P_i$ 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 $5,000 \text{ rpm}$ 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

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

$^{32}\text{PO}_4$ was added one to two cell doublings prior to infection in order to allow equilibrium labelling of the nucleotide pools. At a density of $3 \times 10^8 \text{ cells ml}^{-1}$ the culture was infected at a multiplicity of infection of 10. Five ml samples were taken at the indicated times and passed through 0.45μ Millipore HA membranes. The filters were washed with 2.5 ml each of ice-cold TCS $1 \times P_i$ 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 $8,000 \text{ rpm}$ 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 dH_2O . 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 dH_2O 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 ^{14}C 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

[6-³H]-uracil - Cultures contained 1.0 $\mu\text{Ci ml}^{-1}$ of label and 10 $\mu\text{g ml}^{-1}$ of cold uracil. 0.1 ml samples were taken directly into 1.0 ml of 0.3 NaOH and incubated overnight at 37°. In the morning 1.0 ml of 0.3 N HCl and 0.25 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 μ 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°, overlaid with Toluene-based scintillant and counted.

[³H]-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 ml samples were taken into 1.0 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°. In the

morning, TCA was added to a final concentration of 5 percent. The samples were collected, washed, dried and counted as described above.

[methyl-³H]-TdR - Cultures contained 10 μ Ci [³H]-TdR per ml of culture and 250 μ 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 [5-³H]-uracil

[5-³H]-uracil was added to the cultures containing 10 μ 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 samples 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 Scintiverse.

Enzymatic digestion of DNA

The concentration of the DNA sample was determined spectrophotometrically and the required amount of DNA was precipitated with two volumes of 95 percent ethanol, washed several times with 70 percent ethanol, several times with 95 percent ethanol, then with ether and air-dried. The sample was redissolved in the minimum volume of sterile distilled, deionized 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, ZnSO₄ was added to give a final concentration of 0.1 mM and 10 units of S₁ nuclease were added per μ 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 NH₄CO₃, pH 8.4, and MgCl₂ 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}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 $(\text{NH}_4)_2\text{SO}_4$:1 M Na acetate:isopropanol
(80:12:2 v/v).

Solvent B - t-butanol:2-butanone:conc. HCl:H₂O (40:30:10:20 v/v).

Solvent C - n-butanol:H₂O (86:14 v/v).

Solvent D - n-butanol:H₂O:conc. NH₄OH (86:9:5 v/v).

Solvent E - isobutyric acid:H₂O:conc. NH₄OH (66:20:1 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 S_1 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 $75 \mu\text{g ml}^{-1}$. 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 S_1 -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 3×10^8 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 ($3,000 \text{ g} \times 5$ 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 5×10^9 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 ($400 \text{ } \mu\text{g ml}^{-1}$) 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 $100 \text{ } \mu\text{g ml}^{-1}$ of unlabelled $\phi\text{W-14}$ 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

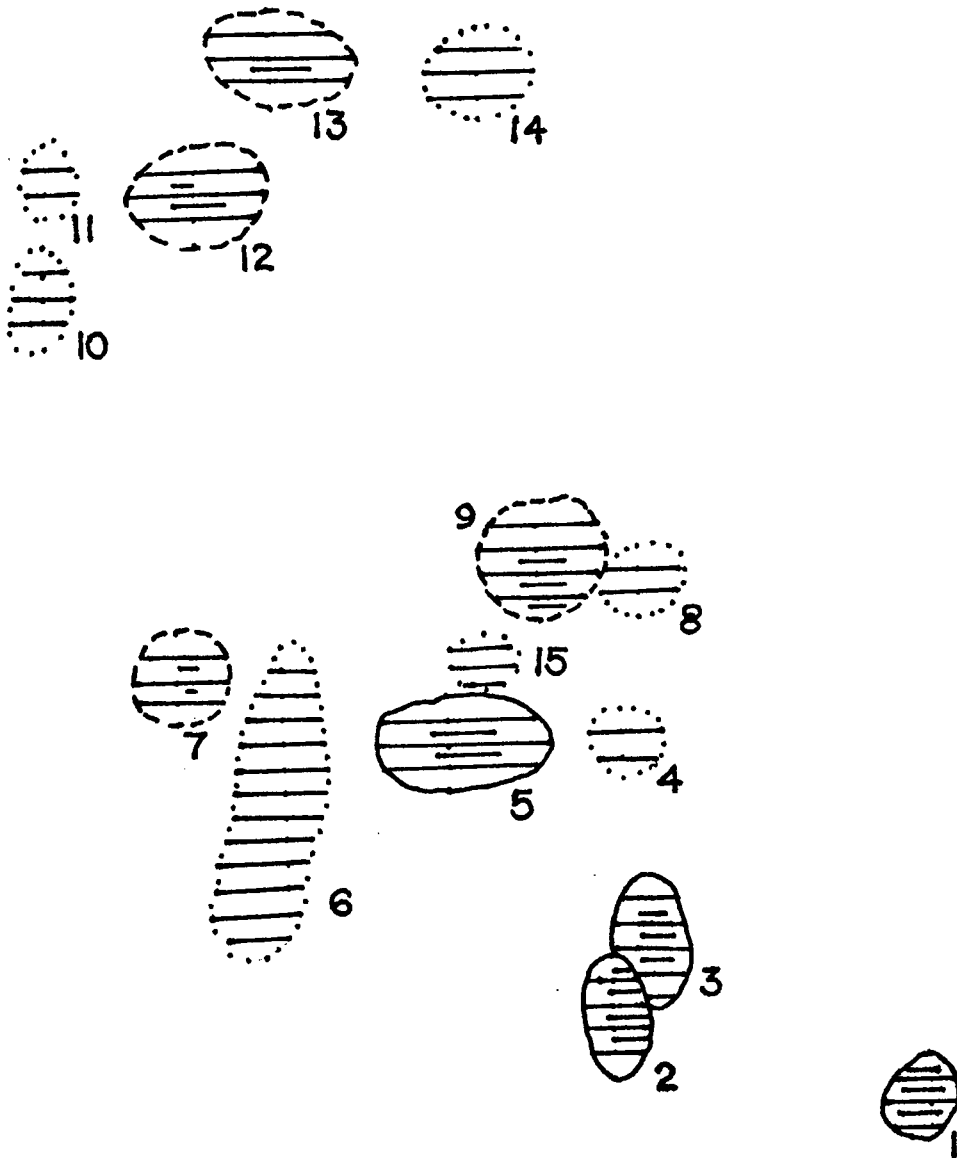
Nucleotide pools

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

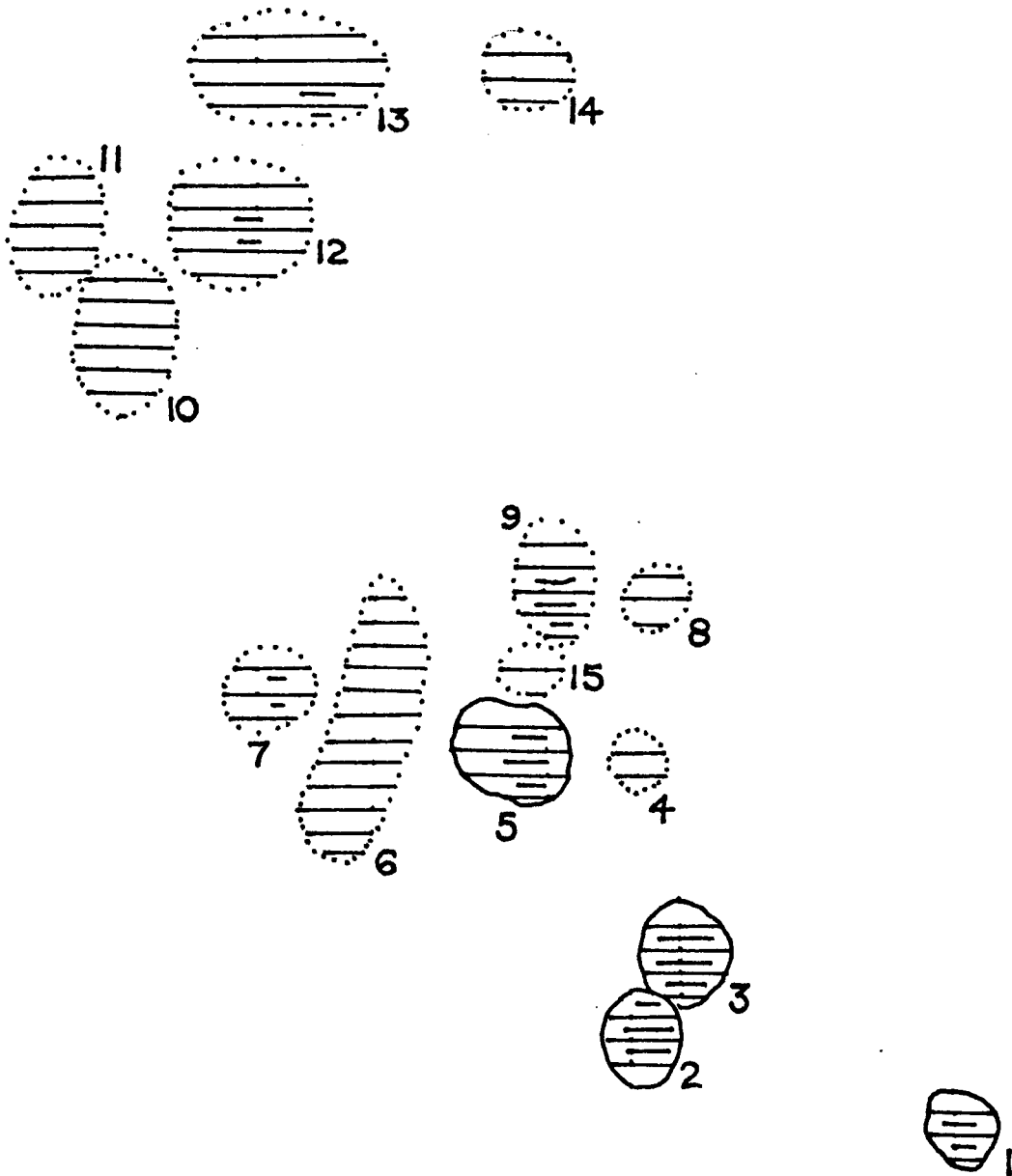
[2- ^{14}C]-uracil labelling of nucleotide pools in infected and uninfected P. acidovorans strain 29 was also undertaken. All uracil-labelled compounds separable using the total pool separation protocol appeared in both infected and uninfected cell extracts (Figure 2). Micromole quantities of labelled nucleotides could have been detected and resolved. Thy, hmUra 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 ØW-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 [2-¹⁴C]-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.



A



B

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 $^{32}\text{PO}_4$ 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 ØW-14-infected
P. acidovorans strain 29.

Nucleotide	Time after infection					
	0 min	10 min	20 min	30 min	40 min	50 min
dGTP	1318	601	366	274	266	218
dATP	429	338	362	-	169	271
dCTP	5555	1281	1376	1961	1225	1181
dUTP/dTTP/hmdUTP	1704	750	414	421	408	236

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 ØW-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 ØW-14 DNA.

Deoxyuridine but not deoxythymidine
was a ØW-14 DNA precursor

Before radioactive deoxyuridine could be used to label ØW-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]-dUdR and [2-¹⁴C]-dUdR 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 ØW-14 infection. The isolates were tested for the ability to incorporate [6-³H]-uracil (Figure 3). Of the five clones, only one, 3L/FU^R₂, was found which did not incorporate [³H]-uracil into TCA insoluble counts in uninfected or infected cells (Figure 3). 3L/FU^R₂ was tested for the ability to incorporate [6-³H]-dUdR into uninfected ØW-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 ØW-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 ØW-14 DNA grown in 3L/FU^R₂ 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 ØW-14 infected 3L/FU^R₂ 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^R2.

At 0 minutes [6-³H]-uracil (1.0 $\mu\text{Ci ml}^{-1}$, 10 $\mu\text{g ml}^{-1}$) was added to cultures of 3L,O; 3L/FU^R2,●; and $\phi\text{W-14}$ infected 3L/FU^R2,▲. Incorporation of the label into TCA precipitable material was followed.

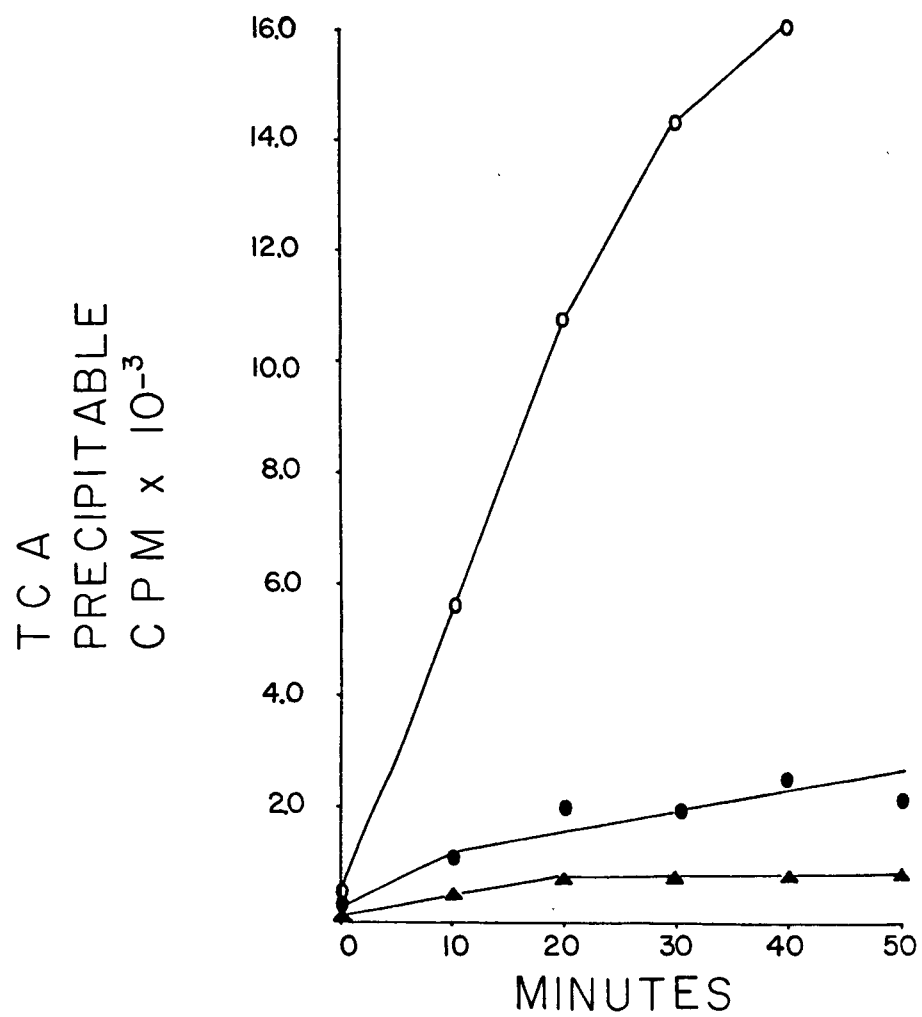


FIGURE 4.--The incorporation of deoxyuridine in ϕ W-14 infected 3L/FU^R2.

A growing culture of 3L/FU^R2 was split into two halves. One half was infected with ϕ W-14. [6-³H]-UdR (4.0 μ Ci ml⁻¹, without carrier) was added at 10 minutes after infection. The incorporation of radioactivity into alkali resistant, TCA precipitable material was followed. ϕ W-14 infected 3L/FU^R2, ●; 3L/FU^R2, ○. The arrow indicates the beginning of cell lysis in the infected culture.

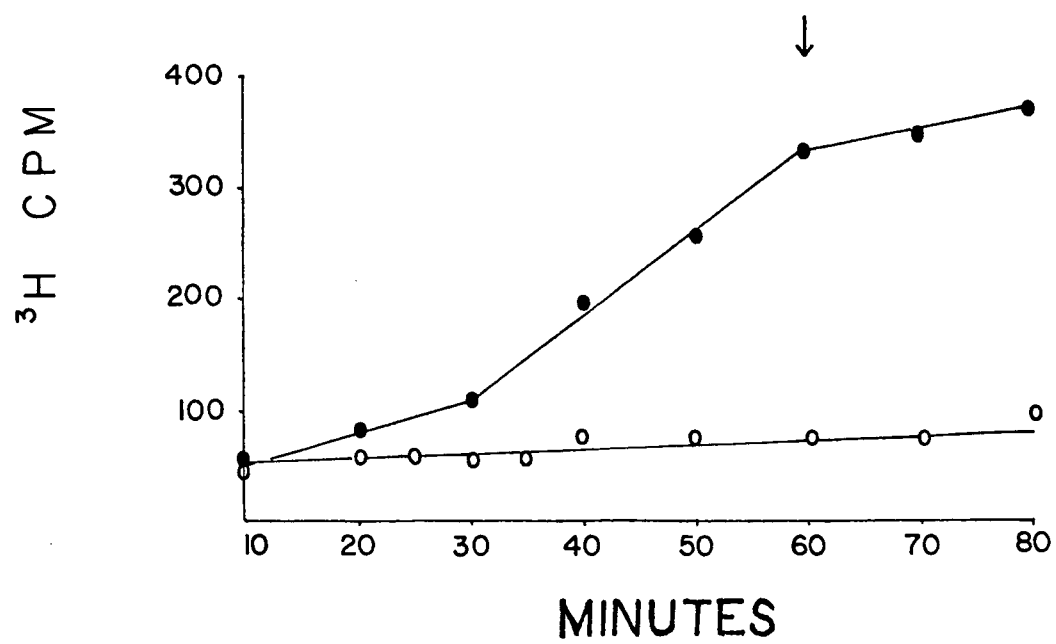


TABLE 4.--Deoxyuridine labelling of bases in ØW-14 infected 3L/FU^R₂.

Base	[³ H]-orn	[6- ³ H]-Ura
putThy	1593 (0.95) ^a	1853 (0.48)
Cyt	37 (0.02)	88 (0.02)
Thy	55 (0.03)	1903 (0.50)

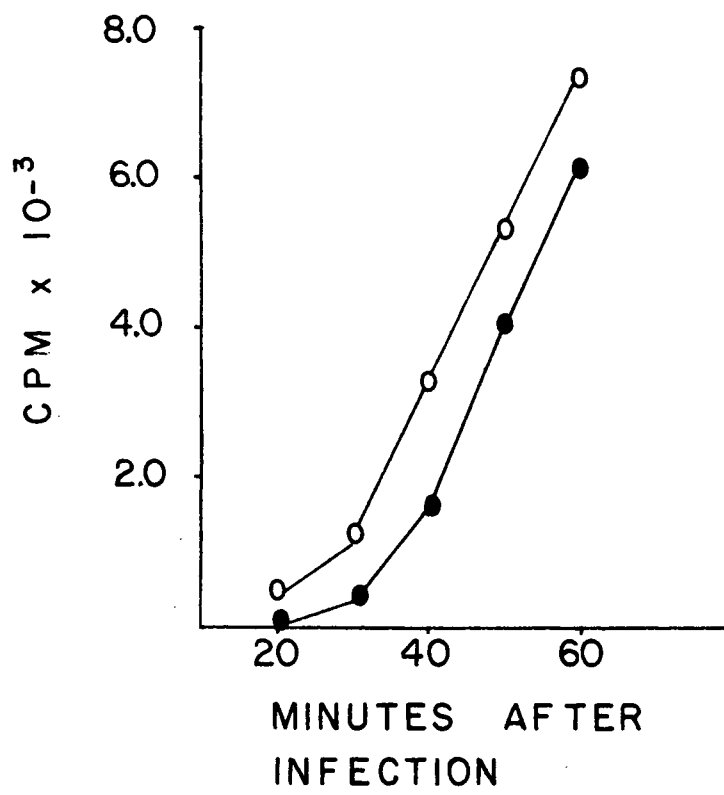
ØW-14 infected cultures of 3L/FU^R₂ were labelled with [³H]-orn (0.1 μ Ci μ g⁻¹) or [6-³H]-dUdR (4.0 μ Ci ml⁻¹). The cultures were allowed to lyse and the DNA was purified from the phage particles. The DNA was acid-hydrolyzed and the bases were separated by one dimensional thin-layer chromatography in 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 ØW-14 DNA.

3L/FU^R₂ was infected with ØW-14 and labelled with [6-³H]-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. ³H-label in Thy, O ; ³H-label in putThy, ●.



Kelln had reported that [methyl-³H]-TdR would label ØW-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 [³H]-TdR is not degraded and reincorporated into ØW-14 DNA (Maltman et al., 1980).

Although the data strongly suggested that ØW-14-infected cells could not incorporate deoxythymidine, it was possible that this was due to an overwhelming preference for endogenously supplied deoxythymidine nucleotides. ØW-14-infected strain 3L produced viable phage bursts after exogenous thymidine was removed by centrifugation and washing (Figure 7). ØW-14-infected 3L was spun down, washed and resuspended in conditioned TCS medium without cold thymidine but containing 25 µCi ml⁻¹ [methyl-³H]-TdR. The infected cells were allowed to lyse and DNA was extracted from the purified phage. A small amount of tritium-labelled ØW-14 DNA was obtained which banded with reference ØW-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 ØW-14 DNA, failed to label the thymine bases of ØW-14 DNA.

FIGURE 6.--Thymidine incorporation in strain 3L and in ØW-14-infected strain 3L.

[Methyl-³H]-thymidine ($0.04 \mu\text{Ci } \mu\text{g}^{-1}$, $250 \mu\text{g ml}^{-1}$) was added to a culture of ØW-14 infected 3L, ●; and to an uninfected culture of 3L, ○. The incorporation of radioactivity into TCA precipitable radioactivity was determined.

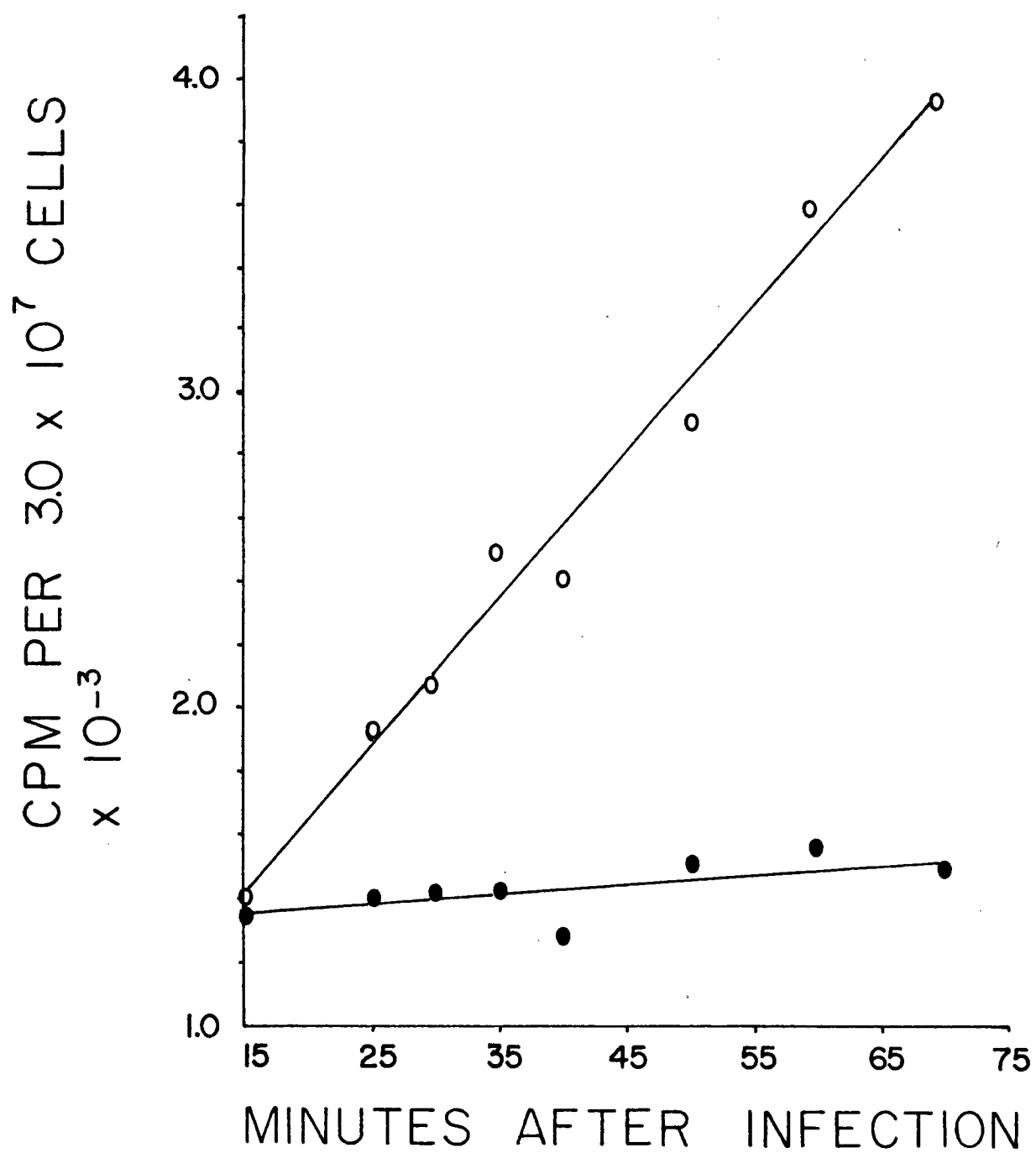


FIGURE 7.--Thymidine was not required for plaque production in ØW-14 infected strain 3L.

At 10 minutes after infection of strain 3L with ØW-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.

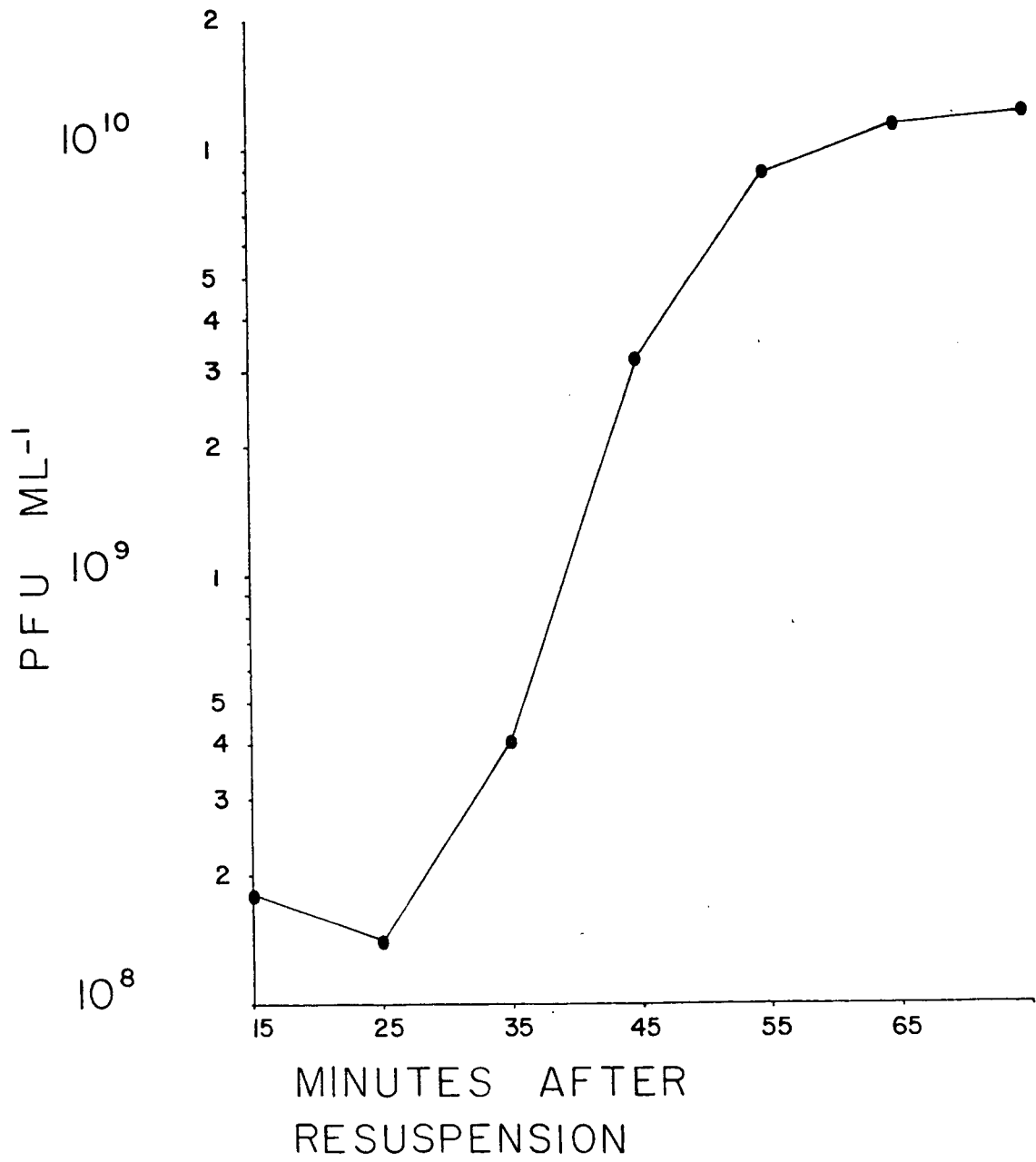
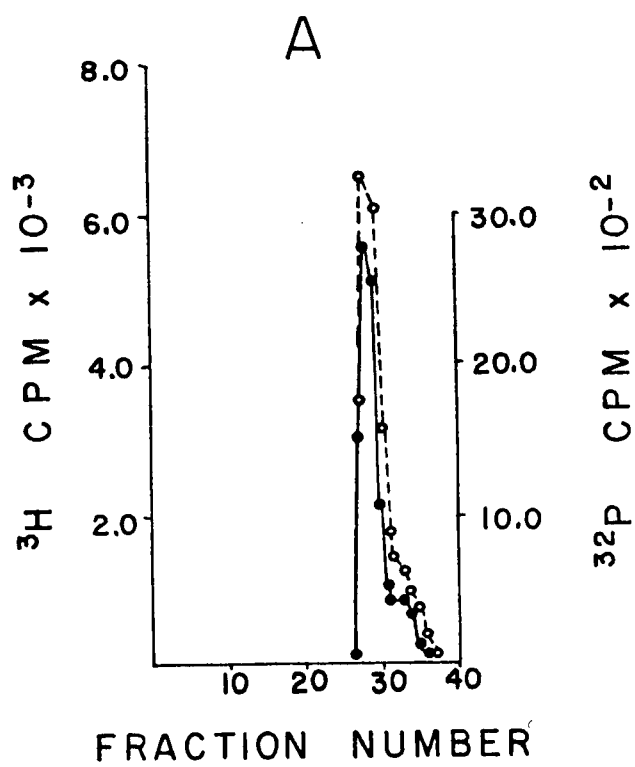


FIGURE 8.--Nonspecific labelling of ϕ W-14 DNA by high specific activity thymidine.

P. acidovorans 3L was infected with ϕ W-14 and at 10 minutes after infection the cells were spun down and resuspended in conditioned medium containing $25.0 \mu\text{Ci ml}^{-1}$ 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; ^{32}P -labelled ϕ W-14 reference DNA, ● .



Attempts were also made to density label ØW-14 DNA with the thymidine analogue, BUdR. 3L grown in TdR was spun down and resuspended in $500 \mu\text{g ml}^{-1}$ of BUdR. The ØW-14 DNA, also labelled with [^3H]-ornithine, was extracted from phage and analyzed on neutral CsCl density gradients (Figure 9). Reference DNA and ØW-14 DNA from phage grown in the presence of BUdR banded at the same density, showing that BUdR was not incorporated into DNA in ØW-14-infected cells (Figure 9).

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

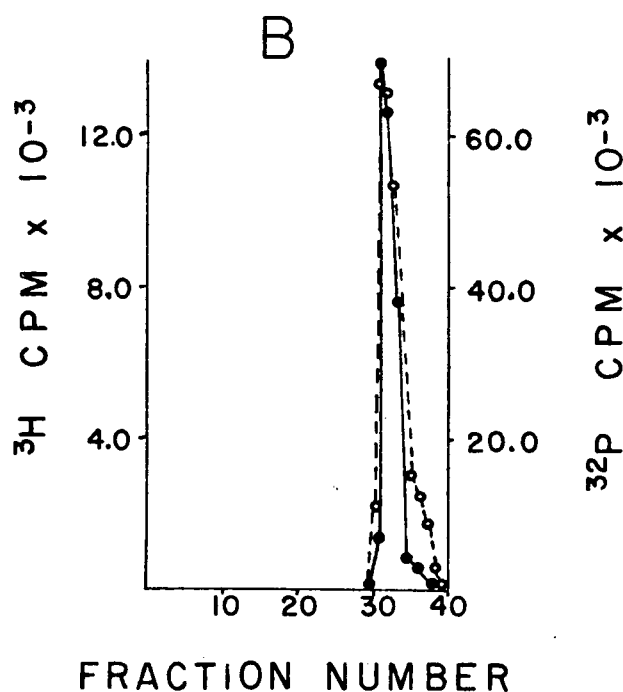
Endogenous thymidine utilization in ØW-14-infected P. acidovorans 3L

Exogenously supplied TdR was not a precursor of thymine in ØW-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 P. acidovorans strain 29 at concentrations greater than $250 \mu\text{g ml}^{-1}$ (Kelln and Warren, 1973). 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 $250 \mu\text{g ml}^{-1}$ 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. $500 \mu\text{g ml}^{-1}$ FUdR did not prevent

FIGURE 9.--Buoyant density of ϕ W-14 DNA synthesized in the presence of BUdR.

ϕ W-14-infected *P. acidovorans* strain 3L was spun down and resuspended in conditioned TCS medium without thymidine. BUdR ($500 \mu\text{g ml}^{-1}$), adenosine ($100 \mu\text{g ml}^{-1}$) and ^3H -ornithine ($1.0 \mu\text{Ci ml}^{-1}$, $10 \mu\text{g ml}^{-1}$) 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, \circ ; ^{32}P -labelled ϕ W-14 w⁺ phage reference DNA, \bullet .



the normal accumulation of plaque-forming units in ØW-14-infected strain 3L (Figure 10). ØW-14 also formed plaques on 3L growing on CAA-M plates containing TdR and FUdR.

The failure of FUdR to inhibit phage formation in ØW-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 [2-¹⁴C]-uracil or [³H]-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 [2-¹⁴C]-Ura and [³H]-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 ØW-14 DNA in 3L/FU^R₂.

FUdR could exert a non lethal effect upon DNA modification in ØW-14-infected cells. FUdR might be incorporated into phage DNA or FUdR might inhibit the accumulation of putThy or Thy residues. [6-³H]-Ura-labelled ØW-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 $250 \mu\text{g ml}^{-1}$ TdR were supplemented with $500 \mu\text{g ml}^{-1}$ FUdR, Δ ; or with $100 \mu\text{g ml}^{-1}$ Tp, \circ ; no addition, \bullet . The cultures were then grown to a density of 3.0×10^8 cells ml^{-1} and infected with ØW-14. The number of plaque forming units in each culture was determined at intervals after infection.

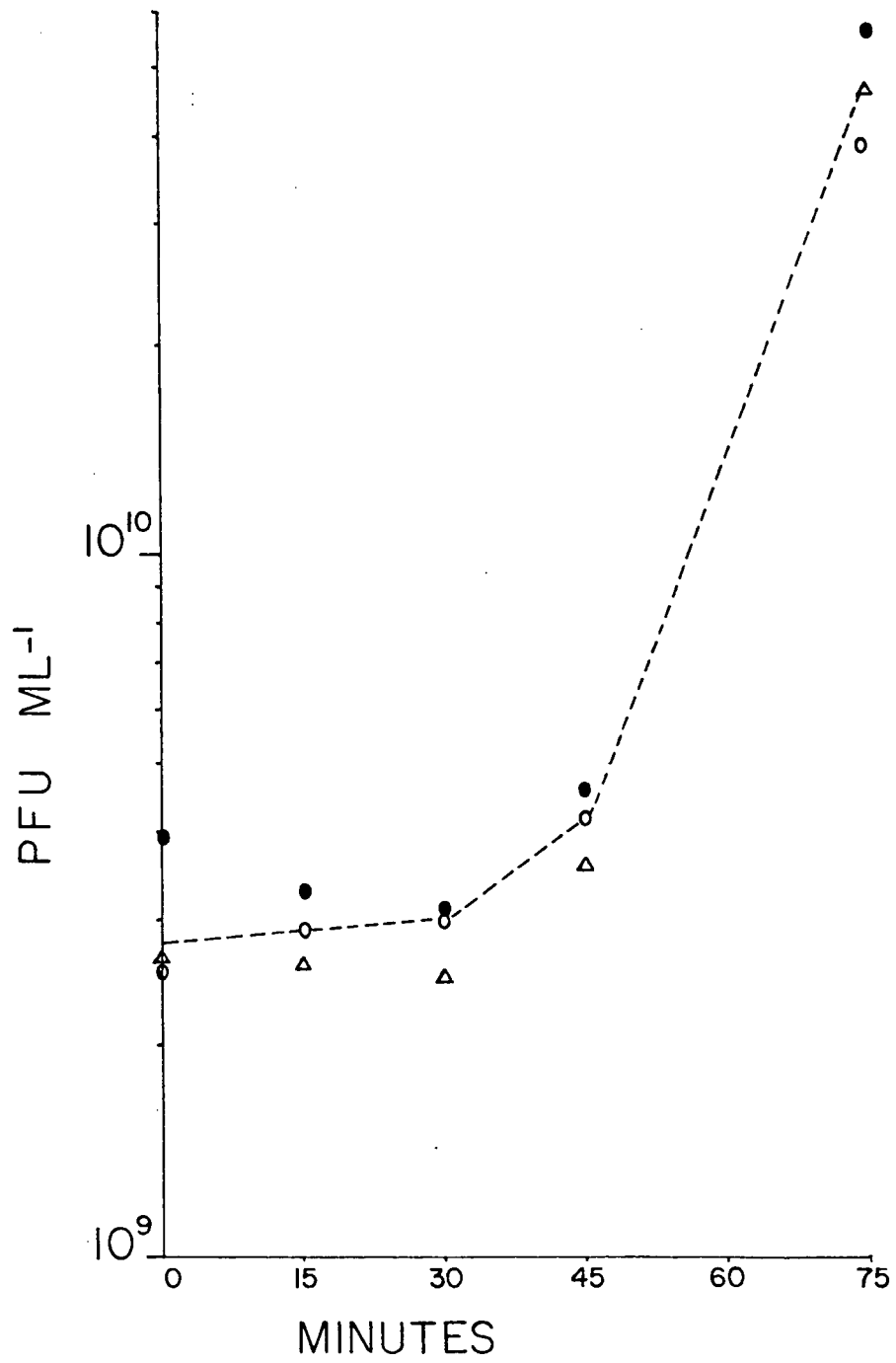


FIGURE 11.--The effect of fluorodeoxyuridine upon DNA synthesis in ϕ W-14-infected P. acidovorans 3L.

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

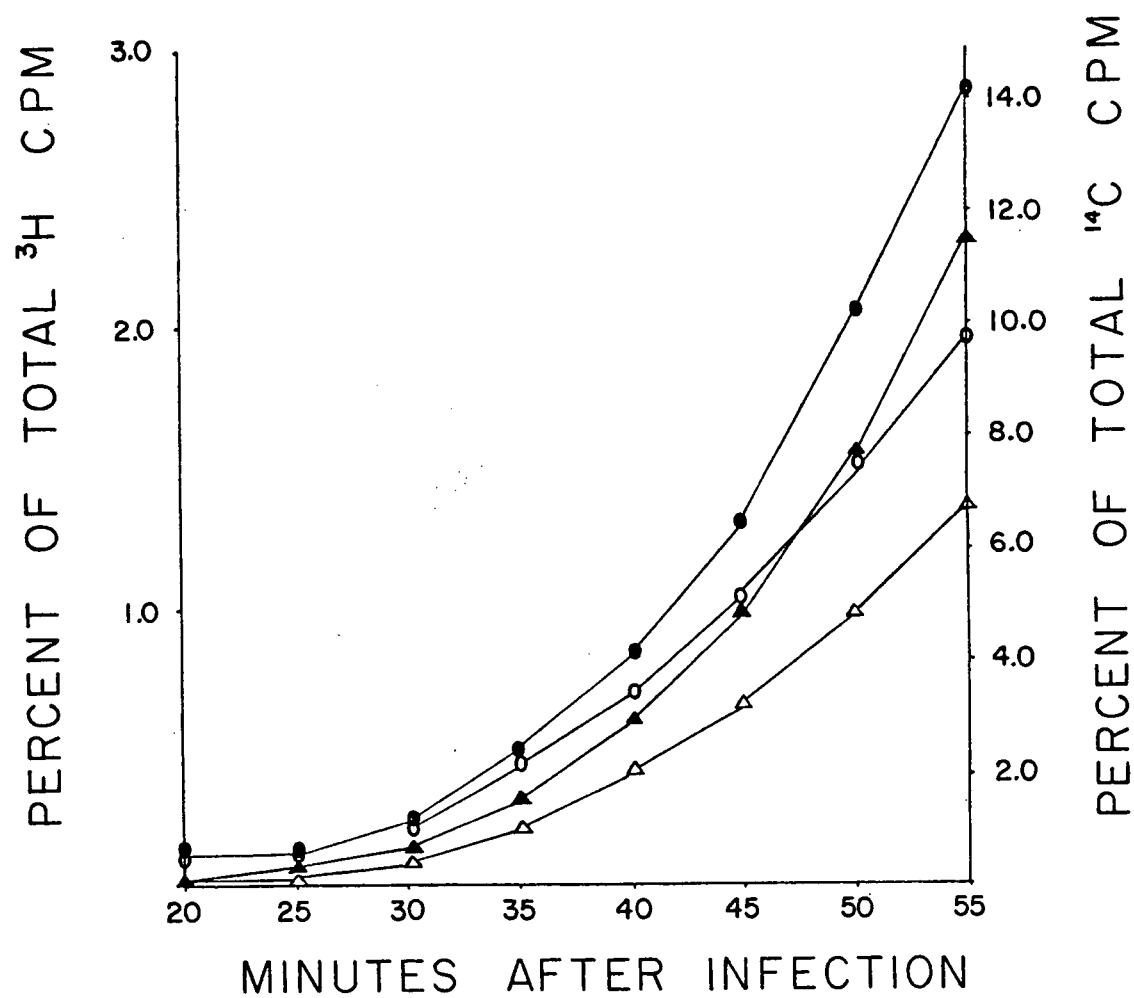


FIGURE 12.--The accumulation of ϕ W-14 DNA in the presence of FUdR.

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.

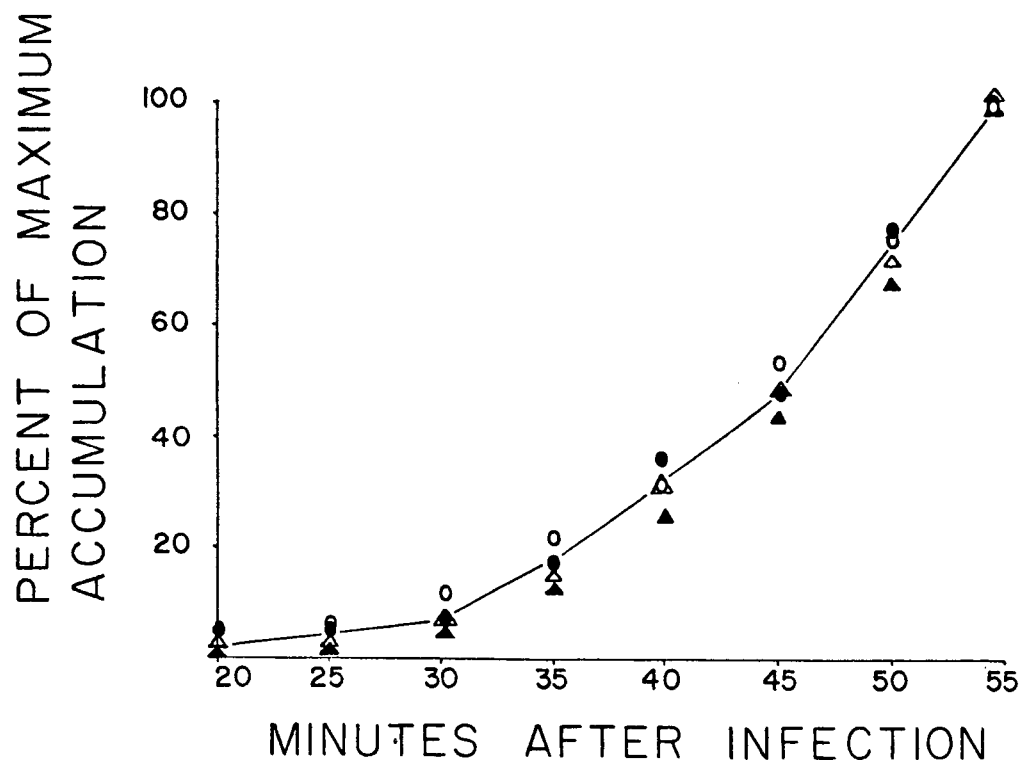


TABLE 5.--The effect of trimethoprim or FUDR upon the buoyant density of ØW-14 DNA synthesized in P. acidovorans

	ØW-14-infected	
	strain 29	strain 3L
Time of addition of FUDR		
0 min.	+ ^a	+
30 min. before infection	+	+
Time of addition of Tp		
0 min.	+	+
90 min. before infection	- ^b	+

The DNA used in these experiments was labelled with [6-³H]-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 ØW-14 reference DNA in neutral CsCl gradients.

b Strain 29 cells with an established inhibition by trimethoprim did not support ØW-14 plaque production or DNA synthesis.

TABLE 6.--The base composition of ϕ W-14 DNA prepared in the presence of Trimethoprim or FUDR.

Phage/Host/Inhibitor	putThy	Cyt	Thy
ϕ W-14 w^+ /29/none	2931 (0.24) ^a	6027 (0.49)	3454 (0.28)
ϕ W-14 w^+ /3L/none	4113 (0.20)	10912 (0.52)	5798 (0.28)
ϕ W-14 w^+ /29/Tp (100 $\mu\text{g ml}^{-1}$)	1787 (0.21)	4027 (0.51)	2255 (0.28)
ϕ W-14 w^+ /3L/Tp (100 $\mu\text{g ml}^{-1}$)	10980 (0.21)	27315 (0.54)	12543 (0.25)
ϕ W-14 w^+ /29/FUDR (500 $\mu\text{g ml}^{-1}$)	27982 (0.22)	65100 (0.52)	31417 (0.25)
ϕ W-14 w^+ /3L/FUDR (500 $\mu\text{g ml}^{-1}$)	34670 (0.22)	81435 (0.53)	38126 (0.25)

The infected cells were labelled from 25 minutes after infection with [6-³H]-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.

^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.

TABLE 7.---ØW-14 DNA modification was independent of the host strain.

Base	host strain	
	29	3L
putThy	4972 (0.24) ^a	13981 (0.23)
Cyt	10858 (0.51)	31661 (0.52)
Thy	5285 (0.25)	15318 (0.25)

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

- a 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 ØW-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 ØW-14 DNA, but as shown earlier, DNA prepared in the presence of FuDR had the same buoyant density as ØW-14 reference DNA.

Therefore, the resistance of ØW-14 DNA replication and modification to FuDR also effectively excluded the possibility that uracil might be a precursor of putThy in ØW-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 ØW-14-infected *P. acidovorans*

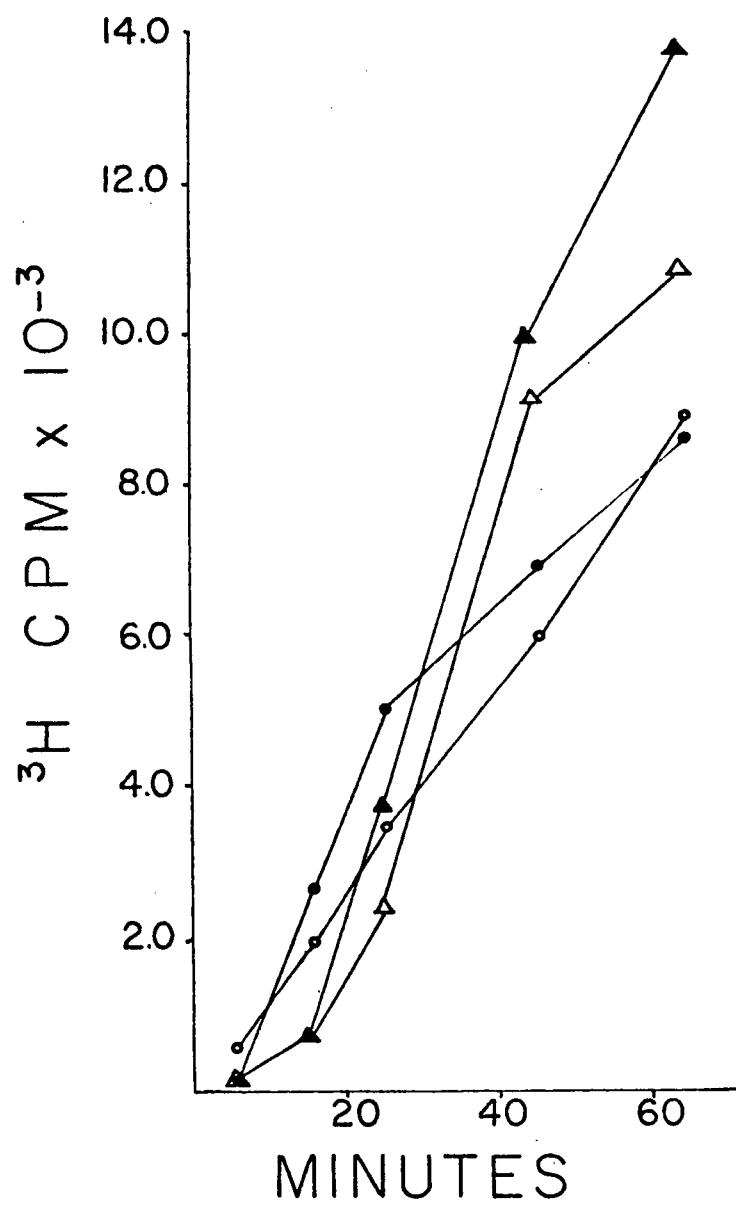
Thymidylate synthase or any enzyme catalyzing the release of tritium from [5-³H]-uracil can be detected in vivo in cells capable of converting uracil to dUMP. In vivo, tritium releasing activity was present in strain 29 but not in strain 3L uninfected cells (Figure 13). Both strains retained the ability 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). ØW-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 ØW-14 also suggested that the

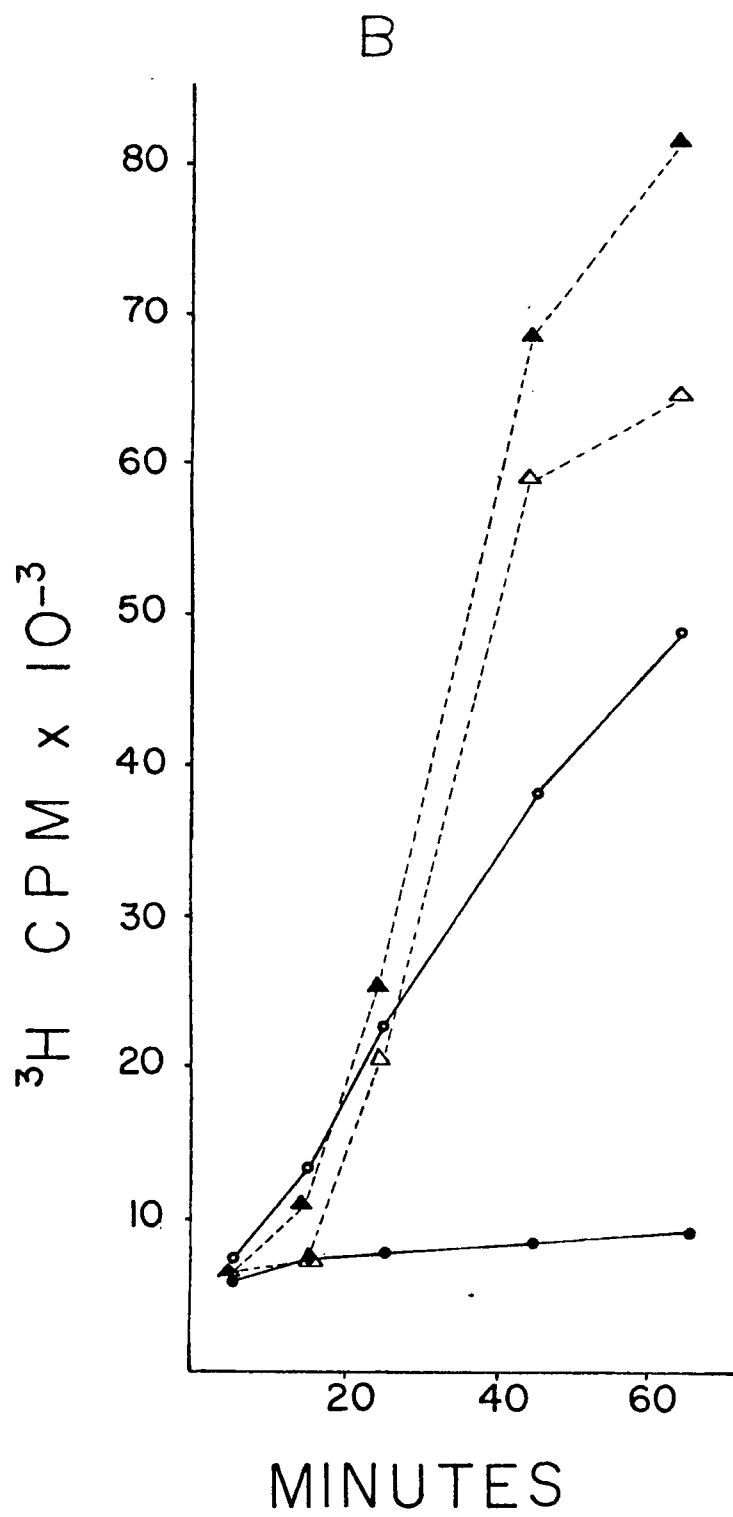
FIGURE 13.--DNA synthesis and tritium release in ØW-14-infected P. acidovorans.

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, ØW-14 infected, Δ; P. acidovorans strain 3L, ØW-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, ØW-14 infected Δ; P. acidovorans strain 3L, ØW-14 infected, ▲.

A





host thymidylate synthase activity was suppressed by bacteriophage infection, otherwise tritium release in ØW-14-infected strain 29 would have been greater than in ØW-14-infected strain 3L.

These results demonstrated the presence of [5-³H] releasing activities in ØW-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 [5-³H]-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 FdUR. Concentrations of 5-FdUMP ten times greater than those inhibiting the host thymidylate synthase do not inhibit the ØW-14 hydroxymethylase activity in extracts (Neuhard and Warren, unpublished observations). The inability of ØW-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 ØW-14 DNA.

Trimethoprim (Tp) and trimethoprim and sulfonamide (sulfa) effects on ØW-14 reproduction

The minimal inhibitory concentration of Tp for P. acidovorans strain 29 is between 2.5 and 5 µg ml⁻¹. 100 µg 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 P. acidovorans strains 29 and 3L.

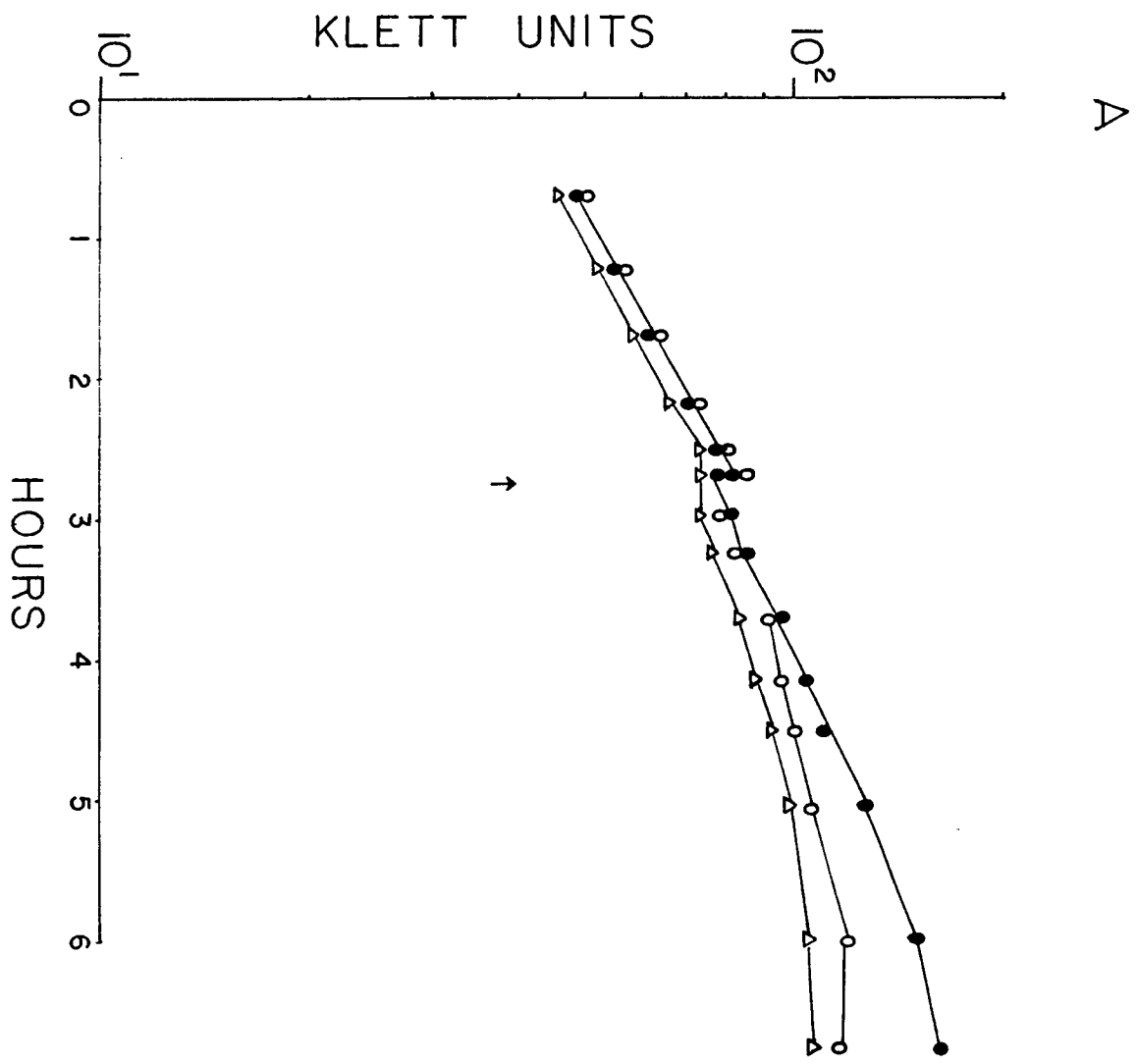
Cultures of strains 29 and 3L were grown in TCS medium supplemented with succinate (2 mg ml^{-1}) or with succinate and Casamino acids (1 mg ml^{-1}), adenosine ($100 \text{ } \mu\text{g ml}^{-1}$), thymidine ($500 \text{ } \mu\text{g ml}^{-1}$), and pantothenic acid ($50 \text{ } \mu\text{g ml}^{-1}$).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

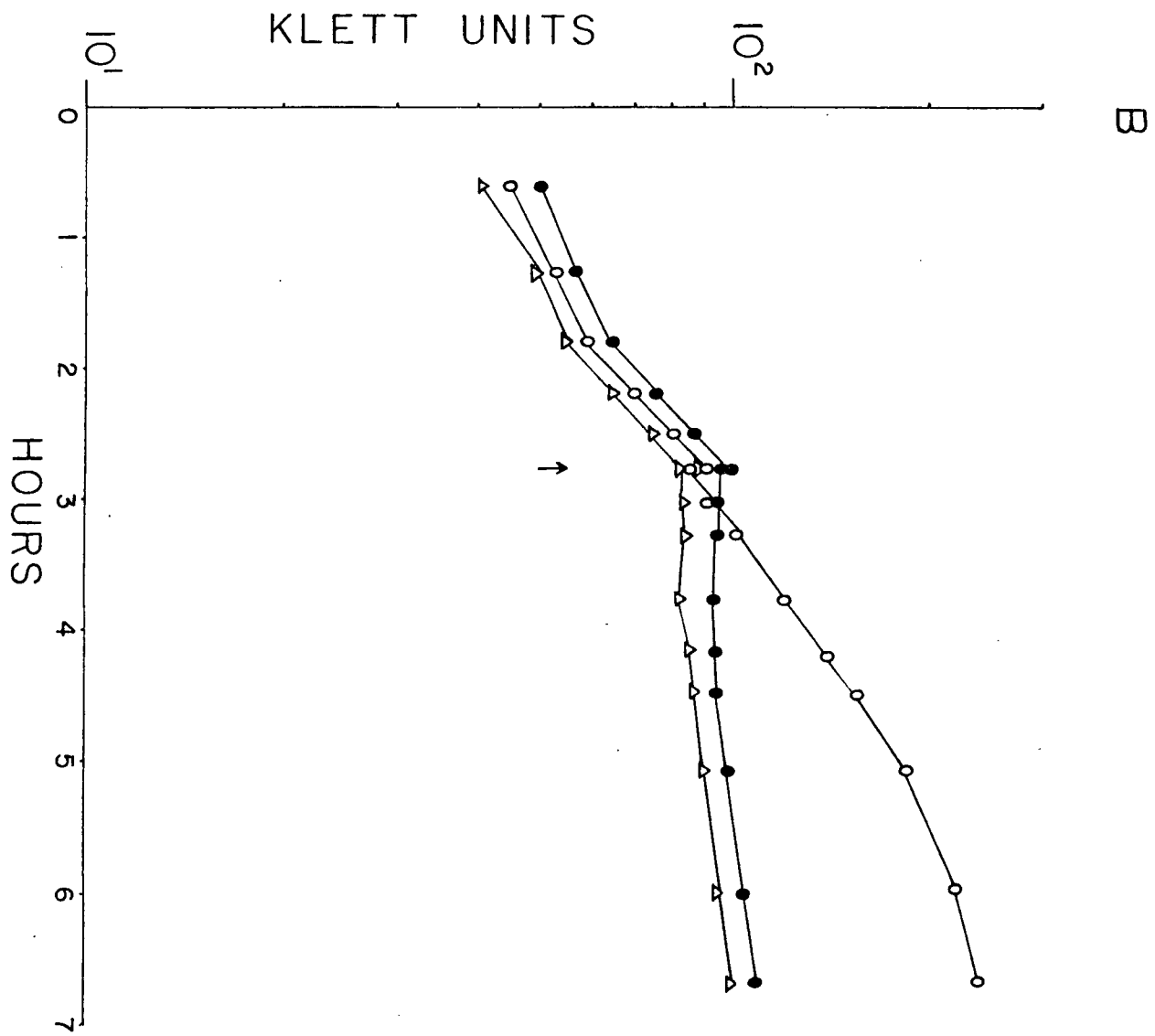
A) Strain 29 grown in succinate; no additions, ●; Tp, ○; Tp and Sulfa, Δ.

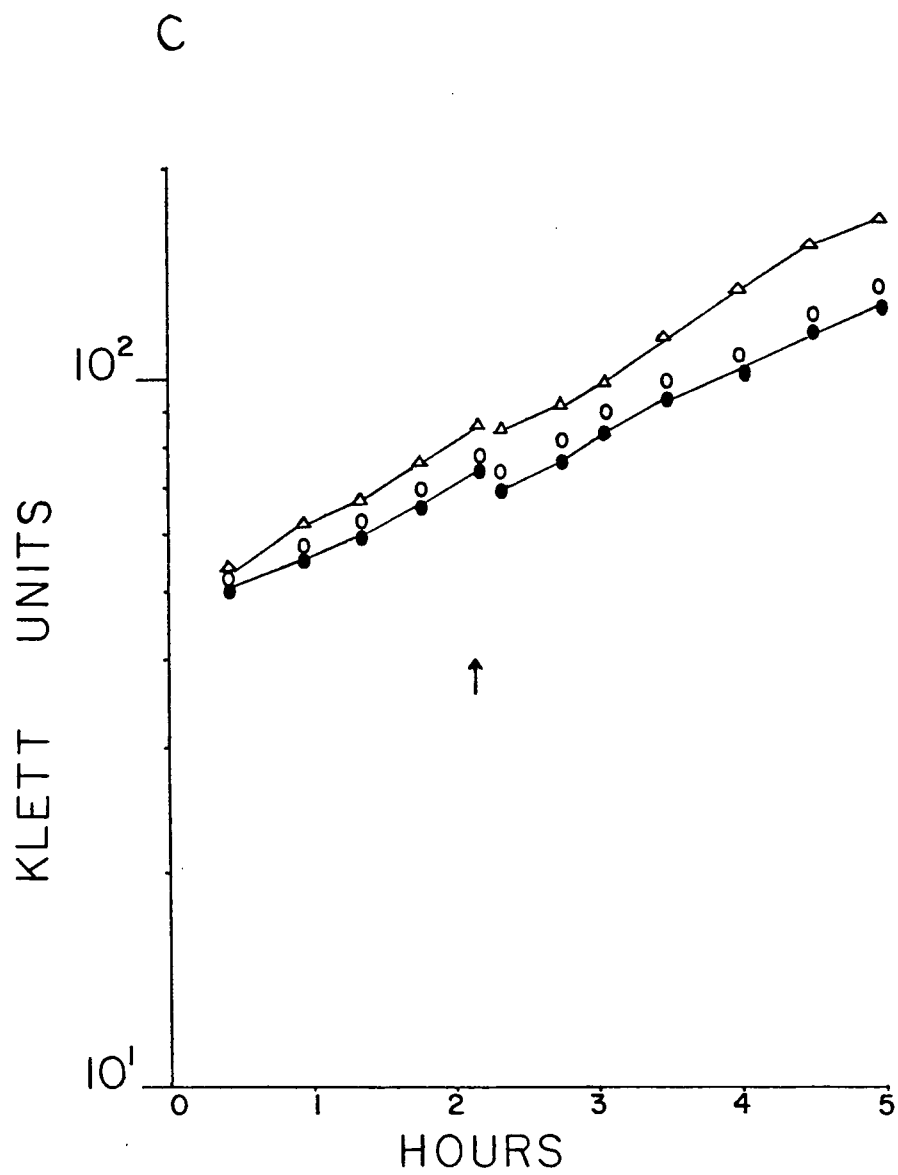
B) Strain 29 grown in succinate, Casamino acids, thymidine, adenosine, and pantothenic acid; no additions, ○; Tp, ●; Tp and Sulfa, Δ.

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

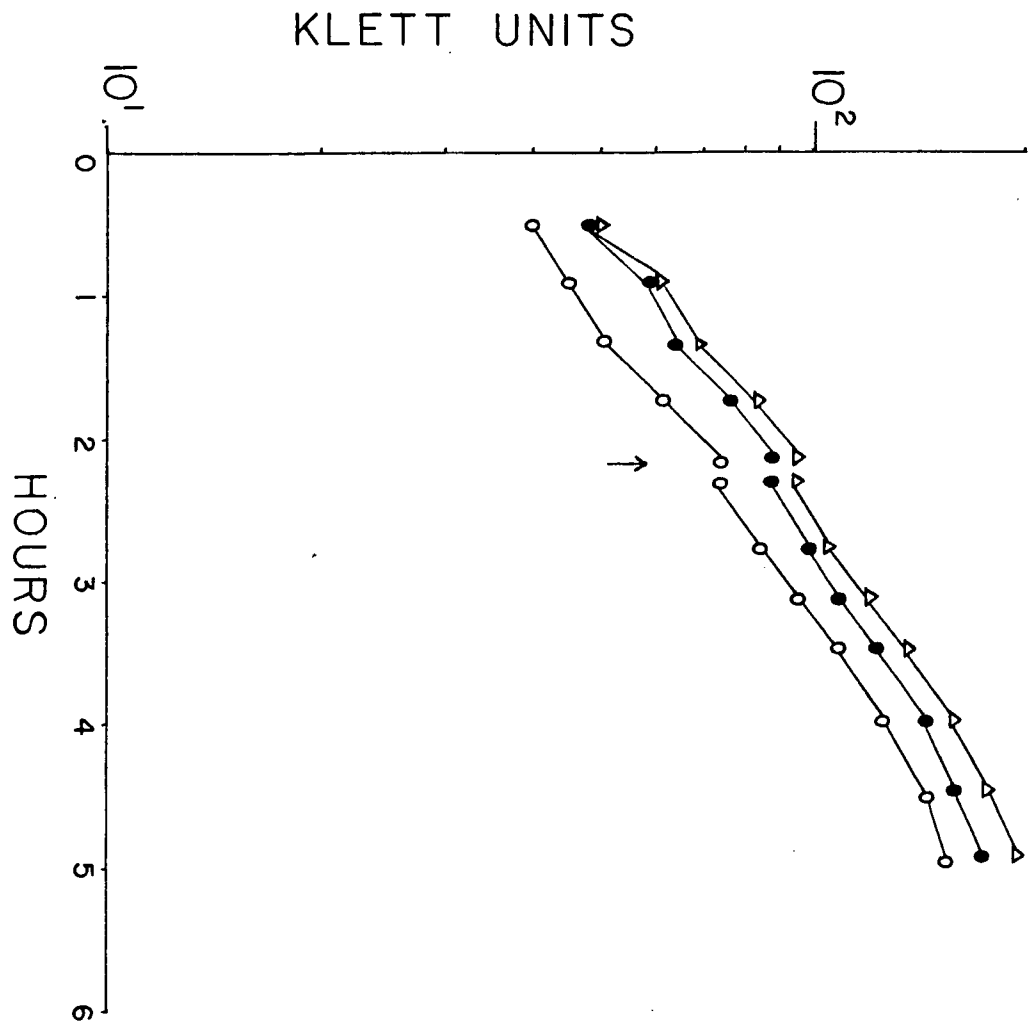
D) Strain 3L grown in succinate, Casamino acids, thymidine, adenosine, and pantothenic acid; no additions, ○; Tp, ●; Tp and Sulfa, Δ.







D



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 ØW-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 ØW-14 DNA in strain 29 (Figures 15 and 16) when the inhibitor was added at the onset of phage infection. The buoyant density of ØW-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 ØW-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 ØW-14 DNA synthesized (Table 5).

[6-³H]-Ura labelling ratios for ØW-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 [6-³H]-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), ○.

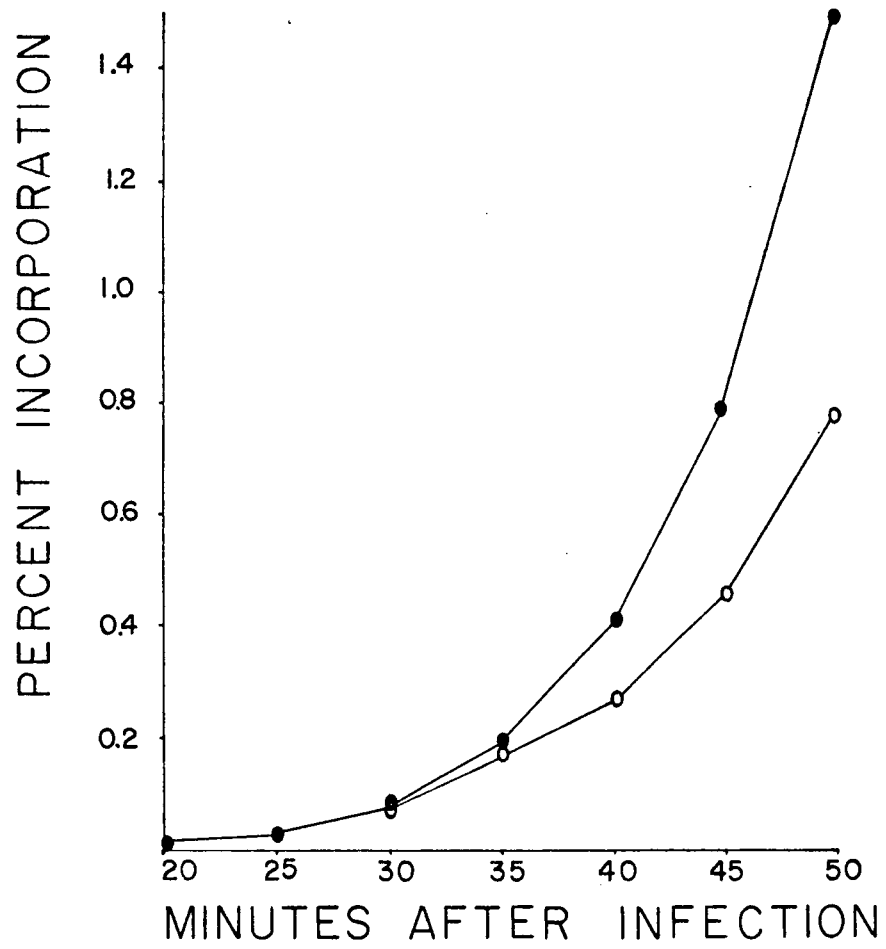
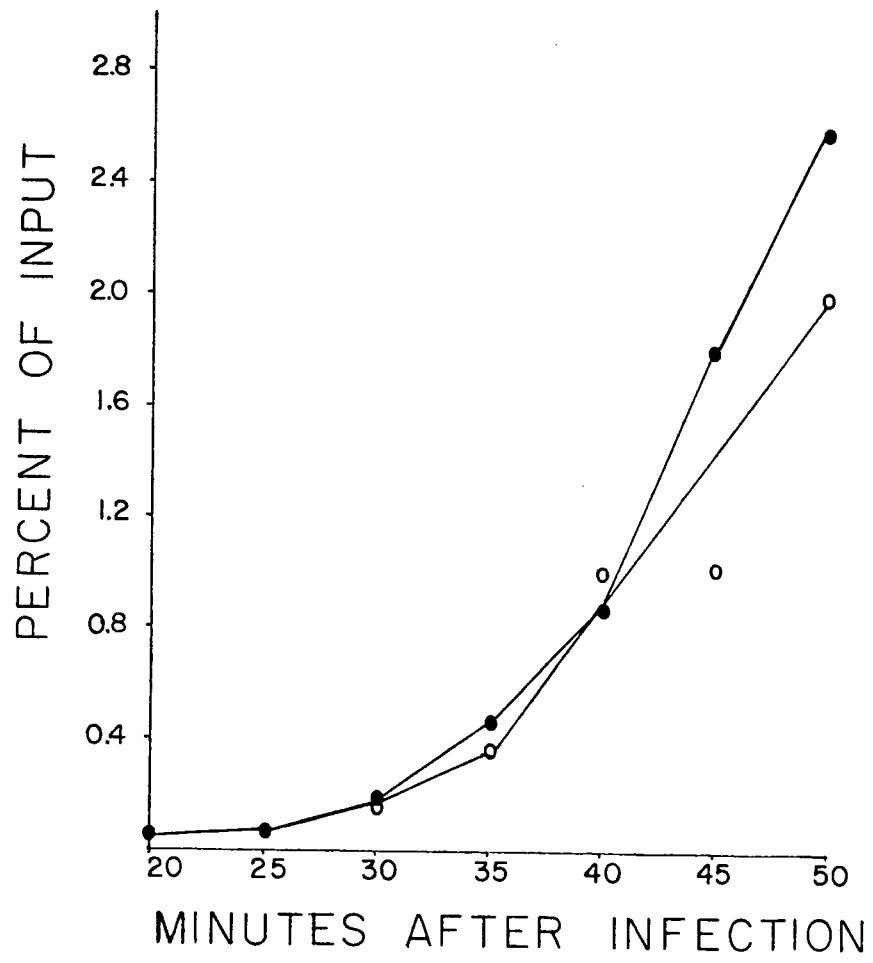


FIGURE 16.--The effect of trimethoprim and sulfonamide upon ØW-14 DNA synthesis.

Tp and Sulfa were added to one-half of a culture at 20 minutes after infection. The incorporation of $[6-^3\text{H}]\text{-uracil}$ into alkali resistant, TCA precipitable material was determined. P. acidovorans strain 29 infected with ØW-14, no additions, ●; P. acidovorans strain 29 infected with ØW-14, Tp ($100\text{ }\mu\text{g ml}^{-1}$), Sulfa ($500\text{ }\mu\text{g ml}^{-1}$), ○.



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 ØW-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 hmdUTP in the soluble nucleotide pools of infected cells (Neuhard et al., 1980). Warren has also shown the induction of a dUTP/dTTPase activity in ØW-14-infected cells. It was concluded that ØW-14 synthesis required the four deoxynucleoside triphosphates; dATP, dGTP, dCTP and hmdUTP, that dTTP was excluded, and that both Thy and putThy in ØW-14 DNA were generated by post-replicative modification of hmUra. These conclusions prompted a detailed evaluation of the nature of replicating ØW-14 DNA. The goals of this work were the demonstration of hmURA in replicating ØW-14 DNA and the elucidation of the biosynthetic pathways for putThy and Thy.

Tritium is not lost from [6-³H]-uracil during ØW-14 DNA synthesis

ØW-14 DNA labelled with [2-¹⁴C]-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 ØW-14 DNA. The biosynthesis of putThy and Thy in ØW-14 DNA preserved the [6-³H]-uracil label. Equilibrium labelling of bases with [6-³H]-Ura accurately reflected their relative levels in ØW-14 DNA.

Replicating DNA

hmdUTP is the nucleotide pool precursor of Thy and putThy in ØW-14 DNA (Neuhard et al., 1980). Acid hydrolysis of ØW-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 ØW-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 α putrescinylothymine levels in ØW-14 DNA (data not presented).

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

Replicating ØW-14 DNA may be examined indirectly using parentally labelled ØW-14 DNA and following it through infection with neutral CsCl gradient analysis (Figure 18). Obviously, the putThy in ØW-14 DNA was

TABLE 8.--Tritium is not lost from [6-³H]-uracil during putThy or Thy biosynthesis.

Base	ØW-14 DNA labelled with:	
	[6- ³ H]-uracil	[2- ¹⁴ C]-uracil
putThy	25840 (0.22) ^a	19089 (0.22)
Thy	31561 (0.26)	23717 (0.28)
Cyt	62229 (0.52)	42260 (0.50)

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.

[2-¹⁴C]-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.

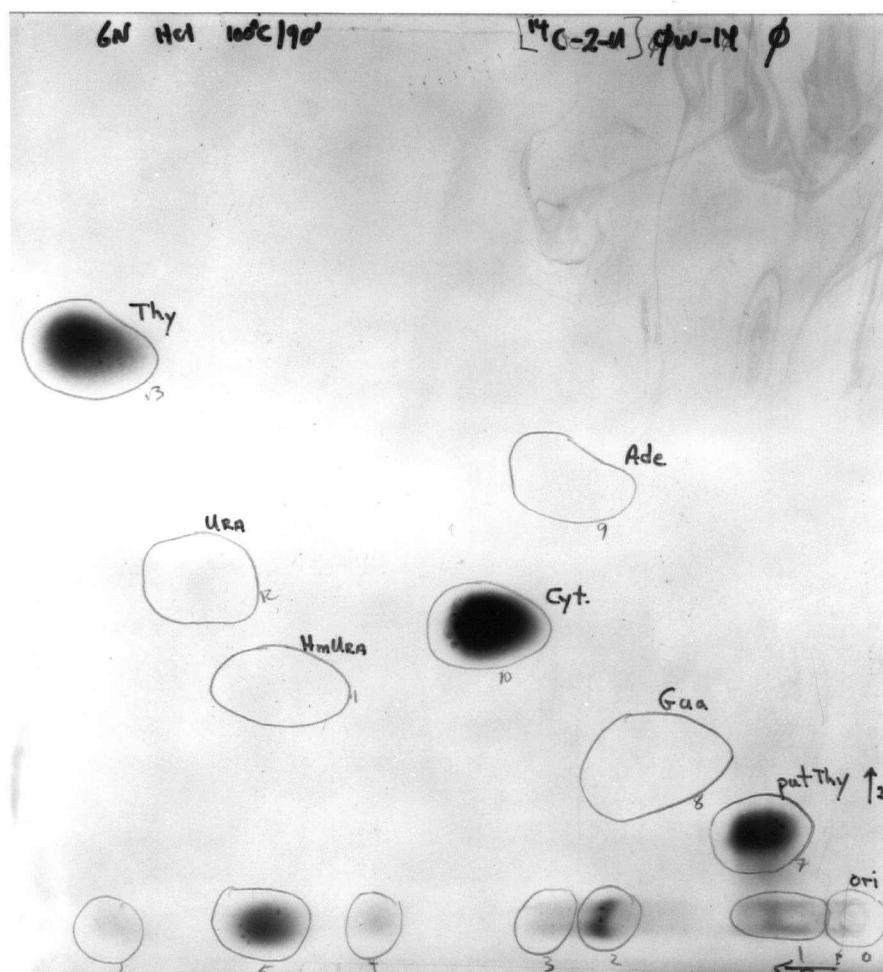
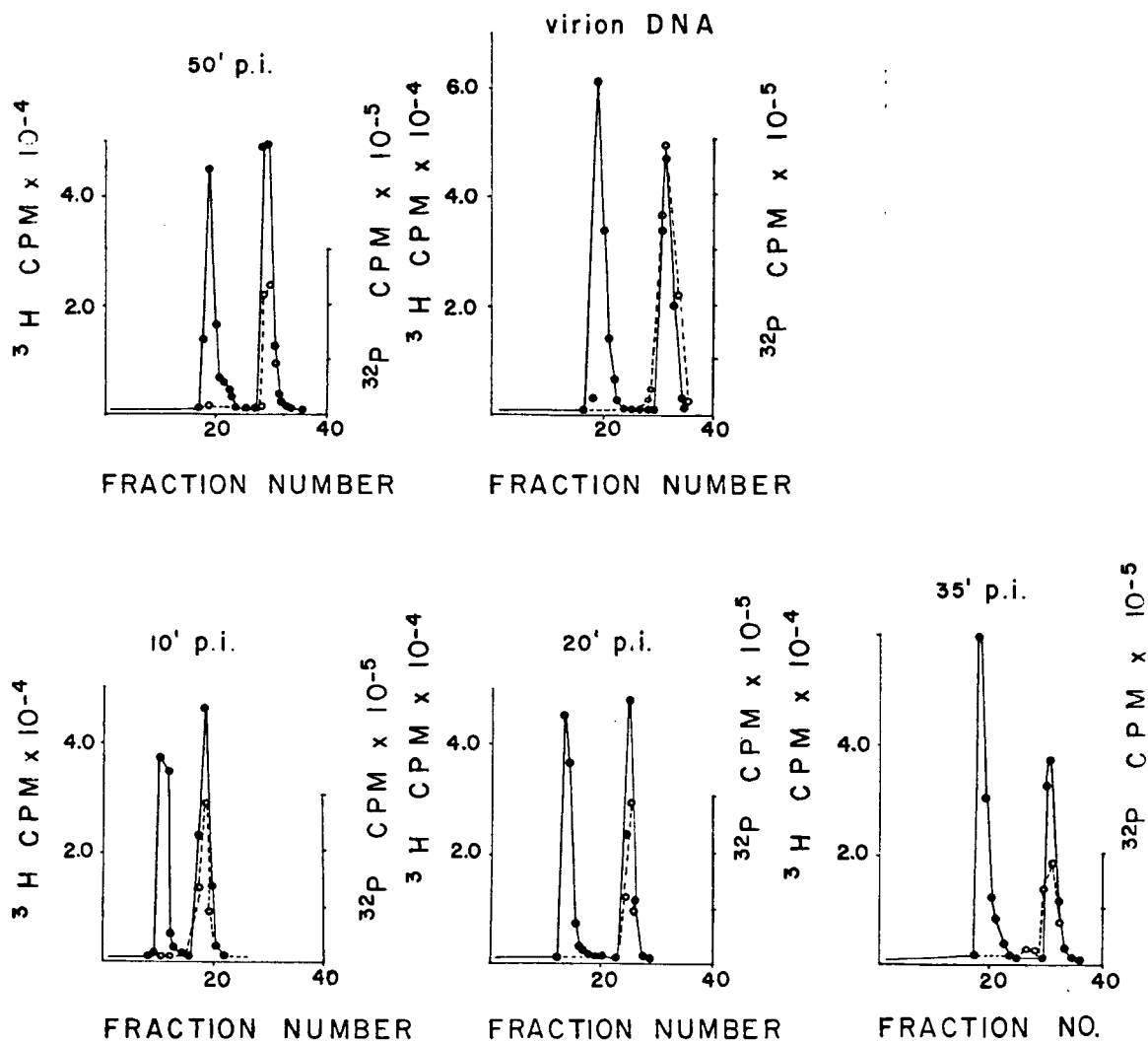


FIGURE 18.--CsCl density gradient analysis of ^{32}P -labelled ØW-14 parental DNA.

^{32}P -labelled ØW-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- ^3H]-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 ϕ W-14 DNA extracted from infected cells allowed similar conclusions regarding the fate of Thy and putThy. Both were stable components of ϕ W-14 DNA (Table 9).

ϕ W-14 intracellular parentally labelled DNA heated to just below the T_m of ϕ 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 ϕ 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 [5-¹⁴C]-ornithine formed a single, homogenous peak before and after heat treatment. ϕ W-14 intracellular DNA prepared from infected cells labelled with [5-³H]-uracil and [5-¹⁴C]-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 ØW-14 DNA.

Base	Sample taken (min. after infection)			
	10 min.	30 min.	50 min.	phage (0 min.)
putThy	3657 (0.23) ^a	3051 (0.21)	2205 (0.21)	9475 (0.21)
Cyt	8972 (0.55)	8220 (0.57)	5719 (0.55)	26949 (0.59)
Thy	3578 (0.22)	3276 (0.22)	2399 (0.23)	9293 (0.20)

[6-³H]-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.

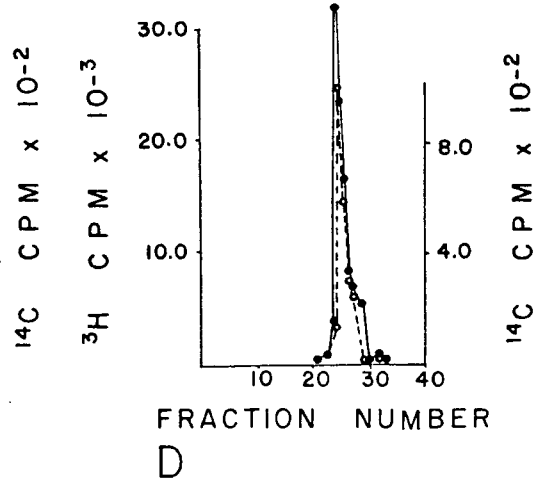
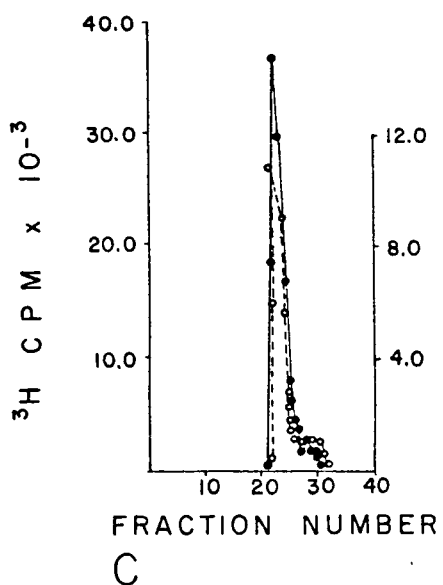
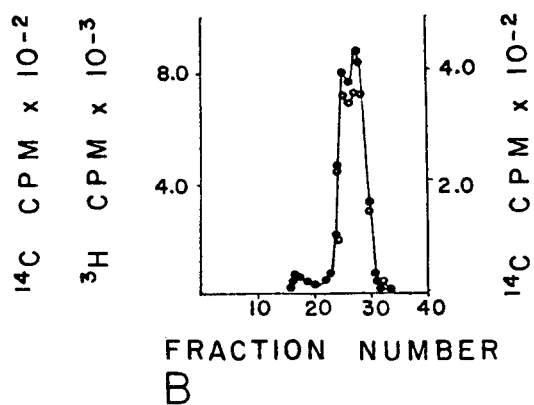
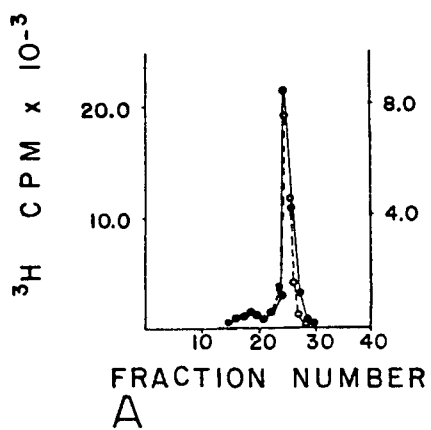
a The values are cpm in the area cut from the chromatogram. The values in parentheses are the fraction of the label recovered in putThy, Thy and Cyt bases.

FIGURE 19.--The effect of heat treatment upon the buoyant density profiles of ϕ W-14 intracellular and virion DNAs.

[5- 3 H]-uracil and [5- 14 C]-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 ϕ W-14 intracellular DNA
- B) heat treated ϕ W-14 intracellular DNA
- C) native ϕ W-14 virion DNA
- D) heat treated ϕ W-14 virion DNA

ϕ W-14 intracellular DNA was purified from infected cells at 35 minutes after infection. [5- 3 H]-uracil, ●; [5- 14 C]-ornithine, ○.



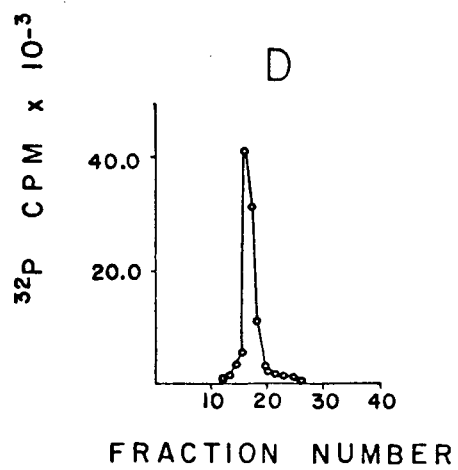
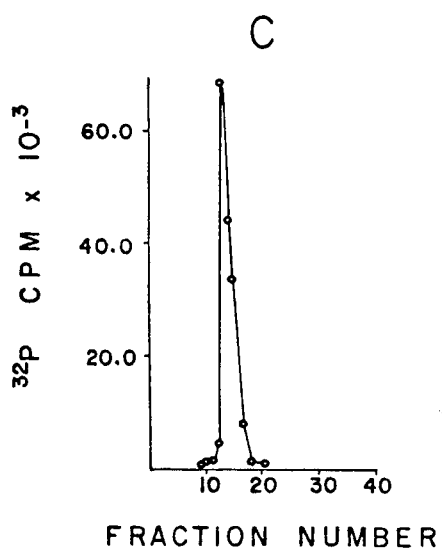
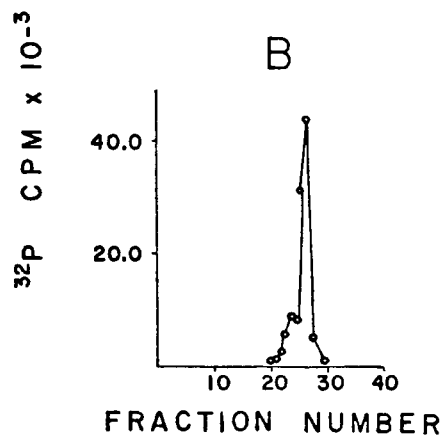
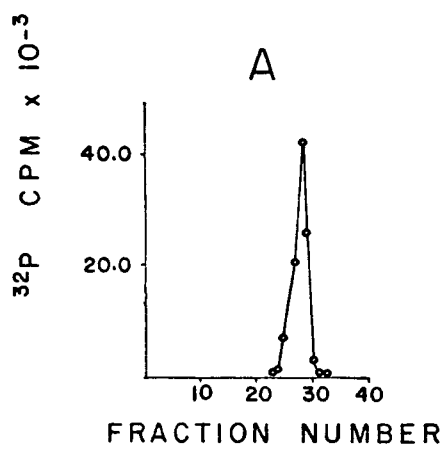
to loading on the gradient split the sample into two peaks containing approximately equal ratios of label. PutThy or Thy-rich regions of the DNA (if there are any) are not preferentially heat-denatured since ornithine or uracil-labelled DNA contributed equally to the slightly denser single-stranded peak. 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 T_m than longer duplex DNA fragments of the same mol % G + C content (Mahler and Cordes, 1971). Reconstruction experiments using purified strain 29 DNA and ϕ W-14 DNA indicated that peak splitting was not due to ϕ W-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 ϕ W-14 DNA banded as a single peak of uniform density.

The effect of shearing upon the buoyant density profile of ϕ W-14 DNA was studied. Peak broadness in neutral CsCl density gradients was a

FIGURE 20.--CsCl buoyant density analysis of ØW-14 virion and P. acidovorans DNA.

- A) ^{32}P -labelled ØW-14 DNA mixed with 50 µg unlabelled host DNA.
- B) ^{32}P -labelled ØW-14 DNA mixed with 50 µg unlabelled host DNA and then heat denatured.
- C) ^{32}P -labelled strain 29 DNA mixed with 50 µg unlabelled ØW-14 DNA.
- D) ^{32}P -labelled strain 29 DNA mixed with 50 µg unlabelled ØW-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. [2-¹⁴C]-uracil and [5-³H]-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 [6-³H]-uracil labelled ØW-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 ØW-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 ØW-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 ØW-14 intracellular DNA extracted at any point during DNA replication.

Thin-layer chromatography of DNA components

The degradative analysis of newly formed replicating ØW-14 DNA was preceded by the evaluation of procedures used for the hydrolysis or

FIGURE 21.--The uniform distribution of putThy in ØW-14 DNA.

[³H]-ornithine-labelled ØW-14 DNA was prepared from infected cells and from purified virions. [2-¹⁴C]-uracil labelled ØW-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.

[³H]-ornithine,--●-- ; [2-¹⁴C]-uracil,--O-- .

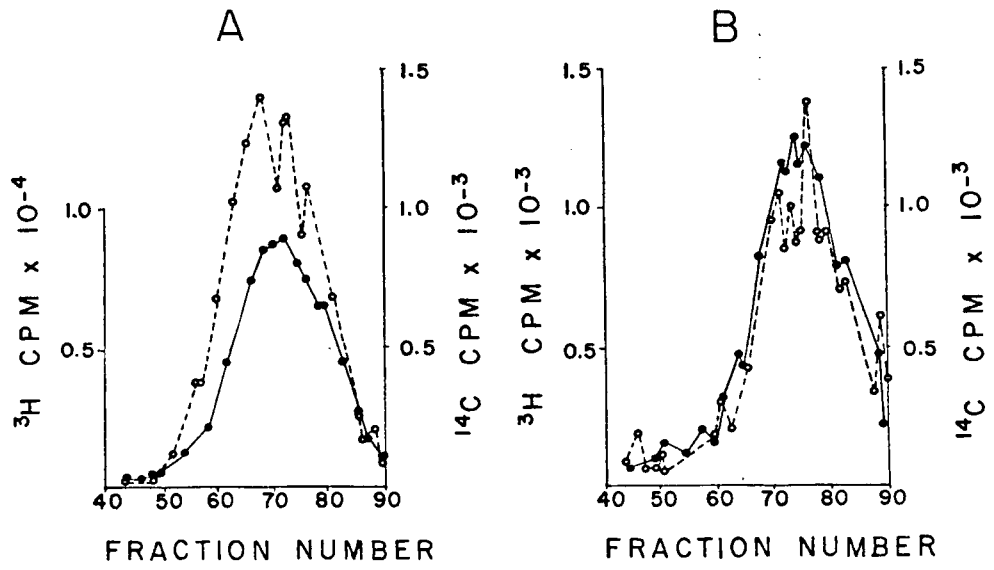


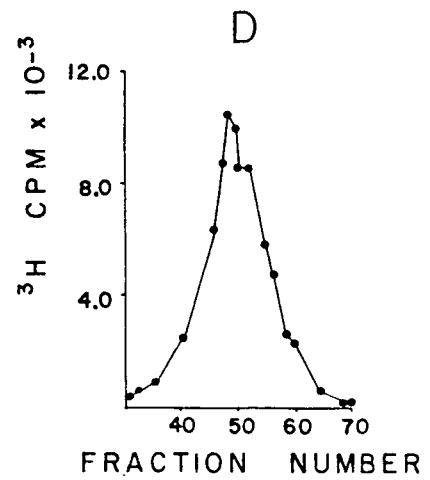
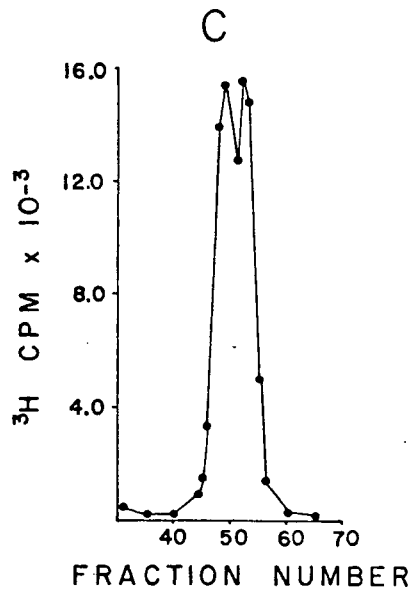
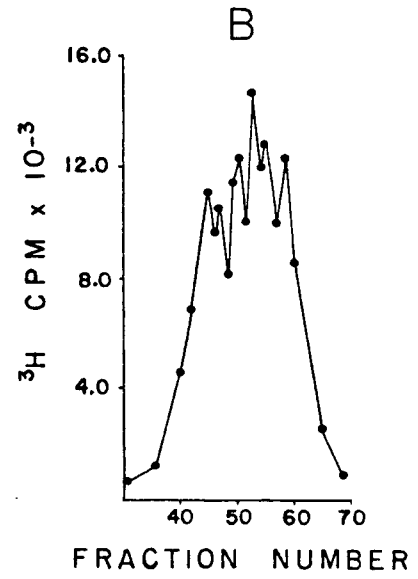
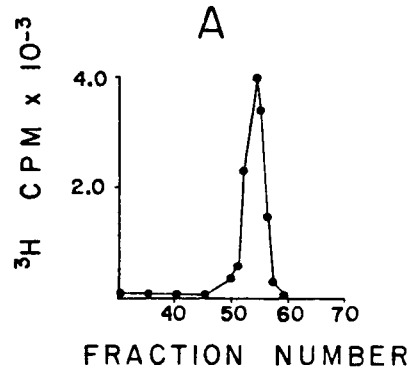
FIGURE 22.--The effect of heat treatment and shearing upon the distribution of label in ØW-14 DNA extracted from infected cells, and banded on CsCl gradients.

[6-³H]-uracil labelled DNA was extracted from ØW-14 infected P.

acidovorans at 35 minutes after infection.

- A) ØW-14 DNA.
- B) ØW-14 DNA, sheared.
- C) ØW-14 DNA, heat treated.
- D) ØW-14 DNA, sheared and heat treated.

The procedure employed is described in the Materials and Methods.



digestion and separation of ØW-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 ØW-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 R_f 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 ØW-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 ØW-14 virion DNA (Figure 17).

FIGURE 23.--Two-dimensional thin-layer chromatography of acid-hydrolyzed, [2- 14 C]-uracil-labelled *P. acidovorans* DNA.

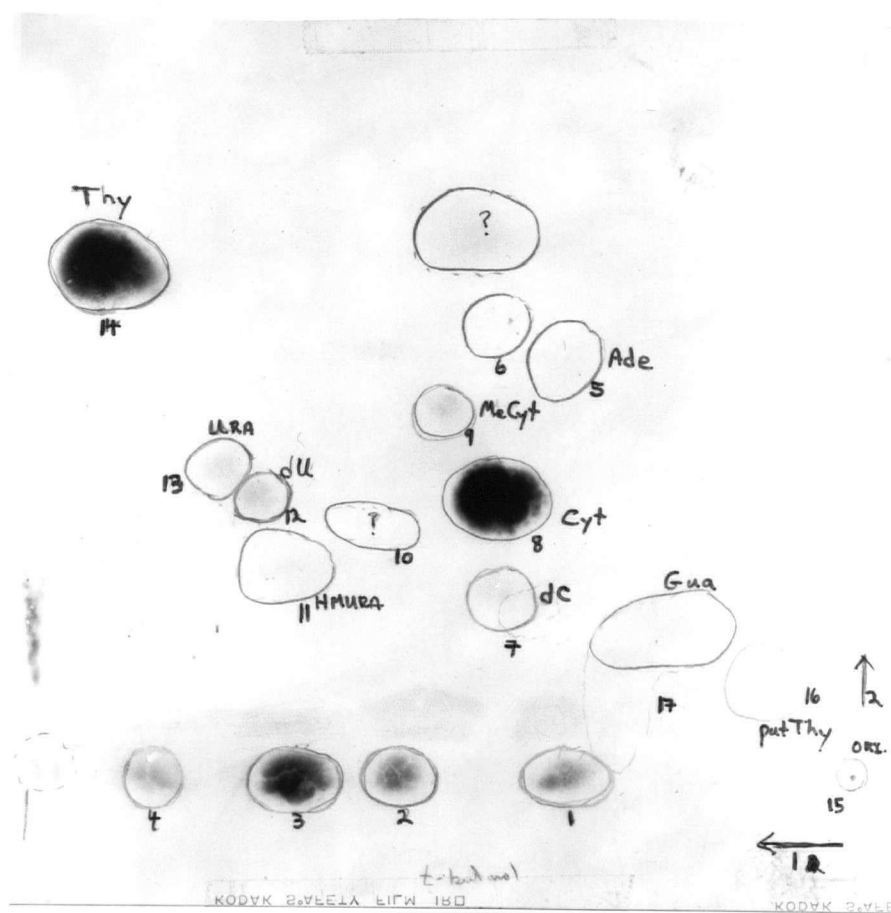


TABLE 10.--The base composition of P. acidovorans strain 29 DNA.

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

[2-¹⁴C]-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 ^{32}P) samples could be acid hydrolyzed and separated. ^3H -labelled bases were completely free of contaminating ^{32}P 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 R_f 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 putrescinyll 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 ØW-14 DNA digests resolved on PEI-cellulose.

	Nucleotide	CPM	(fraction of total label)
1	unknown	611	(0.15)
2	unknown	59	(0.01)
3	unknown	158	(0.04)
4	unknown	63	(0.01)
5	unknown	28	(0.01)
6	unknown	27	(0.01)
7	orthophosphate	1305	(0.31)
8	AMP and dAMP	977	(0.23)
9	UMP and dTMP	257	(0.06)
10	CMP and dCMP	309	(0.07)
11	GMP and dGMP	413	(0.10)

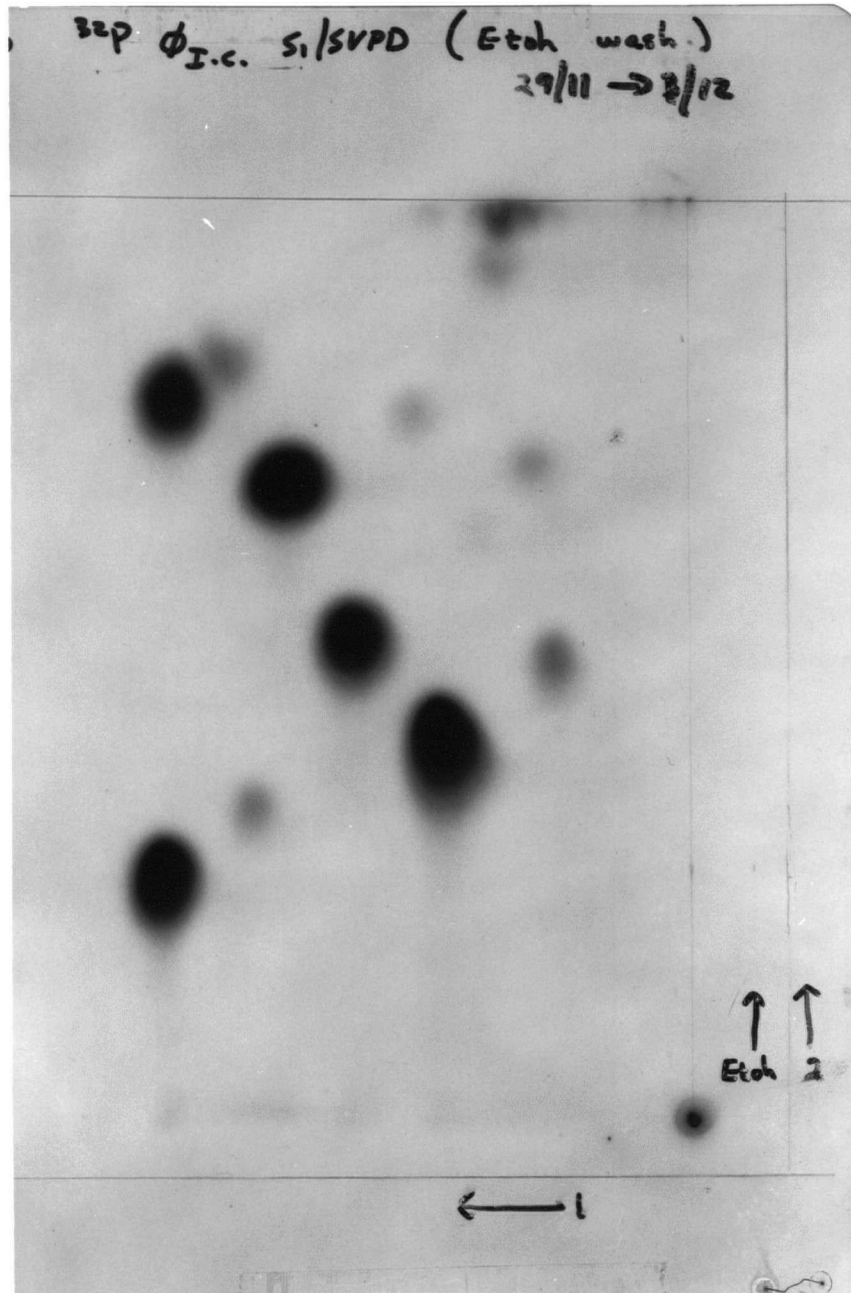
The numbers correspond to the number of the nucleotide or spot on the chromatographic sheet. Radioactivity in each compound was quantitated by cutting the area containing the compound from the sheet and counting it in a toluene-based scintillant. DNA was digested with nuclease S1 and snake venom phosphodiesterase 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 ØW-14 DNA digests resolved on cellulose.

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

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 $\phi\text{W-14}$ 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, dGMP, dCMP and dTMP, but not putdTTP, were liberated in large quantities. The deficiency in putdTTP and the appearance of only three other ^{32}P -labelled spots running as dimers suggested that the distribution of putThy in ØW-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 ØW-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 ØW-14 DNA. The CsCl gradient results presented earlier merely suggested the absence of gross heterogeneity in the distribution of putThy and Thy in ØW-14 DNA.

Pulse-labelling of DNA in ØW-14-infected *P. acidovorans*

Of necessity, the development and analysis of digestion and separation procedures preceded the detailed analysis of nascent ØW-14 DNA.

The tritiated uracil and $^{32}\text{PO}_4$ pulses were performed as described in the Materials and Methods. Acid hydrolysis and two-dimensional thin-layer chromatography of [6- ^3H]-uracil-pulsed ØW-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 $\phi\text{W-14}$ DNA digested with DNase I and SVPD.

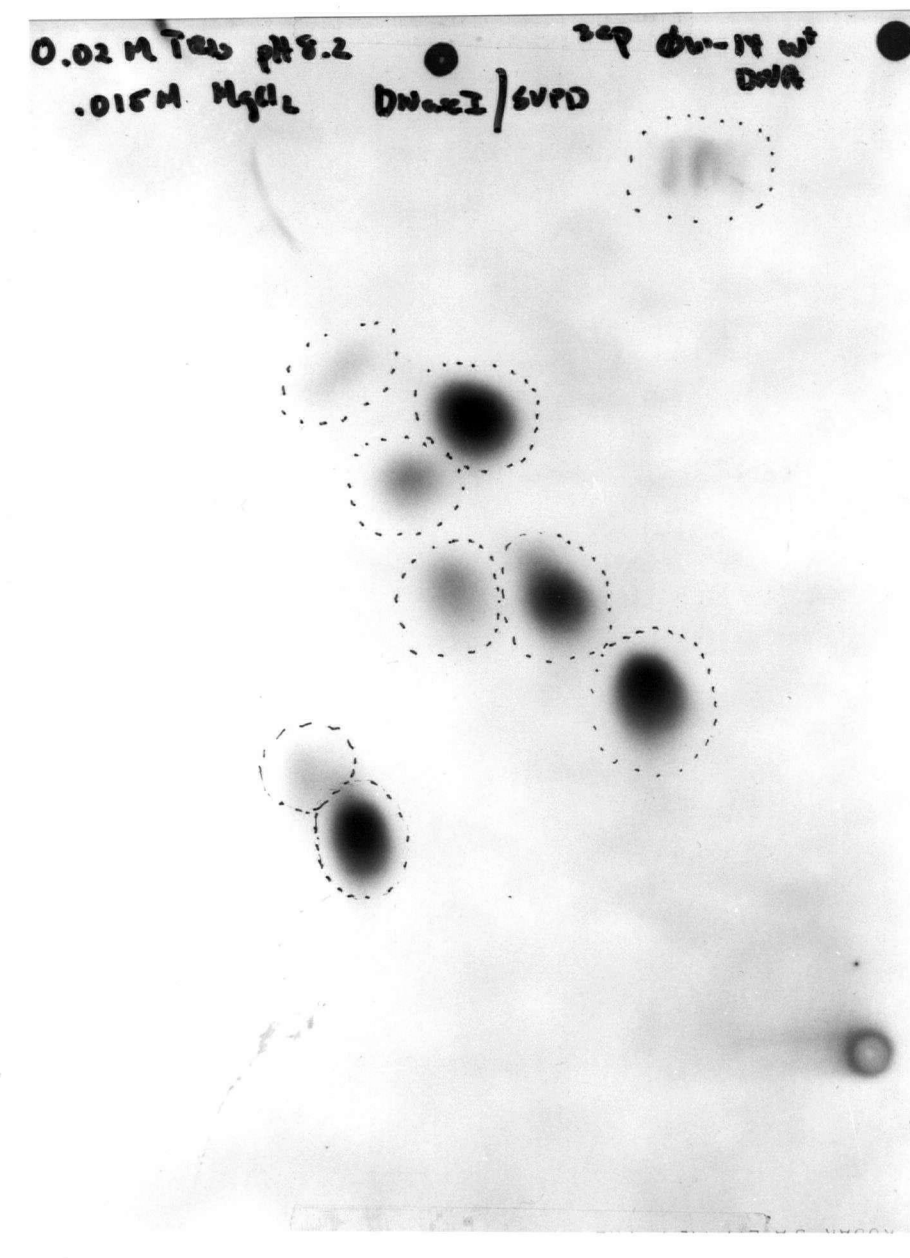


TABLE 12.--Base composition of replicating ØW-14 DNA

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

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 [5-³H]-uracil pulse served as a control. Acid hydrolysis of [5-³H]-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 ØW-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 [5-³H]-uracil pulse-labelled DNA sample was probably a consequence of the contamination of commercial [5-³H]-uracil with [6-³H]-uracil. 2.6 percent of the total pyrimidine label in the [6-³H]-uracil pulse was found in hmUra. This compared to 0.2 percent of the total label in a uniformly labelled ØW-14 intracellular DNA hydrolysate. This result demonstrated that the hmdUTP found in the acid-soluble pools of infected cells was incorporated into ØW-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 putThy and Thy were derived by post-replicative 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 ØW-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.

$^{32}\text{PO}_4$ pulses were also performed. Using $^{32}\text{PO}_4$ to pulse-label DNA facilitated the detection of novel nucleotides and lowered the costs incurred in the pulse experiments. ØW-14 DNA pulse-labelled for 10 seconds with $^{32}\text{PO}_4$ 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- ^3H]-uracil until 35 minutes after infection and pulsed for 10 seconds with $^{32}\text{PO}_4$ 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 ϕ 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 ϕ 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 ϕ W-14 DNA.

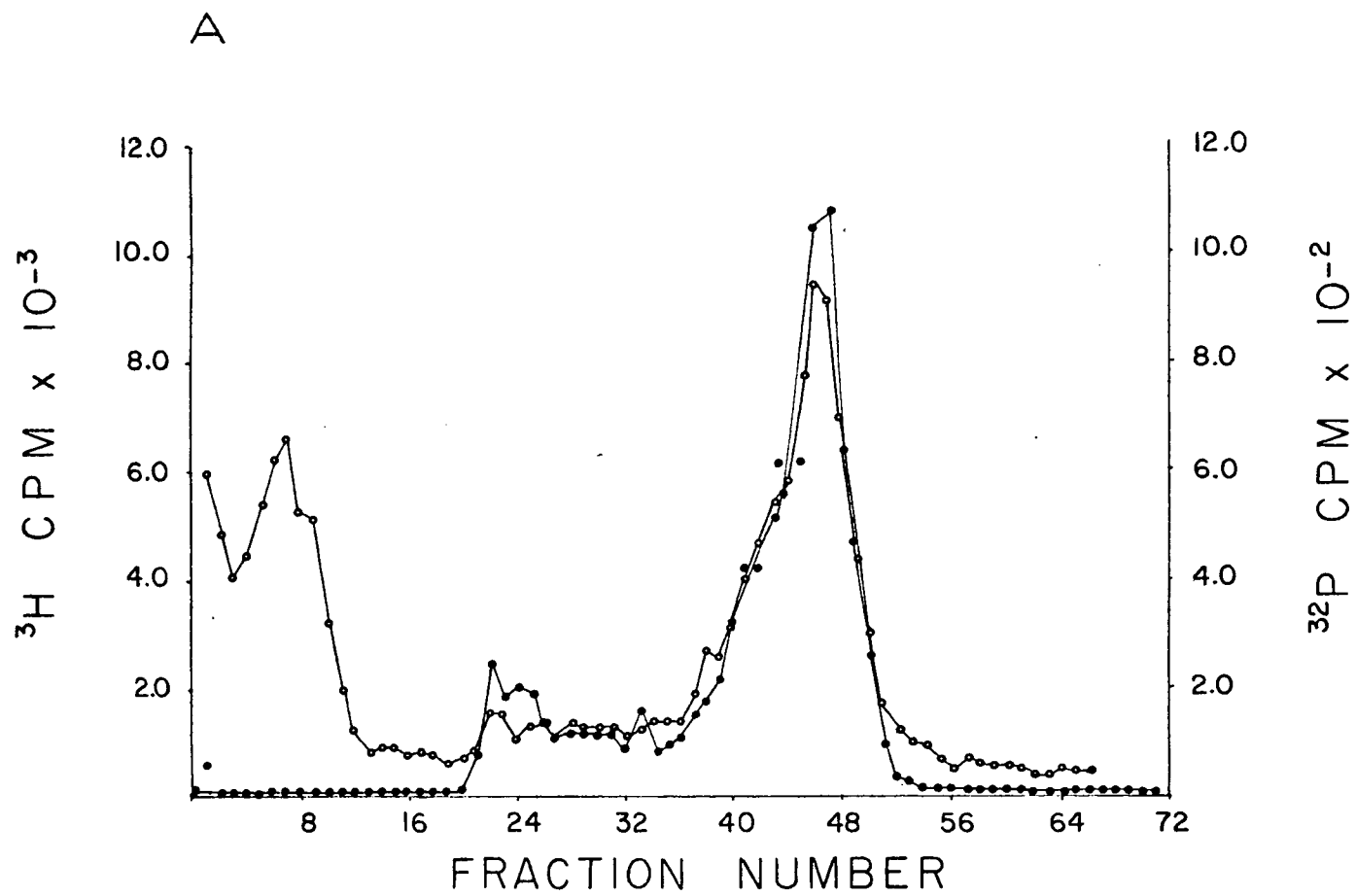
The DNA preparations were used to compare the chemical and physical stability of the pulse-labelled and uniformly-labelled DNA samples (Table 13). ^{32}P -labelled ϕ W-14 DNA served as a control. Both ^{32}P and ^3H label were resistant to the action of pancreatic RNase which was employed during the 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 solubilized when incubated in S1 buffer,

FIGURE 26.--CsCl density analysis of ^{32}P -pulse-labelled ØW-14 DNA.

A) Native DNA. B) Heat treated DNA.

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

$[6\text{-}^3\text{H}]\text{-uracil}$ labelled DNA, ●; $^{32}\text{PO}_4$ pulse labelled DNA, ○.



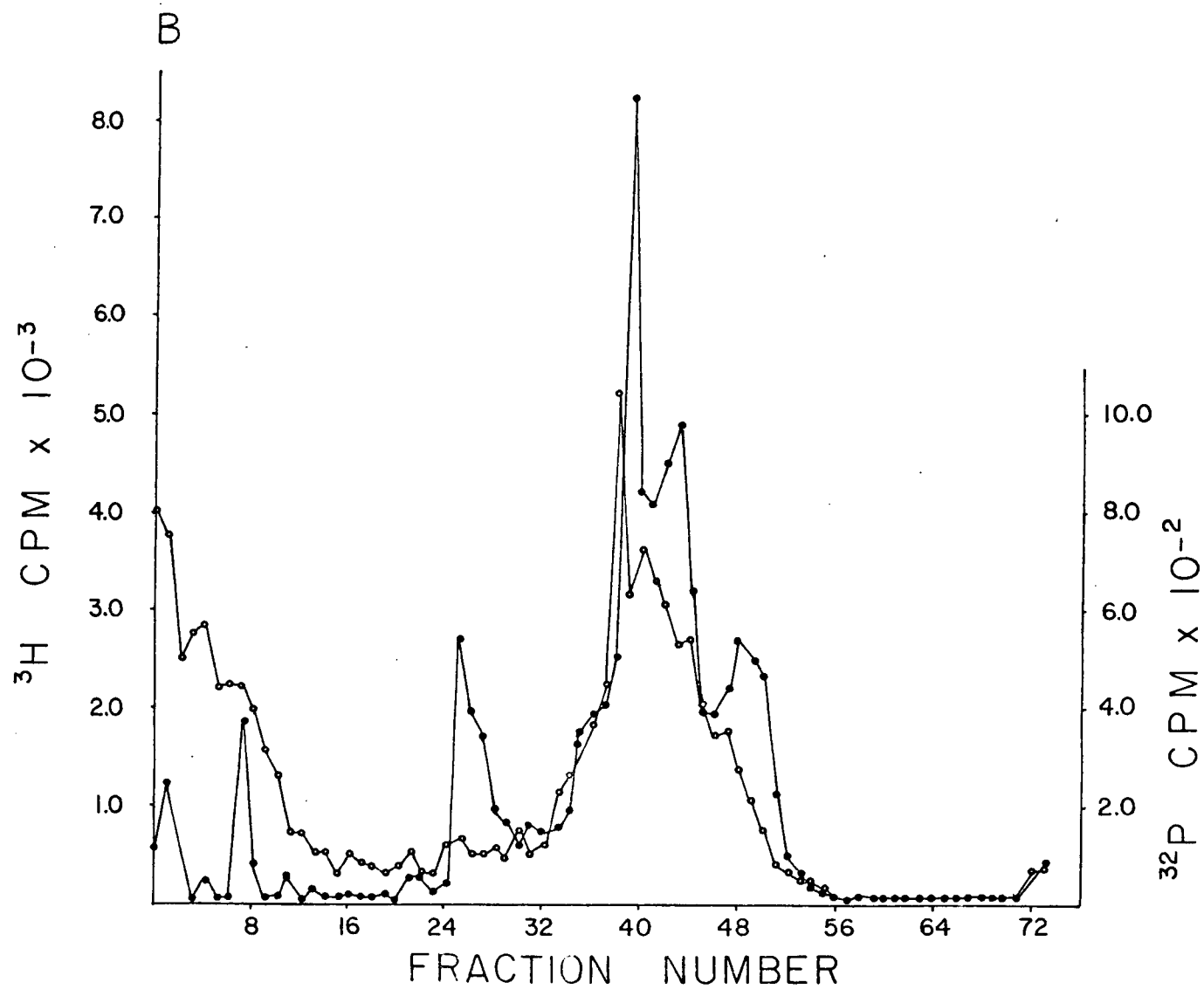


TABLE 13.--The relative chemical lability of pulse-labelled DNA.

[6-³H]-Ura-labelled ØW-14 DNA was extracted from cells 35 minutes after infection. ³²PO₄ was used to pulse label ØW-14 infected P. acidovorans for 10 seconds at 35 minutes after infection. ³²P-labelled ØW-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.

[6- ³ H]-Ura uniformly labelled ØW-14 DNA		³² PO ₄ pulse-labelled ØW-14 DNA		³² PO ₄ uniformly labelled ØW-14 virion DNA	
Treatment	CPM (³ H/ ³² P)	% recovery (³ H/ ³² P)	CPM (³² P)	% recovery	
<u>dH₂O</u>					
(0 min.)	4008/478	90/81	1756	98	
(30 min.)	3581/387		1707		
<u>0.3 N NaOH</u>					
(0 min.)	3733/409	95/91	1816	95	
(30 min.)	3535/370		1713		
<u>0.05 M Na acetate, 0.3 M NaCl, pH 4.5</u>					
(0 min.)	5548/923	100/70	-	-	
(120 min.)	5567/642		-		

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 ØW-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 ØW-14 DNA uniformly labelled with $^{32}\text{PO}_4$ and $[6\text{-}^3\text{H}]\text{-uracil}$ 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 ØW-14 DNA.

A) The DNA was labelled uniformly with $[6-^3\text{H}]\text{-uracil}$, ●; for 20 minutes and then pulse labelled with $^{32}\text{PO}_4$, ○; for 10 seconds.

B) The virion DNA was uniformly labelled with $[6-^3\text{H}]\text{-uracil}$, ●; and with $^{32}\text{PO}_4$, ○.

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.

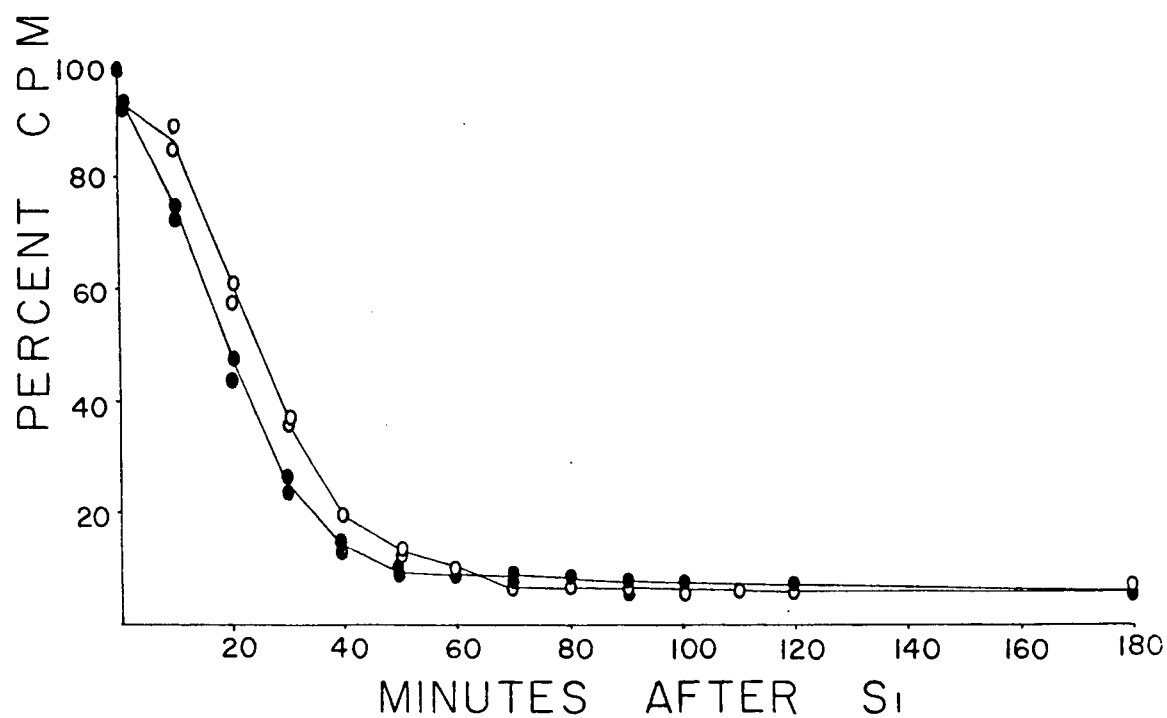
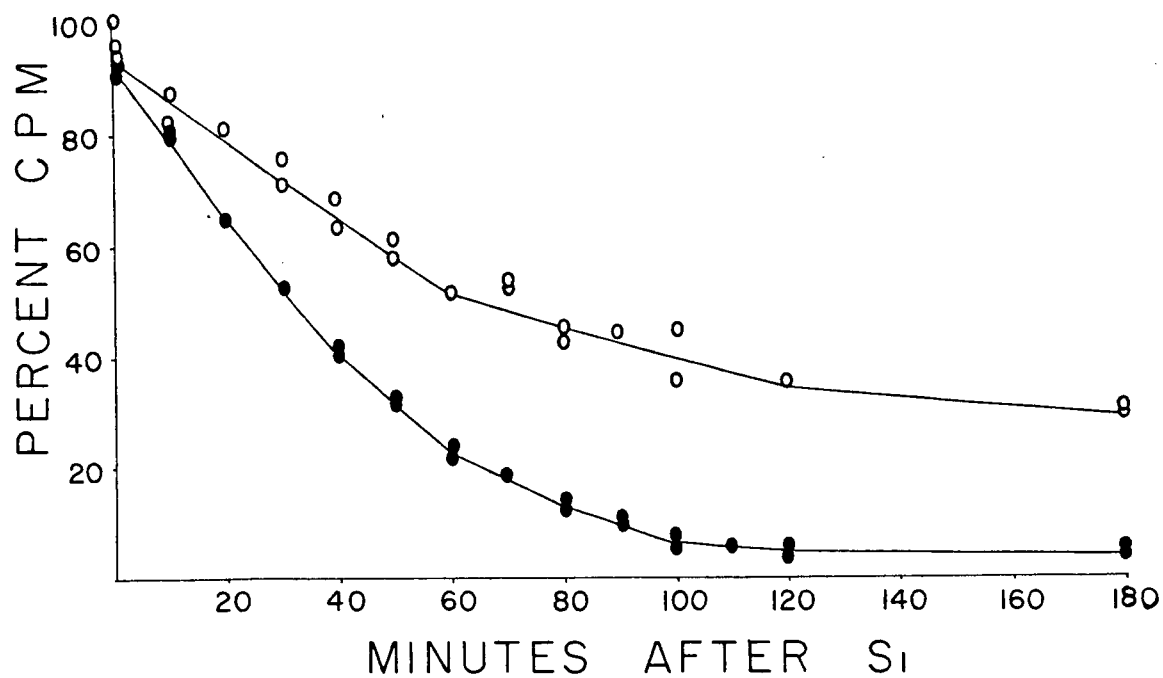


FIGURE 28.--Two dimensional thin-layer chromatography of ^{32}P -pulse-labelled ØW-14 DNA digests.

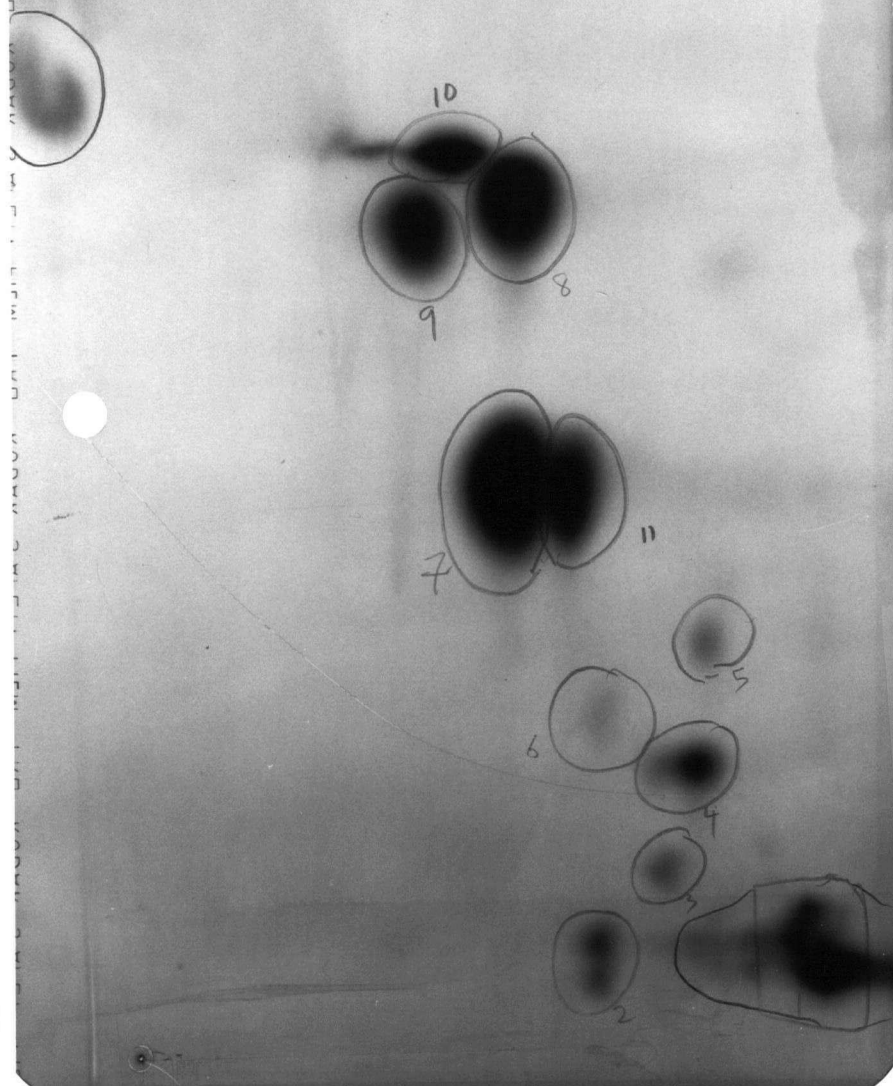
A) PEI-cellulose thin-layer chromatography of ^{32}P -pulse-labelled ØW-14 DNA digested sequentially with nuclease S1 and snake venom phosphodiesterase.

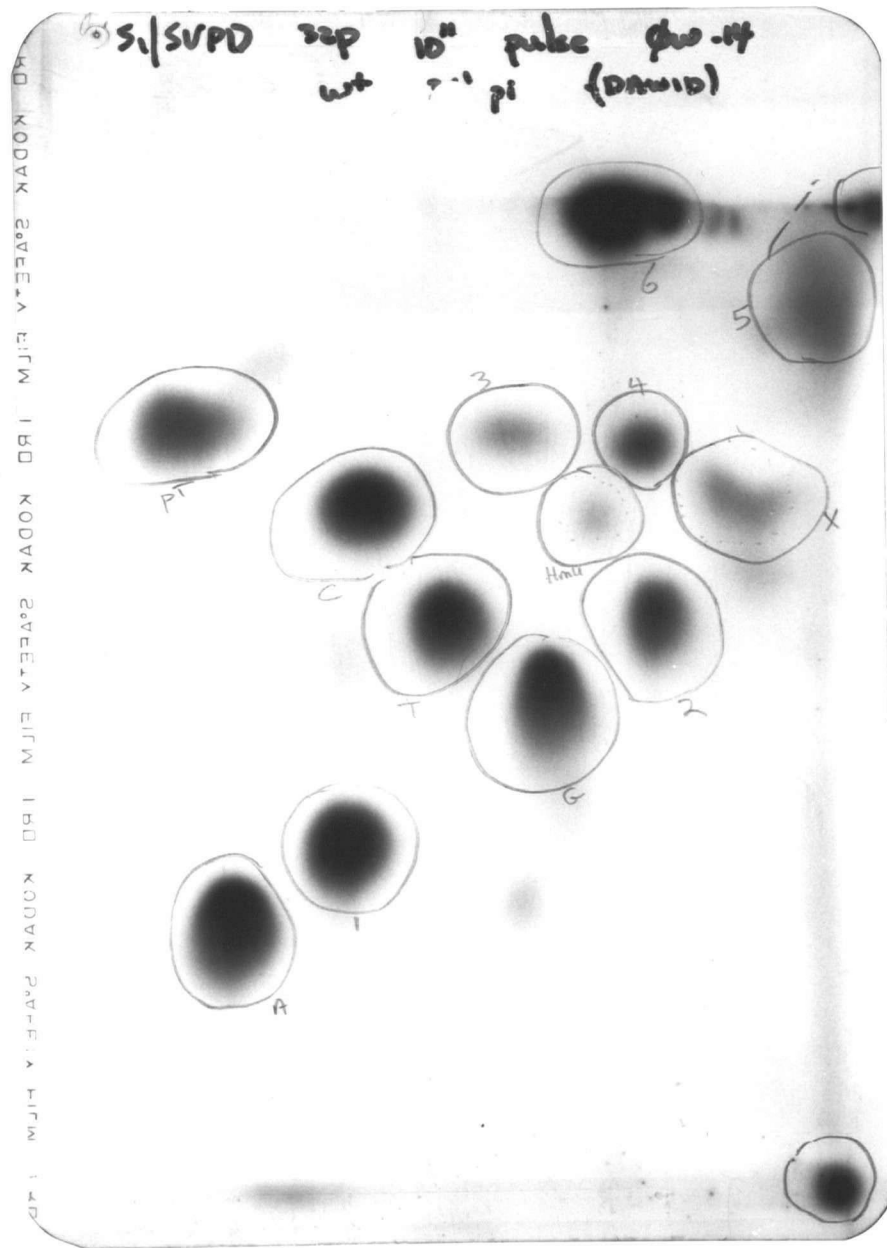
B) Cellulose thin-layer chromatography of ^{32}P -pulse-labelled ØW-14 DNA digested sequentially with nuclease S1 and SVPD.

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

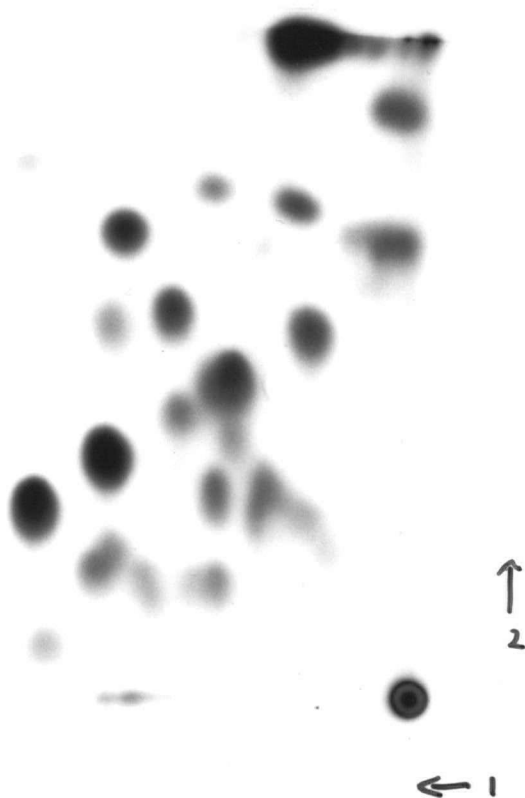
D) Cellulose thin-layer chromatography of ^{32}P -uniformly-labelled ØW-14 and strain 29 DNA digested with nuclease S1 for 2 hours at 55° . The amount of DNA per sample was $50\text{ }\mu\text{g ml}^{-1}$ in 0.05 M NH_4 -acetate pH 5.0, 10^{-4} M ZnCl_2 and with 10 units of S1 per μg of DNA. The digestion and chromatography procedures are described in detail in the Materials and Methods. The numbers on autoradiograms A and B refer to Table 12.

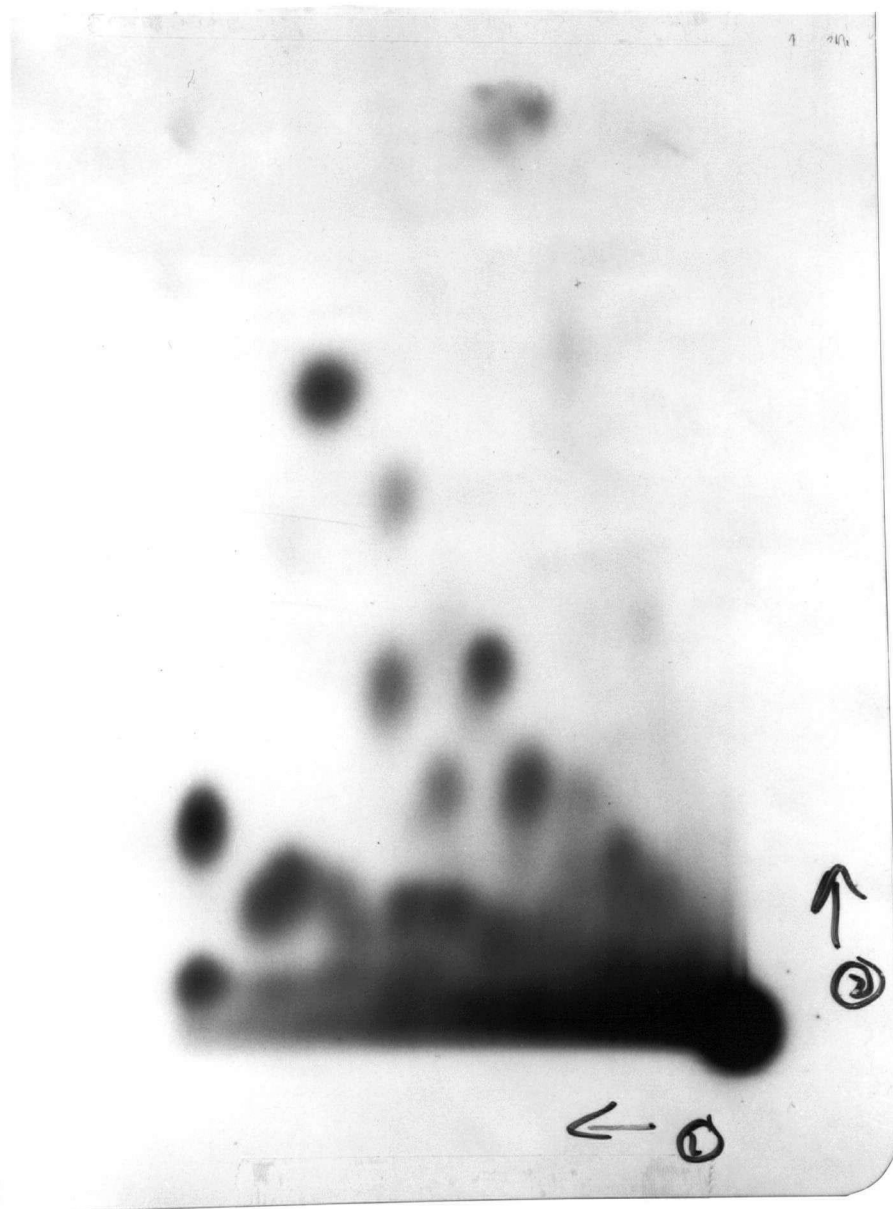
10" 32p pulse Φ W-14 wt
35' pi - RANDEARTH TOTH ⑦





S₁ only 32p 10" pulse ØW-19 wt 35' pi (smio)





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 ØW-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 ØW-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 ØW-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 ØW-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 ØW-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 $^{32}\text{PO}_4$ was also released from DNA digests in much larger amounts than from control S1 and snake venom phosphodiesterase digests of ϕ W-14 DNA. The release of $^{32}\text{PO}_4$ was a consequence of the S1 hydrolysis conditions since release of $^{32}\text{PO}_4$ 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 ϕ 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}P -pulse labelled DNA was lower than that predicted from the $[6\text{-}^3\text{H}]\text{-uracil}$ 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 $[6\text{-}^3\text{H}]\text{-uracil}$ pulse labelled $\phi\text{W-14}$ DNA sample. Uniform labelling of nucleotides in the pulse experiments was unlikely. $^{32}\text{PO}_4$ -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 $\phi\text{W-14}$ DNA synthesis and modification

The major purpose of the inhibitor studies described so far in this thesis was the attempt to demonstrate that $\phi\text{W-14}$ does not utilize thymidine (dTTP) for DNA synthesis. Several other inhibitors were tested in the hope that they might have specific effects upon $\phi\text{W-14}$ DNA replication or modification.

Two inhibitors of ornithine decarboxylase were tested. Ornithine is a precursor of the putresciny 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 ØW-14 DNA. Neither α -methylornithine nor difluoromethylornithine inhibited the growth of P. acidovorans or the ability of the treated cells to support ØW-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 $\mu\text{g ml}^{-1}$. The effect of addition of chloramphenicol upon ØW-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 ØW-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 $\mu\text{g ml}^{-1}$, 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 ØW-14.

Chloramphenicol was added to one-half of a culture at 20 minutes after infection. The incorporation of $[6-^3\text{H}]\text{-uracil}$ into alkali resistant, TCA precipitable material was followed. P. acidovorans strain 29 infected with ØW-14, ●; P. acidovorans strain 29 infected with ØW-14 and treated with chloramphenicol ($100\text{ }\mu\text{g ml}^{-1}$), O. The incorporation of radioactivity is expressed as the percentage of input label which becomes alkali resistant and TCA precipitable.

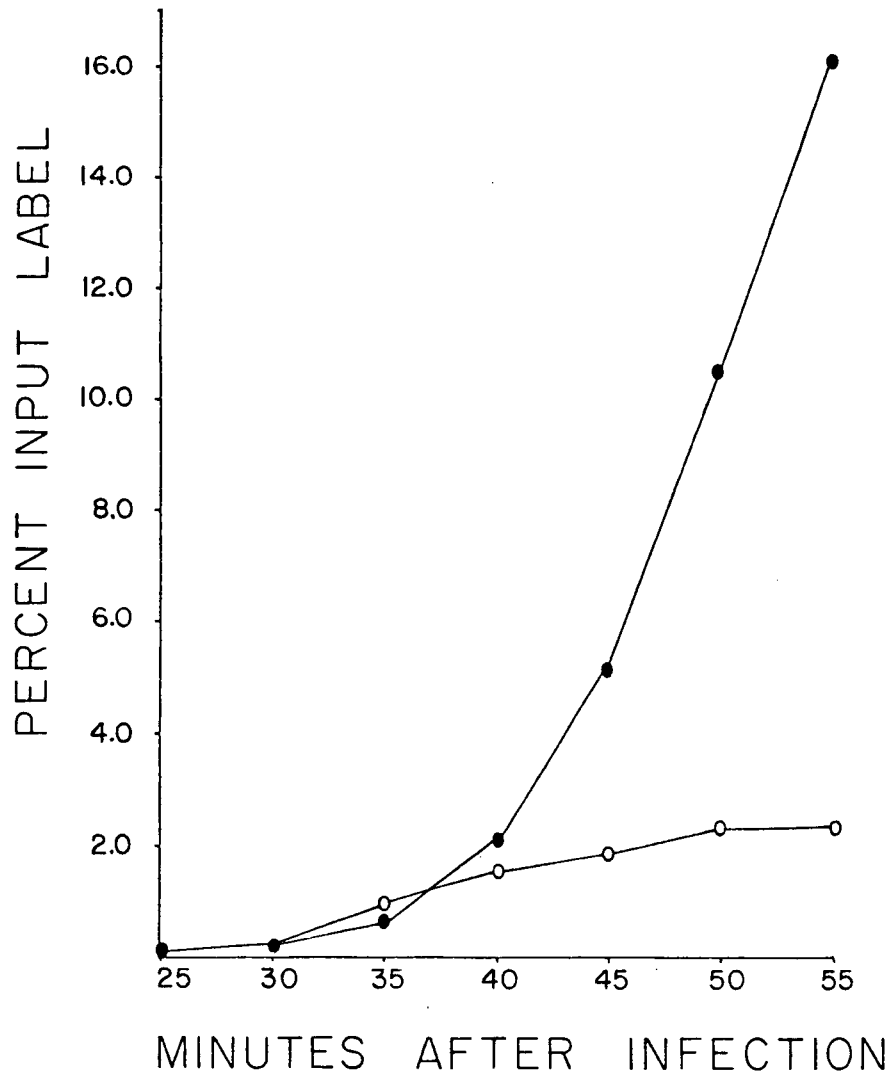


FIGURE 30.--The effect of Netropsin upon the growth of P. acidovorans strain 29.

The cells were grown in TCS medium supplemented with succinate and Casamino acids. Netropsin ($100\text{ }\mu\text{g ml}^{-1}$) 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 ØW-14 at 240 minutes, netropsin treated, Δ.

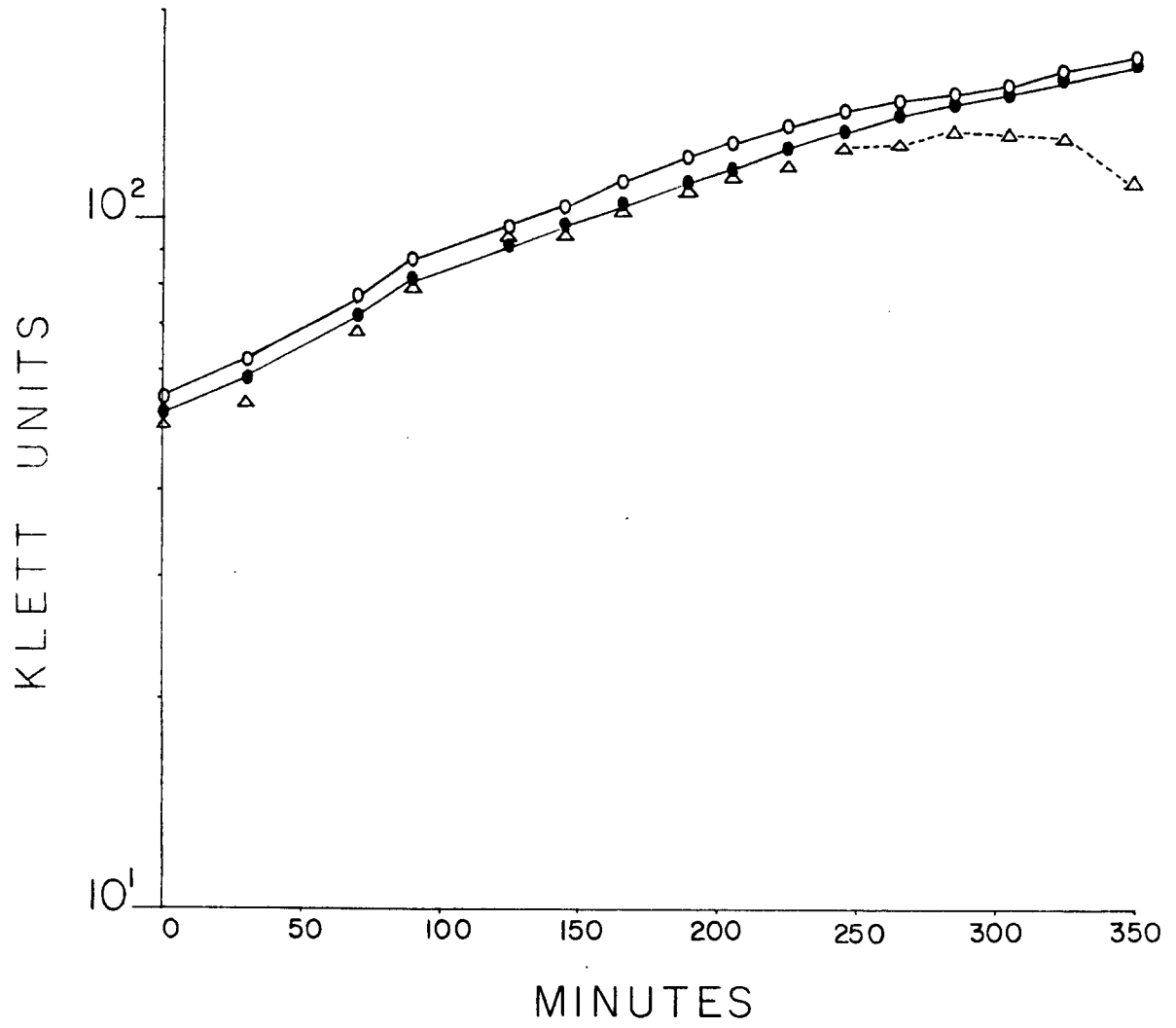
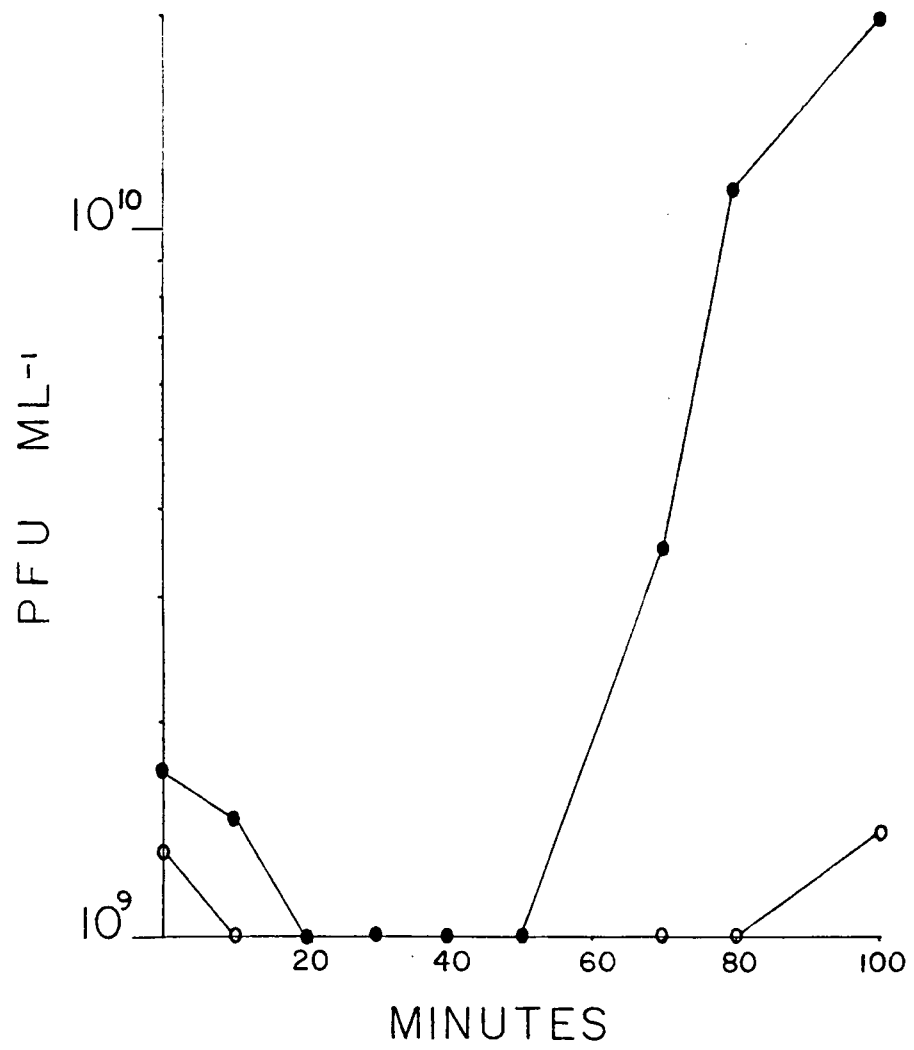


FIGURE 31.--Plaque production in netropsin-treated P. acidovorans strain 29 infected with ØW-14.

A culture of growing cells was infected with ØW-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. ØW-14 infected P. acidovorans strain 29, no additions, ●; ØW-14 infected P. acidovorans strain 29, with netropsin ($100 \mu\text{g ml}^{-1}$), ○.



(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.

ØW-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 ØW-14 DNA were constant and could not be manipulated (K.L. Maltman, unpublished observations).

Seventy temperature-sensitive mutants of ØW-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 ØW-14 DNA extracted from P. acidovorans strain 29 cells treated with netropsin.

Netropsin ($100 \mu\text{g ml}^{-1}$) was added to a growing culture of cells which were then infected with ØW-14. At 25 minutes after infection [$6\text{-}^3\text{H}$]-uracil ($1.0 \mu\text{Ci ml}^{-1}$, $10.0 \mu\text{g ml}^{-1}$) 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. ØW-14 DNA synthesized in the presence of netropsin, ●; ^{32}P -labelled ØW-14 reference DNA, ○.

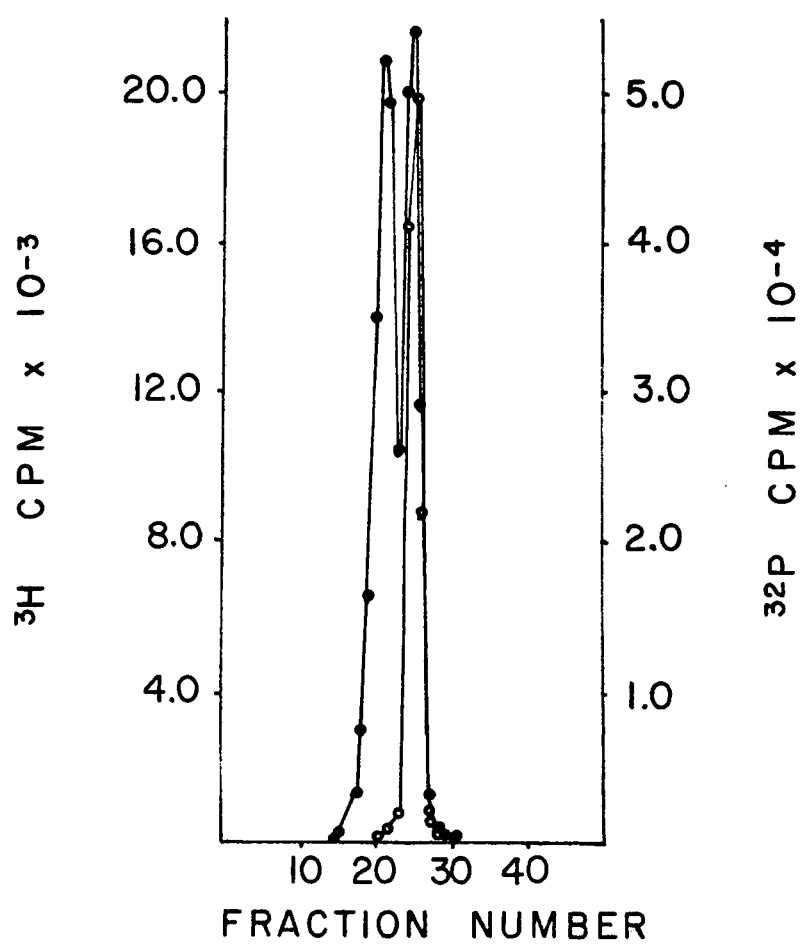


TABLE 14.--The base composition of ØW-14 DNA made in netropsin-treated cells.

Base	cpm in base	fraction of total cpm
putThy	25408	(0.22)
Cyt	55302	(0.49)
HmUra	233	(<0.01)
Ura	1947	(0.02)
Thy	30672	(0.27)
unhydrolyzed nucleotides	86	(<0.01)

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 ØW-14 DNA was described in Figure 32.

different ØW-14 genes. The complementation analysis and grouping is shown in Table 15.

P. Miller has isolated forty-two amber mutants from mutagenized ØW-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).

ØW-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 ØW-14 ts mutants.

Mutant	Group size	Complementation group	<u>ts</u> index	Bases in DNA				% survivors
				putThy	Thy	Hmu	Ura	
1a	1	1	1.5×10^5	nt	nt	nt	nt	33.0
1b	1	2	6.0×10^3	+	+	-	-	21.0
2a	-	-	1.3×10^6	+	+	-	±	14.6
2b	1	3	2.5×10^6	+	+	±	-	33.0
2c	4	4	8.5×10^4	+	+	-	-	27.3
2e	1	5	6.4×10^4	+	+	-	-	<0.1
6a	2	6	$>10^5$	+	+	-	-	14.0
6b	1	7	8.5×10^3	+	+	-	-	12.3
9d	1	8	8.4×10^4	+	+	-	-	10.7
11a	1	9	6.3×10^5	+	+	-	-	<0.1
12a	1	10	4.5×10^4	+	+	-	-	<0.1
19a	4	4	$>10^5$	+	+	-	-	10.0
21b	1	11	4.1×10^5	+	+	-	-	<0.1
24a	1	12	7.2×10^6	+	+	-	-	<0.1
27a	1	13	leaky	+	+	-	-	1.3
29a	-	-	leaky	+	+	-	-	5.9
30a	-	-	leaky	+	+	-	-	16.6
34a	1	14	leaky	+	+	-	-	50.0
A1b	1	15	$>10^4$	+	+	-	-	50.0
A4a	2	16	3.8×10^4	+	+	-	-	2.8
A4c	2	16	5.1×10^6	+	+	-	-	1.1
A4d	1	17	$>10^5$	nt	nt	nt	nt	nt
A4f	1	18	9.5×10^5	+	+	-	-	5.7
A5a	1	19	leaky	+	+	-	-	0.8
A11a	2	6	$>10^3$	nt	nt	nt	nt	nt
A12a	4	4	$>10^3$	nt	nt	nt	nt	nt
A12c	1	20	nt	nt	nt	nt	nt	nt
A17b	4	4	5.0×10^5	+	+	-	-	6.0
A18c	2	21	$>10^5$	+	+	-	-	2.3

TABLE 15.--Continued

Mutant	Group size	Complementation group	<u>ts</u> index	Bases in DNA				% survivors
				putThy	Thy	Hmu	Ura	
A18d	1	22	$>10^4$	nt	nt	nt	nt	nt
A20c	2	21	$>10^5$	nt	nt	nt	nt	nt
A21b	1	23	$>10^5$	nt	nt	nt	nt	nt
A22a	-	-	$>10^5$	nt	nt	nt	nt	nt
B5a	-	-	$>10^4$	+	±	-	-	3.8

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 [6-³H]-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.

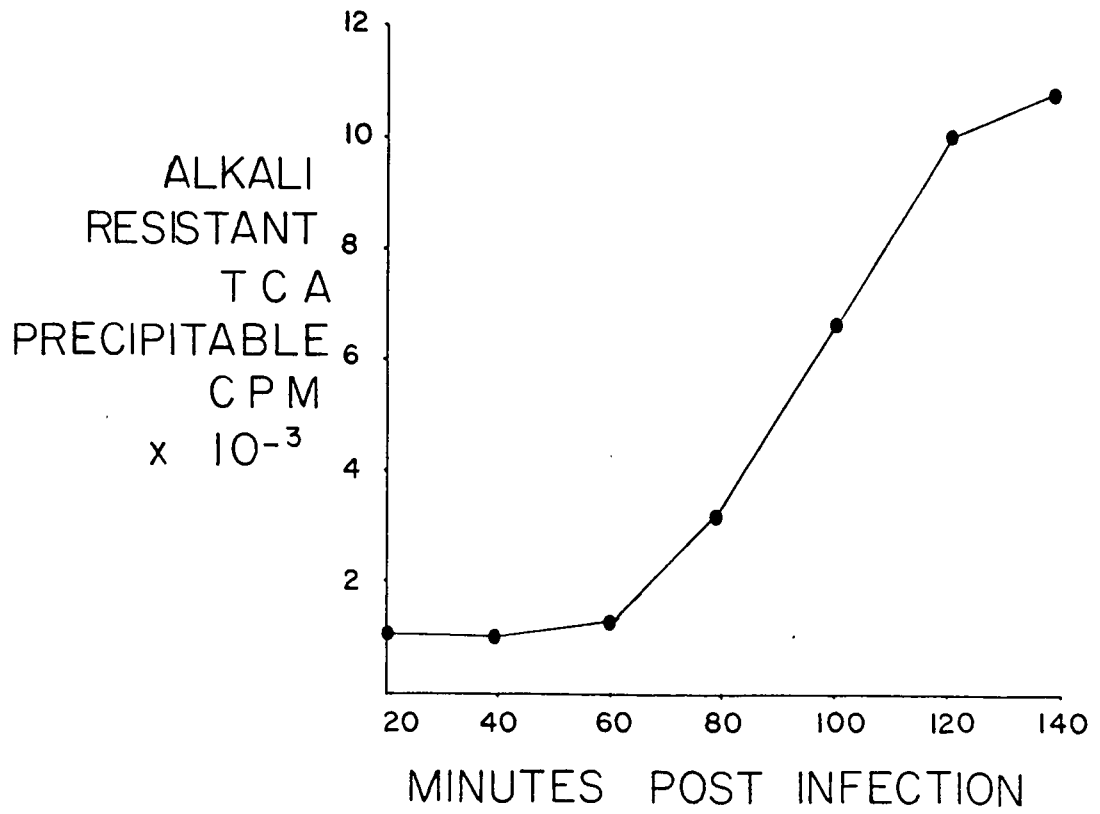
Ts index is the ratio of plaque forming units in a lysate at 20° compared to the number of plaque forming units in the same lysate at 30°. [6-³H]-uracil-labelled bases detected in acid hydrolysates of DNA were scored (+), bases not labelled by [6-³H]-uracil were scored (-), bases present in marginal 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 ØW-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.



[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 ØW-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 ØW-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. [6-³H]-uracil labelled DNA, ●; ³²PO₄ labelled ØW-14 w⁺ phage reference DNA, ○.

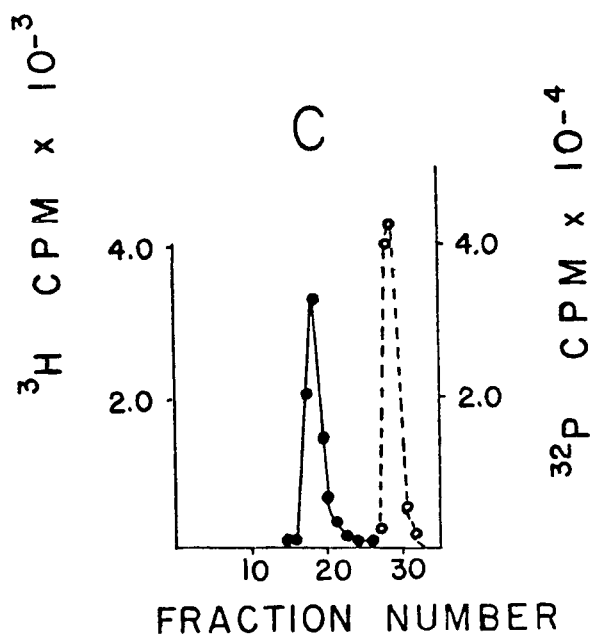
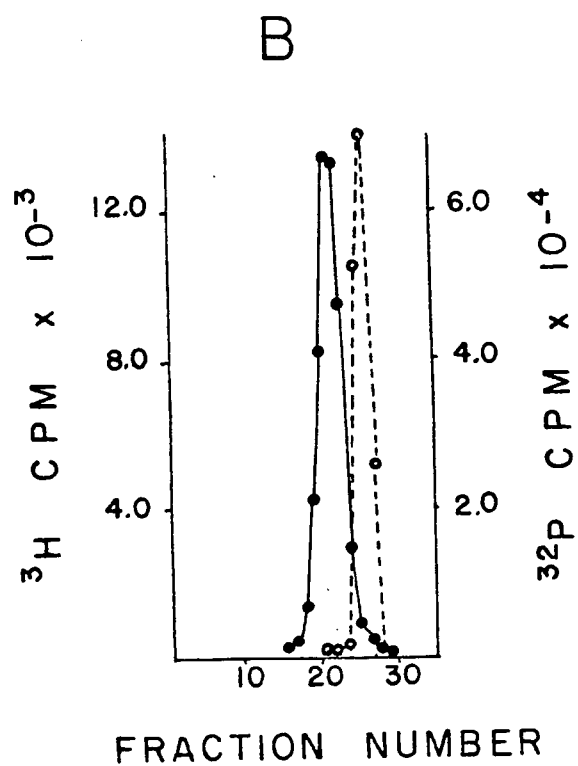
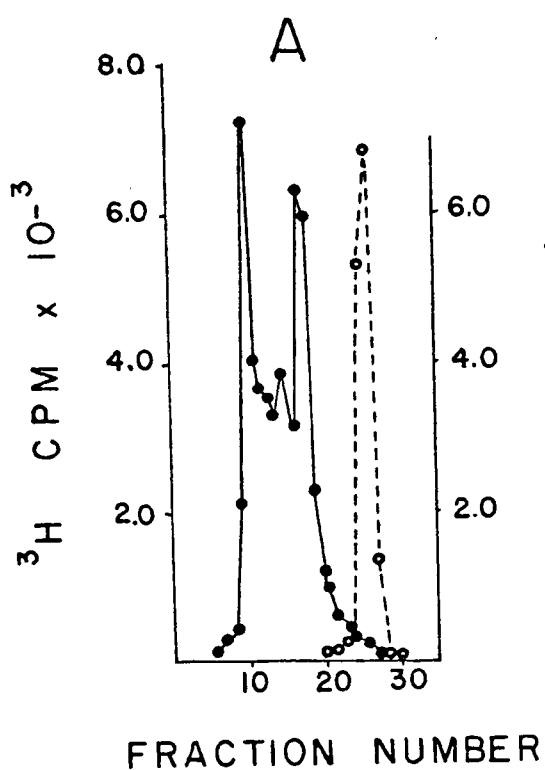


TABLE 16.--The properties of DNA extracted from ØW-14 am-infected P. acidovorans.

P. acidovorans strain 29 was infected with ØW-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.

<u>am</u> mutant	Bases detected in DNA				DNA detected in CsCl			sup index	% survivors
	putThy	Thy	Hmu	Ura	host	ØW-14	other		
2	+	+	-	-	-	+	-	$>10^6$	1.3
3	+	+	-	-	+	+	-	$>10^5$	<1.0
4	+	+	-	-	-	+	-	$>10^5$	8.8
5	+	+	-	-	-	+	-	$>10^6$	0.9
6	-	-	-	-	-	-	-	-	-
7	+	+	-	-	-	+	-	$>10^5$	0.9
8	+	+	-	-	-	+	-	1.6×10^3	0.6
10	+	+	-	-	-	+	-	$>10^5$	0.4
11	+	+	-	-	-	+	-	$>10^5$	<1.0
12	+	+	-	-	+	+	-	$>10^6$	4.9
14	+	+	-	-	-	+	-	$>10^5$	<1.0
15	+	+	-	-	-	+	-	$>10^5$	<1.0
16	+	+	-	-	-	+	-	$>10^6$	0.2
17	+	+	-	-	-	+	-	$>10^5$	<1.0
18	+	+	-	-	-	+	-	$>10^6$	0.5
19	+	+	-	-	-	±	-	$>10^6$	0.4
20	+	+	-	-	nt	nt	nt	$>10^6$	0.6
21	+	+	-	-	+	+	-	1.5×10^5	19.0
22	+	+	-	-	+	+	-	$>10^5$	7.0
23	+	+	-	-	+	+	-	1.4×10^5	9.0
24	+	+	-	-	+	+	-	leaky	>50
25	+	+	-	-	+	+	-	leaky	>50

<u>am</u> mutant	Bases detected in DNA				DNA detected in CsCl			sup index	% survivors
	putThy	Thy	Hmu	Ura	host	ØW-14	other		
26	+	+	-	-	+	±	-	$>10^6$	14.0
27	+	+	-	-	±	+	-	$>10^6$	9.0
28	+	+	-	-	-	+	-	$>10^5$	1.0
29	+	+	-	-	+	+	-	$>10^6$	9.0
30	+	+	-	-	-	+	-	$>10^6$	1.0
31	+	+	-	-	-	+	-	$>10^6$	1.0
32	+	+	-	-	-	+	-	$>10^6$	1.0
33	+	+	-	-	-	+	-	2.5×10^6	1.0
34	+	+	-	-	-	+	-	3.2×10^5	1.0
35	-	+	-	-	+	-	-	$>10^6$	1.0
36	±	+	-	-	+	+	-	leaky	1.0
37	-	+	+	-	+	-	+	2.3×10^5	1.0
38	+	+	-	-	+	+	-	$>10^6$	1.0
39	+	+	-	-	+	+	-	1.8×10^6	8.0
40	+	+	-	-	-	+	-	$>10^5$	1.0
41	+	+	-	-	-	+	-	$>10^4$	1.0
42	+	+	-	-	-	-	+	3.9×10^4	1.0
43	+	+	-	-	+	+	-	3.0×10^5	13.0
44	+	+	-	-	+	+	-	1.5×10^6	9.0
45	-	±	-	-	+	-	-	1.4×10^5	7.0

Twenty-five ts mutants were analyzed by preparation of [6-³H]-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.)

ØW-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 [³H]-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 [6-³H]-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 30°. 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 ØW-14

FIGURE 35.--The incorporation of [^3H]-ornithine in ØW-14 ts 19a-infected P. acidovorans strain 29.

The incorporation of [^3H]-ornithine into pronase resistant, TCA precipitable cpm was measured in ØW-14 ts 19a infected P. acidovorans strain 29 at 30°, ●; and at 20°, O. The procedures used are described in the Materials and Methods. Lysis of the 30° culture occurred at 90 to 100 minutes after infection.

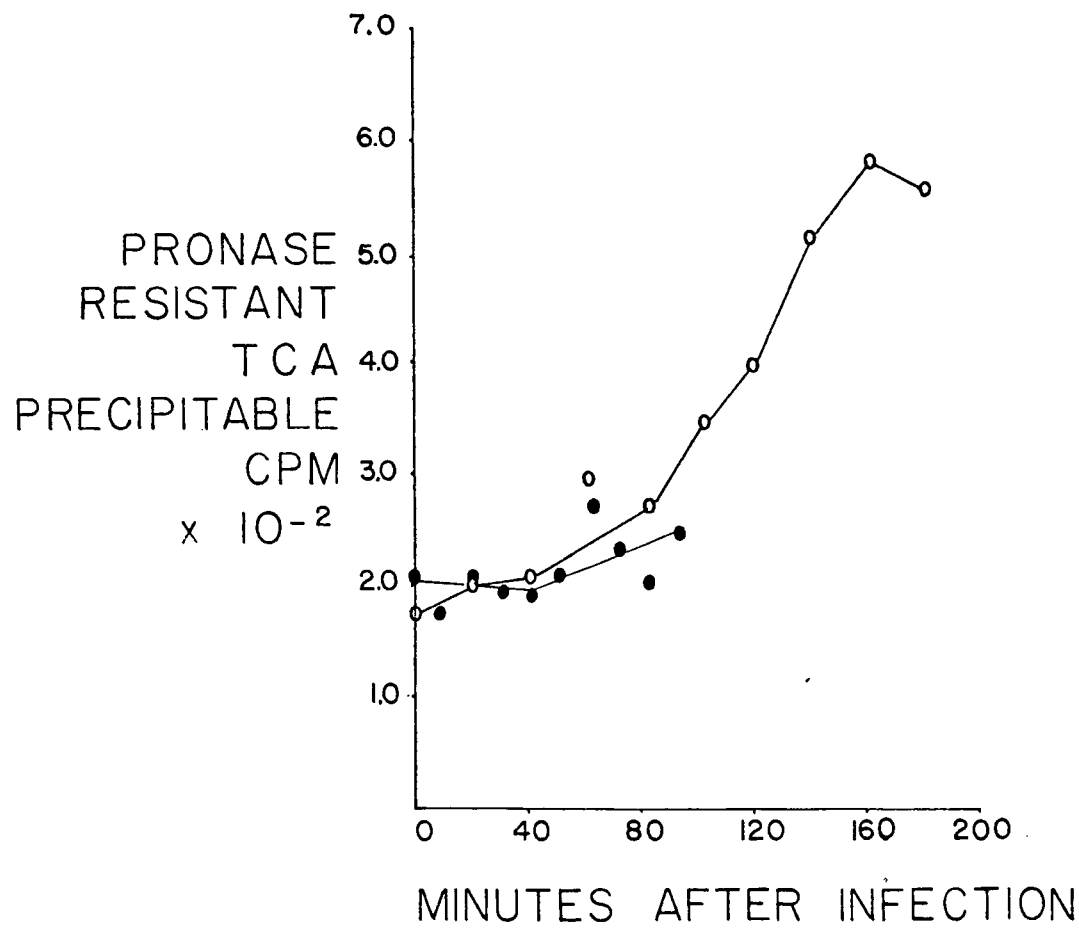


FIGURE 36.--DNA synthesis in ØW-14 ts 19a-infected P. acidovorans strain 3L.

The incorporation of [6-³H]-uracil into alkali resistant, TCA precipitable cpm was determined for ØW-14 ts 19a infected cells at 30°, ○; 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.

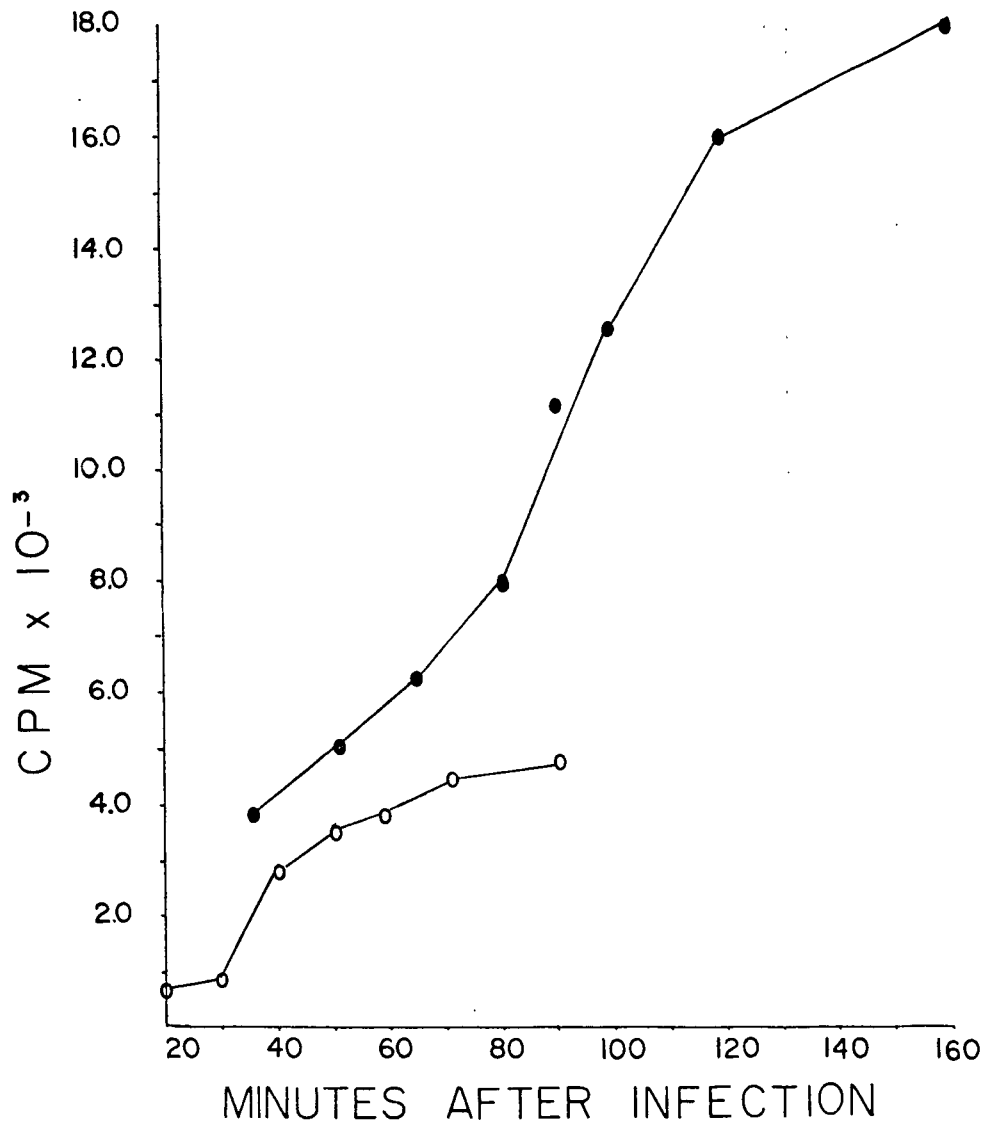


FIGURE 37.--The buoyant density of DNA made in ϕ W-14 ts 19a-infected P. acidovorans strain 29.

[6-³H]-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.

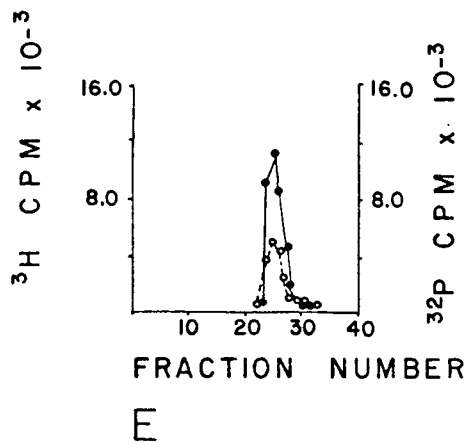
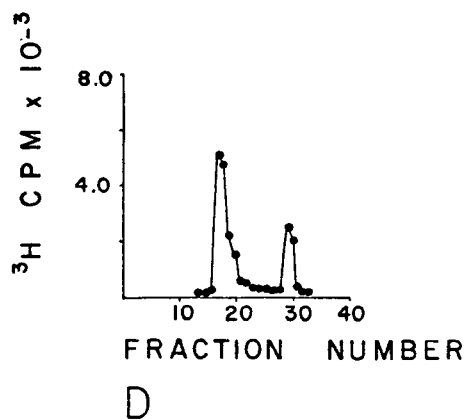
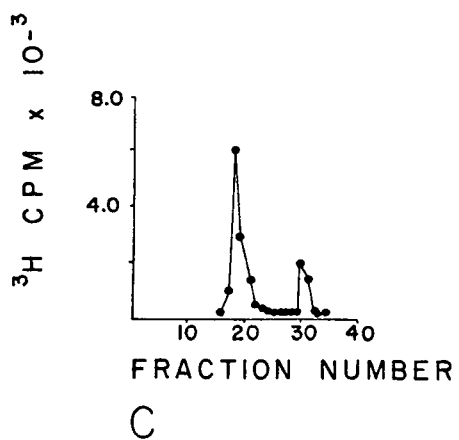
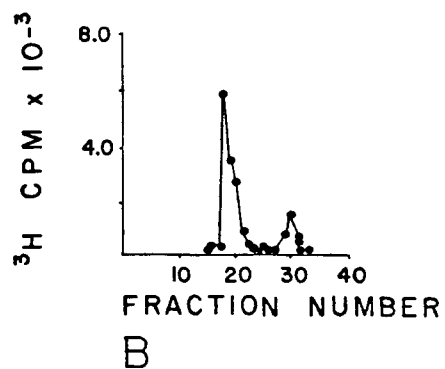
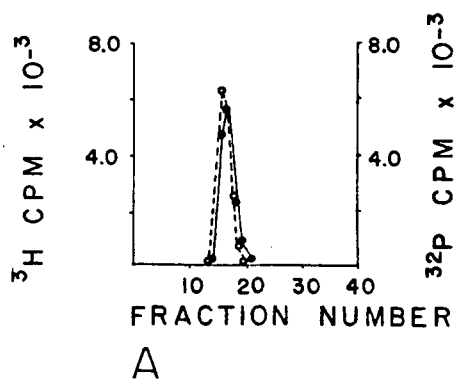
C) The cells were infected at 30° and shifted to 20° at 40 minutes after infection. The DNA was extracted at 75 minutes after infection.

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 ϕ W-14 ts 19a virions.

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

Gradient A includes ³²P-labelled host reference DNA, ○; Gradient E includes ³²P-labelled phage reference DNA, ○.



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). ØW-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.-- ϕ 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 ϕ W-14 ts 19a. The infected culture was incubated at 30°. At 10 minutes after infection [6-³H]-uracil (0.25 μ Ci ml⁻¹, 10 μ g ml⁻¹) was added to the culture. At 35 minutes after infection one-half of the culture was shifted to 20°. DNA synthesis was measured by following the incorporation of radioactivity into alkali resistant, TCA precipitable material. 30°, ●; 30° downshifted to 20°, ○.

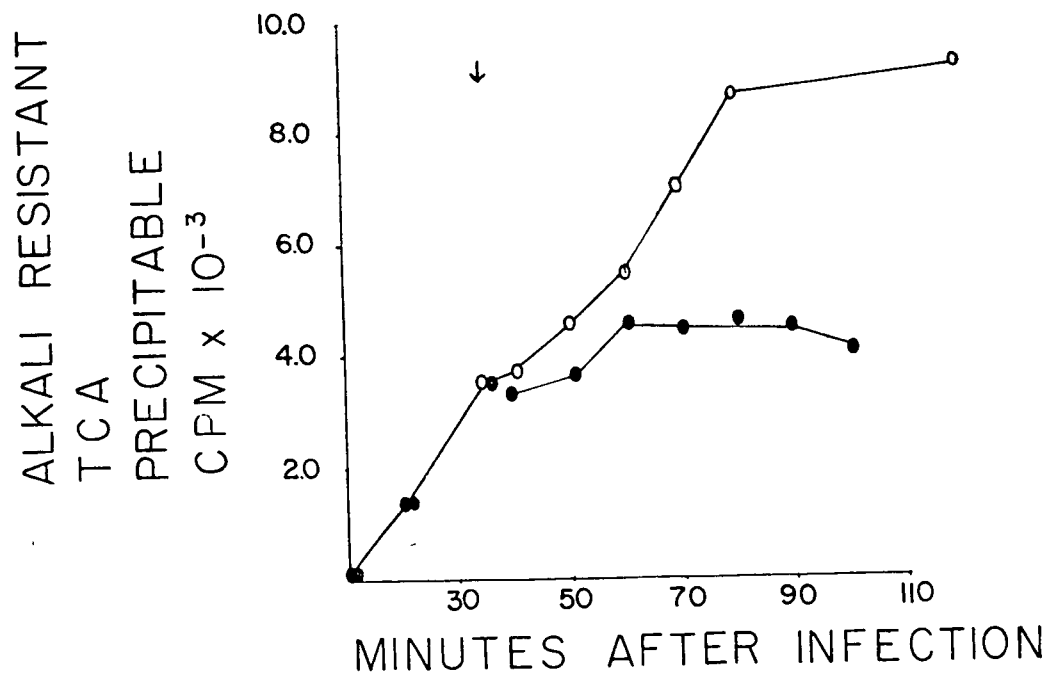


TABLE 17.--The Base composition of DNA made in ØW-14 ts 19a-infected P. acidovorans.

Base	DNA sample		
	35 minutes/30°	90 minutes/20°	35 minutes/30° shifted to 20° for 30 minutes
putThy	407 (<0.01) ^a	32162 (0.18)	10306 (0.05)
Cyt	29799 (0.58)	104744 (0.59)	115418 (0.57)
Thy	21373 (0.41)	39802 (0.23)	77772 (0.38)
Hmu	310 (<0.01)	-	519 (<0.01)
Ura	221 (<0.01)	-	715 (<0.01)

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.

- a 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 ØW-14 am 42-infected P. acidovorans.

The incorporation of [6-³H]-uracil ($1.0 \mu\text{Ci ml}^{-1}$, $10.0 \mu\text{g ml}^{-1}$) 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 CHCl_3 treated lysates.

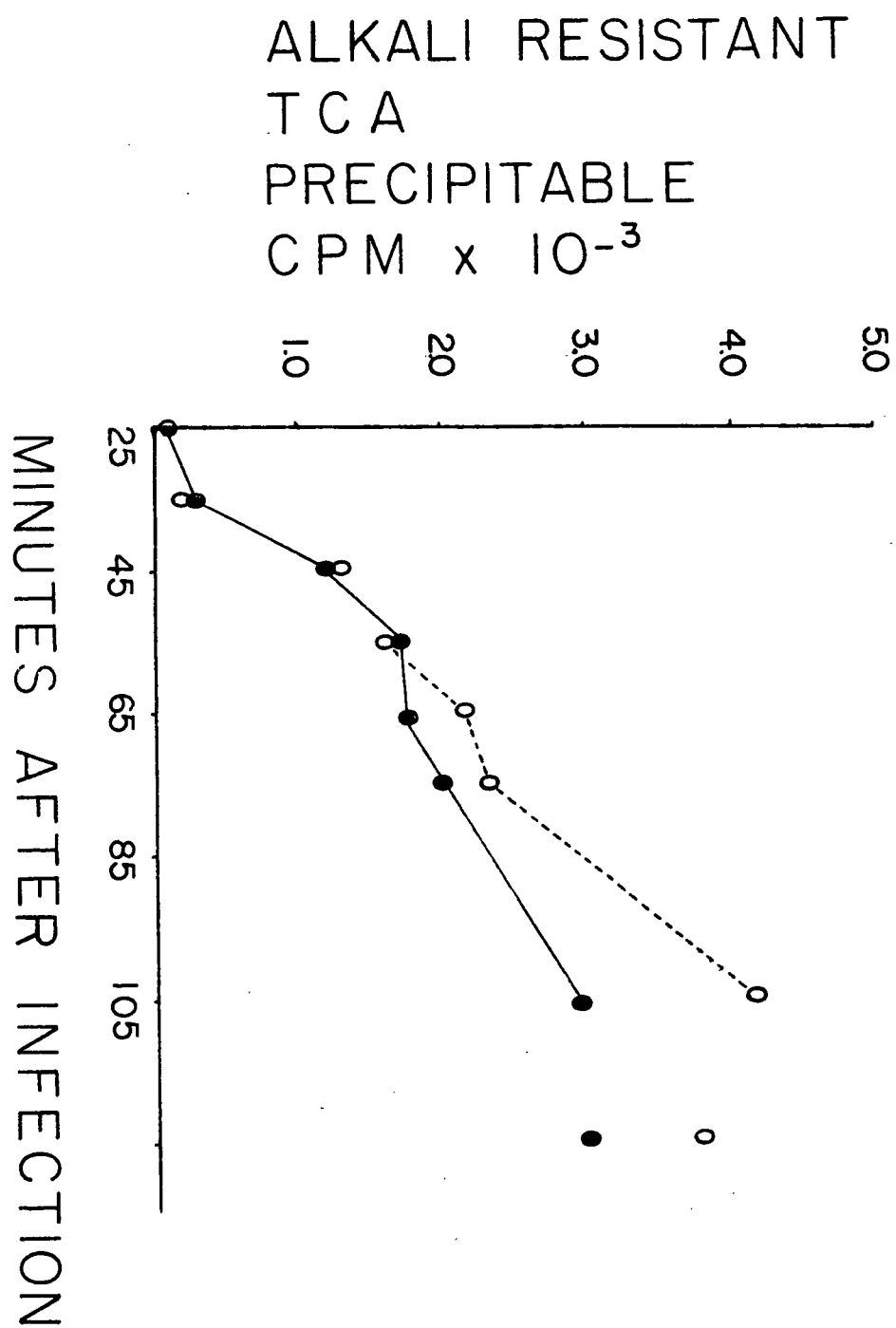


TABLE 18.--The nucleotide composition of ØW-14 am 42 DNA.

Nucleotide	ØW-14 w ⁺ DNA	ØW-14 <u>am</u> 42 DNA
putdTMP	6904 (0.25)	4001 (0.07)
dCMP	14007 (0.50)	29812 (0.52)
dTMP	7219 (0.25)	23639 (0.41)
hmdUMP	none	none
putdTMP/dTMP	0.96	0.17

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 putdTTP and elevated levels of dTTP. The single peak on the CsCl gradient ruled out the possibility that the increase in dTTP 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 ØW-14 DNA are required for ØW-14 viability.

ØW-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 ($\rho = 1.72 \text{ gcc}^{-1}$). ³²PO₄ phage reference DNA ($\rho = 1.666 \text{ gcc}^{-1}$) marks the position where normally-modified ØW-14 should have banded. Two peaks ($\rho = 1.73$ 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). ØW-14 w⁺ plates with high efficiency on

FIGURE 40.--Two-dimensional thin-layer chromatography of [6-³H]-uracil-labelled nucleotides present in am 42 DNA prepared in P. acidovorans strain 29.

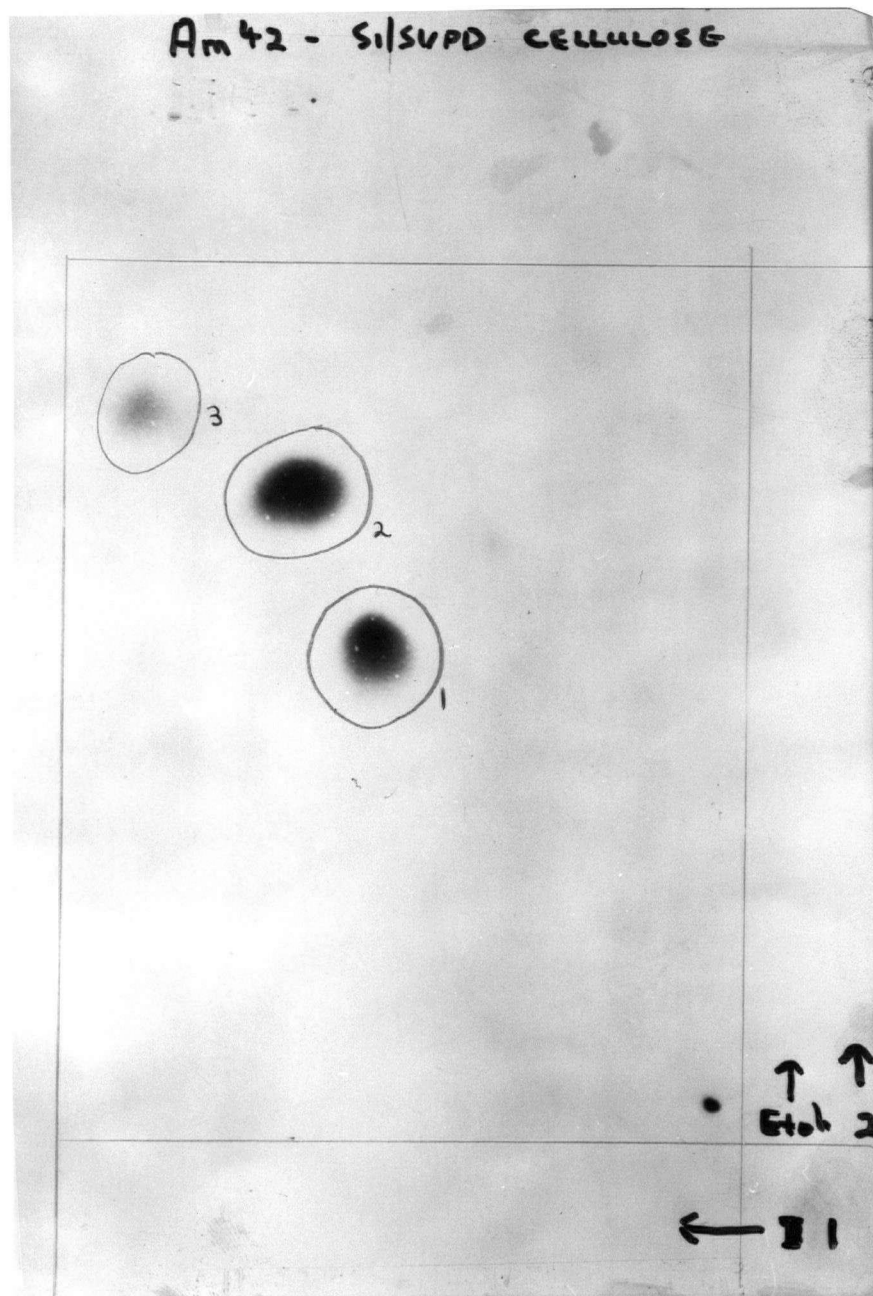


TABLE 19.--The plating efficiency of ϕ W-14 am 37 on P. acidovorans strains.

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

Lysates of ϕ W-14 w⁺ and am 37 phages were plated on various P. acidovorans strains. The values are the numbers of plaque forming units in one milliliter of a lysate. Wild type ϕ W-14 lysates were prepared on P. acidovorans strain 29. ϕ W-14 am 37 lysates were prepared on 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. P. acidovorans sup 2 was the permissive host for ϕ W-14 am 37.

TABLE 20.--Base compositions of ØW-14 and am 37 DNAs.^a

Base			Source of DNA			
	<u>am</u> 37/strain 29		<u>am</u> 37/strain <u>sup</u> ²		ØW-14/strain 29	
putThy	1074 ^b	(2.7) ^c	5062	(19.9)	14430	(18.2)
cytosine	18016	(44.5)	10539	(41.5)	34585	(43.6)
hmUra	5903	(14.6)	1028	(4.0)	157	(0.2)
uracil	1209	(3.0)	176	(0.7)	180	(0.2)
thymine	9353	(23.1)	6173	(24.3)	21976	(27.7)
unhydrolyzed nucleotides	4964	(12.3)	2441	(9.6)	7987	(10.1)

a Replicating DNA was labelled with [6-³H]-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. ØW-14 am 37 plated only on the five sup derivatives of P. acidovorans strain 29. The ØW-14 am 37 reversion frequency is less than 10^{-6} wild-type plaques formed per mutant phenotype plaque.

The burst size of 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 am 37 infected strain 29 cells. The average burst size for 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 am 37 infected cells accumulated some DNA with the same density as ØW-14 w^+ reference DNA. Strain 29 cells infected with 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 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 ØW-14 (Figure 42). These experiments suggested that a gene responsible for some aspect of ØW-14 DNA modification was mutated in am 37. Furthermore, this mutation was responsible for the conditionally lethal phenotype of am 37 phage reproduction.

Am 37 DNA contains a novel nucleotide

Quantitative acid hydrolysis and thin-layer chromatography of [6-³H]-uracil labelled DNA samples was performed (Table 20). DNA

FIGURE 41.--The reproduction of ØW-14 am 37 in 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 P. acidovorans strain sup 2. P. acidovorans was grown in TCS medium supplemented with succinate and Casamino acids. Am 37 infected strain sup 2, O; am 37 infected strain 29, ●.

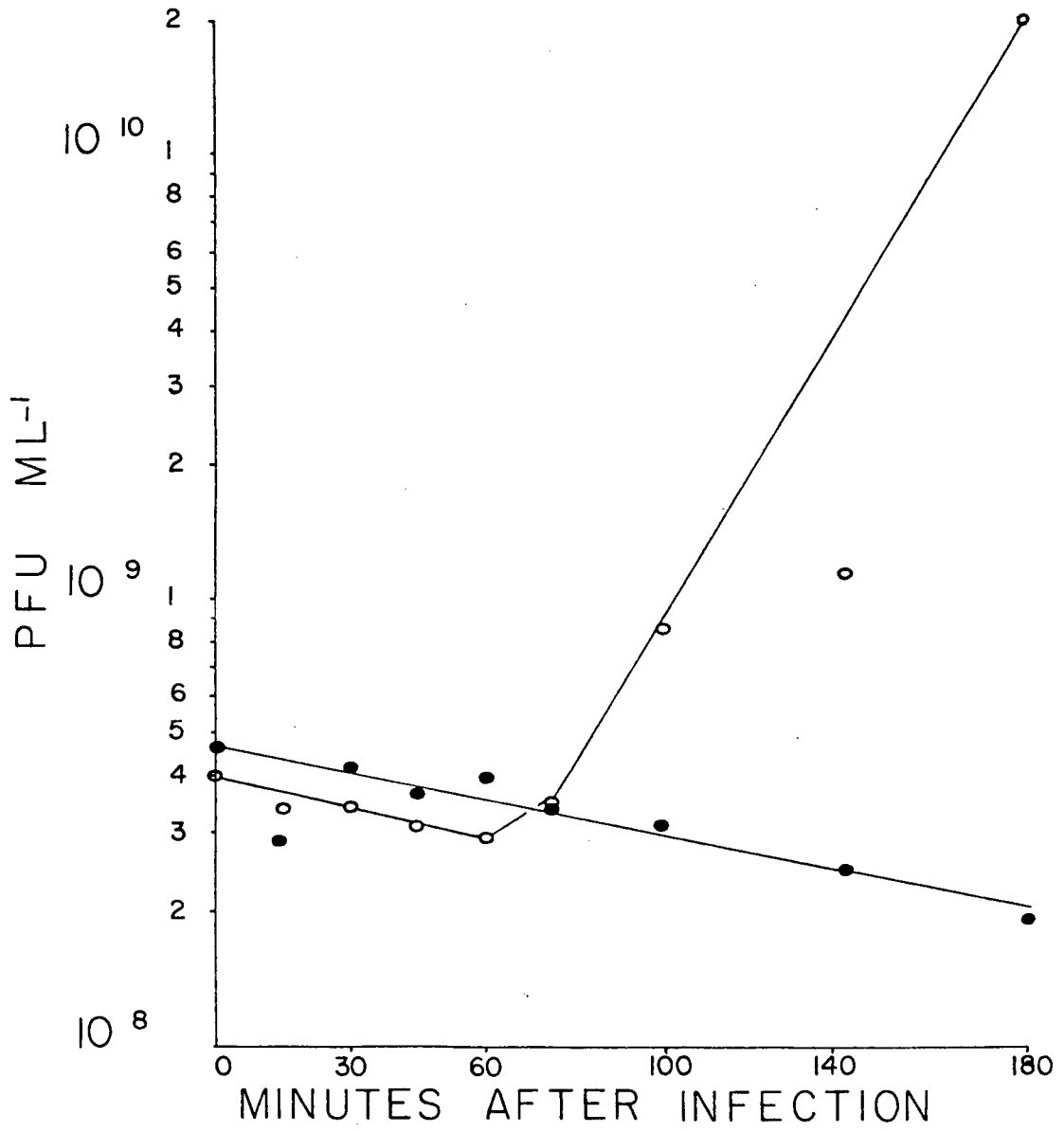
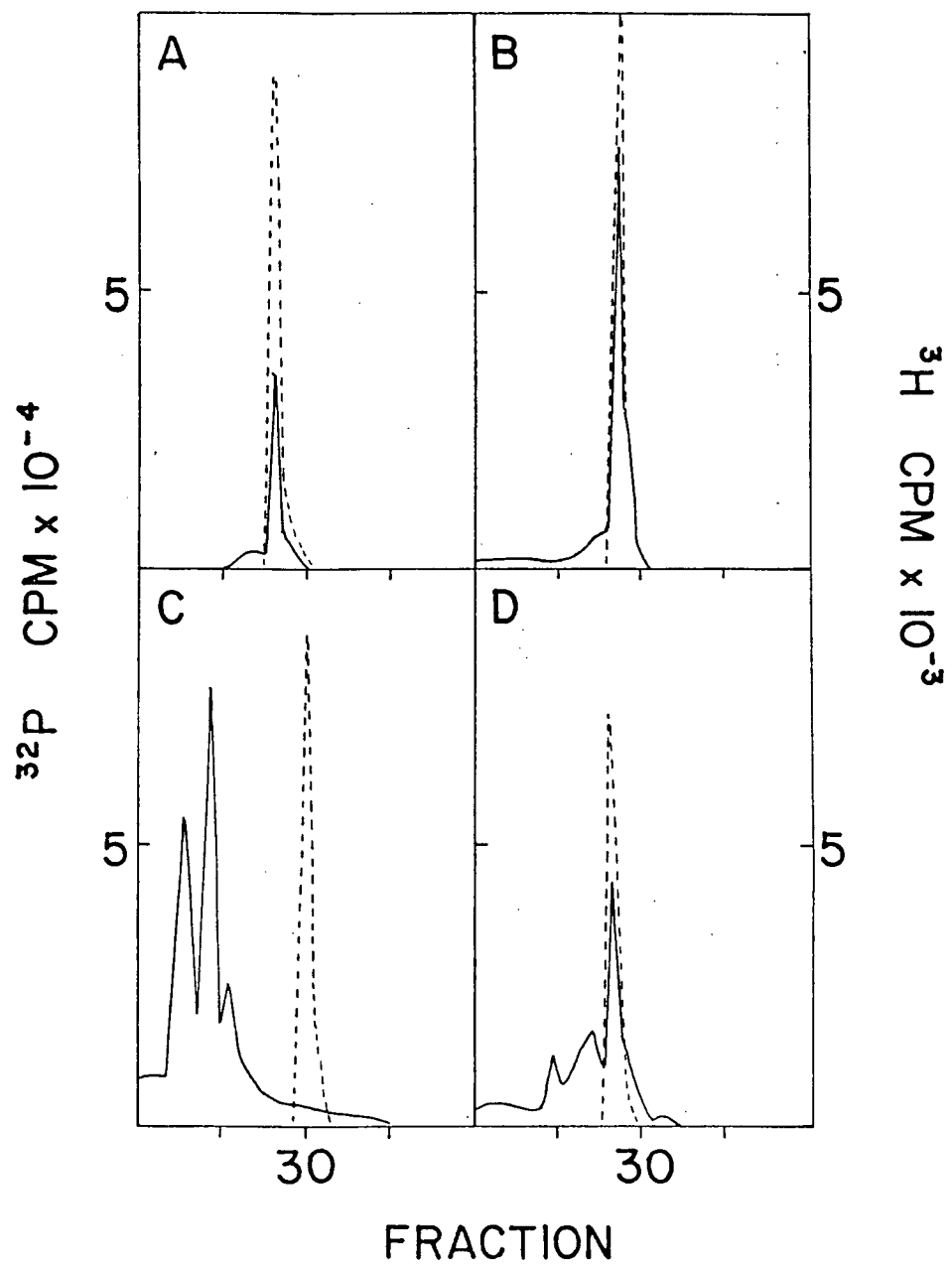


FIGURE 42.--Buoyant density of DNA synthesized by phage-infected cells.

Infected cells were labelled with [6-³H]-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)—— [6-³H]-uracil-labelled DNA; ---- ³²P reference DNA from phage particles. The bottom of the gradient is on the left.



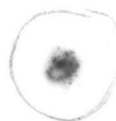
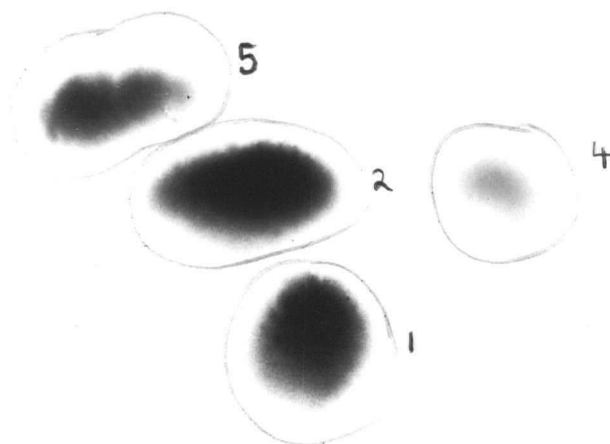
isolated from strain 29 cells infected with am 37 had extremely low levels of putThy when compared to am 37 DNA prepared in the sup 2 host or to wild-type DNA isolated from infection of strain 29. Thymine levels in am 37 DNA prepared in either the permissive or the non permissive host were normal. DNA 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 ØW-14 w^+ infected cells 0.2 percent or less of the total pyrimidine label was found in hmUra residues.

$^{32}\text{PO}_4$ and $[6-^3\text{H}]\text{-uracil}$ labelled am 37 DNA and ØW-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 ØW-14 DNA digests. The two nucleotides shared chromatographic properties with two of the three extra nucleotides detected in ^{32}P -pulse labelled ØW-14 DNA digests. The R_f 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 $^{32}\text{PO}_4$ than with $[6-^3\text{H}]\text{-uracil}$. 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 $^{32}\text{PO}_4$ was also released from ^{32}P -labelled am 37 DNA during

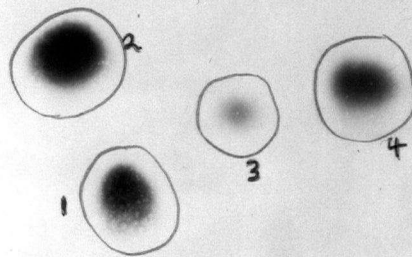
FIGURE 43.--Nucleotides in am 37 DNA.

Cells were labelled with [6-³H]-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.

H-6-U Am 37/sup 27 S1/SUPD DAWIO



SILVAD M-10 CELLULOSE Am 37



↑ ↑ 2
end

← 1

TABLE 21.--Nucleotide composition of ØW-14 and am 37 DNAs.^a

Nucleotide	Source of DNA								Rf ^d	
	<u>am</u> 37/strain 29 ([6- ³ H]-uracil)	<u>am</u> 37/strain 29 (³² P orthophosphate)	<u>am</u> 37/strain <u>sup2</u> ([6- ³ H]-uracil)	<u>am</u> 37/strain <u>sup2</u> (³² P orthophosphate)	ØW-14/strain 29 (³² P orthophosphate)	ØW-14/strain 29 (³² P orthophosphate)	E	A		
dGMP	40 ^b (0.1) ^c	3944 (16.1)	0		13355 (24.1)		26	39		
dAMP	72 (0.2)	3012 (12.3)	0		13921 (25.1)		56	27		
dTMP	8080 (27.6)	2972 (12.1)	41218 (27.0)		7219 (13.0)		36	52		
dCMP	13252 (45.2)	5943 (24.2)	81876 (53.7)		14007 (25.2)		43	70		
putdTMP	385 (1.3)	87 (0.4)	27835 (18.3)		6904 (12.4)		55	79		
hmdUMP	2043 (7.0)	865 (3.5)	256 (0.2)		29 (<0.1)		23	62		
unknown	5430 (18.5)	7750 (31.5)	1264 (0.8)		47 (<0.1)		9	65		

a 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.

b Cpm in the area cut from the chromatogram.

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

d 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 [6-³H]-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 whenever 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 conversion to putThy might answer the question: is putThy biosynthesis absolutely coupled to DNA replication?

Am 37 DNA was labelled with [8-¹⁴C]-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 nucleotide was not a product of the incomplete digestion of am 37 DNA. Samples of ^{32}P -labelled $\phi\text{W-14 w}^+$ DNA were digested with DNase I and snake venom phosphodiesterase. Two dimensional thin-layer chromatography revealed that little putdTMP was released from $\phi\text{W-14 w}^+$ 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 nucleotide and another pyrimidine nucleotide. After two-dimensional thin-layer chromatography of DNase I and snake venom phosphodiesterase digests of $[\text{8-}^{14}\text{C}]$ -adenine labelled am 37 DNA prepared in strain 29 dAMP and dGMP were the only labelled products (Figure 45). Therefore, the radioactive 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 $^{32}\text{PO}_4$ 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 $^{32}\text{PO}_4$ released from am 37 DNA during DNase I

FIGURE 44.--Two-dimensional thin-layer chromatography of [8-¹⁴C]-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.

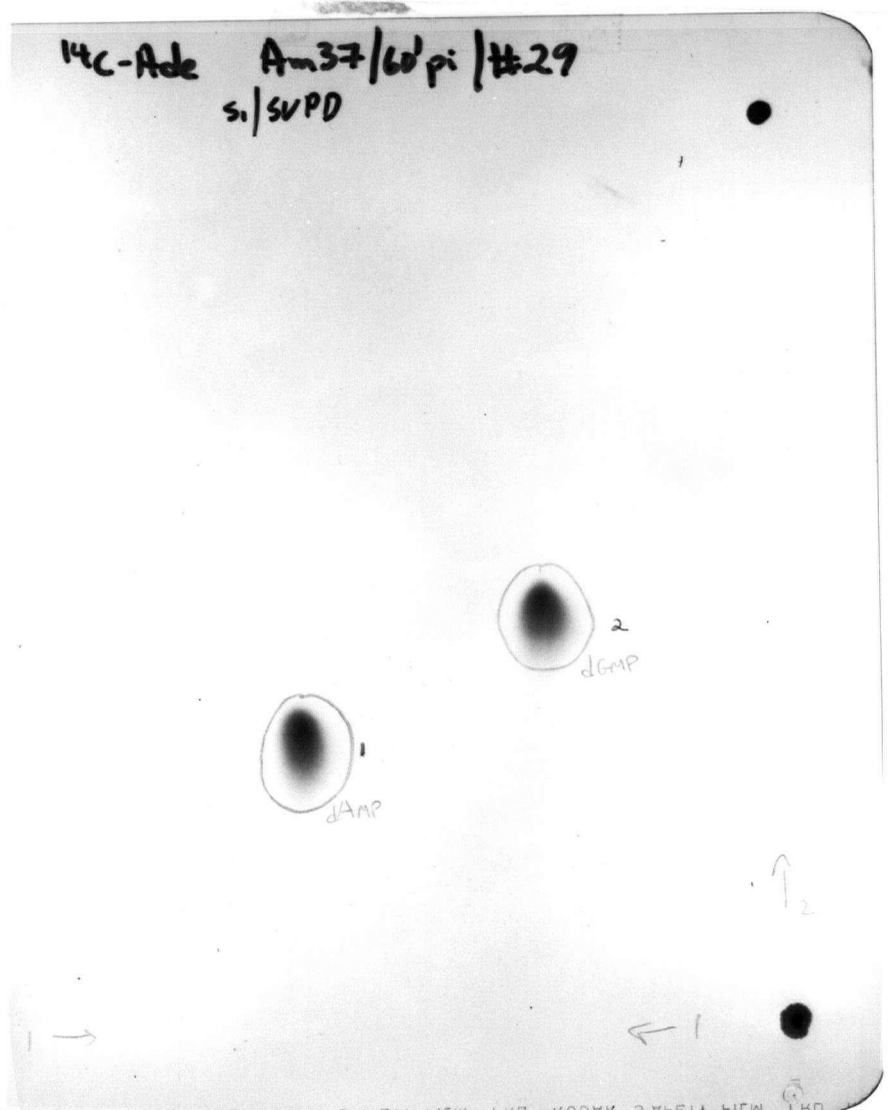
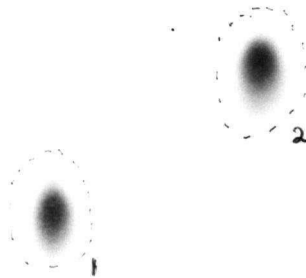


FIGURE 45.--Two-dimensional thin-layer chromatography of [8-¹⁴C]-adenine-labelled nucleotides present in DNase I-SVPD digests of am 37 DNA prepared in P. acidovorans strain 29.

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.

14C-Ade Am37 DNA DNase I / SUPD
0.02M Tris pH 8.2
0.015M MgCl₂



↑↑
16V
← 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 | |

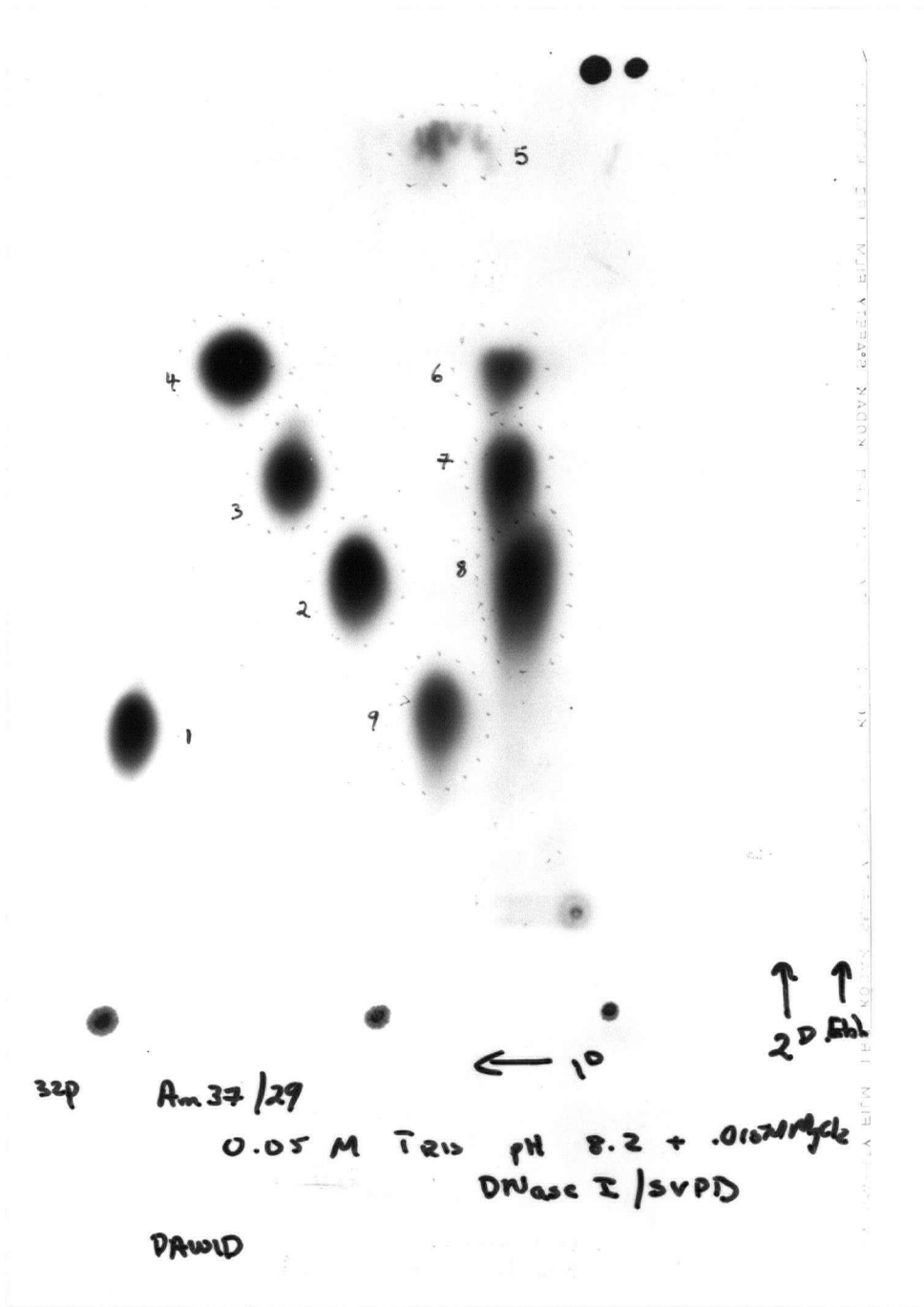


TABLE 22.--ØW-14 am 37 DNA was stable in alkali and S1 buffer.

treatment	cpm	percent recovery
none	16365	
ddH ₂ O, 37°/16 hours	15991	98
none	15993	
0.3 N NaOH, 37°/16 hours	15319	96
none	9742	
S1 buffer, 0.05 M NH ₄ -acetate, pH 5.0, 55°/4 hours	9508	98

³²P-labelled ØW-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 $^{32}\text{PO}_4$ released is not known.

DNA synthesis in ØW-14 am 37 infected-cells

The incorporation of $[6\text{-}^3\text{H}]\text{-uracil}$ 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.

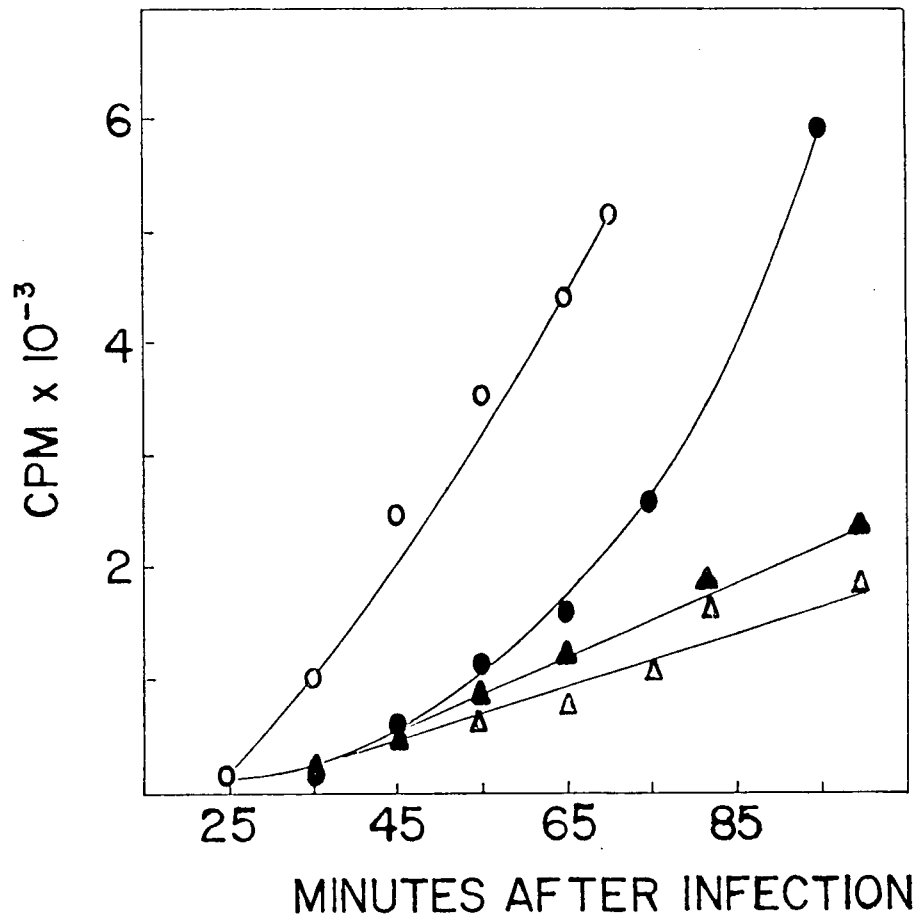
Neither am 37 nor wild-type ØW-14 DNA were labelled by $[2\text{-}^{14}\text{C}]\text{-hmdU}$. Am 37 DNA was not labelled by $[^3\text{H}]\text{-ornithine}$.

Tritium release from $[5\text{-}^3\text{H}]\text{-uracil}$ in am 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 [6-³H]-uracil into alkali-resistant, TCA-insoluble material was determined for wild-type phage in strain 29 (○) and in strain sup 2 (●); and for am 37 in strain 29 (Δ) and in sup 2 (▲). [6-³H]-uracil (1.0 μCi ml⁻¹, 10 μg ml⁻¹ uracil).



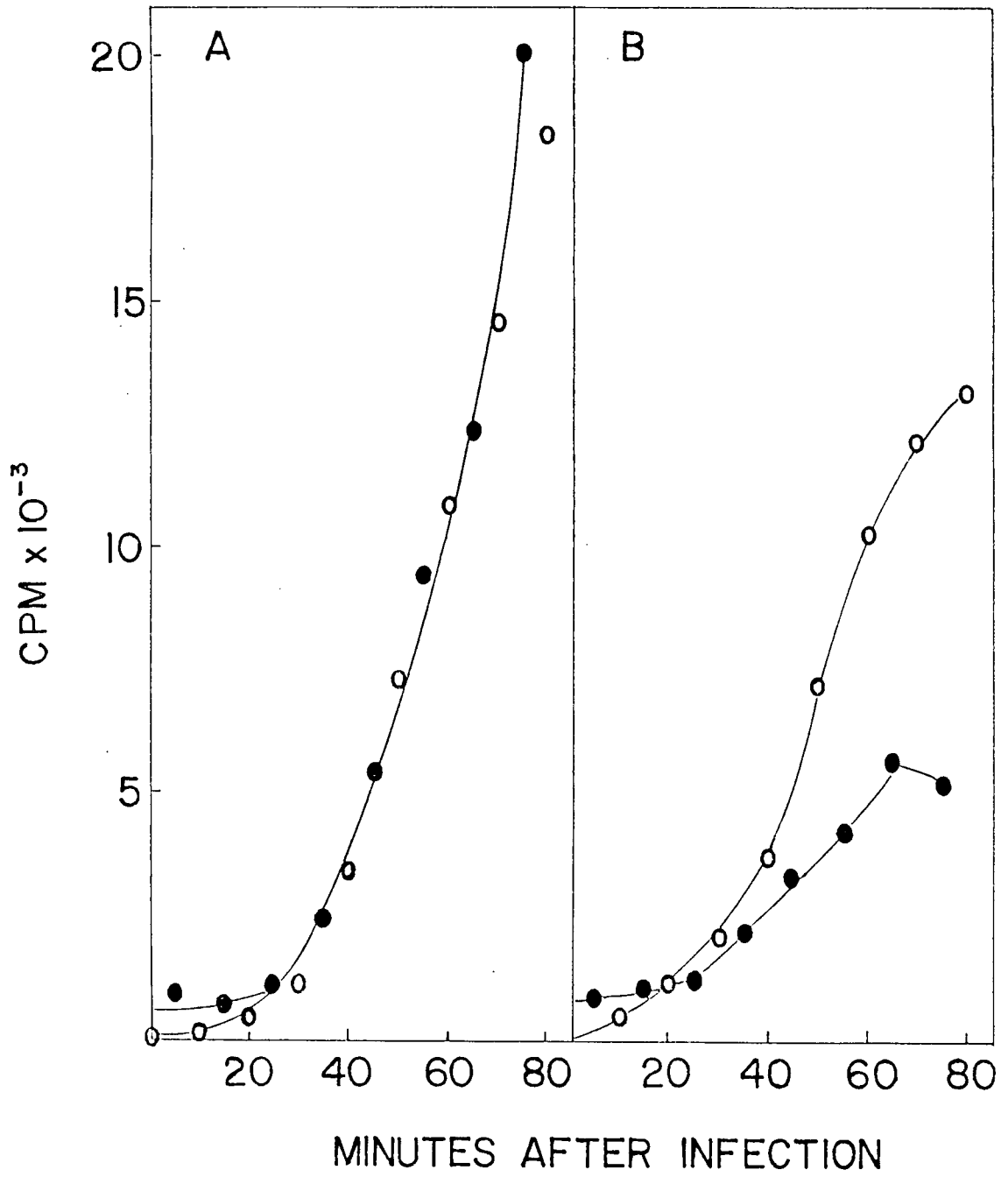
of the accumulation of [5-³H]-uracil label in cytosine bases in DNA and the release of tritium from [5-³H]-dUMP in nucleotide pools. In wild-type ØW-14-infected cells the ratio of tritium release to tritium incorporation was one-to-one (Figure 48). This was not surprising since, in a phage with a DNA 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 ØW-14 D0 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 ØW-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 ³²P-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 [5-³H]-uracil. Samples were assayed for the incorporation of label into alkali-resistant, TCA-insoluble material (●) and for the release of tritium (O). 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 ØW-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.

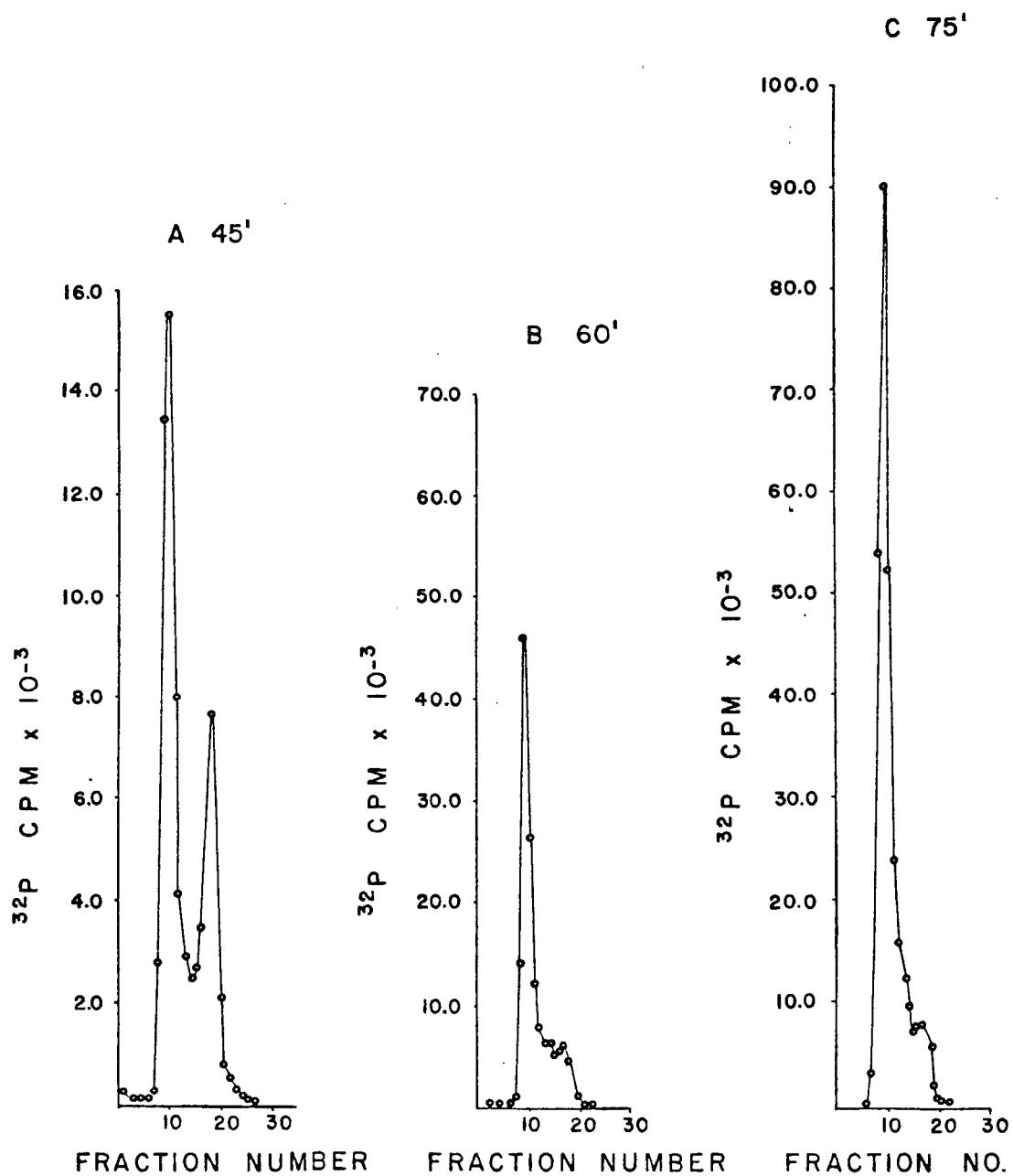


TABLE 23.--The nucleotide composition of ØW-14 am 37 DNA prepared at various times after the infection of P. acidovorans strain 29.

Nucleotide	45 min.	60 min.	75 min.
dTMP	2972 (0.17)	4195 (0.18)	4507 (0.17)
dCMP	5943 (0.34)	8457 (0.36)	9333 (0.35)
putdTMP	87 (<0.01)	117 (<0.01)	105 (<0.01)
hmdUMP	865 (0.05)	1180 (0.05)	1244 (0.05)
unknown	7750 (0.44)	9248 (0.40)	11160 (0.42)

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 $[6\text{-}^3\text{H}]\text{-uracil}$ -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 ϕ W-14 am 37 DNA.

[6-³H]-uracil-labelled ϕ W-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). ³²P-labelled ϕ W-14 w⁺ phage reference DNA, ●; [6-³H]-uracil labelled sample DNA, ○.

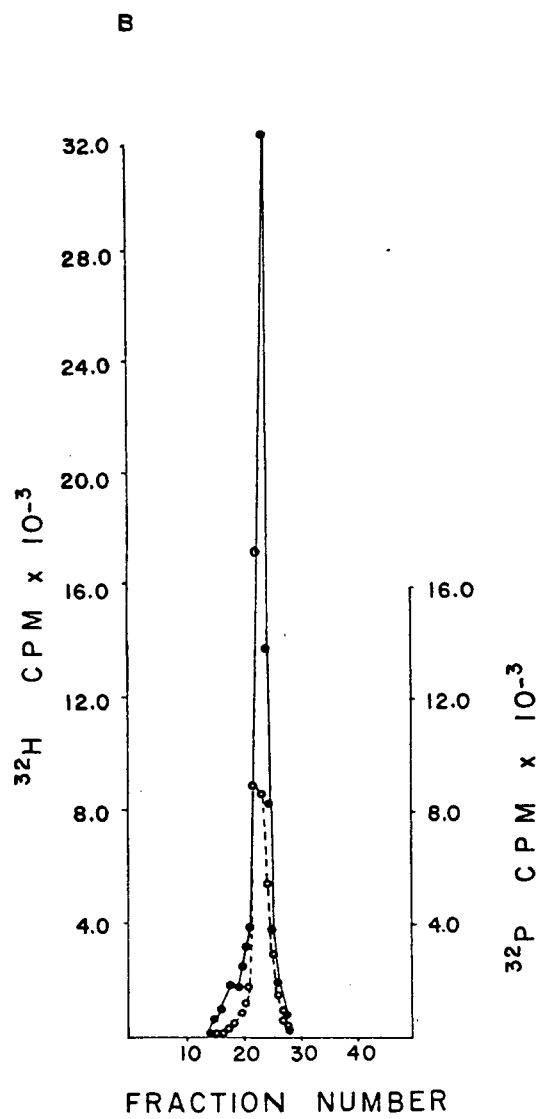
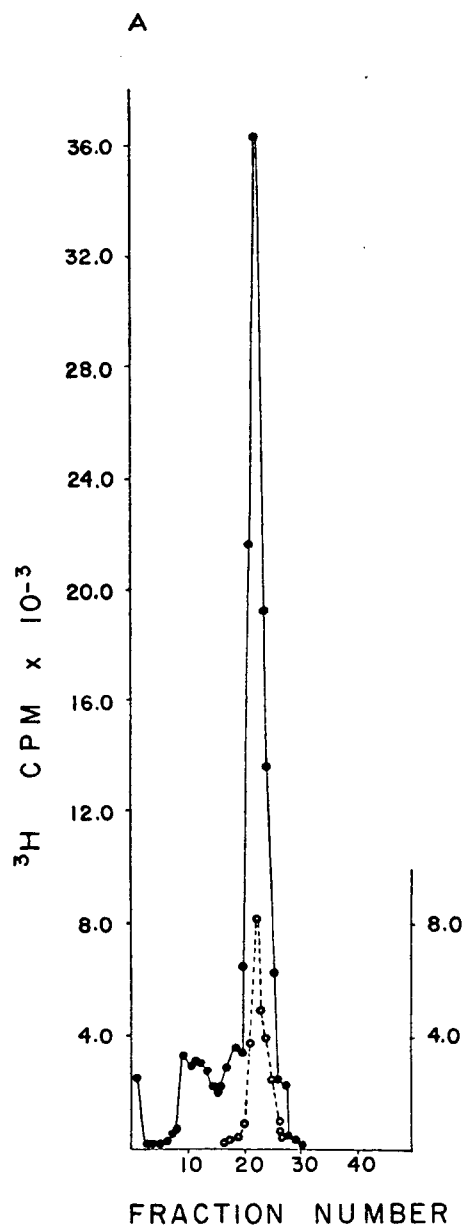
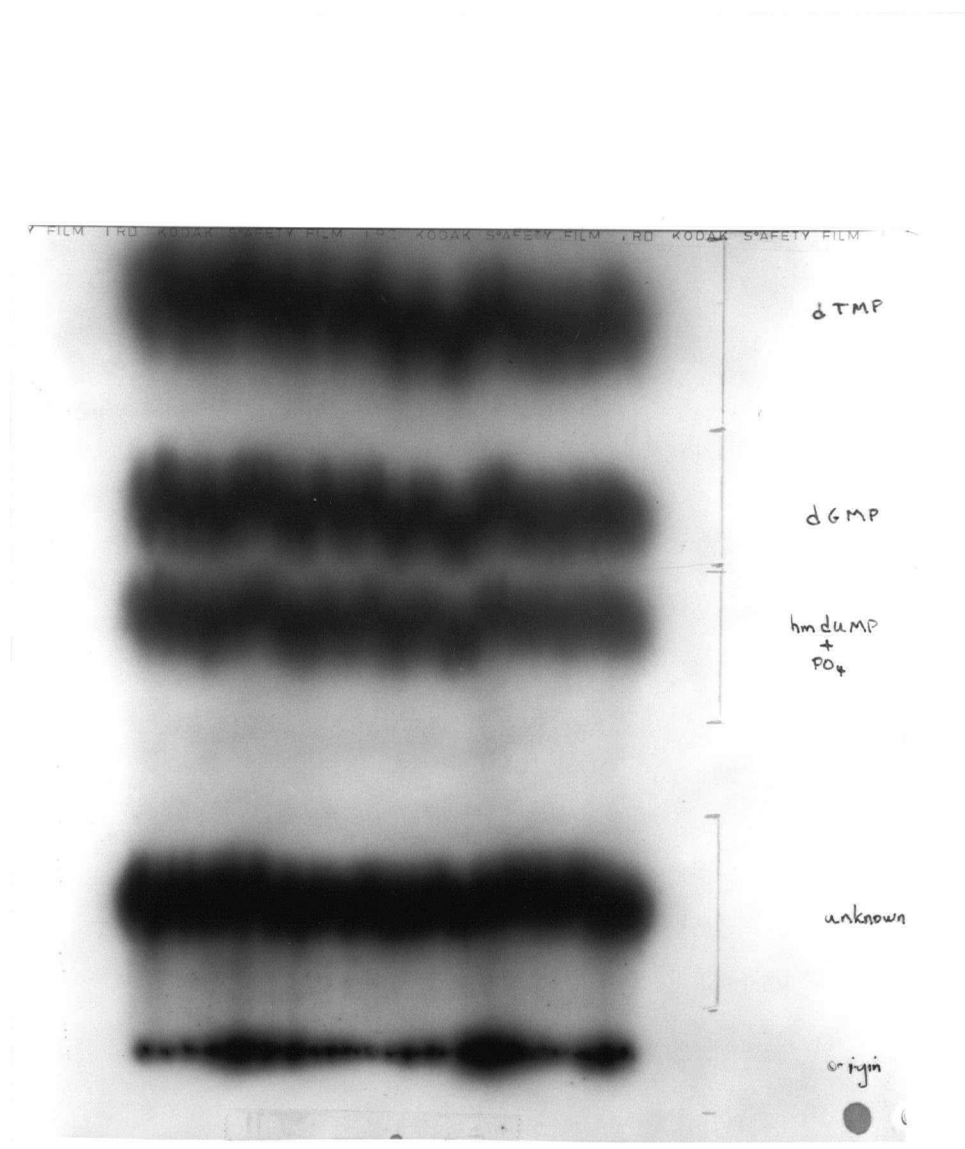


FIGURE 51.--The purification of the novel nucleotide by paper chromatography.

A ^{32}P -labelled ØW-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 ³²PO₄ labelling ratios demonstrated that the novel nucleotide carried extra phosphate residues.

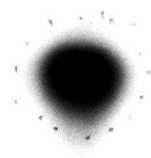
The approximate size of the novel nucleotide was established by gel filtration chromatography on Biogel P₂ columns. All the nucleotides eluted as a single peak, although the early peak fractions contained more of the novel nucleotide and dGMP eluted in later peak fractions (data not shown).

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.

DMWD - JXW?



↑ 2nd

← 1st



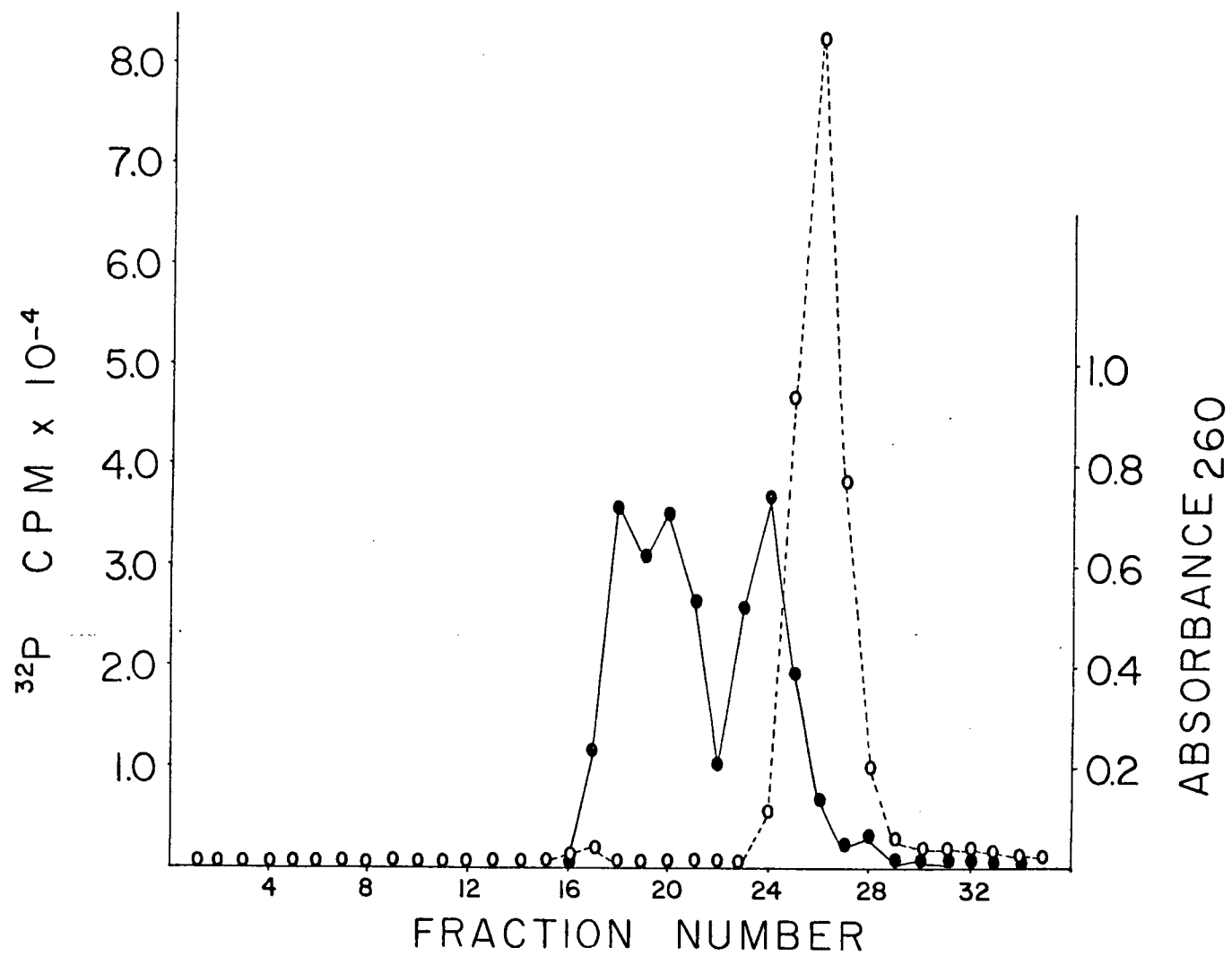
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FIGURE 53.--DEAE-Sephadex column chromatography of the novel nucleotide.

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, ○; A_{260} , ●.



Samples of a [6-³H]-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 hmUra. 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 ³²P 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 [³H]-hmdUra and PO₄ (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 ØW-14
am 37 DNA.

Authentic base	unknown nucleotide	hmdUMP ^b
putThy	12 ^a	0
Cyt	0	17
hmUra	2673	1362
Ura	27	0
Thy	4	0

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 [6-³H]-uracil.

a The values are cpm in the areas cut from the chromatogram.

b Identified as hmdUMP on the basis of its chromatographic properties.

TABLE 25.--Properties of the unknown nucleotide.^a

Treatment ^b	unknown nucleotide			hmdUMP			inorganic phosphate	hmdUra
	³ H	³² P	³² P/ ³ H	³ H	³² P	³² P/ ³ H		
None ^c	2298	6759	2.9					
1 N HCl 37°C 30 min				1903	2040	1.1	4626	
1 N HCL 98°C 7 min				1978	2334	1.2	4550	
alkaline phosphatase							6292	2018

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

b The products were identified by thin-layer chromatography with known standards.

TABLE 26.--Conversion of the unknown nucleotide to its nucleoside.

	-BAP	+BAP
Unknown nucleotide	3747 ^a	19
³² P ₄	138	3422

Identification of the unknown nucleoside by TLC.					
	Rf in Solvent				
	A	B	C	D	E
Authentic Standards					
hmUdR	76	59	78	26	20
TdR	83	46	90	50	49
UdR	83	61	81	38	27
CdR	72	56	51	16	26
Unknown					
-BAP	front	74	unstable	0	0
+BAP	75	58	78	27	19

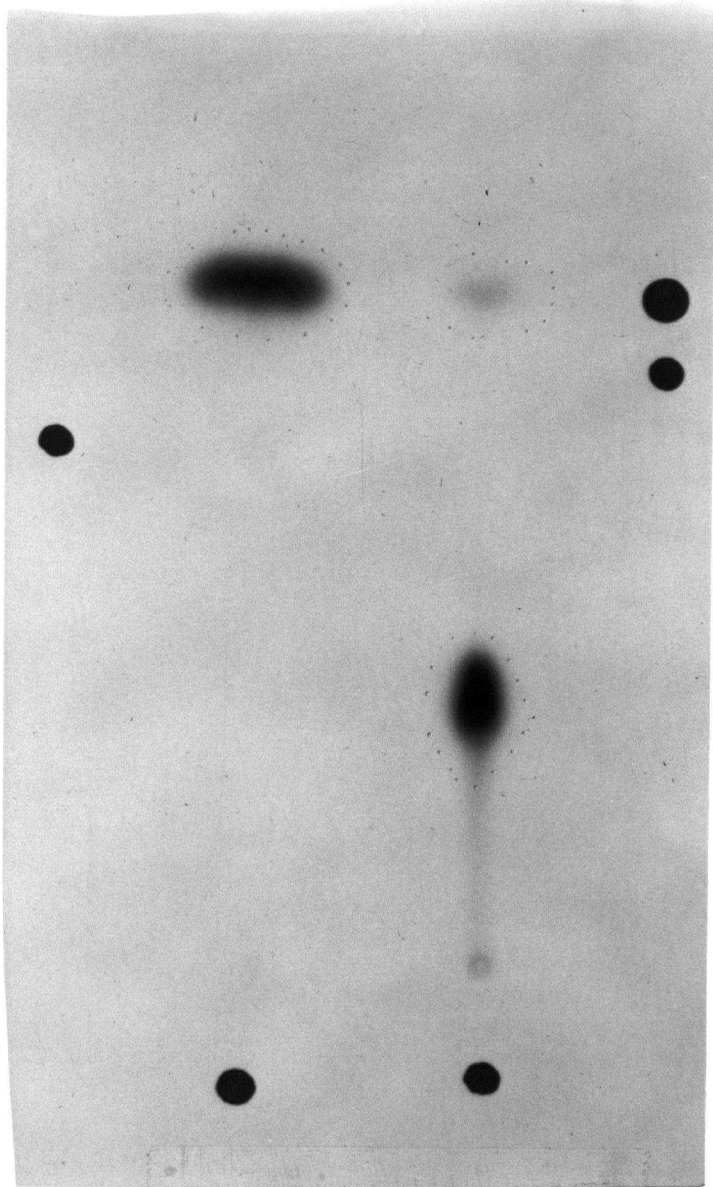
- a A sample of ³²P-labelled nucleotide was treated with BAP. The efficiency of BAP cleavage was monitored by measuring the formation of ³²P₄. The nucleoside was identified by measuring Rf values in 5 different solvents and comparing them to those determined for authentic reference standards. Solvent A, dH₂O; solvent B, (NH₄)₂SO₄:Na acetate (1M):isopropanol, 80/12/2 v/v; solvent C, t-butanol:MEK:HCl:H₂O, 40/30/10/20 v/v; solvent D, n-butanol:H₂O, 86/14 v/v; solvent E, n-butanol:H₂O:NH₄OH, 86/9/5 v/v.

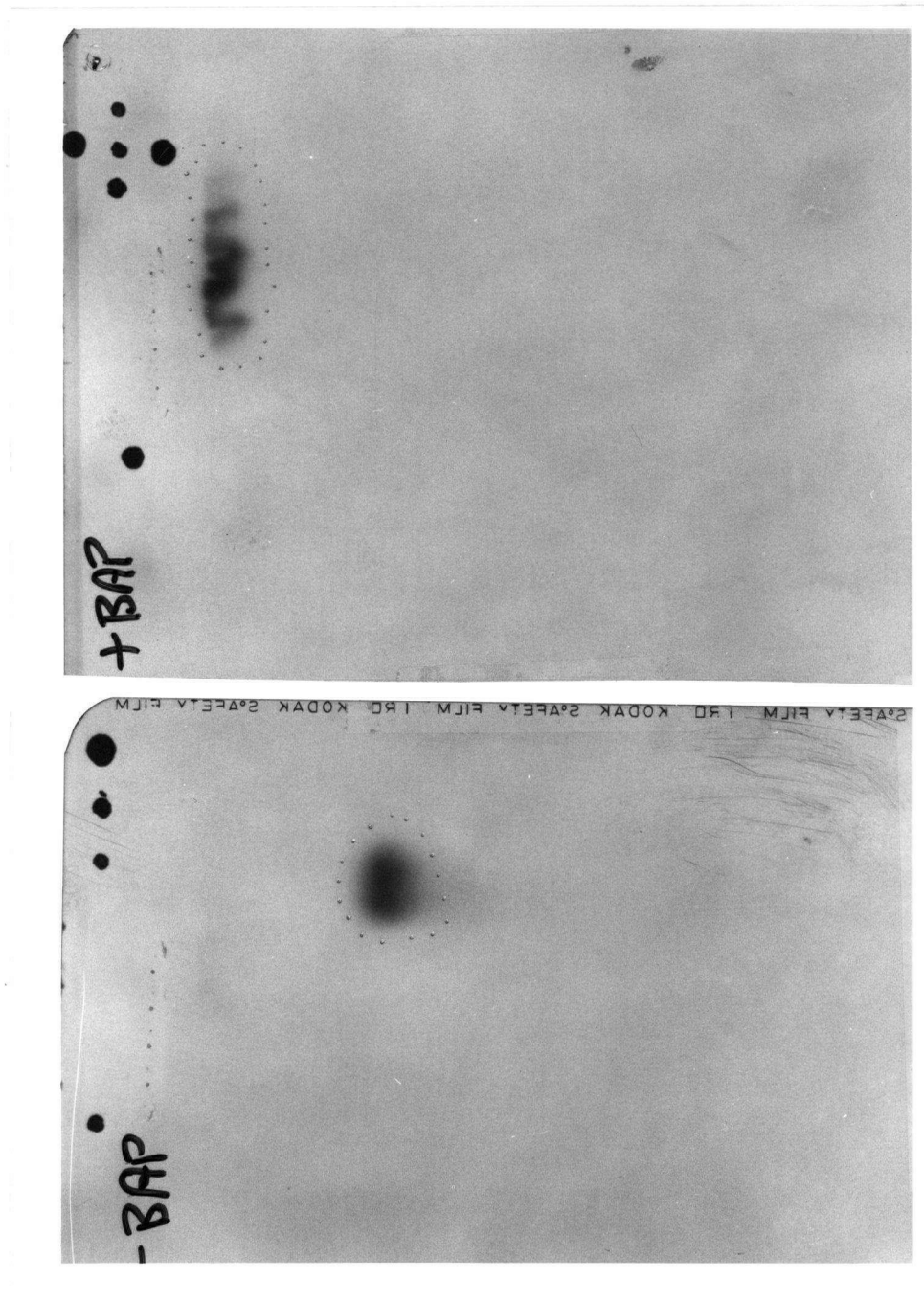
FIGURE 54.--Alkaline phosphatase treatment of the novel nucleotide.

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.

+BAP

- BAP





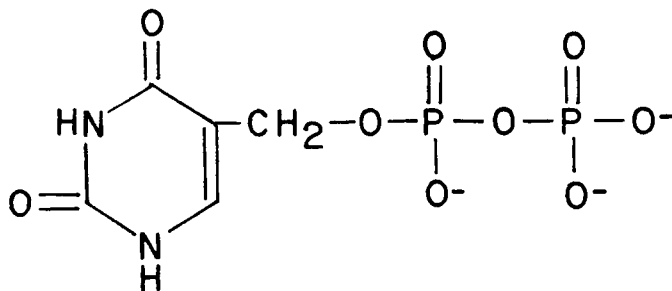
that its base was 5(hydroxymethyl-0-pyrophosphoryl) uracil (Figure 55).

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.₂₆₇ components; however, the second smaller peak is likely protein present in, or added to, the samples during digestion. The other O.D.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).

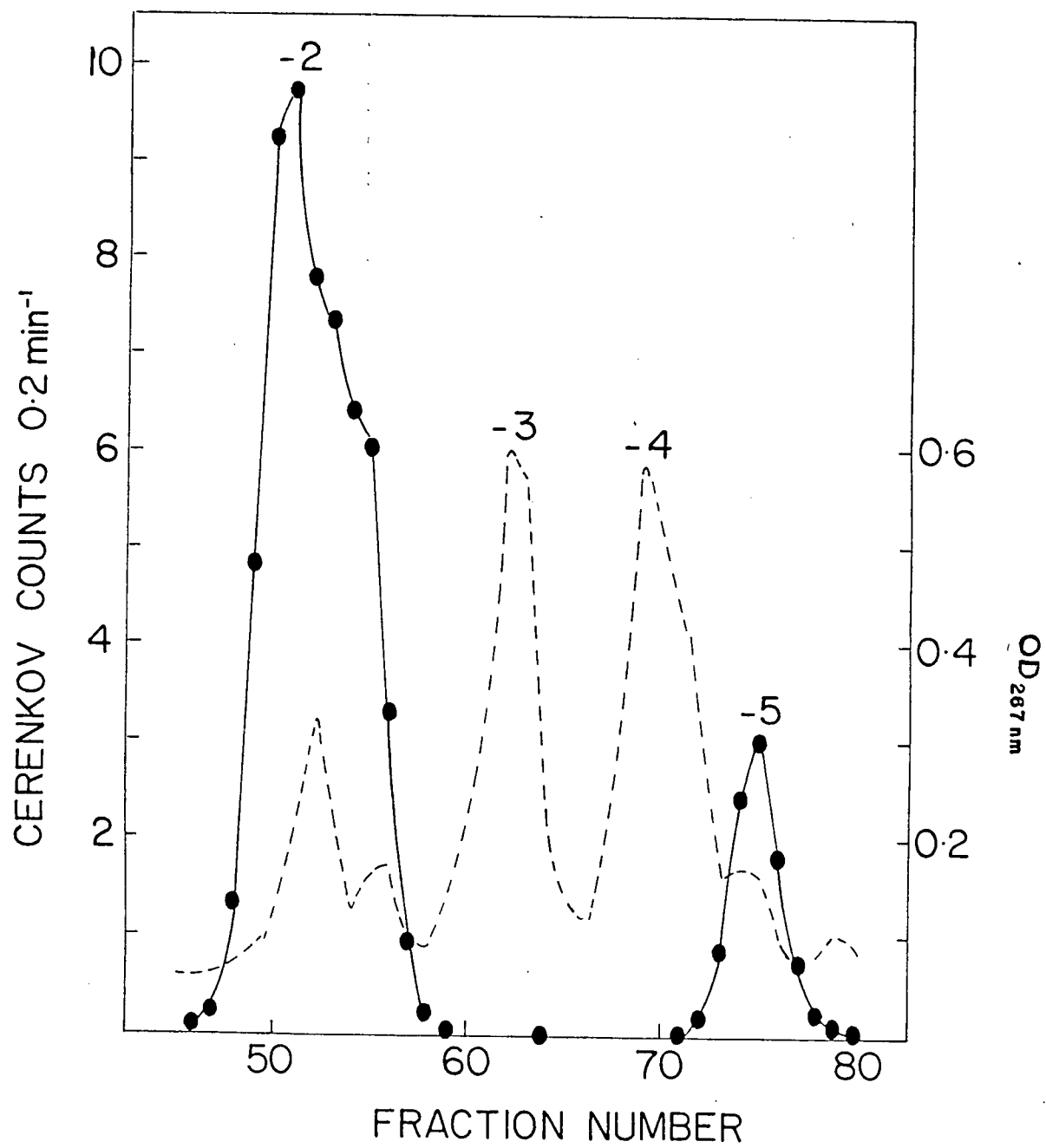
FIGURE 55.--Proposed structure of the novel pyrimidine base.



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

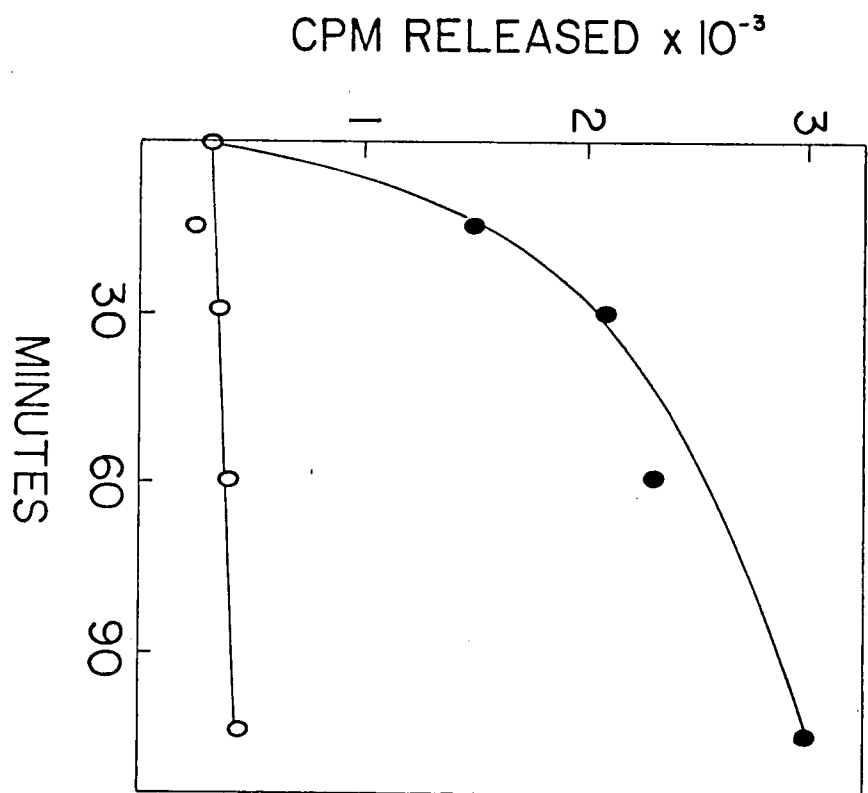
The presence of hmPPUra in am 37 DNA suggested that it was an activated precursor of putThy. Cell-free extracts of strain 29 cultures infected with wild-type ϕ W-14 or am 37 were prepared. ^{32}P -labelled am 37 DNA was used as a substrate in an *in vitro* experiment to test for a precursor and product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

Cell-free extracts of strain 29 cells infected with wild-type ϕ W-14 were capable of releasing $^{32}\text{PO}_4$ 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 ϕ W-14 wild-type extracts were purified, digested to mononucleotides and separated by two-dimensional

FIGURE 57.--Release of radioactivity from ^{32}P -labelled am 37 DNA.

A sample of ^{32}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 (O).



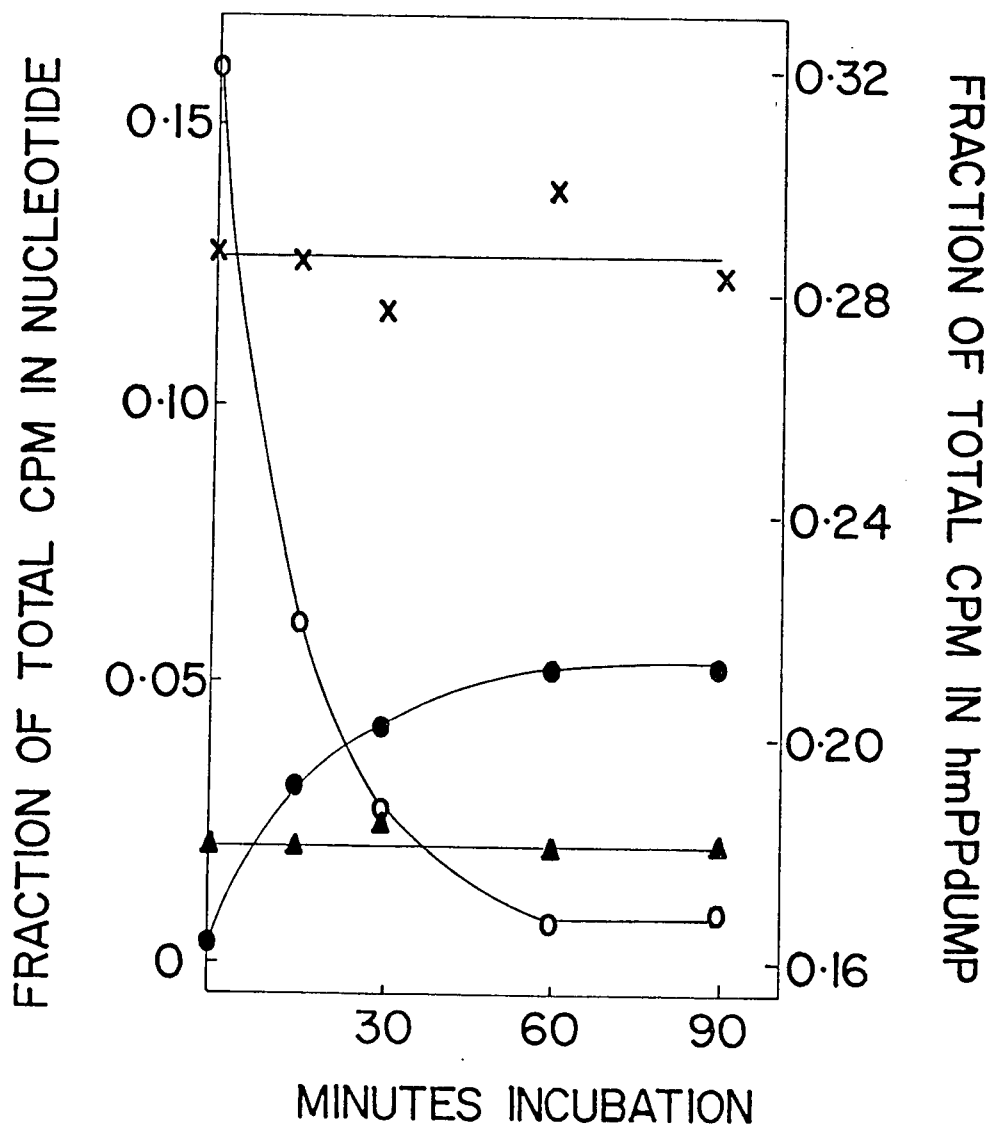
thin-layer chromatography on cellulose. Nucleotides were detected by autoradiography. All nucleotides were cut out and counted. The results were corrected for the loss of $^{32}\text{PO}_4$ during in vitro modification and for other non specific losses during purification, digestion and chromatography. 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 S1 digestion of the DNA samples. DNA samples with 50 percent less hmPPdUMP would yield 50 percent less hmdUMP if the S1 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 ØW-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 (\bullet); hmPPdUMP (O).



successful. Native and denatured ϕ e DNA were tested as substrates in conjunction with γ - ^{32}P ATP and ϕ W-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).

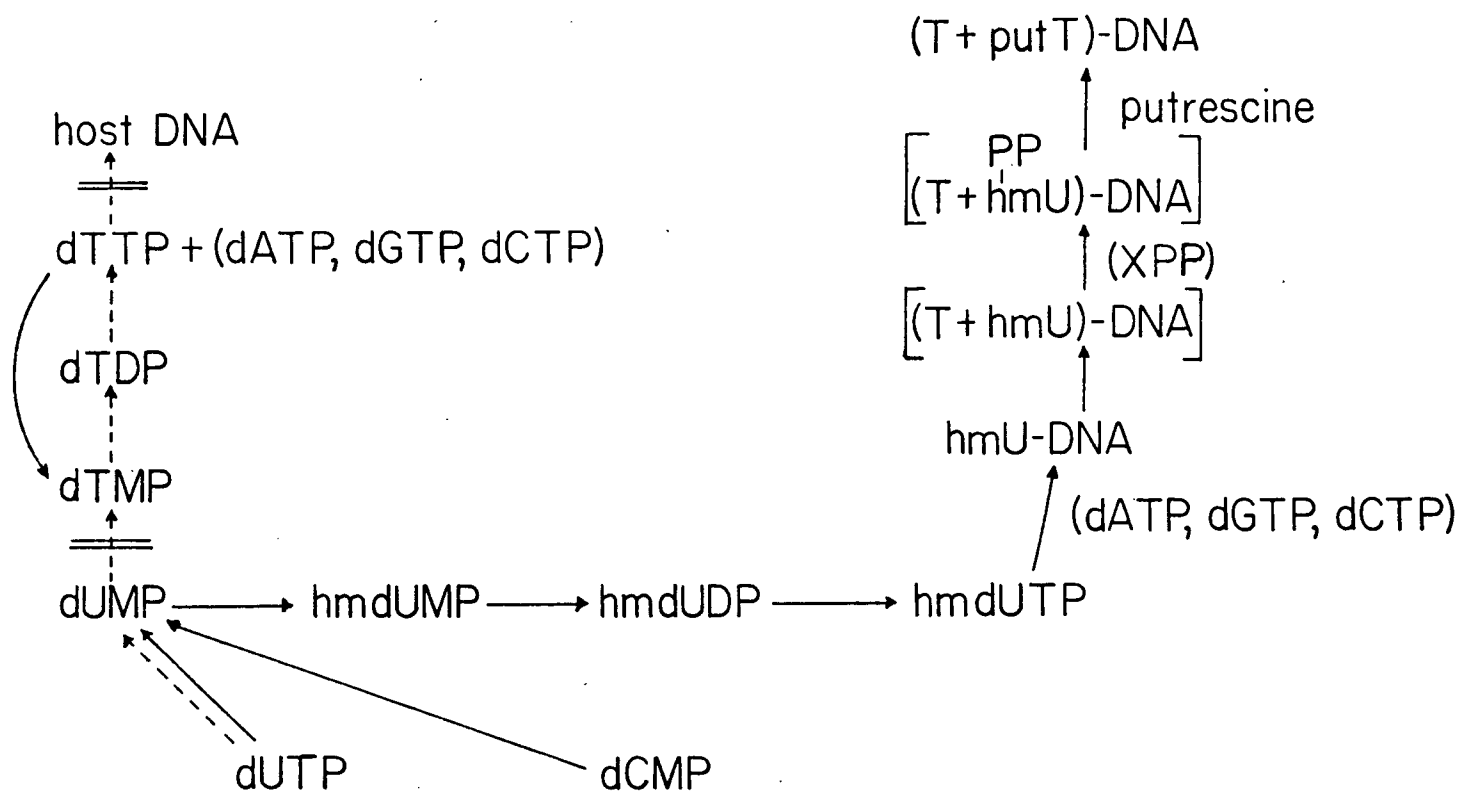
hmUra will alkylate poorly nucleophilic aromatic amines in aqueous alkaline medium (Santi, 1967). The hydroxymethyl group is susceptible to nucleophilic attack. Nucleophilic displacement could be enhanced by the presence of a pyrophosphoryl leaving group. Some other esters of hmUra are also sensitive 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 solubilization of the 6-hydrogen are excluded since [6- ^3H]-uracil labelled putThy and Thy to the same extent as [2- ^{14}C]-uracil (Table 8).

Trimethoprim did not inhibit ϕ W-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 ϕ W-14 DNA precursor synthesis and modification.

This figure summarizes our knowledge of ϕ W-14 DNA metabolism. ϕ 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. ϕ 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 (-----); ϕ W-14 pathways (—).



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