NUCLEIC ACID METABOLISM IN UNINFECTED AND BACTERIOPHAGE ØW-14 INFECTED *PSEUDOMONAS ACIDOVORANS*

by

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ABSTRACT

Pseudomonas acidovorans was shown to be deficient in a number of enzymes of the salvage pathways of nucleic acid metabolism. These included uridine phosphorylase, purine nucleoside phosphorylase, cytidine (deoxycytidine) deaminase, and thymidine phosphorylase. The absence of uridine kinase and deoxycytidine kinase was also indicated.

Pyrimidine requiring mutants were isolated and pyrimidine substitution patterns were examined in these auxotrophs. Concentrations of uracil of 50 µg/ml and greater had the unusual effect of inhibiting the growth of these strains, whereas no effect occurred with the wild-type. An inhibitory effect on the wild-type did result, however, when deoxyadenosine or adenosine was added at critical concentrations.

Obligate thymidine auxotrophs were isolated using an adaptation of the aminopterin technique. These strains required very high concentrations of thymidine (250 to 1000 µg/ml) for growth.

No change in the amount of aspartate transcarbamylase activity occurred during growth in the presence or absence of added uracil. No inhibition of aspartate transcarbamylase activity was evident in the presence of various nucleotides.

Infection of cells by bacteriophage ØW-14 resulted in the apparent synthesis of a phage encoded thymidylate synthetase. In addition, an activity corresponding to deoxyuridylate hydroxymethylase was indicated.

Thymidine was not a precursor for 5-(4-aminobutylaminomethyl) uracil but the base was efficiently labelled by radioactive uracil or deoxyuridine.

Pool studies indicated that the biosynthesis of the base occurred at the nucleotide level rather than the macromolecular level.

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INTRODUCTION

The *de novo* biosynthesis of the pyrimidine nucleotide UMP[1], which is the precursor for all pyrimidine nucleoside triphosphates (see Figure 2), is catalyzed by six enzymes encoded for in *Salmonella typhimurium* and *Escherichia coli* by six unlinked genes, *pyr* A - F. The *E. coli* pathway is diagrammed in Figure 1. This pathway is common to a variety of organisms (O'Donovan and Neuhard, 1970). UMP may also be formed via the so-called salvage pathways (Fig. 2).

As expected for a branched pathway, pyrimidine nucleotide biosynthesis is highly regulated in the enteric bacteria. The regulation is both at the level of enzyme activity and synthesis. Thus, carbamyl phosphate synthetase (CPSase), which is common to pyrimidine and arginine biosynthesis, is feedback inhibited by UMP. Ornithine, whose biosynthesis is controlled by arginine, activates CPSase and

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[1]Bases are identified as follows:
A, adenine; C, cytosine; G, guanine; O, orotate; U, uracil; and T, thymine. Ribonucleosides are identified by prefixing the letter r to the above symbols whereas the common 2'-deoxyribonucleosides are prefixed by the letter d. Thus, rA = adenosine and dA = 2'-deoxyadenosine. The 5'-mono-, di-, and triphosphates of the common ribonucleosides are represented by the customary abbreviations exemplified by AMP, ADP, and ATP. Deoxyribonucleotides are abbreviated as the ribonucleotides except that they are preceded by a "d". Thus, dCTP = 2'-deoxycytidine-5'-triphosphate and dTMP = 2'-deoxythymidine-5'-monophosphate. The rare bases 5-hydroxymethylcytosine and 5-hydroxymethyluracil are represented as HMC and HMU respectively. The deoxyribonucleotide abbreviations used are: dHMP, dHDP, dHTP, 5-hydroxymethyldeoxycytidine-5'-mono, di-, and triphosphate and dHMUMP etc. for the 5'-mono-, di-, and triphosphates of 5-hydroxymethyldeoxyuridine.

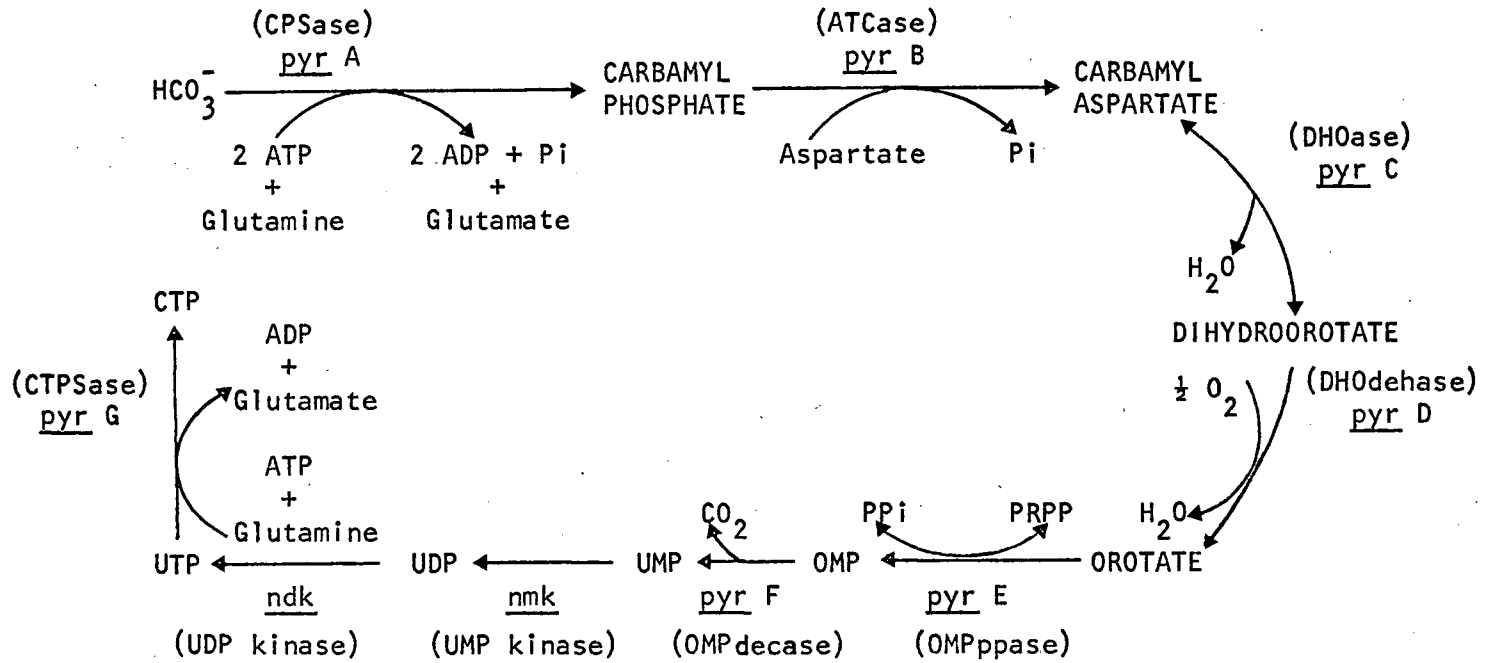

Figure 1. Pathway of pyrimidine biosynthesis. Genetic symbols for the enzymes involved are underlined. The enzymes shown in parentheses are abbreviated as follows: carbamyl phosphate synthetase (CPSase); aspartate transcarbamylase (ATCase); dihydroorotase (DHOase); dihydroorotate dehydrogenase (DHOdehase); orotidine-5'-phosphate pyrophosphorylase (OMPppase); orotidine-5'-phosphate decarboxylase (OMPdecase); uridine-5'-phosphate kinase (UMPkinase); uridine-5'-diphosphate kinase (UDP kinase); cytidine-5'-triphosphate synthetase (CTPSase).

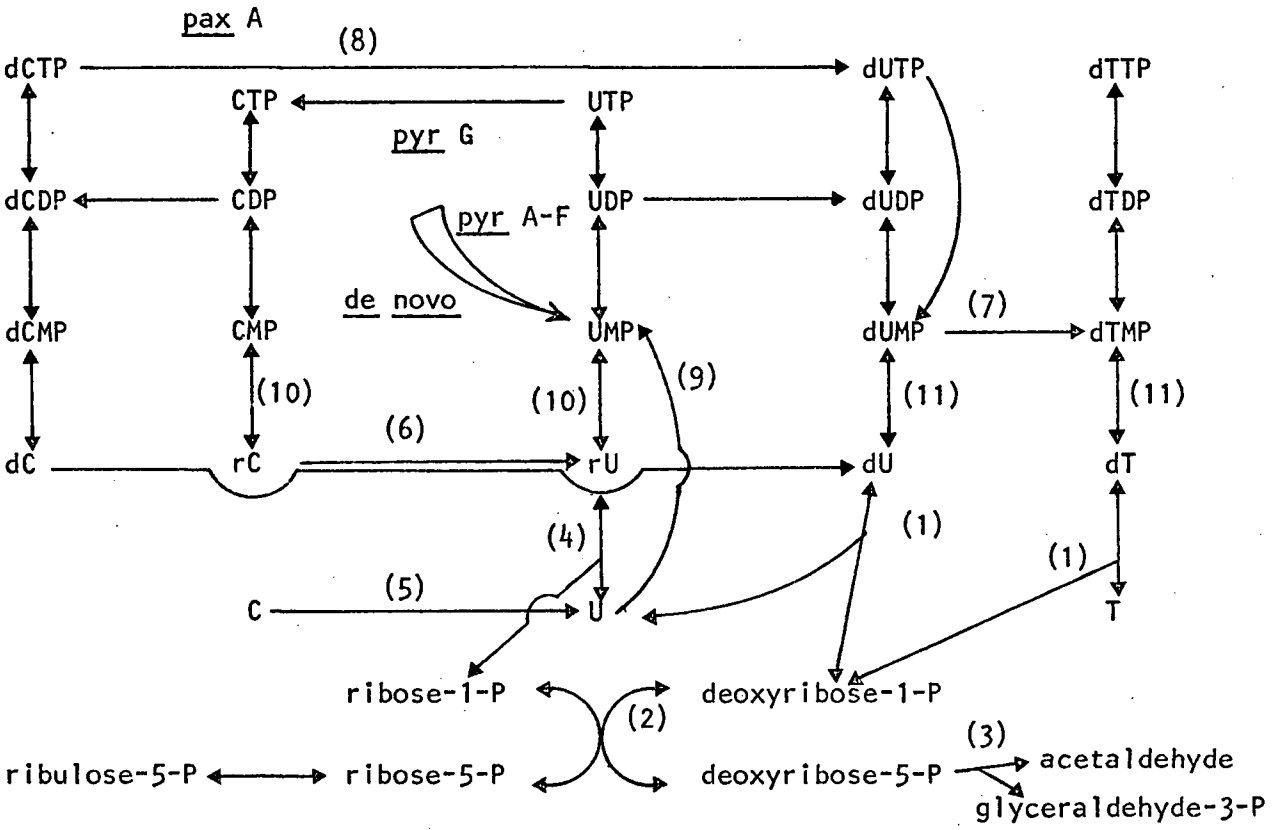

Fig. 2. Pyrimidine nucleotide interconversions and metabolism of pyrimidine bases and nucleosides in Escherichia coli and Salmonella typhimurium. Enzymes are: (1), thymidine phosphorylase; (2), deoxyribomutase; (3), deoxyriboaldolase; (4), uridine phosphorylase; (5), cytosine deaminase; (6), cytidine(deoxycytidine)deaminase; (7), thymidylate synthetase; (8), deoxycytidine triphosphate deaminase; (9), uridine-5'-monophosphate pyrophosphorylase; (10), uridine kinase; (11), thymidine kinase.

antagonizes the effect of UMP (Pierard, 1966). In E. coli and S. typhimurium, aspartate transcarbamylase (ATCase) is feedback inhibited by CTP but activated by a purine nucleotide, namely ATP. The inhibition by CTP is competitive for both substrates and is never complete (Gerhart and Pardee, 1964). In Pseudomonas, Saccharomyces and Neurospora the feedback inhibitor of ATCase is UTP. One would expect the control of CPSase in these organisms to differ from that of the enteric bacteria and indeed it does (for a review see O'Donovan and Neuhard, 1970).

The ATCases of Bacillus subtilis and Streptococcus faecalis are not under feedback control (Bethell and Jones, 1969; Neumann and Jones, 1964). The regulation of the supply of a common intermediate to each of two biosynthetic pathways in these organisms presents an interesting problem.

Regulation at the level of enzyme synthesis was originally reported for E. coli (Yates and Pardee, 1957). Initially, it was thought that four of the six enzymes involved in pyrimidine biosynthesis were linked on the chromosome. The other two, pyr A and pyr B, were known to be well separated from each other and from the group of four. However, it was shown later that these four genes, pyr C - F, were not linked (O'Donovan and Neuhard, 1970). Although all six genes are scattered on the chromosome, the original observation of coordinate repression and derepression of genes pyr C - F remains true. Synthesis of CPSase and ATCase is also controlled, but not coordinately. In

other organisms, a variety of combinations of substrate induction, end product repression and no apparent regulation have been observed (for a review see O'Donovan and Neuhard, 1970).

Significant changes occur in the nucleic acid metabolism of E. coli and B. subtilis following infection by certain bacteriophages (Cohen, 1968; Koerner, 1970; Matthews, 1971).

The genome of bacteriophage T4 contains some 30 genes known to be involved either directly or indirectly in viral deoxyribonucleic acid (DNA) synthesis. Some of them encode for enzymes of DNA precursor synthesis; for example, thymidylate synthetase and ribonucleoside diphosphate reductase. Others encode for enzymes of DNA metabolism at the macromolecular level; for example, DNA ligase and 5-hydroxymethylcytosine glucosyl transferases.

The probable initiation point of studies on nucleic acid metabolism of T-even infected cells was the discovery of HMC in T-even phage DNA (Wyatt and Cohen, 1952). Initially, the presence of HMC was overlooked because perchloric acid (PCA) was used to hydrolyze the phage DNA. PCA destroys a high percentage of the HMC in DNA, but the reason for this is not known. HMC itself is quite stable in PCA. When formic acid was used for hydrolysis good yields of a cytosine-like compound were obtained which subsequently was shown to be HMC.

From the initial discovery of HMC to the solving of its biosynthesis spanned a period of six years. Adequate techniques for obtaining and handling tetrahyrofolic acid (THFA), the active coenzyme

in the transfer of one-carbon fragments, were unavailable before 1956. In spite of this, considerable progress was made in elucidating the route of biosynthesis of HMC. It was shown that HMC was not synthesized in the cell as the free base or nucleoside and that neither HMC, its deoxyribonucleoside, nor dHMP were incorporated into phage DNA when added to an infected cell culture.

Friedkin and Kornberg (1957) demonstrated the in vitro formation of labelled dTMP from dUMP and radioactive formaldehyde in the presence of THFA. The THFA was oxidized to dihydrofolic acid (DHFA) in the course of the reaction. Flaks and Cohen (1957) found that phage infected cells contained a higher level of dTMP synthetase activity than uninfected cells, and that if dCMP was substituted for dUMP the product of the reaction was dHMP, which was formed without the oxidation of THFA. The new enzyme activity was termed dCMP hydroxymethylase. It is only found in infected cells, appearing 2-3 min after infection. Its synthesis stops at the time of initiation of phage DNA synthesis.

If a phage-infected thymine auxotroph is starved for thymine, normal phage production results, and the phage DNA contains normal amounts of T (Barner and Cohen, 1954). Infected cells contain higher levels of dTMP synthetase activity than uninfected cells. Phage infected cells were shown to contain two distinct dTMP synthetases, one of which was phage-specific (Greenberg et al., 1962). Phage strains mutated in the dTMP synthetase gene (td) have been isolated (Simon and Tessman, 1963; Wulf and Metzger, 1963) and are somewhat

impaired in development compared to *td*$^+$ phage (Matthews, 1965).

In T-even phage DNAs all guanine residues are paired with HMC. Therefore, C must be excluded from the phage DNA, and this is achieved by the synthesis of a phage-specific dCTPase (Kornberg *et al.*, 1959). The enzyme is also active on dUTP, dUDP and dCDP. Thus, a single enzyme leads to exclusion of C from the DNA and at the same time generates dCMP, the substrate for dCMP hydroxymethylase.

As might be expected, a phage-specific deoxynucleoside monophosphate kinase appears in infected cells; it is active not only on dHMP, but also on dTMP and dGMP to yield the corresponding diphosphates (Kornberg, *et al.*, 1959). Apparently the host nucleoside diphosphate kinase converts dHDP to dHTP.

The transfer of a one-carbon group in the dTMP synthetase reaction with subsequent reduction occurs at the expense of THFA. The increased dTMP synthetase activity seen in infected cells is paralleled by a twenty-fold increase in the level of dihydrofolate reductase activity (Matthews and Cohen, 1963). The increased activity is not a consequence of derepression of the host enzyme. Phage dihydrofolate reductase mutants (*wh* mutants) give a reduced burst size (Hall *et al.*, 1967), which indicates that the increased dTMP synthetase activity in phage infected cells demands an increased rate of THFA regeneration.

A small amount of dHMUMP, the deamination product of dHMP, appears during the dCMP hydroxymethylase reaction. It is formed by the action of a dCMP deaminase which will deaminate dHMP but to a lesser extent

than dCMP (Flaks and Cohen, 1959). Phage dCMP deaminase mutants (cd mutants) give a burst size about one half that of wild-type (Hall and Tessman, 1966).

The phage-induced deaminase would be expected to reduce the level of dCMP, which is required for the biosynthesis of dHMP, and raise the level of dUMP, which is converted to dTMP. However, the enzyme is activated by dHTP and inhibited by dTTP (Fleming and Bessman, 1965).

In T2-infected cells the ^{32}P incorporated into viral ribonucleic acid (RNA) is subsequently incorporated extensively into viral DNA (Volkin and Astrachan, 1956). It was not clear whether this efficient reduction of ribonucleotides was catalyzed by the bacterial system and/or a new ribonucleotide reductase induced by the phage. Eventually, the increased reductase activity was shown to be due to a phage-specific reductase system (Yeh et al., 1969; Berglund et al., 1969). Yeh et al. (1969) isolated T4 mutants (called nrd) unable to induce the enzyme.

Although the phage-specific dCMP deaminase appears to be regulated by the relative concentrations of dHTP and dTTP, it is not clear why the enzyme is produced at all. Obviously, the enzyme converts dCMP to dUMP which in turn is a substrate for dTMP synthetase, the level of which is also increased by phage infection. But the level of ribonucleotide reductase activity is also increased which will lead to an extremely efficient conversion of ribonucleotides to deoxyribonucleotides. The host organism obtains an adequate supply of dUMP in the absence of dCMP deaminase. It is known now that in E. coli (O'Donovan et al.,

1971) and S. typhimurium (Neuhard and Thomassen, 1971) more than 70% of the cell's dUMP is derived from dCTP by the combined action of a dCTP deaminase and a dUTPase. The other 20 - 30% is obtained by the reduction of UDP, etc. Thus, although the phage dCTPase prevents the synthesis of dUMP via the dCTP deaminase pathway, the presence of dCMP deaminase will compensate for this decrease in dUMP synthesizing capacity.

Hiraga et al. (1967) reported that infection with T2 or T4 induces a new thymidine kinase. Induction of the activity was seen in an E. coli mutant deficient in thymidine kinase, suggesting that the new enzyme is a viral gene product. Okazaki and Kornberg (1964) had previously reported that no change in this activity occurs upon T2-infection. To date these contradictory observations have not been resolved.

Four phage genes, namely wh, td, nrd and cd map very close together on the genome of T4. However, the expression of these genes is not coordinate (Matthews, 1971). None of them is essential since a mutation in any one gene or in all would not be lethal. It should be noted, however, that phage mutants deficient for any one function are at a selective disadvantage as compared to the wild-type. A summary of the enzymology of T-even infection is diagrammed in Figure 3.

The T-even phage DNAs are unusual in that HMC is substituted for C. Base substitutions are also found in the DNAs of certain B. subtilis phages: SP8 (Kallen et al., 1962), Øe (Roscoe and Tucker, 1966), and

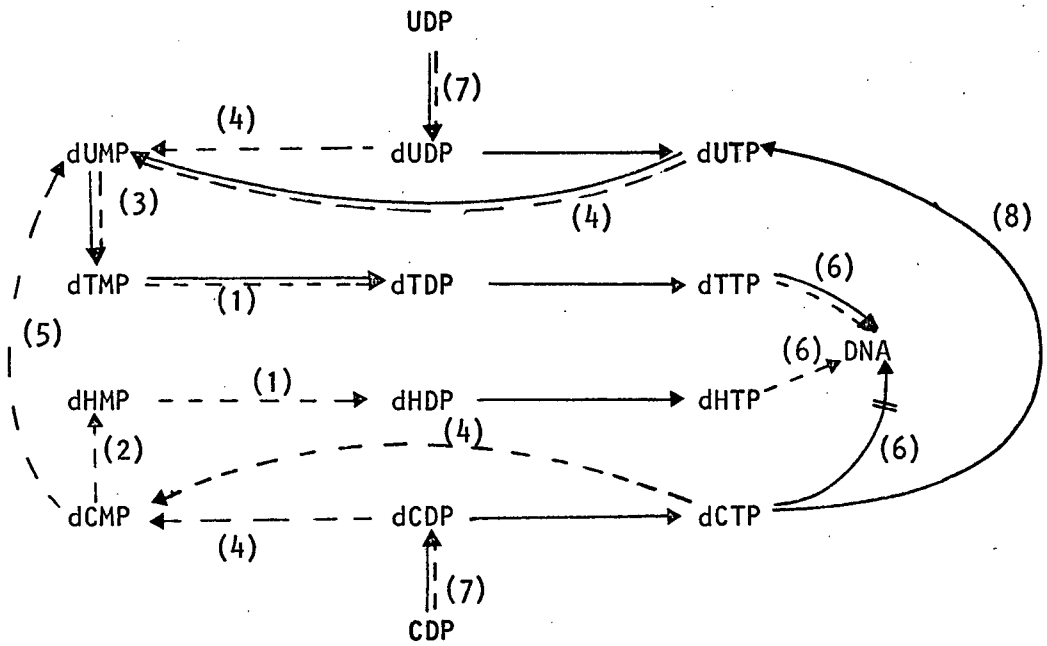

Fig. 3. Nucleotide metabolism in cells infected by T-even bacteriophages. Solid lines represent host enzymes; broken lines represent phage-induced enzymes; solid plus broken lines represent enzymes encoded for by both. Enzymes are: (1), dTMP; dHMP; dGMP kinase; (2), dCMP hydroxymethylase; (3), dTMP synthetase; (4), dCTPase-dUTPase; (5), dCMP deaminase; (6), DNA polymerase; (7), ribonucleoside diphosphate reductase; (8), dCTP deaminase.

SP01 (Okubo et al., 1964) contain HMU instead of T; transducing phages PBS1 and PBS2 (a clear plaque mutant of PBS1) contain uracil instead of T (Takahashi and Marmur, 1963); in SP-15, 5-(4',5'-dihydroxypentyl) uracil replaces about half the thymine residues (Brandon et al., 1972).

Three new enzyme activities are found in cells infected by PBS2: a dUMP kinase, a dTMP phosphatase and a dCTP deaminase (Tomita and Takahashi, 1969). The dUMP kinase probably phosphorylates dUMP to dUDP, which would then be converted to dUTP. The dTMP phosphatase would seem responsible for the exclusion of T from phage DNA. The main route for the formation of dUTP for DNA synthesis may be the deamination of dCTP by the phage-specific dCTP deaminase. Figure 4-1 shows the probable pathways of pyrimidine nucleotide metabolism in PBS1 or PBS2 infected cells.

The mechanism of replacement of T by HMU has been explored by several groups. In cells infected by three different phages, SP8, Øe, and SP5, the mechanism of synthesis of dHMUMP seems identical (Matthews, 1971). A phage-specific dCMP deaminase is induced and this presumably creates a large pool of dUMP which is the substrate for a THFA-dependent dUMP hydroxymethylase. The phosphorylation of dHMUMP, at least to the diphosphate level, is catalyzed by a phage-induced kinase (Kahan et al., 1964). Cells infected with SP8 can incorporate the deoxynucleoside, 5-hydroxymethyldeoxyuridine, but not HMU, into phage DNA (Nishihara et al., 1969), in contrast with T-even infected cells which fail to incorporate the deoxynucleoside of HMC into DNA.

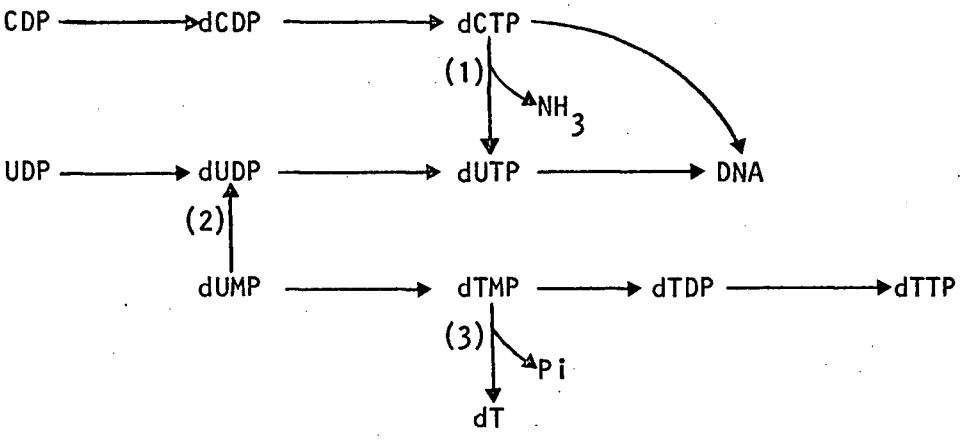

Fig. 4-1. Nucleotide metabolism in cells infected by PBS1 or PBS2. Phage-induced enzymes are: (1), dCTP deaminase; (2), dUMP kinase; (3), dTMP phosphatase.

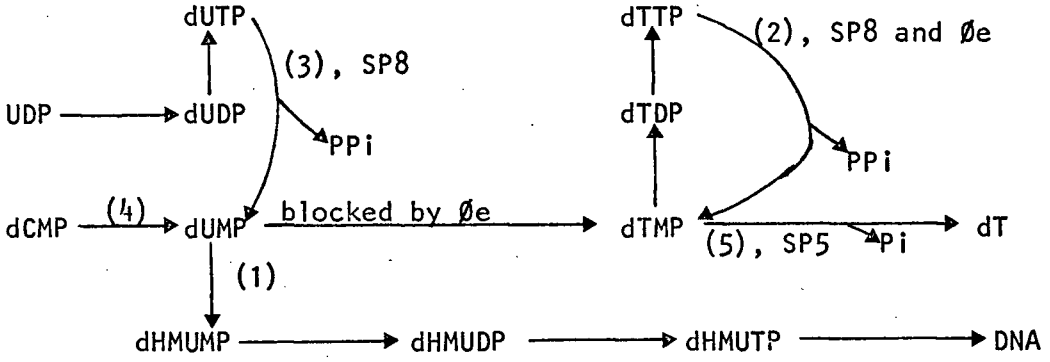

Fig. 4-2. Pathways of HMU nucleotide synthesis and thymine exclusion for various Bacillus subtilis bacteriophages. Phage-induced enzymes are: (1), dUMP hydroxymethylase; (2), dTTPase; (3), dUTPase; (4), dCMP deaminase; (5), dTMP phosphatase.

Although the three phages synthesize dHMUMP in the same fashion, they differ in their mechanisms of T exclusion. Phage SP8 and Øe induce the synthesis of a single enzyme that cleaves dUTP and dTTP to the corresponding monophosphates; in addition, phage Øe forms a protein which acts as a specific inhibitor of dTMP synthetase (Matthews, 1971). SP5 induces a dTMP phosphatase which cleaves dTMP to dT and orthophosphate (Aposhian and Tremblay, 1966). The pathways of HMU nucleotide synthesis and T exclusion are summarized in Fig. 4-2.

Marcus and Newlon (1971) have examined the biological signifigance of the substitution of T with HMU in phage Øe. Infection of a thymine auxotroph of B. subtilis with a mutant of Øe producing a temperature sensitive dTTPase leads to the formation of phage particles in which up to 20% of the HMU is replaced by T. These particles are viable. Mutants of phage SP82G, temperature-sensitive for dUMP hydroxymethylase have recently been isolated (Price et al., 1972). These mutants are defective in DNA synthesis at the restrictive temperature and thus it appears dUMP hydroxymethylase is an essential enzyme for phage DNA synthesis when dTTPase activity is present.

Bacteriophage ØW-14 is a group A type (Bradley, 1967) phage which lyses some strains of Pseudomonas acidovorans (Kropinski and Warren, 1970). The DNA of the phage has a buoyant density of 1.666 g/cm^3 in neutral CsCl and a melting temperature of 99.3 C in standard saline-citrate, values which correspond to 4.5 and 73 moles % G plus C respectively (Kropinski et al., 1973). By chemical analysis the actual

base composition is 56 moles % G plus C. Such discrepancies often result from the presence of an unusual base in the DNA.

The DNA contains five bases; about half the thymine residues are replaced with the fifth base. The base was purified from hydrochloric acid hydrolysates of bacteriophage ØW-14 DNA and shown by chemical, chromatographic and spectral analyses to be 5-(4-aminobutylaminomethyl) uracil (Kropinski et al., 1973). It was given the trivial name N-thyminylputrescine.

The route of biosynthesis of the base became an immediate, intriguing question, coupled with whatever changes in nucleic acid metabolism accompany its synthesis. In order to examine a problem of this nature, it was first necessary to gain some understanding of the nucleic acid metabolism of the host bacterium.

MATERIALS AND METHODS

I. Organisms.

The bacterial strains employed are listed in Table I. In general, stock cultures were maintained at 4 C on standard minimal base slants supplemented with 0.5% yeast extract (Stanier et al., 1966) and were transferred every two months. Slants for the maintenance of thymidine auxotrophs of Pseudomonas acidovorans were supplemented with 1 mg thymidine/ml and 15 µg trimethoprim/ml. Phage ØW-14 stocks were stored at 4 C over chloroform.

II. Media.

Yeast tryptone broth (YT) contained 1% yeast extract (Difco), 0.5% tryptone (Difco), 0.5% NaCl and 0.1% D-mannitol. Dilute mannitol broth (DMB) contained 0.1% tryptone, 0.05% yeast extract, and 0.2% D-mannitol. Minimal medium (M29), a modification of 007 medium (Clark, 1968) contained (g/l): KH_2PO_4, 3.0; Na_2HPO_4, 6.0; $(NH_4)_2SO_4$, 2.0; $MgCl_2$, 0.4; $FeCl_3 \cdot 6H_2O$, 0.0008; $CaCl_2$, 0.017; and succinic acid, 2.0. For solid minimal medium 6.8 g disodium succinate$\cdot 6H_2O$ was substituted for succinic acid. The medium (TCS medium) used for preparing ^{32}P labelled bacteriophage was a modification of the TCG medium described by Kozinski and Szybalski (1959) and contained (g/l): 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; disodium succinate$\cdot 6H_2O$, 6.8; Casamino acids (vitamin free, Difco), 0.5.

Table I. Bacterial strains used in this study

Bacterium	Strain	Derived from	Phenotype	Source
Pseudomonas acidovorans	29		prototrophic	R.Y. Stanier
	3L	29	obligate thymidine requirer	this study
	3L/FU	3L	obligate thymidine requirer; resistant to 5-fluorouracil	this study
	U_1	29	pyrimidine requirer	this study
	U_2	29	pyrimidine requirer	this study
Salmonella typhimurium	LT-2		prototrophic	G.A. O'Donovan

The pH was adjusted to 7.0 with HCl. Solid media were prepared by the addition of 15 g agar/l.

III. Growth of bacteria.

All cultures were grown at 30 C unless stated otherwise. Liquid cultures were incubated in a Metabolyte G-77 shaker water bath (New Brunswick Scientific Co., New Brunswick, N.J.) set at 250 revolutions per min (rpm). Cell density was determined with a Klett-Summerson colorimeter (Klett Manufacturing Co., New York) equipped with a No. 54 filter. Klett units were converted to cell numbers or optical density units by reference to a standard curve.

IV. Titration of phage.

Plaque forming units (pfu)/ml of lysate were determined by the agar layer technique (Adams, 1959) using YT medium. The top and bottom layers contained 0.65 and 1.5% agar respectively.

V. Preparation of high titer lysates.

The host bacterium was grown in 250 ml of YT in 2 liter Erlenmeyer flasks on a New Brunswick G-25 Gyrorotary Shaker set at 250 rpm. At a cell density of 3×10^8/ml, phage were added at a multiplicity of infection (moi) of 0.1. Incubation was continued until lysis was complete. Titers of 10^{11} pfu/ml were routinely obtained.

VI. Purification of phage.

Deoxyribonuclease 1 (DNase) was added to give a final concentration of 1 µg/ml of lysate and digestion was carried out for 1 hr at 30 C. The lysate was freed of any remaining whole cells and debris by centrifugation at 10,000 x g for 20 min. All centrifugations were at 4 C. The supernatant was removed and centrifuged at 17,000 x g for 2 hr. The pelleted material was resuspended in DMB to one tenth of the original volume and centrifuged for 20 min at 10,000 x g. The phage suspension was carefully removed and stored over chloroform at 4 C; the pellet was discarded.

VII. Conditioned medium.

Conditioned medium was prepared by growing the parent strain to a cell density of 2 - 4 x 10^8/ml in M29, then rapidly centrifuging out the majority of cells. The supernatant was decanted and sterilized by filtration through a 0.22 µm Millipore membrane. The collected filtrate was used as conditioned medium.

VIII. Utilization of compounds as carbon or nitrogen source.

(i) Carbon source. Plating medium consisted of M29 basal salts solidified with 15 g/l Ionagar No. 2 (Oxoid). Cells were washed with 10 mM potassium phosphate buffer (pH 7.0) and approximately 5 x 10^6 bacteria were spread on each plate. The test compound (about 1 mg) was added to the center of the plate with a flamed spatula. The plates

were examined for growth daily. A compound was considered to be non-utilizable if growth had not occurred within eight days.

(ii) Nitrogen source. The preparation of plating medium and testing of compounds was as described for carbon source except that $MgSO_4$ was substituted for $MgCl_2$, $(NH_4)_2SO_4$ deleted and disodium succinate added as carbon source.

IX. Analogue sensitivity and pyrimidine substitution patterns.

Minimal plates were seeded with 5×10^6 cells and about 1 mg of a particular analogue was then added to the center of a plate with a flamed spatula. Examinations for a zone of growth inhibition were made within 24 hr. Competition studies between a particular analogue and its biological counterpart were done in M29 at known concentrations of analogue and competitor. Pyrimidine substitution patterns were examined by spreading 5×10^6 washed cells of a pyrimidine requiring strain on minimal plates and then adding the test compound (about 1 mg) to the center of the plate. A compound was considered not to satisfy the pyrimidine requirement if growth had not occurred within eight days.

X. Mutant isolation.

(i) Spontaneous mutants. (a). Thymidine auxotrophic (dT^-) strains were isolated as follows. A culture of the parent strain was grown to 5×10^8 cells/ml in M29 and 0.1 ml of culture was spread on each of a series of minimal medium plates containing the following additions (g/l):

aminopterin (APN), 0.6; trimethoprim (TMBP), 0.015; thymidine, 2.0. Further 0.1 ml samples were spread on plates of the same medium without APN. After incubation, colonies were screened for thymidine auxotrophy. (b). Analogue resistant mutants were selected in the following manner. A culture of the strain in which the mutation was desired, was grown to 5×10^8 cells/ml and 0.1 ml samples spread on plates of minimal medium containing any necessary supplements. Approximately 1 mg of the analogue was added to the center of the plate with a flamed spatula. Colonies occurring within the zone of growth inhibition were screened for the desired traits.

(ii) Mutagenesis with ethane methane sulphonate (EMS). (a). Pyrimidine requiring mutants were isolated from the parent strain after treatment with EMS as described by Hayashi et al. (1964). The parent strain was grown to 4×10^8 cells/ml in 10 ml of M29, then harvested by centrifugation at room temperature, resuspended in 10 ml of 200 mM Tris-chloride (Tris-HCl) buffer (pH 7.5), and recentrifuged. The cell pellet was resuspended in 2 ml of the Tris-HCl buffer. EMS (0.06 ml) was dissolved in 2 ml of the same buffer and added to the 2 ml of washed cells. The suspension (in a tightly sealed flask) was agitated (100 rpm) on a shaking water bath at 30 C for 2 hr. Aliquots (0.1 ml) were transferred to a series of test tubes containing 2 ml of M29 with added uracil (25 µg/ml) and the tubes incubated until the cultures reached saturation. Dilutions were made and 0.1 ml volumes spread for individual colonies on minimal plates supplemented with 0.5 µg

uracil/ml. Any small colonies developing after incubation were tested for pyrimidine auxotrophy. (b). Histidine auxotrophs were selected from a culture of strain 3L mutagenized as described for pyrimidine mutants except that thymidine (final concentration 250 µg/ml) was added to the Tris-HCl buffer and medium. Histidine was added to the medium at 50 µg/ml. After growth to saturation, 0.05 ml samples from each of five culture tubes were transferred to one flask containing 20 ml of supplemented medium and incubated until a cell density of 1×10^8/ml was reached. The cells were collected by centrifugation, resuspended in M29 conditioned medium without added thymidine or histidine and incubated for 6 hr. Dilutions were made and 0.1 ml samples spread for isolated colonies on supplemented minimal plates. Resulting colonies were replicated to the same medium and to medium without histidine. Indicated colonies were further screened with regard to histidine requirement.

(iii) Screening of mutant colonies. Potential mutant clones were treated as follows. The colonies were picked and purified on minimal plates (plus any appropriate supplement), and then tested for the desired characteristics. Auxotrophy was tested simply by streaking cells from the test colony on minimal medium plates, with and without the test requirement and looking for the growth of isolated colonies. Analogue resistance was similarily tested. All mutants were screened for phage ØW-14 sensitivity by spreading a lawn of the mutant on YT medium and streaking a sample of phage lysate across the plate.

XI. Isolation of DNA.

(i) Extraction of DNA from uninfected and phage-infected cells. The DNA was isolated by the sodium dodecyl sulphate (SDS)-Pronase-phenol method (Kozinski and Lin, 1965). The chilled bacteria were harvested by centrifugation at 4 C for 10 min at 10,000 x g, washed once with 10 mM Tris-HCl/150 mM NaCl/15 mM ethylenediamine tetraacetic acid (EDTA), pH 7.6 (TNE buffer) and resuspended to 3 - 6 x 10^8 cells/ml in TNE buffer. The suspension was lysed by the addition of SDS to a final concentration of 0.5% followed by incubation at 40 C for 15 min. An equal volume of 150 mM NaCl/15 mM trisodium citrate, pH 7.0 (SSC, standard saline citrate) was then added. Pronase (self-digested for 30 min at 37 C) was added to a final concentration of 1 mg/ml and digestion carried out for 6 hr at 37 C. The lysate was chilled and extracted with an equal volume of water-saturated phenol at room temperature by revolving in a tube at 40 - 60 rpm at an angle for 1 hr. The mixture was then centrifuged and the aqueous layer removed. Residual phenol in the aqueous phase was extracted by gentle washing (3x) with ether. Two volumes of ice-cold 95% ethanol were added and the nucleic acid precipitates collected on a glass rod and dissolved in 0.1 x SSC/1 mM EDTA. Heat treated (80 C for 10 min) ribonuclease (RNase) was added to a final concentration of 50 µg/ml and digestion carried out at 37 C for 1 hr. DNA was then precipitated from solution as described above, washed (3x) with 70% ethanol and redissolved in a minimal volume of 0.1 x SSC or if the DNA was to be hydrolyzed, it was washed in acetone,

dried and transferred to a glass ampoule after the third ethanol wash.

(ii) Extraction of DNA from phage. The phage lysate was treated as described for purification of phage from high titer lysates, followed by another high speed centrifugation to again pellet the phage particles. The pellet was resuspended in TNE buffer and the DNA extracted by direct addition of water-saturated phenol. After rotation, the aqueous phase was removed, ether washed, and the DNA solution dialyzed (2x) against 0.1 x SSC (4 ml DNA solution/liter of 0.1 x SSC) at 4 C a total of 24 hr. For hydrolysis the DNA was precipitated, washed, and dried as described above.

XII. Hydrolysis of DNA.

A 0.2 ml volume of 6N HCl was added to the glass ampoule containing the DNA sample. The ampoule was sealed under reduced pressure and the DNA hydrolyzed for 90 min at 100 C. The hydrolyzed sample was evaporated (in vacuo over NaOH) to dryness and the residue dissolved in 0.1 N HCl for chromatography.

XIII. Measurement of radioactivity.

(i) Chromatographed hydrolysates of isolated DNA. Chromatograms of DNA hydrolysates were cut in sections, each section transferred to a scintillation vial and the radioactivity per section determined.

(ii) Chromatograms of enzyme reactions. These were treated as described for DNA hydrolysates.

(iii) Intracellular acid soluble material. At specific time

intervals, 3 ml samples of culture were removed and filtered rapidly on a 0.45 µm Millipore membrane; the membrane was washed twice with 5 ml of 0.01 x YT, then transferred immediately to 5 ml of 0.5 N HCl contained in 250 ml beaker on ice. After 1 hr, the supernatant was removed, filtered through a 0.45 µm Millipore membrane and the filtrate evaporated to dryness. The residue was dissolved in 0.2 ml 6N HCl and hydrolysis and chromatography carried out as described for DNA.

(iv) Acid insoluble material. (a). Trichloroacetic acid (TCA) precipitates were collected on 0.45 µm Millipore membranes, washed (3x) with ice-cold 5% TCA, dried and counted. When necessary, RNA was eliminated from the TCA insoluble material as follows. After the addition of TCA, the precipitate was collected by centrifugation at 4 C for 20 min at 10,000 x g, resuspended in 0.3 N KOH and incubated at 37 C for 24 hr. The solutions were chilled and neutralized with 6N HCl. Then, an equal volume of ice-cold 10% TCA was added to each solution. After 2 hr on ice, the RNA-free precipitate was either collected on a 0.45 µm Millipore membrane and treated as described for acid soluble material or extracted with 1 N HCl as follows. The precipitate was collected by centrifugation, resuspended in 1 N HCl and placed at 90 C for 25 min and then placed on ice for 2 hr. The precipitate was collected on a 0.45 µm Millipore membrane and the filtrate was treated as described for acid soluble material. (b). The method of Kleppe et al. (1971) was adapted for the measurement of incorporation of thymidine. Samples were taken from cultures at intervals and spotted on

Whatman 3 MM filter paper discs (2.4 cm in diameter). The discs were immediately immersed in 5% TCA in an ice bath. After 3 hr the discs were washed (3x) with 250 ml ice-cold 5% TCA, once with a mixture of alcohol-ether (1:1) and finally with ether. After drying, the discs were placed in scintillation vials for measurement of radioactivity.

XIV. Counting radioactivity.

After transfer of the samples to scintillation vials, 5 ml of scintillation fluid (Liquifluor, New England Nuclear) was added (except in the case of the tritium release assay) and the radioactivity measured with an Isocap/300 liquid scintillation spectrometer (Nuclear Chicago Corp., Des Plaines, Ill.).

XV. Preparation of bacterial extracts.

Cultures were grown to a density of approximately 3×10^8 cells/ml, centrifuged for 5 min at 10,000 x g and resuspended to one half of the original volume in 40 mM potassium phosphate buffer, pH 7.0. The cell suspension was recentrifuged and the pellet resuspended in sufficient ice-cold 40 mM potassium phosphate/1 mM dithiothreitol buffer, pH 7.0 to give about 10^{10} cells/ml. The cells were disrupted by treatment with a Bronwill sonic oscillator (Bronwill Scientific, Rochester, N.Y.) at a setting of 70 for a total of 60 sec (4 x 15 sec bursts with a 30 sec cooling period between each burst). The extract was centrifuged at 39,000 x g for 1 hr at 4 C, the supernatant removed, dialyzed against 400 volumes of resuspension buffer for 24 hr at 4 C and then used for enzyme assays.

XVI. Protein determination.

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

XVII. Enzyme assays.

All enzyme assays were carried out at 28 C. Changes in absorbance were measured with a Unicam SP. 800 B spectrophotometer (Unicam Instruments Ltd., Cambridge, England) except for the colorimetric measurements in the aspartate transcarbamylase assay where a Beckman Model DU (Beckman Instruments Inc., Fullerton, Calif.) was employed. Extracts of S. typhimurium LT-2 were used as positive controls for testing assay mixtures. All extracts were assayed for fumarase before use and only positive extracts were used for the other assays.

(i) Thymidylate synthetase. The enzyme was measured in three different ways. (a). Spectrophotometrically (Wahba and Friedkin, 1962). Two cuvettes each contained the following: 25 µmoles Tris-HCl buffer, pH 7.4; 50 µmoles mercaptoethanol; 12.5 µmoles $MgCl_2$; 10 µmoles formaldehyde; 0.5 µmole EDTA; 0.1 µmole THFA; extract and water to give a final volume of 0.9 ml. To start the reaction, 0.05 µmole dUMP (0.1 ml of a 0.5 mM solution) was added to one cuvette and the increase in absorbance at 340 nm was followed against the blank cuvette. For the conversion of THFA to DHFA the change in molar extinction at 340 nm was 6.4×10^3. (b). Tritium release. The procedure employed was a combination of the methods of Lomax and Greenberg (1967) and O'Donovan et al.

(1971). The reaction mixtures were incubated for periods of 0, 15, 30 and 45 min. The assay mixture contained the following in 0.3 ml: 10 µmoles Tris-HCl buffer, pH 7.4; 20 µmoles mercaptoethanol; 5 µmoles $MgCl_2$; 4 µmoles formaldehyde; 0.2 µmole EDTA; 0.2 µmole THFA; extract and water. To start the reaction, 0.1 ml of 0.5 mM dUMP-5-^{3}H (1 µCi/ml) was added. The reaction was stopped by the addition of 0.5 ml of a slurry of Norite A (100 mg/ml in 1 mM sodium phosphate/1 mM sodium pyrophosphate, pH 7.4). The Norite with adsorbed dUMP-5-^{3}H was removed by centrifugation. A 0.1 ml amount of the supernatant was mixed with 3 ml Methyl Cellosolve and 6 ml scintillation fluid (Liquifluor) and the radioactivity determined. (c). Incorporation of radioactive formaldehyde. The method described by Roscoe and Tucker (1966) for the assay of dUMP hydroxymethylase was adapted. The reaction mixture contained the following in 0.5 ml: 25 µmoles Tris-HCl buffer, pH 7.4; 0.5 µmole formaldehyde (1 µCi/µmole); 25 µmoles mercaptoethanol; 5 µmoles $MgCl_2$; 0.5 µmole EDTA; 0.1 µmole THFA; 0.5 µmole dUMP; extract and water. After 60 min incubation, 0.2 ml of 30 mM hydroxylamine hydrochloride was added to reaction and control mixtures and the tubes were placed in a boiling water bath in a fume hood for 2 min. Coagulated protein was removed by centrifugation, a portion of the supernatant applied to Whatman 40 SFC paper and the chromatogram developed with solvent D.

(ii) Cytosine (or rC, dC) deaminase. The method was essentially that of Neuhard (1968) for the assay of extracts of S. typhimurium LT-2. The procedure was modified for extracts of Pseudomonas acidovorans;

the reaction mixture contained the following components in a final volume of 5 ml: 125 µmoles Tris-HCl buffer, pH 7.1; 0.5 µmole of substrate; extract and water. At 20 min intervals, 1.0 ml samples were transferred to 1.5 ml 2 N PCA in conical centrifuge tubes on ice and left on ice for 30 min, then centrifuged. Supernatant fluids were read at 290 nm for rC(dC) deaminase assays using a molar extinction of 10.1×10^3. Cytosine deaminase was measured at 282 nm using a molar extinction of 7.3×10^3.

(iii) Adenosine deaminase. The procedure employed was that described by Hoffmeyer and Neuhard (1971), with slight modifications. The reaction mixture contained the following in 1 ml: 25 µmoles Tris-HCl buffer, pH 7.6; 0.05 µmole adenosine; extract and water. The decrease in absorbancy at 265 nm was followed spectrophotometrically and 8.5×10^3 was used as the difference in molar extinction coefficient between adenosine and inosine.

(iv) Thymidine phosphorylase. The assay was carried out as described by Schwartz (1971). The reaction mixture contained in a final volume of 1 ml: 10 µmoles potassium phosphate buffer, pH 7.3; 10 µmoles Tris-HCl buffer, pH 7.3; 5 µmoles thymidine; 1 µmole EDTA; extract and water. At 10 min intervals, 0.2 ml samples were transferred to 0.8 ml 0.1 M NaOH. Samples were read at 300 nm using a molar extinction of 3.6×10^3.

(v) Uridine phosphorylase. The method was essentially that of Neuhard (1968). The reaction mixture contained the following in 1 ml:

10 µmoles potassium phosphate buffer, pH 7.3; 10 µmoles Tris-HCl buffer, pH 7.3; 5 µmoles uridine; 1 µmole EDTA; extract and water. At 10 min intervals, 0.2 ml samples were transferred to 0.8 ml 0.5 M PCA. The precipitated protein was removed by centrifugation and 0.5 ml of the supernatant fluid was mixed with 0.5 ml 1 M NaOH, before reading at 290 nm. The difference in molar extinction coefficient between uracil and uridine was taken as 5.4×10^3.

(vi) Purine nucleoside phosphorylase. The activity was measured spectrophotometrically by coupling the reaction with xanthine oxidase, as described by Kalckar (1947). The reaction mixture contained the following in 1 ml: 170 µmoles potassium phosphate buffer, pH 7.1; 2 µmoles inosine; 0.01 to 0.02 unit of xanthine oxidase (Sigma); extract and water. The increase in absorbancy at 293 nm was followed using a molar extinction of 12.0×10^3.

(vii) Aspartate transcarbamylase. The enzyme was assayed by the method of Gerhart and Pardee (1962). The reaction mixture contained the following in 0.5 ml: 20 µmoles potassium phosphate buffer, pH 7.0; 2 µmoles dilithium carbamyl phosphate; 2.5 µmoles potassium L-aspartate; extract and water. Effectors were added at 0.05 µmole per reaction mixture. The reaction was allowed to proceed for 30 min and the carbamyl aspartate measured colorimetrically.

(viii) Fumarase. The activity was measured spectrophotometrically according to the procedure of Racker (1950), measuring the increase in absorbancy at 240 nm. The reaction mixture contained the following in

1 ml: 40 µmoles potassium phosphate buffer, pH 7.4; 1 µmole L-malic acid; extract and water. For calculations a molar extinction of 2.44×10^3 was assumed.

XVIII. Chromatography.

Samples of DNA hydrolysates, pool materials or enzyme reactions together with reference standards were applied to either thin layer cellulose sheets (Eastman Chromogram Sheet 6064, without fluorescent indicator) or to Whatman 40 SFC grade paper. Chromatograms (descending development for paper) were developed with the following solvents. Solvent A (Bendich, 1957): 2-propanol-concentrated HCl-water (65:16.7:18.3, v/v); Solvent B (Kropinski et al., 1973); 2-propanol-concentrated NH_4OH-water (70:10:20, v/v); Solvent C (Cline et al., 1958): t-butanol-methyl ethyl ketone-concentrated HCl-water (40:30:10:20, v/v); Solvent D (Wahba and Friedkin, 1962): 600 g $(NH_4)_2SO_4$ were added to 1 liter of 0.1 M potassium phosphate buffer, pH 6.8. The solution was filtered through Whatman No. 1 paper and 20 ml n-propanol were added to the filtrate. After development the chromatograms were dried and the ultraviolet absorbing areas detected with a Chromato-vue (Ultra-Violet Products, Inc., San Gabriel, Calif.).

XIX. Density gradient centrifugation.

The DNA sample was mixed with CsCl to give a solution of average density about 1.68 g/ml containing 10 mM Tris-HCl, pH 7.5. The gradient

was formed and the DNA banded by spinning in an International B-60 centrifuge (International Equipment Co., Needham Heights, Mass.) for 48 hr at 20 C in an SB 283 rotor at 30,000 rpm. Approximately 40 fractions were collected from the bottom of each centrifuge tube. The fractions were transferred to glass filters in scintillation vials, dried and the radioactivity per fraction determined.

XX. Chemicals.

Bases, nucleosides, nucleotides and trimethoprim were purchased from Calbiochem (San Diego, Calif.). Aminopterin was obtained from Nutritional Biochemical Co. (Cleveland, Ohio). Tetrahydrofolic acid was purchased from General Biochemicals (Chagrin Falls, Ohio). Other chemicals were obtained from Sigma Chemical Company (St. Louis, Mo.). Synthetic 5-(4-aminobutylaminomethyl)uracil was obtained from A.M.B. Kropinski. The 5-fluoro-analogues were generous gifts from W.E. Scott, Hoffman-La Roche, Inc., Nutley, N.J.

XXI. Radiochemicals.

Radiochemicals were purchased from New England Nuclear (Boston, Mass.) with the exception of dUMP-5-^{3}H which was obtained from Schwarz/ Mann (Orangeburg, N.Y.).

RESULTS AND DISCUSSION

Prior to Kropinski et al. (1973) determining the structure of the novel base, 5-(4-aminobutylaminomethyl)uracil (putThy), it was assumed that the base was a modified pyrimidine. Thus, experiments were initiated to verify or disprove the assumption.

P. acidovorans incorporated exogenous radioactive uracil into acid-insoluble material. Bacteriophage ØW-14 was grown on the wild-type host in the presence of uracil-2-^{14}C. The progeny phage were collected, the DNA isolated, hydrolyzed and chromatographed. Radioactivity was found associated with the cytosine, thymine and putThy areas. No radioactive compound corresponding to putThy was observed in hydrolysates of the DNA from the uninfected host. The radioactive putThy was later shown to co-chromatograph in three solvent systems with the synthetic compound prepared by Kropinski et al. (1973).

Following the uracil labelling experiments, attempts were made to label the DNA of infected and uninfected cultures with radioactive thymine or thymidine using a variety of conditions. These were unsuccessful; little or no incorporation occurred. At this point, it was decided to examine nucleic acid metabolism in the uninfected host before continuing experiments on the biosynthesis of putThy.

Nutritional studies.

The ability of P. acidovorans to utilize various compounds as carbon and/or nitrogen sources was examined (Table II). Nucleosides

Table II. Utilization of various compounds as sole carbon or nitrogen source by Pseudomonas acidovorans

Compound	Ability to grow with compound as[a]	
	Carbon Source	Nitrogen Source
Ribose	-	
Deoxyribose	-	
Thymidine	-	-
Thymine	-	+
Deoxyuridine	-	-
Uridine	-	-
Uracil	nd[b]	+
Deoxycytidine	-	-
Cytidine	nd	-
Cytosine	nd	+
Deoxyadenosine	-	-
Adenosine	-	+
Adenine	nd	+
Deoxyguanosine	-	-
Guanosine	-	-
Guanine	nd	+
Inosine	-	-
Xanthine	nd	+
Hypoxanthine	nd	+

[a] Symbols: +, growth; -, no growth

[b] nd, not done

were ineffective as carbon sources, suggesting that this organism lacked enzymes for the catabolism of these compounds (assuming that the compounds entered the cells). E. coli can utilize deoxyribonucleosides as sole carbon sources but cannot grow on 2-deoxyribose, whereas S. typhimurium can utilize both the sugar and the nucleosides (Hoffee and Robertson, 1969). The difference was explained by the activity of a 2-deoxyribose kinase found in S. typhimurium but not in E. coli. Thus, it was considered important to test the nucleosides as well as the sugars.

Adenosine was the only nucleoside to serve as nitrogen source. Growth with rA was odd in that a lawn of growth around the crystals did not occur, but rather individual colonies developed after several days incubation within a radius of about 20 mm. On closer examination, a zone of growth inhibition (as measured against the weak background of confluent growth) of about 20 mm was evident around the rA crystals. A similar observation was made with dA but no individual colonies developed within the zone. The colonies on the rA plate were probably mutants that had overcome the rA inhibition and were able to use the compound as nitrogen source.

During previous studies on T incorporation, it was observed that the addition of 250 µg dA/ml resulted in pronounced growth inhibition. Concentrations lower than 250 µg/ml had little or no effect.

The toxic effects of dA and rA were readily demonstrated by spreading cells of P. acidovorans on M29 plating medium and adding dA or rA to the

center of the plate. Inhibition of growth in liquid culture by rA required the addition of 500 µg rA/ml. The inhibitory effects of rA and dA are shown in Fig. 5.

The inhibitory effects of nucleosides have been described previously. Shobe and Campbell (Bacteriol. Proc., p.132, 1969) reported inhibition of growth of Micrococcus sodenensis by rA. In vitro experiments demonstrated that ATP completely inhibited 5-phosphoribosylpyrophosphate (PRPP)-amido-transferase activity. Complete inhibition of PRPP-amido-transferase (which is required for the synthesis of the pyrimidine moiety of thiamine as well as for de novo purine synthesis) results in cessation of thiamine biosynthesis. Quantitative studies on thiamine synthesis, in conjunction with the reversal by thiamine, of rA-induced growth inhibition confirmed that the primary effect of rA was the shut-down of thiamine biosynthesis.

When thiamine (10 µg/ml) was added to cultures of P. acidovorans, followed by the addition of rA or dA, growth was still inhibited. Thus, assuming that thiamine entered the cells, the primary effect of dA or rA was not the inhibition of thiamine synthesis.

Two further explanations for the growth inhibition were considered. Firstly, purine nucleoside phosphorylase (pup), which catalyzes the following reactions:

purine deoxyribonucleoside $\rightleftarrows$ deoxyribose-1-phosphate (dRib-1-P) + purine base

or

purine ribonucleoside $\rightleftarrows$ ribose-1-phosphate (Rib-1-P) + purine base

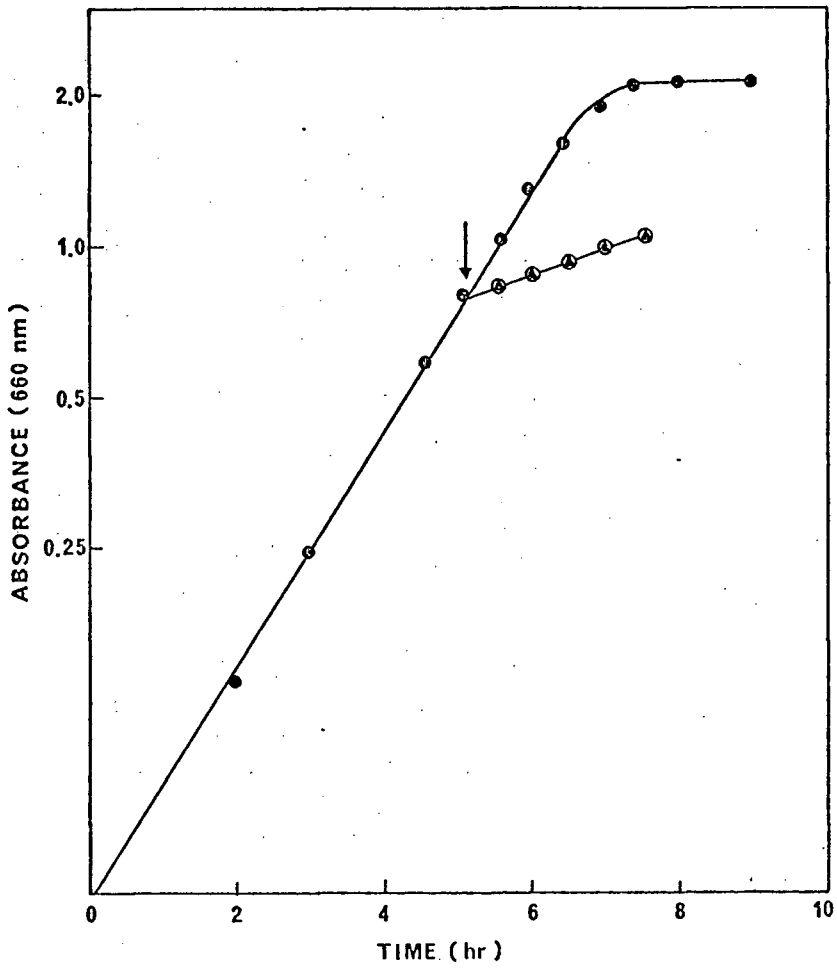

Fig. 5. Inhibition of growth of P. acidovorans in M29 by added adenosine or deoxyadenosine. A culture was grown to 6×10^8 cells/ml, then divided into three portions. Adenosine was added to one and deoxyadenosine to another; the arrow denotes the time of addition. The third served as a control. (O) adenosine (500 µg/ml); (▲) deoxyadenosine (250 µg/ml); (●) control.

was very active and, coupled with deoxyribomutase (drm), resulted in the accumulation of a high level of intracellular dRib-5-P with dA as substrate or Rib-5-P with rA (provided deoxyriboaldolase (dra) was absent). The adenine liberated would serve as nitrogen source and mutation to overcome the toxicity occurred only with rA. Munch-Peterson (1968; 1970) has shown that in dra$^-$ strains of E. coli the addition of deoxyribonucleosides poisoned the cells, apparently due to the accumulation of dRib-5-P. The target site(s) for the inhibition was not resolved. Ribonucleosides were not toxic as any Rib-5-P formed was further metabolized through the pentose-phosphate pathway. However, Rib-5-P may be toxic for P. acidovorans. Secondly, the rA and dA were each phosphorylated to the corresponding nucleotide and a rapid accumulation of a high intracellular pool of the monophosphate (or derivative) resulted; the large pool caused an inhibition of an essential cellular activity. If P. acidovorans could phosphorylate rA or dA, this would be in contrast to S. typhimurium (Hoffmeyer and Neuhard, 1971) and E. coli (Karlström, 1970), which lack such activities.

Ribonucleotide reductase has been shown to be inhibited in vitro by dATP in certain systems (Blakely and Vitols, 1968) which, by extrapolation to the in vivo situation, would mean the cell starves for deoxyribonucleotides in the presence of high dATP pools. Recently, Ingram and Fisher (1972) have demonstrated the selective inhibition

of DNA synthesis by dA in Agmenellum quadruplicatum.

P. acidovorans was assayed for purine nucleoside phosphorylase activity (Table III). Under the conditions of the assay it was concluded that the activity was absent and suggested that the accumulation of dRib-5-P or Rib-5-P was not the cause of growth inhibition. Studies to support or disprove the second hypothesis were not undertaken.

It was considered improbable that the inhibition by rA was a consequence of simple deamination and the resulting inosine being the inhibitory compound since added inosine was not toxic. However, this assumed that P. acidovorans was permeable to inosine. P. acidovorans also lacks adenosine deaminase activity (Table IV).

In view of the toxicity observed with dA and rA it was considered important to test the possible toxicity of the other nucleosides before concluding whether these compounds were non-utilizable as carbon and/or nitrogen sources. No toxicity was observed with any of the other nucleosides listed in Table II.

A significant observation was the ability of T to serve as nitrogen source, whereas dT did not. Thus, a lack of thymidine phosphorylase (tdp) activity was implied, and if so, this would negate the isolation of T auxotrophs in this bacterium. P. acidovorans does appear to lack thymidine phosphorylase activity (Table V).

A thymine auxotroph was first isolated from an E. coli culture that had been exposed to X-rays (Roepke and Mercer, 1947). Subsequently,

Table III. Purine nucleoside phosphorylase activity in extracts of *Pseudomonas acidovorans* and *Salmonella typhimurium*[a]

Bacterium	Specific activity[b] of cells grown in the presence of	
	Adenosine	Deoxyadenosine
Pseudomonas acidovorans	0	0
Salmonella typhimurium	nd[c]	80

[a] Cultures were grown in M29 (0.2% glucose added for growth of *Salmonella*) to a cell density of 6×10^8/ml. The *Pseudomonas* culture was divided into two portions; to one 250 µg/ml of deoxyadenosine was added, to the other 500 µg/ml adenosine. After 1 hr incubation, a fraction of each culture was removed and the cells harvested by centrifugation and extracts prepared.

[b] Expressed as nanomoles of urate formed per minute per milligram of protein.

[c] nd, not done.

Table IV. Adenosine deaminase activity in extracts of *Pseudomonas acidovorans* and *Salmonella typhimurium*[a]

Bacterium	Specific activity[b]
Pseudomonas acidovorans	0
Salmonella typhimurium	19

[a] Extracts were those described in Table III for *Pseudomonas*; both extracts were assayed. The extract of *Salmonella* was prepared from cells grown on minimal medium.

[b] Expressed as nanomoles of inosine formed per minute per milligram of protein.

Table V. Thymidine phosphorylase activity in extracts of *Pseudomonas acidovorans* and *Salmonella typhimurium*[a]

Bacterium	Specific activity[b]
Pseudomonas acidovorans	0
Salmonella typhimurium	28.4

[a] Cells were grown in M29 (plus glucose for *Salmonella*) with added thymidine (250 µg/ml). Log phase cells were collected and extracts prepared.

[b] Expressed as nanomoles of thymine formed per minute per milligram protein.

the folic acid antagonists aminopterin (Okada et al., 1960) and trimethoprim (Stacey and Simson, 1965) were used to select thymine auxotrophs in several genera of bacteria. Bertino and Stacey (1966) and Wilson et al., (1966) independently proposed a mechanism for the selection.

A prerequisite for the isolation of T requiring auxotrophs in microorganisms is the presence of enzymes capable of converting exogenous T to dTMP. Thymidine phosphorylase, an enzyme common to many microorganisms, carries out the reaction: dT + Pi $\rightleftarrows$ T + dRib-1-P. Lactobacilli lack tdp but have an alternative method for the production of dT from T. A trans-N-deoxyribosylase performs the reaction: $base_1$ + $base_2$-deoxyriboside $\rightleftarrows$ $base_1$-deoxyriboside + $base_2$. The dT can be converted to dTMP by thymidine kinase (tdk). An organism having neither tdp nor trans-N-deoxyribosylase is unable to convert T to the nucleoside level and this prevents the isolation of T auxotrophs. However, dT requiring auxotrophs remain possible, providing the organism has a functional tdk.

Cytosine proved to be a most adequate nitrogen source and thus, cytosine deaminase (cod) appeared present. Other bases served as nitrogen source in addition to C but none were as effective as C, including T and U. Cytidine and dC were not nitrogen sources and an absence of cytidine (deoxycytidine) deaminase (cdd) was indicated as was the absence of enzymes capable of degrading the nucleosides and liberating free C.

The assays for cytosine deaminase or cytidine (deoxycytidine) deaminase activity were not carried out until after the isolation of pyrimidine requiring auxotrophs of P. acidovorans. Cytosine deaminase activity was found but compared to S. typhimurium the activity was quite low (Table VI). No cytidine (deoxycytidine) deaminase activity was detected in P. acidovorans extracts (Table VI).

Since U was a nitrogen source but rU failed to support growth, an absence of uridine phosphorylase (udp) activity was indicated. The activity was not observed under the assay conditions (Table VII).

Analogue sensitivity and resistance.

Analogues can be valuable aids as a means of indicating the presence or absence of particular enzyme functions in an organism. Some general principles (O'Donovan and Neuhard, 1970) to consider when working with pyrimidine (and purine) analogues are the following:

(i) The analogue must be converted to the nucleotide level for expression of toxicity.

(ii) Two general types of nucleotide analogue toxicity result: inhibition of one or more enzymes involved in pyrimidine (purine) metabolism and incorporation of the analogue into nucleic acids after conversion to the triphosphate.

(iii) Since analogues are generally metabolized in the same manner, by the same enzymes, as the natural compounds, then it is not surprising

Table VI. Cytosine deaminase and cytidine (deoxycytidine) deaminase activity in extracts of *Pseudomonas acidovorans* strain U_1 and *Salmonella typhimurium*[a]

Bacterium	Specific activity[b]	
	Cytosine deaminase	Cytidine (deoxycytidine) deaminase
Pseudomonas acidovorans	0.11	0
Salmonella typhimurium	3.1	5.2

[a] Cultures of U_1 and LT-2 were grown in M29 (plus glucose for LT-2) with added cytosine (50 µg/ml). Log phase cells were collected and extracts prepared for the assay of cytosine deaminase. The protocol was similar for the preparation of extracts for cytidine (deoxycytidine) deaminase assays, except that 250 µg/ml cytidine was added to each culture and 25 µg/ml of uracil was added to the U_1 culture.

[b] Expressed as nanomoles of product formed per minute per milligram of protein.

Table VII. Uridine phosphorylase activity in extracts of *Pseudomonas acidovorans* and *Salmonella typhimurium*[a]

Bacterium	Specific activity[b]
Pseudomonas acidovorans	0
Salmonella typhimurium	3

[a] *P. acidovorans* was grown in M29 plus added uridine (250 µg/ml). Log phase cells were collected and an extract prepared. The extract of LT-2 employed was that described in Table V. Thus, the basal level of uridine phosphorylase of LT-2 was measured.

[b] Expressed as nanomoles of uracil formed per minute per milligram of protein.

that the addition of the natural substrate reverses or prevents the analogue toxicity through simple competition for conversion to the nucleotide level.

(iv) It is important to understand the interconversions of the natural bases and nucleosides for a particular organism, since many analogues *per se* are not inhibitory but rather become toxic only after being metabolized. For example, 5-fluorocytosine (5-FC) is only toxic for *E. coli* or *S. typhimurium* after conversion to 5-fluorouracil (5-FU). Mutants lacking cytosine deaminase are resistant to 5-FC but remain sensitive to 5-FU. Knowledge regarding interconversions becomes exceedingly crucial when specific radioactive labelling studies are attempted.

P. acidovorans was tested for sensitivity or resistance to a variety of pyrimidine and purine analogues (Table VIII). Compared with the enterics, *P. acidovorans* showed limited sensitivity to the analogues. Purine base analogues were completely ineffective. It was unfortunate that purine nucleoside analogues (especially of dA and rA) were not available.

By extrapolation from previous results, certain of the analogue sensitivities were expected. For example, sensitivity to 5-FU and 6-azauracil (6-AU) was expected since it was already established that *P. acidovorans* was capable of incorporating exogenous uracil, this indicating the presence of UMP pyrophosphorylase (upp). Sensitivity to

Table VIII. Sensitivity of Pseudomonas acidovorans to various nucleic acid analogues

Analogue	Response[a]
5-fluorouracil	S
6-azauracil	S
5-fluoruridine	R
6-azauridine	R
5-fluorodeoxyuridine	S
5-fluorocytosine	S
6-azacytosine	S
5-fluorocytidine	R
6-azacytidine	R
5-fluorodeoxycytidine	R
5-bromouracil	R
5-bromodeoxyuridine	S
8-azaadenine	R
8-azaguanine	R
8-azahypoxanthine	R
2,6-diaminopurine	R
6-azathymine	R

[a] Sensitivity (S) or resistance (R) to an analogue was determined as described in Materials and Methods.

6-azacytosine (6-AC) and 5-FC was also expected as cytosine deaminase activity was indicated from previous studies. Resistance to 6-azathymine (6-AT) was also expected.

Mutants resistant to 5-FU were found to be simultaneously resistant to 5-FC, indicating that toxicity of 5-FC was a consequence of deamination to 5-FU. It should be possible to isolate mutants resistant to 5-FC but remaining sensitive to 5-FU (i.e. cod$^-$ strains). Such strains were not sought in this study.

P. acidovorans did not catabolize nucleosides and, therefore, one would not have expected sensitivity to nucleoside analogues as a consequence of metabolism of the compound and liberation of the free base. However, kinase activities for a variety of nucleosides have been observed in other microorganisms (O'Donovan and Neuhard, 1970) and if such activities were present in P. acidovorans, toxicity to nucleoside analogues would be observed.

It should be pointed out here, as a reminder, that in relating sensitivity and resistance to analogues to the presence or absence of certain enzyme activities, it was assumed that the analogues can enter the cell. In addition, it was assumed that the test conditions were adequate for the demonstration of particular enzyme activities if they were present.

From the results, it was concluded that P. acidovorans, was deficient in kinase activities save for the possibility of thymidine

kinase. As a consequence of certain kinase deficiencies, labelling with radioactive rU or rC appears impossible as does the isolation of rC-requiring mutants (i.e. pyrG mutants). A summary of pyrimidine metabolism in P. acidovorans, as indicated by the results, is diagrammed in Fig. 6.

Conversion of 5-fluorodeoxyuridine (5-FdU) to 5-FdUMP by thymidine kinase results in the inhibition of thymidylate synthetase (thyA) and the cell starves for dTMP. The 5-bromo derivative (5-BdU) exerts its toxic effect as a consequence of phosphorylation to the monophosphate by thymidine kinase, followed by further phosphorylations to the triphosphate and incorporation into DNA.

If thymidine kinase was present in P. acidovorans, the isolation of dT auxotrophs was feasible. Such mutants could prove invaluable in elucidating the biosynthesis of putThy. Therefore, further investigations were carried out to establish the presence or absence of thymidine kinase.

Enzymatic breakdown ot 5-FdU to yield 5-FU and dRib-1-P, with inhibition resulting from the 5-FU was unlikely since uridine phosphorylase activity was not found in extracts. But spontaneous breakdown of the 5-FdU remained possible. The rationale cannot be applied to 5-BdU as 5-BU was non-toxic; furthermore, 5-BU is an analogue of T, not U. Thus, 5-BdU sensitivity was indicative of thymidine kinase activity.

A concentration of 250 µg 5-FdU/ml was required (compare to dA and rA inhibition) to inhibit growth, whereas growth was inhibited by 2.5 µg

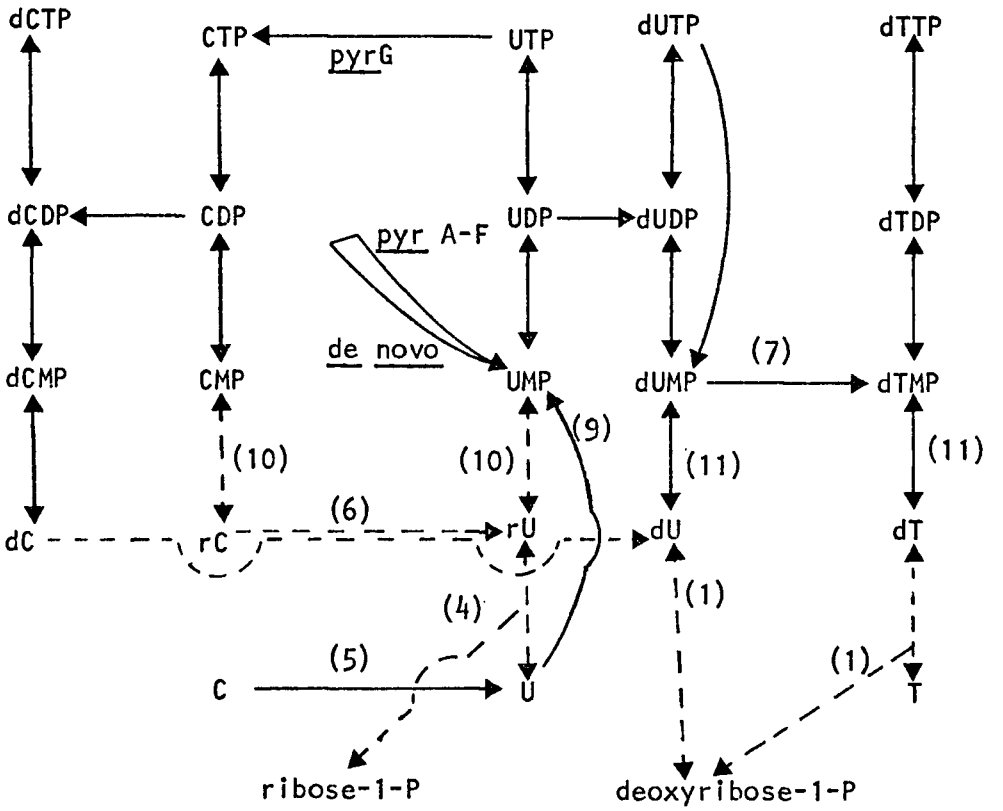

Fig.6. Pyrimidine nucleotide interconversions and metabolism of pyrimidine bases and nucleosides in Pseudomonas acidovorans. Enzymes are as described in Fig. 2. Broken lines represent enzyme reactions that do not occur; solid lines represent most probable reactions. Cytosine triphosphate deaminase is an unresolved reaction.

FU/ml. Simple competition experiments were designed to further clarify the mechanism of 5-FdU toxicity. Thymidine but neither U nor T overcame the 5-FdU toxicity (Table IX). Thus, the isolation of dT auxotrophs was feasible.

Establishing the concentration of 5-FdU (250 µg/ml as compared to 5 µg/ml for E. coli) required for inhibition was most pertinent to the isolation procedure. P. acidovorans dT auxotrophs would probably require at least 250 µg dT/ml for growth and it would be reasonable to expect that concentrations below 250 µg/ml would actually select against such mutants.

Isolation of thymidine auxotrophs.

Preliminary experiments established that APN resistance did not confer resistance to TMBP, and vice versa, nor were such resistant strains dT auxotrophs. Thus, it was predicted that doubly resistant mutants which appeared at appreciable frequency would be dT requiring (i.e. mutated in thyA). The results bore out the prediction. Since dT requiring colonies were not visible on APN plus TMBP medium prior to 4 - 5 days incubation, it seemed reasonable that colonies developing prior to 72 hr incubation on media containing only a single antifolate (i.e. TMBP) would be mutant types other than dT^-, whereas some of the colonies developing after this time would be dT^-. This was also found to be the case.

Table IX. Effect of thymidine, uracil, and thymine on growth inhibition by 5-fluorodeoxyuridine[a]

Culture conditions	Growth response[b]
M29 plus dT	+
M29 plus T	+
M29 plus U	+
M29 plus 5-FdU[c]	-
M29 plus 5-FdU plus dT	+
M29 plus 5-FdU plus T	-
M29 plus 5-FdU plus U	-

[a] Competitors added at 1 mg/ml; 5-fluorodeoxyuridine was added at 250 µg/ml in liquid medium.

[b] Symbols: +, growth; -, no growth

[c] 5-FdU, 5-fluorodeoxyuridine

Characterization of thymidine auxotrophs.

Compared to similar mutants in other bacteria, the P. acidovorans mutants required extremely high levels of dT for growth (Table X). Attempts to isolate secondary mutants of several of these strains which could grow with low levels of thymidine were only partially successful. Strains could be isolated which grew very poorly with 50 µg dT/ml. Thymine concentrations up to 2 mg/ml did not support growth of any of the mutants, not even in the presence of an added deoxyribonucleoside. Therefore, the mutants were obligate dT auxotrophs.

The isolation of a dT requiring mutant in a Pseudomonas species has been reported previously (Espejo et al., 1971). The mutant required a very low level of dT (1 µg/ml) for growth, and it was not determined whether T could substitute for dT. However, this bacterium, a marine halophile, cannot be assigned to any of the subgeneric catagories (Espejo and Canelo, 1968) described by Stanier et al. (1966).

Obligate dT auxotrophs have been isolated in Diplococcus pneumoniae (Brunel et al., 1971; Friedman and Ravin, 1972). These mutants required approximately 20 to 25 µg dT/ml for growth.

Strain 3L was chosen for the following studies: dT starvation, dT incorporation into DNA, uracil labelling of DNA, thymidylate synthetase activity, and use of thymidineless death for the isolation of doubly auxotrophic mutants.

Starvation for dT resulted in exponential cell death (Fig. 7). After 30 min starvation, viability was reduced by 50%.

Table X. Concentration of thymidine required for growth of primary isolates of thymidine auxotrophs of P. acidovorans[a]

Mutants[b]		Thymidine concentrations[c] (µg/ml)				
		2000	1000	500	250	0
1	1A	+	+	-	-	-
2	1B	+	+	-	-	-
3	1C	+	+	-	-	-
4	1D	+	+	-	-	-
5	1E	+	+	-	-	-
6	5A	+	+	+	-	-
7	5B	+	+	-	-	-
8	6A	+	+	-	-	-
9	6B	+	+	+	+	-
10	1S	+	+	-	-	-
11	1L	+	+	+	-	-
12	2S	+	+	-	-	-
13	2L	+	+	+	+	-
14	3S	+	+	-	-	-
15	3L	+	+	+	+	-

[a] Symbols: +, growth in 24 hr; -, no growth in 24 hr

[b] Mutants 1 - 9, selected on medium containing both aminopterin and trimethoprim;
10 - 15, selected on medium containing only trimethoprim

[c] Final concentrations of thymidine in YT.

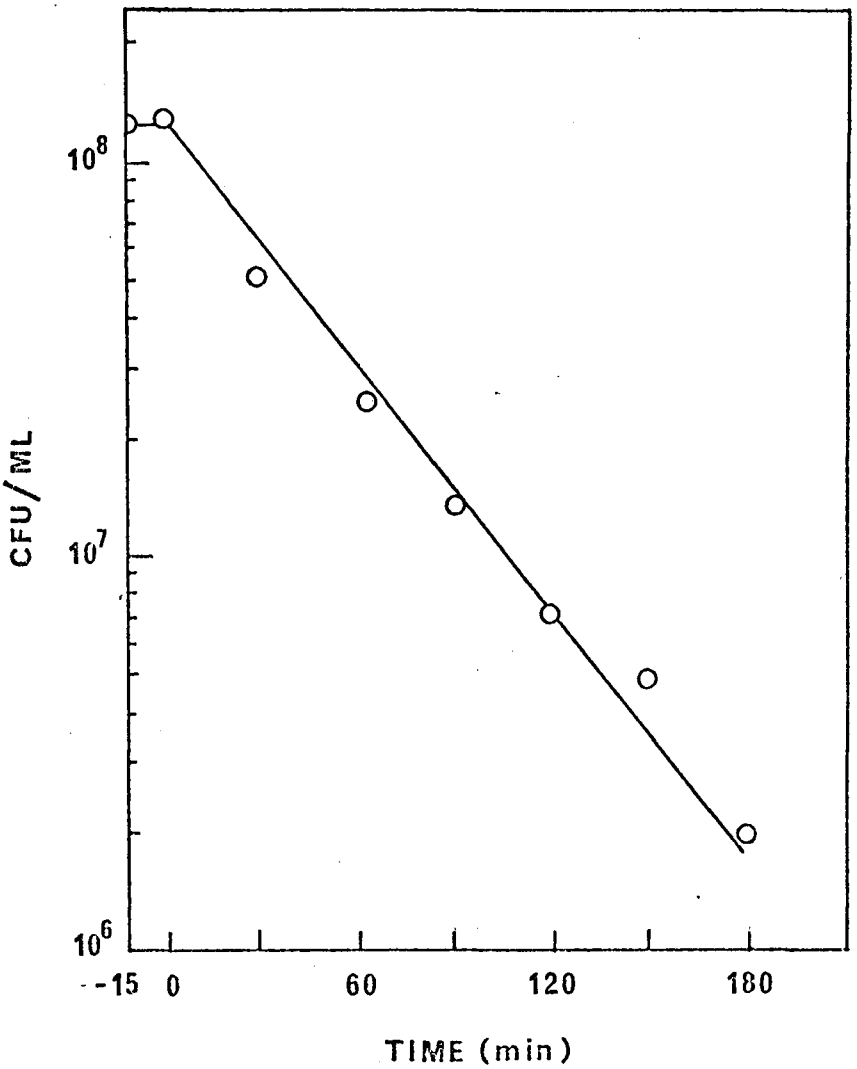

Fig. 7. Thymidineless death of strain 3L. The mutant was grown to 1 - 2 x 10^8 cells/ml in YT plus 250 µg thymidine/ml. The cells were collected by centrifugation and resuspended in YT without dT. Incubation was continued and samples were removed at various times for determination of colony forming units (cfu).

Strain 3L incorporated exogenous ^{3}H-methyl-dT into acid-insoluble material (Fig. 8). As mentioned previously, the wild-type did not incorporate significant amounts of dT.

Purification of DNA from strain 3L grown in the presence of ^{3}H-methyl-dT showed that the incorporated radioactivity was associated with DNA. Chromatographic analyses of hydrolysed DNA from strain 3L and wild-type cultures grown in the presence of uracil-2-^{14}C demonstrated that added U did not label T in the DNA of the mutant, whereas the T residues of wild-type DNA were radioactive (Table XI).

No thymidylate synthetase activity (as measured by tritium release) was detected in extracts of strain 3L, whereas wild-type extracts had excellent activity (Table XII).

The finding that thymineless death in E. coli was sharply curtailed by omission of a carbon and energy source (Barner and Cohen, 1954) or of a required amino acid (Barner and Cohen, 1957), provided a rationale for the use of T starvation to select polyauxotrophic mutants. Bauman and Davis (1957) succeeded in isolating such mutants in E. coli by allowing cells to undergo thymineless death during incubation in minimal medium. Wachsman and Hogg (1964) obtained similar results with Bacillus megaterium. Doubly auxotrophic mutants accounted for the majority of the survivors.

Doubly auxotrophic mutants (dT^{-}, $histidine^{-}$) were isolated in strain 3L using the thymidineless death technique. Approximately 1% of the survivors tested were found to be histidine requiring (His^{-}), a

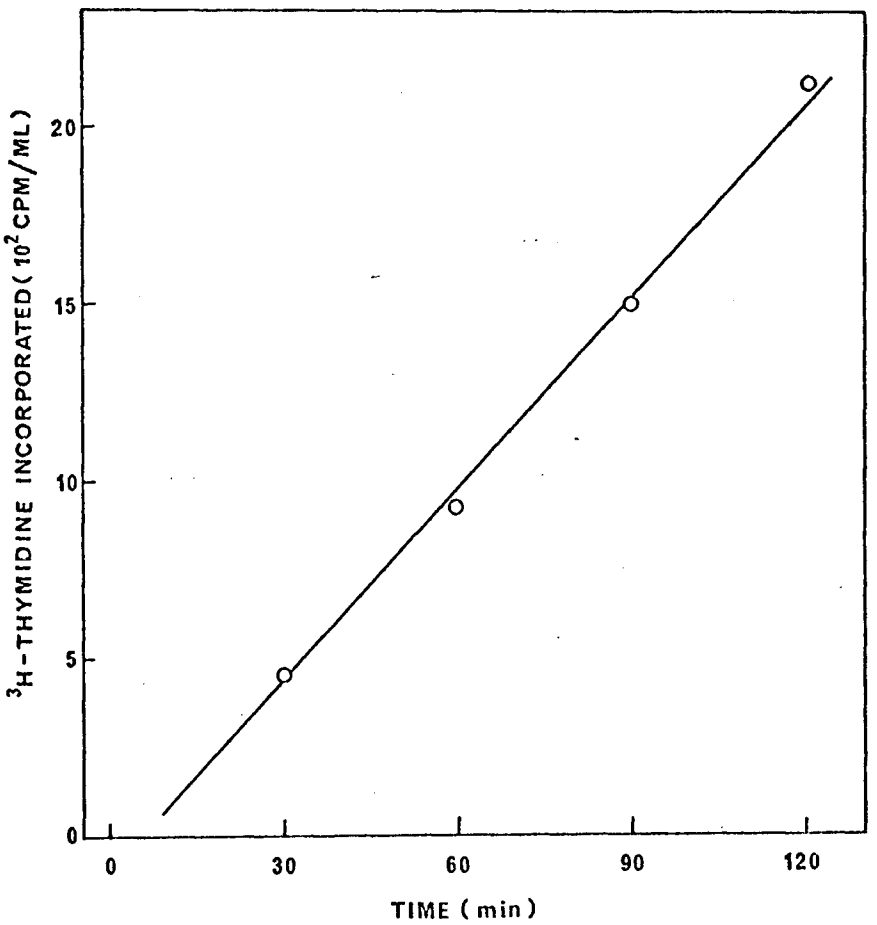

Fig. 8. Incorporation of ^{3}H-methyl-thymidine into acid-insoluble material in strain 3L. The mutant was grown to 3 x 10^8 cells/ml in M29 plus 250 µg/ml thymidine. ^{3}H-methyl-thymidine was added (25 µCi/ml), incubation continued and 0.05 ml samples removed at specific times and treated as described in Materials and Methods.

Table XI. Uracil-2-^{14}C labelling of DNA bases of Pseudomonas acidovorans 29 and strain 3L[a]

Base	Radioactivity[b] in	
	29	3L
Adenine	0	0
Guanine	0	0
Cytosine	1876	3675
Thymine	916	17

[a] Cultures of the parent strain and strain 3L were grown in M29 plus 250 µg thymidine/ml. At a cell density of 2 x 10^8/ml, uracil-2-^{14}C (0.1 µmole/ml; 0.25 µCi/ml) was added. Incubation was continued for 2 hr, the cells were collected and the DNA isolated and treated as described in Materials and Methods.

[b] Values are expressed as total counts per minute in the spot cut from the chromatogram. Backgrounds have been subtracted.

Table XII. Thymidylate synthetase activity in extracts of *Pseudomonas acidovorans* 29 and strain 3L

Strain	Specific activity[a]
29	0.28
3L	0

[a] Expressed as nanomoles dUMP converted per minute per milligram of protein.

lower percentage than expected. On closer examination of the thymineless death curve of B. megaterium, it was observed that the decrease in colony forming units (cfu) began about 1 hr after T removal. In other words, it took time for the cells to use up residual thymine before thymineless death started. Once the amino acid pools are depleted in any auxotrophic cells, those cells become stringent and survive. Thus, there would be an enrichment for auxotrophs as a consequence of the time required for thymine depletion.

Loss of cfu with strain 3L began virtually immediately after removal of dT (refer to Fig. 7). Thus, many His^- cells were probably not in a stringent state and subsequently lost viability. This hypothesis was not investigated further.

Isolation and characterization of pyrimidine requiring mutants.

From thirty colonies tested, two were found to be pyrimidine-requiring (Pyr^-). The mutants were designated U_1 and U_2.

Various compounds including intermediates of the de novo pathway were tested for the ability to substitute for the pyrimidine requirement (Table XIII). None of the intermediates of the de novo pathway supported growth of either mutant. Assuming that P. acidovorans is permeable for these compounds, then the mutants were defective in either or both of the following: pyrE (OMP pyrophosphorylase) or pyrF (OMP decarboxylase).

Isaac and Holloway (1968) found that Pyr^- mutants of P. aeruginosa affected in genes pyrB, C and D were unable to grow on any of the de novo

Table XIII. Ability of various compounds to substitute for the pyrimidine requirement of strains U_1 and U_2

Compound	Growth response[a]
carbamyl aspartate	-
dihydroorotate	-
orotate	-
uridine-5'-monophosphate	-
orotidine-5'-monophosphate	-
uracil	+
uridine	-
deoxyuridine	-
cytosine	+
cytidine	-
deoxycytidine	-
thymine	-
5-hydroxymethylcytosine	-
5-hydroxymethyluracil	-
5-methylcytosine	-

[a] Symbols: +, growth; -, no growth

intermediates. P. acidovorans was possibly impermeable to the intermediates as well.

Strain U_1 has been tentatively identified as a pyrD mutant (i.e. mutated in dihydroorotate dehydrogenase) and strain U_2 tentatively identified as a pyrE mutant (G.A. O'Donovan, personal communication). Therefore, no intermediate of the pathway could have supported growth of U_2 but strain U_1 should have used orotate, provided the compound entered the cell. Apparently, it did not.

Growth of the mutants was supported only by U or C. Cross-feeding of the mutants by the wild-type did not occur, indicating that the parent strain did not excrete U or C in appreciable amounts.

A rather unexpected observation was made with regard to growth on uracil. A large zone of growth inhibition was observed around the added uracil. Upon continued incubation, the zone gradually decreased in size until it was eventually grown over. Apparently, the mutants were inhibited by high U concentrations, and the inhibition was specific for U since no inhibition occurred with C (which reflects the low activity of cod). The wild-type was not inhibited by added uracil.

The uracil-inhibition phenomenon was evident for both strains in liquid culture. Inhibition by U of Strain U_1 is shown in Fig. 9; U_2 gave similar results. The increased concentration of U (at least up to 100 µg/ml) did not result in a slower growth rate, but rather, increased the lag period. Addition of arginine or ornithine or both failed to overcome the inhibitory effect of U.

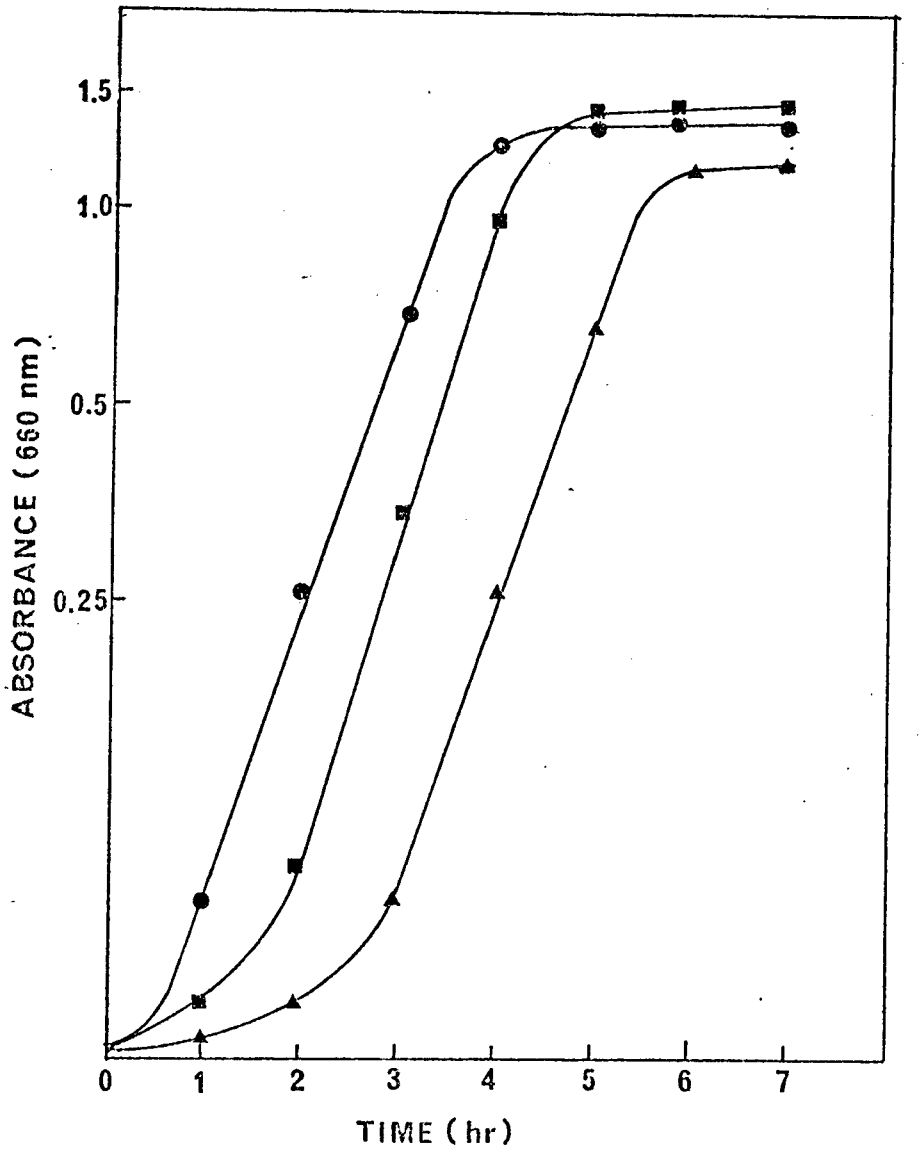

Fig. 9. Effect of added uracil on growth of strain U_1. The mutant was grown in M29 with (●) 20 µg, (■) 50 µg, and (▲) 100 µg of uracil/ml.

The Pyr$^-$ strains of P. aeruginosa were not affected by U at 100 µg/ml (Isaac and Holloway, 1968). Experiments employing higher concentrations of U were not reported.

Repression of ATCase.

It has been demonstrated for many biosynthetic pathways in a variety of organisms that the rate of synthesis of the enzymes involved in the biosynthesis of a compound can be markedly decreased by the presence of the particular compound (or readily converted precursor) in the medium.

Growth of P. acidovorans was completely inhibited by the addition of 5-FU to M29 at a final concentration of 2.5 µg/ml. Since such a low concentration of this analogue inhibited growth, it seemed likely that the pyrimidine biosynthetic pathway in P. acidovorans was regulated by mechanisms responsive to added U.

Recently, regulatory mutants of the pyrimidine pathway have been isolated in S. typhimurium (O'Donovan and Gerhart, 1972). Constitutive mutants of the pyrimidine pathway (ATCase derepressed at least 20-fold) were obtained by selection for strains simultaneously resistant to both 5-FU and 5-FrU or by a screening procedure that tested for pyrimidine excretion. In addition, mutants in which ATCase was partially desensitized to its feedback inhibitor, CTP, were isolated by screening for pyrimidine over-producers (i.e. excreters), but mutants of this type were not isolated through selection for double drug resistance.

A completely desensitized ATCase mutant might be the only variety of desensitized mutant capable of surviving the conditions of selection. However, as evidenced from the results of these workers, if such mutants are possible, under the conditions of selection, they must occur at low frequency.

The level of ATCase activity in P. acidovorans was determined after growth in M29 and M29 plus uracil (Table XIV). There was no evidence of repression of the enzyme. A similar lack of repression of the enzymes involved in pyrimidine biosynthesis was observed for P. aeruginosa (Isaac and Holloway, 1968).

Three possible explanations for the inability to demonstrate repression can be suggested.

(i) ATCase in P. acidovorans is simply non-repressible. However, other enzymes of the pathway may be subject to repression.

(ii) Intracellular levels of pyrimidine compounds in the wild-type cell grown in minimal medium are sufficient to effect repression of ATCase in the absence of exogenous uracil.

(iii) Repression is not a major mechanism of control of the pyrimidine pathway in P. acidovorans.

Feedback inhibition of ATCase.

ATCase from E. coli is subject to feedback inhibition and stimulation by various nucleosides and nucleotides (Gerhart and Pardee, 1962). The effects are competitive with respect to L-aspartate.

Table XIV. Amount of aspartate transcarbamylase activity in extracts of Pseudomonas acidovorans grown in the presence and absence of uracil[a]

Medium	Specific activity[b]
M29	13.0
M29 plus uracil	13.1

[a] Uracil was added to a final concentration of 25 µg/ml.

[b] Expressed as nanomoles of carbamylaspartate formed per minute per milligram of protein.

The ATCase of Pseudomonas fluorescens is inhibited as much as 90% by 7 mM UTP and 60% by 7 mM ATP (Neumann and Jones, 1964). These inhibitions were competitive with respect to carbamyl phosphate and non-competitive with respect to L-aspartate.

The ATCase from P. aeruginosa (Isaac and Holloway, 1968) is inhibited by ATP and UTP. However, in this study, the high concentrations of effector nucleotides used, would appear non-physiological when compared with the sizes of the nucleotide pools in E. coli. The same criticism can be directed at the studies of Neumann and Jones (1964). Thus, it is not possible to relate the significance of the in vitro results to the in vivo situation. Determination of the nucleotide pool sizes in these organisms would be helpful in assessing the significance of the observed inhibitions.

The effect of various nucleotides(added at a final concentration of 0.1 mM) on the activity of ATCase from crude extracts of P. acidovorans was examined (Table XV). No marked inhibition was observed with any nucleotide tested. CTP, if anything, was stimulatory; uridine nucleotides may have caused slight inhibition.

The studies on regulation of pyrimidine metabolism were of an extremely limited nature. However, it seems that the regulation of the pyrimidine biosynthetic pathway in P. acidovorans and other pseudomonads is unlike that seen in the enteric bacteria.

Table XV. Influence of uridine nucleotides and cytidine triphosphate on aspartate transcarbamylase activity from crude extracts of Pseudomonas acidovorans

Nucleotide[a]	Specific activity[b]
None	13.1
UMP	11.8
UDP	12.6
UTP	12.6
CTP	13.5

[a] Nucleotides were added to a final concentration of 0.1 mM.

[b] Expressed as nanomoles carbamyl aspartate formed per minute per milligram of protein.

Preliminary studies on the biosynthesis of 5-(4-aminobutylaminomethyl) uracil.

As mentioned previously, the putThy, C and T residues of progeny phage DNA were labelled when bacteriophage ØW-14 was propagated in a medium containing radioactive uracil. This was achieved prior to determination of the structure of putThy and substantiated that the novel base was a modified pyrimidine. Adequate separation of C, T and putThy was possible in a variety of solvent systems (Table XVI), and thus, it was unlikely that the radioactivity observed in the putThy area was due to trailing radioactivity from the other pyrimidines. In addition, chromatograms prepared from uninfected control cultures contained no radioactivity in the putThy area.

Before proceeding with experiments on putThy biosynthesis, the efficiency of infection of P. acidovorans by bacteriophage ØW-14 was studied (Fig. 10). At 8 min after phage addition, only 8% of the cells were viable colony forming units; at 2 min after phage addition, 40 - 50% of the cells were converted to infective centers and no increase in detectable infective centers occurred with continued incubation. At this stage, an adequate explanation cannot be offered for only some 50% of the infected cells yielding progeny.

Bacteriophage ØW-14 infection of thymidine auxotrophs.

Strain 3L lacked thymidylate synthetase activity since radioactive uracil did not label the T residues of its DNA.

Table XVI. Chromatography of nucleic acid bases

Base	Rf[a] in solvent[b]		
	A	B	C
Adenine	0.33	0.63	0.32
Guanine	0.21	0.38	0.22
Cytosine	0.47	0.60	0.43
Thymine	0.80	0.77	0.86
putThy	0.15	0.53	0.10

[a] Rf, ratio of distance travelled by the compound from the origin to the distance travelled by the solvent from the origin.

[b] Letters correspond to the solvent systems as described in Materials and Methods.

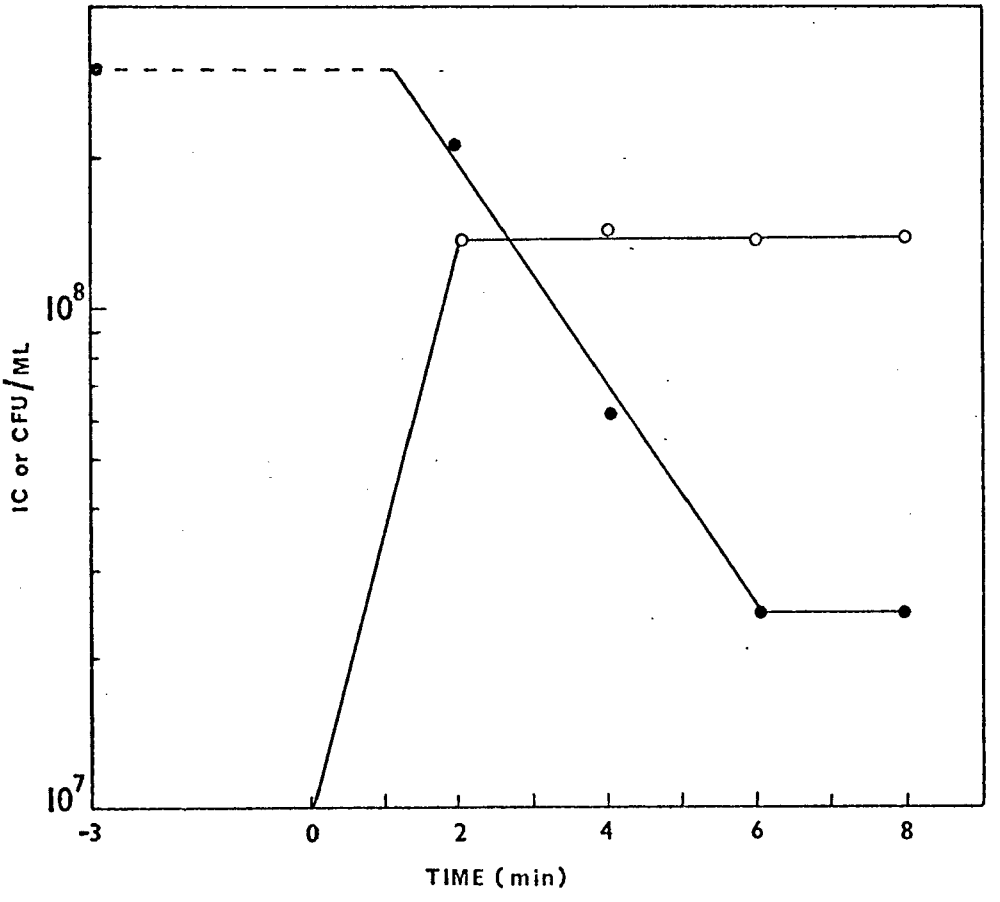

Fig. 10. P. acidovorans was grown in M29 to a cell density of 3×10^8/ml and then infected with phage ØW-14 at a moi of 10. At intervals after infection, samples were removed and diluted 100-fold into medium containing phage antiserum (a gift from A.B.M. Kropinski). The phage antiserum had been preadsorbed with uninfected bacterial cells. The colony forming units (cfu) and infective centers (ic) were determined for each sample. (●) cfu; (O) ic.

If putThy was derived from T (regardless of whether modification occurred at the nucleotide or macromolecular level), then the DNA of phage grown on 3L in the presence of radioactive uracil should be labelled in the C but not in the T and putThy residues. However, if putThy was not derived from T, then the C and putThy but not the T residues should be labelled unless the phage induced a phage-specific thymidylate synthetase. Induction of a phage thymidylate synthetase would prevent any conclusion being made with regard to the precursor of putThy.

The experimental results suggested that the phage does encode for a thymidylate synthetase (Table XVII). In addition, the ratios T/C and putThy/T for phage DNA remained virtually constant regardless of the host strain, indicating the preferential incorporation of "de novo" dTMP rather than that formed from added dT. Therefore, infected wild-type cells would contain both the host and the phage enzymes, whereas infected cells of strain 3L, would contain only the phage enzyme. The effect of the loss of the host activity on phage development was not examined.

The results did not offer any insight into the precursor question. Two approaches to the problem remained. One was the isolation of a phage mutant deficient in thymidylate synthetase, the second was to label the phage DNA with radioactive dT.

Initially the isolation of a phage thyA mutant was considered as

Table XVII. Labelling of bacteriophage ØW-14 and host DNAs with uracil-2-^{14}C[a]

Strain	Base[b]	Radioactivity in uninfected host DNA[c]	Radioactivity in isolated progeny phage DNA[d]	Ratios in phage DNA	
				$\frac{T}{C}$	$\frac{putThy}{T}$
wild-type	Adenine	0	0		
	Guanine	0	0		
	Cytosine	5276	6789	0.42	0.86
	Thymine	2387	2877		
	putThy	0	2451		
3L	Adenine	0	0		
	Guanine	0	0		
	Cytosine	8822	7844	0.42	0.83
	Thymine	0	3492		
	putThy	0	2759		

[a] Wild-type and strain 3L were grown in M29 (250 µg dT/ml was added to the 3L culture) to 3×10^8 cells/ml, then uracil-2-^{14}C was added (0.1 µmole/ml; 0.25 µCi/ml). Each culture was divided into two portions and 10 min later one portion of each infected with bacteriophage ØW-14 at a moi of 10. At 65 min a sample of each uninfected culture was transferred to 10% TCA on ice. Infected cultures were incubated until lysis was complete (approximately 120 min). Analyses of incorporated label were carried out as described in Materials and Methods.

[b] Bases were separated by thin layer chromatography with solvent A.

[c] Values are for alkali-resistant, acid-insoluble material and are expressed as counts per minute in the section cut from the chromatogram.

[d] Values are for isolated DNA and are expressed as for the uninfected host.

a relatively easy task. Such a mutant was of interest other than just as a tool for elucidating the pyrimidine precursor of putThy. Wild-type cells were sensitive to TMBP and APN whereas thyA mutants were resistant. It seemed likely that the phage thymidylate synthetase would render infected cells of strain 3L sensitive to the antifolates, so that the analogues should block development of the phage. Cells infected by phage thyA mutants would remain resistant and thus, phage development would continue.

The effect of the antifolates at a variety of concentrations on wild-type phage development was tested by simply diluting phage stocks and then assaying for the number of plaque forming units (pfu) in the presence and absence of the drugs. No difference was observed in the plating efficiences and the project was abandoned.

Two explanations are possible for the inability of the drugs to inhibit phage development. Firstly, by analogy with T-even infections, bacteriophage ØW-14 may encode for a dihydrofolate reductase. If so, the increased amount of dihydrofolate reductase activity could circumvent the inhibitory effect of the drugs; APN and TMBP are inhibitors of dihydrofolate reductase. Secondly, the synthesis of T by the phage enzyme occurs not through thymidylate synthetase activity per se, but through some other mechanism that does not result in the oxidation of THFA to DHFA.

As a consequence of the apparent phage encoded thymidylate synthetase and the preferential usage of "de novo" dTMP, labelling of phage DNA

with radioactive dT was expected to be rather marginal. The amount of radioactive thymidine-2-^{14}C incorporated into progeny phage DNA was, indeed, small but nonetheless sufficient to allow the conclusion that putThy was not derived from dT (Table XVIII). Experiments using thymidine-6-^{3}H gave similar results, but in these experiments it was necessary to use strain 3L/FU as host (Table XVIII). The commercial dT-6-^{3}H contained contaminants (most probably uracil) and with the quantities of radioactivity used (500 μCi), these impurities posed a problem. Uracil was suspected as the problem contaminant since in preliminary experiments with strain 3L as host, radioactivity was found associated with C, T and putThy. The C and T areas contained about equal amounts of radioactivity, whereas the putThy area contained about a third of that seen in T. With 3L/FU as host, only the T residues were radioactive.

The dT-2-^{14}C posed no problem; all the incorporated label was in T when strain 3L was the host. When the experiment was repeated with strain 3L/FU as host, similar results were obtained.

These results were of profound interest. The data implied that phage DNA biosynthesis occurred through the polymerization of five nucleotides, two purine nucleotides and three pyrimidine nucleotides.

Besides dT, deoxyuridine is also a substrate for thymidine kinase. The resulting dUMP can be converted to dTMP by thymidylate synthetase so that it is possible to label DNA indirectly with radioactive dU.

Bacteriophage ØW-14 was propogated on strain 3L/FU in the presence of radioactive dU. Strain 3L/FU was chosen as host because the commercial

Table XVIII. Labelling of bacteriophage ØW-14 deoxyribonucleic acid with thymidine-2-^{14}C and thymidine-6-^{3}H[a]

Radioactive compound added	Radioactivity[b] in DNA bases of progeny phage				
	Adenine	Guanine	Cytosine	Thymine	putThy
Thymidine-2-^{14}C[c]	0	0	0	582	0
Thymidine-6-^{3}H[d]	0	0	0	648	0

[a] Cultures were grown in M29 plus 250 µg dT/ml to 3 x 10^8 cells/ml and then infected with bacteriophage ØW-14 at a moi of 10. The labelled compounds were added at the time of the inoculation of the growth medium. After lysis, the progeny phage were collected and analyses of incorporated label carried out as described in Materials and Methods.

[b] Bases were separated by descending paper chromatography with solvent C. Values are expressed as counts per minute in the section cut from the chromatogram.

[c] Thymidine-2-^{14}C was added at 2.5 µCi/ml to a culture of strain 3L.

[d] Thymidine-6-^{3}H was added at 25 µCi/ml to a culture of strain 3L/FU.

dU-2-^{14}C contained 1% contaminating U-2-^{14}C. Deoxyuridine proved to be a more effective DNA precursor than dT as judged by the total amount of label incorporated, and both putThy and T were labelled (Table XIX). Therefore, it seemed that putThy was synthesized from a deoxyuridine nucleotide and that the biosynthesis of putThy occurred as a deoxynucleotide rather than as a ribonucleotide with subsequent reduction. The ratio putThy/T was consistent with that observed for other experiments.

Role of tetrahydrofolate in putThy biosynthesis.

The transformation of serine to glycine is accomplished by serine transhydroxymethylase in the presence of pyridoxal phosphate and Mn^{2+}. The acceptor for the hydroxymethyl group is THFA. The nitrogen atoms at the 5- and 10-positions function as the active sites of THFA. In glycine formation the reaction, schematically, is the following:

$$HO\text{-}CH_2\text{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}\text{-}COOH + THFA \rightleftarrows \underset{\displaystyle NH_2}{\underset{|}{CH_2}} - COOH + N_{10}\text{-hydroxymethyl-THFA}$$

The N_{10}-hydroxymethyl-THFA is readily cyclized by loss of water between the N-5 and N-10 positions to yield N_5,N_{10}-methylene-THFA, the cofactor required for dTMP biosynthesis.

Thymidylate synthetase catalyzes a THFA-dependent methylation of dUMP to yield dTMP, by transfer of the C_1-unit from N_5,N_{10}-methylene-THFA to the 5-position of dUMP. The C_1-unit is reduced to the methyl

Table XIX. Labelling of bacteriophage ØW-14 DNA with deoxyuridine-2-^{14}C[a]

Base[b]	Radioactivity in DNA of progeny phage[c]	Ratio of putThy / T
Adenine	0	
Guanine	0	
Cytosine	0	
Thymine	5625	0.89
putThy	4974	

[a] Conditions were as described in Table XVIII, using strain 3L/FU as host. Deoxyuridine-2-^{14}C was added at 2.5 µCi/ml.

[b] Bases were separated by paper chromatography in solvent C.

[c] Values are expressed as counts per minute in the section cut from the chromatogram.

group of dTMP during transfer, THFA being the hydrogen donor and DHFA the resulting second reaction product. Therefore, the THFA serves as both the C_1-unit carrier and as the reductant. The reaction can be summarized as follows:

$$dUMP + N_5,N_{10}\text{-methylene-THFA} \xrightarrow{Mg^{2+}} dTMP + DHFA$$

The resulting DHFA can be reduced to THFA by the enzyme dihydrofolate reductase. APN and TMBP, as mentioned previously, are potent inhibitors of dihydrofolate reductase. Since thymidylate synthetase oxidizes THFA to DHFA during the course of the reaction, the continued action of the enzyme in the presence of APN and/or TMBP depletes the cell of THFA. Since THFA derivatives are required for the biosynthesis of purines, some amino acids, and the initiation of protein synthesis, RNA, DNA and protein synthesis cease in the presence of the antifolates. Therefore, cells are only able to grow in the presence of these analogues and T and/or dT if thymidylate synthetase is inactive. Since T-or dT-requiring mutants can grow in the presence of APN and/or TMBP plus T or dT, it appears that inhibition of dihydrofolate reductase is incomplete allowing the synthesis of catalytic amounts of THFA (for review see O'Donovan and Neuhard, 1970).

Bacteriophage ØW-14 was grown on the parent strain in the presence of serine-3-^{14}C. The progeny phage were collected and the DNA isolated and analyzed (Table XX). Both putThy and T were heavily labelled, which suggested the involvement of a THFA-cofactor in putThy biosynthesis. The ratio of putThy to T again was consistent with previous work.

Table XX. Labelling of bacteriophage ØW-14 deoxyribonucleic acid with serine-3-^{14}C and serine-2,3-^{3}H[a]

Radioactive compound added	Radioactivity[b] in DNA bases of progeny phage					Ratio of $\frac{putThy}{T}$
	Adenine	Guanine	Cytosine	Thymine	putThy	
Serine-3-^{14}C	3624	10493	0	2133	1796	0.88
Serine-2,3-^{3}H[c]	1095	800	0	639	579	0.90

[a] The wild-type was grown to 3 x 10^8 cells/ml in M29 supplemented with 20 µg serine, histidine and methionine/ml and 300 µg adenosine/ml. Radioactive serine was added at 0.125 µCi/ml, followed by the addition of bacteriophage ØW-14 at a moi of 10. Cultures were incubated until lysis and the analyses of incorporated label carried out as described in Materials and Methods.

[b] Bases were separated by two dimensional thin layer chromatography: first dimension, solvent C; second dimension, solvent B. Values are expressed as counts per minute in the section cut from the chromatogram.

[c] Data were obtained from E. Karrer, (personal communication).

What was the oxidative state of the group actually transferred from THFA to the deoxynucleotide during putThy biosynthesis? More specifically, was transfer at the level of oxidation of methylene or formyl? If at the formyl level (via N_{10}-formyl-THFA) only one hydrogen atom of the original hydroxymethyl group donated by serine would be retained; if at the methylene level both hydrogens on carbon-3 would be retained.

Bacteriophage ØW-14 was grown on the wild-type in the presence of serine-2,3-^{3}H and the DNA of the progeny phage analyzed (Table XX). If a methylene group was transferred then the ratio of putThy to T with respect to incorporated radioactivity would be the same as that observed for serine-3-^{14}C, but if a formyl group was transferred the ratio would be halved. It was obvious that a formyl group was not involved in putThy biosynthesis since the ratio putThy/T was unchanged. This experiment was conceived and conducted by E. Karrer.

Karrer (personal communication) has also established that the putrescine moiety of putThy can be derived from ornithine-5-^{14}C. Ornithine-1-^{14}C did not label putThy but this was not unexpected since the 5-methylene group was donated via a THFA cofactor. Unfortunately, P. acidovorans is not permeable to putrescine and the direct testing of this compound as a precursor to putThy is not yet possible.

Analysis of intracellular DNA during phage infection.

With the biosynthesis of putThy partially established, attention

was again focussed on the question of the state of its formation: i.e. was it at the mononucleotide or the polynucleotide level?

The low buoyant density of the phage DNA is consistent with the covalent bonding of putrescine to the DNA (Kropinski et al., 1973). Therefore, if the putrescine was cleaved from the phage DNA, resulting in T residues for each original putThy, the buoyant density would be increased to a value corresponding to 56 moles % G plus C. In other words, a sharp increase in buoyant density would occur. This would also be true if putThy residues were substituted by 5-hydroxymethyl-uracil; in fact, the buoyant density would correspond to a value greater than 56 moles % G plus C.

If during replication of ØW-14 DNA the putrescine moiety was added after polymerization of the DNA, the early synthesized progeny DNA should be of a higher buoyant density than the native DNA of mature phage. Extraction of replicating DNA from infected cells, followed by CsCl density gradient centrifugation would show if replicating DNA was of a different buoyant density than the DNA of mature virus.

Prior to undertaking such an investigation, it was necessary to establish the pattern of DNA synthesis in phage-infected cells. This was accomplished by measuring the incorporation of radioactive uracil into alkali-resistant, acid-insoluble material during the latent period (Fig. 11). DNA synthesis continued at the control rate for about 12 - 14 min at which time DNA synthesis stopped for approximately 6 min. Between 18 - 20 min DNA synthesis resumed, but at a faster rate.

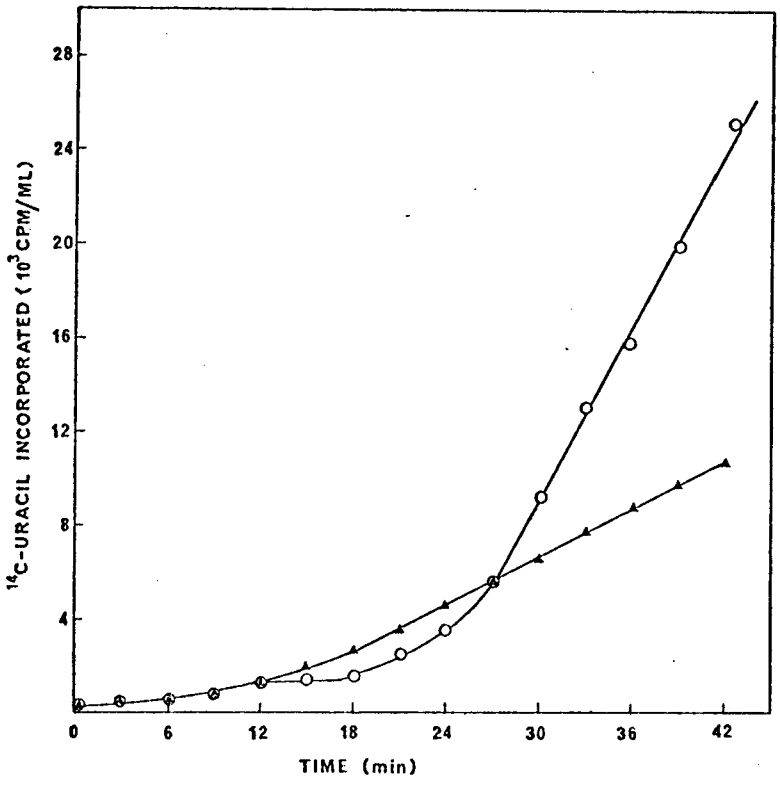

Fig. 11. Deoxyribonucleic acid synthesis in uninfected and phage ØW-14 infected cells. P. acidovorans was grown in M29 to 3×10^8 cells/ml and then uracil-2-^{14}C (0.1 µmole/ml; 0.25 µCi/ml) was added. The culture was divided into two portions and one of them was infected with phage ØW-14 at a moi of 10 after 10 min. At intervals, samples of infected and uninfected bacteria were transferred to an equal volume of 10% TCA on ice. RNA was removed and the radioactivity in alkali-resistant, acid-insoluble material measured as described in Materials and Methods. (O) infected; (▲) uninfected.

PutThy was not detected in alkali-resistant, acid-insoluble material prior to the resumption of DNA synthesis at 18 - 20 min.

Infection of cells with ^{32}P labelled bacteriophage ØW-14 and extraction of the intracellular DNA at various times after infection with subsequent sonication and CsCl density gradient centrifugation of the DNA showed that the buoyant density of the parental label remained unchanged (Fig. 12). This indicated that the buoyant density of the replicating DNA was the same as mature phage DNA.

Two problems were inherent in the experiment. One was a function of using a parental input label. If, prior to or during transcription of the infecting DNA, putrescine was cleaved from the DNA, then an increase in buoyant density would be observed, presenting difficulty with regard to interpretation of the results. However, none of the early DNA samples exhibited any change in buoyant density, indicating that the removal of the putrescine moiety was not required for transcription of the infecting DNA. The second problem is the possibility that the putrescine is added so rapidly after polymerization of the precursor deoxynucleotide that the quantity of non-putrescine containing DNA is inadequate for detection under the experimental conditions. This second problem cannot be resolved within the limits of the experiment.

Analysis of nucleotide pools of phage-infected cells.

A direct approach as to whether putThy was synthesized at the mononucleotide level, was to examine the nucleotide pools and observe if putThy was present in the absence of phage-specific DNA synthesis;

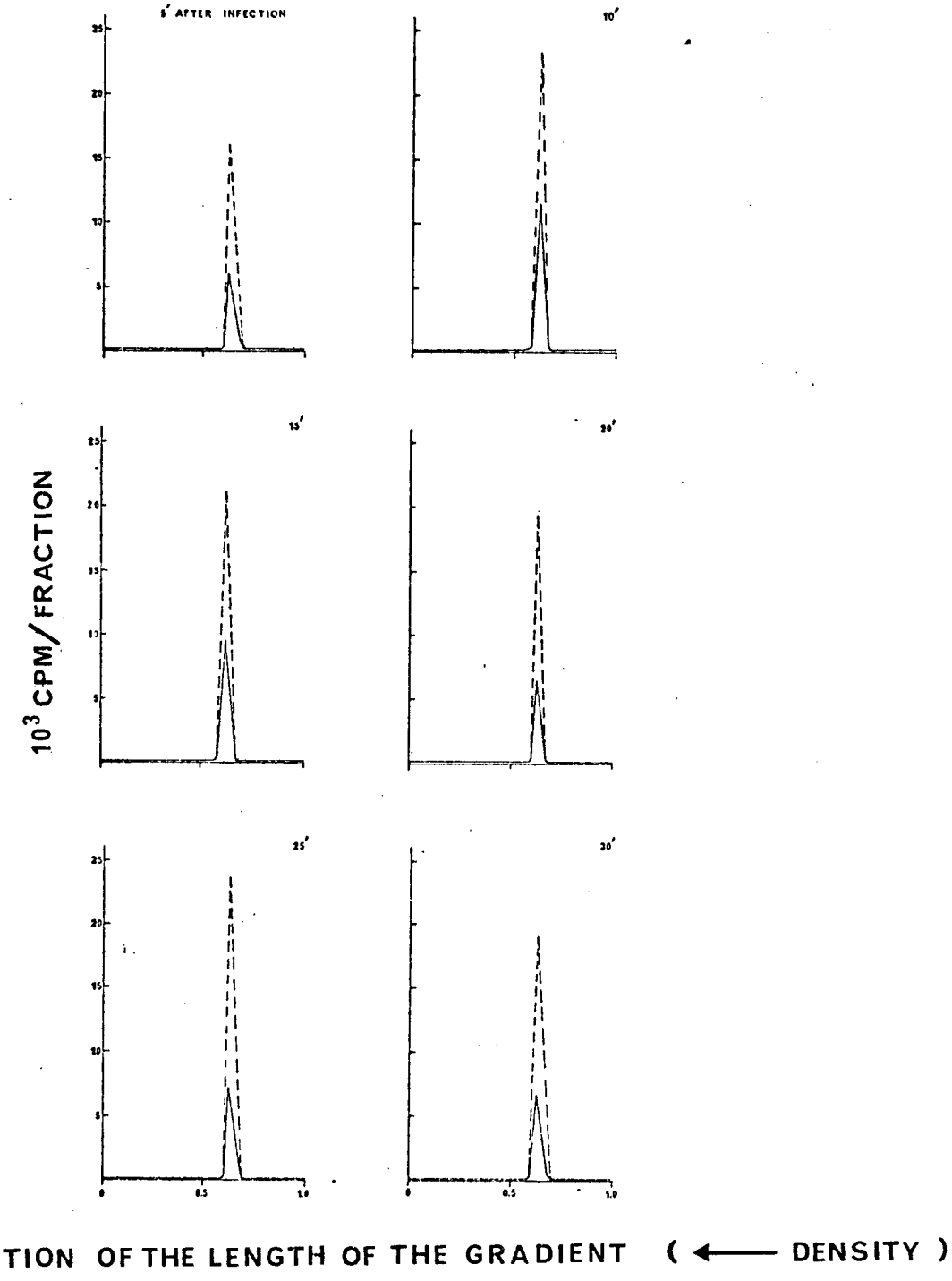

Fig. 12. Cesium chloride density gradient centrifugation analyses of replicating bacteriophage ØW-14 DNA. A culture of P. acidovorans was grown on M29 to 3 x 10^8 cells/ml and then infected with ^{32}P labelled phage ØW-14 at a moi of 2. The ^{32}P phage were prepared by propagating the phage on cells growing in TCS with added ^{32}P (10 µCi/ml). The intracellular DNA was extracted and analyzed as described in Materials and Methods. The tritiated reference ØW-14 DNA was prepared from phage propagated on cells growing in TCS plus added uracil-6-^3H (0.02 µmole/ml; 2.5 µCi/ml). Broken line represents the tritiated reference DNA profile and the solid line represents the ^{32}P intracellular DNA profile. Average recovery of label applied to the gradients was 85%.

phage-specific DNA being defined as DNA containing putThy. It was considered important to find putThy in the absence of phage-specific DNA to circumvent the argument that any putThy in the pools was a consequence of DNA editing and repair during replication.

Strain U_1 was chosen for the experiments, since the choice of label was radioactive uracil and with strain U_1 no dilution of the specific activity of the added label would occur as a result of the de novo pathway. Infection of strain U_1 in the presence of radioactive uracil, followed by extraction of the pool material, demonstrated the presence of putThy in the absence of phage-specific DNA synthesis (Table XXI). This suggests that putThy is synthesized at the nucleotide level and then incorporated into DNA.

In infection by wild-type T4 phage, there are no significant changes in ribo- or deoxyribonucleoside triphosphate pool sizes, except for replacement of dCTP by dHTP (Mathews, 1972). By contrast, infection by T4 DNA-negative mutants causes up to 30-fold expansion of dATP and dTTP, with dHTP accumulating to similar extent. The dGTP pool, however, does not expand to a significant degree. Following infection of P. acidovorans with bacteriophage ØW-14, the cytosine nucleotide pool appeared to be expanded about 5-fold (Table XXI). The significance of such an expansion is unclear. Other pool sizes were not measured.

Enzyme activities of infected cell extracts.

Infection of P. acidovorans with bacteriophage ØW-14 apparently

Table XXI. Labelling with uracil-2-^{14}C of the pyrimidine bases in the nucleotide pools and of alkali-resistant, acid-insoluble material: uninfected and bacteriophage ØW-14 infected cultures of strain U_1[a]

Base[b]	Minutes after Infection	Radioactivity[c] in the areas cut from the chromatograms			
		Uninfected culture		Infected culture	
		Pool Materials	Alkali-resistant acid-insoluble material	Pool Materials	Alkali-resistant acid-insoluble material
Cytosine	12	1794	12288	5766	15959
	18	2590	22814	9830	26960
	24	2132	44823	11820	41307
putThy	12	0	0	635	0
	18	0	0	1075	48
	24	0	0	1519	1317

[a] Strain U_1 was grown in M29 plus 15 µg uracil/ml to a cell density of 3×10^8/ml. The culture was divided into two portions and uracil-2-^{14}C was added to each (4 µCi/ml), immediately followed by the addition of bacteriophage ØW-14 at a moi of 10 to one culture. Samples were removed at specific times and treated as described in Materials and Methods.

[b] Bases were separated by paper chromatography with solvent C.

[c] Values are expressed as counts per minute in the section cut from the chromatogram.

resulted in the synthesis of phage-specific enzymes involved in deoxynucleotide metabolism; eg. thymidylate synthetase and an enzyme(s) for putThy deoxynucleotide synthesis.

Thymidylate synthetase was assayed spectrophotometrically in four different extracts (Table XXII). Excellent activity was observed with the uninfected wild-type; the infected wild-type extract gave comparable activity. This was somewhat surprising since the earlier in vivo studies had indicated the induction of a phage-specific thymidylate synthetase after infection, and an increase in total activity was anticipated in the infected extract. The uninfected strain 3L extract was inactive, as expected. The strain 3L phage-infected extract also appeared to be inactive, which again was surprising even though it agreed with the observation made with the wild-type infected extract.

The apparent lack of phage-specific thymidylate synthetase activity might have resulted from: (a) the assay conditions being unsuitable for the detection of the phage enzyme; possibly an effector or specific cofactor was required, (b) only a low level of phage-specific enzyme being produced.

The extracts were assayed using dUMP-5-^{3}H as substrate (Table XXII). The spectrophotometric assay is limited in that any activity with dUMP as substrate can only be measured if the reaction results in oxidation of THFA, any non-oxidative reactions involving dUMP would not be detected. Under these conditions any activity would be measured which involved addition at the 5-position of dUMP with displacement of the tritium,

independent of THFA oxidation. The infected cell extracts were shown to be highly active when assayed in this manner; dUMP was obviously a substrate and the tritium release was THFA dependent (Table XXII). However, the reaction(s) did not result in detectable oxidation of THFA. The biosynthesis of dHMP in T-even infections or of dHMUMP in Øe-infections is THFA-dependent, but the THFA is not oxidized to DHFA. Substantial tritium release was also observed with the uninfected wild-type, whereas no activity was seen with strain 3L extract. The tritium release observed with the wild-type extract was assumed to be the result of thymidylate synthetase activity. The reaction rate assayed by tritium release was 85% of that observed spectrophotometrically; a reduction of similar magnitude was reported for Lactobacillus casei (Crusberg et al., 1970) and E. coli (Lomax and Greenberg, 1967) and attributed to an isotope effect.

Infected cell extracts mediated a THFA-dependent release of tritium with dUMP-5-^{3}H as substrate without detectable oxidation of THFA. Since in vivo experiments had indicated that putThy was formed at the mononucleotide level, infected cell extracts were assayed for the conversion of dUMP to other deoxynucleotides.

A non-enzymatic reaction between formaldehyde and THFA generates N_5,N_{10}-methylene-THFA. Since tritium release from dUMP-5-^{3}H was THFA dependent, it seemed reasonable to assume that any nucleotide formed could be effectively labelled by radioactive formaldehyde via the THFA cofactor.

Table XXII. Thymidylate synthetase activity in uninfected and bacteriophage ØW-14 infected cells[a]

Extract	Specific activity[b]		
	Spectrophotometric assay	Tritium release assay	
		Complete reaction mixture	minus THFA[c]
wild-type, uninfected	0.33	0.28	0
wild-type, infected	0.30	0.80	0
3L, uninfected	0	0	0
3L, infected	0	1.0	0

[a] Extracts were prepared as described in Materials and Methods. For infected extracts, bacteriophage ØW-14 was added at a moi of 10 and the infective centers collected after 20 min incubation.

[b] Expressed as nanomoles DHFA formed per minute per milligram of protein for the spectrophotometric assay and nanomoles of dUMP converted per minute per milligram of protein for the tritium release assay.

[c] Water was substituted for THFA.

Assays of infected and uninfected 3L extracts under these conditions revealed a phage encoded thymidylate synthetase: the infected extract produced labelled dTMP but the uninfected extract did not (Table XXIII). Furthermore, a significantly greater amount of label appeared to be associated with dUMP. In solvent system D, 5-hydroxymethyldeoxyuridine-5'-monophosphate (dHMUMP) has the same relative mobility as dUMP (Roscoe and Tucker, 1966). The unknown labelled compound was assumed to be dHMUMP.

The formation of dHMUMP in infected extracts is in agreement with the *in vivo* data using serine-2-3-^{3}H as label, which indicated that the biosynthesis of putThy proceeded through a 5-hydroxymethyldeoxyuridine nucleotide intermediate rather than a 5-formyl intermediate. Also, the addition of a hydroxymethyl group to the 5-position of dCMP or dUMP does not result in the oxidation of THFA which would explain the inability to detect any activity by the spectrophotometric procedure. After acid hydrolysis of the compound, the radioactivity co-chromatographed with synthetic HMU.

The amount of thymidylate synthetase activity measured in infected extracts by the radioactive formaldehyde assay was marginal, and this level of activity would be difficult to detect using the spectrophotometric assay. If the assay time was extended for a prolonged period, such low activity might be detected.

A pathway of putThy biosynthesis in bacteriophage ØW-14 infected cells is proposed in Fig. 13. Limited attempts at the *in vitro* synthesis of a putThy deoxynucleotide were unsuccessful.

Table XXIII. Incorporation of radioactive formaldehyde into deoxynucleotide monophosphates in uninfected and bacteriophage ØW-14 infected cells[a]

Extract	Radioactivity[b] in area corresponding to	
	dTMP	dUMP[c]
3L, uninfected	0	0
3L, infected	508	2994

[a] Extracts were as described in Table XXII.

[b] Expressed as counts per minute per millilitre of reaction mixture per milligram of protein.

[c] With solvent system D, the position of radioactive dHMUMP is the same as dUMP and the latter (Rf = 0.59 compared with 0.47 for dTMP) was easily detected in ultraviolet light.

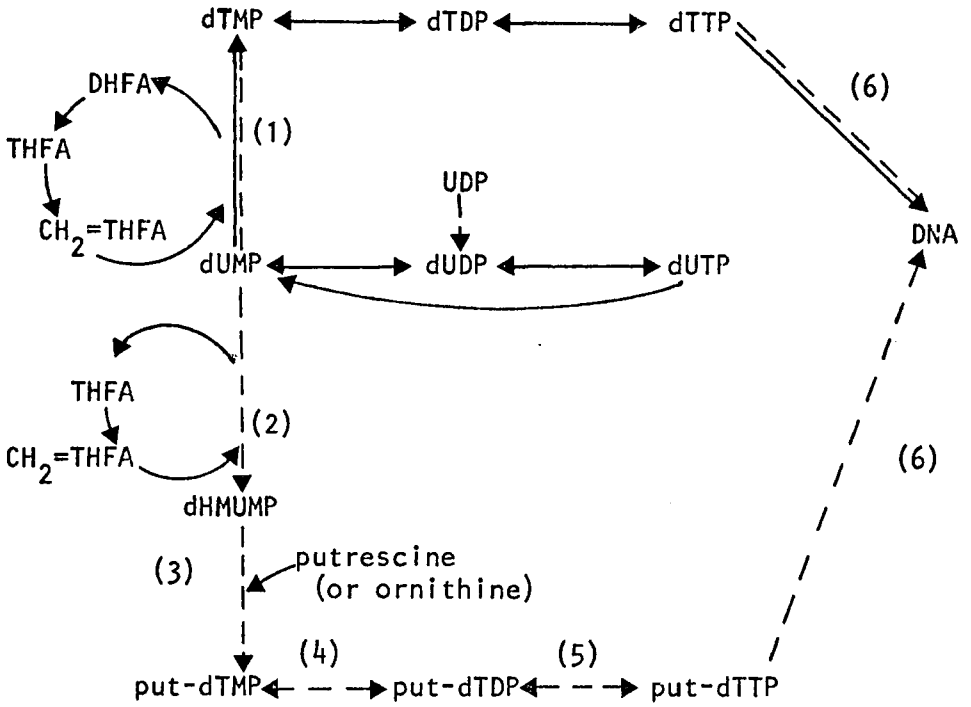

Fig. 13. Proposed pathway of thymine and putThy deoxynucleotide metabolism in bacteriophage ØW-14 infected Pseudomonas acidovorans. Solid lines represent host enzymes; broken lines represent phage-induced enzymes; solid plus broken lines represent enzymes encoded for by both. Enzymes are: (1), thymidylate synthetase; (2) dUMP hydroxymethylase; (3), dHMUMP putrescine transferase; (4), put-dTMP kinase; (5), put-dTDP kinase; (6), DNA polymerase. Abbreviations are: put-dTMP etc., the 5'-mono-, di-, and triphosphates of 5-(4-aminobutylaminomethyl)deoxyuridine; CH_2=THFA, N_5, N_{10}-methylene-THFA.

SUMMARY

Pseudomonas acidovorans is deficient in the salvage pathways of nucleic acid metabolism since none of the following activities was detected in crude extracts of the organism: uridine phosphorylase, purine nucleoside phosphorylase, cytidine (deoxycytidine) deaminase and thymidine phosphorylase. The organism does have thymidine kinase activity.

Obligate thymidine auxotrophs were isolated using an adaptation of the aminopterin technique. The mutants have lost thymidylate synthetase activity, die when starved for thymidine, and thymidineless death was shown to be an effective selective technique for the isolation of other auxotrophic mutants of P. acidovorans.

Pyrimidine requiring mutants of P. acidovorans were isolated and used to examine pyrimidine substitution patterns. Only cytosine and uracil satisfy the pyrimidine requirement. Growth of the mutants is inhibited by high concentrations (50 µg/ml and greater) of uracil.

The aspartate transcarbamylase of P. acidovorans is not repressed by uracil. Neither is the activity of the enzyme in crude extracts affected by various nucleotides. Infection of P. acidovorans with bacteriophage ØW-14 results in the appearance of a phage-specific thymidylate synthetase, and a tetrahydrofolate-dependent activity which causes extensive release of tritium from dUMP-5-^{3}H without concomitant oxidation of the tetrahydrofolate to dihydrofolate. Since extracts

of infected cells catalyze the formation from dUMP and formaldehyde of a compound which behaves chromatographically like dHMUMP, it is assumed that infection also results in the appearance of a phage-specific dUMP hydroxymethylase.

The novel base 5-(4-aminobutylaminomethyl)uracil which partially replaces thymine in the DNA of ØW-14, is not derived from a thymidine nucleotide, but rather from a deoxyuridine nucleotide, since it is labelled by deoxyuridine but not by thymidine. Nucleotide pool studies indicate that the base is synthesized at the mononucleotide level. Therefore, the synthesis of ØW-14 DNA involves five rather than four nucleotides.

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