Thymine Metabolism in *Pseudomonas aeruginosa* Strain 1: The Presence of a Salvage Pathway

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Exogenous thymine was found to be taken up very slowly by *Pseudomonas aeruginosa* in comparison to other pyrimidines, and most of it was catabolized by the cell. The existence of a functional, although inefficient, thymine salvage pathway was demonstrated and this pathway operated more effectively when *de novo* thymidine nucleotide biosynthesis was inhibited by trimethoprim or methotrexate. The mechanism of thymine salvage by *P. aeruginosa* appears to be different from that of *Escherichia coli* and *Pseudomonas acidovorans* as thymidine was not incorporated into the DNA. Like *P. acidovorans*, *P. aeruginosa* lacked thymidine phosphorylase activity. Unsuccessful attempts were made to isolate thymine auxotrophs.

INTRODUCTION

In most bacteria, pyrimidine deoxyribonucleotide biosynthesis can occur via a *de novo* pathway and a salvage pathway (Kornberg, 1974). In the latter system, thymine and 2'-deoxyribosylthymine (thymidine) have been shown to be exclusively incorporated into the DNA of bacteria and this has provided a useful tool for specifically labelling the DNA with radioisotopes (Carmody & Herriott, 1970). Whilst thymine is not readily incorporated by some bacteria because of the unavailability of endogenous deoxyribosyl donors (Breitman & Bradford, 1964; O'Donovan & Neuhard, 1970), this difficulty can be overcome by adding deoxyribonucleosides to the growth medium or alternatively by isolating thymine auxotrophs (Boyce & Setlow, 1962; O'Donovan & Neuhard, 1970). It is also known that thymidine is incorporated for only a short time because of its degradation by the enzyme thymidine phosphorylase (EC 2.4.2.4). The period of incorporation can be extended, however, if an exogenous source of deoxyribonucleosides is present, as this leads to competitive inhibition of thymidine phosphorylase (Boyce & Setlow, 1962; Budman & Pardee, 1967).

A *de novo* pathway of pyrimidine biosynthesis has been established in *Pseudomonas aeruginosa* and appears to be similar to that found in *Escherichia coli* (Isaac & Holloway, 1968). There is, however, little information about the salvage of pyrimidines by *P. aeruginosa* (see Fig. 1 for salvage reactions in other organisms). It is known that thymidine is not incorporated at all by *P. aeruginosa*, even with the addition of deoxyribonucleosides to the growth medium (Holloway, 1975; Pemberton & Clark, 1973). Pemberton & Clark (1973) attempted to isolate mutants capable of incorporating thymidine, as well as thymine and thymidine auxotrophs, but with no success. Therefore, no DNA-specific labelling system is readily available for studies involving *P. aeruginosa*.

Kelln & Warren (1973, 1974) reported that *Pseudomonas acidovorans* lacks a number of pyrimidine salvage enzymes, including uridine phosphorylase (EC 2.4.2.3), purine-nucleoside phosphorylase (EC 2.4.2.1), cytidine deaminase (EC 3.5.4.5), thymidine

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Fig. 1. Pyrimidine salvage and interconversions in *Escherichia coli*, *Salmonella typhimurium* and *Pseudomonas acidovorans*. Reactions that are absent from *P. acidovorans* are shown by an oblique line (/) across the reaction, while those absent from *P. aeruginosa* are denoted by an oblique line downwards (\). Reactions which are missing in both *P. acidovorans* and *P. aeruginosa* are denoted by an 'X'. Enzymes are: (1) thymidine phosphorylase (EC 2.4.2.4); (2) deoxyribomutase (EC 2.7.5.-); (3) uridine phosphorylase (EC 2.4.2.3); (4) cytosine deaminase (EC 3.5.4.1); (5) cytidine (deoxycytidine) deaminase (EC 3.5.4.5); (6) thymidylate synthetase (EC 2.1.1.b); (7) deoxycytidine triphosphate deaminase; (8) uridine-5'-monophosphate pyrophosphorylase (uracil phosphoribosyl-transferase, EC 2.4.2.9); (9) uridine kinase (EC 2.7.1.48); and (10) thymidine kinase (EC 2.7.1.21). Bases are identified as C, cytosine; U, uracil; and T, thymine. Ribonucleosides are identified by the letter 'r' in front of the base, and deoxyribonucleosides by placing the letter 'd' in front of the base. Data are from Kelln & Warren (1974), O'Donovan & Neuhaud (1970), Sakai *et al.* (1976) and this paper.

phosphorylase (EC 2.4.2.4), and probably uridine kinase (EC 2.7.1.48) and deoxycytidine kinase (EC 2.7.1.74) (Fig. 1). They were unable to label the DNA of *P. acidovorans* with isotopic thymine because of the lack of thymidine phosphorylase activity, but thymidine auxotrophs were isolated by aminopterin selection (Kelln & Warren, 1973). These mutants lacked thymidylate synthetase (EC 2.1.1.b) activity and required an unusually high exogenous thymidine concentration for growth, greater than 250 µg ml⁻¹.

The present study was undertaken in an effort to determine some of the factors involved in pyrimidine uptake and metabolism in *P. aeruginosa*. We were particularly concerned with the existence of a thymine salvage pathway, and our ultimate goal was the development of a DNA-specific labelling system.

METHODS

Bacteria. All strains used are derivatives of *P. aeruginosa* strain 1. The parent strain was *P. aeruginosa* OT15 (Loutit, 1969) which is prototrophic and carries the plasmid FP2. Strain OT700 is defective in the conversion of thymine to dihydrothymine, the first step of thymine catabolism (*dht*), and was derived from strain OT15 by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis. Strain OT710 is a spontaneous 5-fluorouracil-resistant *upp* derivative of strain OT15.

Media. The minimal medium of Davis & Mingioli (1950) was routinely used, except that 0.2% (w/v) sodium citrate, dihydrate, was used as the carbon source. Where different carbon and nitrogen sources were used, the same basal salts [without $(\text{NH}_4)_2\text{SO}_4$] were provided and carbon and nitrogen sources were added to final concentrations of 0.2% (w/v) and 0.1% (w/v), respectively. The type of medium is referred to by listing the principal nitrogen source first followed by the principal carbon source (for example, glutamate/citrate medium contained glutamate as the nitrogen source and citrate as the carbon source). Difco Brain Heart Infusion supplemented with 4 g of $\text{KNO}_3 \text{ l}^{-1}$ (nitrate/BH) was routinely used as a complete liquid medium. For all solid media, Davis agar was added to 2.0% (w/v). Amino acids were added to a concentration of 1.0 mM when required.

Radioisotopes. $[6\text{-}^3\text{H}]\text{Thymidine}$, $[6\text{-}^3\text{H}]\text{uracil}$, $[5\text{-}^3\text{H}]\text{uracil}$ and $[\text{methyl-}^3\text{H}]\text{thymine}$ were obtained from Amersham.

Labelling of cells and DNA extraction. A culture (10 ml) of *P. aeruginosa* strain OT15 grown overnight in nitrate/BH was washed and inoculated on to 200 ml of minimal agar supplemented with $50 \mu\text{Ci}$ ($6.25 \text{ Ci mmol}^{-1}$; $0.23 \text{ TBq mmol}^{-1}$) of radioisotope in a $25 \times 15 \text{ cm}$ tray. The cells were incubated overnight at 37°C and harvested in 50 ml of saline/EDTA (0.15 M-NaCl, 0.1 M-EDTA, pH 7.0). DNA was extracted by the procedure of Marmur (1961) and was stored in 10 ml of phosphate/EDTA (50 mM-sodium phosphate, 10 mM-EDTA, pH 7.0) at 4°C .

DNA hydrolysis. DNA solutions in phosphate/EDTA were concentrated to 0.5 ml by rotary evaporation or lyophilization, and these were added to thick-walled tubes with constricted necks, and dried. After adding 2.5 ml of trifluoroacetic acid, the tubes were sealed and refluxed in cyclohexane at 158°C for 1 h, cooled in an ice/NaCl mixture to reduce pressure, and the necks were carefully cut off. The trifluoroacetic acid was evaporated under a stream of air and the hydrolysed material resuspended in $20 \mu\text{l}$ of sterile distilled water. This procedure is basically that of Carrier & Setlow (1971).

Separation of base components. The hydrolysed DNA ($10 \mu\text{l}$) was applied to a piece of Whatman no. 1 chromatography paper along with reference spots of adenine, guanine, cytosine, thymine and uracil. The chromatogram was placed in a tank with a small amount of solvent (isopropanol/HCl/water, 171:41:39, by vol.) in the bottom and was left to equilibrate for 4 h. After equilibration, the solvent was added to the reservoir and the descending chromatogram was run for 36 h. After drying, the positions of the reference bases were determined by viewing under ultraviolet light. The section containing the hydrolysed DNA was cut into $1.2 \times 2.5 \text{ cm}$ strips and the radioactivity was measured under 5.0 ml of butyl-PBD scintillation fluid (6 g litre^{-1} in toluene) in a Packard Tri-Carb Liquid Scintillation Spectrometer, model 2002. The counting efficiency under these conditions was 12%.

Kinetics of pyrimidine uptake. Cultures grown overnight in nitrate/BH were washed three times with sterile 0.85% (w/v) saline and 30 ml of liquid minimal medium in 250 ml sidearm flasks were inoculated ($10^7 \text{ cells ml}^{-1}$). These were incubated with shaking at 37°C and the labelled pyrimidines were added when the cells were in the mid-exponential phase (generally 3.5–4.5 h). Samples were removed at appropriate intervals and the radioactivity was measured. For the detection of radioactivity in macromolecules, a 0.5 ml sample of cells was added to 10 ml of ice-cold 5% (w/v) trichloroacetic acid (TCA) and this was held on ice for 30 min. The suspension was filtered through a Millipore filter (HAWP, $0.45 \mu\text{m}$ pore size), washed once with 10 ml cold 5% (w/v) TCA and once with 3 ml of 70% (v/v) ethanol. Filters were dried at 70°C for 60 min and the radioactivity measured as described above. In order to detect radioactivity in compounds other than nucleic acids, a sample of cells was added to 10 ml of 10% (w/v) TCA and heated at $70\text{--}80^\circ\text{C}$ for 60 min. It was then treated as described for cold TCA-insoluble samples. The counting efficiency was 23% under these conditions.

Uptake of thymine in an overnight culture. Glutamate/citrate medium (1 ml) containing thymine and $[\text{methyl-}^3\text{H}]\text{thymine}$ was inoculated with 10^7 cells and grown for 16 h. The radioactivity was measured as described above for hot or cold TCA-insoluble samples.

Analogue sensitivity. Minimal agar plates with various carbon and nitrogen sources were inoculated on the surface with 10^4 to 10^7 cells. A crystal of the pyrimidine analogue to be tested was placed on the surface of the plate which was incubated at 37°C for 48 h. In some cases, liquid minimal medium solutions containing varying concentrations of the analogue were inoculated with 10^7 cells per ml and growth was determined after overnight incubation at 37°C .

Effect of folate antagonists. A culture of *P. aeruginosa* strain OT700, grown overnight in nitrate/BH, was washed twice and $10 \mu\text{l}$ samples were inoculated into two series of tubes, each containing 1 ml of glutamate/citrate medium supplemented with $[\text{methyl-}^3\text{H}]\text{thymine}$ at $1.0 \mu\text{Ci}$ and $10 \mu\text{g}$ of unlabelled thymine ($12.7 \text{ mCi mmol}^{-1}$; $0.47 \text{ GBq mmol}^{-1}$). Trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)-pyrimidine] was added to one series of tubes at concentrations ranging downwards from $200 \mu\text{g ml}^{-1}$ (200, 100, 50, 25, 12, 6, 3, 1.5, $0 \mu\text{g ml}^{-1}$), and methotrexate (4-amino- N^{10} -methylpteroyl-glutamic acid) was added to the other series at concentrations ranging downwards from $1250 \mu\text{g ml}^{-1}$ (in doubling fashion as for trimethoprim). After growth for 16 h, the cold TCA-insoluble radioactivity was determined. In some experiments, methionine and/or vitamin B12 was added and trimethoprim was then used at a concentration of $25 \mu\text{g ml}^{-1}$.

Fig. 2. Labelling of the DNA of *P. aeruginosa* strain OT15 with (a) isotopic uracil and (b) thymine. The cells were grown on 200 ml of minimal agar containing 50 μCi ($6.25 \text{ Ci mmol}^{-1}$) of the appropriate isotope and the DNA was extracted, hydrolysed, and separated by paper chromatography. The positions of adenine (A), cytosine (C), thymine (T) and uracil (U) are indicated. (a) $\bullet$, $[6\text{-}^3\text{H}]\text{uracil}$; $\circ$, $[5\text{-}^3\text{H}]\text{uracil}$; (b) $\bullet$, $[\text{methyl-}^3\text{H}]\text{thymine}$.

RESULTS

Establishment of pyrimidine salvage pathways

When *P. aeruginosa* strain OT15 was grown on solid medium containing $[5\text{-}^3\text{H}]\text{uracil}$, $[6\text{-}^3\text{H}]\text{uracil}$ or $[\text{methyl-}^3\text{H}]\text{thymine}$ and the DNA was extracted, hydrolysed and separated into its base components, radioactivity was shown to be present in the DNA, and the distribution of radioactivity in the bases can be seen in Fig. 2. Exogenously added $[6\text{-}^3\text{H}]\text{uracil}$ supplied the cells with both dTTP and dCTP, while only the cytosine moiety of the DNA was labelled when $[5\text{-}^3\text{H}]\text{uracil}$ was provided. This suggested that dTMP was formed by methylation at the C-5 position on the pyrimidine ring of dUMP (Friedkin, 1973). The incorporation of $[\text{methyl-}^3\text{H}]\text{thymine}$ into the DNA demonstrated that a functional thymine salvage pathway does exist in *P. aeruginosa* and therefore it should be possible to label the DNA specifically with isotopic thymine.

Pyrimidine uptake

The rates of incorporation of thymine, thymidine, uracil and uridine into the acid-insoluble fraction of strain OT15 are shown in Fig. 3. Both uracil and uridine were incorporated constitutively, and the lack of uridine uptake by strain OT710 (*upp*) indicated that uridine kinase is not active in *P. aeruginosa*. Uridine phosphorylase was quite active in strain OT15 as indicated by the efficient incorporation of uridine.

$[6\text{-}^3\text{H}]\text{Thymidine}$ was not incorporated at all and $[\text{methyl-}^3\text{H}]\text{thymine}$ incorporation was only slight (Fig. 3). The addition of deoxyribose or ribose donors to the growth medium did not stimulate uptake of either compound. The deoxyribose donors tested were deoxyadenosine and deoxyguanosine, and the ribose donors were adenosine and uridine. These compounds were employed at varying concentrations up to 1 mg ml^{-1} . In addition, the acid-soluble fraction of cells labelled with isotopic thymine and analysed by thin-layer and paper chromatography (Denhardt, 1969) had no radioactivity associated with the area on the chromatogram normally occupied by thymidine (data not shown). Labelled dTMP appeared to be present, however, indicating that there was a functional salvage pathway. Further evidence that thymidine was not involved in salvage was provided by a growth experiment in

Fig. 3

Fig. 4

Fig. 3. Pyrimidine uptake by *P. aeruginosa* strains OT15 and OT710. Bacteria were grown in liquid NH_4^+ /citrate medium supplemented with isotopic uracil ($2.5 \mu\text{g ml}^{-1}$, $48.5 \text{ mCi mmol}^{-1}$), uridine ($2.5 \mu\text{g ml}^{-1}$, 98 mCi mmol^{-1}), thymine ($4 \mu\text{g ml}^{-1}$, $31.5 \text{ mCi mmol}^{-1}$) or thymidine ($2.5 \mu\text{g ml}^{-1}$, $97.1 \text{ mCi mmol}^{-1}$). The radioactivity was measured in cold TCA-insoluble material. (a) ●, [methyl-³H]thymine and ○, [6-³H]thymidine uptake by strain OT15; (b) ○, [6-³H]uridine, OT15; □, [6-³H]uracil, OT15; ●, [6-³H]uridine, OT710 *upp*; ■, [6-³H]uracil OT710 *upp*.

Fig. 4. Incorporation of thymine into a hot TCA-insoluble fraction by *P. aeruginosa* strain OT15 grown in various media. The cells were diluted 100-fold into 30 ml of the appropriate liquid medium supplemented with unlabelled thymine ($3 \mu\text{g ml}^{-1}$). After 3.5 h incubation, [methyl-³H]thymine was added ($20 \mu\text{Ci}$, 28 mCi mmol^{-1}) and the hot TCA-insoluble radioactivity in 0.5 ml samples was measured. Values are corrected to a standard cell density. (a) ●, Glutamate/citrate; ○, glutamate/glucose; ■, NH_4^+ /citrate; □, NH_4^+ /glucose. (b) ○, Glutamate/glutamate; ●, glutamate/acetate; □, alanine/acetate; ■, nitrate/BH.

which we showed that *P. aeruginosa* was unable to utilize thymidine as a carbon or nitrogen source when supplied at a concentration ranging from 0.01% (w/v) to 1.0% (w/v). Nucleosides such as uridine, adenosine and deoxyadenosine are catabolized efficiently by *P. aeruginosa*. Finally, we were unable to demonstrate either thymidine kinase or phosphorylase activity (results not shown). Consequently, we looked at thymine uptake under various conditions in an effort to determine the optimum conditions for its incorporation by the cell.

Effect of growth medium on thymine uptake

Most of the thymine which entered strain OT15 was catabolized since more than 70% of the radioactivity could be recovered in a hot TCA-insoluble fraction when cells were grown overnight in the presence of various carbon and nitrogen sources. As can be seen in Fig. 4, thymine incorporation was poor in the presence of NH_4^+ , probably due to nitrogen metabolite repression. When NH_4^+ was supplied at a growth limiting concentration, thymine catabolism was observed with all carbon sources tested (Table 1). Both glutamate and alanine allowed thymine degradation when used as nitrogen sources, and thymine degradation occurred when citrate or acetate was the principal carbon source. Glutamate/citrate was the medium which allowed maximum thymine uptake by strain OT15. In all experiments there was a lag of 2–3 h before thymine was catabolized.

Optimum conditions for labelling

The optimum ratio of isotopic to unlabelled thymine was investigated in order to improve the labelling efficiency. Isotopic and unlabelled thymine were added to cultures of strain

Table 1. Incorporation of [methyl-³H]thymine into a hot TCA-insoluble fraction by *P. aeruginosa* strain OT15 growing in various media

Portions (10 µl) of a washed overnight culture were inoculated into 1 ml portions of various media supplemented with [methyl-³H]thymine (5 µg ml⁻¹, 0.25 Ci mmol⁻¹). The cells were grown overnight, the cell density measured and the radioactivity in a hot TCA-insoluble fraction determined.

Carbon source	Thymine incorporation*		
	NH ₄ ⁺ (10 mM)	NH ₄ ⁺ (1 mM)	Glutamate (10 mM)
Glucose	39	128	82
Citrate	44	132	174
Acetate	39	193	42
Glutamate as carbon and nitrogen source	ND	ND	70
Minimal medium	19	ND	ND

ND, Not done.

* Units are c.p.m. corrected to a standard cell density [c.p.m. (Eel nephelometer unit)⁻¹]. The conversion factor is 100 Eel nephelometer units = 2 × 10⁹ cells ml⁻¹.

Fig. 5. Effect of exogenous thymine concentration on thymine uptake by *P. aeruginosa* strain OT15. The cells were grown for 16 h in glutamate/citrate medium supplemented with [methyl-³H]thymine (31.5 mCi mmol⁻¹) and unlabelled thymine at various concentrations. The radioactivity in a cold TCA-insoluble fraction was measured.

OT15 in the exponential phase at various ratios and after 3 h the radioactivity in the acid-insoluble fractions was measured. The ratio which yielded the greatest uptake was between 31.5 mCi mmol⁻¹ (1.17 GBq mmol⁻¹) and 42.0 mCi mmol⁻¹ (1.55 GBq mmol⁻¹).

The extent to which exogenous thymine is catabolized or incorporated into the DNA is largely dependent upon its concentration in the growth medium. *Escherichia coli*, in the presence of deoxyribosyl supplements, shows saturation of thymine utilization at a concentration of less than 7 µg ml⁻¹ but saturation occurs at a concentration greater than 50 µg ml⁻¹ in the absence of these supplements (Kammen, 1967). In *P. aeruginosa*, thymine utilization was not saturated until an exogenous concentration of greater than 200 µg ml⁻¹ was reached (Fig. 5) and as mentioned above, the addition of deoxyribosyl donors to the growth medium had no effect on thymine uptake.

Pyrimidine analogue sensitivity

Pseudomonas aeruginosa was found to be sensitive to most uracil analogues and resistant to thymine analogues, reflecting the efficiency of incorporation of the two compounds. A

Fig. 6

Fig. 7

Fig. 6. Effect of trimethoprim on the growth and incorporation of thymine by *P. aeruginosa* strain OT700. The cells were labelled with [*methyl*- ^3H]thymine ($10\text{ }\mu\text{g ml}^{-1}$, $12.7\text{ mCi mmol}^{-1}$) in the presence of trimethoprim. Further details are given in Methods. The radioactivity in a cold TCA-insoluble fraction was measured. $\circ$, Thymine incorporation; $\bullet$, growth (measured in Eel nephelometer units; $100\text{ Eel units} = 2 \times 10^9\text{ cells ml}^{-1}$).

Fig. 7. Effect of methionine and vitamin B12 on the trimethoprim-stimulated incorporation of thymine by *P. aeruginosa* strain OT700. The cells were labelled with [*methyl*- ^3H]thymine ($10\text{ }\mu\text{g ml}^{-1}$, $12.7\text{ mCi mmol}^{-1}$) in the presence of trimethoprim, methionine and/or vitamin B12 as described in Methods. The radioactivity in a cold TCA-insoluble fraction was measured. $\bullet$, Trimethoprim + methionine; $\circ$, trimethoprim + methionine + vitamin B12; $\square$, methionine alone.

gradient of toxicity was observed with 5-fluorouracil, 5-chlorouracil and 5-bromouracil in which the sensitivity was inversely related to the ionic radius of the halogen at the C-5 position. Thus, 5-fluorouracil was very toxic while 5-bromouracil had no apparent effect on the cells. The chlorine atom, therefore, gave rise to a toxicity between 5-fluorouracil and 5-bromouracil.

Mutation to 5-fluorouracil resistance resulted in the cessation of uracil uptake and the acquisition of 6-azauracil resistance, which indicated that a functional uracil phosphoribosyl-transferase was absent from the resistant strain (O'Donovan & Neuhaud, 1970). Only one level of resistance to 5-fluorouracil was observed and resistant strains could not be sensitized to lower fluorouracil concentrations in the presence of exogenous adenosine or deoxyadenosine, which suggested that the enzymes uridine kinase, thymidine kinase and thymidine phosphorylase were absent (Ahmad & Pritchard, 1969).

In view of the absence of deoxythymidine kinase it is not surprising that 5-fluorodeoxyuridine was not inhibitory at concentrations as high as 1 mg ml^{-1} , since it functions as an inhibitor only after conversion to 5-fluorodeoxyuridine monophosphate. This last compound functions as an inhibitor of thymidylate synthetase.

Effect of folate antagonists

Trimethoprim and methotrexate are potent inhibitors of dihydrofolate reductase (Friedkin, 1973; Seydel *et al.*, 1972) and the biosynthesis of tetrahydrofolate is inhibited in the presence of these compounds. The *de novo* formation of dTMP requires a tetrahydrofolate-dependent methylation of dUMP and therefore one would expect the thymine salvage pathway to operate more efficiently in the presence of trimethoprim or methotrexate.

The increase in thymine incorporation by strain OT700 in the presence of trimethoprim is illustrated in Fig. 6. Methotrexate yielded the same result, although it did not appear to be as

toxic as trimethoprim. Both of these compounds stimulated thymine incorporation, although trimethoprim did not appear to promote thymine uptake at concentrations greater than $25 \mu\text{g ml}^{-1}$ because of low cell yields. The strain OT700 is defective in thymine catabolism and therefore the increase in thymine incorporation must have been due to salvage. In support of this, no increased hot TCA-insoluble radioactivity was observed in the presence of the antifolate compounds.

Tetrahydrofolate is also required for methionine biosynthesis (Hatch *et al.*, 1961) and therefore the effect of exogenous methionine and vitamin B12 on the trimethoprim-stimulated incorporation of thymine was examined. Vitamin B12 is a necessary co-factor for the *metH* protein of *E. coli* (Cauthen *et al.*, 1966). When the two compounds were added to the growth medium, thymine incorporation by strain OT700 decreased to the basal level (Fig. 7). Neither methionine nor vitamin B12 had any effect on thymine salvage in the absence of trimethoprim (only the result for methionine is shown), and therefore the incorporation of thymine by strain OT700 must have been the result of thymine salvage.

Thymine auxotrophs

Our results suggested that *P. aeruginosa* was able to incorporate thymine and convert it to the corresponding nucleotide. Since the thymine salvage pathway operated more efficiently when the *de novo* biosynthesis of dTMP was impaired, the isolation of thymine auxotrophs should be possible. Trimethoprim has been used to select for thymine auxotrophs in many genera (O'Donovan & Neuhaud, 1970) but efforts have been unsuccessful with *P. aeruginosa* (Holloway, 1975; Pemberton & Clark, 1973).

Minimal agar plates containing various carbon and nitrogen sources and concentrations of folate antagonists were inoculated with 10^9 , 10^8 or 10^7 viable cells from washed cultures grown overnight in nitrate/BH. Normally, cells were not treated with a mutagen, but in some cases *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was used as previously described (Loutit, 1969). A total of 6×10^{12} cells were screened and no thymine auxotrophs were obtained. However, some clones exhibiting the ThyA phenotype of *B. subtilis* (Neuhaud *et al.*, 1978) were isolated on NH_4^+ /citrate agar containing trimethoprim, sulphadiazine and thymine at 80, 20 and $300 \mu\text{g ml}^{-1}$, respectively, and also from glutamate/citrate agar supplemented with thymine at $300 \mu\text{g ml}^{-1}$ and trimethoprim + sulphadiazine at $160 + 40 \mu\text{g ml}^{-1}$, respectively. The parental strains were OT15 and OT700. These mutants required thymine for growth only in the presence of trimethoprim (the ThyA phenotype) but changed within one or two subcultures to a trimethoprim-resistant thymine-prototrophic phenotype indistinguishable from other isolates. This suggested that there may be more than one mechanism for the *de novo* biosynthesis of dTMP and presumptive *thyA* mutants were all subjected to a second round of selection with trimethoprim and sulphadiazine present at 750 and $80 \mu\text{g ml}^{-1}$, respectively. No thymine auxotrophs were detected.

DISCUSSION

DNA-specific labelling should be possible in bacteria provided a functional thymine salvage pathway exists. We have demonstrated such a pathway in *P. aeruginosa* and shown that thymine incorporation can be stimulated by the inhibition of *de novo* dTMP biosynthesis with trimethoprim. Most of the thymine taken up by the cell was normally catabolized, but under certain conditions which did not permit catabolism, all of the thymine taken up entered the DNA. Thymine incorporation into the DNA was most efficient when the cells were grown in liquid minimal medium containing NH_4^+ , which repressed thymine catabolism, and isotopic thymine at a specific activity of $31.5 \text{ mCi mmol}^{-1}$. Alternatively, a strain unable to catabolize thymine (*dht*) incorporated more thymine into the DNA. The amount of thymine incorporated under these conditions was small and there was a 2–3 h lag before it entered the

cell, making it impossible to pulse-label the DNA. The folate antagonists trimethoprim and methotrexate stimulated thymine salvage, and since methotrexate does not appear to affect the growth rate of *P. aeruginosa* when supplied at a concentration of 1 mg ml^{-1} , it may prove useful when one requires a greater labelling efficiency.

Previous attempts to isolate thymine auxotrophs of *P. aeruginosa* were unsuccessful (Holloway, 1975; Pemberton & Clark, 1973) and ours were no exception. The *de novo* biosynthesis of dTMP could be blocked by the addition of trimethoprim to the growth medium, but perhaps the salvage pathway does not operate efficiently enough to give thymine auxotrophs a growth advantage over normal cells. Alternatively, there may be more than one pathway for the *de novo* biosynthesis of dTMP. Neuhaud *et al.* (1978) demonstrated that *B. subtilis* possessed two genetically distinct thymidylate synthetases, one of which was heat-labile. We have been able to isolate *thyA* mutants of *P. aeruginosa* (requiring thymine only in the presence of trimethoprim), but they were extremely unstable. This suggested to us that a second trimethoprim-sensitive mechanism for thymine nucleotide biosynthesis may be active in *P. aeruginosa*. This would explain the difficulties encountered in thymine auxotroph isolation and the high level of resistance of clinical isolates of *P. aeruginosa* to trimethoprim/suphamethoxazole (Bushby, 1973).

Although a functional thymine salvage exists, it would appear to be different from that found in *E. coli* and *P. acidovorans* since thymidine was not utilized. In support of this lack of ability to utilize thymidine, it has not been possible to demonstrate the presence of either deoxythymidine kinase or phosphorylase in *P. aeruginosa*. Further evidence for the absence of deoxythymidine kinase was provided by the lack of toxicity of 5-fluorodeoxyuridine even when this compound was present at a concentration of 1.0 mg ml^{-1} . 5-Fluorodeoxyuridine must be converted to the monophosphate derivative by deoxythymidine kinase to be toxic, but it had no effect on *P. aeruginosa*.

An alternative explanation for the lack of thymidine incorporation may be that *P. aeruginosa* is unable to transport the compound into the cell. This, however, is doubtful in light of the observation that thymidine kinase activity was not present in cell-free extracts. Also, Pemberton & Clark (1973) unsuccessfully attempted to isolate mutants capable of thymidine uptake and thymidine auxotrophs following mutagenesis. Therefore, it seems more probable that thymidine cannot be converted to the nucleotide by *P. aeruginosa* strain 1.

Kelln & Warren (1973, 1974) showed that *P. acidovorans* lacks thymidine phosphorylase activity but thymidine phosphorylase activity is present. They were therefore able to isolate thymidine auxotrophs in which the DNA could be labelled with isotopic thymidine. *Pseudomonas aeruginosa* appears to be similar in that deoxythymidine phosphorylase activity is absent, but the two species probably have different mechanisms for thymine salvage since the DNA of *P. aeruginosa* can be labelled with isotopic thymine, whereas *P. acidovorans* has no mechanism for converting thymine to the nucleotide (Kelln & Warren, 1974).

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