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PYRIMIDINE METABOLISM IN *BREVIBACTERIUM HELVOLUM* AND GENETIC MANIPULATION OF THIS ORGANISM TO OVERPRODUCE PYRIMIDINE NUCLEOSIDES

Thesis submitted to the Nottingham Trent University in partial fulfilment of the requirement for the degree of

Master of Philosophy

by

Satwinder Kaur Kalirai, BSc.

The Nottingham Trent University
Nottingham
October 1992

To my Parents,

Sucha Singh and Kirpal Kaur

and my Family

ABSTRACT

Studies were carried out on *Brevibacterium helvolum* in order to isolate a thymidine overproducing strain. This required a knowledge of the pyrimidine metabolic pathway in this bacterial species.

A mutant strain of *B. helvolum* from ICI was employed that had already undergone some genetic manipulation and was found to be capable of producing and excreting more thymidine than the wild type.

Studies on the wild type strain show that *B. helvolum* can utilise both purine and pyrimidine (deoxy)nucleosides as alternative sources of carbon. These studies suggest that the organism possesses the enzymes necessary to degrade thymidine, deoxyuridine, uridine, cytidine, guanosine, adenosine and deoxyadenosine, but not the sugar deoxyribose. It was also shown to be sensitive to the pyrimidine analogues 5-fluorouracil, 5-fluoro-deoxyuridine and, to a limited extent, 2-thiouracil.

The ICI mutant strain is thought to be a dUMP overproducer. It was found to be resistant to high levels of fluorouracil and fluorodeoxyuridine. It could not utilise uridine as a sole source of carbon implicating the absence of a functional uridine phosphorylase. Resistance to fluorouracil was proposed to be a combination of three factors; a defective UMP pyrophosphorylase and uridine phosphorylase and, substrate competition between uracil (produced as an end product of dUMP degradation) and fluorouracil for thymidine phosphorylase.

In the course of the studies undertaken a thymidine overproducing strain was isolated in which the breakdown of thymidine was found to be reduced. HPLC studies showed this strain could produce and excrete 100% more thymidine and deoxyuridine than the original mutant strain. The thymidine auxotroph (probable genotype *thyA deoC deoA*) from which the thymidine overproducing strain was derived was found to produce and excrete almost seven times the level of deoxyuridine produced by the original mutant.

Studies also suggest the existence of an activity *viz* deoxyuridine phosphorylase in *B.helvolum* which is not known to be present in *E. coli*.

In addition, a super high thymine requirer of the probable genotype *thyA deoD deoR* (analogous to the super high thymine requirer of *E. coli*) was isolated during the course of the investigations undertaken.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

Acet	acetaldehyde
AR	adenosine
C	cytosine
CR	cytidine
dCTP	deoxycytidine triphosphate
dRib-1-P	deoxyribose-1-phosphate
dRib-5-P	deoxyribose-5-phosphate
dTDP	deoxythymidine diphosphate
dTMP	deoxythymidine monophosphate
dTTP	deoxythymidine triphosphate
dUDP	deoxyuridine diphosphate
dUMP	deoxyuridine monophosphate
dUTP	deoxyuridine triphosphate
Gly-3-P	glyceraldehyde-3-phosphate
FU	5-fluorouracil
FUdR	5-fluoro-2'deoxyuridine
PPi	pyrophosphate
Pur	purine ribonucleotide
Pur dR	purine deoxyribonucleoside
Pur R	purine ribonucleoside
T	thymine
TdR	(deoxy)thymidine
TU	2-thiouracil
U	uracil
UR	uridine
UDP	uridine diphosphate
UdR	deoxyuridine
UMP	uridine monophosphate

CHAPTER 1: INTRODUCTION

INTRODUCTION

1.1 AIDS and AZT

Acquired immune deficiency syndrome (AIDS) was first recognised as a disease in 1981. It is caused by a retrovirus known as human immunodefeciency virus (HIV). Currently about 12 million people worldwide are infected by HIV and the World Health Organisation predicts that up to 40 million people will be infected by the year 2000 (Brown, 1992)

HIV attacks the CD4 T-lymphocytes of the immune system, resulting in an acute depression of natural immune defences (Barre-Sinoùssi *et al*, 1983). This renders the patient extremely vulnerable to overwhelming secondary infections. It is these secondary infections, such as *pneumocystis carinii*, pneumonia and taxoplasmosis of the brain that ultimately kill people with AIDS.

In 1987 Zidovudine (also known as Retrovir, Azidothymidine or simply AZT) was the first drug to be licenced for the treatment of AIDS. The drug was originally isolated in 1964 as a potential anti-cancer agent. It has since been found to inhibit the replication of retroviruses, including HIV (Gallo *et al*, 1984; Furman *et al*, 1985). The USA approved the use of AZT for HIV infection in 1989 (1990 in Britain) after clinical trials had shown the drug was able to slow down the course of AIDS in people who had already developed

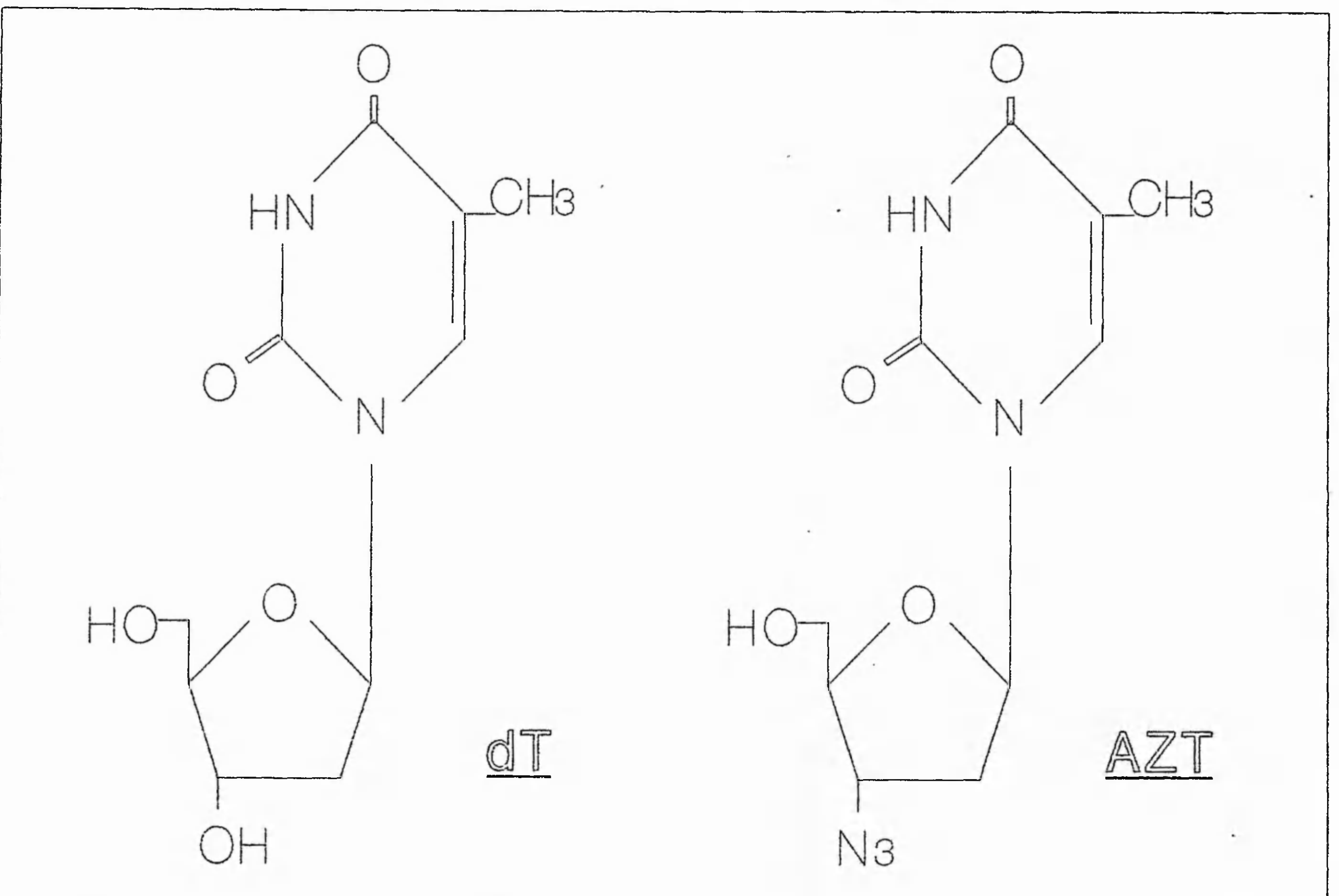
the disease (Mitsuya *et al*, 1985). It has since been found that AZT also delays the onset of the disease in symptom-free people but, as yet, there is no evidence that it prolongs life in these people.

The chemical name for AZT is 3'-azido 3'-deoxythymidine. It is a nucleoside analogue of the naturally occuring DNA base thymidine. The OH group from the third position of the deoxyribose of thymidine is substituted by N_3 in AZT (Fig. 1). The drug interferes with HIV's reverse transcriptase and elongation of the viral DNA chain in which AZT is incorporated in place of thymidine. This results in the inhibition of viral replication.

Thymidine is the precursor of AZT. It is chemically altered to produce AZT. AZT is an expensive product (£254.60 /g from SIGMA) and one of the reasons for this is because thymidine itself is expensive to produce. Thus, there is a demand to find cheaper alternative ways of producing thymidine which could subsequently lead to the lower cost of AZT.

In this study attempts were made to genetically manipulate a strain of B. helvolum to overproduce thymidine (and deoxyuridine). This also involved elucidating the pyrimidine metabolic pathway in this bacterial species.

Fig.1 Structure of AZT and Thymidine



1.2 *Brevibacterium helvolum*

B. helvolum is the microorganism which has been employed in this research programme. This organism belongs to a group of microbes known as Coryneform bacteria which are widely distributed in nature. B. helvolum is most commonly found in the soil.

Coryneform bacteria are commercially important and have made a significant contribution to the biotechnology industry. For example, they are involved in the production of amino acids, nucleotides, and emulsifying agents. They are also involved in various industrial processes including cheese processing, degradation of hydrocarbons and bioconversion.

B. helvolum was first isolated by O.E.R. Zimmermann in 1890. Since then several workers have attempted to classify the organism (Lehmann and Neumann, 1896; Kissalt and Berend, 1918; Conn, 1928). As a result of this work the following generic names were considered as synonyms; Bacillus helvolus, Bacterium helvolum, and Flavobacterium helvolum (Lochhead, 1955).

In 1953 Breed established a new genus, Brevibacterium, that included short unbranched Gram positive non-spore forming rod shaped bacteria, reproducing by simple cell division. It was into this new genus that Zimmermann's organism was placed, designated as B. helvolum. Bergey's manual of

systemic bacteriology describes the genus Brevibacterium as follows:

> Irregular, slender rods, variable in length and from 0.6-1.0 um in diameter. Many of the cells are arranged at an angle to give V forms. Primary branching may occur but a true mycelium is not produced. A marked rod-coccus cycle during growth on complex media. Cells from older cultures (3-7 days) are composed largely or entirely of coccoid cells 0.6-1.0 um in diameter or occasionally of coccal rods. On transfer to a suitable fresh medium these forms grow out to give the characteristic rods of exponential phase cultures. Both rod and coccoid forms are Gram positive. Not acid fast. No endospores are produced. Non-motile. Optimum temperature is 20-30^{0}C or 37^{0}C depending on species and strain. Colonies become larger 2-4mm after 4-7 days incubation. Brevibacterium strains also produce yellow to deep orange-red colonies when cultured on a variety of media but for the majority of the strains pigment production is light dependent.

B. helvolum produces yellow pigmentation when grown on minimal synthetic medium. This was used as an identifying characteristic for this bacterial species.

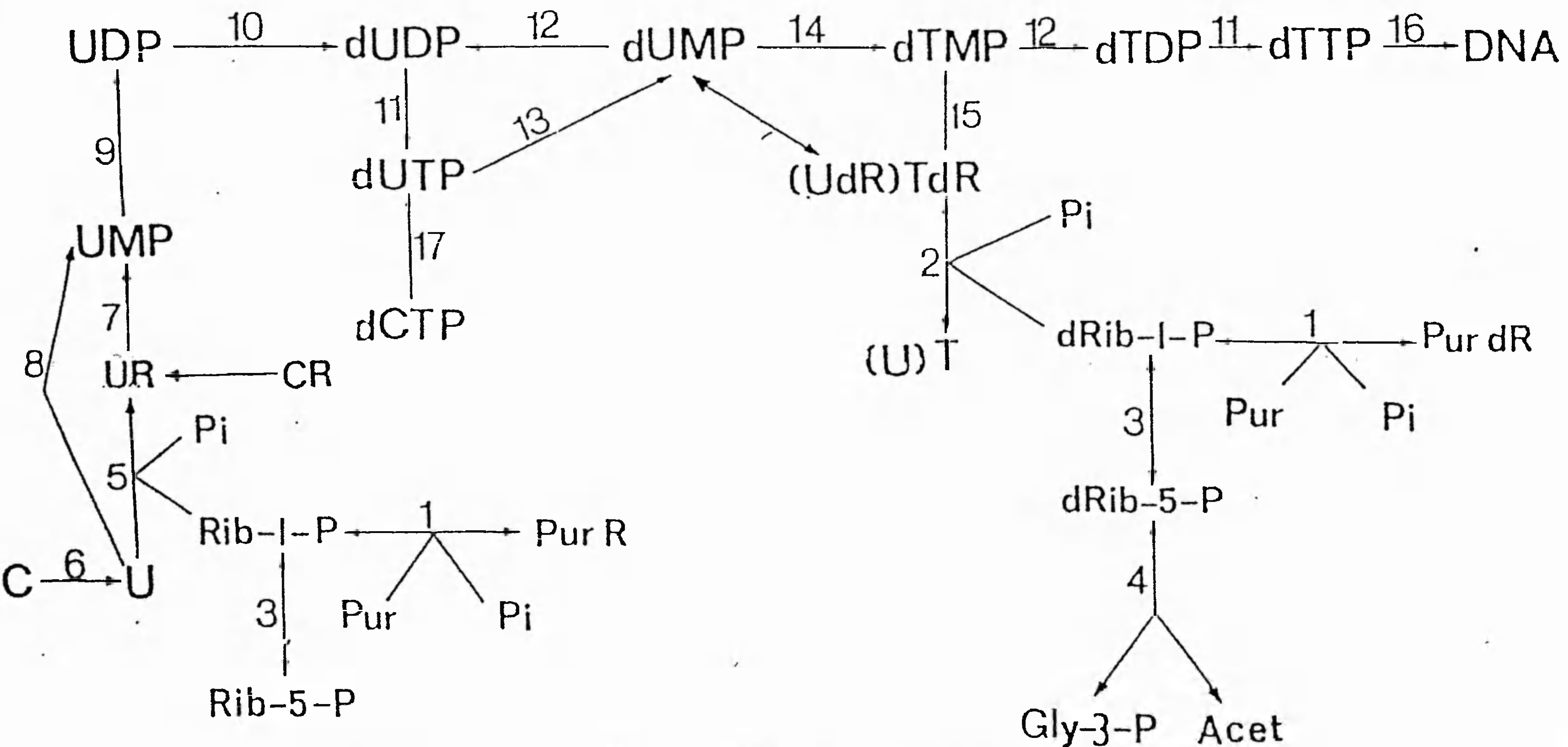
Like other Coryneform bacteria B. helvolum has the potential to become an industrially important strain. However, there is a need to learn more about its biochemical and genetic characteristics before this potential can be realised. This research programme aims to look into the pyrimidine metabolic pathway in B. helvolum with a view to comparing it with E. coli and to use this knowledge in the isolation of a thymidine overproducing mutant.

1.3 Pyrimidine Metabolism in Bacteria

The pyrimidine metabolic pathway is the branch of the biosynthesis of pyrimidine nucleotides, leading to the synthesis of the nucleotides CTP and UTP and the deoxynucleotides dTTP and dCTP. Little is known about the pyrimidine metabolic pathway in B. helvolum. However, in depth studies have been carried out on this pathway in other bacteria, notably E.coli and S.typhimurium (O'Donovan and Neuhard 1970). These studies provide the basis for elucidation of this pathway in other bacteria. Therefore to understand the metabolic pathway for pyrimidine biosynthesis in B. helvolum it is important to look first into this pathway in E.coli.

In E.coli two distinguishable pathways for dTTP synthesis exist (Fig.2). One is involved in the synthesis of deoxyuridylate (dUMP) and is known as the *de novo* pathway. The other is a salvage pathway, responsible for the uptake of exogenous sources of dTMP (thymidine and thymine) in the absence of a functional thymidylate synthetase. The catabolic enzymes of this latter pathway; thymidine phosphorylase, deoxyribomutase and deoxyriboaldolase, also enable the the cell to degrade and utilise deoxynucleosides as sole sources of carbon and energy.

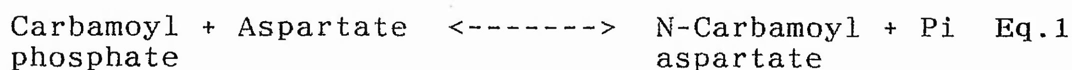
Fig. 2 The Pyrimidine Metabolic Pathway in *E.coli* (Dr. S. I. Ahmad)

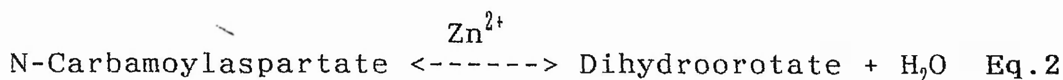


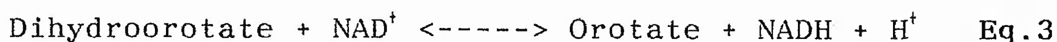
1. Purine nucleoside phosphorylase (*deo D*)
2. Thymidine phosphorylase (*deo A*)
3. Deoxyribomutase (*deo B*)
4. Deoxriboaldolase (*deo C*)
5. Uridine phosphorylase (*udp*)
6. Cytosine deaminase (*cod*)
7. Uridine kinase (*udk*)
8. UMP pyrophosphorylase (*upp*)
9. UMP kinase
10. Nucleoside diphosphate reductase
11. Nucleoside diphosphate kinase
12. Deoxyribonucleotide kinase
13. dUTP pyrophosphatase
14. Thymidylate synthetase (*thyA*)
15. Thymidine kinase (*tdk*)
16. DNA polymerase (*pol*)
17. dCTP deaminase

1.3.1 The *de novo* Pathway for UDP Synthesis

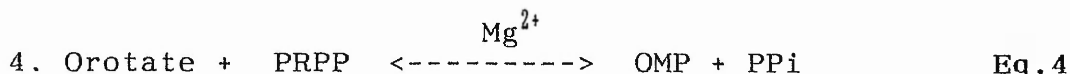
The committed step in the *de novo* synthesis of pyrimidine nucleotides is the formation of N-carbamoylaspartate from aspartate and carbamoyl phosphate. This reaction is catalysed by aspartate transcarbamoylase (Equation (Eq) 1). This is followed by the reversible cyclodehydration of carbamoylaspartate, carried out by dihydroorotase, to yield dihyroorotate (Eq. 2). Orotate (a free, ultra violet absorbing pyrimidine), is then formed by dehydrogenation of dihydroorate by dihydroorotate dehydrogenase (Eq. 3).





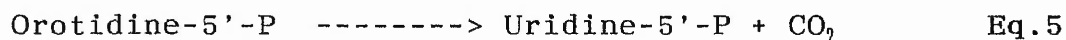


The next step in the pathway is the formation of orotidylate (Orotidine-5'-phosphate, OMP) from orotate and 5-phosphoribsyl-1-pyrophosphate (PRPP). This is a reversible reaction catalysed by OMP pyrophosphorylase (Eq. 4).

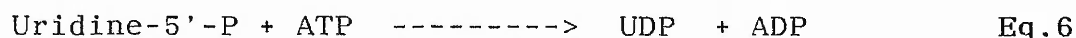


Orotidylate is then decarboxylated to uridylate (Uridine-

5'-phosphate, UMP) in an irreversible reaction catalysed by orotidylate (OMP) decarboxylase (Eq. 5)


Orotidine-5'-P ---------> Uridine-5'-P + CO_2 Eq.5

Finally UDP is formed from UMP by the action of UMP kinase (Eq. 6). UDP can then be converted to UTP, which acts as an intermediate in the synthesis of CTP and dCTP or, it can be used in the synthesis of dTTP for DNA synthesis. It is the latter pathway which is the subject of investigation in this research programme with regards to the synthesis of thymidine in B. helvolum.

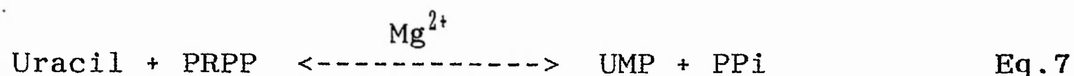

Uridine-5'-P + ATP ----------> UDP + ADP Eq.6

1.3.2 Salvage Pathway for the synthesis of UDP

A salvage pathway exists to synthesise UDP from exogenous sources (*eg*. uracil, uridine, cytosine or cytidine). The enzymes of this salvage pathway enable mutants that lack the ability to synthesise UDP by the *de novo* pathway, to grow normally so long as their absolute requirement for UDP is satisfied by the exogenous source.

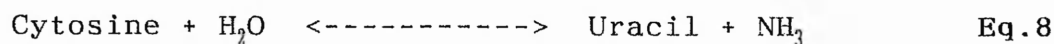
In this salvage pathway the precursor of UDP is UMP. UMP is synthesised from uracil and phosphoribosyl pyrophosphate (PRPP) and is catalysed by UMP pyrophosphorylase (encoded

by the *upp* gene) (Eq.7). UMP is then converted to UDP by UMP kinase.



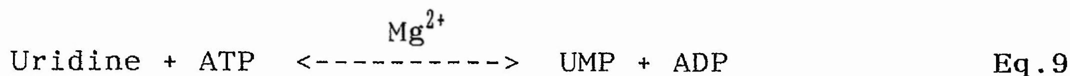
$$\text{Uracil} + \text{PRPP} \xleftrightarrow{Mg^{2+}} \text{UMP} + \text{PPi} \qquad \text{Eq.7}$$

Exogenous cytosine can also be used as a source of UMP but first it must be converted to uracil by cytosine deaminase (*cod*) (Eq. 8).



$$\text{Cytosine} + H_2O \longleftrightarrow \text{Uracil} + NH_3 \qquad \text{Eq.8}$$

If uridine is provided as a source of UMP, it can be converted directly to UMP by uridine kinase (Eq. 9). Alternatively, it can be broken down to uracil by uridine phosphorylase (Eq. 10), and then converted directly to UMP via UMP pyrophosphorylase.



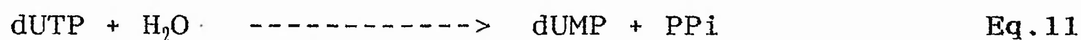
$$\text{Uridine} + \text{ATP} \xleftrightarrow{Mg^{2+}} \text{UMP} + \text{ADP} \qquad \text{Eq.9}$$

$$\text{Uridine} + \text{Pi} \longleftrightarrow \text{Uracil} + \text{Rib-1-P} \qquad \text{Eq.10}$$

1.3.3 The *de novo* Pathway for dUMP Synthesis

In E. coli dUMP is the immediate precursor to thymidine nucleotide synthesis. It is therefore important for the cell to maintain an adequate supply of dUMP.

Biosynthesis of dUMP can occur by one of two ways. It can be achieved by converting dCTP to dUTP by the action of dCTP deaminase, followed by the conversion of dUTP to dUMP, catalysed by dUTP pyrophosphatase (Eq.11). dUMP synthesis by this means is derived from a cytosine compound.



dUTP + H_2O ------------> dUMP + PPi   Eq.11

dUMP can also be synthesised from UDP. Ribonucleoside diphosphate reductase reduces UDP to dUDP and subsequently, dUDP is phosphorylated to dUTP before being rapidly degraded to dUMP by dUTP pyrophosphatase. In this case dUMP is derived from a uracil compound. The two pathways share dUTP as a common intermediate.

In 1967 Karlstrom and Larsson determined that dCTP deaminase provides the major source of dUMP (80%), while UDP reductase provides the rest. In the absence of dCTP deaminase, UDP reductase is able to take over the role of providing the cell with an adequate source of dUMP for normal growth.

1.3.4 <u>Salvage Pathway for the Synthesis of dTMP</u>

Mutants lacking thymidylate synthetase activity (*thyA*) cannot convert dUMP from the *de novo* pathway into dTMP. Instead they rely on a salvage pathway for the synthesis of dTMP and ultimately, dTTP. As they have an obligate requirement for an exogenous source of dTMP they are known as thymine or thymidine auxotrophs depending on the mutant genotype. If thymidine is provided as the source of thymidylate it can be converted to dTMP by thymidine kinase. Alternatively if thymine is provided, it must first be converted to thymidine by thymidine phosphorylase (*tpp* or *deoA*) (Eq. 12).

ThyA mutants lacking thymidine phosphorylase are solely dependent on thymidine as a source of dTMP and are known as thymidine auxotrophs. In contrast thymine auxotrophs can assimilate both thymine and thymidine. In <u>E. coli</u> thymidine phosphorylase is located near the surface of the cell (Kammen, 1967), possibly in the the periplasmic space and is also capable of catabolising deoxyuridine to uracil and dRib-5-P.

Thymine + dRib-1-P <----------> Thymidine + Pi Eq.12

The incorporation of exogenous thymine into DNA is dependent on the supply of endogenous dRib-1-P groups. This deoxyribosyl group is necessary for the conversion of

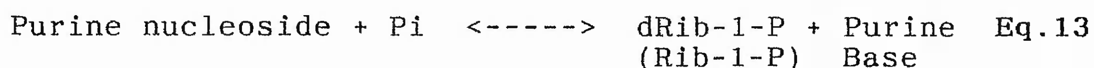
thymine to thymidine. In wild type E. coli the incorporation of exogenous thymine into its DNA does not occur because of a lack of an endogenous dRib-1-P pool. However, thymine assimilation will occur if a source of this compound is provided exogenously. Deoxynucleosides can act as sources of dRib-1-P since they are degraded by phosphorylase enzymes to yield a nucleoside base plus dRib-1-P. A number of workers have shown the simultaneous addition of a deoxynucleoside into the growth medium, such as deoxyadenosine, results in extensive thymine incorporation in wild type E. coli (Boyce *et al*, 1962; Budman and Pardee, 1967; Kammen, 1967; Munch-Petersen, 1967). It has also been shown that purine deoxyribonuceosides are more effective than pyrimidine deoxynucleosides in promoting the assimilation of exogenous thymine (Munch-Petersen, 1967).

In *thyA* mutants, an increase in catabolism of deoxynucleosides is believed to occur, providing a source of dRib-1-P for the utilisation of exogenous thymine. The most likely source of this dRib-1-P is dUMP, the catabolism of which is increased as result of the loss of thymidylate synthetase activity (Beacham and Pritchard, 1971).

1.3.5 <u>Utilisation of Nucleotides as Sole Sources of Carbon</u>

Wild type <u>E. coli</u> is capable of utilising nucleosides and deoxynucleosides as sole sources of carbon and energy due to enzymes capable of catabolising the ribosyl groups generated by the various nucleoside phosphorylases.

The *in vivo* degradation of purine deoxynucleosides occurs via the enzyme purine nucleoside phosphorylase (*pup* or *deoD*), yielding a deoxyribosyl group and the purine base (Eq.13).

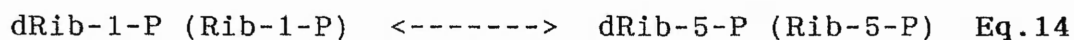


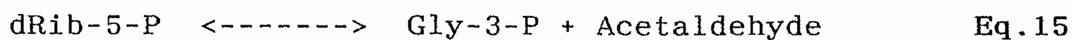
Purine nucleoside + Pi <-----> dRib-1-P (Rib-1-P) + Purine Base   Eq.13

In <u>E. coli</u> thymidine phosphorylase is responsible for the breakdown of both thymidine and deoxyuridine to their respective pyrimidine bases plus dRib-1-P (Eq. 12). Uridine on the other hand is degraded via uridine phosphorylase to uracil plus Rib-1-P (Eq. 10).

There are two enzymes involved in degrading ribosyl moieties for the purpose of generating energy. The first, deoxyribomutase, can degrade ribose-1-P and dRib-1-P to their respective ribosyl-5-P and deoxyribosyl-5-P groups (Eq. 14). Ribose-5-P is then fed directly into the pentose phosphate pathway to produce energy. In contrast, dRib-5 -P must be degraded to glyceraldehyde-3-P and acetaldehyde before it can be used via the glycolytic pathway as a

source of energy. This reaction is carried by the second enzyme, deoxyriboaldolase (Eq. 15)


dRib-1-P (Rib-1-P) <-------> dRib-5-P (Rib-5-P) **Eq.14**


dRib-5-P <-------> Gly-3-P + Acetaldehyde **Eq.15**

1.4 The *Deo* Operon of *E. coli*

The *Deo* operon of E. coli encodes a cluster of four structural genes and a number of regulatory genes specifying the enzymes thymidine phosphorylase (*deoA*), purine nucleoside phosphorylase (*deoD*), deoxyribomutase (*deoB*) and deoxyriboaldolase (*deoC*) (Fig.3). Although these genes are closely linked, they are believed to be part of two overlapping operons (Ahmad and Pritchard, 1969; Albrechtsen *et al*, 1976). One of the operons is thought to transcribe all four genes while the other expresses *deoB* and *deoD* only. Two promoter-operator regions are located to the left of *deoC*. They are *deoPO-cytPO* and are responsible for controlling the expression of the tetracistronic mRNA transcribing *deoC*, *deoA*, *deoB* and *deoD*. A third internal promoter-operator region, designated PO-3, is believed to be located between *deoA* and *deoB*. This is thought to transcribe the distronic mRNA for *deoB* and *deoD*.

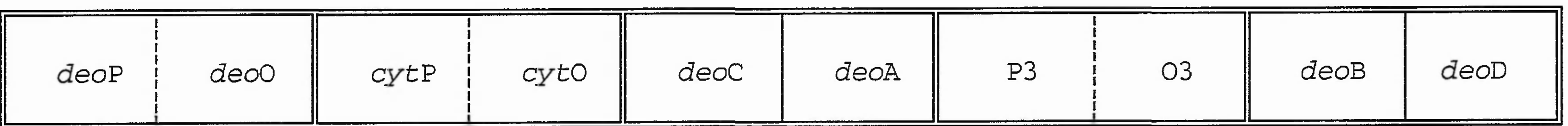
Hammer-Jesperson and Munch-Peterson (1975) showed that at *cytPO*, cyclic AMP (cAMP) + cAMP receptor protein (CRP) is needed for the efficient binding of mRNA. The *deoPO* site is independent of cAMP regulation but appears to be under the control of negative regulation by a repressor protein encoded by *deoR*, located at 18.5 min on the E.coli chromosome (Ahmad and Pritchard, 1971). This repressor protein binds to the *deo* operator site preventing the operon from being transcribed. Induction (derepression) of the system can be achieved by the presence of an inducer

(effector) molecule which renders the repressor protein inactive and unable to bind to the operator. Various workers have shown that the four enzymes encoded by the *deo* operon are inducible and that they share the same inducer molecule, namely deoxyribose-5-P (Rachmeler, Gerhardt and Rosner 1961; Breitman and Bradford 1967, 1968; Barth *et al* 1968; Munch-Petersen *et al*, 1972). Negative regulation is exerted on the *cytO* by a *cytR* gene product (located at 87 min on the E. coli chromosome) in a similar way to that of the *deoR* gene product on the *deoO* site (Jorgensen *et al* 1977). In this case, cytidine (and possibly adenosine) is the effector molecule which prevents the binding of the *cytR* repressor protein to the *cytO* site (Munch-Petersen *et al* 1972).

As d-Rib-5-P is generated by the degradation of deoxynucleosides all four enzymes can be induced by both purine and pyrimidine deoxynucleosides. In addition, purine nucleoside phosphorylase and deoxyribomutase are also induced by purine ribonucleosides such as inosine and guanosine (Hammer-Jepersen *et al*, 1971; Munch-Petersen *et al*, 1972). This is evidence for the existence of additional control on these two enzymes by the third regulatory unit PO3.

Fig. 3 The *Deo* Operon In E.coli

(Hammer-Jesperson and Munch-Petersen, 1975; Jorgensen *et al*, 1977)



Interacting Molecules:

deoP	deoO	cytP	cytO
RNA Pol	Deo R Rep	RNA Pol + cAMP-CRP	Cyt R Rep

RNA Pol = RNA Polymerase

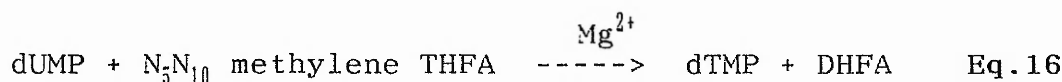
Rep = Repressor Molecule

cAMP-CRP = cyclic AMP and cAMP Receptor Protein

1.5 Thymidylate Synthetase and the Isolation of Thymine Auxotrophs

As thymidylate synthetase plays such a central role in dTTP synthesis it is worth considering how its loss triggers the cell into using the salvage pathway.

Thymidylate synthetase (TS), encoded by the *thyA* gene, was first isolated by Friedkin and Korneberg (1957). It catalyses the tetrahydrofolate (THFA) dependent methylation of dUMP to dTMP (Eq.16).



$$dUMP + N_5N_{10} \text{ methylene THFA} \xrightarrow{Mg^{2+}} dTMP + DHFA \qquad \text{Eq.16}$$

This C1 transfer reaction is unique in that THFA acts as both the C1-unit carrier and as the reductant. The C1-unit is transferred to the 5 position of dUMP, and THFA is oxidised to dihydrofolate (DHFA). Thus TFHA is consumed during the reaction (Fig. 4).

In order to replenish supplies of N_5N_{10} methylene TFHA, DFHA is converted back to TFHA by dihydrofolate reductase. TFHA is subsequently converted to N_5N_{10}- methylene THFA by the action of serine transhydroxymethylase.

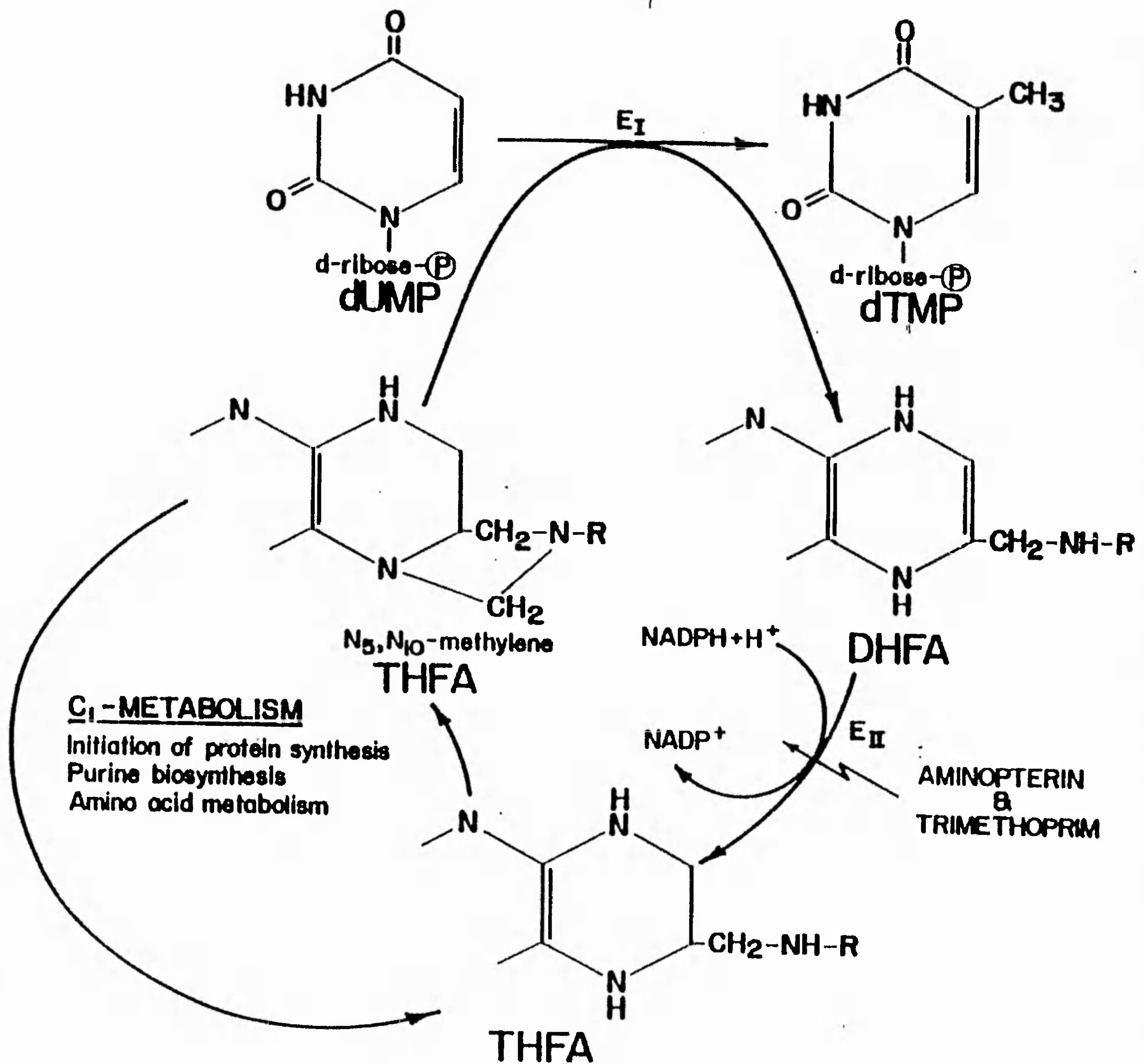
Thymine auxotrophs can be isolated if cells are grown in the presence of a folate antagonist (eg. trimethoprim or aminopterin) provided that thymine or thymidine as a source dTMP is also present. Successful isolation can be

attributed to a mutation in *thyA* enabling the cell to counteract the activity of the antifolate drug. In this research programme trimethoprim was employed in the isolation of thymine auxotrophs.

Trimethoprim (TM) is an antibiotic which has strong anti-folate activity. It is a structural analogue of the pteridine portion of DHFA. It acts as a potent inhibitor of dihydrofolate reductase, rendering the cell incapable of replenishing supplies of THFA. Since TFHA is the key compound in C1 metabolism, many essential biochemical processes such as initiation of protein synthesis, amino acid metabolism and purine metabolism can no longer function and cell death occurs. It therefore becomes a selective advantage for cells grown in the presence of trimethoprim to lack thymidylate synthetase, thereby preventing the consumption of the short supply of THFA. This can only occur if an exogenous source of thymidylate is also provided, in this case thymine. In addition to the supply of thymine, *thyA* mutants require sufficient THFA since the other biochemical processes mentioned above are dependent on a supply of this compound. This is made possible because the inhibition of dihydrofolate reductase by the folate antagonist is incomplete (Wilson et al, 1966; Bertino and Stacey, 1966).

Fig. 4 The Relationship between Thymidylate Synthetase and Dihydrofolate Reductase.

(O'Donovan and Neuhard, 1970)



1.6 Classes of *thyA* mutants

Essentially there are four types of thymine auxotrophic mutants. Each class of mutant requires different concentrations of thymine. The minimum concentration of thymine required by any strain is that compatible with normal growth (Pritchard, Barth and Collins, 1969; Pritchard and Zaritsky, 1970).

1.6.1 Thymine High Requiring Mutants (THR)

ThyA mutants, selected in the presence of a folate antagonist, typically require 20 µg/ml of exogenous thymine for growth. Their genotype is *thyA* because they lack thymidylate synthetase. The *thyA* mutation results in an accumulation of dUMP. In E.coli the accumulated dUMP is first degraded to deoxyuridine and subsequently catabolised to uracil and dRib-1-P by the action of thymidine phosphorylase (Breitman and Bradford, 1964; Beacham, Barth and Pritchard, 1968). It has also been shown that dRib-1-P in *thyA* mutants is produced almost exclusively from the degradation of pyrimidine deoxynucleotides and that uridine phosphorylase (contrary to previous assumptions (O'Donovan and Neuhard 1970)), in addition to thymidine phosphorylase, plays an important role in this breakdown (Krentinsky, Barclay and Jacquez, 1964; Beacham and Pritchard, 1971). Uridine phosphorylase has been shown to accept deoxyuridine

and, to a lesser extent, thymidine as a substrate (Kretinsky *et al*, 1964). The dRib-1-P produced as a result of these reactions is then available for the assimilation of thymine.

The relatively high concentration of thymine required by the *thyA* mutant for growth is necessary to allow thymidine phosphorylase to compete successfully with dRib-1-P catabolism by deoxyribomutase.

1.6.2 Thymine Low Requiring Mutants (TLR)

When THR strains are grown under conditions of 2 µg/ml thymine spontaneous secondary mutations arise to enable the cell to assimilate the limited thymine for dTTP synthesis. Two genotypic classes of these TLR mutants can be distinguished. One type has no deoxyribomutase activity as a result of the second mutation, whereas the other type loses the function of deoxyriboaldolase. The genotypes of these two double mutants are *thyA deoB* and *thyA deoC* respectively. In both cases deoxyribosyl degradation is affected, resulting in the accumulation of intracellular dRib-1-P. Consequently, thymidine phosphorylase no longer has to compete for dRib-1-P, enabling TLR strains to grow with thymine concentrations of 2 µg/ml.

The two types of TLR mutants can be phenotypically

differentiated from one another by their response towards high levels of deoxynucleosides. TLR strains of the genotype *thyA deoC* are sensitive to high levels of deoxyribonucleosides, for example thymidine, added to the growth medium (Beacham *et al*, 1968). It is irrevelant if a strain is *thyA* or *thyA+* for thymidine sensitivity to occur. All that is required for thymidine sensitivity is a strain to exhibit the *deoC* mutation (O'Donovan, 1978). This phenomenon has been ascribed to the endogenous accumulation of dRib-5-P which reaches toxic levels (Beacham *et al*, 1968; Lomax and Greenberg, 1968). At present however, it is unclear exactly how the increase in dRib-5-P is toxic to the cell's growth. Strains of the genotype *thyA deoB* are insensitive to thymidine at high levels simply because dRib-5-P cannot be accumulated (Alikhanian *et al*, 1966; Beacham *et al*, 1968).

The ability of TLR to utilise various nucleosides as sole sources of carbon is dependent on the nature of the second mutation. TLR of the genotype *thyA deoB* cannot degrade deoxyribonucleosides or ribonucleosides as sole sources of energy. In contrast, TLR of the genotype *thyA deoC* cannot catabolise deoxyribonucleosides as carbon sources but, are capable of utilising ribonucleosides by virtue of a functional deoxyribomutase.

1.6.3 Super Low and Super High Thymine Requirers (SLTR and SHTR)

A class of TLR exists in which a further mutation results in the ability of a strain to grow on thymine concentrations as low as 0.2 µg/ml. This class of genetically heterogeneous *thyA* mutants are known as super low thymine requirers (SLTR).

One group of SLTR can be isolated from TLR strains of the genotype *thyA deoB*. In this group the additional mutation is not linked to the genes in the *deo* operon. One such mutant was isolated by Okada in 1966 and analysed by Munch-Petersen in 1968. This mutant was found to be constitutive for the enzymes thymidine phosphorylase and deoxyriboaldolase. Constitutivity did not appear to be linked by P1 transduction to the *deoB* locus (Ahmad and Pritchard, 1971), instead it was shown to be due to a mutation in a distant regulatory gene. This mutation, originally denoted by Ahmad and Pritchard as *nucR* (Ahmad and Pritchard, 1971), has now been designated *deoR* (Albrechtsen *et al*, 1976). Thus, the mutant isolated by Okada would have a genotype *thyA deoB deoR*.

The very low thymine requirement of the *thyA deoB deoR* strain can be ascribed to the constitutive activity of thymidine phosphorylase resulting in the rapid degradation of deoxyuridine to yield dRib-1-P (and uracil). Since no further breakdown of dRib-1-P can occur due to the lack of

deoxyribomutase activity, a high level of deoxyribosyl groups is maintained for thymine assimilation.

When a *thyA deoB deoR* strain, Y-70-22, was transduced to *deoB*+ by Ahmad and Pritchard (1971) they found that the *thyA deoB+ deoR* transductant, instead of being a SLTR, had become a super high thymine requiring strain (SHTR), requiring about 50-100 µg/ml thymine. In this strain the *deoR* mutation had rendered thymidine phosphorylase deoxyribomutase and deoxyriboaldolase constitutive, resulting in a rapid depletion of the dRib-1-P pool, thus significantly raising the threshold requirement of exogenous thymine.

From these studies it is clear that the *deoR* mutation can alter the thymine requirement in both *deoB* and *deoB*+ strains to produce a SHTR or a SLTR, depending on the genetic characteristics of a particular mutant strain.

1.7 Pyrimidine Analogues

Certain pyrimidine (and purine) analogues, toxic to bacterial growth, have been used extensively as biochemical tools to elucidate the pathways involved in DNA and RNA biosynthesis. They enable the selection of mutants defective in particular enzymes. The isolation and study of such mutants have provided important information in determining the role of these enzymes within the overall functioning of these metabolic pathways. Some of the analogues which inhibit the pyrimidine pathway in E.coli are described below.

In general, nucleobase analogues must be metabolised to the nucleotide level before any toxicity can be observed. Toxicity can be expressed in one of two ways; conversion of the analogue to the triphosphate level and subsequent incorporation into nucleic acids, or by inhibition of one or more enzymes involved in the pyrimidine pathway. Consequently, two classes of mutant can arise as a result of analogue toxicity.

In one class of mutant, conversion of analogues to the nucleotide level cannot take place because the enzyme responsible for that conversion is lacking or is defective. A mutant of this type can occur on exposure to 5-fluorouracil (FU) toxicity. The cells become resistant to FU if the activity of the enzyme UMP pyrophosphorylase is lost (O'Donovan and Neuhard, 1970). Similarly, mutants

lacking the enzyme cytosine deaminase are resistant to 5-fluorocytosine (Ahmad and Pritchard, 1970).

The other class of mutant are the pyrimidine overproducers. These mutants counteract analogue toxicity by excreting natural pyrimidine bases and nucleosides which compete with the analogue for the conversion to the nucleotide level. For example, 5-fluorodeoxyuridine (FUdR) is converted to FdUMP by thymidine kinase. FdUMP is a potent inhibitor of thymidylate synthetase. Thus, an accumulation of dUMP and its subsequent breakdown to deoxyuridine could successfully compete with FUdR for thymidine kinase and prevent expression of FUdR toxicity (O'Donovan and Neuhard 1970).

Another possible way in which resistance to FUdR could occur is through a double mutation resulting in the cell losing the activity of two enzymes *eg*. thymidine kinase and thymidine phosphorylase. In this double mutant, FUdR cannot be converted to FdUMP, nor can it be degraded to FU and subsequently converted to FUMP via UMP pyrophosphorylase. In a similar way, mutations in UMP pyrophosphorylase and uridine kinase would be expected to result in a FU resistant, fluorouridine (FUR) resistant double mutant since fluorouridine is converted to FUMP via uridine kinase (O'Donovan and Neuhard 1970).

1.8 Aims and Investigation Strategy

The aim of this study is to genetically manipulate a strain of B. helvolum to obtain overproduction of the nucleoside thymidine (and deoxyuridine). Since little is known about pyrimidine metabolism in B. helvolum the manipulation process will be carried out using the pyrimidine metabolic pathway in E. coli as a template.

There are a number of ways in which the bacterial strain can be manipulated to overproduce thymidine.

a. By increasing the production of dUMP and its subsequent conversion to thymidine via dTMP.

b. By blocking degradation of thymidine through mutating thymidine phosphorylase (*deoA*).

In this study a mutant of B. helvolum was used, in which ICI Biological Products had possibly already introduced a mutation(s) resulting in an increase production of dUMP. They isolated the mutant during an extensive research programme involving a series of screening regimes and manipulation techniques. Of the wide range of bacterial species screened B. helvolum proved the most promising in the excretion of thymidine into the surrounding medium. A mutant derived from B. helvolum ($N^{0.}$ 2.977) yielded 3 mg/ml thymidine under industrial fermentation conditions. This yield was considered insufficient for commercial production

so the mutant was employed for the basis of our research to further increase the thymidine excretion.

In this research programme the second option was to be pursued ie. to select a *deoA* mutant. The first step in the investigation strategy involves the isolation of thymine auxotrophic mutants using trimethoprim and thymidine. Subsequently these thymine auxotrophs would be mutated for deoxyriboadolase to obtain a *thyA deoC* mutant. Such a mutant should be sensitive to deoxynucleosides. Selection pressure could then be applied on the *thyA deoC* strain to isolate a mutant resistant to pyrimidine deoxynucleosides. The deoxynucleoside resistant mutant could have one of three genotypes; *thyA deoC+*, *thyA deoA deoC* or *thyA deoB deoC* (the latter genotype cannot produce dRib-5-P). In the final stage of the isolation process any mutants with potential *thyA deoA deoC* genotype would be selected for, and reverted back to, *thyA+*. Any *thyA+ deoA deoC* mutants isolated as a result of this process would subsequently be tested for their ability to excrete thymidine (and deoxyuridine) using reverse phase HPLC.

CHAPTER 2: METHODS AND MATERIALS

MATERIALS

2.1 General Reagents

Ethanol, methanol (HPLC grade), peptone, thiamine-HCl, tri-sodium citrate, magnesium chloride, magnesium sulphate, sodium dihydrogen sulphate, disodium hydrogen orthophosphate, sodium hydroxide, sodium chloride, ammonium chloride, ferric chloride, potassium dihydrogen orthophosphate, hydrochloric acid, EDTA, TRIS-HCl, D-glucose, 2-Deoxy-D-ribose, and D-ribose were purchased from **B.D.H. Chemicals Ltd.**, Poole.

Chloroacetic acid crystals were purchased from **Fisons**, Ipswich.

Biorad dye reagent and bovine serum albumin were purchased from **Sigma Chemicals** St. Louis, USA.

2.2 Pyrimidine Analogues

5-fluorouracil (5-FU), 5-fluoro-2'-deoxyuridine, 6-amino-uracil (6-AU), 5-AU, 5-aza-U, 3-deaza-U, 5-chloro-U, 6-methyl-U, dihydro-6-methyl-U, 5-nitro-U and 2-thio-U were purchased from **Sigma Chemicals** St. Louis, USA.

2.3 Nucleobases and Nucleosides

Thymine, uracil, adenosine, thymidine, deoxyuridine, deoxyadenosine, uridine, cytidine and guanosine were purchased from **Sigma Chemicals** St. Louis, USA.

2.4 Antibiotics

Trimethoprim lactate was purchased from **Wellcome Foundation** Ltd. London.

2.5 Growth Media

Nutrient agar, nutrient broth and Bacteriological Agar $N^0$1 were purchased from **Oxoid**, Basingstoke.

2.6 Specialized Equipment

HPLC: Beckman System Gold
Pump 126, 166 Detector Module.
Column: Ultrasphere 150mm x 5µ
S.P. Packaging: C_{18} (octadecylsilyl/silica)

Beckman Laboratories High Wickham, Bucks.

Fig. 5 Beckman System Gold HPLC

2.7 Bacterial Strains

Strain	Phenotypic Characteristics	Probable Genotype
E.coli		
KL16	Wild Type	
S145	Thymidine Auxotroph	*thyA deoA*
B.helvolum		
SK1	Wild Type ICI 315	
SKM1	ICI mutant 2.977	*upp udp*
SKM18	Thymine Low Requirer of SKM1	*thyA deoC*
SKM8	Thymine Low Requirer of SKM1	*thyA deoC*
SKM28	Thymidine Auxotroph of SKM1	*thyA deoC deoA*
SKM46	Super High Thymine Requirer (SHTR) derived from SKM8	*thyA deoD deoR*
SKM47	Thymine High Requirer derived from SKM46	*thyA deoD*
SKM48	*thyA+ deoD+* strain derived from SKM46 able to grow on deoxyadenosine as a Sole Carbon Source	*thyA+ deoD+ deoR*
SKM50	Thymidine Overproducer derived from SKM28	*thyA+ deoC deoA*

METHODS

2.8 Preparation of Media

2.8.1 Minimal Synthetic Medium/Agar (MSM/A)

Sterile distilled water (SDW)	90ml
Sterile M9X10 (see below)	10ml
Sterile 0.01 M $CaCl_2$	1ml
Sterile 0.1 M $MgSo_4$	1ml
Sterile 20% Glucose	2ml

For MSA 2 g of Bacteriological Agar Number 1 were dissolved in the 90 ml of distilled water and sterilised at 121^0C.

2.8.2 M9X10

The following were added, in order, to 100 ml of distilled water, allowing each one to dissolve fully before the next addition;

Component	g/100ml
Na_2HPO_4	6.0
KH_2PO_4	3.0
NaCl	0.5
NH_4Cl	1.0

The M9X10 solution was autoclaved at $121^{0}C$ for 15 min and stored at room temperature.

2.8.3 Modified GM Medium

Component	g/l
Distilled Water	1.0 l
Ammonium sulphate	5.0
Di-potassium hydrogen orthophosphate	10.5
Potassium dihydrogen orthophosphate	4.5
Peptone (Oxoid)	10.0
Magnesium sulphate	1.0
Tri-sodium citrate	0.5
0.972% Ferric chloride solution	0.2 ml
* Biotin	30.0 mg
* Thiamin HCl (Vit. B1)	10.0 mg
* Glucose	50.0
* Fison's trace elements	5.0 ml

* Made up as sterile solutions and added separately after the remainder had been autoclaved at $121^{0}C$ for 15 min.

The GM medium was adjusted to pH 7.0 with sterile HCl and NaOH.

2.8.4 Trace Elements

Component	mg
Distilled Water	100 ml
Magnesium sulphate	40.60
Zinc sulphate	44.00
Copper sulphate	7.86
Calcium chloride	734.00

The trace elements solution was autoclaved at 121^0C for 15 min and stored at room temperature.

2.9 Measurement of Bacterial Growth

From a overnight grown culture 1ml was used to inoculate 10 ml of MSM in a 250 ml conical flask. The flask was incubated in a shaking incubator set at 25^0C or 30^0C, 150 rpm. Growth was detected either by taking OD readings at 600 nm at timed intervals or by doing viable counts. The latter were done using either serial dilutions of the culture on spread plates, or by using the Miles and Misra technique (see 2.9.1).

A semi-logarithmic plot was made of OD_{600nm} or viable count against time and the mean generation time was determined.

2.9.1 Miles and Misra Method

Serial dilutions of the culture were made down to 10^{-7}. Using thoroughly dried MSM agar plates the base of each plate was marked into sectors for the 5 highest dilutions. Using separate pasteur pipettes, 1 drop of each dilution was transferred to the relevant sectors on the plates. A pasteur pipette delivers the same volume per drop of culture (1/30 of a ml) when held vertically at a height of 2 cm from the surface of the plate. The plates were allowed to dry and then incubated at 30^0C for 48 hours. When colonies appeared, the dilution which gave a statistically significant number of colonies (5-30 colonies) was used to calculate the viable count, taking into consideration the dilution and inoculation volume used.

2.10 Growing Cells for Thymidine Excretion

1 ml of an overnight grown culture was inoculated into 20 ml of GM medium (at pH 7.5) in 250 ml conical flasks. Incubated at 25^0C in shaking incubator set at 200 rpm. At 24 h time intervals 1ml of the batch culture was removed aseptically, and placed in a sterile microfuge tube. The sample was microfuged for 5 min at high speed to obtain a cell free supernatant. This supernatant was stored at 4^0C ready for the cross feeding test or HPLC analysis.

2.11 Bacterial Growth on Alternative Carbon Sources

MSA plates were prepared as described previously with the omission of glucose. The alternative carbon source was placed in a petri dish to give a final concentration of 5 mM. Molten MSA was poured into the plates dissolving and dispersing the carbon source.

10 ml of an overnight grown culture of B.helvolum was spun down at 5000 rpm for 5 min and the supernatant removed. The pellet was re-suspended in 5 ml of saline and 20 µl was streaked or spotted onto the agar plates.

2.12 Selection of Thymine Auxotrophs

MSA plates were prepared containing trimethoprim (TM) at 20 µg/ml and thymidine (TdR) 50 µg/ml. 10 ml of an overnight culture of B.helvolum was centrifuged at 5000 rpm for 5 min and the supernatant removed. The pellet was re-suspended in 1 ml of saline and 0.1 ml aliquots were spread on 4 MSA + TM + TdR plates. The plates were incubated at 30^{0}C for 72 hours until colonies appeared.

2.13 Determination of Thymine Requirement of SKM18

An exponentially growing culture of the SKM18 was diluted by 10^{-5} and 0.1 ml aliquots were spread onto MSM plates

supplemented with various concentrations of thymine, ranging from 0.1 µg/ml to 10 µg/ml. The plates were incubated for 3 days at 30^{0}C. The number of colonies appearing on each plate were recorded and the thymine requirement of the strain was determined as the lowest concentration of thymine on which TLR colonies could grow.

2.14 Detection of Thymidine Excretion

2.14.1 Thymidine Cross-Feeding by Disc Assay

Detection of thymidine excretion by B.helvolum mutants was achieved as described:

MSA plates containing vitamin B1 at 10 µg/ml were made and left to solidify on a level platform. 4.5 ml of 0.4 % molten soft agar (Bacteriological Number 1) were added to 0.5 ml of an overnight E. coli thymidine auxotrophic culture (S145) and poured on top of the MSA to form a soft agar overlay. 20 µl of the supernatant collected from a culture grown in GM was absorbed into a sterile Whatman disc (antibiotic size). Up to 5 discs, including a control disc containing a standard concentration of thymidine, were placed equidistantly onto the surface of the soft agar and left to incubate for 48 h at 37^{0}C.

If any thymidine is present in a disc it should diffuse into the soft agar seeded with S145 resulting in a circular

zone of auxotrophic growth. This growth should be proportional to the amount of thymidine excreted by the B.helvolum sample. Diameters of the S145 growth around the sample discs were taken and compared with the thymidine standard disc measurements.

2.14.2 Reverse Phase HPLC

Reverse phase HPLC was used to detect excretion of thymidine, and any other nucleosides or nucleobases, by various mutants of B.helvolum.

2.14.2.1 HPLC Conditions

Wavelength: 254 nm

Eluent: Methanol plus water (adjusted to pH 2.5-3.0 using Chloroacetic acid crystals)

Flowrate: 1 ml/min

Injection volume: 15 µl

Programmes: DNA 1; Isocratic: 10% methanol, 90% water
Run Time: 15 min

DNA 2; Methanol Gradient: 11 min 2% Meth upto 25% Meth in 2 min, held for 0.5 min, return to 2% Meth in 2min
Run Time: 15.5 min

DNA 1 was used mainly to detect thymidine.

DNA 2 was specifically programmed to separate thymine from deoxyuridine which was not possible with DNA1. Both programmes were capable of detecting uracil without any modification.

2.14.2.2 Preparation of the HPLC Reagents

All reagents were made using double distilled water. The methanol used was HPLC grade. The methanol and water were filtered before use. The HPLC was primed using 100% methanol followed by 100% water. The system was set to running conditions and allowed to reach equilibrium over 15 min before analysing the first sample. Samples consisted of 0.1 ml of the culture supernatant in 0.9 ml of double distilled water (pH 2.5-3.0).

After each run the sample injection apparatus was purged with the eluent to prevent any carryover of the previous sample.

Standards of thymidine, deoxyuridine, thymine, and uracil at a concentration of 100 µg/ml were run during each session on the HPLC. Standard readings of these four components were also made for reference.

2.15 Determination of Thymidine and Deoxyuridine degrading ability of various *B. helvolum* strains

The procedure adopted for assaying the thymidine and deoxyuridine degrading ability of B. helvolum was based on that used for detecting thymidine phosphorylase activity in E. coli (Schwartz, 1971).

2.15.1 Preparation of Cultures for Enzyme Assay

1 ml of an overnight culture was inoculated into 50 ml of MSM medium in a 250 ml conical flask. This flask was incubated at 30^{0}C in a shaking incubator set at 150 rpm. After about 20 hours incubation the culture was harvested and centrifuged at 5000 rpm for 5 min at 4^{0}C (MSE High Speed 18). The supernatant was discarded and the pellet gently re-suspended in 5 ml of Tris-HCL buffer (10 mM, pH 7.1) containing EDTA (2 mM) and kept on ice prior to sonication and the enzyme assay. OD of 1 ml of the unsonicated cell suspension was taken at 550 nm.

2.15.2 Sonication

The culture suspension was sonicated using 2 cm diameter pre-cooled sonicating probe. The amplitude was set to 6 microns peak to peak. Sonication was done in 5-10 one minute bursts with 1 min rest intervals until the suspension had became clear, as judged by visual means. The suspension thus obtained was referred to as the 'cell free extract'. Periodic cooling of the sonicating probe was undertaken during the process by submerging in iced water for a short period to avoid overheating.

2.15.3 Enzyme Assay

0.3 ml of potassium phosphate buffer (0.1 M, pH 7.1) was added to 1.55 ml of the cell free extract at 0^0C. The mixture was then transferred to a 37^0C waterbath and incubated for 2 min. The reaction was started by the addition of the substrate, 0.15 ml thymidine (100 mM) or deoxyuridine (100 mM).

At 5 min intervals, 0.3 ml samples were transferred to 0.7 ml sodium hydroxide (0.5 N). The amount of thymine or uracil formed was determined by measuring at 300 nm and 290 nm respectively, in a UV spectrophotometer.

CHAPTER 3: RESULTS

RESULTS

3.1 Growth Characteristics of B. helvolum

Preliminary growth studies were carried to determine the growth characteristics of the wild type strain of B.helvolum, designated SK1, and to compare them with the mutant strain, SKM1. The utilisation of alternative carbon sources, growth in GM medium and sensitivity to pyrimidine analogues were undertaken.

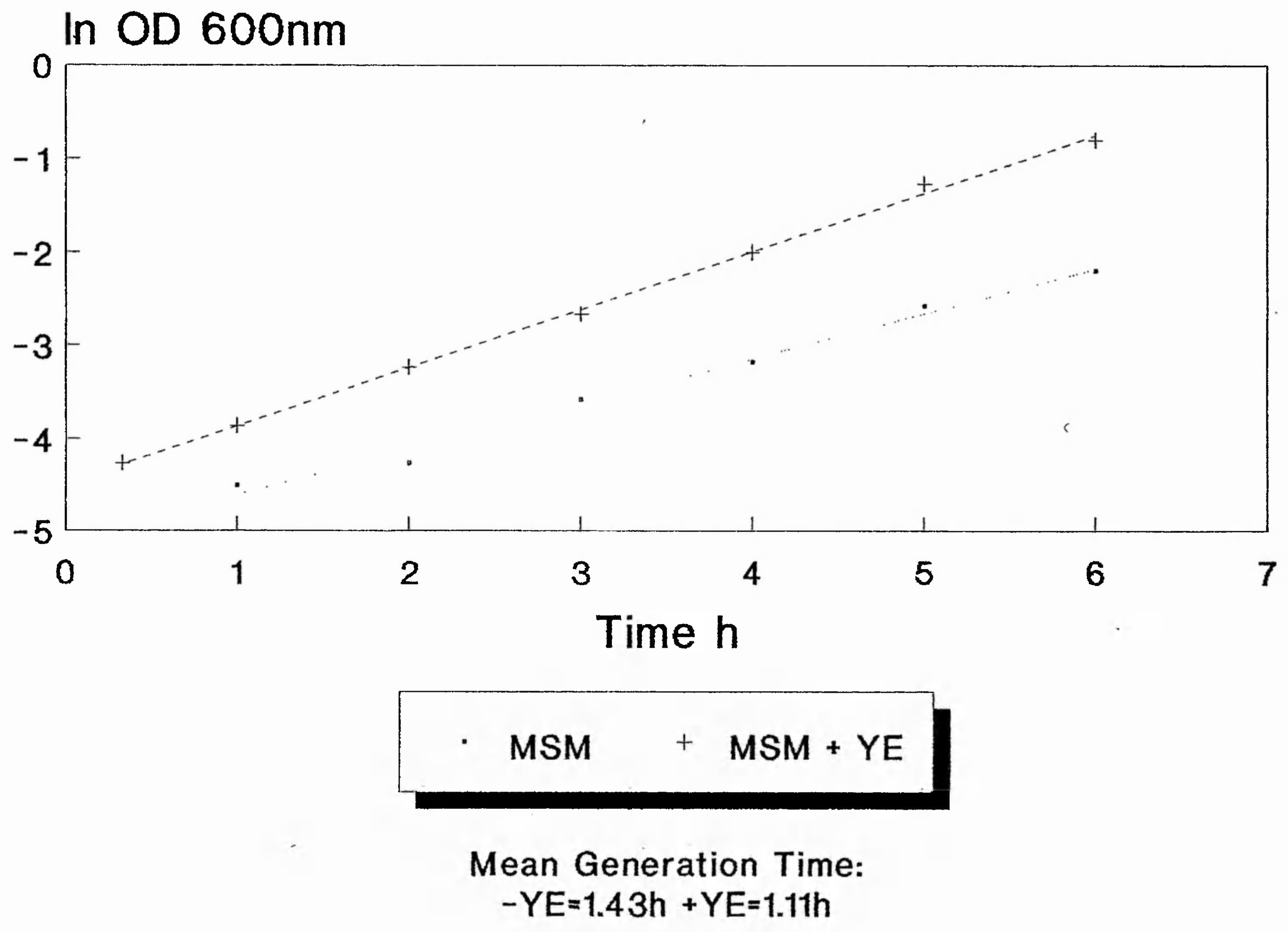
B. helvolum is capable of growing in minimal synthetic medium (MSM). The growth was found to be enhanced in the presence of yeast extract (0.05%) (Fig. 6). However, a reduction in the mean generation time (MGT) from 1.43 to 1.11 hours in the presence of yeast extract, was not sufficient to warrant the inclusion of this complex nitrogen source as part of the nutritional requirements of this bacterium. Also the uncertainty of how the addition of yeast extract would affect the isolation programme justified the continued use of minimal synthetic medium.

In an effort to optimise the conditions for excretion of thymidine by B. helvolum, ICI developed an enriched growth medium called GM. SK1 was found to grow well in GM with a lag phase of 2 hours and a MGT of 1.21 hours (Fig. 7). In contrast, SKM1 had a lag phase of about 3 hours and a MGT of 1.82 hours. These results show that SKM1 had a slower growth rate than SK1, with a longer lag phase and slower in

growth during the exponential phase. This suggests that the genetic changes that have taken place to produce SKM1 have had an unfavourable effect on its growth rate.

The growth rate the thymidine overproducing strain (SKM50) in GM was compared with that of SKM1 (Fig. 8). Over a 96 hour batch culture, SKM50 had a MGT of 1.56 hours compared with 1.7 hours for SKM1. Both cultures reached the stationary phase after 30 hours of incubation. Thereafter only moderate increases in the viable count occured up until 70 hours. The cultures then entered the death phase and viable counts began to decline. These growth curves provided important information with regards to the timing of sampling for HPLC analysis since optimum excretion of pyrimidine catabolites was noted to occur during the stationary phase.

Fig. 6 Growth of SKM1 in MSM +/- Yeast Exract



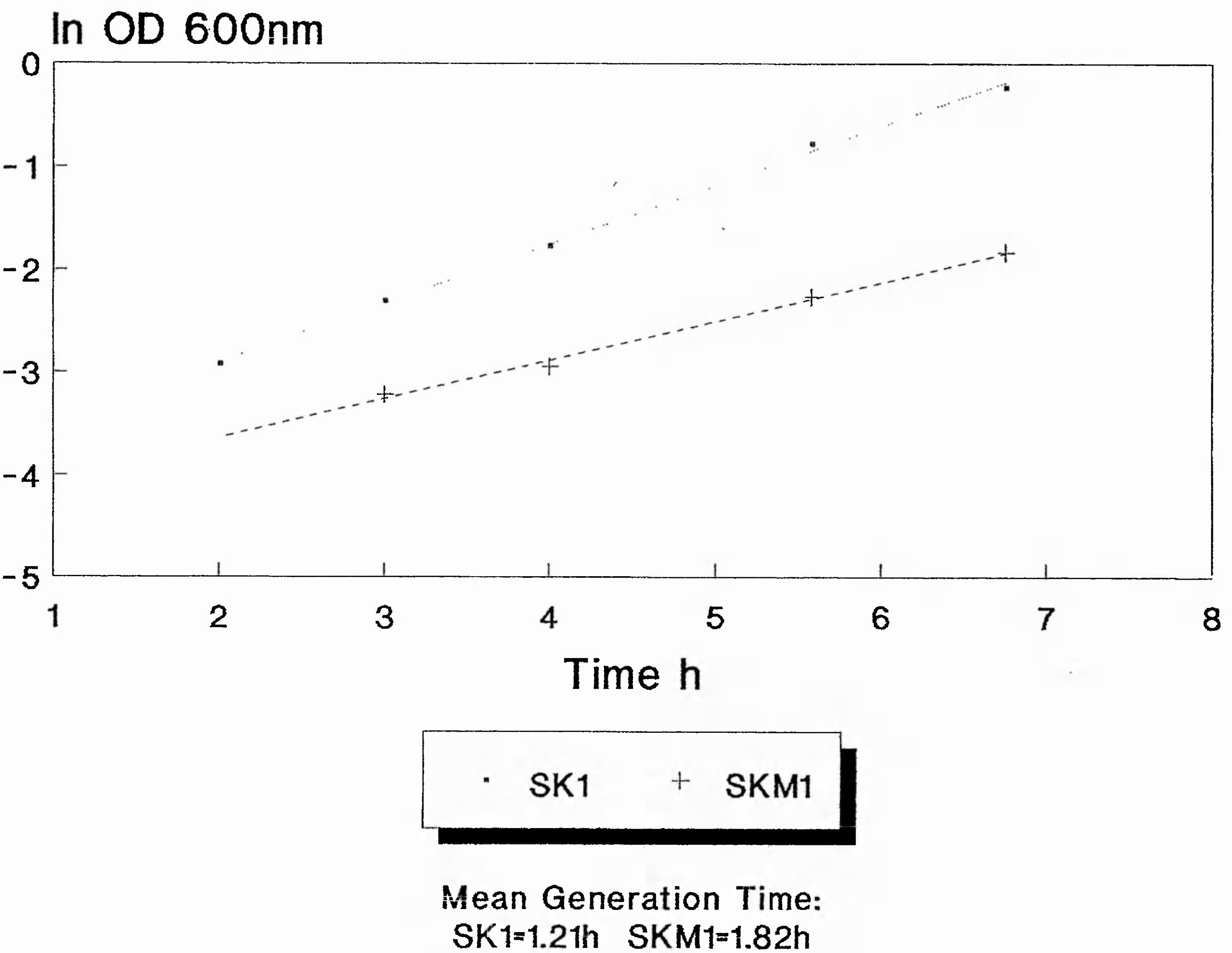
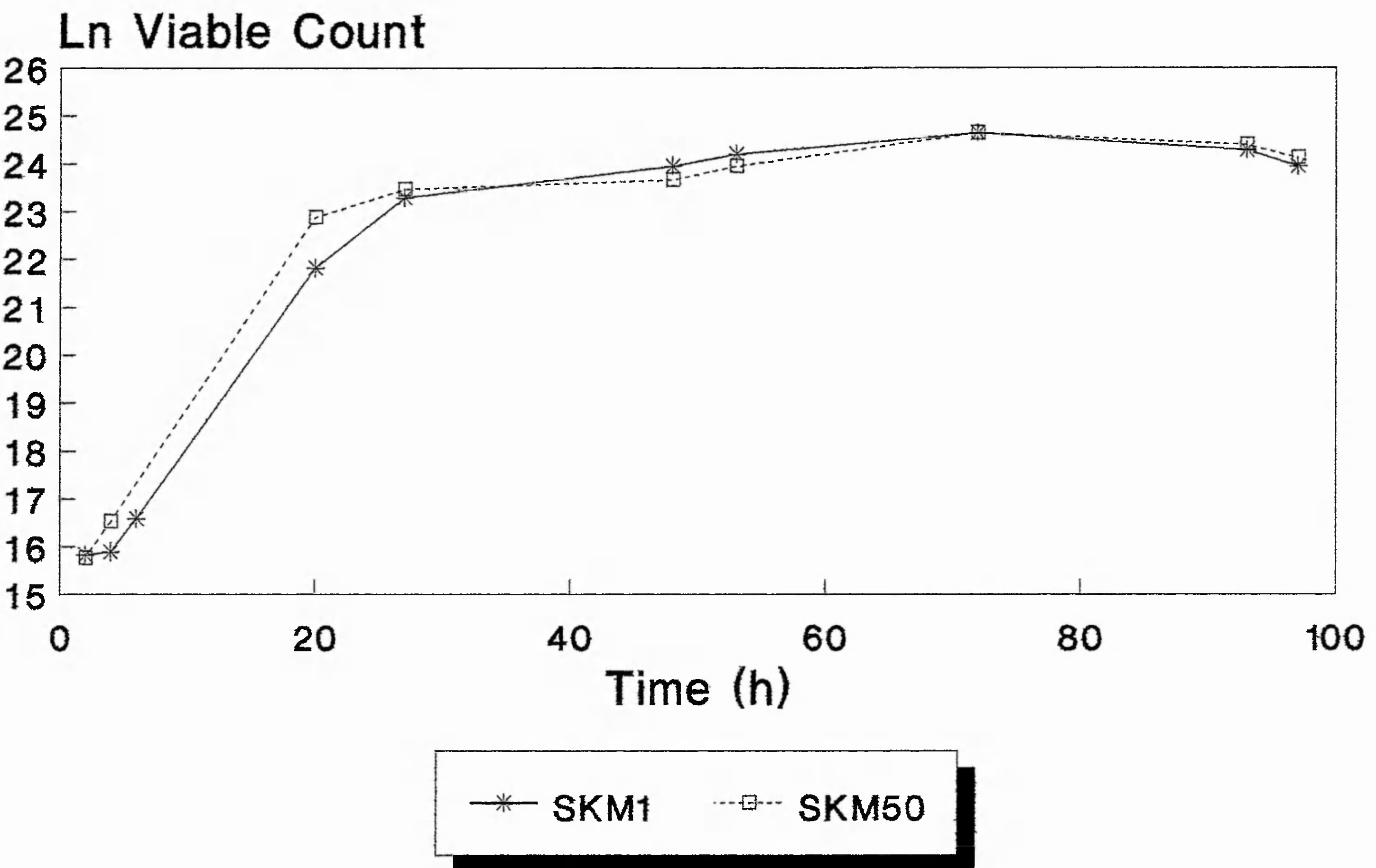


Fig. 7 Growth of SK1 and SKM1 in GM

Fig. 8 GROWTH OF SKM1 AND SKM50 IN GM



Grown in GM Medium at 30oC
Mean Generation Time:
SKM1=1.7 h SKM5O=1.56 h

3.2 Growth of B. helvolum on various Carbon Sources

SK1 was able to utilise both purine and pyrimidine nucleosides and deoxynucleosides as sole carbon and energy sources (Table 1). Generally the growth response on these carbon sources was weaker and took longer (7-10 days) to show any observable growth when compared with growth on glucose as a sole carbon and energy source (5 days).

SK1 was able to grow on cytidine, guanosine, adenosine and uridine as ribonucleoside sources of carbon and energy. In addition, the utilisation of thymidine, deoxyuridine and deoxyadenosine indicated SK1 was also capable of using deoxyribonucleosides as sole sources of carbon and energy. It was noted however that the growth of SK1 was more pronounced on deoxyuridine than on thymidine as a carbon source. No growth was observed when deoxyribose was used as a source of carbon and energy.

SKM1 was capable of utilising adenosine and deoxyadenosine as sole sources of carbon and energy as efficiently as SK1. SKM1 was also able to grow on deoxyuridine and thymidine as sole sources of carbon and energy. However, more prominent growth on deoxyuridine as a carbon source than on thymidine, as with SK1, was observed. SKM1 was unable to degrade uridine as a carbon source.

Table 1. Utilisation of Alternative Carbon Sources by SK1 and SKM1

Carbon Source	SK1	SKM1
Thymidine TdR	+	+/-
Deoxyuridine UdR	++	+
Deoxyadenosine AdR	+	+
Uridine UR	+	-
Cytidine CR	+	NT
Adenosine AR	+	+
Guanosine GR	+	NT
d-Ribose	-	NT

++ confluent growth + good growth +/- Weak growth

- no growth NT = Not Tested

3.3 <u>Brevibacterial Sensitivity towards Pyrimidine Analogues</u>

For all of the analogue tests the bacterial strains were spot inoculated onto the surface of the plates containing the analogue using a 1/10 dilution of a fresh overnight culture.

In this study wild type B. helvolum was found to be resistant to FU up to a concentration of 2.5 µg/ml. In contrast, SKM1 was resistant to this analogue up to a concentration of 400 µg/ml (Tables 2 and 4). SK1 was found to be sensitive to FUdR at a concentration of 10 µg/ml whereas SKM1 was resistant up to 50 µg/ml of FUdR (Table 3).

Tests for base analogue toxicity were also carried out on SK1 and SKM1, using a variety of uracil analogues (400 µg/ml) in the presence and absence of deoxyadenosine (200 µg/ml) (Table 4). The latter was added as a source of dRib-1-P groups. SK1 and SKM1 were found to be resistant to 5-fluorouracil, 5-azauracil, 3-deazauracil, 5-aminouracil, 4-amino-2,6-dihydroxypyrimidine, 5-chlorouracil, 5-methyluracil, dihyro-6-methyluracil and 5-nitrouracil in the presence and absence of deoxyadenosine. SKM50 was also found to be resistant to fluorouracil in the presence and absence of deoxyadenosine.

Table 2. Fluorouracil Sensitivity in SK1 and SKM1

Strain	Fluoro Uracil ug/ml								
	400	100	50	25	10	5	2.5	1.0	0.5
SK1	-	-	-	-	-	-	-	2 col	3 col
SKM1	+++	+++	+++	+++	+++	+++	+++	+++	+++

+++ Confluent Growth - No Growth col Resistant Colonies

Table 3. Fluorodeoxyuridine Sensitivity of SK1 and SKM1

Strain	FUdR μg/ml 50	30	20	10
SK1	-	-	1 col	+/-
SKM1	++	+++	+++	+++

+++ Confluent Growth ++ Good Growth +/- Weak Growth

- No Growth col Resistant Colony

Table 4. Growth of SK1 and SKM1 in the presence of various Uracil Analogues +/-Deoxyadenosine

Uracil Analogue 400 µg/ml +/- Deoxyadenosine 200 µg/ml	Strain SK1	 SKM1
5-Azauracil 5-Azauracil + Deoxyadenosine	+ +	+ +
3-Deazauracil 3-Deazauracil + Deoxyadenosine	+ +	+ +
5-Aminouracil 5-Aminouracil + Deoxyadenosine	+ +	+ +
4-Amino-2,6-dihydroxypyrimidine 4-A-2,6-diHPyr + Deoxyadenosine	+ +	+ +
5-Chlorouracil 5-Chlorouracil + Deoxyadenosine	+ +	+ +
6-Methyluracil 6-Methyluracil + Deoxyadenosine	+ +	+ +
Dihydro-6-methyluracil DiH-6-MU + Deoxyadenosine	+ +	+ +
5-Nitrouracil 5-Nitrouracil + Deoxyadenosine	+ +	+ +
2-Thiouracil 2-Thiouracil + Deoxyadenosine	+/- +/--	+/- +/--
5-Fluorouracil 5-Fluorouracil + Deoxyadenosine	- -	+ +

+ Growth +/- Weak Growth +/-- Poor Growth - No Growth

SKM50 also resistant to FU +/- Deoxyadenosine

Apart from SK1's sensitivity to FU, which has already been acknowledged, the only other analogue to produce any sign of sensitivity was 2-thiouracil. Thiouracil (TU) appeared to be toxic for SK1, SKM1 and SKM50. This toxicity appeared to be slightly enhanced in the presence of deoxyadenosine (Table 5).

Further work on SKM1 and SKM50 using the analogue thiouracil suggested that its toxicity effect was short-lived and occurred only when high concentrations of the analogue were used, ie 400 µg/ml (Table 6).

Table 5. Sensitivity of SK1, SKM1 and SKM50 to Thiouracil +/- Deoxyadenosine

Strain	MSM	Thiouracil 400µg/ml	Thiouracil + AdR 200µg/ml
SK1	+++	+	+/-
SKM1	++	+/-	-
SKM50	+++	+	+/-

+++ Confluent Growth ++ Good Growth + Poor Growth

+/- Weak Growth - No Growth

NB. the toxicity displayed was short-lived

Table 6. Thiouracil Sensitivity Range for SKM1 and SKM50

Culture Dilution	Thiouracil ug/ml					
	0	10	50	100	200	400
SKM1 10^{-3}	+++	+++	+++	+++	++	14 col
10^{-4}	++	++	++	++	+	5 col
10^{-5}	12 col	7 col	10 col	9 col	4 col	-
SKM50 10^{-3}	+++	+++	+++	+++	+++	-
10^{-4}	++	++	++	++	+	-
10^{-5}	22 col	25 col	22 col	-	-	-

+++ Confluent Growth ++ Good Growth + Growth - No Growth col = no. of colonies

3.4 Isolation of a Thymidine Overproducing Mutant of *B. helvolum*

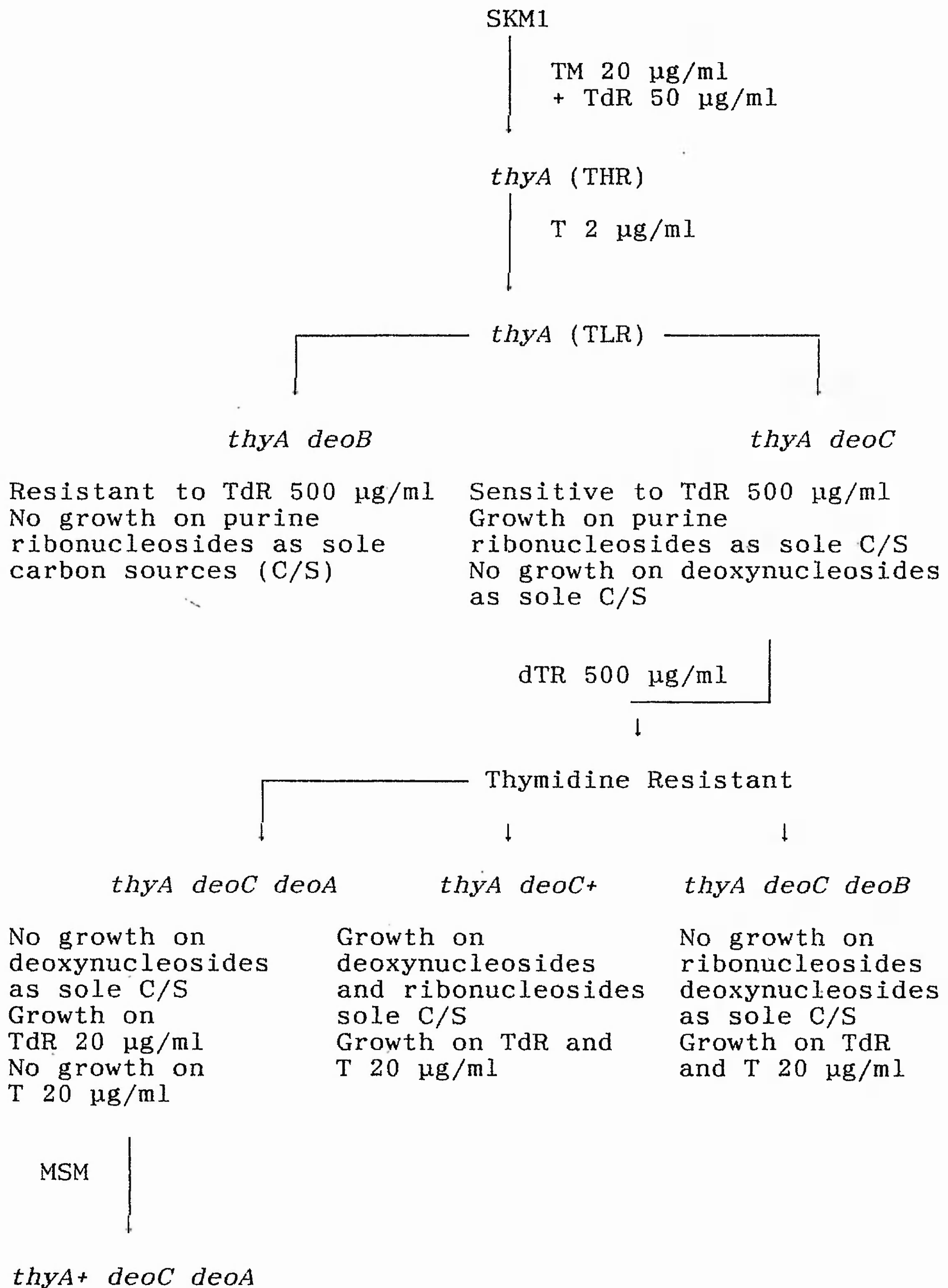
The isolation of a thymidine overproducer of SKM1 was carried out using a specific programme of selection and genetic manipulation which was based on the isolation of thymidine auxtrophic mutants from E. coli (Fig. 9).

In the first step towards isolating a thymidine overproducing strain *thyA* mutants of SKM1 were required. Preliminary work on wild type B. helvolum (SK1) had showed that it was possible to isolate thymine auxotrophs of this bacterial species. Subsequently, the mutant strain, SKM1, was spread on plates containing trimethoprim (20 µg/ml) and thymidine (50 µg/ml), as a source of thymidylate, and incubated for 7 days at 30^{0}C.

Colonies that appeared on these plates were tested for their thymine auxotrophic requirement by streaking them on thymine (20 µg/ml) supplemented and thymine free minimal agar plates. Out of 24 colonies tested only 4 showed a true requirement for thymine. These were designated thymine high requirers (THRs).

The next stage of the strategy was to select a *thyA deoC* mutant. This was achieved by isolating thymine low requirers (TLRs) from the THRs as follows. The four THR mutant strains were spread onto plates containing thymine 2 µg/ml and incubated for 3 days at 30^{0C}.

Fig. 9 Plan of Isolation for a Thymidine Overproducer from *B. helvolum* strain SKM1



Subsequently, colonies (14) were picked from the plates and streaked for purity on fresh thymine 2 µg/ml plates. Of the two types of TLR genotypes that can occur; *thyA deoB* and *thyA deoC*, identification of the *thyA deoC* genotype was achieved by testing their sensitivity to high concentrations of thymidine (500 µg/ml). Those TLRs of the genotype *thyA deoC* are sensitive to thymidine at high concentrations since they are defective in the enzyme deoxyriboaldolase. The degradation of the thymidine leads to an accumulation of intracellular dRib-5-P which reaches a toxic level. TLRs of the genotype *thyA deoB* lack deoxyribomutase so dRib-5-P accumulation does not occur. The ability of the TLRs to grow on (deoxy)nucleosides as sole sources of carbon and energy (with exception of uridine due to an assumed absence of uridine phosphorylase in SKM1) was also used to distinguish between the two types of TLRs. Those TLRs which lack deoxyribomutase (*thyA deoB*) cannot utilise ribonucleosides as sole sources of energy. On the other hand, if the TLR is a *thyA deoC* mutant it should be capable of utilising ribonucleosides as sources of carbon and energy but not deoxyribonucleosides.

Based on this knowledge of isolating E. coli TLRs, the 14 colonies were tested to determine their likely genotypes. Subsequently, three potential *thyA deoC* mutants were selected for further study. These were found to ultilise adenosine but not thymidine as a source of carbon and energy and, were sensitive to thymidine 500 µg/ml.

Table 7. Thymine Requirement of SKM18 (TLR)

Thymine Concentration µg/ml	Number of Colonies SKM18
0.1	0
0.25	0
0.5	0
1.0	0
2.0	0
4.0	10
6.0	35
8.0	45
10.0	35

Inoculum = 3.8×10^7 cfu/ml (cfu colony forming unit) which should give approximately 40 colonies on a medium containing sufficient thymine for growth.

The threshold thymine requirement for SKM18 appears to be 4 µg/ml.

During the selection process it was noted that one of the THR, designated SKM18, produced a heterogeneous population on the thymine 2 µg/ml plates with some colonies being larger than others. In an experiment to determine SKM18's thymine requirement (see section 2.13) it was found to be about 4 µg/ml (Table 7). From this result it was postulated that SKM18 could be a double mutant which had become a TLR in a single step selection. When SKM18 was tested for growth on thymidine at 500 µg/ml it was found to be sensitive. Furthermore, growth occurred on adenosine as a sole source of carbon but on thymidine, it was much reduced compared with SKM1. These findings suggested that SKM18 was a TLR of the possible genotype *thyA deoC*. As a result SKM18 was selected, along with the other three potential *thyA deoC* TLRs, to undergo further manipulation to select a *thyA deoC deoA* mutant.

The next stage of this selection procedure was to obtain thymidine resistant colonies from the thymidine sensitive (*thyA deoC*) mutants. This was done by spreading concentrated cultures of the four TLRs onto minimal agar plates supplemented with thymidine at a concentration of 500 µg/ml. Since the accumulation of dRib-5-P is the cause of thymidine sensitivity in a TLR of the genotype *thyA deoC*, resistance can be achieved by selecting mutants unable to accumulate this ribosyl moiety. For example in E. coli, a further mutation leading to either a defective *deoA* gene or *deoB* gene would convey resistance to thymidine. Alternatively, dRib-5-P accumulation could be prevented if

the strain was to revert to *deoC+*, since any dRib-5-P generated from thymidine catabolism could then be degraded to glyceraldehyde-3-P and acetaldehyde. The desired strain is one that has lost thymidine phosphorylase (*thyA deoC deoA)* and therefore unable to degrade thymidine. This type of mutant requires thymidine as a source of dTMP and is known as a thymidine auxotroph. Furthermore, a mutant of the genotype *thyA deoC deoA* cannot utilise deoxynucleosides as sole sources of carbon and energy, but can use ribonucleosides because deoxyribomutase remains functional.

A total of 42 thymidine resistant colonies were randomly selected from the thymidine 500 µg/ml plates for further testing. To identify possible *thyA deoC deoA* mutants from amongst these thymidine resistant colonies, cultures were spot inoculated on two sets of minimal agar plates, one supplemented with thymidine (20 µg/ml) and the other with thymine (20 µg/ml). Those mutants capable of growth on the thymidine plate, but could not assimilate thymine as a source of dTMP, were considered to be defective in thymidine phosphorylase (*deoA*). In contrast, *thyA deoC deoB* or *thyA deoC+* mutants were considered to be those isolates able to grow on both the thymine and thymidine supplemented plates. Furthermore, *deoC+* strains can utilise deoxynucleosides and ribonucleosides as sources of carbon and energy, while *deoB* mutants cannot utilise either type of energy source.

Table 8. Frequency of Reversion by Thymidine Resistant Strains of SKM18

TdR Resistant strains	TdR 20 ug/ml cfu/ml	T 20 ug/ml cfu/ml	Revertant Frequency
SKM28	2.1×10^7	9.0×10^3	1 in 2333
SKM25	1.35×10^7	1.5×10^4	1 in 900
SKM20	1.13×10^7	5.6×10^3	1 in 2018

cfu Colony Forming Unit

Only 3 of the 42 isolates that were tested (SKM20, SKM28, and SKM18) indicated they were likely to be mutants unable to degrade thymidine.

Analysis of the thymidine resistant mutants showed that the majority of isolates grew confluently on the thymine supplemented plates, indicating a *deoA+* phenotype (27 out of the 42 tested). However, in those cases where a mutant did not grow on the thymine plate, as would be expected if the mutant had been a *deoA* thymidine auxotroph, the absence of growth was not absolute. Instead, a number of revertant colonies were found to be present where the cultures had been spot inoculated. The frequency of revertants for three such mutants was determined by spreading serial dilutions of each culture on a set of thymine and thymidine supplemented minimal agar plates, and comparing the colony counts obtained on the two sets of plates. The rate of revertant frequency ranged from 1 in 1000 to 1 in 2000 mutant cells (Table 8). These colonies, capable of utilising thymine were not studied further but were assumed to be *deoA+* revertants.

In the final step of the isolation programme three putative *thyA deoA deoC* strains; SKM20, SKM25 and SKM28, were reverted to *thyA+* by spreading a heavy inoculum of culture of each isolate onto a set of four MSM plates and incubating at 30^{0}C for five days. Re-establishing thymidylate synthetase activity enables the cell to convert dUMP to dTMP *de novo* and the subsequent conversion of dTMP

to thymidine (and dTTP, for DNA synthesis). After the incubation period only one colony was isolated, from a plate seeded with SKM28. This *thyA+* revertant was designated SKM50. A characteristic Gram staining pattern during various stages of growth (see page 6) and yellowing of the culture on exposure to light confirmed that this strain was *B. helvolum*.

Preliminary studies to detect thymidine excretion from SKM50 were undertaken using the cross-feeding test and reverse phase HPLC (see section 2.14). The cross-feeding test indicated that SKM50 was excreting more thymidine than SKM1 (data not shown). This was based primarily on the increased density of growth of the *E. coli* around the SKM50 disc compared with SKM1. These findings were confirmed by HPLC studies (see section 3.7). However, when SKM50 cells were tested on thymidine as a sole source of carbon, growth did occur, although it was much weaker compared to that of SKM1. This indicated thymidine degradation was still occuring in this strain (Table 10).

3.5 Analysis of a Super High Thymine Requirer of *B. helvolum*

An attempt was made to isolate another mutant in which thymidine degradation could be completely blocked. For this the TLR mutant SKM8 was used, and the isolation programme was repeated to try to isolate a thymidine resistant strain which would not revert from thymidine auxotroph (*thyA deoC deoA*) to thymine auxotroph (*thyA deoC deoA+*) with a high rate.

SKM8 was derived from SKM18. SKM18 is a thymine auxotrophic mutant subsequently found to have become a TLR. Such double mutants isolated by single step selection are not uncommon and have been isolated in *E. coli* (Okada, 1966). SKM8 was one of the larger colonies in a heterogeneous population that grew when SKM18 was spread on thymine 2 µg/ml. Whether SKM8 and the other larger colonies differed genetically from SKM18 is not known but growth of SKM8 on various test plates suggested it had a *thyA deoC* genotype. SKM8 was selected as a TLR for subsequent studies because it had been isolated from a thymine 2 µg/ml plate and had a lower thymine requirement than SKM18 (4 µg/ml thymine requirement).

SKM8 was plated on a minimal agar plate supplemented with thymidine 500 µg/ml and resistant colonies were isolated. About 50 of these colonies were tested on thymine and thymidine supplemented plates. Forty isolates grew

confluently on both plates. Nine isolates showed no growth on the thymine 20 µg/ml plate except for the presence of thymine revertant colonies. Only one isolate, designated SKM46, showed a complete absence of growth on the thymine plate. At the time, the complete absence of thymine assimilation suggested that SKM46 lacked a functional thymidine phosphorylase (later shown to be incorrect). It was therefore assumed to be of the genotype *thyA deoC deoA*, like the nine isolates described above but, unlike these isolates its thymidine auxotrophic character was thought to be unrevertable (ie. to *deoA+*). SKM46 and 4 of the 9 thymidine resistant strains with lowest reversion frequencies (as judged by simple spot test), were selected for further study.

The five potential *thyA deoC deoA* isolates were tested on a variety of plates, including sensitivity to deoxyuridine (Table 9). Except for SKM46, the remaining four isolates behaved in exactly the same manner, showing confluent growth on thymidine 20 µg/ml, revertants on thymine 20 µg/ml and resistance to deoxyuridine 500 µg/ml. This pattern of growth suggests that these strains may have reverted to *deoC+*. SKM46 showed no revertants on the thymine 20 µg/ml plate, grew much weaker than the other strains on thymidine 20 µg/ml and, was sensitive to deoxyuridine at 500 µg/ml. Sensitivity to deoxyuridine was assumed to be due to this deoxynucleoside being degraded to dRib-5-P by another phosphorylase, possibly specific for deoxyuridine.

Table 9. Plate Growth of Five SKM8 Thymidine Resistant Strains

Strain No.	MSM	T_{20} Revertant Number	/ TdR_{20}	TdR_{500}	UdR_{500} + TdR_{20}
SKM36	-	22	+++	+++	+++
SKM39	-	30	+++	+++	+++
SKM40	-	30	+++	+++	+++
SKM45	-	17	+++	+++	+++
SKM46	-	0	+/-	+++	-

+++ Confluent Growth   +/- Weak Growth   - No Growth

The possible existence of an enzyme capable of degrading deoxyuridine (in addition to thymidine phosphorylase and uridine phosphorylase) led to attempts to select a mutant in which this activity was blocked. This was hoped to be achieved by selecting a deoxyuridine resistant mutant of SKM46. The principle of selection was analogous to that of selecting a thymidine phosphorylase mutant.

From strain SKM46 colonies resistant to deoxyuridine were isolated in the presence of 500 µg/ml deoxyuridine plus thymidine (20 µg/ml) (represented in this study by the strain SKM47). SKM47 was subsequently tested for its ability to grow on thymidine and deoxyuridine as sole sources of carbon as an indication that deoxyuridine resistance had resulted in the complete inability of this strain to degrade pyrimidine deoxynucleosides. This however proved not to be case since SKM47 was found to be able to utilise both these deoxynucleosides as sole sources of carbon and energy (Table 10).

Further studies on SKM46 revealed that this strain, like SKM47, was capable of ultilising both thymidine and deoxyuridine as sole sources of carbon and energy (Table 10). It was also found to be capable of growth on medium supplemented with thymine 50 µg/ml or thymidine 50 µg/ml (Table 11). These additional findings suggested that SKM46 was not a *thyA deoA deoC* mutant as suggested by earlier findings and consequently, SKM47 could therefore no longer be regarded as a deoxyuridine resistant derivative of SKM46

(see section 4.4).

Another finding was that SKM46 was unable to utilise deoxyadenosine as a sole carbon and energy source. It was therefore thought to lack a functional purine nucleoside phosphorylase (*deoD*). However it was possible to select *deoD+* revertants from SKM46 using a concentrated culture spread on plates in which deoxyadenosine was present as the sole energy source. The *deoD+* colonies that were selected were subsequently found to grow in the absence of an exogenous source of thymidylate (thymidine or thymine) and were therefore regarded to be *thyA+ deoD+* revertants of SKM46 (represented by mutant strain SKM48).

Table 10. Utilisation of Alternative Carbon Sources by various B.helvolum Strains

Strain	Carbon Source (5mM)				
	TdR	UdR	AdR	UR	AR
SK1	+	++	+	+	+
SKM1	+/-	+	+	-	NT
SKM18	+/-	+/-	+/-	NT	+
SKM28	+/---	+/--	-	NT	NT
SKM50	+/---	+/-	+/-	-	NT
SKM8	+/-	+/-	+/-	NT	+
SKM46	+	+	-	NT	NT
SKM47	+	++	-	NT	NT
SKM48	+	+	+	NT	NT

++ Good Growth + Growth +/- Weak Growth

+/-- Very Weak Growth +/--- Scanty Growth

NT Not Tested

Table 11. Growth Characteristics of Important Strains of B. helvolum

Strain	MSM	T_2	T_{20}	T_{50}	TdR_{20}	TdR_{50}	TdR_{500}	AdR_{500} + TdR_{20}	UdR_{500} + TdR_{20}
SK1	+++	+++	+++	+++	+++	+++	+++	+++	+++
SKM1	+++	+++	+++	+++	+++	+++	+++	+++	+++
SKM18	-	+	++	+++	+++	++	+/-	+	-
SKM28	-	-	-	-	+++	+++	+++	NT	-
SKM50	+++	+++	+++	+++	+++	+++	+++	NT	+++
SKM8	-	++	+++	+++	+++	++	+/--	+	+/--
SKM46	-	-	-	+	+/-	++	+++	NT	-
SKM47	-	-	+/-	++	+++	+++	++	NT	+
SKM46 AdR	+++	NT	+++	NT	+++	NT	+++	NT	+++

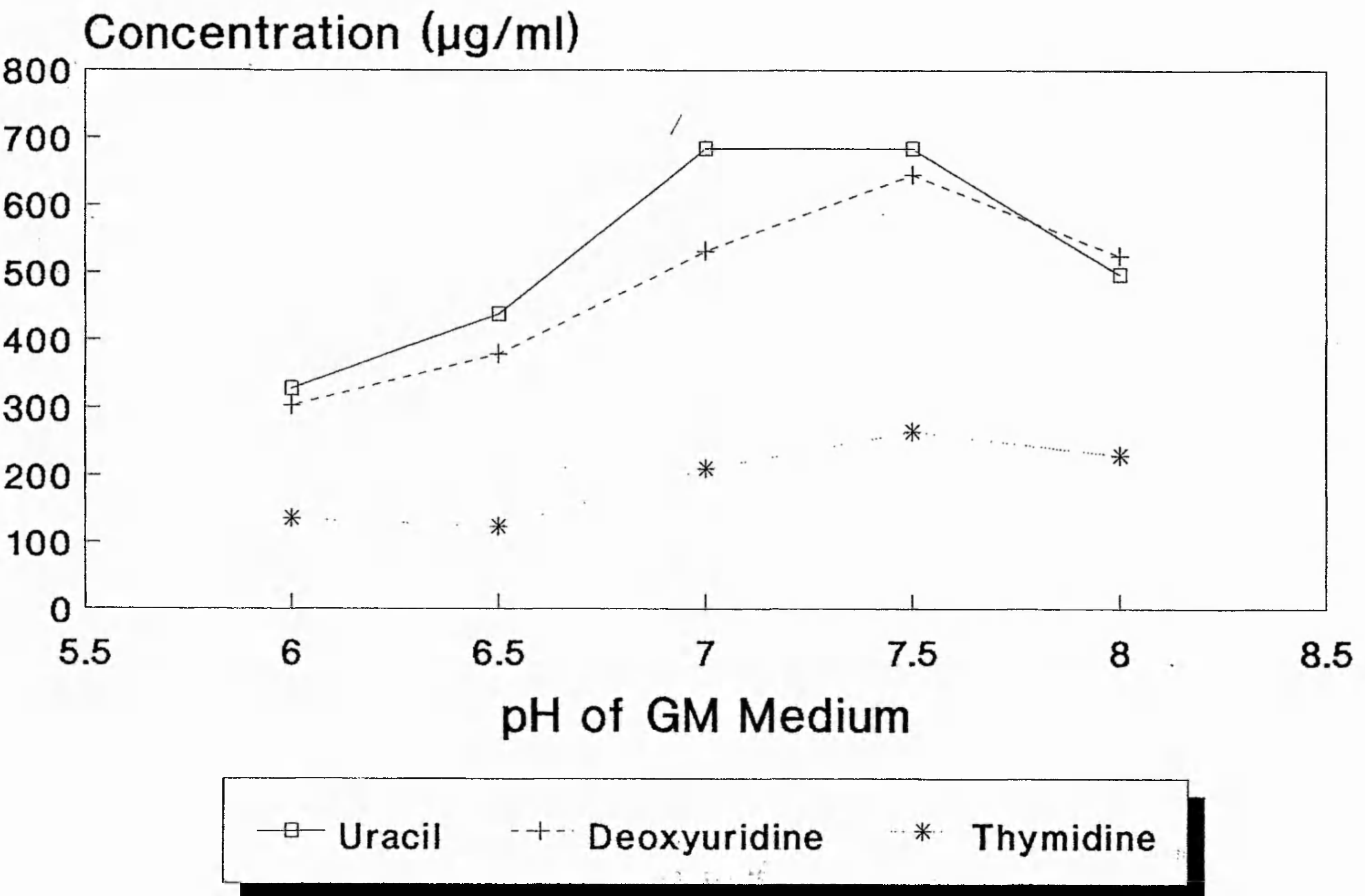
NT Not Tested +++ Confluent Growth + Growth +/-- V. Weak Growth - No Growth

3.6 The Effect of Starting pH of GM on Excretion of Pyrimidine Catabolites by SKM50

The pH of GM medium was normally adjusted to 7.0 prior to use in batch cultures. Studies on this medium by ICI, who developed the medium for the purpose of optimising the growth of B. helvolum cells and excretion of nucleosides, showed that during prolonged batch cultures (six days) the pH dropped significantly from 6.9 to 5.3 (ICI personal communication 1989). Due to the difficulty in maintaining the pH of batch cultures over such long periods a change in the starting pH was tested for its effect on the excretion of pyrimidine catabolites. The effect of starting pHs of 6.0, 6.5, 7.0, 7.5, and 8.0 on the excretion titres of thymidine, deoxyuridine and uracil from SKM50 were examined (Fig. 10).

In general it was found that a starting pH less than 7.0 had detrimental effects on the excretion yields, whereas a slightly alkaline starting pH (pH 7.5) was beneficial. Any further increases in alkalinity resulted in a decline in yields, but not as great as the equivalent move to acidity. Thymidine excretion increased by 26% benefiting most from a change of the starting pH from 7.0 to 7.5. Deoxyuridine excretion also increased by 21% at the same pH. The level of uracil excretion however did not benefit from the change in pH, remaining at the level observed for pH 7.0. As a result of these findings all subsequent batch cultures were set up using a starting pH of 7.5.

Fig. 10 Effect of pH on Excretion of Pyrimidine Catabolites



3.7 Pyrimidine Catabolite Excretion Studies using HPLC

Once the conditions for growing the cultures in GM medium and detecting nucleosides using the HPLC had been correctly established, it was possible to determine the levels of thymidine excreted by the B. helvolum mutant strains. In addition to thymidine, the levels of deoxyuridine (dUR), thymine and uracil excreted by various strains were examined. The amount of a particular pyrimidine metabolite eluted on the HPLC column was converted from peak area to concentration using a standard curve (Fig. 11) or, an internal standard.

HPLC studies were carried out on the strains SKM1, SKM18 (TLR derived from SKM1), SKM28 (thymidine auxotroph derived from SKM18) and SKM50 (thymidine overproducing strain derived from SKM28) (Fig. 12).

All the excretion data was obtained from samples taken from 72 hour batch cultures grown in GM with a starting pH of 7.5.

SKM1 was shown to overproduce uracil (386 µg/ml) and yield relatively high levels of deoxyuridine (158 µg/ml). These findings correlate with the possible overproduction of dUMP and its subsequent catabolism to deoxyuridine and uracil in SKM1. Thymidine and thymine were excreted in almost equal amounts (104 µg/ml and 100 µg/ml respectively), and were found to be about 30% less than the deoxyuridine yield.

These findings support the assumption that excretion of thymidine (and thymine) is also linked to an overproduction of dUMP in SKM1, particularly since the amount of thymidine excreted is comparable to the deoxyuridine excretion level.

In SKM18 (TLR) the level deoxyuridine excretion was found to be five-fold (776 µg/ml) that of SKM1, whereas the level of uracil excreted (380 µg/ml) was similar to the uracil level of SKM1. The absence of thymidine or thymine in SKM18 (and SKM28) is attributed to this strain being a *thyA* mutant and therefore unable to convert dUMP to dTMP, which is a precursor thymidine. This also accounts for the large increase in the excretion of deoxyuridine since the loss of thymidylate synthetase activity means all the dUMP is catabolised to deoxyuridine. The level of uracil (380 µg/ml) remained comparable to that of SKM1, indicating the deoxypyrimidine catabolising ability of this strain did not alter as a result of becoming a *thyA* mutant.

In the thymidine auxotroph, SKM28, deoxyuridine excretion was almost seven times (1071 µg/ml) the level found in SKM1 and, about 30% higher than that found for its parent strain SKM18. The amount of uracil (309 mg/ml) was about 20% lower than that produced by SKM18. The decrease in uracil excretion and increase in deoxyuridine excretion in SKM28 is assumed to be due to the loss of a functional thymidine phosphorylase. However, since the strain still is capable of degrading deoxyuridine, as indicated by the continued excretion of uracil, another enzyme is thought to be active

in deoxyuridine degradation.

The HPLC data on SKM50 showed thymidine excretion (226 µg/ml) to be double the level of this compound found in SKM1. The level of thymine (22 µg/ml) was about 20% of that found in SKM1, whereas the amount of uracil excreted (398 µg/ml) was slightly higher than the level found in SKM1. The reduction in the level of thymine excretion and increase in thymidine supports the proposed loss of thymidine phosphorylase activity in SKM50.

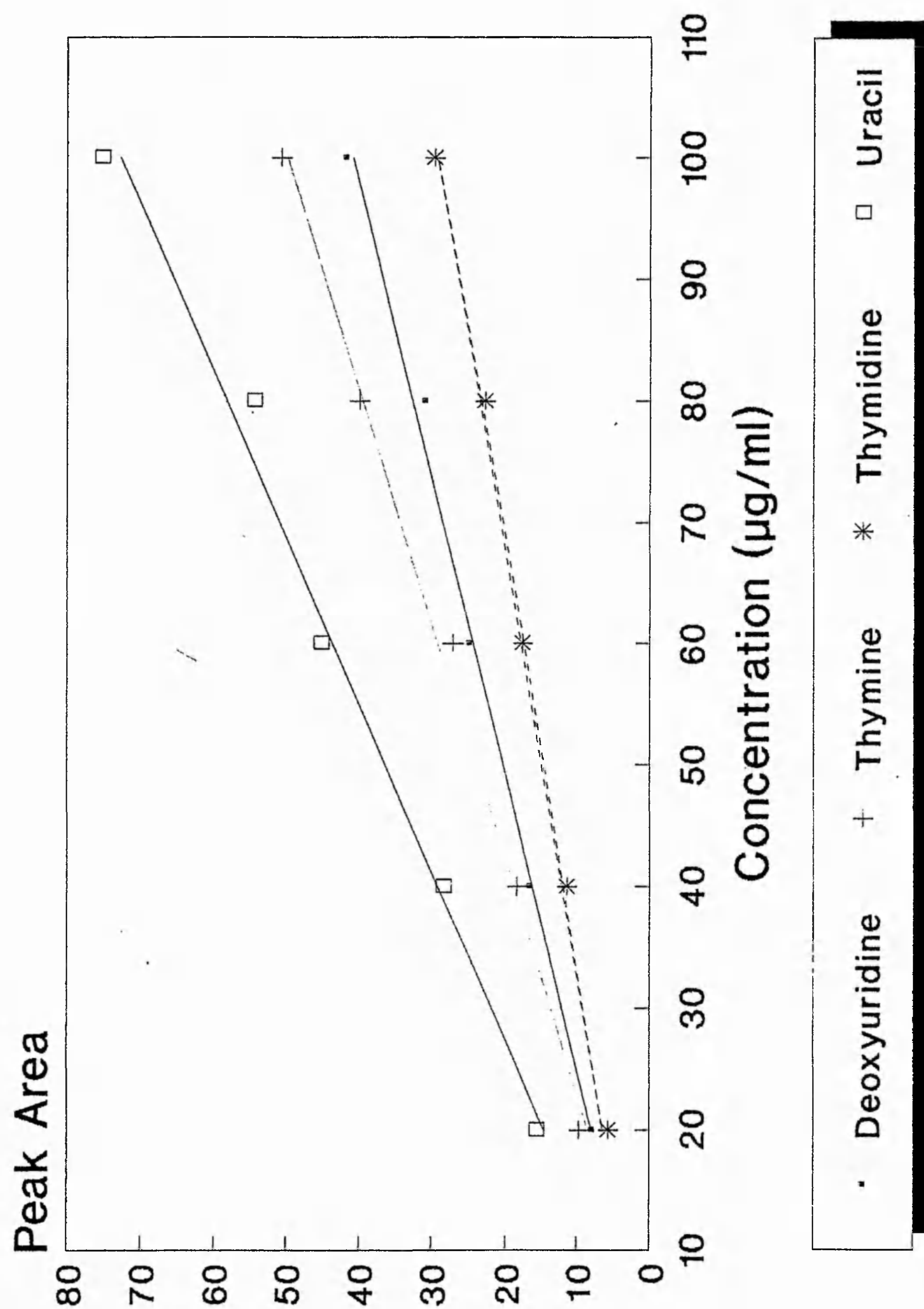
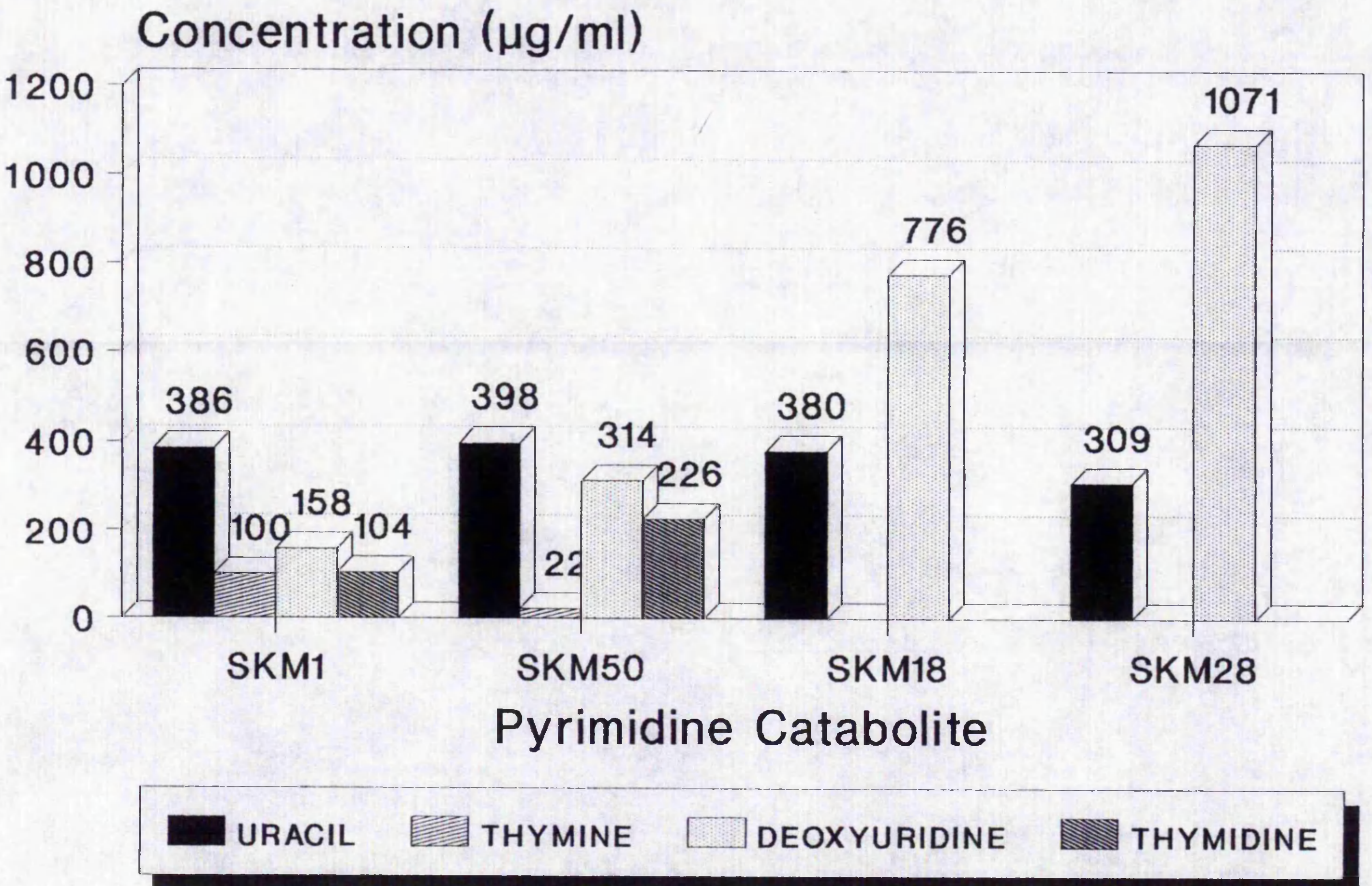


Fig. 11 HPLC Standard Curves

Fig. 12 Pyrimidine Catabolite Excretion from SKM1, SKM18, SKM28, and SKM50



3.8 <u>Comparative Study of the Thymidine and Deoxyuridine degrading ability of a number of Mutant Strains of *B. helvolum*</u>

The ability of various strains to breakdown thymidine and deoxyuridine was determined using an enzyme assay used on E. coli extracts to measure thymidine phosphorylase activity (Schwartz, 1971).

The thymidine degrading ability of SK1 was found to be ten fold less than that found in wild type E. coli (KL16) (Fig. 13 and Table 12). In addition, the mutant strain of B. helvolum, SKM1, was found to have a thymidine degrading ability five fold less than wild type E. coli (Fig. 14 and Table 12). One explanation may be that the two species of bacteria have thymidine phosphorylases of different specificity (possibly with differing affinity for their natural substrate, thymidine). Alternatively, this difference may be due to a lower level of thymidine phosphorylase synthesis in B. helvolum compared with E. coli.

SKM1 was found to have thymidine degrading ability (Fig. 16 and Table 13) higher than that found in wild type B. helvolum (SK1). This suggests that the level of thymidine phosphorylase in SKM1 may be above basal levels normally present in the wild type. Since no thymidine is excreted by the wild type and only negligible amounts of thymine are excreted (ICI personal communication), the increased

excretion of thymidine by SKM1 (Fig. 12) is unlikely to be the result of a reduced thymidine phosphorylase activity. The higher thymidine degrading ability of SKM1 does however support the proposed increase in dUMP production in this strain by virtue of a requirement for an increased pyrimidine deoxynucleoside activity to catabolise the increase in thymidine and deoxyuridine levels.

The breakdown of deoxyuridine in SKM1 (8.94×10^{-4} µmol uracil/min/550nm) (Table 14) was found to be higher than the value for thymidine degradation in SKM1 (7.03×10^{-4} µmol thymine/min/550nm) (Table 12). This correlates with earlier findings which suggested SKM1 was capable of utilising deoxyuridine more efficiently than thymidine when used as a sole source of carbon and energy (Table 10).

Thymidine breakdown was found to occur in SKM50 but at a much reduced rate compared with SKM1 (Fig. 16 and Table 13). The continued excretion of thymine by SKM50, albeit at reduced level, suggests thymidine degradation is not completely blocked. This could indicate that if a mutation has affected thymidine phosphorylase it has only hindered thymidine degradation and not removed it altogether. Alternatively, if is postulated thymidine phosphorylase activity has been completely lost through a mutation, the lower level of thymidine degradation could be attributed to the existence of another phosphorylase enzyme, possibly a deoxyuridine phosphorylase. This postulation appears to be supported by Fig. 18 in which SKM50's ability to degrade

deoxyuridine is not diminished when compared with SKM1, as found with thymidine degradation, but is infact shown to be more effecient than SKM1.

Thymidine degradation in SKM46 (SHTR) was found to be consistently higher than in SKM1 (Fig. 15 and Table 12). This correlated with the concept of SKM46 as SHTR, with a constitutive thymidine phosphorylase.

Deoxyuridine degradation in SKM46 was higher than in SKM1 (Fig. 17 and Table 14). This may be the effect of a constitutive thymidine phosphorylase increasing its contribution to the breakdown of deoxyuridine.

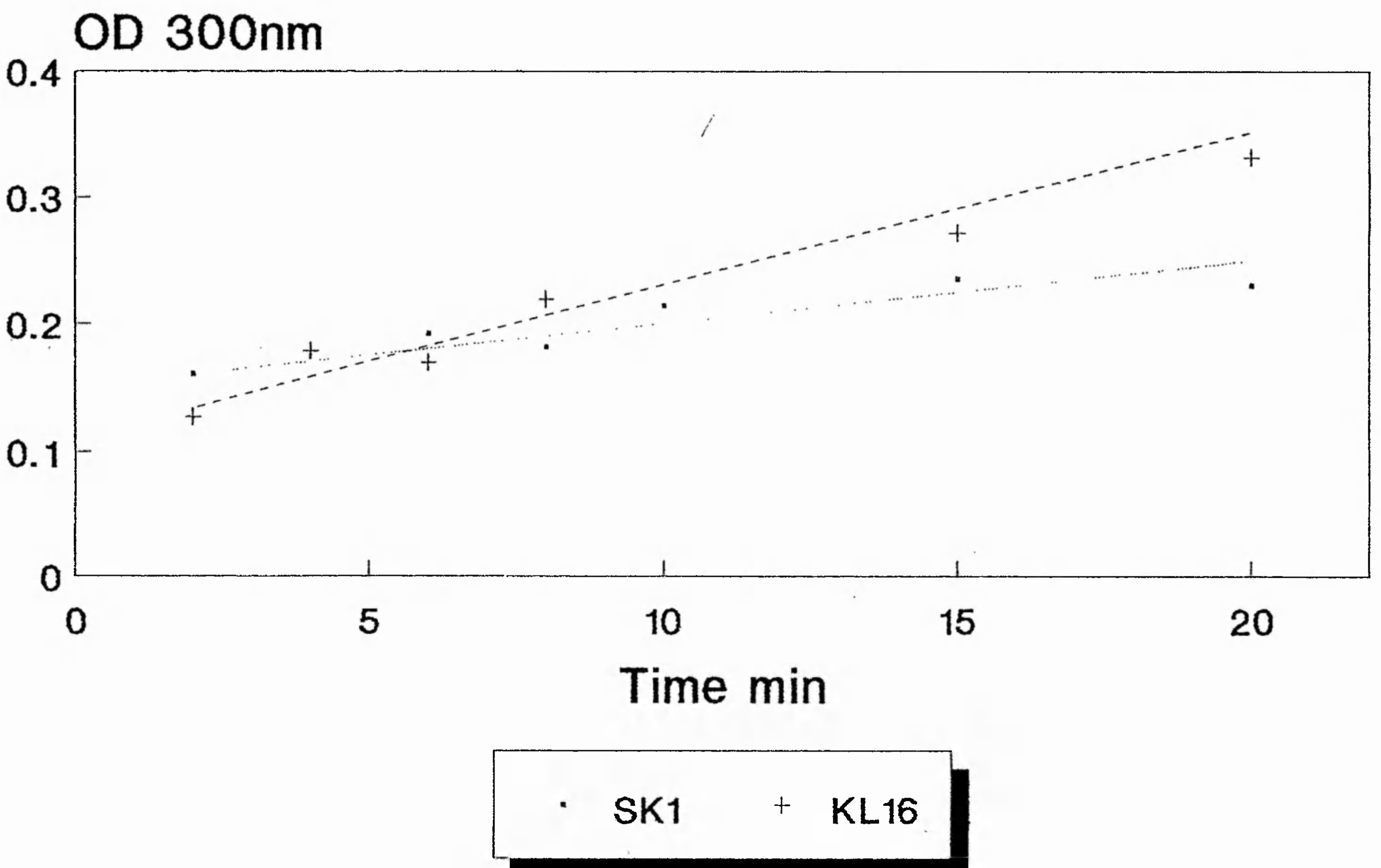
SKM47 had a lower thymidine degrading ability than SKM46 (Fig. 15 and Table 12). This supports the assumption that SKM47 is no longer constitutive for thymidine phosphorylase and has reverted to a thymine high requirer (THR).

Table 12. Thymidine degradation in *E. coli* and various Strains of *B. helvolum*

Strain	Gradient OD_{300}/min/ml	OD 550nm	TdR Degradation µmol T/min/550nm
SKM1	4.01×10^{-3}	1.41	8.36×10^{-4}
KL16	1.78×10^{-2}	1.39	3.77×10^{-3}
SKM1	3.29×10^{-3}	1.36	7.11×10^{-4}
SKM46	4.29×10^{-3}	0.76	1.65×10^{-3}
SKM47	2.73×10^{-3}	0.71	1.13×10^{-3}
SKM1	3.75×10^{-3}	1.57	7.03×10^{-4}
SKM46	2.14×10^{-3}	0.286	2.20×10^{-3}
SKM47	1.93×10^{-3}	0.366	1.24×10^{-3}
SKM1	1.69×10^{-3}	0.99	5.03×10^{-4}
SKM46	1.52×10^{-3}	0.53	8.41×10^{-4}
SKM47	2.22×10^{-3}	0.84	7.76×10^{-4}
SK1	5.88×10^{-3}	2.80	6.56×10^{-4}
Kl16	1.42×10^{-2}	0.695	6.41×10^{-3}

Gradients were calculated using MINITAB

Fig. 13 Thymidine degradation in SK1 and KL16



Thymidine Degradation (umol T/min/550nm)
SK1=6.56E-4 KL16=6.41E-3

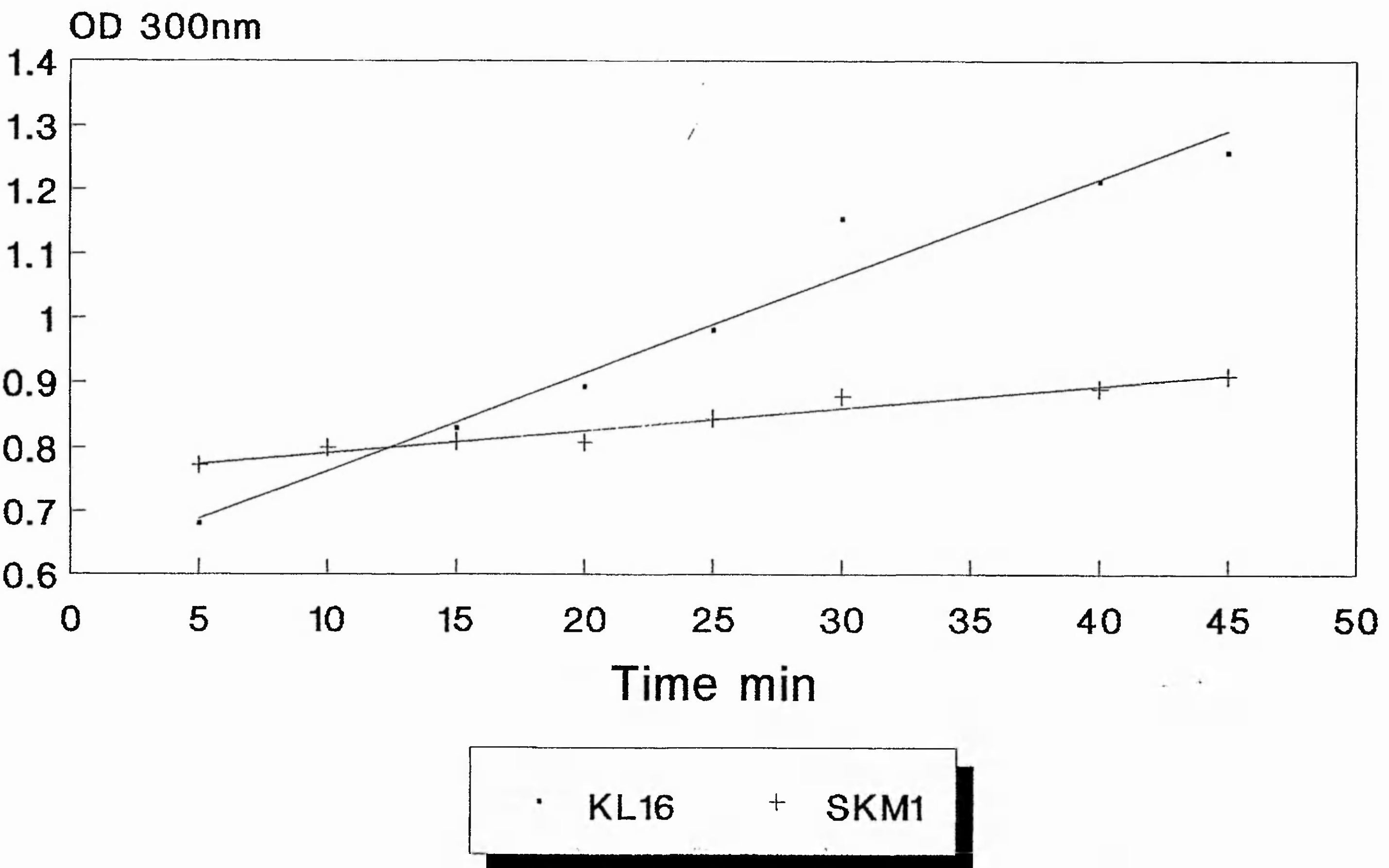
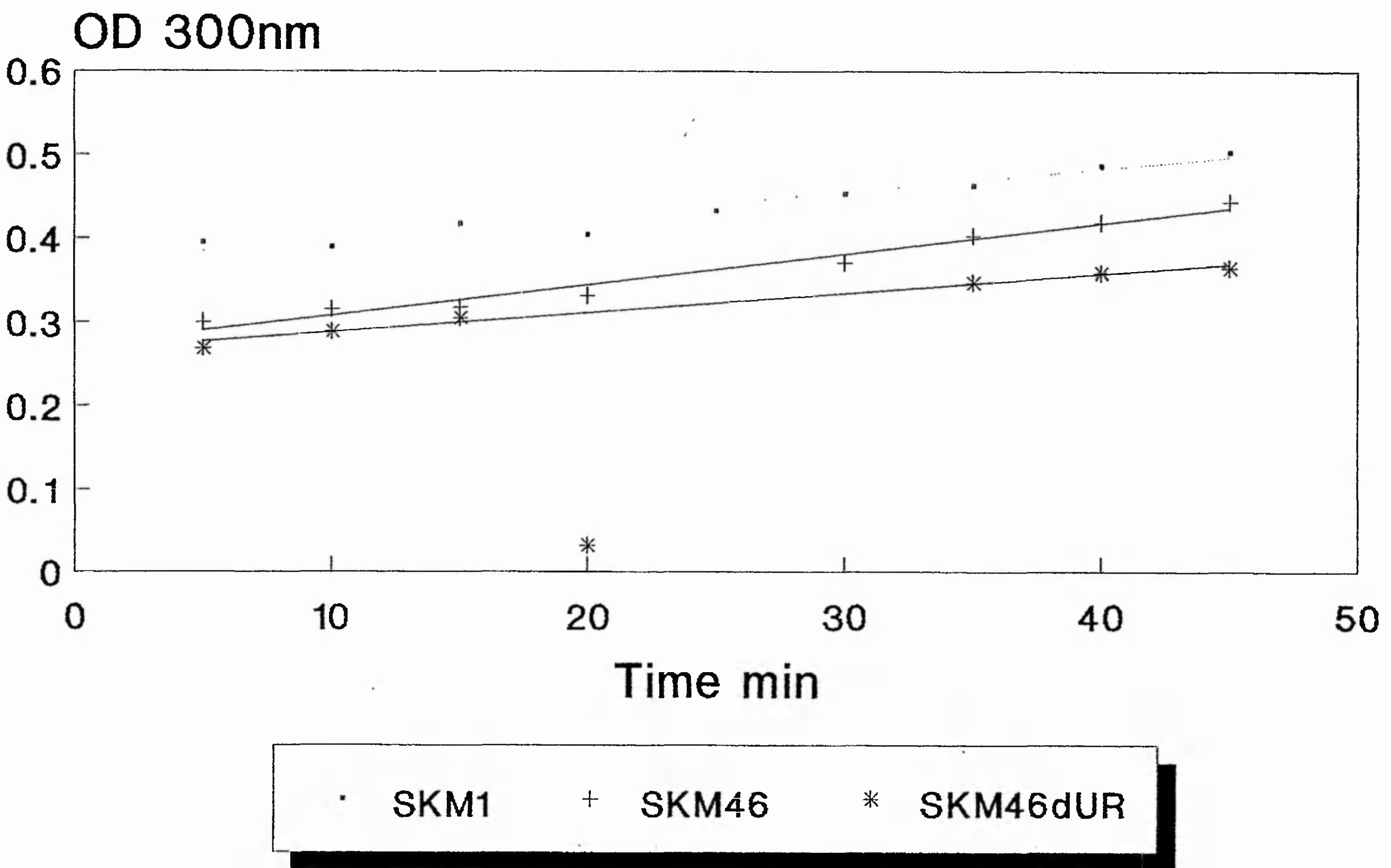


Fig. 14 Thymidine degradation in SKM1 and KL16
OD 300nm
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0
5
10
15
20
25
30
35
40
45
50
Time min
KL16
SKM1
Thymidine Degradation (umol T/min/550nm)
SKM1=8.36E-4 KL16=3.77E-3

Fig. 15 Thymidine degradation in SKM1 SKM46 and SKM47



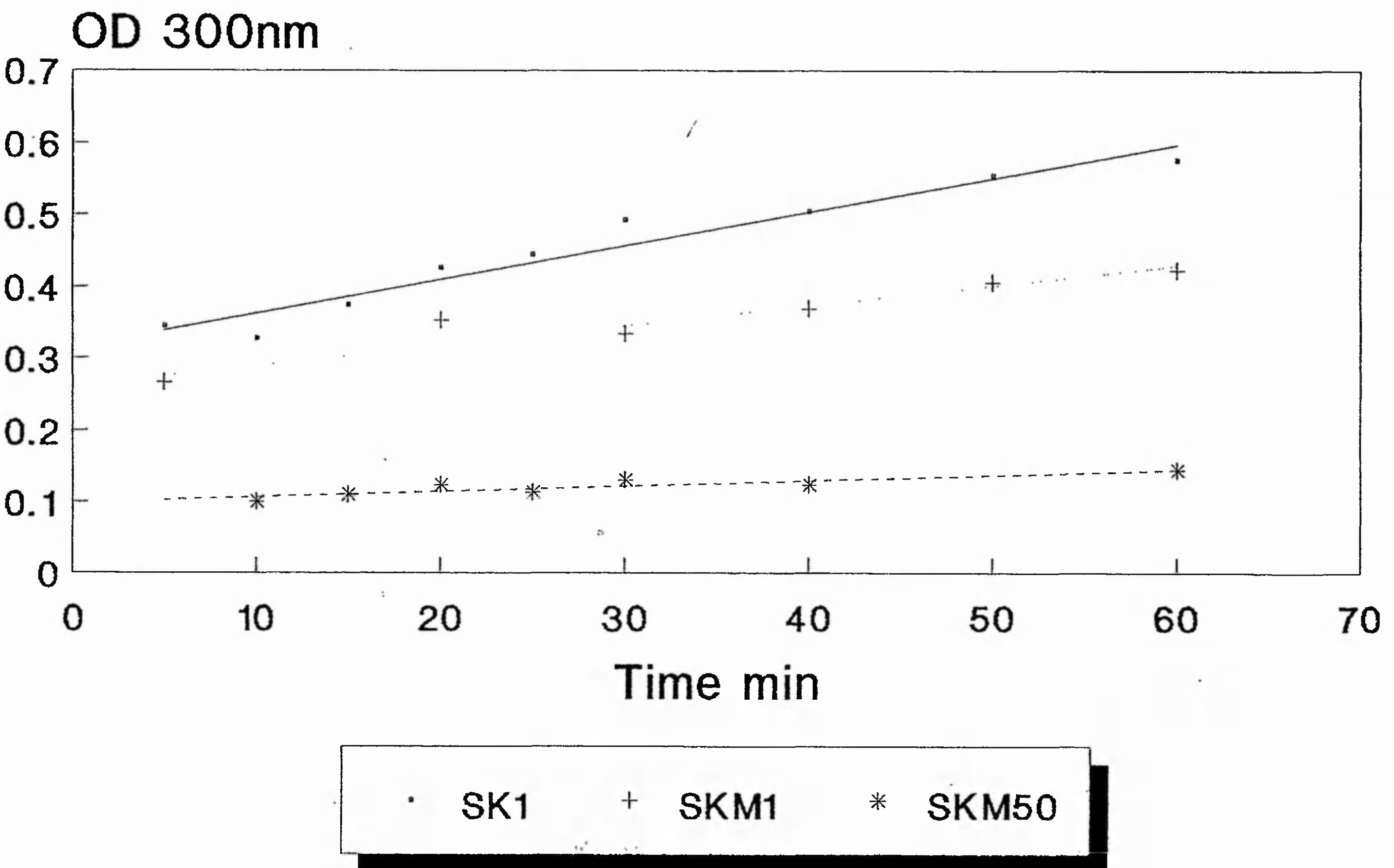
Thymidine Degradation (umol T/min/550nm)
SKM1=7.1E-4 SKM46=1.7E-3 SKM47=1.1E-3

Table 13. Thymidine degradation in SK1, SKM1 and SKM50

Strain	Gradient OD_{300}/min/ml	OD 550nm	TdR Degradation µmol T/min/550nm
SK1	5.51×10^{-3}	4.12	3.93×10^{-4}
SKM1	3.29×10^{-3}	2.18	4.44×10^{-4}
SKM50	9.09×10^{-4}	1.06	2.52×10^{-4}

Gradients were calculated using MINITAB

Fig. 16 Thymidine degradation in SK1 SKM1 and SKM50



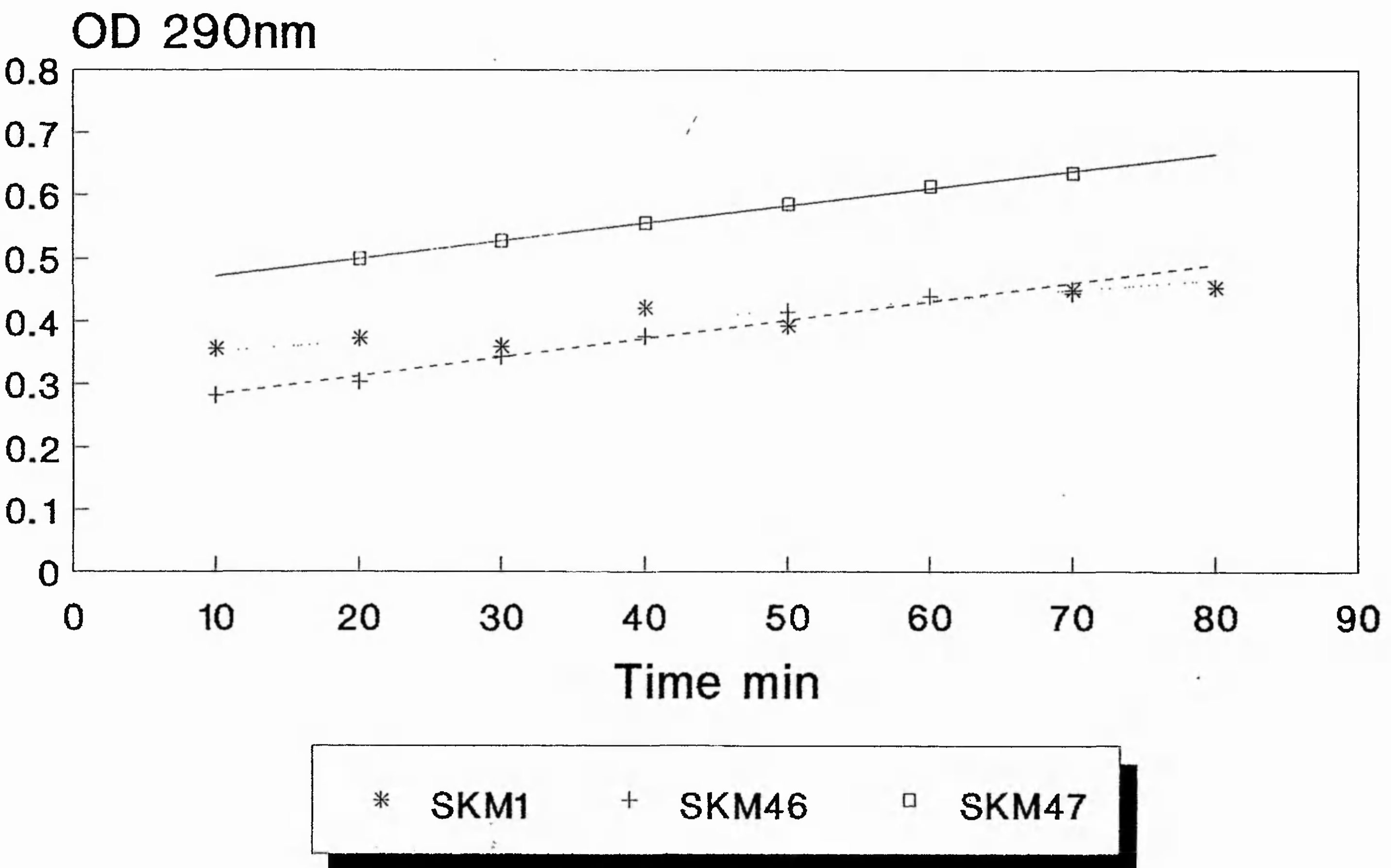
TdR Degradation (umol T/min/550nm)
SK1=3.9E-4 SKM1=4.4E-4 SKM50=2.5E-4

Table 14. Deoxyuridine degradation in various Strains of B. helvolum

Strain	Gradient OD_{290}/min/ml	OD 550nm	UdR Degradation µmol U/min/550nm
SKM1	7.58×10^{-3}	1.57	8.94×10^{-4}
SKM46	1.24×10^{-3}	0.286	8.00×10^{-4}
SKM47	4.69×10^{-3}	0.366	2.37×10^{-3}
SKM1	1.91×10^{-3}	0.99	3.57×10^{-4}
SKM46	3.49×10^{-3}	0.53	1.22×10^{-3}
SKM47	3.25×10^{-3}	0.84	7.19×10^{-4}
SKM1	3.00×10^{-3}	0.76	7.30×10^{-4}
SKM47	1.07×10^{-2}	1.30	1.52×10^{-3}
SKM50	2.04×10^{-3}	0.22	1.72×10^{-3}

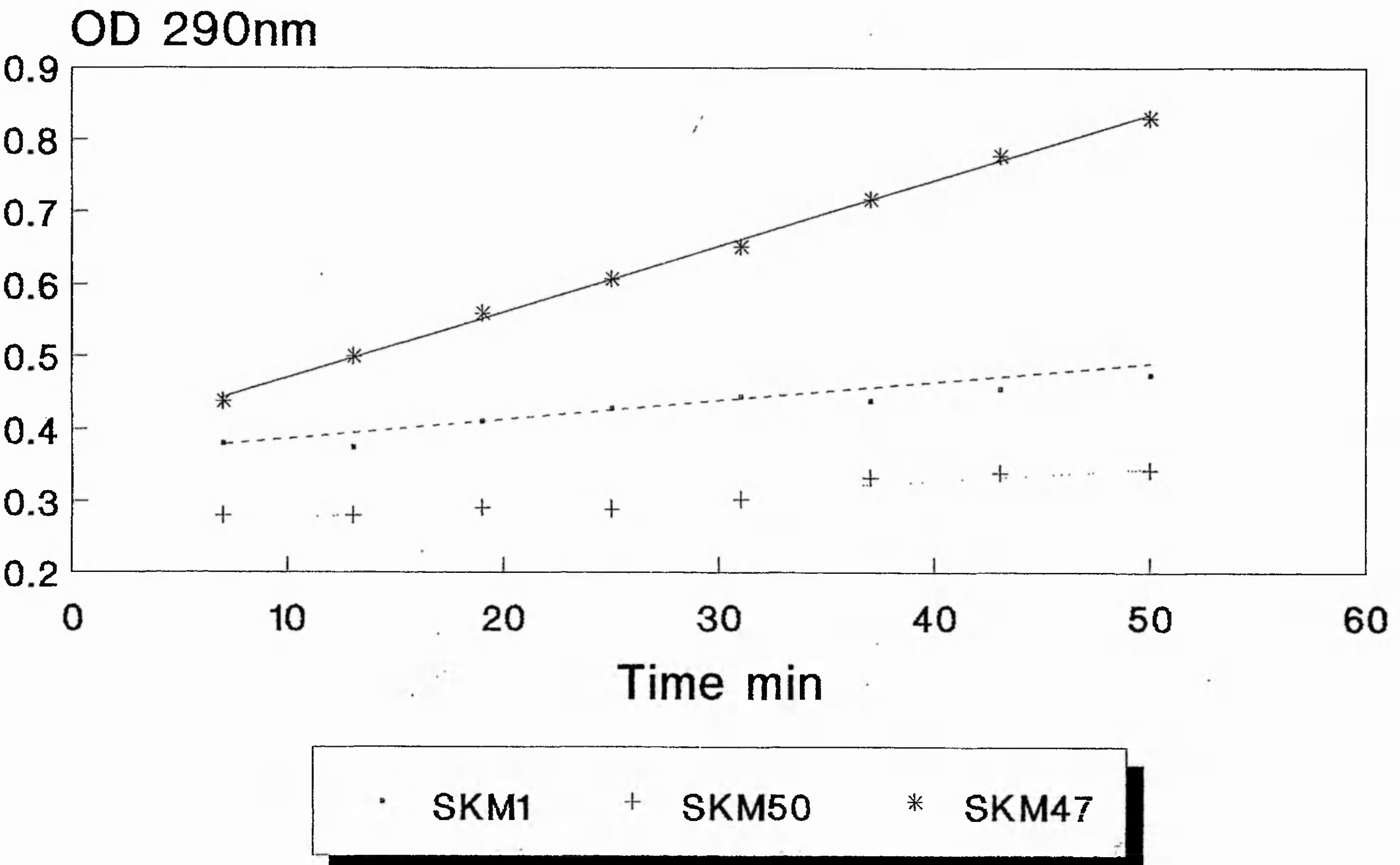
Gradients were calculated using MINITAB

Fig. 17 Deoxyuridine degradation in SKM1, SKM46 and SKM47



dUR Degradation (umol U/min/550nm)
SKM1=3.6E-4 SKM46=1.2E-3 SKM47=7.2E-3

Fig. 18 Deoxyuridine degradation in SKM1, SKM50 and SKM47



UdR degradation (umol U/min/550nm)
SKM1=7.3E-4 SKM50=1.7E-3 SKM47=1.52E-3

CHAPTER 4: DISCUSSION

DISCUSSION

4.1 Pyrimidine Metabolism in Wild Type *B. helvolum*

Work on wild type B. helvolum (SK1) contributed to the understanding of pyrimidine metabolic pathways in this bacterial species. Studies included growth on alternative carbon sources and pyrimidine analogue toxicity tests with various nucleoside and nucleobase analogues.

The ability of SK1 to utilise both purine and pyrimidine (deoxy)ribonucleosides as sole carbon and energy sources (Table 1) suggested the existence of a number of catabolic pathways, corresponding to those known to be present in E. coli (Fig. 2).

The growth of SK1 on cytidine indicated that B. helvolum is capable of deaminating cytidine and utilising it as a source of carbon. The breakdown of uridine, guanosine and adenosine as sole carbon and energy sources showed that organism is capable of catabolising ribonucleosides for energy purposes. In addition, the presence of enzymes involved in deoxynucleoside catabolism were implicated by the utilisation of thymidine, deoxyuridine, and deoxyadenosine as sources of carbon and energy. However, it was noted that the growth of B. helvolum appeared to be more prominent on deoxyuridine as a carbon source than on thymidine. This suggested the possibility that in B. helvolum deoxyuridine was being degraded by different

enzymes to those responsible for thymidine catabolism. Alternatively, it could be a result of differing substrate specificity of the same enzyme for the two deoxynucleosides. Indirect evidence exists to support the former possibility (see section 4.5).

SK1 was unable to utilise deoxyribose as a sole source of carbon and energy. This suggests that B. helvolum, like E. coli, but unlike Salmonella typhimurium, does not possess an ATP deoxyribokinase to convert deoxyribose to deoxyribose-5-P (O'Donovan, 1978). Alternatively, the inability to utilise deoxyribose may be the result of permease mutation preventing the entry of this pentose sugar into the cell.

Pyrimidine analogue studies indicated wild type B. helvolum (Tables 2 and 3) was sensitive to both 5-fluorouracil (FU) and 5-fluorodeoxyuridine (FUdR). Since analogue toxicity occurs at the nucleotide level these findings indicate the presence of enzymes in B. helvolum responsible for the conversion of FU and FUdR to their respective nucleotides. In E. coli, these are the salvage enzymes responsible for the assimilation of exogenous sources of pyrimidine nucleobases and (deoxy)nucleosides for the synthesis of pyrimidine nucleotides; UMP pyrophosphorylase, uridine phosphorylase, uridine kinase and UMP kinase in the synthesis of UDP and, thymidine phosphorylase and thymidine kinase in the synthesis of dUMP and dTMP respectively.

4.2 Characterisation of *B. helvolum* Mutant Strain SKM1

The ICI mutant (SKM1) used in this study was already capable of excreting significant levels of thymidine before the investigations began. However, the underlying genetic changes that resulted in this increased production of thymidine are unknown.

The growth pattern of SKM1 on various purine and pyrimidine (deoxy)nucleosides as sole sources of carbon and energy (Table 1) were similar to those of the wild type with one major exception; SKM1 was incapable of growing on uridine as a carbon source indicating a possible defect in one of the enzymes involved in uridine catabolism. Growth on adenosine as a carbon source indicated that SKM1 was capable of utilising ribonucleosides suggesting the enzymes involved in the catabolism of Rib-1-P were functional in this strain. This, therefore, suggested that the most likely site of a defect preventing utilisation of uridine as a sole source of carbon and energy was the first step in the catablic process, namely uridine phosphorylase (Fig. 2).

In an attempt to explain resistance of SKM1 to FUdR it is postulated that in SKM1 an overproduction of dUMP exists. This is supported by the HPLC results which show that SKM1 produces high levels of deoxyuridine and uracil (Fig. 12), which are both catabolites of dUMP degradation. The precise nature of how this occurred and what mutations were

involved is not known.

In *E. coli* FUdR is converted to its toxic form (FdUMP) by thymidine kinase. The analogue can also be degraded to FU by the action of thymidine phosphorylase. It has been proposed resistance to FUdR requires both thymidine kinase and thymidine phosphorylase to be absent, such that FUdR cannot be converted to the nucleotide level or be degraded to the nucleobase (FU), (O'Donovan and Neuhard 1970). Thymidine phosphorylase activity in SKM1 has been shown enzymically (Tables 12 and 13) and by SKM1's ability to utilise thymidine as a sole carbon source (Table 1). This suggests FUdR resistance cannot have arisen from an absence of this enzyme. Similarly, the isolation of thymine auxotrophs from SKM1 indicates the presence of a functional thymidine kinase in this mutant strain. However if FUdR was to be degraded to FU by thymidine phosphorylase in SKM1, it is unlikely that it would cause analogue toxicity at the ribonucleotide level because of the suggested block on the subsequent conversion of FU to FUMP (see FU resistance later in this section). Thus, FUdR resistance appears to be consistent with an overproduction of dUMP and its subsequent catabolism in SKM1 whereby such resistance could occur by means of substrate competition, preventing the conversion of FUdR to the nucleotide level.

Out of the possible routes for FU incorporation; via uridine phosphorylase, UMP pyrophosphorylase and via thymidine (deoxyuridine) phosphorylase, it is likely that

the first two pathways are blocked in SKM1. With a functional thymidine phosphorylase and an adequate supply of dRib-5-P available (from dUMP catabolism) the third route should still be operative and the cells should not become completely resistant to FU. However, since SKM1 is highly resistant to FU, the most likely explanation for resistance along this third route, is preventing the conversion of the analogue to FdUMP by substrate competition (as with FUdR resistance) at the level of thymidine phosphorylase and the enzyme responsible for the conversion of FUdR to FUMP in B. helvolum.

Thus it appears that very high resistance to FU in SKM1 may be the result of three contributing factors; the absence of a functional uridine phophorylase, a defective UMP pyrophorphylase and, substrate competition as a result of dUMP overproduction and its subsequent catabolism to deoxyuridine and uracil.

These findings suggest SKM1 (probable genotype of *upp udp*) is another example of a mutant in which very high resistance to FU has been achieved by a combination of resistance factors. Those mutants already known to exist include for example, an FU resistant mutant of the genotype *upp deoA udk* isolated by Brockman and his co-workers in 1968, while Pritchard and Ahmad (1970) isolated a mutant of the genotype *upp deoA udp* which was also found to have a high resistance to FU.

Having established that the most likely route by which a uracil analogue could cause toxicity in SKM1 is by overcoming the resistance posed by substrate competition, it is difficult to find an explanation for the apparent 2-thiouracil toxicity observed in SKM1 (and SKM50) (Tables 4,5 and 6). The situation is further complicated by taking into consideration thymidine phosphorylase's specificity for the 2- and 4-position oxo-groups of uracil analogues. Since 2-thiouracil is a 2-position substituted uracil analogue it has been shown to bind poorly to this enzyme (Niedzwicki *et al*, 1983). In comparison, 5-fluorouracil consists of an electron withdrawing group substituted at the 5-position which is believed to enhance the binding (two-fold better than uracil) of this analogue to thymidine phosphorylase (Niedzwicki *et al*, 1983). On this basis, fluorouracil would have been expected to be more likely to cause analogue toxicity in SKM1 than 2-thiouracil. Thus, the fact that SKM1 is highly resistant to 5-fluorouracil, further questions 2-thiouracil sensitivity in this strain. A possible explanation to this inconsistency may lie in the fact that the sensitivity to thiouracil occurred only at very high concentrations of the analogue, and when it did occur, it was found to be short-lived. However, further studies to determine the mechanism of 2-thiouracil resistance in B. helvolum are needed before any proposals can be made as to why such sensitivity exists in this mutant strain.

4.3 Mutants involved in the Isolation of the Thymidine Overproducing Strain of *B. helvolum*

SKM50 is the thymidine overproducing strain of B. helvolum isolated from SKM1 by a process of genetic manipulation and selection. The mutants involved in this isolation process showed distinctive characteristics, some of which are described below.

SKM1 is thought to be a dUMP overproducer. It is thought to be defective in UMP pyrophosphorylase and uridine phosphorylase activities (*upp udp*). It is clearly able to excrete thymidine and deoxyuridine into the surrounding growth medium (Fig. 12). However, because both these deoxynucleosides are still being degraded in this strain, as indicated by the presence of thymine and uracil excretion, further manipulations were necessary to block thymidine phosphorylase activity.

SKM18 is the thymine low requiring strain isolated from SKM1. It appears to have undergone mutations in *thyA* and *deoC* genes. The TLR character of this strain is consistent with its sensitivity to high levels of thymidine and deoxyuridine (Table 11) and its ability to grow on thymine (4 µg/ml) at a low concentration (Table 7) as a source of thymidylate. However, weak growth of SKM18 on thymidine and deoxyuridine as sole sources of carbon indicated the *deoC* mutation was leaky. HPLC studies show this strain to be capable of excreting five times more deoxyuridine than

SKM1. The increase in the excretion of this deoxynucleoside is thought to be linked to the loss of thymidylate synthetase.

SKM28 is the thymidine auxotroph isolated from SKM18 by selecting for thymidine resistance. Its inability to assimilate thymine as a source of thymidylate (Table 13) and the almost complete absence of growth on thymidine and deoxyuridine as sole sources of carbon (Table 10) suggested it possessed a *thyA deoC deoB* genotype. HPLC studies show that the excretion level of deoxyuridine is increased in SKM28 whereas the level of uracil decreased compared with the levels found in SKM18 (Fig. 12). These findings indicated the loss of some deoxynucleoside catabolising activity in SKM28. This was ascribed to the loss of a functional thymidine phosphorylase. The level of deoxyuridine excretion was found to be almost seven times greater than the level found in SKM1. It is postulated that such high level of deoxyuridine would not have been attained had there not been an overproduction of dUMP occuring in this strain. Thus, it is assumed that dUMP overproduction and the loss of thymidylate synthetase were prerequisite to the high level of deoxyuridine excretion in SKM28. The subsequent loss of thymidine phosphorylase only served to augment the existing conditions for deoxyuridine excretion by reducing its catabolism.

SKM50 was isolated by reverting SKM28 to *thyA+*. It should have retained the *deoC deoA* mutations present in SKM28 and

therefore be unable to degrade thymidine and utilise it as a sole source of carbon and energy. However, such studies indicate that SKM50 is capable of weak growth on thymidine and deoxyuridine as sole sources of carbon (Table 10). This is supported by enzyme studies that suggest the thymidine degrading ability of SKM50 is significantly reduced compared with SKM1 but not completely absent (Table 13). In contrast, SKM50's ability to degrade deoxyuridine is found to be higher compared with SKM1 (Table 14 annd Fig 18). Thus, the continued ability of SKM50 to catabolise pyrimidine deoxynucleosides (particularly deoxyuridine) in the assumed absence of thymidine phosphorylase and uridine phosphorylase led to proposal that another deoxypyrimidine phosphorylase existed in B. helvolum that is not known to occur in E. coli (see section 4.5).

The existence of another phosphorylase in B. helvolum may also explain why it has not been possible to reach the commercial target of isolating a mutant capable of excreting thymidine atleast three to five times the level found in SKM1.

4.4 Super High Thymine Requirement in *B. helvolum* and Reversion to Thymine High Requirement

SKM46 is a SHTR strain of B. helvolum that was isolated by chance during the course of investigations to isolate the thymidine overproducing strain. It had originally been assumed to be a thymidine auxotroph (*thyA deoC deoA*) similar to SKM18, with the exception that it did not produce any *deoA*+ revertant colonies, when grown on agar plates supplemented with thymine 20 µg/ml. However subsequent studies, including ability to grow on thymidine and deoxyuridine as sole carbon sources and, growth on plates supplemented with thymine 50 µg/ml or thymidine 50 µg/ml, revealed this was not the case. Instead these growth characteristics were found to be more akin to a SHTR strain of E. coli. Thus it was concluded that SKM46 had probably undergone regulatory gene mutation, leading to constitutivity in the *deo* enzymes thymidine phosphorylase, deoxyribomutase and deoxyriboaldolase *ie*. it had become a *thyA deoD deoR* mutant.

The earlier difficulty in recognising SKM46 as a SHTR stemmed from attempting to find an explanation for its apparent sensitivity to deoxyuridine at high concentrations, and the subsequent isolation of 'deoxyuridine resistant' mutants (SKM47). As a SHTR strain, SKM46 should be capable of degrading deoxyuridine. Therefore, the most likely explanation for SKM46 being unable to grow on deoxyuridine 500 µg/ml is an

insufficiency in the amount of thymidine provided, rather than due to a toxic accumulation of 5-dRib-P. Retrospectively, the provision of thymidine 20 µg/ml as a source of thymidylate may have been limiting in the light of SKM46 being a SHTR.

In a SHTR the threshold requirements for both thymine and thymidine are expected to be increased because of competition between a constitutive thymidine phosphorylase and the activity of thymidine kinase for the available exogenous thymidine and, because of the limited supply of endogenous deoxyribsoyl groups due to constitutive deoxyribomutase and deoxyriboaldolase. These circumstances are consistent with SKM46 being able to grow better on plates supplemented with thymine 50 µg/ml and thymidine 50 µg/ml, but unable to grow on thymine 20 µg/ml and only weakly on thymidine 20 µg/ml.

Based on the theory above the most likely explanation for the subsequent isolation SKM47 from the deoxyuridine 500 µg/ml plate, is that SKM47 has lost the mutation (*deoR*) responsible for consitutivity in SKM46 and reverted to a thymine high requirer(THR). As a THR (*thyA*), SKM47 was able to grow in the presence of deoxyuridine 500 µg/ml because the available thymidine (20 µg/ml) was sufficient to meet its requirements for a exogenous source of dTMP. There is some evidence to support SKM47 as a THR revertant in the enzyme studies (Table 14) which indicate a reduction in the thymidine degrading ability of SKM47 compared with SKM46.

The growth of SKM47 on thymine 20 µg/ml also appears to support the idea that this strain has lost the constitutive element for the enzymes thymidine phosphorylase, deoxyribomutase and deoxyriboaldolase (Table 13). This is because the competition between thymidine phosphorylase and deoxyribomutase for dRib-5-P is likely to be reduced, providing SKM47 with the necessary supply of dRib-5-P for the assimilation of the thymine available.

Although the growth characteristics of SKM47 described above suggest that it is likely to be a thymine high requiring revertant of SKM46, this proposal does not explain why SKM47 is able to degrade deoxyuridine more efficiently than SKM47 and SKM1, as suggested by enzyme studies (Table 16) and supported by the growth of SKM47 on deoxyuridine as a sole source of carbon and energy (Table 12). The reason for this disparity is not known and requires further enzymic studies before it can be resolved.

Another type of mutant derived from SKM46 were the SKM46 *deoD+* strains. These were SKM46 colonies that were capable of growing on deoxyadenosine as a sole source of carbon, without a source of thymidylate present (*ie* no thymidine added to the growth medium). The isolation procedure was analogous to the way *thyA* strains are converted to *thyA+* (isolating colonies capable of growth on thymine free plates), except in this case deoxyadenosine was used in place of glucose as a source of carbon. Thus, these isolates were considered to be *thyA+ deoD+* revertants of

SKM46. They are assumed to have retained the constitutive element for the enzymes thymidine phosphorylase deoxyribomutase and deoxyriboaldolase since their ability to utilise thymidine as a sole source of carbon is similar level to SKM46 but more efficient compared with SKM1 (Tables 12 and 13).

4.5 Evidence for the Existence of a Putative Deoxyuridine Phosphorylase in *B. helvolum*

The following findings suggest the presence of an enzyme in B. helvolum which is not known to occur in E. coli. This enzyme is thought to be a putative deoxyuridine phosphorylase that is responsible for catabolising deoxyuridine, and to a lesser extent thymidine, in this bacterial species.

The growth of SKM18, SKM28, and SKM50 on thymidine and deoxyuridine as sole carbon sources suggest that B. helvolum is able to discriminate between these two deoxynucleosides as sole sources of carbon and energy (Table 12). Deoxyuridine appears to be a utilised more efficiently as a carbon source than thymidine. This had previously shown to be the case in SK1 and SKM1. This difference in ability to degrade these two deoxynucleosides was most apparent in SKM50, and least in the wild type, SK1.

If it is considered just one enzyme is responsible for the degradation of pyrimidine deoxyribonucleosides in B. helvolum and that this enzyme is thymidine phosphorylase, (as in E. coli) one may expect substrate specificity to ensure thymidine is utilised more efficiently than deoxyuridine since hydrophobic groups such as the methyl group of thymidine enhance substrate binding to thymidine phosphorylase (Niedzwicki *et al*, 1983). Instead, the

opposite was found to be the case. This suggests the possibility of separate pyrimidine phosphorylases to catabolise the two deoxynucleosides.

The resistance of SKM28 to thymidine 500 µg/ml but sensitivity to deoxyuridine 500 µg/ml (Table 13), strongly suggests there must be another route via which deoxyuridine is degraded, particularly since this strain is thought to be of the genotype *thyA deoC deoA*.

The HPLC data on SKM50 (Fig. 12) show that thymidine and deoxyuridine excretion (226 µg/ml and 314 µg/ml respectively) was double the level of these deoxynucleosides found in SKM1. The level of thymine (22 µg/ml) was about 20% of that found in SKM1, whereas the amount of uracil excreted (398 µg/ml) was slightly higher than that excreted by SKM1. The reduction in the level of thymine excretion lends further weight to the assumption that thymidine phosphorylase activity is inactive. Consequently, the small amount of thymine excreted could be attributed to the activity of a putative deoxyuridine phosphorylase.

The continued excretion of uracil in SKM28 and SKM50, in the assumed absence of thymidine phosphorylase also supports the existence of a deoxyuridine phosphorylase, particularly since the other enzyme capable of degrading deoxyuridine, uridine phosphorylase, is thought to be absent in these strains. In addition, the fall in the level

of uracil excreted, and the corresponding increase in the excretion of deoxyuridine in SKM28 compared with SKM18 (Fig. 12), suggests that some deoxynucleoside degrading activity has been lost in SKM28, presumably that of thymidine phosphorylase. Therefore, the most likely origin of the uracil produced by SKM28 is from deoxyuridine catabolism via a putative deoxyuridine phosphorylase. These findings provide indirect evidence for thymidine phosphorylase being involved in the degradation of deoxyuridine, but also imply that it is unlikley to be the sole enzyme responsible for the breakdown of deoxyuridine in B. helvolum.

Despite an increase in the level of uracil in SKM50, the level deoxyuridine (314 µg/ml) excreted was almost double that found in SKM1. Assuming the loss of thymidine phosphorylase is the cause of the increase in deoxyuridine excreted by SKM50, this suggests thymidine phosphorylase contributes significantly to deoxyuridine catabolism in B. helvolum.

It is necessary to point out that the existence of deoxyuridine phosphorylase has been based on the HPLC studies and growth tests only. Confirmation is required by isolation and purification of this enzyme followed by enzymic studies.

4.6 Thymidine and Deoxyuridine Degradation

Thymidine and deoxyuridine degrading ability of different mutant strians of *B. helvolum* was measured using an enzyme assay used to determine thymidine phosphorylase activity in *E. coli* (Schwartz 1971). Since the aim was to obtain a mutant lacking the ability to degrade thymidine (and deoxyuridine) the assay was hoped to have provided an 'all or nothing' response when a particular strain was tested. However, it became apparent that the strain (SKM50) which was thought to have lost the ability to degrade thymidine as a result of the manipulation programme, was still capable of catabolising thymidine (Table 13). This introduced the possibility of the existence of another pyrimidine phosphorylase, perhaps specific for deoxyuridine (see section 4.5), being present in *B. helvolum*. Consequently the enzyme assay could no longer be regarded as a simple qualitative means of establishing the loss of a particular enzyme, namely thymidine phosphorylase.

If it is proposed that two pyrimidine deoxynucleotide degrading enzymes exist in *B. helvolum*, it would be difficult to detect the contribution made by either enzyme in degrading a particular substrate using this assaying procedure unless each enzyme is assayed separately. Consequently, without using an isolated, purifed enzyme extract for assaying, the possibility of cross-reactivity between two such enzymes for a substrate exists. However, an activity could be attributed to a single enzyme if the

strain being assayed is known to have mutation in a gene transcribing one of the enzymes, leading to complete loss of activity. Thus, it could be proposed that if thymidine phosphorylase activity (and uridine phosphorylase activity) has been completely lost in SKM50, any thymidine and deoxyuridine degradation occuring in this strain should be contributed solely to the activity of a second deoxypyrimidine phosphorylase.

Another complication was encountered during the preparation of the cell extract for the enzyme assay. Ideally the culture should have been completely broken up by sonication before assaying. As a Gram positive organism B. helvolum required a longer sonication exposure time than for example a Gram negative organism such as E. coli. In addition, since the level of cell disruption was judged by visual means, the number of cells disrupted by this treatment may not have been 100%. This further highlights the qualitative nature of the assay employed, and the necessity that should a more quantitive approach be adopted, the sonicated extract be centrifuged prior to carrying out the enzyme assay and the soluble protein level of the cell free extract be determined.

4.7 Reverse Phase HPLC and the Cross Feeding Test for Thymidine Detection

Both reverse phase HPLC and the cross feeding test were used in this study to detect thymidine excretion by various mutant strains of B. helvolum.

The cross feeding test using a thymidine auxotroph of E. coli is a relatively simple test. It involves excretion of thymidine by a test organism into the surrounding soft agar growth medium and growth of the thymidine auxotrophic E. coli. The advantages of this test are that it is easy to set up; a large number of samples can be tested at any one time and it is simple to interpret. The disadvantages are that thymidine excretion cannot be detected in a direct manner but relies on the growth of another organism. Another disadvantage is that 24-48 hours incubation time is necessary before the results can be read. In addition, the diameter of the ring growth of the E. coli culture gives only an approxiamate evaluation of the level of thymidine present in a given sample since this estimation is complicated by the density of growth present. Due to these factors the cross feeding test was used only as a screening test for potential thymidine excreting strains.

Reverse phase HPLC has a stationary phase consisting of alkylsilyl (C_{18}) groups bonded to silica to give a non-polar, hydrophic surface. The mobile phase is polar, consisting of water mixed with methanol, so solutes are

eluted in order of decreasing polarity. This is because solute retention is mainly due to hydrophobic interactions between the solutes and the hydrocarbonaceous stationary phase. Reverse phase HPLC has several advantages over the cross feeding test. Firstly, it is capable of detecting pyrimidine compounds other than thymidine *eg.* deoxyuridine, uracil or thymine. In addition, these compounds can be detected simultaneously in a single sample run. Another advantage is that HPLC can analyse and provide the results within minutes of injecting a sample into the system. As results are presented in the form of a chromatogram with peak areas already calculated by a computer integration package, operator error is minimized.

Although there were many benefits to using HPLC for the detection of pyrimidine compounds the technique is not simple. For example, each programme had to be tailored to meet the requirements for the compounds investigated. Problems were encountered with regards to separating the various components in the samples obtained from the bacterium cultures grown in GM medium. Consequently, two HPLC programmes were developed for the detection of the pyrimidine compounds analysed. The DNA1 programme had a relatively short run time and was able to separate thymidine and uracil with good resolution, but was unable to separate the deoxyuridine peak from the thymine peak. Therefore another programme, DNA2, was developed to separate thymine from deoxyuridine, in this case the thymidine peak (which is the last to be eluted) was removed

from the column using a methanol gradient to avoid a run time that was too long (1 hour). Despite these problems the HPLC's effeciency more than compensated for any inconveniences encountered during the investigations. Thus, for accurate analysis of pyrimidine catabolic excretion by a particular strain reverse phase HPLC was preferred as the method of analysis.

4.8 Future Investigations

In this study the pyrimidine metabolic pathway of E. coli was used as a template for the genetic and physiological manipulation of this pathway in B. helvolum. As a result of the findings obtained it is clear that there are many similarities in pyrimidine metabolism in the two organisms. However, there also appear to be exist certain contrasts between the two organisms for example, they may differ in the way certain pyrimidines are catabolised. This may be one of the reasons why the project was unable to meet the commercial target of isolating a mutant strain capable of excreting at least a further 3-5 fold increase in thymidine excretion compared with the ICI mutant SKM1. It is clear more work is necessary to understand the enzymes of this pathway in B. helvolum before any differences which may exist to E. coli pyrimidine metabolism can be confirmed and, before the full potential of the mutants isolated in this study can be realised.

Areas of study that could be exploited include:

1. Further studies to confirm the assumed overproduction of dUMP in SKM1 and to locate any possible mutations that have led to this condition. This would involve determining the cellur levels of dUMP in SKM1 and measuring the enzyme activities of thymidylate synthetase and dUTP pyrophosphatase.

2. Further genetic, pyrimidine analogue, and enzymic studies to confirm that uridine phosphorylase and UMP pyrophosphorylase are defective in SKM1.

3. Further improving the yield of thymidine from SKM50 by cloning the *thyA* gene in a high-copy-number plasmid to be inserted into SKM50. This may increase the breakdown of dUMP to dTMP and increase thymidine production. Alternatively, SKM50 could be further genetically manipulated to increase the activity of thymidylate synthetase.

4. Evidence presented in this thesis for a deoxyuridine phosphorylase in B. helvolum is weak, but sufficient data is presented to warrant further study on this enzyme. For this, it is important to isolate and purify the enzyme before carrying out enzymic studies to confirm its identity as a separate deoxypyrimidine catabolising enzyme to that of thymidine phosphorylase. Once the existence of such an enzyme have been confirmed, mutants defective in this enzyme could be isolated and analysed. Such mutants should have reduced catabolism of thymidine and subsequently thymidine excretion could be further increased.

REFERENCES

Ahmad S.I. and Pritchard R.H. (1969); A map of four genes specifying enzymes involved in the catabolism of nucleosides and deoxynucleosides. Molec Gen Genetics **104**: 351-359

Ahmad S.I. and Pritchard R.H. (1970); A regulatory mutant affecting the synthesis of enzymes involved in the catabolism of nucleosides in *E. coli*. Molec Gen Genetics **111**: 77-83

Albrechtsen H. and Ahmad S.I. (1980); Regulation of the synthesis of nucleoside catabolic enzymes in *E. coli*: Further analysis of a *deo*O^{c} mutant strain. Molec Gen Genetics **179**: 457-460

Albrechtsen H., Hammer-Jespersen K., Munch-Petersen A. and Fiil A. (1976). Multiple regulation of nucleoside catabolising enzymes: Effects of polar *dra* mutation on the *deo* enzymes. Molec gen Genetics **146**:139-145

Alikhanian S.I., Iljina T.S., Kaliaeva E.S., Kamenva S.V. and Sukhodolec V.V. (1966); A genetical study of thymineless mutants of *E. coli* K12. Genet Res **8**: 83-100

Barre-Sinoussi F., Chermann J.C., Rey F. *et al.* (1983);

Isolation of a T-lymphotrophic retrovirus from a patient at risk from AIDS. Science **220**: 868-871

Barth P.T., Beacham I.R., Ahmad S.I. and Pritchard R.H. (1968); The Inducer of the deoxynucleoside phosphorylases and deoxyriboaldolase in *E. coli*. Biochim Biophys Acta **161**: 554-557

Beacham I.R., Barth P.T. and Pritchard R.H. (1968); Constitutivity of thymidine phosphorylase in *dra-* strains: dependence of *thy* requirement and concentration. Biochim Biophys Acta **166**: 589-592

Beacham I.R., Eisenstark A., Barth P.T. and Pritchard R.H. (1968); Deoxynucleoside-sensitive mutants of *S. typhimurium*. Molec Gen Genetics **102**: 112-127

Beacham I.R. and Pritchard R.H. (1971); The role of nucleoside phosphorylases in the degradation of deoxyribonucleosides by thymine requiring mutants of *E. coli*. Molec Gen Genetics **110**: 289-298

Bertino J.B. and Stacey K.A. (1966); A suggested mechanism for the selective procedure for isolating thymine requiring mutants in *E. coli*. Biochem J. **101**: 32-33c

Breed R.S. (1953); The *Brevibacteriaceae* fam. nov. of order *Eubacteriales*. Riassunti d. Communicaz., VI Cong. Internaz.

di Microbiol. 1: 13-14

Breitman T.R. and Bradford R.M. (1964); The induction of thymidine phosphorylase and excretion of deoxyribose during thymine starvation. Biochem Biophys Res Commun **17**: 786-791

Breitman T.R. and Bradford R.M. (1967); The absence of deoxyriboaldolase activity in thymineless mutant of *E. coli* strain 15: a possible explanation for the thymine requirement of some thymineless strains. Biochim Biophys Acta **138**: 217-220

Breitman T.R. and Bradford R.M. (1968); Inability of low thymine requiring mutants of *E. coli* lacking phosphodeoxyribomutase to be induced for deoxythymidine phosphorylase and deoxyriboaldolase. J. Bacteriol **95**: 2434-2435

Brockman R.W., Davis J.M., Stutts P. (1960): Metabolism of uracil and 5-fluorouracil by drug-sensitive and drug resistant bacteria. Biochim biophys Acta (Amst.) **40**: 22

Brown P. (1992); Aids: The challenge of the future. New Scientist, Inside Science No. **54**

Boyce R.P. and Setlow S.B. (1962); A simple method of increasing the incorporation of thymidine into the DNA of *E. coli*. Biochim Biophys Acta **61**: 618-620

Budman D.R. and Pardee A.B. (1967); Thymidine and thymine incorporation into DNA: Inhibition and repression by uridine or thymidine phosphorylase of *E. coli*. J Bacteriol **94**:1546-1550

Conn H.J. (1928); A type of bacterium abundant in productive soils, but apparently lacking in certain soils of low productivity. New York Agric. Exp. Sta. Geneva Bull. **138** pp 3-26

Freidkin M. and Kornberg A. (1957); The enzymatic conversion of deoxyuridylic acid to thymidylic acid and the participation of tetrahydrofolic acid. In: A Symposium on the Chemical Basis of Heredity (eds; McElroy W.D. and Glass B.). John Hopkins Press, Baltimore 609-613

Furman P.A., St Clair M., Weinhold K. *et al.* (1985); Selective inhibition of HTLV-III by BW A509U. In: Program and abstracts of the Twenty-fifth International Conference on Antimicrobial Agents and Chemotherapy, Minneapolis, October 1985. American Society of Microbiology. **1985**: 172 abstract.

Gallo R.C., Salahuddin S.Z., Popovic M. *et al.* (1984); Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk from AIDS. Science **224**: 500-503

Hammer-Jespersen K. and Munch-Petersen A. (1975); Multiple

regulation of nucleoside catabolising enzymes: Regulation of the *deo* operon by *cytR* and *deoR* gene products. Molec Gen Genetics 137: 327-335

Hammer-Jespersen K., Munch-Petersen A., Nygaard P. and Schwartz M. (1971); Induction of enzymes involved in the catabolism of deoxyribonucleosides and ribonucleosides in *E. coli* K12. Eur J Biochem (1971) 19: 533-538

Jorgensen P., Collins J. and Valentin-Hansen P. (1977); On the structure of the *deo* operon of *E. coli*. Molec Gen Genetics 155: 93-102

Kammen H.O. (1967); Thymine metabolism in *E. coli* I. Factors affecting in utilisation of exogenous thymine. Biochim Biophys Acta 134: 301-311

Karlstrom O. and Larsson A. (1967); Significance of ribonucleotide reduction in the biosynthesis of deoxyribonucleotides in *E. coli*. Eur J Biochem 3: 164-170

Kissalt K. and Berend E. (1918); Untersuchungen uber die Gruppe der Diphtheroiden (Corynebakterien). Centr. Bakt. Parasitenk. Abt. 1, Orig. 91: 444-447

Kretinsky T.A., Barclay M., and Jacquez J.A. (1964); Specificity of mouse uridine phosphorylase. J Biol Chem 239: 805-592

Lehmann K.B. and Neuman R.O. (1896): Atlas und Grundriss der Bakteriologie und Lehrbuch der speciellen Backteriologische Diagnostik 1st edit. J.F. Lehmann, Munchen pp 1-448

Lochhead A.G., (1955); *Brevibacterium helvolum* (Zimmermann). Comb. Nov. International Bulletin of Bacteriological Nomenclature and Taxonomy. vol. 5, 3: 115-119

Lomax M.S. and Greenberg G.R. (1968); Characteristics of the *deo* operon: role in thymine utilisation and sensitivity to deoxynucleosides. J Bacteriol **96**:501-514

Mitsuya H., Weinhold K.J., Furman P.A. *et al.* (1985); 3'azido-3'-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotrophic virus type III/lymphadenopathy-associated virus *in vitro*. Proc Natl Acad Sci USA **82**: 7096-7100

Munch-Petersen A., Nygaard P., Hammer-Jepersen K. and Fiil N. (1972); Mutants constitutive for nucleoside catabolising enzymes in *E. coli* K12: Isolation, charaterisation and mapping. Eur J Biochem **27**: 208-215

Munch-Petersen A. (1967); Thymidine breakdown and thymine uptake in different mutants of *E. coli*. Biochim Biophys Acta **142**: 228-237

Niedzwicki J.G., Mahmoud H.K., Chu S.H. and Cha S. (1983); Structure-activity relationship of ligands of the pyrimidine nucleotide phosphorylases. Biochemical Pharmacology, Vol. **32**, No. 3, pp 399-415

O'Donovan G.A. (1978); Thymidine metabolism in bacteria In: DNA Synthesis; Present and Future. Eds. Molinex I., Kohiyama M. Plenum Publishing pp. 219-253

O'Donovan G.A. and Neuhard J. (1970); Pyrimidine metabolism in microorganisms. Bacteriological Reviews American Society for Microbiology. vol **34**, No. 3, pp 278-343

Okada T. (1966); Mutational site on the gene controlling quantitative thymine requirement in *E. coli* K12. Genetics **54**: 1329-1336

Pritchard R.H. and Ahmad S.I. (1971); Fluorouracil and the isolation of a mutant lacking Uridine Phosphorylase in *E. coli*: Location of the Gene. Molec Gen Genetics **111**: 84-88

Pritchard R.H., Barth P.T., and Collins J. (1969); XIX Microbial growth Symposium. Gen Microbiol pp 263-297

Pritchard R.H and Zaritsky A. (1970); Effect of thymine concentration on the replication velocity of DNA in a thymineless mutant of *E. coli*. Nature **226**; 126-131

Rachmeler M., Gerhardt J., and Rosner J. (1961); Limited thymidine uptake in *E. coli* due to an inducible thymidine phosphorylase. Biochim. biophys. Acta (Amst.) 49; 22

Schwartz M. (1971); Thymidine phosphorylase from *E. coli*. Methods in enzymology Vol.I Nucleic acid metabolism. ed. Hoffee P.A., Jones M.E. (1978) Academic Press pp 442-445

Wilson M.C., Farmer J.L., and Rothman F. (1966); Thymidylate synthesis and aminopterin resistance in *B. subtilis*. J Bacteriol 92: 186-196

Zimmermann O.E.R. (1890); Bakerien unserer Trink- und Nutzwasser. Chemnitz. 1: 52-53