The University of Leicester

Department of Genetics

A Genetic and Physiological Study of Enzymes Involved in the Catabolism of Nucleosides in *Escherichia coli*

By

Shamim Iqbal Ahmad

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

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Statement

The experimental work in this thesis has been carried out by the author in the laboratories of the Genetics Department of the University of Leicester, Leicester.

This work has not been presented and is not being presented for any other degree.

Shamim Ahmad

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INTRODUCTION

Cultures of wild type Escherichia coli can utilise both purine and pyrimidine bases present in the medium as nucleic acid precursors, instead of synthesising these compounds de novo (Bolton and Reynard 1954; Siminovitch and Graham 1955). The first reaction involved in the incorporation of purine bases (adenine, guanine or hypoxanthine) was initially demonstrated in an extract from yeast (Kornberg et al. 1955) and later in Salmonella typhimurium (Kalle and Gots 1961) viz.

$$\text{Purine base} + \text{PRPP} \longrightarrow \text{Purine nucleotide} + \text{PPi} \quad (1)$$

PRPP (5-phosphoribosyl-1-pyrophosphate) is an activated ribose -5-phosphate derivative synthesised by the following reaction (Kornberg et al. 1955[a]):

$$\text{ATP} + \text{Rib-5-P} \longrightarrow \text{PRPP} + \text{AMP} \quad (2)$$

In a recent study Krenitsky et al. (1970) have found that the purine nucleotide pyrophosphorylase activity of E.coli catalysing reaction (1) could be chromatographically separated into three distinct components. One is specific for adenine, one for hypoxanthine and one for guanine or xanthine.

Among pyrimidine bases a similar pyrophosphorolytic reaction was observed with uracil in an extract of E.coli W (Crawford et al. 1957).

$$\text{Uracil} + \text{PRPP} \longrightarrow \text{UMP} + \text{PPi} \quad (3)$$

UMP-pyrophosphorylase catalysing reaction (3) was subsequently studied by Molloy and Finch (1969) in a dialysed crude extract of E.coli ALBA. It was observed that the enzyme was strongly activated by GTP and inhibited by UMP.

A reversible reaction, similar to (1) and (3) operates in the de novo synthesis of orotidine monophosphate from orotic acid in the presence of OMP-pyrophosphorylase (Lieberman et al. 1955):

$$\text{Orotic acid} + \text{PRPP} \longleftrightarrow \text{OMP} + \text{PPi} \qquad (4)$$

The pyrophosphorolytic conversion of orotic acid to OMP was observed simultaneously in Neurospora (Lieberman et al. 1955) and in E.coli (Hurlbert and Reichard 1955). Exogenous orotic acid has been found to serve as a substitute for uracil in a pyrimidine requiring mutant of E.coli (Beckwith et al. 1962). The pathway by which its incorporation occurs may involve as its first step pyrophosphorylation to OMP (reaction 4).

No enzyme capable of catalysing a pyrophosphorolytic or phosphorolytic reaction with cytosine has been detected either in E.coli (Karlstrom and Larsson 1967) or in S.typhimurium (Neuhard 1968). It would appear that the first reaction involved in the incorporation of exogenous cytosine is its deamination to uracil, catalysed by cytosine deaminase (Hahn and Lentzel 1923,

Chargaff and Kream 1948).

$$\text{Cytosine} + H_2O \longrightarrow \text{Uracil} + NH_3 \qquad (5)$$

since the uracil requirement of a mutant of S.typhimurium (in which carbamyl phosphate synthetase activity was absent) was found to be satisfied by cytosine, but the cytidine requirement of the same mutant (due to lack of CTP synthetase activity) could not be satisfied by cytosine (Neuhard 1968). In addition, mutant strains lacking cytosine deaminase activity were found to be resistant to 5-fluorocytosine (FC) but sensitive to 5-fluoro-uracil (FU). (FC and FU are both growth inhibitory compounds).

From these observations in E.coli it is clear that free bases, which are not synthesised de novo (except orotic acid), can be utilised from exogenous sources. Their incorporation occurs by well controlled mechanisms which are presumably important in sparing the de novo synthesis of various nucleic acid precursors, since the presence of these bases in the growth medium is associated with inhibition of the de novo synthesis of the corresponding precursors. Thus Gots and Chu (1952) and Love and Gots (1955) found that addition of purines to the culture medium of purine-requiring E.coli mutants caused inhibition of formation of two purine precursors. Beckwith et al. (1962) showed that growing a uracil auxotroph of E.coli in the presence of either

uracil, orotic acid or deoxycytidylic acid led to repression of all five enzymes involved in the synthesis of pyrimidine nucleotides from aspartic acid.

In contrast to their ability to utilise uracil, wild type strains of E.coli incorporate only negligible amounts of thymine into their DNA, even when very high concentrations of thymine are present in the growth medium (Bolton and Reynard 1954, Siminovitch and Graham 1955, Munch-Petersen 1968). This inability of the bacteria to incorporate thymine is not due to permeability restrictions or lack of a mechanism capable of doing so, since there is evidence that thymine is extensively incorporated provided a deoxynucleoside is present in the growth medium along with thymine (Boyce and Setlow 1962, Kammen 1967, Kammen and Strand 1967, Munch-Petersen 1968). Also Kammen (1967) has shown that a brief exposure of cells to low concentrations of EDTA (which brings about a change in permeability - Leive, 1965- rendering the cells sensitive to actinomycin D; a compound to which E.coli is normally insensitive) does not affect the incorporation of labelled thymine.

In spite of this behaviour of wild type E.coli, mutant strains can be isolated which incorporate exogenous thymine and utilise it for DNA synthesis. Such a thymine-requiring mutant

was initially isolated by Roepke et al. (1947) using x-ray mutagenesis, and subsequently by Okada et al. (1961, 1962) and Stacey and Simson (1965) using aminopterin and trimethoprim respectively as selective agents to which thymine-requiring (thy) mutants are resistant. Further biochemical studies of thy mutants indicated that they were lacking thymidylate synthetase activity (Barner and Cohen 1959) and therefore, had lost the ability to synthesise thymidylate de novo.

In E.coli de novo thymidylate is synthesized via the following route:

$$dUMP + N_5\ N_{10}\ \text{methylenetetrahydrofolate} \longrightarrow dTMP + \text{dihydrofolate} \qquad (6)$$

Thymidine monophosphate is subsequently converted to the corresponding diphosphate and triphosphate and incorporated into DNA. The reactions are catalysed by thymidylate synthetase (reaction 6, Friedkin and Kornberg 1957), dTMP kinase (Nelson and Carter 1969) and dTDP kinase (see Lehman et al. 1958) respectively.

An alternative to the pathway shown above for the synthesis of dTTP in E.coli B was claimed by Forster and Holldorf (1965) involving the methylation and subsequent deamination of dCTP according to the following two reactions:

dCTP + 5,10-methylenetetrahydrofolate ⟶

5-methyl dCTP + dihydrofolate (7)

5-methyl dCTP ⟶ dTTP + NH_3 (8)

The enzyme catalysing the methylation of dCTP was claimed to be distinct from thymidylate synthetase since the two activities could be separated chromatographically. In addition thy mutants were reported to lack both the enzymes (reaction 7 and 8) and thymidylate synthetase.

In order to trace this second pathway involved in dTTP synthesis, Karlstrom and Larsson (1967), and Neuhard (1968) performed independent experiments using E.coli and S.typhimurium respectively. They used mutant strains in which cytidine was unable to be converted to uridine due to the lack of cytidine deaminase activity (Wang et al. 1950):

Cytidine + H_2O ⟶ Uridine + NH_3 (9)

The purpose of using this type of mutant was to block the conversion of cytidine to uridine and therefore, to separate the pathway for synthesis of uridine nucleotides from that of cytidine nucleotides. Cells were grown either in the presence of ^{14}C cytidine or in the presence of ^{14}C uridine. The nucleic acids were hydrolysed to release the component nucleotides and these were separated from each other. As much as 75 % of the thymine

nucleotide was shown to be derived from cytidine and only 25 % from uridine. These observations show that thymine is mainly synthesised from cytidine compounds.

To further elucidate these results Neuhard and Thomassen (1971) produced a strain of S.typhimurium having mutations in carbamyl phosphate synthetase (requiring arginine and uracil), cytidine triphosphate synthetase (unable to convert UTP to CTP) and cytidine deaminase (unable to convert cytidine to uridine). This mutant strain, besides requiring arginine and uracil, specifically required cytidine for growth. Also, due to the mutation, independent pathways for the synthesis of uracil and cytosine nucleotides were operating. When a thy mutation was introduced into this strain, it was observed that it relieved the uracil requirement of the strain. A further analysis revealed that the thy mutation enabled the cell to increase the production of endogenous uracil which in turn was utilised via UMP-pyrophosphorylase (reaction 3). Increase in the intracellular dCTP concentration was observed and a new enzyme dCTP deaminase, catalysing the conversion of dCTP to dUTP, was found. Further to confirm the existence of this reaction, bacteria were grown in presence of ^{3}H cytidine, ^{32}P orthophosphate and unlabelled uracil. Analysis of the distribution of radioactive ^{3}H and

^{32}P in various nucleotides revealed that about 70-90 % of dUMP was supplied by cytosine compound without equilibrating with the uridine nucleotide pool.

Based on these observations Neuhard and Thomassen (1971) explained the previous results (Neuhard 1968; Karlstrom and Larsson 1967), indicating dCTP as the major precursor of dUMP, as due to the fact that in thy mutants an endogenous rise in the dCTP pool occurs which leads to an enhanced deamination of dCTP to dUTP via dCTP deaminase. The dUTP is subsequently converted to dUMP by dUTP pyrophosphatase (Bertani et al. 1963; Greenberg and Somerville 1962).

In addition their observations also explain the previous finding (Biswas et al. 1965) that UDP is a poor substrate of nucleoside diphosphate reductase which catalyses the conversion of nucleoside diphosphates to their corresponding deoxynucleoside diphosphates; hence only 10-30 % of the dUMP formation is the result of the direct reduction of UDP.

When a mutant of E.coli lacking dCTP deaminase was analysed (O'Donovan et al. 1971), it was observed that the dTTP level of this mutant was two fold reduced and the dCTP pool was 5-10 fold higher than the level in the parent strain. Analysis of the distribution of label in dTMP after growing the mutant

and parent strains in ^{3}H uracil, ^{12}C cytidine and ^{32}P orthophosphate indicated that about 52 % of the dTMP was derived from CMP in the parent strain. In the mutant strain on the other hand 100 % of the dTMP was derived from UMP. It was therefore concluded that a similar pathway to that observed in S.typhimurium was operating in E.coli which involved the deamination of dCTP and its subsequent conversion to dUMP.

These observations, although clearly indicating that about 70-90 % of endogenous dUMP is synthesised from dCTP which in turn may be a major source of thymidylate, do not necessarily solve the dilemma of the existence of the pathway proposed by Forster and Holldorf.

Genetics studies of thy mutants indicated that in E.coli the thyA gene is located between lysAB and argA loci (Ishibashi et.al. 1964; Kitsuji 1964) on the linkage map (Taylor 1970). In a detailed genetic study of 134 thy mutants, Alikhanian et al. (1966) observed that all thy mutations were clustered in a short region and were distributed over 17 sites. Since all thy mutants so far investigated lack thymidylate synthetase activity and map at a single location on the genome, it seems probable that the thy locus is identical with the structural gene for this enzyme. If there were two pathways for dTTP synthesis, a double mutational event would be necessary to achieve thymine auxotrophy i.e. a

mutation in thyA (thymidylate synthetase) as well as a mutation in the gene which would have enabled the strain to synthesise dTTP via the pathway proposed by Forster and Holldorf. The genetic studies of thy mutants therefore oppose the existence of a second pathway for thymidylate synthesis.

The consequential gain of the ability of thy mutants of E.coli to incorporate exogenous thymine is a pleiotropic effect. A major part of our work in this laboratory including my own genetic studies have been to elucidate the nature of this pleiotropy.

In contrast to thymine, thymidine is efficiently incorporated by wild type E.coli (Rachmeler et al. 1961), indicating that unlike thymine there is a scavenging route for the utilisation of thymidine. By isolating an E.coli mutant lacking thymidine kinase activity Hiraga et al. (1967) have shown that this mutant was unable to incorporate exogenous labelled thymidine into its DNA; thus the only route by which thymidine is incorporated is the first step conversion of thymidine to dTMP.

$$dT + ATP \longrightarrow dTMP + ADP \qquad (10)$$

The existence of this scavenging route is often masked in laboratory cultures; thymidine incorporation being only short lived. This is because the inducible enzyme thymidine phosphorylase

(Rachmeler et al. 1961) degrades thymidine to thymine and dRib-1-P. However, thymidine incorporation occurs for prolonged period in strains lacking thymidine phosphorylase or when thymidine phosphorylase is inhibited by addition of uridine, adenosine or cytidine to the growth medium (Budman and Pardee 1967, Doskocil and Paces 1968, Boyle and Jones 1970, Yagil and Rosner 1970). Razzell and Casshyap (1964) suggested that dRib-1-P, a degradation product of thymidine, might be the inducer of thymidine phosphorylase. (Our observation that dRib-5-P is the inducer of thymidine phosphorylase will be discussed later).

The incorporation of thymine by thy mutants has been shown (Fangman and Novick 1966) to involve its conversion to thymidine via thymidine phosphorylase.

$$\text{Thymine} + \text{dRib-1-P} \longleftrightarrow \text{dT} + \text{Pi} \qquad (11)$$

since a thy mutant of E.coli lacking thymidine phosphorylase activity (Fangman and Novick 1966) was unable to grow on thymine as a thymine source, instead of thymidine (Fangman 1969).

Although the results obtained by Fangman (1969) clearly indicate that an intact thymidine phosphorylase is necessary for thy mutants to utilise thymine, my own observation is that the thymidine requirement of a thy$^-$ thymidine phosphorylase mutant can be satisfied by thymine, if along with thymine, deoxyadenosine

(200 µg/ml) is added to the growth medium. This incorporation may, however, be occuring via uridine phosphorylase, which in addition to catalysing the conversion of uridine to uracil and Rib-1-P (Paege and Schlenk 1952) has also been shown to cleave thymidine to a small extent (Krenitsky *et al.* 1964, Beacham and Pritchard 1971).

An alternative to the phosphorolytic pathway for thymine incorporation in *thy* mutants was claimed to be deoxyribosyl transferase reaction (Munch-Petersen 1967):

$$\text{dRib-Base} + \text{Thymine} \longleftrightarrow \text{dT} + \text{Base} \qquad (12)$$

In this reaction, in contrast to the phosphorolysis of thymine, dRib-1-P was not involved as an obligatory intermediate. Although there have been several confirmed reports of the existence of a transferase reaction in *Lactobacilli* (MacNutt 1952, Roush and Betz 1958, Kanda and Takagi 1959, Beck and Levin 1963) one has never been demonstrated in *E.coli*. In a recent report (O'Donovan and Neuhard 1970) however, it has been claimed that neither in *E.coli* nor in *S.typhimurium* does such a reaction exist which could catalyse the transferase activity.

If wild type *E.coli* can incorporate thymidine and not thymine, and the incorporation of exogenous thymine in *thy* mutants involves as a first step its conversion to thymidine, then *thy*

mutants must clearly have gained the ability to carry out the reaction converting thymine to thymidine. It has been known for some time that thy mutants of E.coli are of two classes which differ in the minimum amount of thymine required for their growth. Mutants of the thyA class, lacking thymidylate synthetase activity, require about 20 µg/ml of thymine. Mutants of the second thymine-requiring class are normally derivatives of the thyA mutants and require only about 2 µg/ml of thymine to grow. In an early study, Breitman and Bradford (1964) analysed these two classes of thy mutants. They observed that when the thymine low requiring mutant was incubated in the absence of thymine, a large amount of deoxyribose was excreted into the medium and thymidine phosphorylase was also induced. Based on these properties, it was proposed that the secondary mutation in the thy auxotroph may have resulted in greater production of deoxyribonucleotides, which ultimately resulted in greater production of deoxyribose phosphate due to the cleavage of the former compounds. The deoxyribose phosphate not only induced thymidine phosphorylase, but its large intracellular synthesis resulted in its degradation to deoxyribose and excretion into the medium. Wild type E.coli on the other hand was assumed to have too little dRib-1-P to promote the conversion of thymine to thymidine. In subsequent

studies (Breitman and Bradford 1967, 1968) the nature of the thymine low requiring (Tlr) mutants was understood more clearly. It was observed that these mutants lacked either deoxyriboaldolase (*dra*$^-$) or deoxyribomutase (*drm*$^-$) activities. From the known pathways of deoxynucleoside catabolism (Manson and Lampen 1951):

$$\text{Deoxynucleoside} + \text{Pi} \xleftrightarrow[\text{nucleoside phosphorylase}]{\text{Purine or Pyrimidine}} \text{dRib-1-P} + \text{Base} \qquad (13)$$

$$\text{dRib-1-P} \xleftrightarrow{\text{Deoxyribomutase}} \text{dRib-5-P} \qquad (14)$$

$$\text{dRib-5-P} \xleftrightarrow{\text{Deoxyriboaldolase}} \text{Acetaldehyde} + \text{Glyceraldehyde-3-Phosphate} \qquad (15)$$

it was postulated that a block in either the catabolism of deoxyribose-1-phosphate (*drm*$^-$) or deoxyribose-5-phosphate (*dra*$^-$) results in an enhanced pool size of dRib-1-P permitting thymine to be converted to thymidine efficiently and incorporated into DNA. An implication of the high thymine requirement (20 μg/ml) of *thy*$^-$ *drm*$^+$ *dra*$^+$ strains is that their intracellular concentration of dRib-1-P is comparatively lower than that of *thy*$^-$ *drm*$^-$ or *thy*$^-$ *dra*$^-$ strains.

If only *thy* mutants can form large intracellular concentrations of dRib-1-P, the next questions to arise are

what are the precursors, pathways and the mechanisms involved in its formation. For some time several laboratories (Beacham et. al. 1968, 1971; Breitman and Bradford 1964, 1967, 1968; Munch-Petersen 1968, 1970; Neuhard 1966, 1967, 1968; O'Donovan et. al. 1971) have been trying to resolve these problems. Their proposed hypothesis could be summarised as follows.

Let us consider a thy mutant which is unable to synthesise thymidylate de novo due to the lack of thymidylate synthetase activity (reaction 6). Immediately after the mutational event, when its intracellular concentration of dRib-1-P is presumably very low, the mutant will be unable to incorporate thymine and consequently a fall in thymidylate pool will occur. Since it has been shown (Biswas et. al. 1965) that dTTP represses nucleoside diphosphate reductase, catalysing the conversion of nucleoside diphosphates to their corresponding deoxynucleoside diphosphates (Reichard 1962), a derepression of the enzyme will occur due to low concentration of dTTP. A consequence of the rise in the dCTP pool will be its deamination (O'Donovan et. al. 1971) to dUTP and from this high concentrations of dUMP will form. Since thy mutants cannot catalyse the conversion of dUMP to dTMP, the degradation of dUMP will increase the endogenous pool of deoxyuridine and from this the pool of dRib-1-P. Exogenous thymine will

therefore be incorporated much more efficiently and dTTP synthesis will occur.

It has been repeatedly observed (Munch-Petersen 1970; Neuhard 1966; Beacham et al. 1971) that the endogenous dTTP pool of thy mutants never reaches to the wild type level even when very high concentrations of thymine were provided along with deoxyguanosine to the growing culture. This is consistent with the above hypothesis, since if the dTTP pool in thy mutants rose to the level of wild type strains, an immediate cessation in the supply of dRib-1-P would occur. Thus DNA synthesis would depend on maintenance of a low dTTP pool. Beacham et al. (1971) observed, however, that even if the requirement for endogenous synthesis of dRib-1-P was removed (e.g. by growing cultures in the presence of thymidine or thymine plus deoxyguanosine) the internal dTTP pool could still not be raised to the wild type level. In fact they showed that the low dTTP pool seems to be due to the fact that thymidine itself inhibits its own conversion to dTTP. The above regulatory hypothesis is therefore not established.

Neuhard and Munch-Petersen (1966), Neuhard (1966) and Munch-Petersen (1970) observed that when thy mutants of E.coli are grown in absence of thymine, the level of both pyrimidine

(dCTP) and purine (dATP) nucleotides rises. This observation led to the supposition (Beacham and Pritchard 1971) that purine as well as pyrimidine may both be the precursors of dRib-1-P. To resolve this problem, they produced mutants of E.coli blocked in pyrimidine nucleoside degradation (lacking uridine phosphorylase, thymidine phosphorylase (reaction 11) or both the enzyme activities) or purine nucleoside degradation (lacking purine nucleoside phosphorylase activity catalysing the following reaction (Koch 1956):

$$\text{Purine nucleoside} + \text{Pi} \longleftrightarrow \text{Purine base} + \begin{matrix}\text{dRib-1-P} \\ \text{or} \\ \text{Rib-1-P}\end{matrix} \quad (16)).$$

These mutants were grown in the absence of thymine and the excretion of deoxyribose in the medium was measured. Results indicated that in thy mutants the sole source of dRib-1-P is pyrimidine deoxynucleosides, since mutants lacking thymidine phosphorylase or uridine phosphorylase activities excreted decreased amount of deoxyribose and mutants lacking both the enzyme activities excreted negligible deoxyribose, but addition of a pup (purine nucleoside phosphorylase (reaction 16)) mutation to these mutant strains had no effect on deoxyribose excretion.

Genetic studies of thymine low requiring mutants indicated that the drm and dra genes are located close to the

left of thr locus in both E.coli (Alikhanian et al.1966, Okada 1966) and S.typhimurium (Eisenstark et al. 1968).

In the studies of Alikhanian et al. (1966) it was observed that some of the thymine low requiring mutants were inhibited when grown in presence of 10 µg/ml or higher concentrations of thymidine. They designated these mutants Td^s (thymidine sensitive). When Td^s mutants of both S.typhimurium (Beacham et al. 1968[a]) and E.coli (Lomax and Greenberg 1968, Munch-Petersen 1968) were further examined, it was found that only the dra^- class of Tlr mutants showed a significant reduction in growth if the growth medium was supplemented with a deoxynucleoside, although drm^- mutants were slightly inhibited. Purine deoxynucleosides were less effective inhibitor than pyrimidine deoxynucleosides, and phosphorolysis of the added deoxynucleoside was necessary. In the absence of deoxyribonucleoside phosphorylases no inhibition was observed in dra mutants by the corresponding deoxynucleosides (Munch-Petersen 1970).Addition of a nucleoside, or prolonged incubation of the culture, allowed the growth of the inhibited cells to be resumed (Beacham et al. 1968[a], Alikhanian et al. 1966). It was also confirmed that the thymidine sensitivity and utilisation of low external concentrations of thymine (dra^-) were two effects of

a single mutation (Beacham et al. 1968[a]). In considering the fact that only dra mutants were inhibited in growth and not the drm mutants, it was proposed (Beacham et al. 1968[a]) that the inhibition was due to the accumulation of dRib-5-P. Another suggestion, that the inhibition in growth was due to the deficiency of oxygen uptake (Lomax and Greenberg 1968), was ruled out by the observation (Munch-Petersen 1970) that in E.coli the inhibition was not affected when mutants were grown anaerobically in the presence of thymidine.

We have observed by now that the four enzymes viz. thymidine phosphorylase (reaction 11), uridine phosphorylase, deoxyribomutase (reaction 14) and deoxyriboaldolase (reaction 15) are involved in the utilisation of thymine. These enzymes and purine nucleoside phosphorylase are also capable of catalysing the breakdown of nucleosides and use of the deoxyribose moiety as carbon source. A question of interest concerning these enzymes arised as to what are their biological roles. Are they concerned with breakdown rather than synthesis since (i) they are inducible (ii) wild type strains of E.coli cannot convert thymine to thymidine (iii) they are released into the medium (Kammen 1967; Munch-Petersen 1968; Beacham 1969) when cells are osmotically shocked (Neu and Heppel 1965) and therefore, they may be

associated with the other degradative enzymes which are surface localised (e.g. alkaline phosphatase). If they are degradative enzymes one possibility might be that they are part of a larger system of enzymes whose function is the degradation of foreign DNA entering into the cell as a result of phage infection or conjugation. Consistent with this possibility is the location of hsp (host specificity) gene (Arber 1968) which appears to be located close to the Tlr loci (Glover and Colson 1969).The work reported in this thesis was undertaken to examine this possibility and as a part of more detailed study of thymine metabolism. The results described in this thesis may be summarised as follows.

(1) The pup gene which specifies purine nucleoside phosphorylase is also linked to the thr locus and is located to the left of it.

(2) The relative locations of the four genes viz. dra (deoxyriboaldolase), drm (deoxyribomutase), tpp (thymidine phosphorylase) and pup (purine nucleoside phosphorylase) are, in order, hsp - dra - tpp - drm - pup - serB - thr.

(3) A regulatory gene has been located which maps near the gal region and a mutation in this gene renders at least deoxyriboaldolase, thymidine phosphorylase and purine nucleoside

phosphorylase constitutive.

(4) The probable inducer of purine nucleoside phosphorylase is dRib-5-P.

(5) The four genes viz. *dra*, *tpp*, *drm* and *pup* constitute at least two operons and *dra* and *tpp* genes belong to the same operon, which has its operator located to the left of *dra*.

(6) A fifth gene, *udp*, specifying uridine phosphorylase, which is also probably involved in the catabolism of nucleoside, is not linked to the other four genes and maps near *met*E on the linkage map of *E.coli*.

(7) Location of the gene (*cod*) specifying cytosine deaminase has been determined and it has been shown that the only pathway involved in the utilization of exogenous cytosine is the first step its conversion to uracil.

(8) A selective technique for the isolation of a variety of mutants has been devised. This was found to be useful in not only isolating several classes of mutants but was also useful in elucidating a part of the information regarding nucleoside catabolism.

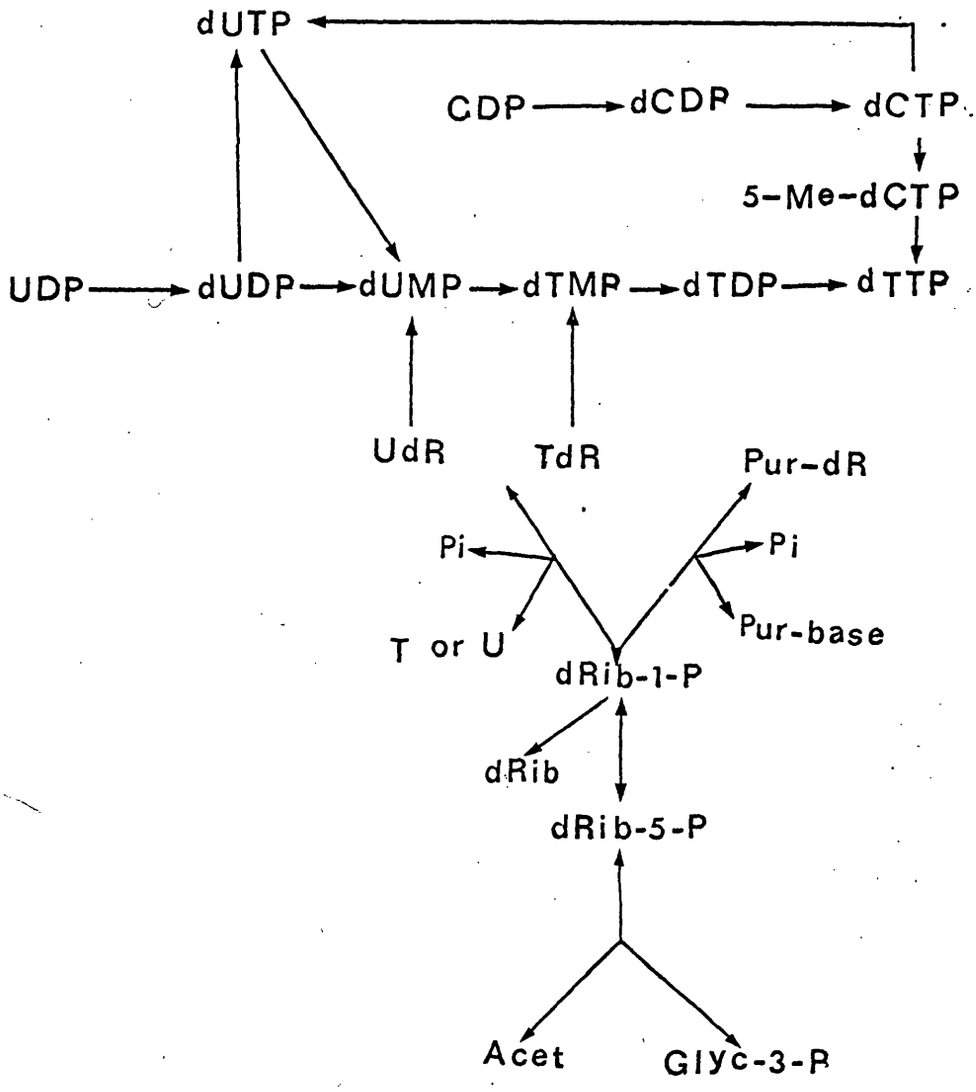


Fig 1 Pathways Involved in dTTP Synthesis

MATERIALS AND METHODS

List of bacterial strains

Strain	Mating type	Genotype	Origin
P226	Hfr R4	met$^-$	see Hayes 1964
P227	Hfr R4	met$^-$ upp$^-$	from P226
P228.2	Hfr R4	met$^-$ upp$^-$ pup$^-$	from P227
P57	Hfr H	thi$^-$ thy$^-$ lac$^-$	see Hayes 1964
P152	Hfr H	thi$^-$ thy$^-$ lac$^-$ leu$^-$ drm$^-$	as above
P293	Hfr H	thi$^-$	as above
SA6	Hfr R4	met$^-$ upp$^-$ tpp$^-$	from P227
SA10	Hfr H	thy$^-$ thi$^-$ lac$^-$ drm$^-$	P228.2XP152
SA53	Hfr R4	met$^-$ upp$^-$ tpp$^-$ udp$^-$	from SA6
SA74	Hfr R4	met$^-$ cod$^-$	from P226
SA83	Hfr C	metB$^-$ pyrE$^-$	AT 2243
SA92	Hfr H	galE$^-$ thi$^-$ lac$^-$	
SA144	Hfr H	thi$^-$ thy$^-$ dra$^-$ lac$^-$	from P57 as T1r
SA145	Hfr H	thi$^-$ thy$^-$ dra$^-$ lac$^-$ tpp$^-$	from SA144
SA153	Hfr KL16-99	recA$^-$ thi$^-$	see Low 1968
SA156	Hfr H	thy$^-$ thi$^-$ lac$^-$ tpp$^-$	SA144 X SA6
BW114	Hfr P4X	thi$^-$ uvrA$^-$	from Dr Wilkins

F⁻ Strains	Genotype	Origin
P203	thr⁻ leu⁻ thi⁻ arg⁻ his⁻ trp⁻ str^r lac⁻ gal⁻ xyl⁻ man⁻ mal⁻ T1^r	
SA37	thi⁻ serB⁻ drm⁻ hsp⁻ azu^r str^r	from SA49
SA41	thi⁻ pup⁻ drm⁻ hsp⁻ azu^r str^r	P228.2 X SA37
SA44	thr⁻ leu⁻ thi⁻ serB⁻ hsp⁻ azu^r str^r	from 4K
SA46	thi⁻ pup⁻ dra⁻ azu^r str^r hsp⁻	SA41X SA48
SA47	leu⁻ thi⁻ tpp⁻	SA6 X SA48
SA48	thr⁻ leu⁻ thi⁻ lac⁻ dra⁻	P228.2 X P162
SA48.1	thr⁻ leu⁻ thi⁻ lac⁻ dra⁻ tpp⁻	from SA48
SA48.2	thr⁻ leu⁻ thi⁻ lac⁻ dra⁻ tpp⁻	as SA48.1
SA49	thi⁻ serB⁻ drm⁻ str^r hsp⁻	SA10 X 4K
SA73	thr⁻ leu⁻ thi⁻ thy⁻ lac⁻ ara⁻	thy⁻ derivative of E17
SA75	thr⁻ leu⁻ thi⁻ thy⁻ lac⁻	drm⁺ derivative of Y-70-22
SA79	thi⁻ udp⁻ tpp⁻ met⁻ his⁻ trp⁻ str^r	SA53 X P203
SA89	thr⁻ leu⁻ thi⁻ thy⁻ lac⁻ str^r	from SA75
SA91	thr⁻ leu⁻ thi⁻ galE⁻ thy⁻ lac⁻ str^r	SA89 X SA92
SA107	metE⁻ rha⁻ lac⁻ mal⁻ str^r	thy⁺ derivative of JG 123
SA112	leu⁻ thi⁻ thy⁻ lac⁻	from Y-70-22
SA114	thr⁻ leu⁻ thi⁻ thy⁻ galE⁻ str^r	from SA89

F⁻ strains	Genotype	Origin
*SA115	thr⁻ leu⁻ drm⁻ dra⁻ thy⁻ thi⁻	from SA119 as Tlr
*SA116	thr⁻ leu⁻ drm⁻ thy⁻ thi⁻	as above
SA119	thr⁻ leu⁻ thi⁻ thy⁻	thy⁻ derivative of C600
SA139	leu⁻ thy⁻ thi⁻	from SA115
*SA141	thr⁻ leu⁻ thi⁻ dra⁻ drm⁻	thy⁺ derivative of SA115
*SA142	thr⁻ leu⁻ thi⁻ dra⁻	drm⁺ derivative of SA141
SA143	thr⁻ leu⁻ thi⁻	SA6 X SA142
*SA147	thr⁻ leu⁻ thi⁻	SA145 X SA142
SA150	thr⁻ leu⁻ thi⁻ pro⁻ his⁻ arg⁻ lac⁻ gal⁻ ara⁻ str^r recA⁻	AB2463, see Low 1968
*SA152	thr⁻ leu⁻ thi⁻ thy⁻ drm⁻ dra⁻ str^r	from SA115
*SA154	thr⁻ leu⁻ thi⁻ drm⁻ dra⁻ recA⁻	SA153 X SA152
*SA155	thr⁻ leu⁻ thi⁻ drm⁻ dra⁻ recA⁻ ara⁻	ara⁻ derivative of SA154
SA161	thr⁻ leu⁻ thi⁻ arg⁻ his⁻ trp⁻ cod⁻ lac⁻ gal⁻ xyl⁻ man⁻ mal⁻	SA74 X P203

*is constitutive for thymidine phosphorylase

Strain	Mating type	Genotype	Origin
SA168	F^-	*thr*$^-$ *leu*$^-$ *thi*$^-$ *arg*$^-$ *his*$^-$ *trp*$^-$ *pyrE*$^-$ *cod*$^-$ *str*^{r} *lac*$^-$ *gal*$^-$ *man*$^-$ *mal*$^-$	SA83 X SA161
SA157	F'	F'*thr*$^+$ *leu*$^+$ *tpp*$^-$/SA150	SA156 X SA150
SA169	F'	F'*thr*$^+$ *leu*$^+$ *tpp*$^-$/SA155	SA156 X SA155
4K	F^-	*thr*$^-$ *leu*$^-$ *thi*$^-$ *serB*$^-$ *str*^{r} *hsp*$^-$	from Dr S. Glover
4K-4	F^-	*thr*$^-$ *leu*$^-$ *thi*$^-$ *arg*$^-$ *azu*^{r} *str*^{r}	from 4K
Y-70-2	F^-	*thr*$^-$ *leu*$^-$ *thi*$^-$ *thy*$^-$ *lac*$^-$	from Dr Munch-Petersen
Y-70-22	F^-	*thr*$^-$ *leu*$^-$ *thi*$^-$ *thy*$^-$ *lac*$^-$ *drm*$^-$	as above
JG123	F^-	*metE*$^-$ *thy*$^-$ *rha*$^-$ *lac*$^-$ *mal*$^-$	from Dr J.Gross
E17	F^-	*thr*$^-$ *leu*$^-$ *thi*$^-$ *lac*$^-$ *gal*$^-$ *ara*$^-$	
C600	F^-	*thr*$^-$ *leu*$^-$ *thi*$^-$	see Barth *et.al* (1968)
CR34	F^-	*thr*$^-$ *leu*$^-$ *thi*$^-$ *thy*$^-$ *dra*$^-$	as above

Media.

M9 salt (stock solution): Na_2HPO_4, 6.0 g; KH_2PO_4, 3.0 g; NaCl, 0.5g; NH_4Cl, 1.0 g; water to 100 ml.

CaMg (stock solution): $CaCl_2$, 0.01 M; $MgSO_4$, 0.1 M.

Glucose solution: Glucose, 20.0 g; water 100 ml.

Minimal synthetic medium:

M9 salt, 10.0 ml; CaMg, 1.0 ml; Glucose soln., 2.0 ml; water, 100 ml.

For synthetic agar medium, Davis Japanese agar (1.5 g) was first dissolved in 100 ml of water and was adjusted to pH 8.5. M9 salt, 10 ml; CaMg, 1.0 ml; and glucose solution 2.0 ml were then added.

Nutrient medium:

Oxoid Number 2 nutrient broth powder, 2.5 g; water 100 ml. To solidify, 1.25 g of Davis Japanese agar was added.

Tryptone agar medium:

New Zealand agar, 1.1 g; Oxoid tryptone, 1.0 g; NaCl, 0.5 g; water, 100 ml.

Arbo top agar:

Oxoid Number 1 agar, 0.75 g; water, 100 ml (pH 7.0).

Laboratory phosphate buffer:

KH_2PO_4, 0.3 g; Na_2HPO_4 (anhydrous), 0.7 g; NaCl, 0.4 g;

$MgSO_4$, $7H_2O$, 0.01 g; water, 100 ml.

Sodium phosphate buffer:

Na_2HPO_4, 0.2 M; NaH_2PO_4, 0.2 M; 39 ml of NaH_2PO_4 solution + 61 ml of Na_2HPO_4 solution gives pH 7.0.

Tris-HCl buffer:

Tris (hydroxymethyl) methyl amine, 0.05 M solution was prepared and adjusted to pH 7.4 with concentrated HCl.

Succinate-arsenate buffer:

KH_2AsO_4 or Na_2HAsO_4 (0.5 M) and succinic acid (0.5 M) was prepared and adjusted to pH 6.0 using strong KOH solution.

Phage buffer:

Na_2HPO_4, 10.5 g; KH_2PO_4, 4.5 g; NaCl, 7.5 g; 0.01 M $MgSO_4$, 15.0 ml; 0.01 M $CaCl_2$, 15.0 ml; 1 % gelatin, 1.5 ml; water, 1470 ml.

Methods

Cultural condition:

For aerated growth, cultures were shaken on New Brunswick gyrotory shakers, either in a warm room or in a water bath. All cultures were grown at 37°C, unless otherwise specified.

Measurement of cell growth:

Particle numbers were counted in a Coulter Counter (model A, Coulter Electronic Company, England) after diluting 0.1 ml of growing culture with 25 ml of NaCl (0.9 per cent) solution (Normal saline, Polyfuser product). The threshold was set at 11 and the sensitivity control at 4.

Preparation of cell extracts:

Cell extracts were prepared either by sonication or by toluenization, and for both these preparations cultures grown overnight in minimal synthetic medium were diluted (1 : 20) with fresh synthetic medium and grown aerobically. When the cell titre reached about 5×10^8 cells/ml, the cultures were harvested by centrifugation at 7000 rpm (Sorval Superspeed RC 2-B) for 10 min. Pellets were resuspended in Tris-HCl buffer (0.02 M, pH 7.4) and again centrifuged. Finally phosphate, succinate arsenate or Tris-HCl buffer (1/10th. - 1/50th. of the original volume) was added. For toluenization a drop of toluene was added, shaken well and left at room temperature (between 20-22°C) for 20 minutes. For

sonication, cells were disrupted at 0°C with an ultrasonicator and extracts were centrifuged at 15,000 rpm for 20 minutes at -4°C.

Measurement of enzyme activities:

Thymidine phosphorylase: Enzyme activity was determined by the rate of the formation of thymine from thymidine in the presence of inorganic phosphate. Assay mixture containing appropriate amounts of enzyme and phosphate buffer (0.01 M, pH 7.4) was incubated for 10 minutes at 37°C. Thymidine (final concentration 10 mM) was added to the assay mixture at zero time. Samples (0.05 ml) were withdrawn at intervals and added to 0.45 ml of 0.1 N NaOH. The increase in absorption at 300 nm due to thymine formation was determined, assuming the difference in molar extinction coefficient between thymine and thymidine to be $4.3X10^3$ (Hotchkiss 1948).

Uridine phosphorylase: The assay was essentially that used for thymidine phosphorylase. The formation of uracil from uridine was followed by measuring the increase in absorption at 290 nm at pH 13. (Assuming the difference in molar extinction coefficient between uracil and uridine to be $5.41X\ 10^3$ (Razzell and Khorana 1958)).

Deoxyriboaldolase: Enzyme activity was determined by measuring

the disappearance of diphenylamine reacting material. The reaction mixture contained, in a final volume of 0.5 ml, 22.5 µmole Tris-HCl buffer (pH 7.4), dRib-5-P, 1.0 µmole and enzyme. The reaction was started by the addition of dRib-5-P. Samples (0.1 ml) were taken at intervals and added to 0.9 ml of 0.5 M perchloric acid. To each of these 2.0 ml of diphenylamine reagent (diphenylamine 1.5g, glacial acetic acid 100 ml, concentrated H_2SO_4 1.5 ml, 0.1 ml of glyceraldehyde, 16 mg/ml added for each 20 ml required just before use (Burton 1956)) was added and incubated at room temperature in the dark overnight. Disappearance of blue colour was determined at A_{600} nm.

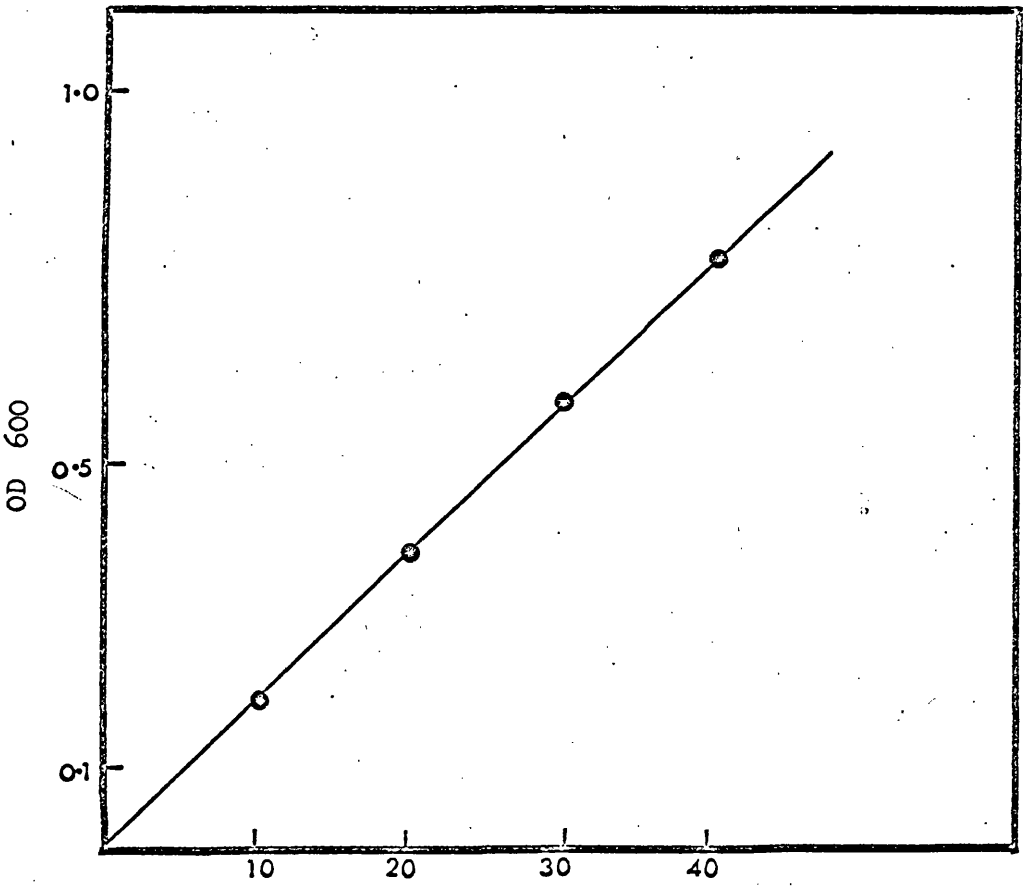
Purine nucleoside phosphorylase: The assay employed was a modification of the assay of Tsuboi and Hudson (1957) using guanosine or deoxyguanosine as substrate. The substrate (50 mM) was dissolved in 50 mM NaOH. Enzyme extract was prepared either in succinate-arsenate buffer (pH 6.0) or this buffer was added in the reaction mixture. Reaction mixture containing appropriate amount of extract and substrate (to a final concentration of 5.0 mM) was incubated at 37°C. Samples (0.05 ml) were withdrawn at intervals and added to 0.35 ml of 2 per cent Na_2CO_3 in 0.1 N NaOH solution. Folin's reagent (0.05 ml, diluted 1:1 with water) was then added with shaking. A_{600} nm was measured after 3 hours.

a calibration curve relating guanine concentration to the absorption of A_{600} nm was plotted (Fig 2). Enzyme activity was calculated according to the amount of guanine formed from its nucleoside. Negligible activity was observed if arsenate, substrate or extract was omitted from the reaction mixture.

UMP-pyrophosphorylase assay: The reaction mixture, incubated at 37°, contained appropriate amounts of sonicated extract, 5-phosphoribosyl-1-pyrophosphate (0.02 ml, 2.0 mM solution prepared in 5.0 mM EDTA + 0.1 M tris adjusted to pH 7.5 with HCl), $MgCl_2$ (0.01 ml, 30.0 mM), guanosine triphosphate (0.01 ml, 10 mM, this was added 5 minutes before the addition of uracil) and ^{14}C-uracil (0.002 μ moles of uracil, specific activity 52.5 mc/m Mole). At intervals a small quantity of sample was withdrawn in a micro-pipette and spotted on a pre-marked area on chromatography paper (cellulose M N 300 polyethylenimine, Machery-nagel and Co., Durn, Germany). Five spots of each sample were superimposed on the paper following each with drying under a stream of hot air. Finally each spot was superimposed by a drop of cold uracil (1.0 M). After this dried, chromatograms were run in a closed jar containing the solvent, water, to a height of 0.7 mm. After the solvent had ascended to a height of 18-19 cm. the sheet was dried under warm air. The area of the uracil spots were detected under

Fig 2

Standard guanine curve



Guanine concentrations in mμmoles

a u.v. light and they were excised. The radioactivity present in these areas and that present at the point of origin was determined in a scintillation counter (Packard Tri-carb liquid scintillation spectrometer). Each vial contained 10.0 ml of scintillation fluid.

Cytosine deaminase: Enzyme activity was determined by the rate of the formation of uracil from cytosine. Assay mixture containing appropriate amount of enzyme and tris-maleic buffer (0.05 M, pH 7.0) was incubated for 10 minutes at 37°C. Cytosine (final concentration 5.0 mM) was added to the assay mixture at zero time. Samples (0.05 ml) were withdrawn at intervals, added to 0.95 ml of 0.5N perchloric acid (0°C) and the mixture was centrifuged. The extinction of the supernatant fluids was measured at 282 nm. A difference in molar extinction coefficient between cytosine and uracil was assumed to be 7.3×10^3 (Neuhard 1968).

Protein estimation: Total protein in the extracts were estimated according to Lowry et. al. (1951) as follows: 0.1 ml of 2 % Na-K-tartrate and 0.1 ml of 1 % $CuSO_4$ was added to 9.8 ml of 2 % Na_2CO_3 dissolved in 0.1 N NaOH solution. Aliquots (0.5 ml) of the mixture were mixed with the protein sample (0.1 ml). Folins reagent (0.05 ml, Folin and Ciocalteu's reagent, diluted 1:1 in water) was then added with shaking and the A_{600} nm was measured after 3 hrs.

Selection of mutants:

Selection of thy mutants: To each of five test tubes containing 5.0 ml of minimal synthetic medium supplemented with appropriate growth requirements and 250 µg of thymine, were added approximately $5X10^2$- $1X10^3$ thy^+ cells (freshly grown in synthetic media) and 0, 25, 50, 100 or 250 µg of trimethoprim respectively. The tubes were then incubated for 48 hrs at 37°C. The cells grew well in the medium without trimethoprim, and the turbidity of the cultures decreased with the increase in trimethoprim concentration. Cells from each tube were diluted several fold and from each dilution 0.1 ml of culture was spread onto a synthetic agar plate containing thymine (50 µg/ml). The thy^- colonies were identified by the replica plating technique. Results indicated that at the optimum concentration of trimethoprim (10 µg/ml) about 50 per cent of the cells were thymine-requirer.

Selection of streptomycin resistant mutants: Streptomycin-sensitive cells were grown in 10 ml of minimal synthetic medium to stationary phase. The culture was centrifuged, resuspended in 0.2 - 0.4 ml of laboratory phosphate buffer and spread onto minimal agar plates supplemented with streptomycin (100 µg/ml). Streptomycin-resistant colonies appeared after 48 hrs incubation.

Transduction:

Preparation of P1 lysates: Cultures (10 ml) were grown in nutrient broth to approximately $5X10^8$ cells /ml. Calcium chloride (0.1 ml of 100 mM) was then added and incubated for 10 minutes. This was then divided into 1.0 ml aliquots to which phage ($1X10^6$ plaque forming units) was added and further incubated for 10 min. To each sample 0.4 ml of melted arbo top agar (diluted 1:1 in distilled water) was added and the mixture poured onto a tryptone agar plate (thymine was added to the tryptone agar when preparing lysate of thy$^-$ strains). After overnight incubation the top layer agar was collected, and 2.5 ml of phage buffer was added. This was shaken well for 30 minutes and centrifuged (10,000 rpm) for 10 minutes to remove the agar. The titre of the phage was usually determined using the host culture. Phage P1 prepared by this technique usually gave titre of $1X10^{10}$ - $5X10^{10}$ plaque forming unit/ml.

P1 transduction: Cultures (10 ml, $5X10^8$- $1X10^9$ cells/ml) of the recipient strain grown in nutrient broth were resuspended in the fresh medium and 0.1 ml of 100 mM $CaCl_2$ was added. After five minutes incubation at 37° phage was added (multiplicity of infection 0.1 - 0.02) and the incubation continued for an additional 20 minutes. The mixture was then centrifuged and

resuspended in 1/10th volume of laboratory phosphate buffer supplemented with sodium citrate (0.25 per cent). Transductants were selected by plating the mixture on M9 synthetic agar without $CaCl_2$ but with sodium citrate (0.25 per cent) and either glucose or an appropriate nucleoside or deoxynucleoside (5 mM) as carbon source.

Mating experiments:

When the titre of an Hfr strain (growing without shaking) reached approximately $4X10^8$ cells/ml and an F^- strain (growing with aeration) approximately $1X10^8$ cells/ml in nutrient broth, these were mixed in the ratio of 1 Hfr : 5 F^- cells, and aerated slowly. For the interrupted mating experiment 0.1 ml of this mating mixture was withdrawn at various intervals, appropriately diluted and added to arbo top agar (4.5 ml, molten condition maintained at 45°C). This was agitated vigorously for 8-10 seconds and then poured on selective synthetic agar media.

To select recombinants, the mating mixture was normally left for about one hour and then appropriate dilutions were spread on selective agar media.

Plating technique to classify strains:

Mutant strains were discriminated from non-mutant strains by making suspensions of cultures in laboratory phosphate buffer ($5X10^7$- $1X10^8$ cells/ml) and placing a drop on selective agar media. This technique was found to be very useful when the cells were grown on poor carbon source.

Use of mutagens:

Use of N-methyl-N-Nitro-N-Nitrosoguanidine (NTG), ethyl methane sulphonate (EMS) or 2-aminopurine (2-AP) for induced reversion.

Whatman filter discs (about 6 mm in diameter) were cut, using a paper perforating machine. These discs were dipped in NTG (25.0 mg/ml, dissolved in water), EMS (undiluted, commercially supplied concentration) or 2-AP (concentrated solution prepared in warm water) and were placed on selective agar plates containing a lawn of bacteria resuspended in arbo top agar. After 48 hrs incubation, a clear zone of no growth appeared around each discs. Revertants were obtained as colonies growing in the clear zone.

Classification for *hsp*:

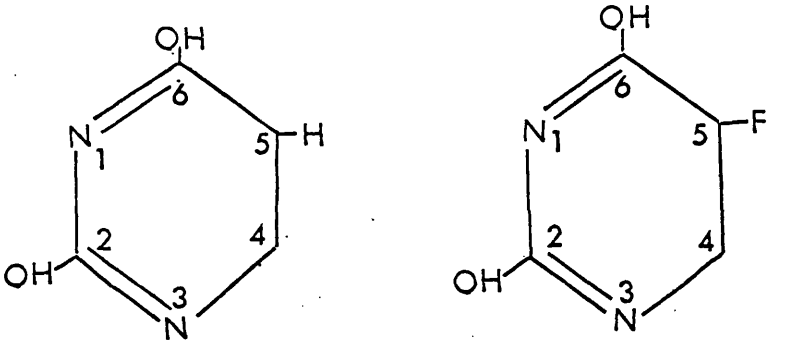
Suspensions of approximately equal titre of two known cultures - *hsp*$^+$ (strain P227) and *hsp*$^-$ (strain 4K) were spotted (about one inch diameter) on several tryptone agar plates. After these had dried a drop of various concentrations of a virulent mutant of bacteriophage lambda, grown on *hsp*$^-$ host, was superimposed on the centre of each spot. After overnight incubation at 37°, at a particular concentration of lambda, host spots with an *hsp*$^-$ mutation gave a clear zone, whereas an *hsp*$^+$ host showed only isolated plaques. This concentration of lambda was always used in classifying *hsp*$^+$ and *hsp*$^-$ transductants. Titres of the bacterial suspensions were approximately equal in all the experiments and each set of experiments was accompanied by two control spots for reference.

CHAPTER I

Isolation of Mutants

Use of 5-fluorouracil in the isolation of mutants lacking nucleoside phosphorylases and UMP-pyrophosphorylase activities.

Fluorouracil (an analogue of uracil) differs from uracil at position 5 of the pyrimidine ring at which hydrogen is substituted by fluorine (Fig 3).



URACIL FU

Fig 3

This compound, if added to the growth medium of E.coli, considerably inhibits cell growth (Heidelberg et al. 1957). A proportion of the cells grown in the presence of fluorouracil (FU) have been found to develop resistance to the compound (Brockman et al. 1960). On further analysis, it was observed that these FU resistant mutants were lacking some of the enzymes involved in the metabolism of uracil, uridine and deoxyuridine, indicating that FU itself is not the inhibitor.

In the light of these properties of FU, we considered that this compound could successfully be used to select specifically a variety of mutants of E.coli lacking various enzymes involved in pyrimidine metabolism. Before describing the method used in each case, it will be helpful to summarize the known pathways involved in the incorporation of FU into nucleic acid and its conversion to inhibitory compounds. Since we have not been interested in further analysis of the mode of action of FU and other pyrimidine analogues used by us, only a very brief account of its inhibitory activity is given in this thesis.

Cohen et al. (1958) observed that FU produces a bacteriostatic and occasionally a weak bacteriocidal effect on E.coli. The latter effect was shown to be associated with thymidylate biosynthesis. When the cells were exposed to FU,

they found that deoxyribonucleotide of FU was synthesised from FU, and this nucleotide inhibited thymidylate synthetase, an enzyme which catalyses the conversion of dUMP to dTMP (reaction 6). Fluorodeoxyuridine monophosphate (FdUMP) was isolated from the acid-soluble fraction of cells treated with FU and fluorouridine, and it was observed that this compound combines irreversibly with the enzyme. The presence of thymine in the medium was found to reverse the bacteriocidal action of FU, but did not markedly stimulate growth, indicating that this bacteriocidal effect of FU may be analogous to "thymineless death" - a previous observation (Cohen and Barner 1954), that when thy mutants are grown in the absence of thymine, death of the cells follows cessation of DNA synthesis. Thymine did not reverse the bacteriostatic effect of FU, implying that there are other effects of FU on bacterial metabolism. The addition of uracil however, restored almost normal growth and Horowitz and Chargaff (1959) have shown that when E.coli is grown in the presence of FU, a substantial amount of FU is incorporated into RNA, replacing up to 50 % of the uracil content. This incorporation however did not markedly inhibit RNA synthesis (Cohen et. al. 1958; Horowitz et. al. 1960). Presumably FU inhibits cell growth by causing the synthesis of fraudulent RNA and consequently the synthesis of

inactive proteins (Lowrie and Bergquist 1968; Andoh and Chargaff 1965). A third possible mechanism of FU inhibition in E.coli K12 was suggested by Thomasz and Borek (1962). They found that FU induced an osmotically sensitive state in growing culture, and this caused 90-99 % of the cells to disintigrate. This cell disintigration was assumed to be due to an inhibition of cell wall synthesis. Addition of uracil or uridine to the medium, or an increase in osmotic strength, restored the viability of the cells. Among the few other pyrimidine analogues they checked, only 6-aza-uracil showed an identical effect.

In brief, FU is known as a strong bacteriostatic and a weak bacteriocidal agent. The known mechanisms by which the effects are produced are either by replacing uracil of RNA, inhibition of thymidylate synthetase or possibly by inhibition of cell wall synthesis.

Pathways involved in the incorporation of FU.

Analysis of the pathways involved in the metabolism of FU by biological system has been carried out using mammalian cells in most cases and has been reviewed in great detail (see Heidelberg 1965). A part of the work however, done with bacteria, indicates that at least three routes exist in E.coli via which FU is metabolised to products which inhibit cell growth. These pathways are those known to be primarily involved in the metabolism of free uracil (see Fig 4). The order of their description here is not significant.

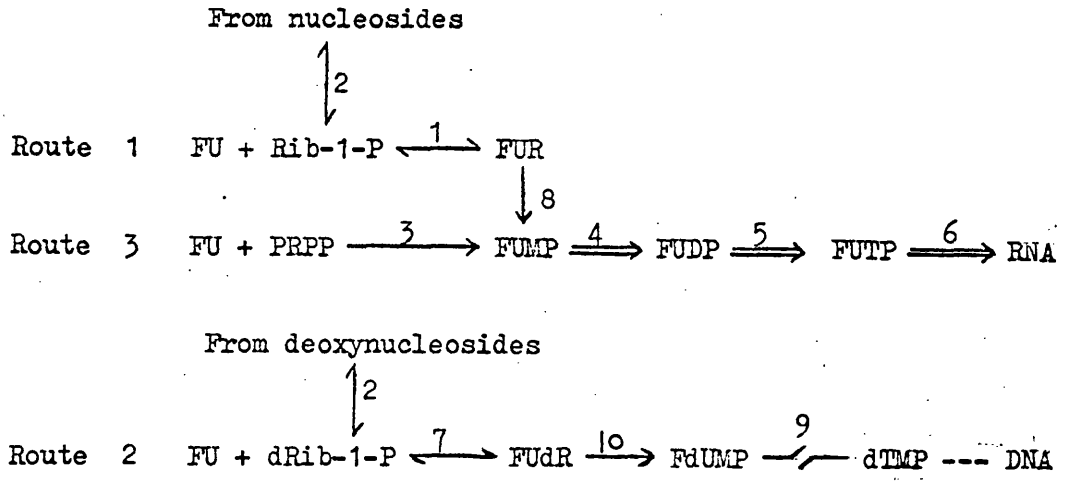
The first route involves as its first step the conversion of FU to its ribonucleoside, catalysed by uridine phosphorylase (Skold O, 1958):

$$FU + Rib\text{-}1\text{-}P \longleftrightarrow FUR + Pi \qquad (17)$$

Fluorouridine (FUR) is subsequently converted to fluorouridine monophosphate, fluorouridine diphosphate and fluorouridine triphosphate (see Heidelberg 1965). Finally fluorouridine triphosphate is incorporated into RNA (Horowitz and Chargaff 1959; Gros et al. 1961). Although enzymologically the existence of this pathway has only been demonstrated in mammalian systems, my own data (which will be given later) and the data presented by

Figure 4

Pathways via which FU may be incorporated into the bacterial metabolic system.



1. Uridine phosphorylase
2. Nucleoside phosphorylase
3. UMP-pyrophosphorylase
4. Uridine monophosphate kinase
5. Nucleoside diphosphate kinase
6. RNA polymerase
7. Thymidine phosphorylase
8. Uridine kinase
9. Thymidylate synthetase
10. Thymidine kinase

═══ de novo routes

——— scavenging routes

O'Donovan and Neuhard (1970) indicate that this pathway also exists in E.coli.

The second route by which FU may be incorporated into the cell's metabolic system involves, as the first step, conversion of FU to fluorodeoxyuridine (FUdR).

$$FU + dRib\text{-}1\text{-}P \longleftrightarrow FUdR + Pi \qquad (18)$$

FUdR is subsequently converted to fluorodeoxyuridine monophosphate (Cohen et al. 1958). Although the corresponding deoxynucleotide of uracil is subsequently converted to thymidine monophosphate and finally incorporated into DNA, no deoxynucleotide di-or triphosphate of FU has been detected in cells grown in the presence of FU (Heidelberg 1965). It would appear that fluorodeoxyuridine monophosphate and fluorouridine diphosphate are not substrates for thymidine monophosphate kinase and nucleoside diphosphate reductase respectively and therefore, there is no incorporation of the FU derivative into DNA.

A third pathway by which FU is metabolised in E.coli has been shown (Brockman et al. 1960) to involve the direct conversion of FU to its nucleotide, catalysed by UMP-pyrophosphorylase:

$$FU + PRPP \longrightarrow FUMP + PPi \qquad (19)$$

Fluorouridine monophosphate (FUMP) is subsequently metabolised via route (1) and incorporated into RNA.

Data presented in this thesis indicate that the third route is primarily involved in FU metabolism, if cells are grown in media supplemented with low concentrations of this compound. This observation has not been made before.

METHODS

Initial attempts to isolate mutants of *E.coli* lacking various enzymes involved in purine and pyrimidine metabolism, using FU, were made with the knowledge that FU is metabolised in bacteria via three distinct routes. However, the order of importance of these routes was not known. We therefore first isolated FU resistant mutant at the lowest concentration of FU which effectively inhibited the cell growth on agar plates, assuming that such mutants would indicate the pathway by which FU is most readily metabolised.

The inhibitory effect of various concentrations of FU in liquid media was determined initially. Exponentially growing cells of strain P226 in minimal synthetic medium supplemented with glucose (0.4 per cent) and methionine (20 μg/ml) were transfered into various flasks containing minimal media supplemented with 0.25, 0.5, 1.0, 2.5 or 5.0 μg/ml of FU and growth was followed. At intervals cultures were appropriately diluted in laboratory phosphate buffer and 0.1 ml of this dilution was taken into melted arbo top agar and spread onto minimal agar media without FU. Colonies were counted after 24 hours incubation at 37°C.

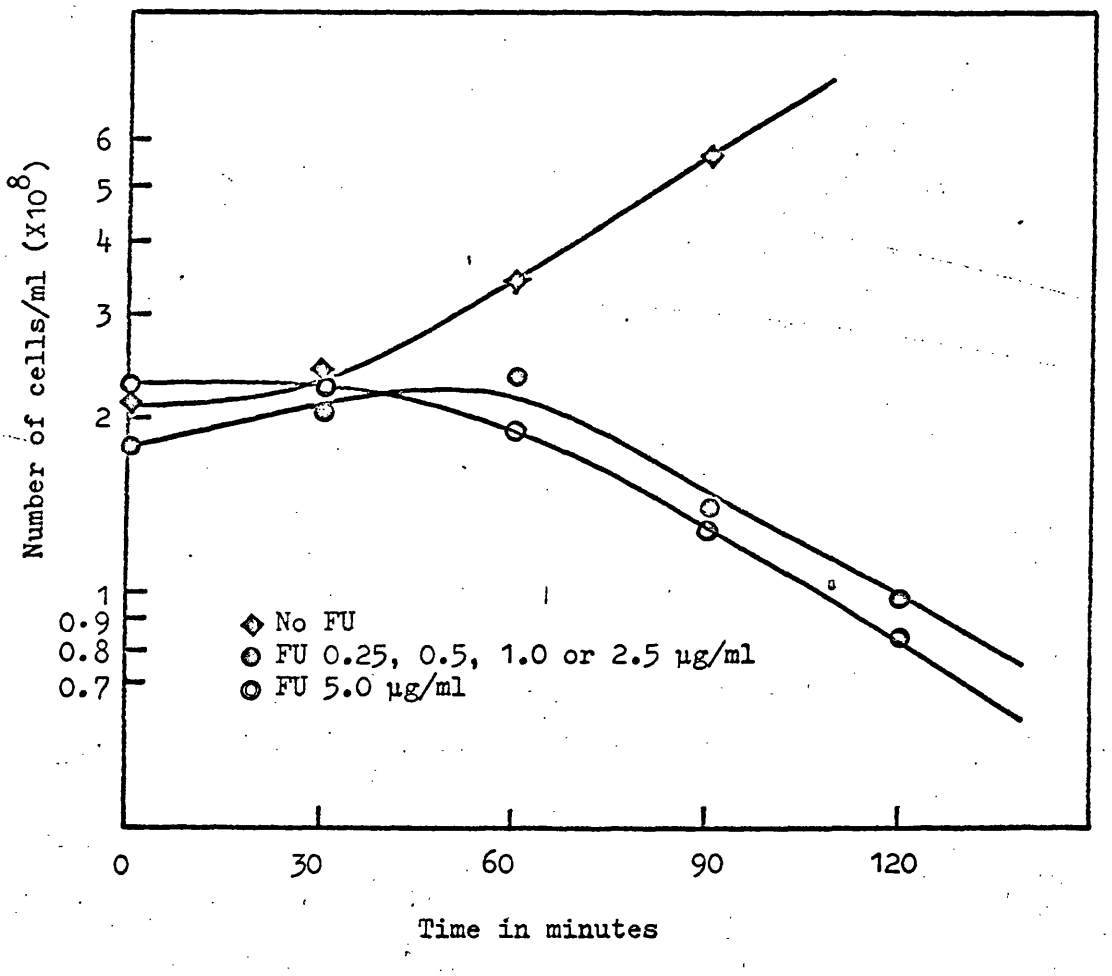
Results shown (Fig 5) indicate that as little as 0.25 μg/ml of FU was bacteriocidal. There is no significant difference in the effect on colony forming ability of higher concentrations of FU. Mutants resistant to 0.25 μg/ml of FU were therefore isolated by spreading $5X10^7$ - $1X10^8$ cells growing exponentially in minimal glucose medium, onto synthetic agar medium containing 0.25 μg/ml of FU. After 48 hrs incubation at 37°C, resistant colonies appeared on the plates at a frequency of 10^{-7}. These were purified on similar medium and were tested for resistance to various concentrations of FU as described in the Materials and Methods section. It was found that, although these resistant mutants were initially isolated as colonies resistant to 0.25 μg/ml of FU, they were resistant to 2.5 μg/ml of FU.

It was assumed that these mutants lacked UMP-pyrophosphorylase activity (upp$^-$), since they were still sensitive to a mixture of FU (2.5 μg/ml) plus deoxyadenosine (100 μg/ml) or adenosine (200 μg/ml). Addition of purine nucleosides along with FU presumably provides a supply of dRib-1-P and Rib-1-P via purine nucleoside phosphorylase (reaction 16) and results in potentiation of the metabolism of FU via reaction (18) and (17) respectively.

A similar and presumably analogous effect was observed

Figure 5

Growth inhibition in strain P226 by various concentrations of fluorouracil.

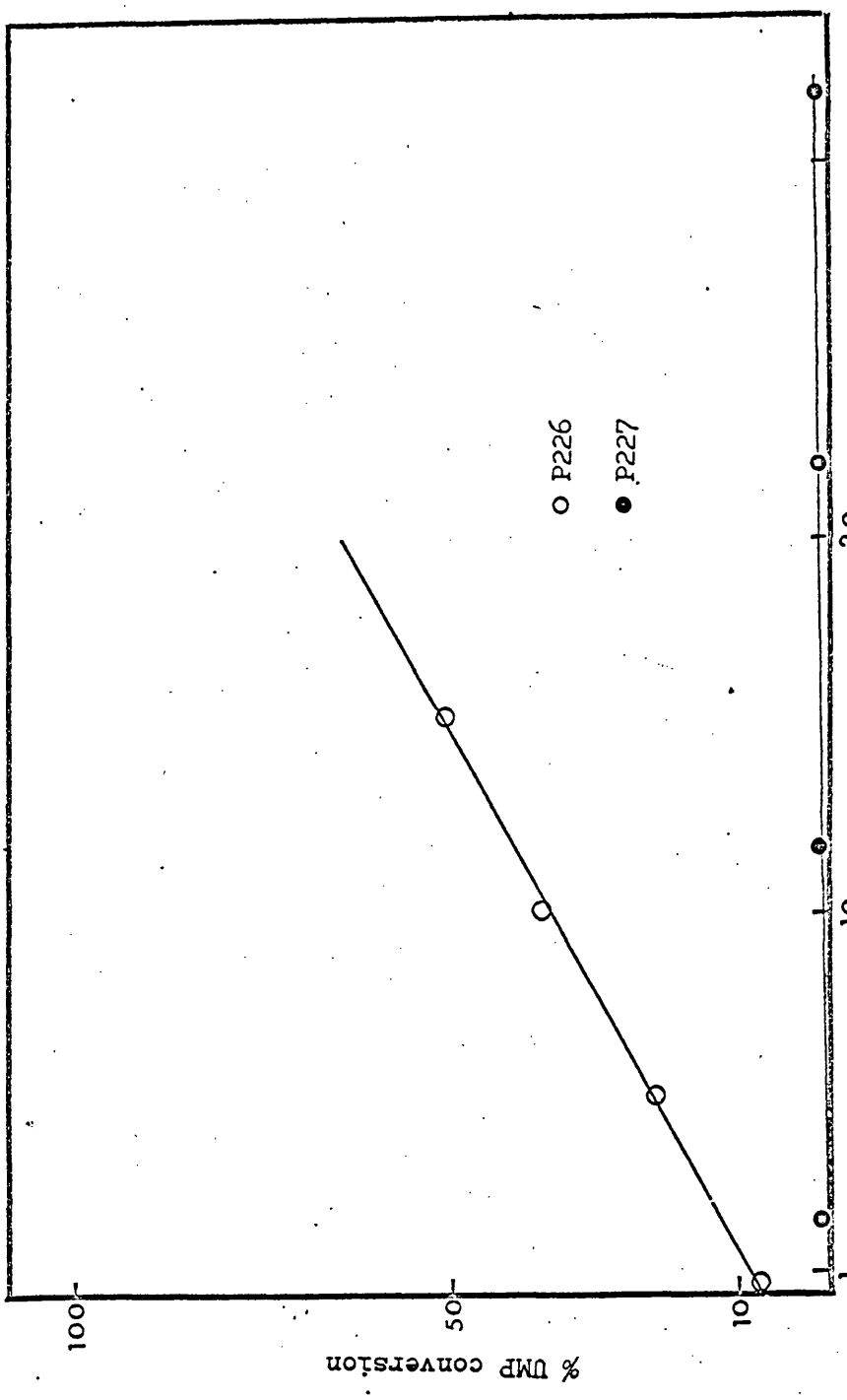


earlier by Boyce and Setlow (1962). They found that although wild type strains of E.coli cannot incorporate exogenous thymine they may do so if along with thymine a source of dRib-1-P, such as deoxyadenosine, was provided in the growth medium.

Absence of UMP-pyrophosphorylase activity in one such resistant strain (P227) was later confirmed (Fig 6) by measuring the percentage of labelled UMP formed from ^{14}C uracil and PRPP in the presence of extract prepared by ultrasonication (Molloy and Finch 1969; see Materials and Methods also).

Fig 6

UMP-pyrophosphorylase activity in wild type and mutant strains.



Isolation of thymidine phosphorylase and purine nucleoside phosphorylase mutants of E.coli.

Strain P227 still posseses thymidine phosphorylase and purine nucleoside phosphorylase activities. This was judged by the fact that the strain was sensitive to a deoxyadenosine (dA) plus FU mixture. In addition the mutant is also able to grow on thymidine (5 mM) or deoxyadenosine (5 mM) as sole carbon sources.

If our hypothesis relating the two reactions (numbers 18 and 20) and sensitivity to FU is correct, then among mutants resistant to the FU plus dA mixture a proportion should lack thymidine phosphorylase activity, since such mutants would be unable to synthesise FUdR and from this FdUMP. Some resistant strains may also lack purine nucleoside phosphorylase activity, since in this case the availability of dRib-1-P is restricted due to the lack of reaction (16).

Exponentially growing cells ($5X10^7$- $1X10^8$) of strain P227 were spread on synthetic agar medium containing FU (2.5 µg/ml) and dA (100 µg/ml) and incubated for 48 hrs. Resistant colonies were purified and tested nutritionally for growth on dA (5 mM) or dT (5 mM) as sole carbon source. Mutants lacking thymidine phosphorylase activity cannot grow on thymidine but can grow on

deoxyadenosine, and mutants lacking purine nucleoside phosphorylase activity can grow on thymidine but not on adenosine. In the first attempt 20 resistant colonies were tested and one of them possessed the phenotype predicted for a strain lacking purine nucleoside phosphorylase activity (*pup*$^-$). The experiment was repeated and a presumptive thymidine phosphorylase mutant (*tpp*$^-$) was isolated likewise. Absence of relevant enzyme activities in strain P228.2 (*pup*$^-$) and strain SA6 (*tpp*$^-$) was later confirmed by enzyme assays (Table 1).

A proportion of the remaining FU plus dA resistant mutants perhaps lack thymidine kinase or a permease for FU incorporation.

Table 1

Thymidine phosphorylase, purine nucleoside phosphorylase and uridine phosphorylase activities in strain P227 and its derived mutants

Strain	Thymidine as inducer	Purine nucleoside phosphorylase	Thymidine phosphorylase	Uridine phosphorylase
P227	-	55.4	143	
	+	142	535	
P228.2	-	>1.5		
	+	>1.5		
SA6	-		0.6	175
	+		1.1	
SA53	-			not detected

Enzyme activities are expressed in m μmoles/base/min/mg protein

Isolation of uridine phosphorylase (*udp*$^-$) mutants.

The selective procedure used to isolate uridine phosphorylase mutants was an extension of the technique already described for the isolation of mutants lacking the other nucleoside phosphorylase. Growth of strain P227, resistant to FU (2.5 µg/ml), was found to be inhibited if adenosine (200 µg/ml) was added to the growth medium along with FU. Presumably this is because the synthesis of FUR is now possible due to the operation of the reaction (17) catalysed by uridine phosphorylase. Added adenosine in the growth medium apparently serves as the source of Rib-1-P (reaction 16). It also indicates that the intracellular concentration of Rib-1-P in strain P227 is too small to potentiate the conversion of FU to FUR. If mutants resistant to an FU plus adenosine mixture are now isolated, some of them should lack uridine phosphorylase activity (*udp*$^-$) and hence be unable to grow on uridine as sole carbon source.

In two preliminary tests of this method some mutants of strain P227, resistant to FU (2.5 µg/ml) and adenosine (200 µg/ml), were isolated. These were purified and tested nutritionally for the uridine phosphorylase negative phenotype. It was found

that, although some of these mutants did grow poorly on uridine as sole carbon source, a clear discrimination between *udp*$^+$ and *udp*$^-$ classes was difficult. It seemed probable that these were *udp*$^-$ mutants but that their ability to grow on uridine was due to conversion of this compound to uridine monophosphate which could then be used as a carbon source via the following route: uridine diphosphate, deoxyuridine diphosphate, deoxyuridine monophosphate, deoxyuridine and thence via thymidine phosphorylase, deoxyribomutase and deoxyriboaldolase to glyceraldehyde and acetaldehyde. An alternative to this was the possibility that thymidine phosphorylase by itself partially degraded uridine to uracil and Rib-1-P.

Consistent with these possibilities was the observation that when the strain used to isolate the *udp* mutant was lacking both UMP-pyrophosphorylase activity (*upp*$^-$) and thymidine phosphorylase activity (*tpp*$^-$) (using 10 µg/ml FU and 200 µg/ml adenosine, since these double mutants are resistant up to 10 µg/ml of FU), a class of mutant was obtained which showed negligible growth on uridine as sole carbon source. Out of 32 resistant mutants isolated from the *upp*$^-$ *tpp*$^-$ strain, 22 were found to have lost the ability to grow on uridine as sole carbon source and presumably lacked uridine phosphorylase

activity. Absence of the enzyme activity was confirmed in one such mutant (strain SA53, Table 1). Three of the remaining 10 mutants were unable to grow on adenosine as sole carbon source. They presumably lacked purine nucleoside phosphorylase and their resistance to the FU plus adenosine mixture would consequently be due to their failure to generate Rib-1-P from adenosine. The remaining mutants were left unclassified. Some of them may have lacked uridine kinase activity.

Isolation of mutants lacking deoxyribomutase and deoxyriboaldolase activities.

Mutants of these classes were selected as secondary thy mutants able to grow on low concentrations of thymine. This was achieved by spreading a lawn of thyA mutants on appropriately supplemented minimal glucose agar media containing 2.0 µg/ml of thymine (this concentration was used at all times unless otherwise specified). After 24 hrs incubation, colonies able to grow at this concentration appeared. It was found unnecessary to mutagenise the culture to isolate the mutants, since (a) the rate of appearance of spontaneous mutants was reasonably high in the strains used (about $1X10^{-6}$). (b) a direct selective technique was available. Thymine low requiring mutants were purified on similar medium and classified into drm$^-$ and dra$^-$ classes on the basis of their ability to grow on nucleoside or deoxynucleoside as sole carbon source. Mutants lacking deoxyribomutase activity (drm$^-$) are unable to grow on any nucleoside or deoxynucleoside, whereas mutants lacking deoxyriboaldolase activity are only unable to grow on deoxynucleosides. A majority of thymine low requiring mutants were drm$^-$ and only a small proportion were dra$^-$.

Isolation of mutants lacking thymidine phosphorylase activity among mutants lacking deoxyriboaldolase activity.

The technique employed in the isolation of thymidine phosphorylase mutants made use of the fact that *dra* mutants are sensitive to deoxynucleosides. A *tpp*$^-$ *dra*$^-$ or *drm*$^-$ *dra*$^-$ double mutant, on the other hand, is resistant to these compounds since the double mutants are unable to form dRib-5-P, the accumulation of which most probably inhibits the cell growth (Beacham *et al.* 1968[a]). In a typical experiment approximately $8X10^7$ exponentially growing cells of a *dra* mutant were spread on synthetic glucose agar medium supplemented with appropriate requirements and thymidine (500 µg/ml). Resistant colonies appearing after 18-20 hrs incubation were purified and tested for their sensitivity to deoxyadenosine and thymidine. From 46 such resistant mutants 21 were found to be sensitive to deoxyadenosine. These were presumably still *drm*$^+$ since deoxyadenosine in these strains could be degraded to dRib-1-P and subsequently to dRib-5-P. Their resistance to thymidine, on the other hand, suggested that they lacked thymidine phosphorylase. Two such deoxyadenosine sensitive thymidine resistant mutants were tested by enzyme assay. They were found to lack the relevant enzyme.

CHAPTER II

Characterization of Mutants

Properties of various FU resistant mutants of E.coli.

It was observed that the upp tpp mutant (SA6) was more resistant to FU alone than was the upp$^-$ strain (P227) itself. The former strain was able to grow on medium containing up to 10 µg/ml of FU. This was surprising since it implied that E.coli could synthesise FUdR in the absence of an added deoxynucleoside as dRib-1-P source. At first sight this suggests that in E.coli the intracellular concentration of dRib-1-P is sufficiently high to facilitate the synthesis of FUdR. This is an apparent paradox since all previous studies (Bolton and Reynard 1954; Siminovitch and Graham 1955; Crawford 1958; Munch-Petersen 1968) have suggested that wild type strains of E.coli are virtually incapable of incorporating exogenous thymine into their DNA, whatever concentration is present in the growth medium. This is because these cells have an insufficiently high dRib-1-P pool.

In order to clarify this paradoxical result, the sensitivity to FU of a set of mutant strains was determined. These strains lack one or more of the three enzymes which catalyse the first step in each of the three routes implicated in the incorporation of FU and subsequent inhibition in cell growth. The results (Table 2) confirm that there are only three

Table 2

Sensitivity of various mutants to 5-fluorouracil

Strain	Genotype				FU concentrations (µg/ml)			
	pup	tpp	udp	upp	0.25	2.5	10	100
P226	+	+	+	+	-	-	-	-
P227	+	+	+	-	++++	+++	+	-
P228.2	-	+	+	-	++++	+++	+	-
SA6	+	-	+	-	++++	++++	++++	+
SA53	+	-	-	-	++++	++++	++++	++++
4K-4	+	+	-	-	++++	+++	++	-

Cells grown on solid synthetic medium were resuspended into 0.5 ml of laboratory phosphate buffer. The suspension was spotted on solid synthetic medium containing various concentrations of fluorouracil. Growth was recorded after 48 hrs of incubation at 37°. The number of +'s indicates the relative amount of growth at this time.

routes (as shown in Fig 2) by which FU can inhibit the cell growth of E.coli, since the mutant strain (SA53) lacking UMP-pyrophosphorylase, uridine phosphorylase and thymidine phosphorylase activity is resistant to a very high concentration (100 µg/ml) of this compound. The mutant (4K-4) lacking UMP-pyrophosphorylase and uridine phosphorylase activities is still sensitive to more than 2.5 µg/ml of FU, indicating that sufficient synthesis of FUdR and subsequently of FdUMP occurs to inhibit the cell growth (Cohen et al. 1958). Therefore a thy^+ strain of E.coli should have an intracellular pool of dRib-1-P which is perhaps so small that it is not usually detected by other techniques. The failure to detect incorporation of thymine in wild type strains is no doubt due in part to competition from de novo synthesis of thymidylate. Another plausible explanation is that the very small intracellular concentration of dRib-1-P initially synthesises small amounts of FUdR and then FdUMP and this partially inhibits thymidylate synthetase. In consequence a reduction in dTTP synthesis would occur. This latter compound is known to be associated with enhanced breakdown of certain deoxynucleotides (Beacham et al. 1968; Beacham and Pritchard 1971). The breakdown would then increase the supply of dRib-1-P and hence of dUMP. In other words, synthesis of this inhibitory compound would be self-amplifying.

Pathways involved in the metabolism of 6-azauracil.

Strain P227 was tested for resistance to 6-azauracil (Azu) and it was found that as much as 1.0 mg/ml of this compound did not inhibit the cell growth. It was therefore assumed that in contrast to FU, azauracil is incorporated solely via UMP-pyrophosphorylase. However, this is not so, since we later observed that addition of adenosine (200 µg/ml) or deoxyadenosine (100 µg/ml) to upp$^-$ strains will inhibit cell growth. Hence Azu may also be incorporated via the other two pathways of FU incorporation. At this point it is difficult to understand the observation (Cihak et al. 1963) that in cell-free extracts of wild type E.coli Azu did not serve as substrate for UMP-pyrophosphorylase in the presence of 5-phosphoribosyl-1-pyrophosphate.

Properties of purine nucleoside phosphorylase mutants.

Mutants of E.coli lacking purine nucleoside phosphorylase activity have lost the ability to grow on adenosine, guanosine or deoxyguanosine as sole carbon source and growth is greatly reduced on deoxyadenosine. They have negligible purine nucleoside phosphorylase activity (Table 1) even when the culture is grown in the presence of thymidine as inducer. These properties of the pup⁻ mutation lend weight to the supposition (Koch 1956) that in E.coli both ribosides and deoxyribosides are substrates for purine nucleoside phosphorylase. Thus although the selective procedure used in the isolation of mutants demanded that the mutant isolated should only be unable to degrade deoxyadenosine, the mutant isolated has simultaneously lost the ability to degrade all purine nucleosides and deoxynucleosides. The possibility that our selective procedure has demanded all the purine nucleoside phosphorylase enzymes (if there were more than one) to be missing, was ruled out in further genetic studies of this mutant strain (to be shown later).

The inducer of purine nucleoside phosphorylase.

Rachmeler et al. (1961) observed that extracts prepared from E.coli cultures grown in presence of 500 µg/ml thymidine had greater thymidine phosphorylase activity than those prepared from bacteria grown in absence of thymidine, i.e. thymidine phosphorylase was induced by thymidine. Razzell and Casshyap (1964) suggested that it was probably dRib-1-P which induced the enzyme, since the induction was observed after addition of both purine and pyrimidine deoxyribosides. This observation led us to ask whether purine nucleoside phosphorylase is also induced by thymidine. Strain P227 and P228.2 were grown in synthetic media without and with thymidine (2 mM) as inducer. Cultures were washed after centrifugation with 0.05 M arsenate-0.05 M succinate buffer (pH 6.0) and finally resuspended in 1/10th volume of this buffer. A drop of toluene was added, shaken well and left at room temperature (about 20°C) for about 10 minutes. Purine nucleoside phosphorylase activity was determined as described in Materials and Methods section. Guanosine or deoxyguanosine were used as substrate.

The results shown (Table 3) indicate that thymidine also induces purine nucleoside phosphorylase, although the observed induction ratio is not as high as that of thymidine

Table 3

Purine nucleoside phosphorylase activity in *pup*$^+$ and *pup*$^-$ strains

Strains	Substrate	Enzyme activity (m μmoles/min per mg protein)	
		Uninduced	Induced
P227	Guanosine	55.4	142.0
	Deoxyguanosine	197.9	526.9
P228.2	Guanosine	<1.5	<1.5
	Deoxyguanosine	<1.5	<1.5

phosphorylase (e.g. see Table 14).

Breitman and Bradford (1967) observed that thymidine also induced deoxyriboaldolase. This observation, and the knowledge of the degradative pathway for thymidine and the availability of drm and dra mutants led us to ask which degraded product of thymidine was the inducer of purine nucleoside phosphorylase. If dRib-1-P is the inducer, the enzyme should be maximally induced by thymidine in drm mutants, since dRib-1-P should accumulate in this strain. If, on the other hand, dRib-5-P is the inducer, we should observe purine nucleoside phosphorylase to be hyper-induced in dra mutants, since in this strain due to blockage in further degradation of dRib-5-P this compound should accumulate. In addition, if dRib-5-P is the inducer we should not observe any induction in drm mutants because these mutants cannot degrade thymidine to dRib-5-P.

To distinguish between these alternatives purine nucleoside phosphorylase activity was determined in P152 (drm^-), CR34 (dra^-) and C600 (drm^+ dra^+) strains after growth in the absence and presence of thymidine (2 mM) as inducer. Results (Table 4) show that purine nucleoside phosphorylase is not induced in a drm mutant or hyper-induced in a dra mutant. It was therefore concluded that dRib-5-P is the inducer. Determi-

Table 4

Inducibility by thymidine of purine nucleoside phosphorylase

Strain	Induced	Enzyme activity m μmoles/min per mg protein
C600	-	82
	+	255
P152	-	73
	+	78
CR34	-	343
	+	520

nation of the induction of thymidine phosphorylase, deoxyribomutase and deoxyriboaldolase in the presence of thymidine in these strains showed that dRib-5-P is also the inducer of these enzymes (Barth et al. 1968).

Similar results were also obtained in S. typhimurium (Robertson et al. 1970) and in E.coli (Munch-Petersen 1968). In their studies these authors also observed that purine nucleoside phosphorylase and deoxyribomutase besides being induced by thymidine are also induced by inosine and guanosine. Uridine, on the other hand, did not induce purine nucleoside phosphorylase. The actual inducers in this case were interpreted as the purine ribonucleosides themselves. By growing a drm mutant and a pup mutant in the presence of inosine and determining purine nucleoside phosphorylase and deoxyribomutase activities Robertson et al. (1970) showed that even in pup mutants, which cannot degrade inosine, deoxyribomutase was induced. They therefore concluded that purine nucleoside (inosine and guanosine) themselves are inducers of purine nucleoside phosphorylase.

CHAPTER III

Mapping of the genes

Mapping of the genes specifying purine nucleoside phosphorylase, thymidine phosphorylase, deoxyriboaldolase, deoxyribomutase and uridine phosphorylase.

As has been shown earlier in this thesis, mutants of E.coli able to grow on a low concentration (2 µg/ml) of thymine belong to two classes. One is dra, the other drm. These genes were classified in early studies (Okada 1966; Alikhanian et. al. 1966) as a single phenotypic class designated Tlr, and were found to be located close to and to the left of the thr locus. Mutants of E.coli sensitive to thymidine (Tds) were considered (Alikhanian et. al. 1966) to be distinct from Tlr but were located close to Tlr. In later studies (Breitman and Bradford 1967, 1968; Beacham et. al. 1968; Lomax and Greenberg 1968) however, when it became clear that Tlr and Tds were in fact the genes which specify deoxyribomutase and deoxyriboaldolase, we proposed the gene symbols drm and dra respectively for them (Barth et. al. 1968). Fangman and Novick (1966) isolated a mutant of E.coli which showed reduced thymidine phosphorylase activity. The gene specifying this enzyme was also shown (Dale and Greenberg 1967) to be located close to and to the left of the thr locus. They used the gene symbol deoA to specify thymidine phosphorylase and subsequently (Lomax and Greenberg 1968)

deoB and deoC to specify deoxyribomutase and deoxyriboaldolase. The designation deo clearly signifies deoxy compounds. We observed that out of the four enzymes viz. thymidine phosphorylase, deoxyriboaldolase, deoxyribomutase and purine nucleoside phosphorylase, at least the latter two are also involved in ribonucleoside catabolism. Hence the symbol deo is not an appropriate designation. In addition, if the four genes constitute two operons (as our data suggest), both of them would share identical nomenclature. Therefore we proposed (Ahmad and Pritchard 1969) tpp, dra, drm and pup as gene symbol for thymidine phosphorylase, deoxyriboaldolase, deoxyribomutase and purine nucleoside phosphorylase respectively until the regulatory relationship of these genes was established. Location of the pup gene was determined in strain P 228.2 as follows:

Mapping of *pup* gene.

in a preliminary mapping experiment to locate *pup* gene, strain P228.2 (Hfr R4, *pup*$^-$, *upp*$^-$, *met*$^-$, *str*^{s}) was mated with strain P203 (F$^-$, *thr*$^-$, *leu*$^-$, *arg*$^-$, *his*$^-$, *trp*$^-$, *str*^{r}). Mating was done as described in the Materials and Methods section. The mating mixture was appropriately diluted and spread on selective minimal agar media containing glucose (0.4 %) and all the other growth requirements of the F$^-$ strain (leucine 40 µg/ml, thiamine 10 µg/ml and other amino acids 20 µg/ml) except the amino acid for which the recombinants were to be selected. Streptomycin (100 µg/ml) was added to the selective media for counter selection. Selection was made for *thr*$^+$*leu*$^+$, *arg*$^+$, *his*$^+$ and *trp*$^+$ recombinants. Recombinants colonies appeared after 24 hrs. incubation at 37° C were purified and cultured on solid minimal media in the form of patches. A loopful of culture from each patch was resuspended in laboratory phosphate buffer and spotted with a pipette on selective agar media containing adenosine (5.0 mM) as sole carbon source. The number of *pup*$^-$ (unable to grow on this medium) and *pup*$^+$ recombinants was recorded after 48 hrs incubation at 37°.

The results (Table 5) indicate that among the four selected classes of recombinants, the *thr*$^+$*leu*$^+$ class carries

Table 5

Location of the *pup*$^-$ mutation (preliminary mapping)

Donor	Recipient	Selected marker	Total	*pup*$^-$ recombinants	Per cent *pup*$^-$ recombinants
P228.2	P203	*thr*$^+$ *leu*$^+$ *str*^{r}	70	65	93
		arg$^+$ *str*^{r}	84	68	81
		his$^+$ *str*^{r}	84	9	14
		trp$^+$ *str*^{r}	70	2	6

the largest percentage of pup$^-$ individuals, indicating that the pup gene is closer to thr leu loci than to any other gene studied in this experiment. The observed linkage between thr leu and pup is about 93 %.

To locate the pup gene with respect to thr and leu loci the experiment was repeated as before, but this time either thr$^+$ or leu$^+$ recombinants were selected. The order of the genes pup, thr and leu could thus be determined by three-point analysis.

The data (Table 6), indicate that the most probable order of the three genes is pup - thr - leu. This has been deduced as follows:

The three possible locations of the pup gene with respect to thr and leu genes are (i) pup - thr - leu (Fig 7) (ii) thr - pup - leu (Fig 8) and (iii) thr - leu - pup (Fig 9). Among thr$^+$ selection (Table 6) we see that the least frequent class which requires 4 cross-overs is leu$^-$ pup$^+$. This indicates that pup is not located according to Fig 8 or 9, since in the case of the gene order shown in Fig 8 it should give pup$^+$ leu$^+$ as the least frequent class and in the case of the gene order shown in Fig 9 the least frequent class should be pup$^-$ leu$^-$. The finding that thr$^-$ pup$^-$ is the least frequent class when selection is made for leu$^+$ recombinants (Table 6) lends further weight to the gene

Table 6

Location of the *pup*$^-$ mutation

Selected recombinants		Genotype				Total
		leu$^+$ *pup*$^-$	*leu*$^-$ *pup*$^+$	*leu*$^+$ *pup*$^+$	*leu*$^-$ *pup*$^-$	
thr$^+$ *str*^{r}	Number obtained	72	3	16	6	97
	Per cent of total	74.2	3.1	16.5	6.2	100
		thr$^+$ *pup*$^-$	*thr*$^-$ *pup*$^+$	*thr*$^+$ *pup*$^+$	*thr*$^-$ *pup*$^-$	
leu$^+$ *str*^{r}	Number obtained	152	110	39	2	303
	Per cent of total	50.2	36.3	12.8	0.7	100

order shown in Fig 7, since it is the pup - thr - leu gene order which requires 4 cross-overs to give $pup^{-}thr^{-}$ recombinants. Thus the data from two selections stongly indicate that the most probable order of the genes is pup - thr - leu.

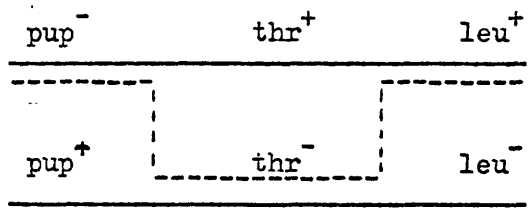


Fig 7

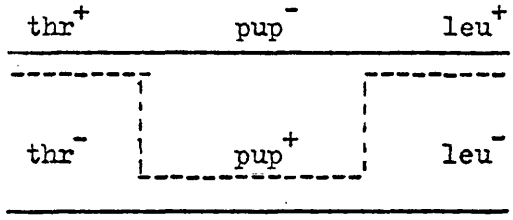


Fig 8

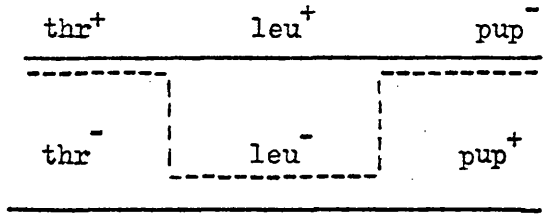


Fig 9

Mapping of *tpp*, *dra*, *drm* and *pup* genes in their respective order.

The relative locations of the four genes viz. *tpp*, *dra* *drm* and *pup*, specifying thymidine phosphorylase, deoxyriboaldolase, deoxyribomutase and purine nucleoside phosphorylase respectively, were determined by P1 transduction. Transductants were purified on minimal agar media containing sodium citrate (2.5 mg/ml) and no calcium chloride. Purified single colonies of each transductant were then cultured in the form of patches on minimal agar media with calcium chloride and no sodium citrate. Transductants carrying a mutation in one of the four genes were classified on the basis of their growth properties on deoxynucleosides or nucleosides as sole carbon source. Mutants of the *pup* class are unable to grow on any purine nucleoside or deoxynucleoside as sole carbon source, mutants of *tpp* class are unable to grow on thymidine, mutants of *dra* class on any deoxynucleoside and *drm* class on any nucleoside or deoxynucleoside (Table 7). Transductants of *drm*$^-$ *tpp*$^-$ and *drm*$^-$ *tpp*$^+$ classes were, however, difficult to classify in this way. This is because both these classes of mutant are unable to grow on all deoxynucleosides and nucleosides as sole carbon source. We thus made use of the fact

Table 7

Gro wth properties of strains with different genotypes

Genotypes					Sole carbon source			Inhibition by		
pup	drm	tpp	dra	upp	Thy-midine	Adeno-sine	Deoxy-adenosine	Thy-midine	Deoxy adenosine	FU+de-oxyade-nosine
+	+	+	+	-	+	+	+	R	R	S
-	+	+	+	-	+	-	-	R	R	R
+	-	+	+	+	-	-	-	R	R	S
+	+	-	+	-	-	+	+	R	R	R
+	+	+	-	+	-	+	-	S	S	
+	-	+	+	-	-	-	-			S
-	-	+	+	-	-					R
+	-	-	+	-		-				R
+	+	-	-	+	-		-	R	S	
+	-	+	-	+	-		-	R	R	

Only those responses which have been confirmed experimentally are shown in the Table

that if pup$^-$ or tpp$^-$ mutants lack in addition UMP-pyrophosphorylase activity (upp$^-$), then they are resistant to a mixture of FU (2.5 µg/ml) plus deoxyadenosine (100 µg/ml), irrespective of their being drm$^+$ or drm$^-$ class (see Table 7).

Another technique used to classify tpp$^+$ and tpp$^-$ classes of transductants carrying the dra mutation was the sensitivity of dra$^-$ tpp$^+$ class to both thymidine and deoxyadenosine and of the dra$^-$ tpp$^-$ class to deoxyadenosine only.

RESULTS

In two experiments (Table 8) the co-transduction frequency between *dra* and *thr* was found to be 29 per cent and 54 per cent, but that between *dra* and *tpp* was more than 95 per cent. Thus *dra* and *tpp* are more closely linked to each other than either of them is to *thr*. If we assume that transductants requiring a minimum of four exchanges between donor and recipient DNA will occur significantly less frequently than those requiring a minimum of two, then the discrepancy between the number of transductants in the reciprocal classes *dra*$^+$ *tpp*$^+$ and *dra*$^-$ *tpp*$^-$ indicates that the order of the three genes is *dra* - *tpp* - *thr*.

The location of *pup* with respect to these three loci can be deduced from the data given in Table 9. In the four-factor crosses numbered 3 and 4, both *tpp* and *thr* were unselected markers. In both crosses the *tpp*$^+$: *tpp*$^-$ ratio among *thr*$^+$ transductants is not significantly different from the corresponding ratio among *thr*$^-$ transductants. This apparent absence of linkage implies that *tpp* does not lie between *thr* and *dra* or *thr* and *pup*. In cross 5, on the other hand, the ratio *pup*$^+$: *pup*$^-$ is significantly different among *thr*$^+$ and *thr*$^-$ transductants implying that *pup* does lie between *thr* and *dra* or *thr* and *tpp*. The data from these three experiments are therefore compatible either with

the gene order dra - tpp - pup - thr or with the order tpp - dra - pup - thr. In addition, the fact that the proportion of tpp^+ transductants is higher in cross 4 than it is in cross 3 implies that tpp-3 is closer to dra than is tpp-2.

Data from four three-factor crosses are given in Table 10. They provide evidence that drm lies between dra and pup but do not define the position of tpp with respect to the other three loci unambiguously. The gene order given in the lower column of this table are those compatible with the data if we are correct in assuming that transductants requiring four exchanges will be significantly less frequent than those requiring two. Comparison of these possible orders shows that two gene arrangements are consistent with the data. They are dra - tpp - drm - pup and dra - drm - pup - tpp (or the inverse of these orders). If we now compare these gene orders with those compatible with the data in Table 8 and 9 we find that only one gene order is consistent with all the data reported. It is dra - tpp - drm - pup - thr.

The data in Table 11 provide evidence bearing on the location of serB. Among ser^+ transductants in cross 10 there were only four recombinants between drm and pup, all of which had the genotype drm^- pup^-. Transductants of this type require

Table 8

Analysis of P1 transduction

Cross	Donor	Recipient	Selected transductants	Unselected marker		Total
				tpp$^+$	tpp$^-$	
1	SA6 *tpp*-1$^-$	SA48 *dra*$^-$ *thr*$^-$	*thr*$^+$	*dra*$^+$	0	503
				dra$^-$	425	4	932
2	SA6 *tpp*-1$^-$	SA48 *dra*$^-$ *thr*$^-$	*dra*$^+$	*thr*$^+$	0	72
				thr$^-$	8	165	245

Table 9

Cross	Donor	Recipient	Selected transductants	Unselected marker			Total
					thr^+	thr^-	
3	P228.2 *pup*$^-$	SA48.1*tpp*-2$^-$ *dra*$^-$ *thr*$^-$	*dra*$^+$ *pup*$^+$	*tpp*$^+$	5	68	
				tpp$^-$	5	40	128
4	P228.2 *pup*$^-$	SA48.2*tpp*-3$^-$ *dra*$^-$ *thr*$^-$	*dra*$^+$ *pup*$^+$	*tpp*$^+$	14	211	
				tpp$^-$	3	27	255
5	P228.2 *pup*$^-$	SA48.2*tpp*-3$^-$ *dra*$^-$ *thr*$^-$	*dra*$^+$ *tpp*$^+$	*pup*$^+$	1	12	
				pup$^-$	59	46	128

Table 10

Cross	Donor	Recipient	Selected transductants	Unselected marker				Total
				dra$^+$	dra$^-$	pup$^+$	pup$^-$	
6	P152drm$^-$	SA46pup$^-$dra$^-$	drm$^+$pup$^+$	11	99			110
7	SA48dra$^-$	SA41pup$^-$drm$^-$	dra$^+$drm$^+$			107	20	127
8	SA6tpp$^-$	SA46pup$^-$dra$^-$	tpp$^+$dra$^+$			24	135	159
9	SA6tpp$^-$	SA41pup$^-$drm$^-$	tpp$^+$drm$^+$			52	8	60

Cross	Compatible gene order[a]
6	drm-dra-pup or dra-drm-pup
7	drm-pup-dra or dra-drm-pup
8	dra-pup-tpp or dra-tpp-pup
9	tpp-pup-drm or tpp-drm-pup

[a]The inverse of each gene order shown is also compatible with the data.

Table 11

Cross	Selected transductants		Unselected marker				Total
			drm$^+$*hsp*$^+$	*drm*$^-$*hsp*$^+$	*drm*$^+$*hsp*$^-$	*drm*$^-$*hsp*$^-$	
10	*ser*$^+$	*pup*$^+$	0	3	0	21	
		pup$^-$	17	0	274	4	319
11	*ser*$^+$	*tpp*$^+$	1	1	5	12	
		tpp$^-$	16	0	219	1	255

Cross	Donor	Recipient
10	P228.2 *pup*$^-$	SA37 *ser*$^-$ *drm*$^-$ *hsp*$^-$
11	SA6*tpp*-1$^-$	SA37 *ser*$^-$ *drm*$^-$ *hsp*$^-$

two exchanges and the absent reciprocal recombinant class four exchanges if the gene order is drm - pup - ser. Similarly in cross 11 there were seven recombinants between tpp and drm, six being tpp^+ drm^+ and tpp^- drm^-. By an analogous argument the indicated gene order is tpp - drm - ser.

Finally, in cross 12 it was found that the drm^+: drm^- ratio was not significantly different among thr^+ and thr^- transductants, implying that drm is not located between ser and thr. The data also show that drm is closer to ser than it is to thr and the indicated gene order is therefore drm - ser - thr.

The only gene order compatible with the data from all twelve crosses is: dra - tpp - drm - pup - ser - thr (Fig. 10).

If this order has been correctly determined we should expect that the hsp locus should lie to the left of dra since Glover and Colson (1969) have shown that this locus lies to the left of ser and gives only about 20 per cent co-transduction with it. The data in Table 11 seem to be incompatible with this order. Among ser^+ transductants the proportion which carry the donor allele drm^+ is smaller among those which are hsp^+ than it is among those which are hsp^- in both crosses. This result by itself would indicate that the gene order was hsp - ser - drm. In neither case is the difference in the proportion of drm^+

Table 12

Cross	Donor	Recipient	Selected transductants	Unselected marker		Total
				thr^+	thr^-	
12	SA48 *dra*$^-$ *thr*$^-$	SA37 *ser*$^-$ *drm*$^-$	*ser*$^+$	*drm*$^+$	137	78	
				drm$^-$	5	2	222

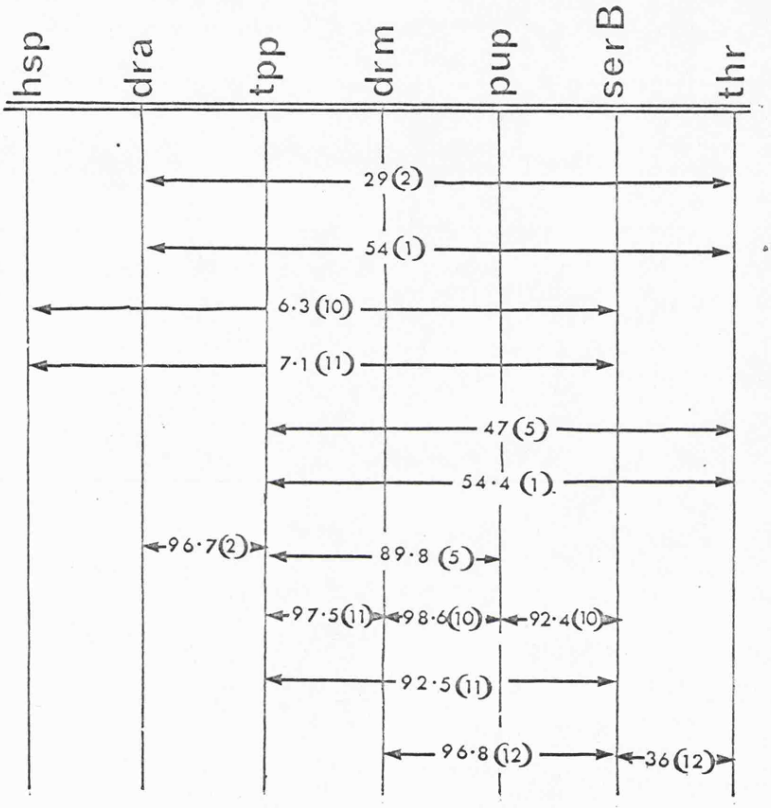


Fig 10

Linkage map. The gene order given is based on the criteria described in the thesis. The number in brackets after each of the given co-transduction frequncies refer to the cross number from which the frequencies are calculated. Co-transductant frequencies were not calculated from crosses in which the selected transductants required an exchange between a donor and recipient marker to avoid negative interference complication (Gross and Englesberg 1959)

transductants among hsp$^+$ and hsp$^-$ classes statistically significant however. In addition, the co-transduction frequency between hsp and ser found in cross 10 and 11 (6.3 and 7.2 per cent respectively) is lower than that found by Glover and Colson (1969). They observed about 20 per cent co-transduction although the hsp$^-$ ser$^-$ strain used by them was the parent of the one used here. It is therefore possible that the presence of the additional mutations being mapped by us in these strains interfered with the expression of the Hsp$^-$ phenotype as determined under the conditions described.

Mapping of udp (uridine phosphorylase) gene.

In preliminary mapping to locate the udp gene two interrupted mating experiments were performed (as described in the Materials and Methods section), using strains Hfr P4X (thi^-, str^s) and SA79 (F^-, thi^-, udp^-, tpp^-, $metB^-$, str^r). Conjugants were interrupted at various intervals and plated on selective minimal agar media to select met^+ and udp^+ recombinants in the presence of streptomycin. The udp^+ recombinants were selected by making use of their ability to grow on uridine (5.0 mM) as sole carbon source. In the first experiment (Fig 11) the data plotted gave the number of met^+ and udp^+ recombinants colonies appearing after 24 hrs incubation. In the second experiment (Fig 12) selection was made for only met^+ recombinants after interruptions at various intervals. A proportion of the recombinant colonies from each sample were then tested for their ability to grow on uridine. From this result the total number of udp^+ recombinants among met^+ recombinants selected was calculated at each blending time. Results obtained by these two experiments indicated that the udp gene is located near metB and about 2-3 minutes distal to it (the origin of Hfr P4X is near the proB marker and transfer is counter-clockwise).

A more accurate indication of the location of the udp

Fig 11

Interrupted mating curve to locate udp

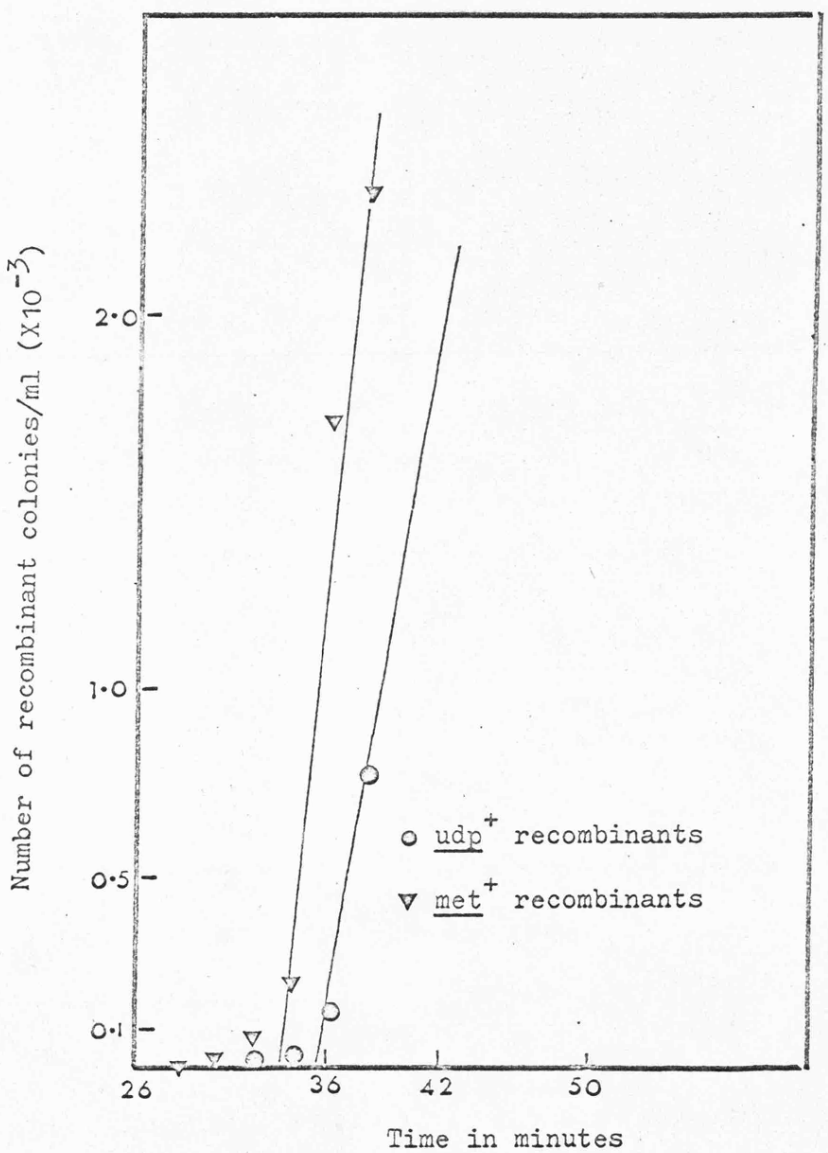
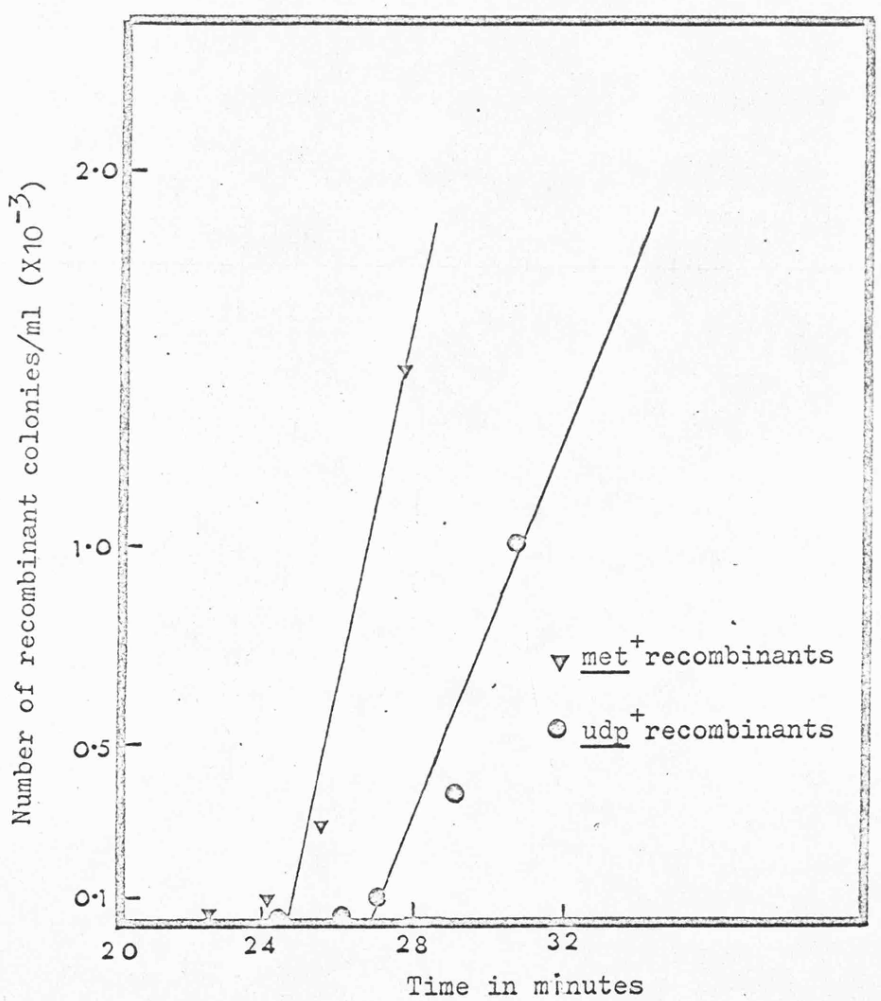


Fig 12

Interrupted mating curve to locate udp



gene was obtained by P1 transduction. A virulent mutant of P1 grown on strain SA53 (*metB*$^-$, *udp*$^-$, *tpp*$^-$, *upp*$^-$) was used to transduce strain SA107 (*metE*$^-$, *rha*$^-$). Selection was made for either *met*$^+$, *rha*$^+$ or *met*$^+$*rha*$^+$ transductants. The selective media contained glucose, rhamnose (10 mM) plus methionine or rhamnose (10 mM) respectively. After purification, the transductants were classified as *udp*$^+$ or *udp*$^-$ by their ability to grow on uridine as sole carbon source. The results (Table 13) show that the frequency of co-transduction between *udp* and *met* is 66 per cent and that between *udp* and *rha* is about 4 per cent. This indicates that *udp* is closer to *metE* than to *rha* . Among *metE*$^+$ transductants (selection 1) the frequncy of *rha*$^+$*udp*$^+$ class is lowest, which indicates that the most improbable gene order is *met* - *rha* - *udp*. Among *rha* transductants (selection 2), *met*$^+$*udp*$^+$ is the least frequent class, thus the gene order *udp* - *met* - *rha* is also least probable. The most probable gene order deduced by these two results is therefore, *metE* - *udp* - *rha*. The result obtained (selection 3) when selection was made for *met*$^+$*rha*$^+$ transductants lends further weight to the deduced gene order, since the majority of *met*$^+$*rha*$^+$ transductants are *udp*$^-$ which requires two cross-overs and the corresponding *met*$^+$*rha*$^+$ class requires four cross-overs if the gene order is *met* - *udp* - *rha*.

Table 13

Linkage between metE, rha and udp

Selection	Selected transductants	Unselected marker	udp$^+$	udp$^-$	Total
1	met$^+$	rha$^+$	1	4	
		rha$^-$	98	185	288
2	rha$^+$	met$^+$	0	3	
		met$^-$	92	1	96
3	met$^+$rha$^+$		4	10	14

The donor strain was SA53. The recipient (SA107) had the following genotype: metE$^-$ rha$^-$.

CHAPTER IV (a)

Analysis of regulatory mutation affecting nucleoside catabolic enzymes.

We have shown that the four genes, dra, tpp, drm and pup specifying deoxyriboaldolase, thymidine phosphorylase, deoxyribomutase and purine nucleoside phosphorylase respectively, are contiguously linked to each other at position 89-90 minutes on the linkage map of E.coli. All these enzymes are induced by a common inducer, which appears to be dRib-5-P. Purine nucleoside phosphorylase and deoxyribomutase, in addition, are induced by purine ribonucleosides, and a recent report (Munch-Petersen, personally forwarded manuscript) indicate that the actual inducers are guanosine and inosine. Adenosine can induce only after prior deamination to inosine.

The biological role of these enzymes in wild type strains is probably mainly the breakdown of nucleosides rather than their synthesis. In thymine-requiring mutants, however, thymidine phosphorylase has an additional role. In these strains, due to a high endogenous dRib-1-P pool, thymine is utilised via thymidine phosphorylase (reaction 11) and incorporated into DNA. Absence of deoxyriboaldolase or deoxyribomutase activity enhances the ability of thy mutants to utilise thymine for growth. This is because one route by which dRib-1-P is degraded is blocked and the availability of this compound for synthesis of thymidine is consequently increased. Biosynthesis of thymidine from thymine is

nevertheless an artefact since wild type strains cannot utilised exogenous thymine due to the lack of endogenous dRib-1-P.

The very close linkage of the four genes viz. dra, tpp, drm and pup (Fig 19), suggests that they may be components of a single unit of transcription. Consistent with this possibility is the fact that these four enzymes are induced by deoxynucleosides and share the same inducer, dRib-5-P. On the other hand, although the aldolase and thymidine phosphorylase are coordinately induced, and the mutase and purine nucleoside phosphorylase likewise, the induction ratio of the two pairs is different (Munch-Petersen 1968; Bonney and Weinfeld 1971). In addition, since purine nucleoside phosphorylase and deoxyribomutase are induced by purine ribonucleosides (Munch-Petersen 1968; Robertson et al. 1970) it seems more likely that the two pairs of genes viz. dra, tpp and drm, pup are components of two adjacent operons. The gene order (Fig 10) is consistent with such a possibility.

To further elucidate the biological role of these nucleoside catabolising enzymes and the functional relationship between the genes specifying them , some mutants of E.coli defective in the regulation of the synthesis of these enzymes were analysed. In this chapter the properties of one such regulatory mutant (Y-70-22) are described.

This strain (Y-70-22) originally isolated by Okada (1966) from among a small sample of Tlr mutants was later analysed by Munch-Petersen (1968[a]) and found to have exceptional properties: It lacks detectable mutase activity and unlike the majority of drm⁻ strains (which have low level of the other three enzymes, and are not inducible by deoxyribosides since their catabolism of dRib-1-P is blocked) this mutant is apparantly constitutive for both thymidine phosphorylase and deoxyriboaldolase. Scrutiny of data of Lomax and Greenberg (1968), Hoffee (1968) and Hoffee and Robertson (1969) suggests that mutants of the same type were present among the Tlr mutants they isolated in E.coli and S. typhimurium.

The frequency with which drm⁻ constitutive strains arise among Tlr mutants suggested to us that they were the result of a single mutation. On a single operon model they can hardly be mutants of this type since the drm gene lies between pup and tpp. On a two operon model, however, a mutant with these properties might arise as a result of a deletion encompassing the operator region of the dra tpp operon and extending into the drm gene, thus fusing the two operons together and bringing the dra tpp operon under the control of the operator of the pup drm operon. It also implies

that transcription of both the operons occurs in the counter clockwise direction (Fig 13).

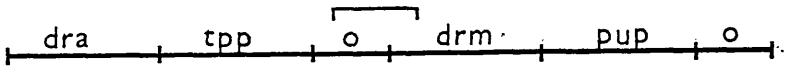


Fig 13

To test the hypothesis that Y-70-22 is a *drm* - O^c deletion, the strain was plated on minimal synthetic medium containing thymidine as sole carbon source. After 48 hrs incubation it was observed that although *drm*$^+$ revertant colonies did not appear spontaneously, they did appear when NTG was used to induce reversion. This result suggested that Y-70-22 does not carry a deletion mutation in the *drm* gene. In addition, thymidine phosphorylase and purine nucleoside phosphorylase were still constitutive (Table 14). This suggested that the *drm* mutation was not the cause of constitutivity.

In order to further investigate the possibility of the constitutivity of the enzymes being due to a mutation linked with the structural genes, strain Y-70-22 was transduced by P1 grown on a *drm*$^+$ *thr*$^+$ strain (P227) and *drm*$^+$ *thr*$^+$ transductants were selected. Several of these transductants were analysed for constitutivity for thymidine phosphorylase and purine nucleoside phosphorylase. The results (shown for one such transductant, SA112, in Table 14) indicated that they were still constitutive for the relevant enzymes and therefore the mutation causing the constitutivity is not linked to *drm* or the other genes involved in the catabolism of deoxynucleosides. Strain Y-70-22 therefore carries at least two lesions in addition to the *thy* mutation; one in the

drm gene, which like other mutations of this type, is co-transducible with the *thr* locus, and a second conferring constitutivity which is not closely linked to *drm*.

The *drm*$^+$ transductants and revertants of Y-70-22 were found to have acquired an unusual property. Unlike typical *thy*$^-$ *drm*$^+$ strains they cannot form colonies on solid media containing 20 µg/ml of thymine. Instead they require about 50 µg/ml of thymine (Table 15). To determine whether this difference was due to the constitutivity mutation, an HfrH strain (SA92) was mated with a *drm*$^+$ *str*^{r} derivative of Y-70-22 (SA89) and selection was made for recombinants which require 20 µg/ml of thymine for growth. Several recombinants carrying Gal$^-$ phenotype were tested for thymidine phosphorylase and purine nucleoside phosphorylase activities with and without thymidine as inducer. Basal levels of both enzymes were found in all recombinants tested and they were again inducible (strain SA91 in Table 14).

With such a high frequency the selection of *drm* constitutive mutants has been reported that it led us to look for an explanation for this. One possibility is that there is a selective pressure which favours the isolation of such double mutants on media containing very low concentration of thymine. To determine if this is so, thymine low requiring mutants were

Table 14

Thymidine phosphorylase and purine nucleoside phosphorylase activities in various strains

Strain	Genotype	Thymidine (as inducer)	Enzyme activities*	
			Thymidine phosphorylase	Purine nucleoside phosphorylase
P152	$\underline{thy}^-\underline{drm}^-$	-	0.15	0.05
		+	0.18	0.07
Y-70-2	$\underline{thy}^-\underline{drm}^+$	-	0.25	0.05
		+	1.2	0.16
Y-70-22	$\underline{thy}^-\underline{drm}^-$	-	3.34	0.16
		+	4.59	0.28
SA89	$\underline{thy}^-\underline{drm}^+$	-	3.36	0.21
		+	3.56	0.23
SA91	$\underline{thy}^-\underline{drm}^+$	-	0.14	0.06
		+	0.76	0.13
SA112	$\underline{thy}^-\underline{drm}^+$	-	3.56	0.24
		+	4.12	0.32

*Enzyme activities are expressed in μmole/base/minute/mg protein

Table 15

Growth characteristics of some thymine requiring strains on various concentrations of thymine.

Concentration of thymine (µg/ml)	Number of colonies of various strains appearing on plates.			
	P152	Y-70-22	SA73	SA89
0.1	0	0		
0.25	0	185		
0.5	0	178		
1.0	128	154		
2.0	163	203		
10.0			0	0
20.0			134	0
30.0			110	0
50.0			159	289
100.0			138	281
Nutrient agar plus thymine (100 µg/ml)	153	193	114	202

isolated in a thy⁻ strain (SA119) on minimal agar media containing 5.0, 2.5 or 1.0 µg/ml of thymine. These mutants were classified (a) nutritionally to distinguish between dra⁻ and drm⁻ classes by growing them either on adenosine (5.0 mM) or thymidine (5.0 mM) as sole carbon source and (b) according to their thymine requirement (Table 16). The majority were found to be drm⁻ and among these some isolated on 2.5 µg/ml and 1.0 µg/ml of thymine were able to grow on 0.5 µg/ml of thymine. The specific activity of thymidine phosphorylase was determined in 38 of these drm⁻ mutants and 2 were found to be constitutive. Both these mutants were isolated on 1.0 µg/ml of thymine and they were able to grow on 0.5 µg/ml. Thus it appears that Tlr mutants are heterogenous with respect to their thymine requirement and that some of those with an exceptionally low thymine requirement (super-lows) are constitutive like Y-70-22. In our previous selection, the failure to obtain such a variety of mutants has no doubt been due to the fact that we have used relatively high concentrations (2.0-5.0 µg/ml) of thymine. The other thymine super-low requirers which are non-constitutive for thymidine phosphorylase are probably like the well known thymine requiring strain E.coli 15T⁻ (555-7). Strain 15T⁻ is also a drm⁻ super-low (see Pritchard and Zaritsky 1970) its exceptionally low thymine requirement is due to a

Table 16

Properties of Tlr mutants isolated from a *thy*$^-$ strain (SA119) plated on different concentrations of thymine.

Concentration of thymine (µg/ml)	Number isolated	*drm*$^-$				*dra*$^-$	
		Total	No. able to grow on 0.5µg/ml thymine	No. tested for constitutivity	No. constitutive	Total	No. able to grow on 0.5 µg/ml thymine
5.0	18	17	0			1	0
2.5	51	37	12	19	0	14	0
1.0	25*	21	5	19	2	4	0

*One among these was *thy*$^+$ revertant.

difference in the efficiency with which thymidine is converted to thymidine triphosphate, and is not associated with a constitutivity mutation (Beacham et al. 1971).

From these results we conclude that constitutivity of nucleoside catabolic enzymes alters the thymine requirement. A possible explanation for the very high (50 µg/ml) thymine requirement of strain SA89 (drm$^+$ derivative of Y-70-22) is that the high specific activity of nucleoside catabolic enzymes (including presumably deoxyribomutase) leads to a rate of breakdown of nucleoside so large that the supply of deoxynucleosides becomes rate limiting. The effect of this in a drm$^+$ strain would be to reduce the intracellular concentration of dRib-1-P and thus raise the thymine requirement. If our explanation for the very high thymine requirement is correct then strain Y-70-22 should have an exceptionally low requirement for thymine, because not only will the degradation of dRib-1-P be blocked but its rate of production, which we know to be from deoxynucleosides (Beacham and Pritchard, 1971), will be enhanced by the high level of deoxynucleoside phosphorylases. This consequently will lead to an exceptionally large intracellular accumulation of dRib-1-P.

In keeping with this hypothesis, strain Y-70-22, a drm$^+$ derivative of it (SA89), and typical thy$^-$ (SA73) and thy$^-$ drm$^-$

(P152) strains were grown on several thymine concentrations. Exponentially growing cultures (approximately $1X10^8$ cells/ml) were diluted by 10^5 and 0.1 ml spread on glucose synthetic agar containing the indicated concentrations of thymine (Table 15) and other growth requirements or on nutrient agar supplemented with thymine. The number of colonies was recorded after 30 hrs incubation at 37°. Results (Table 15) indicate that whereas a typical thy⁻drm⁻ (P152) strain can only form colonies on 1.0 μg/ml of thymine, the thy⁻drm⁻ constitutive strain (Y-70-22) does so on medium containing as little as 0.25 μg/ml of thymine. (Note that although colonies of P152 appeared on plates containing 1.0 μg/ml of thymine, growth is only "normal" (see Pritchard and Zaritsky 1970) in liquid culture if the medium contains at least 2.0 μg/ml).

It seems likely that the constitutivity mutation in strain Y-70-22 has occured in a true regulator gene rather than in a gene which indirectly alters the intracellular concentration of dRib-5-P since the high level of thymidine phosphorylase is similar in drm⁺ and drm⁻ strains and the specific activity is as high as can be obtained under optimal inducing conditions (incubation of a dra⁻ culture in the presence of a deoxynucleoside) in non-constitutive strains. We proposed to name this gene

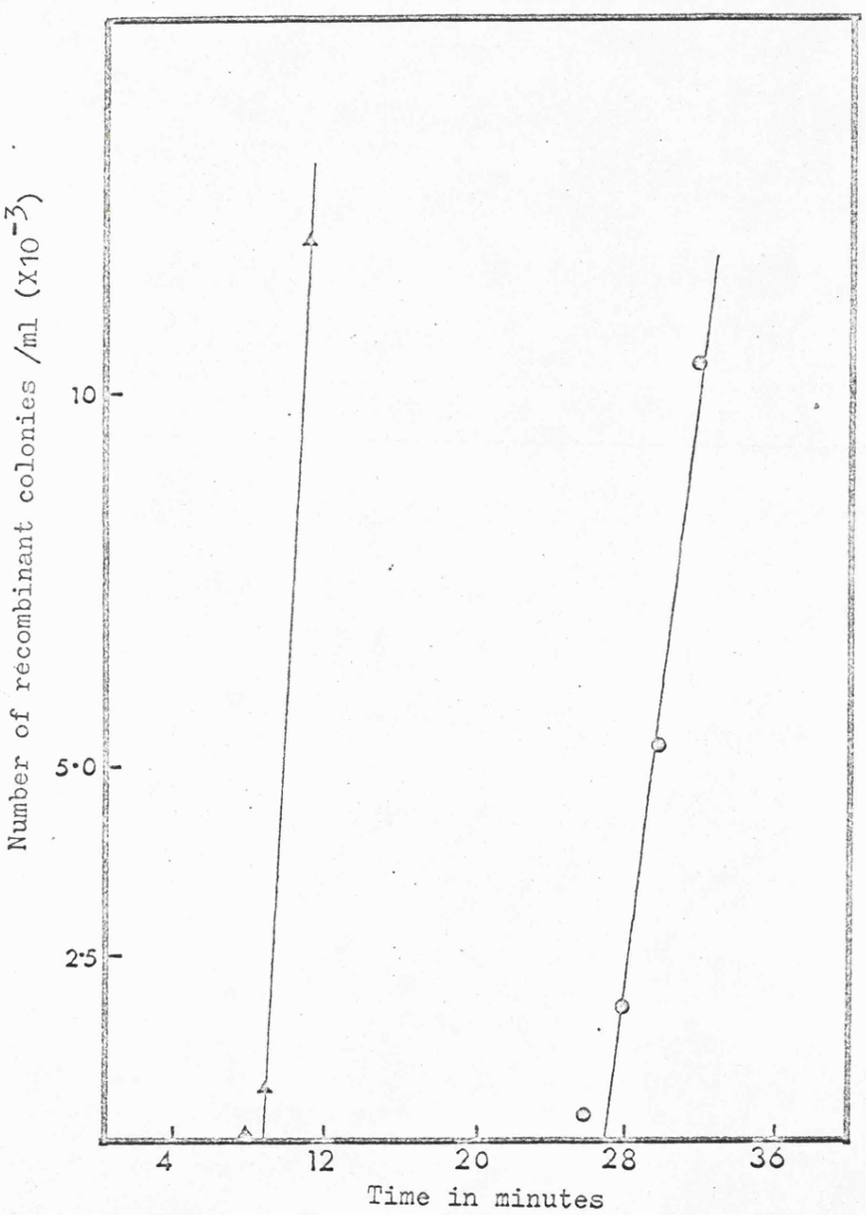
nucR since it regulates the synthesis of a set of enzymes all of which are concerned with the catabolism of nucleoside (Ahmad and Pritchard 1971).

Mapping of nucR gene.

Nucleoside regulatory (nucR) gene was located by interrupted mating experiments using strain P293 (HfrH, thi$^-$, str^s) and strain SA89 (a drm$^+$, str^r derivative of Y-70-22). Selection was made for leu$^+$ recombinants and recombinants which were able to grow in the presence of 20 µg/ml of thymine. Results (Fig 14) indicate that the nucR gene enters approximately 17-18 minutes after leu reading clockwise round the E.coli linkage map. The experiment was repeated using the same Hfr and an F$^-$ strain (SA114) carrying a galE mutation in addition to the mutation in the nucR gene. No discrepancy with the previous result was observed (Fig 15). The conclusion was drawn that the nucR locus is located 2-3 minutes distal to galE reading clockwise.

Fig 14

Interrupted mating curve to locate nucR

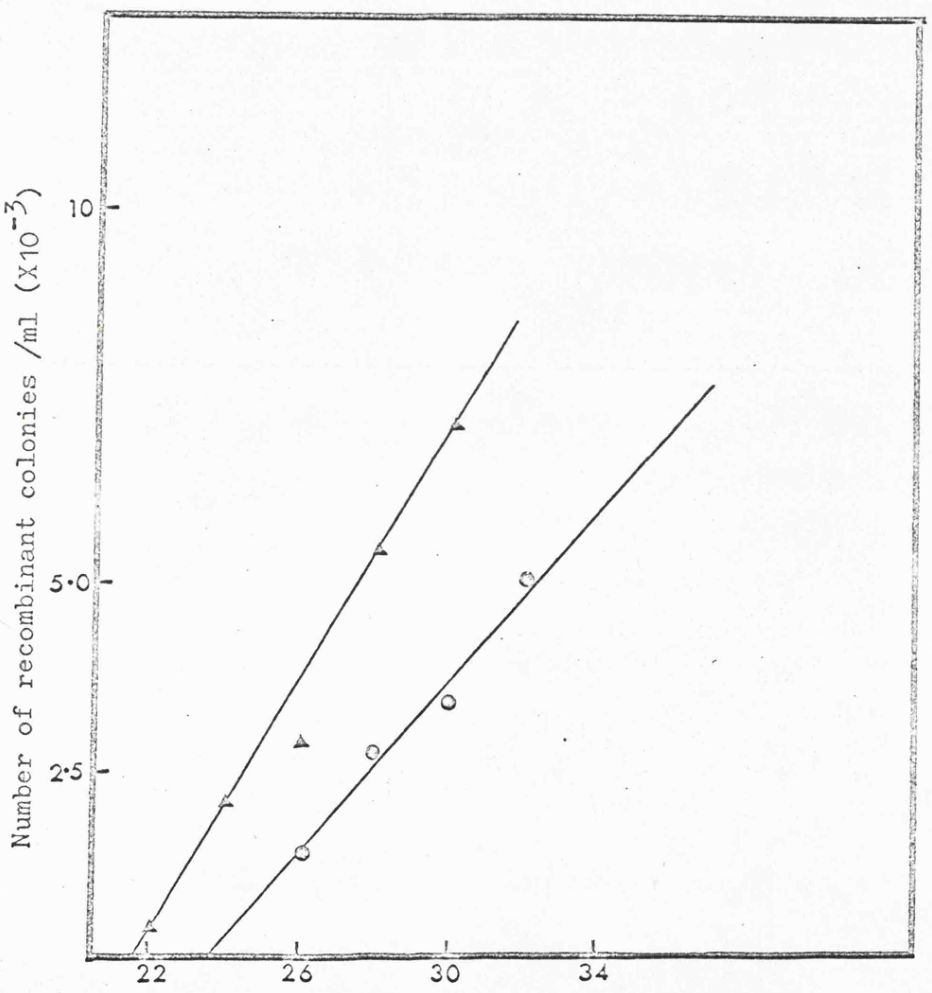


▽ leu⁺ recombinants

○ Recombinants able to grow in the presence of 20 µg/ml of thymine

Fig 15

Interrupted mating curve to locate nucR



▲ galE⁺ recombinants

○ Recombinants able to grow in the presence of 20 µg/ml of thymine

CHAPTER IV (b)

In the previous chapter it has been shown that when thy mutants are plated on media containing low concentrations of thymine (1.0 - 5.0 μg/ml), thymine low requiring mutants able to grow at these concentrations appear. The majority of these are either drm⁻ or dra⁻. Further analysis of 38 of these Tlr mutants, led to the observation that two of the drm mutants were constitutive for thymidine phosphorylase. Both these mutants (SA115 and SA116) were able to grow on 0.5 μg/ml of thymine and were obtained among Tlr mutants able to grow on 1.0 μg/ml of thymine.

In this chapter a further investigation of one of the two mutants (SA115) is described. The results indicate that, although this strain appears to possess some of the properties of strain Y-70-22, such as the ability to form colonies on very low concentration (0.5 μg/ml) of thymine and in being constitutive for thymidine phosphorylase, the mutations determining constitutivity of thymidine phosphorylase are different. The mutation in strain SA115 rendering thymidine phosphorylase constitutive, is similar to some operator constitutive (O^c) mutations of the lac operon (Jacob and Monod 1964). The former mutation does not affect purine nucleoside phosphorylase activity and it has therefore been assumed that at least pup

does not belong to the same operon as tpp. The putative operator mutation is located to the left of dra. These inferences were made as follows:

To compare whether the mutation leading to constitutivity in strain SA115 is similar to the nucR mutation in strain Y-70-22, specific activities of purine nucleoside phosphorylase, thymidine phosphorylase and deoxyriboaldolase in strain SA115 were determined in the absence and presence of thymidine as inducer. Results (Table 17) indicate that unlike Y-70-22, strain SA115 is not constitutive for purine nucleoside phosphorylase and lacks deoxyriboaldolase activity.

The next question therefore to ask was whether the mutation conferring constitutivity is linked with the structural genes. To examine this, thr^+ transductants of strain SA115 were produced, using P1 grown on strain P227. These transductants were classified nutritionally into dra^+drm^+ and dra^-drm^- classes (The observed co-transduction frequency between dra drm and thr was 53 %). Six of these $thr^+dra^+drm^+$ transductants were then assayed for thymidine phosphorylase activity. They all were found to have the basal level of activity and the enzyme was further inducible by thymidine (the specific activity in one such transductant, SA139, is given in Table 17). From the

results it was postulated that the mutation in strain SA115 rendering thymidine phosphorylase constitutive is linked with the thr locus. Determination of the location of the mutation was considered to be the next important step.

In order to find the locus, a spontaneous drm$^+$ revertant (SA142) was first isolated from a thy$^+$ derivative (SA141) of SA115. It was necessary to use a thy$^+$ derivative of the strain since in the thy$^-$ dra$^-$ class, in contrast to thy$^+$ dra$^-$ strains (Munch-Petersen 1970), the uninduced level of thymidine phosphorylase is normally very high (Barth et al. 1968). The drm$^+$ revertants were selected by spreading strain SA141 on plates containing adenosine (5 mM) as sole carbon source. One such thy$^+$drm$^+$ revertant (SA142) which did not revert to dra$^+$ (since it is still unable to grow on any deoxynucleoside as sole carbon source) was analysed for thymidine phosphorylase, deoxyribo-aldolase and purine nucleoside phosphorylase activities. No alteration in the level of these enzymes due to the thy$^+$drm$^+$ reversion was observed (Table 17). We therefore concluded that the constitutivity of thymidine phosphorylase is not due to the mutation in drm. Also the putative O^c mutation is not an O^c-drm deletion (Fig 13).

To determine the location of the O^c mutation, strain

Table 17

Thymidine phosphorylase, purine nucleoside phosphorylase and deoxyriboaldolase activities in strain SA119, P57 and their derivatives.

Strain	Thymidine (as inducer)	Enzyme activities*		
		Thymidine phosphorylase	Purine nucleoside phosphorylase	Deoxyribo-aldolase
SA119	-	123	96	143
	+	1122	264	1382
SA115	-	968	84	n.d.
	+	1353	103	n.d.
SA139	-	189	139	200
	+	2637	281	2568
SA142	-	1139	143	n.d.
SA143	-	55	53	80
	+	1229	193	2155
P57	-	73		64
SA147	-	1090		385

*The specific activities are expressed in m µmoles/base/min/mg protein and were determined in extracts prepared by ultrasonication.

n.d. = not detected

SA142 was transduced by P1 grown in a $\underline{tpp}^-$ strain (SA6). Selection was made for $\underline{tpp}^+\underline{dra}^+$ transductants using thymidine as sole carbon source. It was assumed that if the mutation rendering constitutivity is located between tpp and drm then the majority of $\underline{tpp}^+\underline{dra}^+$ transductants would remain constitutive for thymidine phosphorylase. (Fig 16). Analysis of 10 such transductants (the specific activity

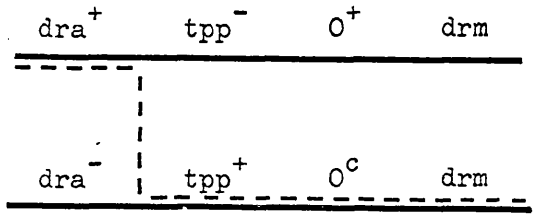


Fig 16

in one such transductant, SA143, is given in Table 17) revealed that they have acquired the basal level activity of thymidine phosphorylase and deoxyriboaldolase and these enzymes were further inducible. The results indicated that the constitutivity mutation is not located between the tpp and drm loci.

To determine whether the constitutivity mutation is located to the left of dra or in between dra and tpp, several dra mutants of an HfrH strain (P57) were first isolated. These were analysed to find a suitable mutant which would intragenically recombine with the dra mutation of strain SA142. Strain SA144 was found to be able to do so giving rise to dra^+ recombinants. Preparation of this dra^- donor was completed by isolating a tpp^- mutation of it, as described in the Materials and Methods section. Addition of the tpp mutation was necessary in order to determine the relative positions of the dra mutations with respect to tpp. The tpp^- strain was discriminated due to its inability to grow on thymine (20 μg/ml) and ability to grow on thymidine (30 μg/ml) as a thymine source. Strain SA145 (HfrH, dra^-, tpp^-) was then mated with strain SA142 (drm^+ derivative of SA115) and selection was made for intragenic dra^+ recombinants using deoxyadenosine as sole carbon source. Sixty four such dra^+ recombinants were classified as either tpp^+ or tpp^-. Fifty eight of them were tpp^+.

The result shows that the dra mutation in the donor strain is closer to tpp than the dra mutation in the recipient strain is to tpp (Fig 17). Determining thymidine phosphorylase activity in 17 recombinants, showed that 16 of them have acquired the basal level activity. One recombinant (SA147) however, has high levels of thymidine phosphorylase and deoxyriboaldolase activities. (Table 17). From these results it was concluded that the location

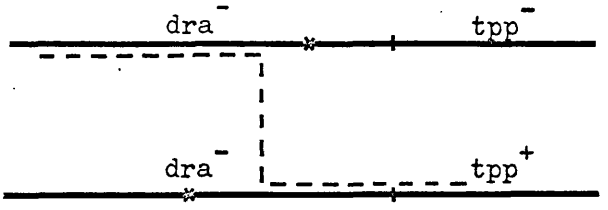


Fig 17

of the mutation leading to constitutivity of deoxyriboaldolase and thymidine phosphorylase is located to the left of *dra* and since the mutations in the operator and *dra* are seggregatable, it is not an O^c- *dra* deletion.

In order to determine the dominance relationship in the heterozygous condition, a *recA*$^-$ *ara*$^-$ derivative of strain SA115 was first isolated. The presence of *rec*$^-$ mutation was considered to be necessary in order to produce a stable diploid strain. The presence of the *ara* mutation is useful in discriminating between the colonies carrying F-primes and those which are F$^-$.

A *recA*$^-$ derivative (SA154) of SA115 was obtained by mating with SA153 (Hfr KL16-99, *recA*$^-$, *str*^{s}) and selecting *thy*$^+$ recombinants. Two out of 12 *thy*$^+$ recombinants were u.v. sensitive. They were considered as *recA*$^-$ mutants. An *ara*$^-$ derivative (SA155) of strain SA154 was then obtained by 2-aminopurine treatment and selecting *ara*$^-$ colonies on arabinose (1.0 %) plus tetrazolium (0.005 %) plates.

Strains harbouring F-primes which carry the *thr*$^+$, *leu*$^+$ and *tpp*$^-$ loci were produced using the technique of Brooks Low (1968). The donor strain was HfrH, *tpp*$^-$ (SA156) and the recipient was SA150 (*thr*$^-$, *leu*$^-$, *recA*$^-$ *str*^{r}). Twelve out of 13 diploids

selected in this experiment were found to be able to transfer the thr and leu markers to an F^-, thr^- leu^- strain. One of these diploid strains (SA157) was then mated with strain SA155 ($recA^-$, ara^- derivative of SA115) and selection was made for the colonies able to grow in the absence of threonine and leucine. A further analysis revealed that they were heterozygous strains since they produced Ara^+ and Ara^- colonies when streaked on arabinose plus tetrazolium plates. They were phenotypically Dra^+ also.

Determining the enzyme activities in one of these heterozygous strains (SA169) it was found (Table 18) that thymidine phosphorylase was still constitutive. The level of deoxyriboaldolase activity was basal and the enzyme was further inducible by thymidine.

Thus the constitutivity mutation in strain SA115 is dominant with respect to thymidine phosphorylase and recessive with respect to deoxyriboaldolase. The simplest interpretation of these results is that the mutation in strain SA115 is cis-dominant affecting both thymidine phosphorylase and deoxyriboaldolase. Therefore the genes specifying thymidine phosphorylase and deoxyriboaldolase constitute a single operon with the direction of transcription being clockwise. To confirm this interpretation, however, a further analysis of the dominance relationships is considered to be necessary.

Table 18

Thymidine phosphorylase and deoxyriboaldolase activities in strain SA119 and *thy*$^+$ *recA*$^-$ derivative of SA115 in haploid and diploid conditions.

Strain	Genotype	Thymidine (as inducer)	Enzyme activities	
			Thymidine phosphorylase	Deoxyribo-aldolase
SA119	*thy*$^-$	-	79	97
		+	926	1144
SA155	*thy*$^+$ *rec*$^-$ *drm*$^-$	-	519	not detected
		+	775	"
SA169	F′ *dra*$^+$ *tpp*$^-$ / *dra*$^-$ *tpp*$^+$	-	566	92
		+	931	1182

Enzyme activities were determined in extracts prepared by toluenisation and are expressed in m μmoles/base/minute/mg protein.

CHAPTER V

Analysis of cytosine deaminase mutants

Cytosine deaminase and the location of the gene specifying the enzyme.

In E.coli and several other bacteria e.g. Proteus vulgaris, Corynebacterium, Mycobacterium, Lactobacillus, S. typhimurium, the first step in the metabolism of cytosine has been shown (Chargaff and Kream 1948; Bresnick et al. 1960; Hayashi 1952) to be deamination to uracil (reaction 5).

The enzyme catalysing this reaction, cytosine deaminase, is believed to be the only enzyme involved in the first step of utilisation of exogenous cytosine.

Mutants of S. typhimurium lacking cytosine deaminase activity were isolated (Neuhard and Ingraham 1968) as colonies resistant to 5-fluorocytosine. This cytosine analogue inhibits cell growth only after its conversion to FU, this reaction being catalysed by cytosine deaminase. When bacterial mutants resistant to fluorocytosine (FC) are isolated, the class of mutant lacking cytosine deaminase activity remains sensitive to FU.

As far as present knowledge goes, no mutant of E.coli lacking cytosine deaminase activity has yet been isolated. In this chapter isolation of E.coli mutants lacking cytosine deaminase activity and location of the gene (cod) specifying this

enzyme on the E.coli linkage map is described.

Our data support the previous observation that in E.coli exogenous cytosine can only be metabolised via its conversion to uracil, catalysed by cytosine deaminase. This has been deduced from the isolation of a mutant strain which, in addition to lacking orotidylic acid pyrophosphorylase activity (pyrE$^-$, unable to grow in absence of cytosine or uracil or their nucleosides) also lacks cytosine deaminase activity. Such a mutant is unable to grow on cytosine but can grow on cytidine, uridine or uracil as sole pyrimidine sources.

Preparation of a pyrimidine requiring mutant lacking cytosine deaminase activity.

Fluorocytosine resistant mutants were first isolated in strain P226 by spreading a lawn of culture on minimal agar plates supplemented with 20 µg/ml of FC and selecting resistant colonies which appeared after 48 hrs incubation. On further examination it was observed that some of these mutants were resistant and some sensitive to FU (0.25 µg/ml). Determination of cytosine deaminase activity (Table 19) in one such FC-resistant and FU-sensitive mutant (SA74) indicated that this strain lacked the relevant enzyme (cod$^-$).

Table 19

Cytosine deaminase activity in strain P226 and SA74.

Strain	Enzyme activity m µmole/base/min/mg protein
P226	84.6
SA74	not detected

Applying a similar selective technique to the isolation of a cytosine deaminase mutant in a multiple auxotroph P203 (*thr*$^-$, *leu*$^-$, *arg*$^-$, *his*$^-$, *trp*$^-$, *xyl*$^-$, *str*^{r}), it was observed that (for some unknown reason) isolation of mutants resistant to FC was not easy in this strain. No colony able to grow at 10 or 20 µg/ml of FC was observed after 96 hrs incubation. Strain P203 was therefore transduced using P1 grown on strain SA74 and selection was made for FC-resistant transductants. About 20 transductants were purified and tested for their sensitivity to FU (0.25 µg/ml). They all were FU-sensitive. It was assumed that the colonies found after P1 treatment were true transductants since a control plate of P203 not pretreated with P1 had no visible colonies.

In order to prepare a *cod*$^-$ *pyrE*$^-$ derivative of one of these F$^-$ strains (SA161), it was mated with strain SA83 (HfrC, *metB*$^-$, *pyrE*$^-$, *str*^{s}; *pyrE* is located near *xyl* at 72 minutes on *E.coli* linkage map). Selection was made for *xyl*$^+$ recombinants using 0.4 % xylose as sole carbon source. Selective media in addition to containing the growth requirements of the F$^-$ strain, contained uridine (50 µg/ml). The *xyl*$^+$ recombinants were then tested for inability to grow in the absence of uridine (*pyrE*$^-$ derivatives). Four of the *xyl*$^+$ *pyrE*$^-$ derivative thus isolated

were further examined for their ability to grow on cytosine as pyrimidine source. None of them showed any growth. Strain SA168 (one of the four recombinants) however, is able to grow on uracil, uridine or cytidine as sole pyrimidine source. We assume that the lack of cytosine deaminase activity renders the strain unable to utilise cytosine for growth.

Location of the cod (cytosine deaminase) gene.

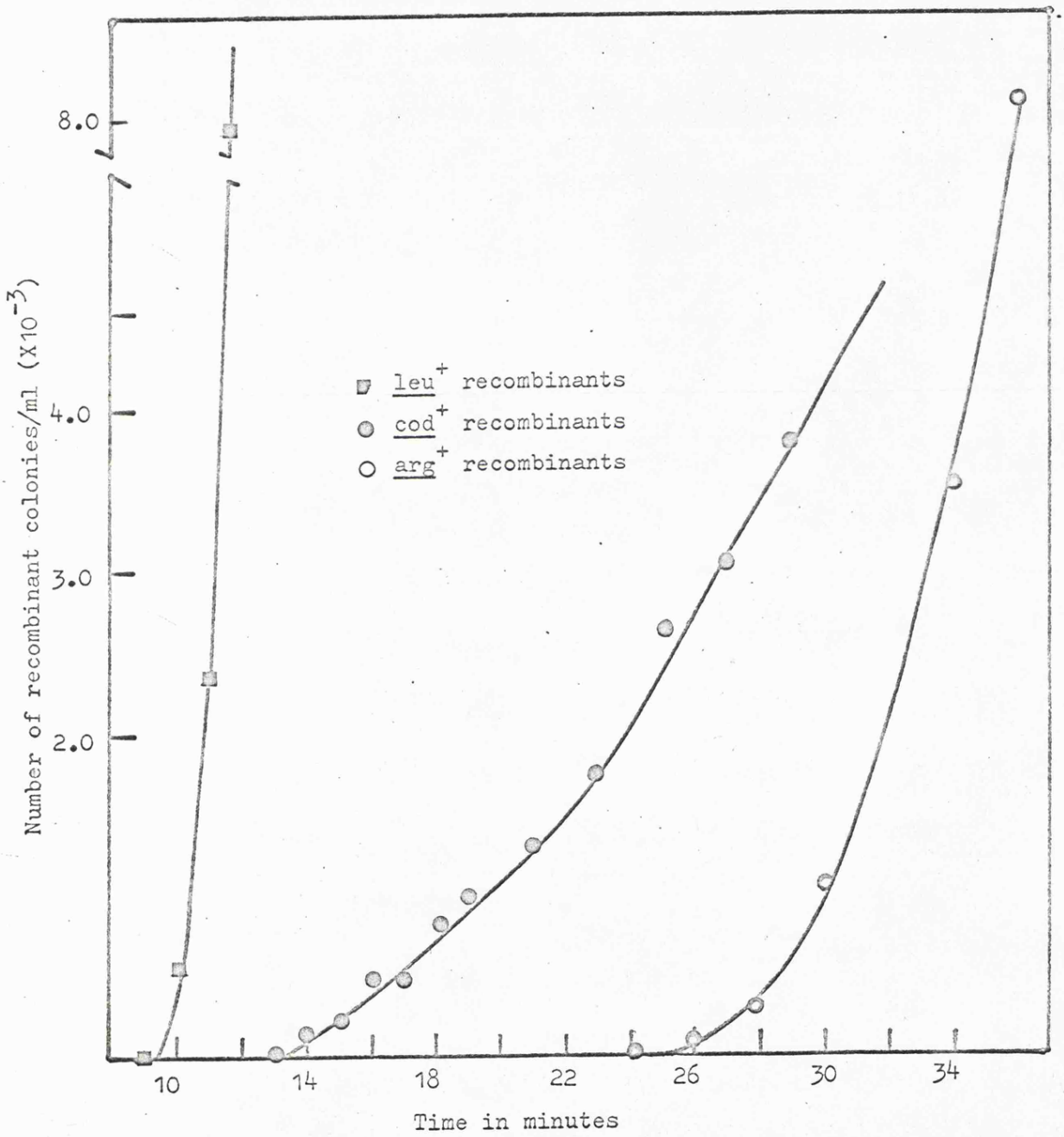
The location of the cod gene was determined by an interrupted mating experiment using HfrP4X (thi$^-$ str^s, orientation of transfer proB - leu - thr) and strain SA168. The number of recombinant colonies able to grow in the presence of cytosine (cod$^+$) or in the absence of arginine (arg$^+$) or in the absence of leucine (leu$^+$) was recorded after 48 hrs incubation.

Result shown (Fig 18) indicate that cod$^+$ recombinants appear 13 minutes after the mixing of Hfr and F$^-$ cells, whereas leu$^+$ and arg$^+$ appear after 9 and 24 minutes respectively. From this result we concluded that the cod gene is located around 87 minutes on the E.coli linkage map.

Production of a pyrE$^-$ cod$^-$ mutant of E.coli which is unable to utilise cytosine but is able to utilise uracil as its pyrimidine source, led to the conclusion that the only pathway

Fig 18

Interrupted mating experiment to locate cod gene



by which cytosine is metabolised involves as its first step, conversion to uracil. Although there has been some indirect evidence (see O'Donovan and Neuhard 1970), the data presented in this chapter constitute further evidence to support the specific involvement of cytosine deaminase in the metabolism of cytosine. Our evidence nevertheless is based on the assumption that the mutation in strain SA168 is a single mutation affecting the cod gene. If there is more than one mutation they must at least be closely linked, since the cod mutation in strain SA74 is transducible by P1.

DISCUSSION

The clustering of the four genes viz. dra, tpp, drm and pup, the induced synthesis of the enzymes they specify by a common inducer, dRib-5-P, and the mutation in the regulator gene (nucR mutation of Y-70-22) affecting all four enzymes, led us to assume initially that all these genes belonged to one unit of transcription. On the other hand, the following evidence suggests that the four genes might constitute at least two operons: (I) The synthesis of purine nucleoside phosphorylase and deoxyribomutase is induced by both purine ribonucleosides and dRib-5-P, whilst the synthesis of deoxyriboaldolase and thymidine phosphorylase is induced only by dRib-5-P. (II) When the mutant strain (SA115) which is defective in the regulation of two of the four enzymes was genetically analysed, the results indicated that the four genes constitute at least two units of transcription. One unit containing of dra and tpp and the other pup and drm.

The occurrence or not of coordinate induction has been examined carefully by Bonney and Weinfeld (1971). They found that thymidine phosphorylase and deoxyriboaldolase activities are co-ordinately induced. On the other hand, they interpreted their data for thymidine phosphorylase and deoxyribomutase as indicating that these two enzymes were not coordinately induced. A careful examination of the data they have presented however indicates that their claim for non-coordinate induction of these two

enzymes may be incorrect. Thus it seems significant that when various compounds which will lead to the production of dRib-5-P are used as inducers the data strongly suggest that induction is coordinate. The data obtained with ribosides as inducers do not fall on the same curve but this seems to be a self evidence prediction from the fact that thymidine phosphorylase is not induced by ribonucleosides.

However, the coordinate induction phenomenon is not sufficient proof in itself that the enzymes must be specified by the same operon, since enzymes specified by different operons which have a common regulatory gene may be coordinately induced. Therefore, although conclusive proof of the existence of two operons is still required, let us assume, on the basis of the weight of the evidence available at this point, that there are two operons.

By analogy with the Jacob and Monod (1961) model of "a negative controlling system in the lac operon" we could postulate that the regulatory mechanism involved in the synthesis of nucleoside catabolic enzymes is also negatively controlled i.e. the product of a regulator gene, the repressor molecule, in the absence of inducer, interacts with the operators and switches off both the operons. Similarly the presence of an inducer which

we now know is dRib-5-P interferes with the binding of the repressor molecule to the operator (Gilbert and Muller-Hill 1967) and this results in the induced synthesis of all the enzymes.

If we now examine the data obtained from the studies with the regulatory mutant (strain Y-70-22) in which three enzymes (and presumably the fourth, deoxyribomutase) are constitutively synthesised, the results suggest that this strain produces an altered repressor molecule (no repressor) which is most probably unable to bind normally to the operator of either operon. In other words, the repressor molecules of wild type strains normally bind to both operators.

The observations that a regulatory gene may be unlinked and that it can control the expression of more than one operon (I, dra tpp and II, drm pup) are not new. Thus it has previously been shown that the regulatory genes of the tryptophan (Ito et al. 1969) and galactose (Saedler et al. 1968) systems are unlinked to their respective operons. Also Maas (1961) and Kadner and Maas (1971) have found that although the eight genes involved in the synthesis of arginine constitute at least five independent units of transcription, their control is determined by a single regulatory gene, argR.

One unusual property of the regulation of the synthesis

of nucleoside catabolic enzymes is that purine nucleoside phosphorylase and deoxyribomutase (operon II) are induced by a second inducer, purine ribonucleoside. Before discussing this unusual property, it would be relevant to point out an identical system which has been observed in the histidine degrading pathway of Salmonella typhimurium (Smith et al. 1971). Four enzymes viz. histidase, urocanase, amidohydrolase and formiminohydrolase are involved in the degradation of histidine to ammonia, glutamate and formamide. All these enzymes are induced by urocanic acid or by histidine since the latter is converted to urocanic acid in the cell. A study of induction and repression in this system revealed that these four enzymes were not induced proportionately. Histidase and urocanase were induced by histidine 100 fold whereas the other two enzymes were induced only 4-5 fold. The four corresponding genes H, U, I and G, specifying histidase, urocanase, amidohydrolase and formiminohydrolase respectively were mapped as shown in Fig 19. Genes (PR) and Q were found to be specifically involved in the regulation of H and U and gene M in the regulation of I and G. In contrast, gene C was found to be involved in the regulation of all four structural genes. From the genetic order it was deduced that the (PR)QUH cluster constituted one operon and the MIG cluster another operon. It was postulated that the C gene in order to act at (PR)Q and M sites, specified a

Fig 19

diffusible repressor molecule.

In order to explain how the product of a regulatory gene can control the expression of more than one operon and that two distinct inducers can affect the synthesis of nucleoside catabolic enzymes, a hypothesis for the regulatory system involved in nucleoside catabolism is now proposed.

According to this hypothesis, the regulator gene (nucR) directs the synthesis of a repressor molecule. This molecule can bind to the operator of both the operons (operon I, dra tpp, operon II, drm pup). It is also suggested that the two operators

differ in their base sequences leading to different binding affinities for the repressor. The difference in binding, however, does not display any significant effect when cells are grown either without an inducer or with deoxynucleoside as inducer. In the absence of inducer all four enzymes are equally repressed and in the presence of dRib-5-P all four enzymes are equally induced. However, according to the model, when purine ribonucleoside is present as inducer the difference in the binding affinity of the repressor for the two operators is expressed. This latter inducer binds with the repressor molecule in such a way that it displaces the molecule from the operator of the drm pup operon only. With the operator of the dra tpp operon, either it cannot bind or, if it binds, it fails to displace the repressor from the operator. Thus in the presence of purine ribonucleoside only deoxyribomutase and purine nucleoside phosphorylase are induced.

It would now be interesting to try to explain the nature of the mutation in strain SA116 in terms of the above model. During the study of Tlr mutants, it was observed that strain SA116 has an unusual property. Genetic analysis of this strain, which is constitutive for only thymidine phosphorylase and deoxyriboaldolase, showed that the mutation determining constitutivity is not linked to the structural genes dra and tpp. It

is assumed therefore, that strain SA116 carries a mutation in the unlinked regulator gene which directs the synthesis of an inactive repressor molecule. This molecule then has lost the affinity to bind with the operator of dra tpp operon but retains the ability to bind with the operator of the drm pup operon. A further genetic analysis is however necessary to determine if the mutation has occured in the true regulator gene.

The above model presented for the regulation of nucleoside catabolism has as yet little experimental basis. Further biochemical and genetic study of a variety of heterogenous Tlr mutants is therefore necessary. Their repressor molecules have to be isolated and characterised.

The physiological role of the nucleoside metabolic enzymes.

In discussing the physiological role of the enzymes involved in nucleoside metabolism, it is necessary to first consider whether these enzymes are catabolic or anabolic. The evidence favouring catabolic properties are overwhelming.

(1) The enzymes are inducible. It is believed that in E.coli inducible enzymes are mainly those which degrade exogenous substrates, of which β-galactosidase (Monod and Cohn 1952), penicillinase (Kogut et. al. 1956) and the enzymes involved in galactose utilisation (Kalckar et. al. 1959) are good examples.

(2) They are released into the medium by osmotic shock (Kammen 1967; Munch-Petersen 1968; Beacham 1969). Release of enzymes in this way (Neu and Heppel 1965) has been suggested to be indicative of a periplasmic location and is a property shared by many degradative enzymes. Alkaline phosphatase, ribonuclease I and 5'-nucleotidase are some of the examples (Heppel 1967). In a recent study (Beacham et. al. 1971), however, it has been observed that the four enzymes involved in deoxynucleoside catabolism do not possess all the properties of typical periplasmic enzymes, since they are only partially released when subjected to osmotic shock and are not released when cells are converted to spheroplasts. It is therefore suggested that they are not

periplasmic enzymes but are associated in some unknown way with the inner surface of the cytoplasmic membrane (Beacham et al. 1971).

(3) Deoxyriboaldolase has a rather low affinity for acetaldehyde (Racker 1952). It is therefore most unlikely that the synthesis of dRib-5-P (the reaction in reverse) normally occurs by the condensation of acetaldehyde and glyceraldehyde-3-phosphate.

(4) The endogenous level of dRib-1-P in wild type strains of E.coli is too low to promote the synthesis of any deoxynucleoside from the corresponding base. The fact that E.coli is normally unable to incorporate thymine has already been mentioned. In addition Karlstrom (1970) has shown that there is apparently no deoxycytidine, deoxyadenosine or deoxyguanosine kinase in E.coli. The exogenous purine bases therefore seems to be only metabolised via the pyrophosphorolytic reactions.

(5) The pathway is subject to catabolite repression (Beacham 1970, Ph.D. thesis): a property of catabolic systems (Magasanik 1961).

(6) Using labelled glucose or acetate, no evidence was obtained to show that the de novo synthesis of deoxyribose can occur via deoxyriboaldolase (Sable 1966).

(7) Nucleosides of purines and pyrimidines (except orotic acid) are not synthesised de novo.

(8) Since mutants of E.coli defective in one or more components of the metabolic pathways of nucleosides have normal growth pattern, the presence of the enzymes does not seems to be essential for the biosynthesis of nucleic acids.

Assuming that the enzymes involved in the metabolism of nucleosides are catabolic, three possible roles for these enzymes can be postulated.

(I) They are involved in the degradation of foreign DNA molecules entering into the cells as a result of phage infection or conjugation. This possibility has been suggested to us by the fact that the hsp loci (Glover and Colson 1969) and the locus involved in the restriction of phage P1 DNA (por, Wu 1969) are located close to the nuc operons. Although it appears that the hsp loci do not belong to the nuc operon, our knowledge of the functional relationships between these genes is still limited.

(II) It is possible that nucleoside catabolic enzymes are involved in the degradation of endogenous nucleotides which may be over-produced as a result of disturbance of normal metabolic pathways. The degradation of nucleotides in this way as a carbon source would not only be economical but in certain circumstances it may protect the cell from the accumulation of a metabolic inhibitor. Such an example is the accumulation of deoxyadenosine

triphosphate which has been shown to inhibit nucleoside diphosphate reductase (Reichard 1967). As a consequence of this, the synthesis of other deoxynucleoside diphosphate is blocked. Removal of such inhibitory compounds by the nucleoside catabolic enzymes under these conditions would be essential.

(III) Finally the existence of nucleoside catabolic pathway in E.coli may be attributed to evolutionary adaptation. Since E.coli is an enteric bacterium it is possible that various nucleosides are frequently encountered by the organism in nature. It is likely that the amounts of nucleosides in the natural environment are greater than those required for the synthesis of nucleic acid. Hence it would be advantageous to use the surplus nucleosides as a source of energy. In this connection it is interesting that nucleoside catabolic enzymes are induced by dRib-5-P unlike other catabolic systems (e.g. lactose) where substrates normally induce the enzymes involved in the degradation. Deoxynucleosides do not induce deoxynucleoside catabolic enzymes. A rationale of this phenomenon can be presented. Since thymidine and deoxyuridine are involved in the synthesis of DNA, their anabolism is more advantageous than their catabolism if these deoxynucleosides are present at low amounts in the natural environment. If thymidine or deoxyuridine induced the catabolic

enzymes then these enzymes would have competed with the anabolic ones. However, when the cell continues to obtain these deoxynucleosides then the level of dRib-5-P rises and consequently induction of catabolic enzymes occurs and the rate of breakdown of thymidine and deoxyuridine also rises. Similar reasoning perhaps can be applied to the purine deoxyribonucleosides. In this case the cells catabolise the low amounts of deoxynucleosides using the basal level of purine nucleoside phosphorylase and use the released base for nucleic acid synthesis and the sugar moiety as an energy source. On the other hand, when cells are exposed to a large supply of purine deoxyribonucleosides, an increase in the dRib-5-P pool enhances the breakdown of the deoxynucleosides. Perhaps, under the latter conditions, the cells would be unable to use all the released bases and therefore excrete it into the medium.

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Summary

In *Escherichia coli* thymidine phosphorylase, purine nucleoside phosphorylase, deoxyribomutase and deoxyriboaldolase are involved in the catabolism of nucleosides and deoxynucleosides and the sugar moiety is used as a carbon source. All four enzymes are induced by deoxynucleosides and the actual inducer appears to be dRib-5-P. Purine nucleoside phosphorylase and deoxyribomutase are also induced by purine ribonucleosides. Overwhelming evidence is available which favours the catabolic role of these enzymes.

Four genes viz. *tpp*, *dra*, *drm* and *pup* specifying thymidine phosphorylase, deoxyriboaldolase, deoxyribomutase and purine nucleoside phosphorylase respectively were mapped by transduction with phage P1. All pairs showed greater than 90% co-transduction. The gene order was found to be *dra-tpp-drm-pup* and the gene cluster lies between the *hsp* and *ser*B loci on the chromosome map of *E.coli*.

A regulatory mutant which leads to constitutive synthesis of at least three of the four enzymes (and perhaps the fourth, deoxyribomutase) was analysed. The regulatory gene is designated *nuc*R and is located near *gal*E. The amount of thymine required for growth (colony formation) of *thy*$^-$ strains is affected by the *nuc*R mutation. The amount required by the *thy*$^-$ *drm*$^-$ strain is reduced about four fold if it carries the constitutivity mutation. The amount required by a *thy*$^-$ *drm*$^+$ strain is increased at least two

fold. These differences in nutritional requirement provide a method for selecting constitutive from non-constitutive strains and vice versa. By this method a thymidine phosphorylase constitutive mutant was selected. The data obtained from the analysis of this second regulatory mutant indicate that it is similar to an O^c type mutation of the lac operon. The mutation of the putative operator, which is located to the left of the dra, renders only thymidine phosphorylase and deoxyriboaldolase constitutive. Purine nucleoside phosphorylase is not affected by this mutation and therefore it is believed that the four genes constitute at least two operons, one operon containing dra and tpp and the other pup and drm.

A double mutant of E.coli was produced which lacks cytosine deaminase activity and requires pyrimidine for growth. This mutant is not able to grow on cytosine as sole pyrimidine source. This observation indicates that the only pathway involved in the incorporation of exogenous cytosine is via its first step conversion to uracil.

A gene, udp, specifying uridine phosphorylase and a gene, cod, specifying cytosine deaminase were located at 74-75 and around 87 minutes respectively on the E.coli linkage map.

Fluorouracil was used to isolate various mutants lacking nucleoside and deoxynucleoside phosphorylase activities. Using these mutants, it was shown that there are three routes via which FU can be converted to compounds which inhibit cell growth.