MEASUREMENT OF FEEDBACK INHIBITION IN VIVO AND SELECTION OF ATCase FEEDBACK ALTERED MUTANTS IN SALMONELLA TYPHIMURIUM

DISSERTATION

Presented to the Graduate Council of the University of North Texas in Partial Fulfillment of the Requirements For the Degree of

DOCTOR OF PHILOSOPHY

By

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August, 1989
Aspartate transcarbamoylase (ATCase; encoded by pyrBI genes) is one of the most studied regulatory enzymes in bacteria. It is feedback inhibited by cytidine triphosphate (CTP) and activated by adenosine triphosphate (ATP). Much is known about the catalytic site of the enzyme, not nearly as much about the regulatory site, to which CTP binds. Until now a positive selection for feedback-modified mutants was not available. The selection we have developed involves the use of a pyrA deletion in S. typhimurium. This strain lacks carbamoylphosphate and requires both a pyrimidine and arginine for growth. In this strain citrulline is used to satisfy the pyrimidine and arginine requirements. The minimal flow through the pyrimidine pathway from the citrulline-produced carbamoylphosphate is exquisitely sensitive to feedback control of ATCase by CTP. By elevating the CTP pool, via exogenous cytidine, in a strain that also contains a cytidine deaminase mutant (cdd) growth can be stopped completely, indicating 100% inhibition. It was therefore possible to measure in vivo feedback inhibition of
ATCase among the citrulline users and to isolate a family of ATCase regulatory mutants with either modified or no response to effectors.
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Introduction

Unlike amino acid and vitamin biosynthetic pathways, all of which are not found in all organisms, virtually every organism contains a de novo pyrimidine (and purine) nucleotide pathway. Although the pyrimidine biosynthetic pathway is the same for all organisms, its regulation differs significantly from one organism to the next.

Fig. 1 shows the de novo pyrimidine pathway in \textit{Escherichia coli}, which supplies UTP and CTP for RNA synthesis, and dCTP and dTTP for DNA synthesis. Mutations in any of the six \textit{pyrA-\textit{F}} genes encoding enzymes for the biosynthesis of UMP give a Pyr\textsuperscript{−} phenotype and require a pyrimidine for growth; mutations in \textit{pyrA} have an arginine requirement as well.

Carbamoylphosphate synthetase (CPSase; EC 6.3.4.16) couples the cleavage of two molecules of ATP to the formation of one molecule of carbamoylphosphate from bicarbonate and NH\textsubscript{4}\textsuperscript{+} or glutamine (EC 6.3.5.5) (Abdelal and Ingraham 1975). In the enteric bacteria, carbamoylphosphate is required for pyrimidine biosynthesis and for arginine biosynthesis. A mutation in the gene encoding CPSase (designated \textit{pyrA} in \textit{Salmonella typhimurium} and \textit{carAB} in \textit{E.coli}) would thus have a dual requirement for pyrimidines and for arginine (Anderson
**Fig. 1** A diagram showing the pyrimidine pathway in *S. typhimurium* and *E. coli* with the structures and enzymes indicated. *pyrA* is carbamoylphosphate synthetase, *pyrB* is aspartate transcarbamoylase, *pyrC* is dihydroorotase, *pyrD* is dihydroorotate oxidase, *pyrE* is orotate phosphoribosyltransferase, *pyrF* is orotidylate decarboxylase, *pyrH* is uridylyl kinase, *pyrG* is cytidine triphosphate synthetase, and *ndk* is nucleoside diphosphate kinase.
Transfer of the carbamoyl group from carbamoylphosphate to aspartate produces N-carbamoylaspartate and inorganic phosphate. This step, the first step unique to pyrimidine biosynthesis (Gerhart and Pardee, 1962), is catalyzed by the enzyme aspartate transcarbamoylase (ATCase; EC 2.1.3.2) encoded by the pyrBI genes in E. coli and S. typhimurium. Mutations in the pyrB gene cause a requirement for pyrimidines. Referring to Fig 1, it can be seen that carbamoylaspartate is cyclized with the elimination of water to form dihydroorotate. This step is catalyzed by dihydroorotase (DHOase; EC 3.5.2.3) and is encoded by pyrC in E. coli and S. typhimurium. Dihydroorotate oxidase (DHOdehase; EC 1.3.3.1) is a flavoprotein that oxidizes dihydroorotate to orotate with oxygen as the external oxidant. DHOdehase is encoded by the pyrD gene. Orotate can then combine with the phosphoribosyl group from 5-phosphoribosyl-1-pyrophosphate (PRPP) to form the first pyrimidine nucleotide, orotidine-5'-monophosphate (OMP). This step is catalyzed by orotate phosphoribosyltransferase (OPRTase; EC 2.4.2.10), a product of the pyrE gene. OMP is next decarboxylated to uridine-5'-monophosphate (UMP) by the enzyme OMP decarboxylase (OMPdecase; EC 4.1.1.23). OMPdecase is encoded by pyrF. Mutations in the pyrC, pyrD, pyrE, or pyrF genes cause a single requirement for pyrimidines. UMP is next phosphorylated using ATP to form UDP. This enzyme,
UMP kinase (EC 2.7.4.4), is highly specific and is encoded by the gene \textit{pyrH}. Complete mutations in the \textit{pyrH} gene would be lethal since no nutrient could be fed exogenously. UDP is further phosphorylated to UTP with ATP by the enzyme nucleoside diphosphokinase (EC 2.7.4.6), a non-specific diphosphate kinase encoded by \textit{ndk}. As with \textit{pyrH}, strict mutations in \textit{ndk} would also be lethal, however it has been possible to isolate leaky \textit{pyrH} mutants (O'Donovan and Gerhart, 1974) as well as temperature sensitive \textit{ndk} mutants (Ginther and Ingraham, 1974). The final step of the pathway is the amination of UTP to CTP, with glutamine serving as the amino donor, by the enzyme CTP synthetase (CTPSase; EC 6.3.4.2). Mutations in the \textit{pyrG} gene, which encodes CTPSase, require cytidine (not uracil, uridine, or cytosine) for growth. Since complex medium does not contain sufficient cytidine to satisfy a \textit{pyrG} requirement (typically between 2-10 \(\mu\text{g/ml}\) are found) exogenous cytidine (50 \(\mu\text{g/ml}\)) must be added to all media for the maintenance of \textit{pyrG} strains. Accordingly, all \textit{pyrG} mutants must be isolated in a \textit{cdd}, a strain that lacks cytidine deaminase (EC 3.5.4.5) since the preferred metabolic route of cytidine is deamination to uridine (Fig. 2).

The synthesis of pyrimidine nucleotides de novo does not involve nitrogenous bases or nucleosides as intermediates, but the ability of different \textit{Pyr}^{-} mutants to use pyrimidine
Fig. 2 Pyrimidine nucleotide biosynthesis and salvage pathways in *S. typhimurium*. Abbreviations used are *pyrA* encodes carbamoylphosphate (CP) synthetase (CPSase); *pyrBI* the catalytic (*pyrB*) and regulatory (*pyrI*) polypeptides of aspartate transcarbamoylase (ATCase); *pyrC* encodes dihydroorotase; *pyrD* encodes dihydroorotate dehydrogenase; *pyrE* encodes orotate phosphoribosyltransferase; *pyrF* encodes orotidine 5' monophosphate (OMP) decarboxylase; *pyrH* encodes uridine 5' monophosphate (UMP) kinase; *ndk* nucleotide diphosphokinase; *pyrG* encodes CTP synthetase; *argI* encodes ornithine transcarbamoylase (OTCase); *cdd* encodes cytidine deaminase; *udp* encodes uridine phosphorylase. *U* is uracil, *UR* is uridine, *C* is cytosine, *CR* is cytidine.
bases and nucleosides for growth confirms that pyrimidine salvage pathways are of widespread occurrence (Fig. 2). These salvage pathways are capable of converting pyrimidine bases and nucleosides into pyrimidine nucleotides. The salvage pathways fulfill three physiological functions. (1) To scavenge exogenous preformed pyrimidine bases and nucleosides for nucleotide biosynthesis. (2) To make the pentose portion of nucleosides and the amino group of cytosine available as sources of carbon, energy, and nitrogen. (3) To rephosphorylate pyrimidine bases and nucleosides produced endogenously by turnover of nucleotides from mRNA (Neuhard, 1983).

The salvage pathways are also important because, (1) the extensive use of radioactively labelled pyrimidines for the study of nucleic acid synthesis in vivo requires a detailed knowledge of pyrimidine salvage pathways in order to select the most appropriate radioactive precursor; and (2) the salvage pathways are required to define the sensitivity of an organism to pyrimidine analogues which must be converted to their nucleotide form to be toxic. This is so because most analogues are metabolized by the same enzymes as the natural pyrimidine bases and nucleosides (O'Donovan and Neuhard 1970). Thus the pyrimidine salvage system includes the transport of the pyrimidine base or nucleoside into the cell.
plus its subsequent phosphorylation to the nucleotide level. Pyrimidine salvage pathways are seen in Fig 2.

Upon entering the *E. coli* cell, uracil, generally the pyrimidine of choice for supplying a Pyr− phenotype, is converted to UMP by uracil phosphoribosyl transferase (EC 2.4.2.9, UPRTase) encoded by *upp* (O'Donovan and Neuhard 1970). Fig. 2 (right, top) shows the relevant interconversions of pyrimidine nucleosides and nucleotides. As can be seen from Fig 2, uracil is not the only pyrimidine base that can satisfy the pyrimidine requirement of Pyr− mutants. Indeed, exogenously supplied cytosine is deaminated to uracil by cytosine deaminase (*codA*) (Neuhard, 1968), the uracil being converted to UMP thereafter. Cytidine is deaminated to uridine by cytidine deaminase (*cdd*) (Neuhard, 1968) which is then phosphorolytically cleaved to uracil and ribose-1-phosphate by uridine phosphorylase (EC 2.4.2.3, *udp*) (Neuhard, 1968). Thus cytidine, uridine, cytosine, and uracil can satisfy the pyrimidine requirement in *pyrA–F* mutants. However only cytidine can satisfy the requirement of a *pyrG* mutant (Neuhard and Ingraham 1968).

The pyrimidine biosynthetic pathway is controlled both at the level of enzyme activity and at the level of enzyme synthesis.

The flow through the pyrimidine pathway is regulated at the level of enzyme synthesis in the following manner.
Growth in uracil represses the synthesis of the first six enzymes with a uridine nucleotide acting as the repressing metabolite for genes pyrBEF and a cytidine nucleotide for the pyrACD genes (Kelln et al., 1975, Schwartz and Neuhard, 1975). (Fig 3) There is also control by attenuation of pyrB (Roof et al. 1982; Turnbough et al. 1983; Navre and Schachman 1983; Poulsen et al. 1983; Michaels et al. 1987) and pyrE (Neuhard et al. 1985; Bonekamp et al. 1984).

Control at the level of enzyme activity occurs at three loci (refer to Fig 3). (1) The enzyme CPSase is inhibited by UMP and activated by ornithine, IMP, and PRPP. The activation in vitro with the latter two compounds is about a factor of two (Piérand 1966; Abdelal and Ingraham 1975). The concentration of IMP needed to produce activation of the enzyme in vitro is far above the physiological concentration range. In contrast, the activation seen with PRPP occurs in the range of concentrations where PRPP is found intercellularly, at one μmole per gram dry weight (Sadler and Switzer 1977; Bagnara and Finch 1973,1974) and may be of physiological significance (Abdelal and Ingraham 1975; Jensen et al. 1979; Sadler and Switzer 1977). UMP and ornithine are the main effectors. (2) The second locus of control is the enzyme ATCase which is feedback inhibited by CTP and activated by ATP (Gerhart and Pardee 1962). (3) CTP synthetase, which requires UTP, ATP, and glutamine (or NH4),
Fig. 3 A diagram of the pyrimidine and arginine pathways in *S. typhimurium* and *E. coli*, showing regulation of the intermediate steps within the pathways. --- denotes feedback inhibition; → denotes activation. © indicates repression of this reaction by a 'C' compound, while [U] indicates repression by a 'U' compound.
is inhibited by CTP and activated by GTP (Long and Pardee 1967, Neuhard and Nygaard 1987).

The first indication of regulation at the level of enzyme activity in the pyrimidine biosynthetic pathway came in 1952 from Bolton et al. In isotope competition studies in *E. coli* they found that non-radioactive uracil or uridine added in minute amounts to the growth medium of *E. coli* immediately and extensively suppressed the bacterial synthesis of pyrimidines from $^{14}$C-labeled CO$_2$. Brooke et al. (1964) found the same results in *Aerobacter*. Later Yates and Pardee (1956) showed that uracil-requiring mutants of *E. coli* produced and released large quantities of intermediates of the pyrimidine pathway (Fig. 2) when uracil was absent and these mutants ceased production of the first and all subsequent intermediates within minutes after uracil was added. This experiment suggested that uracil or some pyrimidine compound derived from it (e.g. UMP, UDP, UTP, or CTP) inhibited the earliest enzymatic step of the pathway, namely the formation of carbamoylaspartate from carbamoylphosphate and aspartate. The reaction is catalyzed by ATCase (Fig 3). Using a *pyrC* mutant (lacking DHOase) of *E. coli*, Yates and Pardee starved the Pyr$^-$ cells for uracil and saw massive production of carbamoylaspartate (up to 50% of the cell dry weight in four hours). Minutes after the readdition of uracil (40 ug/ml) the production of
carbamoylaspartate ceased. Since in the \textit{pyrC} mutant the only functional step was that catalyzed by ATCase, it was possible for Yates and Pardee to suggest that ATCase was inhibited by the added uracil. Such experiments led them to propose a feedback control of the pyrimidine biosynthetic pathway based on end product inhibition. They showed that cytidine compounds (e.g. cytidine, CMP, CDP, or CTP) but not uridine compounds, inhibited ATCase. In a similar independent set of experiments Umbarger (1956) showed that L-isoleucine feedback inhibited threonine deaminase. From these two studies it was clear that in vitro studies of feedback inhibition should be pursued further.

Gerhart and Pardee (1962) using ATCase purified by the method of Shepherdson and Pardee (1960), showed that inhibition by CTP was 20 times more effective than CMP while UTP was non-inhibitory. Thus, CTP was discovered to be the feedback inhibitor of the ATCase step in the pyrimidine biosynthetic pathway (JC Gerhart, 1962, PhD. thesis, University of California, Berkeley).

Using \textit{S. typhimurium}, Neuhard and Ingraham (1968) confirmed the role of CTP as the metabolic inhibitor of ATCase by means of experiments carried out in vivo. They isolated mutants lacking CTP synthetase (\textit{pyrG}) which require cytidine for growth. These \textit{pyrG} mutants can synthesize UTP but not CTP, which they must obtain from exogenously supplied
cytidine, as follows, CR->CMP->CDP->CTP. In the absence of cytidine, the intercellular UTP pool increases 10-fold as uracil, uridine and UMP are released into the medium. Despite the 10-fold increase in UTP, the enzymes of the pathway appeared uncontrolled. Such overproduction of uracil and uridine does not occur when cytidine (hence CTP) is provided. Thus CTP and not UTP is the feedback inhibitor of ATCase.

The end product control of pyrimidine biosynthesis in *E. coli* does not depend on the interaction between CTP and ATCase alone because the preceding step, that catalyzed by CPSase, is also inhibited by a different pyrimidine nucleotide, UMP. Indeed, as pointed out by Gerhart (1970) there are frequently growth conditions in *E. coli* when CPSase becomes the primary locus of control of the pyrimidine pathway with ATCase assuming only a secondary role. This follows from the fact that carbamoylphosphate is used for only one other pathway in the cell, namely the carbamoylation of ornithine by OCCase (encoded by *argI*) to produce citrulline for arginine biosynthesis. But when exogenous arginine is available to the bacterium, this use does not apply, because ornithine is not formed. Thus, during bacterial growth in an arginine containing medium (e.g. casamino acids) CPSase becomes the first step unique to pyrimidine biosynthesis, with UMP as its major effector. When arginine is not
supplied extracellularly, ornithine accumulates and activates CPSase as counterinhibitor to UMP (Piérard 1966), thereby supplying carbamoylphosphate for arginine synthesis as well as pyrimidines. Only when both pathways are functioning simultaneously does ATCase become the primary target of control for pyrimidine production. Accordingly, in E. coli it is both CPSase and ATCase together, and not either one alone, that establishes a balanced flow through the pyrimidine pathway. However the relative importance of control by UMP versus control by CTP has never been evaluated in bacteria growing under different nutrient conditions (e.g. plus and minus arginine). Nor is it known, precisely, how much CTP is required to shut down ATCase. In this dissertation feedback inhibition of ATCase by CTP is quantified in vivo.

The evaluation of the effects of varying CTP concentrations in vivo is dependent on the ATCase pyrI gene product and its interaction with the pyrB gene product. Most of the data available on these interactions come from X-ray crystallographic studies, (Monaco et al. 1978; Ladner et al. 1982; Honzatko and Lipscomb 1982; Krause et al. 1985), X-ray scattering studies of the enzyme in transition states (Moody et al. 1979; Hervé et al. 1985) and studies of hybrids (J Houghton.1986, PhD Dissertation Texas A & M University, College Station; K Kedzie 1987, PhD Dissertation Texas A & M
Fig. 4  A schematic representation of the in vivo assembly of the ATCase holoenzyme from the bicistronic pyrBI operon.
<table>
<thead>
<tr>
<th>Property</th>
<th>Native enzyme</th>
<th>mercurials</th>
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<td>Subunit structure</td>
<td>6 C + 6 R</td>
<td>2(3 C) = 6 C + 3(2 R) = 6 R</td>
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<tr>
<td>Molecular weight</td>
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<td>2 (100,000) + 3 (34,000)</td>
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<td>Sedimentation coefficient</td>
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<tr>
<td>Molecular weight of each polypeptide chain</td>
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<td>17,000</td>
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</tr>
<tr>
<td>Number of polypeptide chains</td>
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<td>2</td>
<td></td>
</tr>
<tr>
<td>Total number of polypeptide chains</td>
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<td>2(3) = 6</td>
<td>3(2) = 6</td>
</tr>
<tr>
<td>Number of half-cystines</td>
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<td>8</td>
<td>24-28</td>
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<tr>
<td>Shape of saturation curve</td>
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<td>Hyperbolic</td>
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<tr>
<td>Catalytic activity (binding of substrates)</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Binding of effectors</td>
<td>+</td>
<td>-</td>
<td>+</td>
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*from O'Donovan (personal communication)
University, College Station) and sequencing (Konigsberg and Henderson 1983; Hoover et al. 1983; Schachman et al. 1984).

The enzyme is a dodecameric structure with two catalytic subunits made of three identical polypeptide chains and three regulatory subunits made of two identical polypeptide chains (Fig. 4, Table 1). It is the relationship between the two types of subunits that is the focus of most of the research currently being done on ATCase. One of the most powerful ways to study enzyme structure-function relationships, namely the evaluation of point mutation effects on the function of the enzyme, has not been readily available for this enzyme, because although there are many mutants with altered catalytic subunits (Smith et al. 1986; Schachman et al. 1984, 1980; Feller et al. 1981), only a few mutants with an altered pyrI subunit have been available. Feller et al., in 1981, found a pyrBI with a modified ATCase lacking substrate co-operative interactions and insensitive to feedback inhibition by CTP. This altered pyrI resulted from viral DNA replacing the last eight codons of the pyrI gene in a transducing phage (Cunin et al. 1985). Mutations in the pyrB gene leading to an altered response to CTP were found by O'Donovan and Gerhart (1972) as well as a pyrI deletion mutant (Roof et al. 1982). Eisenstein et al., (1989) used site-directed mutagenesis to obtain mutants with altered pyrI that had changed catalytic and allosteric properties. The
lack of readily available mutants in \textit{pyrI} has been ascribed to various reasons. One reason is the presence of an essential gene (\textit{pyrX}), the 5' start site of which is overlapping with the 3' end of the \textit{pyrI} gene (T. Hoover, personal communication), such that most mutations in the \textit{pyrI} would result in the lethal loss of \textit{pyrX}.

Legrain et al. (1976b), in an attempt to isolate regulatory mutants in the arginine pathway of \textit{E.coli K12} found mutations such that bacteria grown on high concentrations of citrulline were capable of producing enough carbamoylphosphate to satisfy the pyrimidine requirement in a \textit{pyrA} deletion strain. These strains utilized citrulline at a high concentration (1000 \(\mu\text{g/ml}\)) to force the reaction in the reverse direction (Fig. 2). The instability of these strains (See Discussion) precluded their use for selection of \textit{pyrI} mutations.

Citrulline transport is via the same permease system as arginine, with arginine having a much lower Km. This results in citrulline being excluded when there is arginine in the medium, (Maas 1961; Celis 1977a, 1977b; Furlong 1987). Legrain et al. (1976b), used an arginino-succinate synthetase mutant when selecting for strains capable of using citrulline for carbamoylphosphate in sufficient quantity to allow growth of a \textit{pyrA} strain. These strains require arginine for growth, even in the presence of citrulline. Thus a competition
between arginine and citrulline uptake was established, requiring a much greater concentration of citrulline than might be expected to allow these strains to produce enough carbamoylphosphate for their pyrimidine requirement. The selection, accordingly, does not work for obtaining pyrI mutants.

Carbamoylphosphate is used to supply four major nucleotides, CTP, dCTP, UTP, and dTTP. The dTTP pool requirement is met only after the CTP and UTP requirements. The dTTP pathway is supplied 20% via a uridine pathway, starting with UDP and 80% from deoxycytidine triphosphate deaminase (O'Donovan et al. 1971; Neuhard and Thomassen 1971). Altered dTTP pools are known to cause chromosomal rearrangements and damage, some possibly mediated by recA function (Cummings and Mondale 1967; Eisenstark et al. 1968; Kunz and Glickman 1985). Thymineless death is one result of drastic changes in the endogenous dTTP concentration. Alterations in other pyrimidine nucleotide concentrations would result in the same effects due either to direct changes in the pools (dCTP), or secondary changes (dTTP from UDP) (Kunz and Glickman 1985). The parental strain, while able to take up both thymidine and thymine only incorporates labeled thymidine into DNA for a brief period. For long term incorporation it is necessary to start with thymine and a source of deoxyribose-1-phosphate to allow the synthesis of
dTTP. The source of deoxyribose-1-phosphate can be 2'-deoxyguanosine, 2'-deoxyadenosine or 2'-deoxyinosine (O'Donovan 1978). Thus we supplied thymine and deoxyinosine to all citrulline grown cultures.

Without accurate evaluation of the activity of the interconversions of the de novo and salvage pathways discussion of control is moot. Exogenous cytidine can maintain the UTP pools via cytidine deaminase even if the cdd enzyme is not operating in the strain at full efficiency, because the preferred metabolic route for cytidine in S. typhimurium is deamination to uridine and the Km for cytidine kinase is higher than for cytidine deaminase (Wang et al. 1950). The usual assays for nucleotide pathway enzymes are sensitive in the millimolar range, thus, if mutations are leaky, results can appear to be in error. HPLC measurements can be in the nanomolar range, thus allowing an exquisitely sensitive evaluation of the impact of various growth conditions on the pools.

The traditional assays for cdd and cod gene products involve the use of spectrophotometric measurement of decrease in optical density of a solution of partially purified enzyme, substrate, and cofactors. Both assays rely on the disappearance of the substrate, with no indication of its final disposition. Another enzyme involved in the salvage pathway for pyrimidines is nucleoside monophosphate
glycosylase (originally discovered by A Eisenstadt, 1971 PhD thesis University of Copenhagen). Up to now it has been regarded as a relatively unimportant enzyme, but it becomes very important under the conditions of growth in strains devoid of cdd activity, as those in this study. The enzyme allows cytosine to be formed from either exogenous cytidine via CMP in a cdd strain, or from CMP under normal salvage conditions. The new methods described in this dissertation allow the use of HPLC to demonstrate the impact of all of the above enzymes on the nucleotide pools.
MATERIALS AND METHODS

Bacterial Strains and Plasmids

Strains (Table 2) were constructed from *Salmonella typhimurium* KR 1530, [pyrA81, pyrG611(ts), cdd-7, udp-2], a derivative of *S. typhimurium* LT2 obtained from Dr. Rod Kelln, University of Regina, Canada. This strain requires arginine (50 \(\mu\)g/ml) and uracil (or uridine) (50 \(\mu\)g/ml) at all temperatures. In addition, it requires cytidine (50 \(\mu\)g/ml) when grown at 42°C. Constructed strains either have spontaneous mutations allowing growth on citrulline as a source of arginine and carbamoylphosphate to fulfill the uracil requirement or use plasmids containing *arc* genes from *Pseudomonas aeruginosa* which form carbamoylphosphate from arginine. The plasmids were obtained from Dr Dieter Haas (Baur et al. 1987, 1989).

Growth Conditions

Bacterial cells were grown in minimal A medium (Miller, 1972). It contained, per liter (1) of deionized water, 10.5 g potassium phosphate, dibasic, 4.5 g potassium phosphate, monobasic, 1.0 g ammonium sulfate, and 0.5 g sodium-citrate,
<table>
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<th>Organism</th>
<th>Genotype</th>
<th>Growth requirements</th>
<th>Source</th>
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</thead>
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<td>argI-pyrBI, argF</td>
<td>Arginine, Uracil</td>
<td>Roof et al. 1982</td>
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<tr>
<td>S. typhimurium</td>
<td>pyrGts, cdd, pyrA, codB, udp</td>
<td>Arginine, Uracil, Cytidine (43°C)</td>
<td>Rod Kelln</td>
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<td>Citrulline, Cytidine (43°C)</td>
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<td><em>E. coli</em> CM236</td>
<td>Δ(lac, proAB argF') Δ(argI1 thi-1, supE recA1, srl靛Tn10 r-K m-K [pBR322 Ap/Cb Tc Pneo from Tn5 arcB (P.a.))]</td>
<td>Arginine, Thiamine</td>
<td>Baur et al. 1987</td>
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(pH 7.0). After autoclaving, 0.5 ml 1 M magnesium sulfate solution and 1.0 ml of a 2 mM zinc-acetate were added, along with 0.2% (w/v) glucose as carbon source. Appropriate supplements, such as arginine, uracil, and cytidine were generally added at a concentration of 50 μg/ml. For solid media, 15 g agar were added per 1 of medium. Citrulline concentrations varied, depending on the experimental conditions, between 50 and 750 μg/ml. Turbidity for liquid cultures was monitored with a photoelectric Klett-Summerson colorimeter, using a green filter No. 54. Growth was monitored at 42°C (as the pyrG mutation in most of the strains in this work are temperature sensitive) or 37°C, and recorded as Klett Units (KU), where 1 KU = 1 x 10^7 cells/ml. Liquid cultures were grown in a New Brunswick Gyrotory water bath at 200 revolutions/minute.

Plasmid Isolation and Purification

The pKT240 and pBR322 derived plasmids used in this work were extracted from E. coli using the method of Timmis et al., (1978). A 1 l overnight culture was harvested by centrifugation at 7,500 x g for 12 minutes and resuspended in 12 ml of a 25% sucrose solution containing 0.05 M Tris, pH 8.0. To this suspension was added 2 ml lysozyme solution (5 mg/ml lysozyme in 0.25 M Tris, pH 8.0) followed by 4 ml of
0.25 M EDTA pH 8. After 5 minutes 16 ml of Triton detergent solution (2% Triton X-100 in 0.05 M Tris pH 8.0, 0.0625 M EDTA) was added. After incubation at 0°C for 15 minutes the detergent mix was clarified by centrifugation for 15 minutes at 40,000 x g.

Eight ml of the cleared plasmid solution were mixed with 8 g cesium chloride plus 1 ml of ethidium bromide solution (5 mg/ml in water). The solution was then centrifuged to equilibrium at 40,000 x g for 40 hours in a Beckman 50Ti rotor.

After removing the plasmid band from the centrifuge tube with a 19 gauge needle, the ethidium bromide was extracted with an equal volume of 1-butanol several times, until the solution lost its pink color. The plasmid containing solution was then dialyzed against several changes of a solution of 10 mM Tris and 1mM EDTA pH 8.0 (TE).

The plasmid was then concentrated by precipitation in 0.1 volume of 3 M sodium acetate and 2 volumes of 95% ethanol, left on ice overnight, then centrifuged at 4,000 x g for 45 minutes. The plasmid pellet was redisolved in TE.

Transformation of strain KR1530 using the plasmids prepared as above, was accomplished by inoculation of 1 ml of Luria-Bertani (LB) broth (Miller 1972), which contained 10 g tryptone, 5 g yeast extract, and 10 g sodium chloride per 1 water, with a loop from a single colony of strain KR1530.
This was incubated at 37°C over night, then 100 μl was used to inoculate 10 ml of LB broth which was then grown to 100 KU. After 20 minutes on ice the culture was harvested by centrifugation at 2500 x g for 15 minutes at 4°C. The pellet was gently resuspended in 5 ml sterile, 4°C 100 mM calcium chloride and incubated on ice for 30 minutes. Cells were recentrifuged at 2500 x g for 15 minutes at 4°C, and the pellet resuspended in 1 ml 4°C 100 mM calcium chloride. A 300 μl sample of competent cells was mixed with 1-2 ng of plasmid DNA (A260 x 50 = mg/ml), incubated on ice for for 30 minutes and then heat shocked at 42°C for 2 minutes. Cells were allowed to outgrow for 2 hours in LB broth then plated on selective medium, usually LB agar containing 30 μg ampicillin per ml (Bethesda Research Laboratories Manual).

Cell Extract Preparation

An overnight culture of strain KR1530 was used to inoculate one l of glucose, uracil, and arginine medium, with or without cytidine. The culture was grown at 37°C with shaking (200 revolutions/minute). When the culture reached a density of 100 KU, the cells were collected by centrifugation at 4,200 x g for 10 min at 4°C, washed with 40 mM Tris buffer, pH 7.0, and collected after centrifugation at 12,000 x g for
5 min. Cell pellets were stored overnight at -20°C, or used immediately.

The cell pellet collected from one l of culture was resuspended in 8 ml of 40 mM Tris buffer, pH 7.0, 1 mM dithiothreitol (DTT) and 0.02 mM zinc-acetate. Cells were broken by passage through the small cell (3 ml³) of a French Press (SLM-Aminco) at an internal cell pressure of 14,000 lb per square inch (psi). The extract was then centrifuged at 12,000 x g for 10 minutes to remove large cellular debris. The supernatant was centrifuged at 15,000 x g for 1 hour at 4°C to remove any other particulate matter. The remaining supernatant was dialyzed for two hours against two changes of 40 mM Tris buffer, pH 7.0, 0.02 mM zinc-acetate to remove DTT that might interfere with the colorimetric assay. After dialysis, the cell-free extract was assayed immediately or stored at 4°C until further used.

Aspartate transcarbamoylase assay

ATCase activity was determined by the procedure of Prescott and Jones (1969). Each assay contained the following solutions in a final volume of 2.0 ml: 40 mM Tris (pH 7.0), 4 mM carbamoylphosphate (Sigma), 0.2 ml suitably diluted enzyme preparation (see Spot Assay), 1.0 mM - 150 mM potassium-aspartate and, if required, 1 mM ATP or 1 mM CTP. All assay
tubes were prepared in advance (without carbamoylphosphate) and preincubated at 28°C for several minutes. The reaction was initiated by the addition of carbamoylphosphate, which had been prepared just prior to the beginning of the assay. At 10 minute intervals (10, 20, and 30 minutes) 0.5 ml of reaction mix was pipetted into stop tubes that were maintained on ice and contained 0.5 ml deionized water and 1 ml color mix (100 ml of 50 mg antipyrine (Sigma) in 50% sulfuric acid, 50 ml 40 mg monoxime (Sigma) in 5% acetic acid). Individual samples were kept on ice until all the samples had been taken. Color was developed by the incubation of the tubes, capped with marbles to limit evaporation, in a 60°C water bath exposed to room lighting. After 110 minutes the assay tubes were cooled to room temperature and stored in the dark for approximately 2 hours, when their optical density was measured. The absorbance at 466 nm (A-466) was determined in a Beckman DU-40 model spectrophotometer against a buffer blank. Controls to determine contribution of buffer, substrates, and cell extracts were performed.

Spot Assay

A modification of the complete ATCase assay, referred to as the Spot Assay was used to estimate the proper dilution of a
cell extract to use in a complete assay, and to follow the ATCase assay activity in fractions from molecular weight or ion exchange chromatography. A premix containing (final concentration) 40 mM Tris buffer pH 7.0 and 10 mM potassium-aspartate was prepared. A 20 ml sample of the appropriate fraction or cell extract was placed in a tube, and 430 ml of the premix added. The reaction was performed at 28°C and initiated by the addition of 50 µl 0.1 M carbamoylphosphate. After 20 minutes, 1.5 ml of the stop mix was added to the reaction tube, and the mixture incubated at 60°C until color production was observed (between 20 minutes and 2 hours), when its optical density at A466 was checked.

Protein Determination

The method of Lowry et al. (1951) was used to determine the total protein concentration of some samples. The standard curve was made from tubes containing from 0-100 µg bovine serum albumin (BSA) in 10 µg increments in a total volume of 200 µl. The 0 µg tube contained 200 µl distilled water, the 10 µg protein tube contained 190 µl distilled water + 10 µl 0.1% BSA (100 µl 1% dessicated BSA and 900 µl distilled water yields a 1 µg protein per ml solution of BSA), the 20 µg tube contained 180 µl distilled water + 20 µl 0.1% BSA, the 30 µg tube contained 170 µl distilled water + 30 µl 0.1% BSA, etc.
Samples were made from three different dilutions of the cell free extract, 5 µl, 10 µl, and 20 µl made to 200 µl with distilled water. More dilute samples used greater volumes of cell free extract in the final 200 µl. Alkaline copper reagent (0.5 ml 2% sodium-potassium tartrate, 0.5 ml 1% copper sulfide, mixed before adding 49 ml of 2% sodium carbonate in 0.1 N sodium hydroxide) was added (800 µl) to all tubes and they were allowed to stand at room temperature for 10 minutes. Folin reagent (100 µl, 1 N commercial preparation diluted 1:1 with distilled water) was added to all tubes and they were incubated at room temperature for 30 minutes. The absorbance was read at 660 nm on a Beckman DU-40 model spectrophotometer.

Activity Gels

Non-denaturing 4-20% gradient polyacrylamide gels were employed to screen for alterations in the molecular weight of ATCase enzyme from mutants not responding to CTP. The stock solution of acrylamide contained: 30% w/v acrylamide, and 0.8% w/v N, N'-bis-methylene acrylamide in distilled water. The 4% acrylamide solution contained: 1 M Tris, pH 9.0 (3 ml), acrylamide-bisacrylamide stock (4 ml), distilled water (22.67 ml), and 10% ammonium persulfate (0.3 ml). The 20% solution (for the bottom of the gel) contained: 1 M Tris,
pH 9.0 (3 ml), acrylamide-bisacrylamide stock (20 ml), distilled water (6.67 ml), and 10% ammonium persulfate (0.3 ml). After the 4% and 20% solutions were made they were degassed for 20 minutes and 10 μl TEMED added immediately prior to pouring the gel. The upper gel buffer (10X) contained 6 g Tris and 28.3 g glycine made up to 1 l with distilled water. The lower buffer (10X) contained 10.69 g Tris pH 8.1, made up to 100 ml with distilled water. Samples of the cell free extract (25-30 μl) were mixed with 5X acrylimide loading buffer (0.1% Coomassie Blue in absolute alcohol, 0.01% aqueous Bromphenol blue, in a 50% sucrose solution) and loaded onto a gel that was then run at 90-100 volts (15 mamps) for 15-18 hours. The gel was then specifically stained for ATCase activity by a procedure developed by Bothwell (personal communication to G.A. O'Donovan), and modified by K. Kedzie, (1987 PhD dissertation, Texas A & M University College Station) who used lead nitrate rather than lead sulfate. This staining procedure involved the incubation of the gel in cold (4°C) 50 mM Tris pH 7.0, 20 mM potassium-aspartate and 10 mM carbamoylphosphate for 20 minutes. The gel was then washed in 3 changes of ice water and incubated in 3 mM lead nitrate for 5 minutes, or until a white precipitate began to appear. The gel was again washed three times in distilled water, and left overnight, at 4°C to allow the background precipitate to diffuse out. This
activity stain utilized the phosphate released by the enzymatic condensation of carbamoylphosphate with aspartate into carbamoylaspartate, the phosphate being trapped in the gel matrix by its conversion into an insoluble, white lead precipitate. For increased definition the lead phosphate precipitate was then converted into a dark lead sulfide precipitate by the immersion of the gel into 2.5 mM ammonium sulfide solution for 2-3 minutes or until the bands turned a dark brown colour.

Molecular weight standards were run on a 4-20% acrylamide gel as above except they were stained with 0.05% Coomassie Blue in 7% acetic acid to cover the gel and shaken gently on a gyrotory shaker for 1.5 hours, before being destained overnight in several changes of 7% acetic acid.

Extraction of Nucleotides from Bacterial Cultures

Volumes of 80 ml of bacterial culture, grown as described above, to a density of 100 KU were harvested and centrifuged at 4°C at 12,000 x g for 3 minutes. The supernatant was decanted and the cell pellet was washed in minimal medium. One ml of 6% w/v cold trichloroacetic acid was added to the cell pellet and mixed by vortex for 2 minutes and allowed to stand at 4°C for 30 minutes before further centrifugation at 12,000 x g for 10 minutes. The clear supernatant was then
removed and its pH increased by adding an equal volume of cold Freon-amine solution (0.7 M tri-n-octyl-amine (TOA) in Freon 113). The Freon-amine sample mixture was vortex-mixed for 2 minutes and then allowed to separate for 15 minutes at 4°C. The top aqueous layer, containing the nucleotide extract, was removed, filtered through a 0.45 μm ACRO LC13 filter, and frozen at -20°C until analyzed.

**HPLC standards**

Standards for the HPLC were made in 1 mM concentration and 10 μl injected at the start and end of each daily run to establish retention times for all 12 ribonucleoside phosphates.

**Chromatographic Apparatus and Conditions for Separation and Evaluation of Nucleotides**

The Waters HPLC equipment used consists of two Model 510 pumps, a Model 680 automated gradient controller, a U6K injector, and a Model 481 LC spectrophotometer. Nucleotides were detected by monitoring the column effluent at 254 nm with a sensitivity fixed at 0.05 absorbance units full scale (a.u.f.s.). Separations of nucleotides were performed on a Waters Radial-Pak Partisil SAX Cartridge (10 cm x 0.8 cm)
using a Waters radial compression Z-module system. This is a 10 μm particle size column that separates the nucleotides on the basis of charge. The elution buffer system consisted of eluent A, 7 mM ammonium phosphate (pH 3.8), and eluent B, 250 mM ammonium phosphate (pH 4.5) with 500 mM potassium chloride. Eluent A was brought to the correct pH using 1 M hypophosphorus acid, eluent B was brought to the right pH using a 50% ammonium hydroxide solution.

Nucleotide samples (100 μl), obtained as described above, were injected into the column. A linear gradient of eluent A to eluent B was applied for 20 min followed by an isocratic period of 10 min with eluent B buffer. The column was regenerated by washing with 30 ml of eluent A (pH 3.8) buffer. The flow-rate was maintained at 4 ml/min and analysis performed at ambient temperature. Peaks were integrated and areas calculated using a Waters 740 Data Module.

Chromatographic Apparatus and Conditions for Separation and Evaluation of Bases and Nucleosides

Bases and nucleosides in the cdd and cod enzyme assays were separated on a reverse-phase column. The column used for this separation has a 5 μm particle size and is generally referred to as a "high resolution" column. It was chosen not
only because of its greater resolution but because it can be cleaned with acetonitrile, which is important because unpurified cell extract was used for the enzyme assays. Bases and nucleosides were detected by monitoring the column effluent at 254nm with sensitivity fixed at 0.1 a.u.f.s., and were separated on a reverse phase C18 column, run isocratically with a 0.05 M ammonium phosphate, pH 3.5 buffer.

Nucleotides were separated using ion-pair chromatography. Pic A (Pierce Chemical Co.) was used as the ion pairing agent and added at a concentration of 1 ml per 250 ml of buffer. A C18 Radial-pak with 10 µm size particles was used. Nucleotides were eluted isocratically with 95% buffer A (25 mM ammonium dihydrogen phosphate with 1 mM tetrabutyl ammonium phosphate, pH 7.0) and 5% buffer B (15% acetonitrile) at a flow rate of 2 ml/minute. Areas under the peaks were integrated using a Waters 740 Data Module.

**HPLC Enzyme Assays**

Enzyme assays done on HPLC used cell free extracts prepared as above except they were undialysed and centrifuged in microfuge tubes at 4°C, 9,000 x g for 20 minutes, then the supernatent was either used without further treatment or dialysed against 10 mM Tris, 1 mM EDTA, pH 7, to remove
phosphates and other ions. One ml of 1 mM stock solutions of the substrates (cytidine, cytosine, or uridine) was mixed with 20 µl of the cell extract, incubated (depending on the assay) for between 0 and 20 minutes and 10 µl injected into the column as described above.

**HPLC Maintenance**

The system, including the column, was stored in 50:50 (v/v) filtered methanol and distilled water when not in use. The system was flushed with 50 ml of methanol:water mixture at 1 ml/minute. The pumps were flushed separately by opening the reference valve, with 50 ml of filtered distilled water at 9 ml/minute. The injector loop was flushed by setting the injector valve in the inject position. The flow rate was changed to one ml per minute and the reference valve closed to flush the column. When the pressure dropped from 1000 to 600 lb per square inch, the 50:50 methanol:water mixture was completely washed from the system. The flow rate was increased to 2 ml/minute and the injector valve was changed to the load position to insure that both sides of the loop were equilibrated with the filtered distilled water. Pump A was flushed with the starting buffer (filtered 7 mM ammonium phosphate pH 3.8). The injector loop and column were then
flushed as before. Pump B was flushed separately with final buffer, 250 mM ammonium phosphate (pH 4.5) with 500 mM KCl.

Calculations: All nucleotides are reported as μmoles per gram dry weight of cells and computed as follows:

\[
\frac{Sa \times C \times V}{St \times V_i \times D_w}
\]

where \( Sa \) = peak height of sample, \( St \) = peak height of standard, \( C \) = g compound in standard/molecular weight of compound, \( V \) = total volume of extract, \( V_i \) = volume of extract injected and \( D_w \) = dry weight (Dutta and O'Donovan 1987).
Results

Salmonella typhimurium strain KR1530 has the following genotype pyrA cdd udp pyrG(ts). It was used throughout this research. Because its genotype was so important for the experiments carried out, it was necessary to verify this genotype carefully. This was done with reverse phase chromatography on the HPLC. Fig. 5 shows the cytidine and uridine standards: panel a shows the cytidine peak alone, panel b shows the uridine peak, and panel c shows both peaks. Fig. 6 shows the cytosine and uracil standards. Panel a gives uracil alone, panel b gives cytosine alone and panel c gives both cytosine and uracil. Fig. 7 panel a shows that when cytidine (1 ml of 1 mM per 20 μl cell extract) was incubated with the KR1530 extract no uridine was formed, thus confirming the absolute cdd− genotype. However, small amounts of uracil and cytosine appeared. When uridine was incubated with the extract from KR1530 (panel b) some uracil was formed suggesting that the udp− gene was partially functional. Panel c again shows the result of the incubation of cytidine but this time after the extract was dialysized against Tris-EDTA buffer (TE) pH 7.0 overnight. As can be clearly seen in panel c there was no uracil or cytosine present as product. Accordingly, the genotype cdd, udp was confirmed for the KR1530 strain. If the cdd mutation
Fig. 5 HPLC pyrimidine nucleoside standards (a) cytidine (b) uridine (c) cytidine and uridine
Fig. 6 HPLC pyrimidine base standards (a) uracil (b) cytosine (c) cytosine and uracil.
Fig. 7 HPLC cytidine deaminase assay. (a) undialysed KR1530 cell extract added to 1 mM cytidine. Cytidine remains, there is no uridine but cytidine is converted to cytosine (unlabeled small peak to the left of uridine) and, ultimately to uracil. (b) uridine, 1 mM was used with a udp strain, very little uracil formed but the mutant appears to be leaky. (c) cytidine added to a dialysed cell extract, as phosphates were dialysed out there is no cytosine or uracil formed.
is complete as shown in Fig. 7, panel a, how was the cytosine and uracil in the panel formed? To answer this question the experiments shown in Fig. 8 were carried out. Once again cytidine was incubated with an undialyzed extract of KR1530 (panel b). After 12 minutes all the cytidine was broken down into cytosine (75%) and uracil (25%). Since it is known (Beck et al. 1972) that cytidine is not converted to cytosine in *S. typhimurium* by either phosphorylase or cytidine (uridine) hydrolase, some other pathway must be in operation. This pathway operates as follows: cytidine is phosphorylated to CMP by uridine kinase and the CMP so formed is degraded to cytosine and ribose-5-phosphate by nucleoside monophosphate glycosylase (Neuhard and Nygaard 1987). When the extract was dialysed overnight against TE buffer, and cytidine was now added, there was no conversion of cytidine (panel a). This was the expected result because the ATP or GTP (and all phosphates) required for the kinase reaction were eliminated by the dialysis. This pathway is described in more detail in the Discussion. In addition to elucidating the pathway, the above experiments resolved the problem of the strains not growing well on cytosine and being resistant to 5-fluorocytosine. They had a *codB* or cytosine permease mutation.

The *pyrG* mutation in strain KR 1530 is supposed to be temperature sensitive at 42°C. To test this, the strain was grown at 37°C (Fig. 9) and shifted to 42°C. As can be seen
Fig. 8 HPLC CMP glycosylase and cytosine deaminase assay (a) cytidine peak after cytidine was incubated with KR1530 cell extract that had been dialysed, no uridine. (b) cytosine incubated with KR1530 cell free extract and injected on HPLC. codA is functional from the increase in uracil.
Fig. 9 Effect of temperature shift from 37°C to 42°C on the CTP pool (peak 10) of strain KR1530. Top panel: 42°C and bottom panel: 37°C.
Retention Time (Minutes)

0.05 AUFS (absorbance unit full scale)

1 = CMP  7 = ADP
2 = AMP  8 = GDP
3 = UMP  9 = UTP
4 = GMP  10 = CTP
5 = UDP  11 = ATP
6 = CDP  12 = GTP
**Fig. 10** Effect of CTP concentration (peak 11) on UTP pool (peak 10). (a) KR1530 grown in 250 µg/ml cytidine and (b) 50 µg/ml cytidine.
from the figure, the mono-, di, and triphosphates are present at 37°C (panel b) but the CTP concentration (peak 10, panel a) is missing at 42°C. This proves that there is a complete block in the conversion of UTP to CTP only at 42°C.

Fig. 10 shows the effect of high (250 µg/ml) and low cytidine (50 µg/ml) on the same ribonucleotide pools shown in Fig. 9. Arginine but no uracil was offered to strain KR1530. Panel a shows that despite being fed no exogenous uracil for this pyrA, udp, cdd, pyrG(ts) strain, both the UTP and CTP pools were normal. Once again the CR → CMP → C pathway is employed. In panel b, with low cytidine, the CTP pool was normal while the UTP was limited. Thus in strain KR1530 requiring arginine and uracil (which can be supplied by uracil, uridine, cytidine, or cytosine) the reported genotype is confirmed completely.

Strain KR1530 was next used to isolate citrulline users, citrulline for arginine via arginino-succinate and citrulline for pyrimidine requirement (Cpr+) via ornithine trans-carbamoylase in reverse (Fig. 2). Different concentrations of citrulline were used from 50 µg/ml to 750 µg/ml. Colonies appeared after 5-7 days but on purification by restreaking to the same plates many pure colonies were found to be unstable. Since it was thought that long-term starvation for pyrimidines (UTP) would ultimately cause dTTP starvation, thymine (20 µg/ml) and deoxyinosine (100 µg/ml) as a source
of deoxyribose-1-phosphate (O'Donovan 1978) were included in the incubation mixture. Stable Cpr\(^+\) strains emerged. All of these strains that were assayed for ATCase and OTCase were found to be greatly derepressed for both transcarbamoylases.

Ten of these Cpr\(^+\) mutants were selected for further study. The nucleoside triphosphate pools of four of these strains are shown in Table 3. Also included in this Table are the results of adding increasing concentrations of exogenous cytidine. Three points are made regarding Table 3. (1) All nucleoside triphosphates are lower than in the parent strain, KR1530, (2) Addition of cytidine (to a cdd strain) increases the CTP level (mutant AB1009) and (3) more importantly, decreases the UTP pool (mutant AB1009). This proves that feedback inhibition of ATCase by CTP occurs in vivo as evidenced by the decrease in the UTP concentration concomitant with the addition of exogenous cytidine.

It was now reasoned that if the mere dribble of the pyrimidine biosynthetic pathway growing on citrulline were subjected to cytidine (and hence CR → CMP → CDP → CTP) it should be possible to prevent growth entirely. This was achieved. Now in cytidine resistant mutants were sought among the Cpr\(^+\) strains at 37°C. Various concentrations of cytidine were used and several colonies arose. It appeared that each had escaped the stranglehold of cytidine for feedback inhibition by CTP on their stressed ATCases.
Indeed, the most common class was not involved with ATCase at all, but rather was a class of cytidine (uridine) permease mutants which failed to transport cytidine into the cell. This was a surprise. To counteract the selection of this type of mutant, selection was made on cytidine at 42°C. Then, only those strains which obtained CTP from the exogenous cytidine could grow. At least five different classes of regulatory mutants were isolated. Representative samples of each of these are shown in Figs. 12-18, where the parent strain KR1530 and five mutant strains are depicted. In each figure, panel a gives the time course for ATCase activity and panel b the velocity-substrate plots for the ATCases.

Five points are made for Figs. 12-18: 1) The specific activity of the parent ATCase was 50 nmoles per minute per mg protein and inhibition by CTP and activation by ATP were evident. 2) The specific activity of ATCase in all mutant strains was greater than that of KR1530; in some cases (e.g. AB1002) enormous levels of derepression were seen. This proved that carbamoylphosphate was limiting which is in keeping with the almost 100% inhibition that was possible with elevated CTP. 3) One mutant showed no activation by ATP, another showed inhibition by ATP. 4) One mutant (AB1007) was extremely active and displayed Michaelis-Menten kinetics. It had neither inhibition, nor activation by
effectors and had a molecular weight of 100,000 indicating that it lacked the regulatory subunit. 5) Mutant AB1001 had an extremely high Km for aspartate. This made it safe from CTP inhibition because inhibition is diminished at high aspartate (>30 mM) concentrations. Such an aspartate concentration is possible in the *S. typhimurium* strain employed in these studies.

Further details about the results and some of their implications are elaborated on in the Discussion.
Fig. 11  Lowry assay and carbamoylaspartate standard curves  
(a) standard curve for Lowry protein determination (b)  
standard curve for carbamoylphosphate.
Fig. 12 KR1530 enzyme assays (a) Time course of ATCase reaction without effectors •••, and with effectors ⨕⨯ (ATP) and ⨕⨯ (CTP) at 5 mM aspartate for S. typhimurium parent strain KR1530. Ordinate is absorbance at 466 nm. (b) Effect of aspartate concentration on ATCase activity of S. typhimurium strain KR1530. Ordinate is the percentage of maximal activity at 466 nm.
Fig. 13 AB1002 enzyme assays (a) Time course of ATCase reaction without effectors •••, and with effectors ••• (ATP) and ◦◦ (CTP) at 5 mM aspartate for *S. typhimurium* strain AB1001. Ordinate is absorbance at 466 nm. (b) Effect of aspartate concentration on ATCase activity of *S. typhimurium* strain AB1002. Ordinate is the percentage of maximal activity at 466 nm.
**Fig. 14** AB1004 enzyme assays (a) Time course of ATCase reaction without effectors °-°, and with effectors •--• (ATP) and ◦-○ (CTP) at 5 mM aspartate for *S. typhimurium* strain AB1004. Ordinate is absorbance at 466 nm. (b) Effect of aspartate concentration on ATCase activity of *S. typhimurium* strain AB1004. Ordinate is the percentage of maximal activity at 466 nm.
**Fig. 15** AB1005 enzyme assays (a) Time course of ATCase reaction without effectors ◦-◦, and with effectors •-• (ATP) and ◦-◦ (CTP) at 5 mM aspartate for *S. typhimurium* strain AB1005. Ordinate is absorbance at 466 nm. (b) Effect of aspartate concentration on ATCase activity of *S. typhimurium* strain AB1005. Ordinate is the percentage of maximal activity at 466 nm.
**Diagram a**

- Y-axis: Absorbance at 466 nm
- X-axis: Time (minutes)
- Lines:
  - Open circles: Control
  - Filled circles: ATP
  - Filled diamonds: CTP

**Diagram b**

- Y-axis: % relative activity
- X-axis: Aspartate (mM)
- Trend: % relative activity increases with increasing aspartate concentration.
Fig. 16 AB1006 enzyme assays (a) Time course of ATCase reaction without effectors °••, and with effectors ••• (ATP) and °•• (CTP) at 5 mM aspartate for *S. typhimurium* strain AB1006. Ordinate is absorbance at 466 nm. (b) Effect of aspartate concentration on ATCase activity of *S. typhimurium* strain AB1006. Ordinate is the percentage of maximal activity at 466 nm.
### Graph a

- **Y-axis**: 466 nm
- **X-axis**: Time (minutes)
- **Legend**:
  - Control
  - ATP
  - CTP

### Graph b

- **Y-axis**: % relative activity
- **X-axis**: Aspartate (mM)

The graphs show the changes in 466 nm absorbance over time for different treatments. Graph b illustrates the relative activity percentage in relation to varying concentrations of aspartate.
**Fig. 17** AB1007 enzyme assays (a) Time course of ATCase reaction without effectors °-°, and with effectors ··· (ATP) and °-° (CTP) at 5 mM aspartate for *S. typhimurium* strain AB1007. Ordinate is absorbance at 466 nm. (b) Effect of aspartate concentration on ATCase activity of *S. typhimurium* strain AB1007. Ordinate is the percentage of maximal activity at 466 nm.
Fig. 18 Effects of pyrimidine starvation on nucleotide pools of *S. typhimurium* (a) ribonucleotides (b) deoxyribo-nucleotides (Neuhard 1968).
A

UTP
CTP
ATP
GTP

μmoles/g dry wt.

min. after cytidine removal

B

dTTP
dCTP
dATP
dGTP

μmoles/g dry wt.

min. after cytidine removal

>«
Table 3

Triphosphate Pools\(^a\) in Citrulline Utilizing Mutants

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\(^a\)\(\mu\)mol/g dry wt
Discussion

Three separate but related goals have been achieved with this dissertation: (i) To measure feedback inhibition in vivo, (ii) To obtain regulatory mutations in the pyrBI genes showing altered feedback inhibition in ATCase, (Figs 1 and 2), and (iii) To develop a method of accurately evaluating the contribution to the nucleotide pools of the various de novo and salvage pathways. The primary goal of measuring feedback inhibition of ATCase in vivo necessitated the remaining two projects, one of which was the development of a strategy for genetic manipulation of the pyrimidine pathway, in particular the pyrBI gene product, ATCase. The other project was the precise measurement of end products and related enzyme activity of the de novo and salvage pyrimidine pathways to allow realistic interpretation of nucleotide pool results.

To measure feedback inhibition in vivo requires isolation of a system with a functional ATCase independent from other regulatory systems and then the precise measurement of the products, UTP and CTP, of the pathway under various conditions expected to affect feedback inhibition. As can be seen from Fig 2, a source of carbamoylphosphate, not dependent on the regulation of the
pathway by end products was required. Exogenous carbamoylphosphate is not taken up by *Salmonella typhimurium* and has a 30 minute half-life at 37°C (Bethell et al. 1968). To remove any chance of regulation of different enzymes by the end product UMP, a deletion in *pyrA* was used, which allowed eventual flow of carbamoylphosphate through the pathway to be dictated by the supply of its exogenous source, citrulline. Citrulline is synthesized by the action of ornithine transcarbamoylase (OTCase, encoded by *argI*, E.C. 2.1.3.3) and converted to arginine via arginino-succinate synthetase (*argG*) and arginino-succinate lyase (*argH*). Arginine regulates the system in two ways that could have an impact on the current experiments. It represses CPSase (not a factor here because of a *pyrA* deletion) as well as all arginine enzymes, including OTCase, and it inhibits OTCase up to 58% at 5 mM arginine (Glansdorff 1987). To efficiently utilize citrulline as a source of carbamoylphosphate in a *pyrA* strain requires further genetic manipulation. For example, a *pyrG* bradyotroph allows the accumulation of citrulline from exogenous source and keep the level of arginine produced low so that inhibition and repression of OTCase does not occur. Mutations resulting in permanent derepression of OTCase (i.e. promoter mutations) also create circumstances where sufficient carbamoylphosphate is produced to satisfy the pyrimidine requirement as a result of OTCase
acting in reverse as long as citrulline can be maintained in high enough concentration in the cell (Legrain and Stalon 1976; Legrain et al. 1976a,b; Abdelal et al. 1977). Strict mutations in either arginino-succinate lyase or arginino-succinate synthetase would also allow accumulation of citrulline but would require exogenous arginine which has a 10 fold lower Km for uptake than does citrulline (Maas 1961; Celis 1977a, 1981; Furlong 1987).

Previous attempts to isolate stable citrulline users (Legrain et al. 1976) were not successful. These workers began with the assumption that it would take a great deal (about 1000µg/ml) of citrulline to force the ornithine transcarbamoylase (OTCase) in the reverse direction:

\[
\text{Citrulline} + \text{Pi} \xrightarrow{\text{OTCase}} \text{Carbamylphosphate} + \text{Ornithine}
\]

\[
(1000 \, \mu\text{g/ml})
\]

\[
\text{arg}^{+} \xrightarrow{\text{arg}^{+}} \text{arg}^{-}
\]

to produce carbamoylphosphate. The carbamoylphosphate was required to satisfy the pyrimidine requirement of a pyrA (lacks CPSase, Fig. 1) strain. Any carbamoylphosphate so produced would combine with aspartate to produce carbamoyl-aspartate and ultimately UTP and CTP.

The first point observed by Legrain et al. (1976b) was that much of the citrulline was being shunted through arginino-succinate to satisfy the arginine requirement. This eliminated any chance of fulfilling the pyrimidine
requirement because the citrulline was being converted to arginine via arginino-succinate. The amount of arginine produced in this way was sufficient to repress the synthesis of OTCase (and other arginine enzymes). To obviate this problem arginine requiring mutants were used, blocked in the conversion of citrulline to arginine. These cells could now retain high levels of citrulline and make it available for phosphorolysis to carbamoylphosphate. This created a new problem for Legrain et al., due to the fact that now the Arg^{-} cells required arginine for growth. When fed arginine at low concentrations in the presence of high citrulline, problems were encountered in getting adequate citrulline into the cell. This was because the Km for arginine specific permeases is much less (more efficient) for arginine than for citrulline. Thus, under these conditions, it was not possible to get enough citrulline into the E. coli K12 cell.

Another problem encountered was due to the fact that E. coli K12 contains two different genes, argI and argF, which encode ornithine transcarbamoylase (Maas 1961; Legrain et al. 1976c). Even though these two genes are repressed by growth on arginine (Glansdorff 1987) they did not behave similarly in this experiment.

When citrulline users were finally isolated they were found to be unstable. This was probably due to the fact that when a pyrA strain, which requires both arginine and uracil,
is plated on citrulline to satisfy both requirements there is a considerable lag time until sufficient citrulline is concentrated to allow OTCase to act in reverse to produce carbamoylphosphate. In the meantime, the arginine requirement is satisfied but the cells are starved for pyrimidines. Even though UTP and CTP are the pyrimidine nucleotides that need to be satisfied in Pyr− strains, the ultimate pyrimidine is dTTP. Starvation for dTTP is known to cause thymineless death (Cummings and Mondale 1967; Eisenstark et al. 1968; Kunz and Glickman 1985) as well as make strains hypermutagenic and hence unstable. As can be seen in Fig. 19 Neuhard (1968) has shown that when a pyrimidine requiring strain is starved for uracil the initial response is for the dTTP pool to rise significantly. After prolonged starvation however, the dTTP level drops and becomes near zero. This causes thymineless death or produces survivors which are not stable.

The research described in this dissertation was begun before reading the manuscript by Legrain et al. (1976b). Our reasoning in obtaining citrulline users was different from that of Legrain et al. Whereas their plan was to isolate arginine pathway regulatory mutants, the aim of this research was to isolate allosterically modified mutants of ATCase and to create intracellular circumstances to assess feedback inhibition in vivo.
The starting strain in this research was *S. typhimurium* rather than *E. coli*. This was for three reasons (1) *S. typhimurium*, unlike *E. coli* K12, has only one ornithine transcarbamoylase, that encoded by *argI* (2) *S. typhimurium* has much greater feedback inhibition by CTP than *E. coli*, and (3) appropriate mutations were available in *S. typhimurium* and were not available in *E. coli*.

The primary aim was to isolate mutants which contained modified ATCase enzyme in *S. typhimurium*. To achieve this it was critical that a fully functional pyrimidine biosynthetic pathway through ATCase was in use. Other workers (Schwartz and Neuhard 1975) have used pyrG cytidine requiring, temperature-sensitive mutants which, when grown at sub-maximal temperatures, contain pyrimidine pathways that are limiting. Jensen et al. (1986) used UMP to feed pyrimidines slowly to Pyr− strains. This is effective in producing low intercellular levels of UDP, UTP and CTP in cells that therefore have a high expression of ATCase. Neither of these methods was acceptable for the purposes of this research.

Selection of citrulline users in strain KR1530 on 50 - 250 μg citrulline per ml required a week at 37°C and resulted in small or medium sized colonies that when restreaked on citrulline, after suspension in water, resulted in medium or large colonies after 1-2 days, designated Cpr+, for citrulline
for pyrimidine requirement. It is possible that two mutations are required in this strain to allow citrulline use for pyrimidines. The level of OTCase in several of the mutant strains was high (AB1001, AB1003, AB1009) when they were grown on arginine and uracil, or on citrulline medium, as would be explained by a promoter or operator mutation in the regulation of OTCase making its expression constitutive. The first mutants obtained after one week were extremely unstable. After growth in LB medium, or in arginine plus uracil, the mutants were either lost entirely or grew very poorly when reintroduced to citrulline medium. Storage at 4°C on citrulline for more than two days also prevented recovery. This may be due to the fact that the transport system for arginine and ornithine works at 4°C for arginine but not for ornithine, which citrulline more closely resembles (Celis 1981; Furlong 1987; Glansdorff 1987). Some (30%) mutants did not even survive transfer from the original citrulline plate to identical plates. Increase in citrulline concentration in the medium did not increase survival, nor did casamino acid supplementation. As depletion of the dTTP pool is known to cause thymineless death or high mutation frequency, particularly in a recA+ strain, (Cummings and Mondale 1967; Eisenstark et al. 1968; Kunz and Glickman 1985) as KR1530, the possibility of depleted nucleotide pools causing the instability of the mutants was considered.
Addition of thymidine was not sufficient to increase long-term stability of the strains but use of 20 μg thymine per ml and 100 μg deoxyinosine per ml did result in the mutants being recovered after growth in uracil and arginine and in their being able to survive at 4°C on citrulline, thymine, and deoxyinosine. The lack of response to thymidine may be due to its not being incorporated, long term, into DNA, while thymine, as long as deoxyinosine is available to provide deoxyribose-1-phosphate, is incorporated over a long period of time. (O'Donovan 1978)

Starvation of a Pyr− mutant for uracil initially elevated dTTP which fell drastically after several hours (Fig. 19) In mutant strains with significantly lower than normal pools (the majority as in Table 3, AB1002) it would be expected that the dTTP pools (as well as others) would be depleted beyond recovery after a downshift in a medium from conditions that allow repression and inhibition (high UTP, CTP, and arginine) to conditions of minimal medium.

Two different levels of citrulline, 50 μg/ml and 750 μg/ml, were used to select for the mutants to allow for the possibility of two types of mutations being selected. Of the ten mutants selected for further study (five on 50 μg/ml and five on 750 μg/ml) only one, AB1008, a small colony originally selected on 50 μg citrulline per ml, was unable to grow at the other concentration. It grew after three days on
750 µg citrulline per ml and after one day at 50 µg citrulline per ml.

It was subsequently shown (Table 2) that most citrulline users which produced carbamoylphosphate did so at a minimal level, the UTP and CTP pools in such cases were extremely low, and as expected, ATCase was significantly derepressed (See Table 3). Thus, the flow through the pathway was just a trickle and as such the pathway was highly sensitive to end product inhibition. All that was needed to achieve this was to find a mechanism of increasing, and stabilizing the increase, of the end product CTP.

Before describing this step it is important to show how problems of others (O'Donovan and Gerhart 1972; O'Donovan et al. 1972; Legrain et al. 1976b) were avoided. Using the S. typhimurium strain pyrA, pyrG (ts), cdd which contained only one OTCase (Kelln and O'Donovan 1976), different levels of citrulline were used starting with much lower concentrations than those of Legrain et al.(1976b). To ensure that no starvation for dTTP would occur all cultures were inoculated with thymine and deoxyinosine. Deoxyinosine is added as a source of deoxyribose-1-phosphate (O'Donovan 1978) which is the limiting nutrient when thymine is provided to wild type cells. Then when, and if, dTTP becomes limiting before the end of the long-term uracil starvation, the exogenously fed thymine maintains the dTTP levels. It is now certain that
the dTTP pool remained higher. No unstable mutants appeared when cultures were inoculated with thymine and deoxyinosine from the start of the experiment.

The next step after isolation of the mutant colonies was to determine if these cells were sensitive to exogenously fed cytidine. As can be seen from Fig. 2, exogenous cytidine is primarily metabolized by *S. typhimurium* through cytidine deaminase such that all entering cytidine is converted to uridine and thence to uracil within 15 minutes (Beck et al. 1974). For this reason, a mutation in cytidine deaminase (*cdd*) was used to make certain sufficient cytidine could be concentrated in the cell. *S. typhimurium* does not convert cytidine to cytosine by either a uridine (cytidine) phosphorylase or by a uridine hydrolase (Neuhard 1983). However *S. typhimurium* does convert cytidine to CMP which is then phosphorylated further to CDP and CTP (O'Donovan and Neuhard 1970). This CTP is a powerful feedback inhibitor of ATCase (Gerhart and Pardee 1962). In wild type *S. typhimurium* cells the physiological concentration of CTP (about 1 mM) inhibits ATCase 93% (O'Donovan et al. 1972). Increasing the normal concentration two-fold, by feeding cytidine to a *cdd* strain was expected to put extreme pressure on the pathway which was merely trickling through carbamoylaspartate. Indeed, it was possible to stop growth entirely by adding excess cytidine. Table 3 also shows that
as cytidine is increased the CTP pool increases concomitantly with the decrease of UTP. The sensitivity of the enzyme to feedback inhibition is dependent on the amount of carbamoylphosphate available to the enzyme and thus the flow through the pathway (See Table 3 AB1002 vs AB1009).

One final step had to be added to the selection. A temperature-sensitive mutation, at pyrG, was placed in the selection strain. Then by growing the pyrG (ts) cells at 42°C, the restrictive temperature, all cells required cytidine for growth. By doing the selection at 42°C in high cytidine only those cells which obtained CTP for growth were candidates for ATCase mutants, ruling out permease mutations. Sufficient cytidine was added to the citrulline grown pyrA, pyrG, cdd strain at 42°C to swell the CTP pool which satisfied the cytidine requirement but in so doing produced a level of CTP that shut off ATCase completely. The excess CTP bound to the allosteric site on ATCase and stopped flow through the pathway. The only cells which escaped the stifling inhibition by CTP were mutants which did not bind CTP and thus survived. However, it was possible to isolate allosterically modified ATCase mutants without this final step.

A brief description of these mutants is now given. ATCase of S. typhimurium (O'Donovan and Gerhart 1972) is a dodecamer of structure 2c3:3r2. In other words, the enzyme is
composed of two trimeric catalytic subunits each with three identical polypeptide chains encoded by pyrB. It also contains three dimeric regulatory subunits, each with two identical regulatory chains encoded by pyrI. The catalytic subunit binds the substrates aspartate and carbamoylphosphate to produce carbamoylaspartate (Fig. 1). The regulatory subunits do not bind substrate but contain allosteric sites that bind CTP, the feedback inhibitor (and ATP the positive activator) (Gerhart and Schachman 1965). A mutant cell that did not produce the pyrI encoded regulatory subunit would not be inhibited by excess CTP. Such a mutant would contain trimeric enzymes (c3) only, that are not inhibited by CTP. These mutants were identified by checking the molecular weight of their ATCase. Wild type ATCase has a mw of 300,000 (2c3:3r2) while the catalytic trimer of ATCase is 100,000. One such mutant was isolated in this work (AB1007). A similar mutant had been previously isolated in vitro by mutating cloned DNA before returning it on a plasmid to a host strain. (Roof et al. 1982)

A second class of ATCase regulatory mutant contains the dodecameric structure, as the molecular weight was 300,000 the mutation did not delete the entire pyrI coded polypeptide but rather altered its structure. Surprisingly, the most frequent class obtained had 300,000 mw, inhibition by CTP, but instead of activation by ATP, they were inhibited by ATP.
A similar observation was made by Wild et al. (1980) with *Serratia marcescens* ATCase.

In ATCase with six catalytic polypeptide and six regulatory polypeptides the following interactions continuously occur. There are c-c interactions within and between the two trimeric catalytic subunits; there are r-r interactions among and between the three dimer regulatory subunits and most important for the mutations described here, there are r-c interactions between the regulatory and catalytic subunits. According to Ladjimi et al. (1985), this is the most likely and most desirable class of feedback modified mutants.

Much is known about the 3-D structure of ATCase. Many mutations exist in the *pyrB* gene which produce modified or defective catalytic polypeptides. Very few mutations, if any, derived naturally exist in the *pyrI*. One mutant (Feller et al. 1981; Cunin et al. 1985) has been obtained in a lambda lysate.

The positive selection described here is the first method that isolates mutants with modified or no feedback inhibition. The method worked because a barely functional pyrimidine pathway was derived from the phosphorolysis of citrulline. In a suitable strain, the feedback inhibitor CTP was built up. This build up was sufficient to stop growth completely. Any cells escaping this stranglehold had to be
mutants which contained ATCases that no longer bound the inhibitor CTP.

In initial tests KR1530, the parent strain, grew slowly (1 hour 15 minutes doubling time) on cytosine and arginine (see Fig 2) and had a nearly normal doubling time (45 minutes) when grown on cytidine and arginine after an extremely long lag (4-5 hours longer) compared to uridine and arginine. Measuring feedback inhibition of ATCase in vivo requires elevation of the CTP pool without affecting the UTP pool (see Fig. 1) so that any changes in UTP are unambiguously attributable to the inhibition of its de novo synthesis. This led to the development of a way to measure with extreme precision, the contribution of the salvage pathways and of exogenously fed pyrimidines to the nucleotide pools. The use of high performance liquid chromatography (HPLC) to measure nucleotide pools is well established. The pools are generally extracted from cellular lysates using acid or base conditions to remove contaminating protein. Using a method developed for the HPLC (PK Dutta, personal communication) it was possible to measure the precise activity of the salvage enzymes by measuring in vitro product formation, or substrate disappearance. Fig. 7 shows the degradation of substrate, cytidine, and the formation simultaneously of uridine and uracil, or the degradation of cytosine to form uracil (See Figs.7 and 8).
After verification of a functional cytosine deaminase in strain KR1530 it was determined that the strain harbors a defective codB or cytosine permease. This was tested both on the HPLC and by growth of the strain in the presence of 5-fluorocytosine, normally a powerful inhibitor. Growth of the strain on cytidine required that cytidine deaminase be evaluated. HPLC evaluation of substrate and product formation for the enzyme showed this strain to have absolutely no cytidine deaminase activity; uridine was not produced, however cytosine and uracil were, in low concentration. This method of evaluation of enzyme activity is superior to the usual method of monitoring changes in optical density due to the conversion of substrate because it allows the fate of the substrate to be followed through more than one pathway and to calculate the contribution of each of the salvage pathways. This removed any worry of uracil or uridine contamination of cytidine stocks. Normal spectrophotometric curves done without prior separation on the HPLC gave ambiguous results when cytidine was checked for contamination by cytosine.

Pyrimidine monophosphate glycosylase pmg (Neuhard 1983) is known to exist in enterics but is generally assumed to be a minor salvage enzyme. Under conditions of growth on high concentrations of cytidine of a pyrA, cdd strain, it appears to be induced (hence the long lag) to levels that allow it to
become the sole source of pyrimidines at a level that gives repression of ATCase synthesis. This occurs only when cytidine is provided in such a concentration as to swell the CTP pool to a maximal level that allows accumulation of CMP in sufficient quantity to induce the pyrimidine monophosphate glycosylase. (See Figs. 7 and 10) The glycosylase was functional in the parent strain, KR1530, at cytidine concentrations of 250 µg/ml, but not at 50 µg/ml. The amount of UTP at the lower cytidine concentration was a very low (0.92 µmol/g dry wt) while intercellular CTP was 2.30 µmol/g dry wt, at the higher cytidine concentration, UTP rose to 1.7 µmol/g dry wt and CTP fell to 1.6 µmol/g dry wt. The glycosylase activity was verified by doing an enzyme assay using HPLC. Cytidine was mixed with dialysed and undialysed cell extract. The undialysed extract showed a decrease in cytidine level over time and a concomitant increase in cytosine and a substantial increase in uracil (Fig. 7, 8 and 10). The dialysed extract showed no decrease in cytidine and no increase in cytosine, uracil or uridine. Dialysis removes phosphates thereby preventing the phosphorylation of cytidine to CMP and therefore its conversion to other higher nucleotides in the absence of a functional cytidine deaminase.

As a verification of the *pyrG* temperature sensitive mutation HPLC was again used. The intracellular nucleotide pools were measured from cultures grown on uracil and
arginine at 43°C and at 37°C (Fig. 9) CTP levels from cultures grown at 42°C were extremely low but were not entirely depleted, indicating an almost non-functional pyrG at 42°C.

During the course of these studies a paper appeared from Baur et al. (1989) which had particular relevance for the selection problem employed here. Whereas it is difficult to control the amount of carbamoylphosphate produced from high levels of citrulline in S. typhimurium, in Pseudomonas aeruginosa this is not a problem. This is so because P. aeruginosa has two arginine pathways, a biosynthetic pathway just like S. typhimurium, and a catabolic pathway that is unique. Baur et al. (1987, 1989) cloned the genes from P. aeruginosa that convert arginine to citrulline, carbamoylphosphate, and carbamate. The three genes that achieve this are found in an operon, known as arcABC. The system is inducible, being turned on by growth on arginine. Indeed, as shown by Baur et al. (1987), the flow from arginine to carbamoylphosphate can be controlled by the amount of arginine fed. Different combinations of the three genes were prepared by mutation such that it was possible to obtain arcA'+arcB'arcC−, arcA'arcB−arcC−, etc. Dr. Dieter Haas supplied two plasmids for this work, one with a pBR322 derivative plasmid, which can replicate in both P. aeruginosa and enteric bacteria including S. typhimurium. Though it was
difficult to propagate the hybrid plasmid in the \textit{pyrA pyrG cdd}, \textit{S. typhimurium} strain, it became possible eventually. In this strain using varying concentrations of arginine it was possible to produce appropriate concentrations of carbamoylphosphate as well. Future experiments to isolate modified mutants of ATCase should use this powerful technique.
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