THE REGULATION OF THE PUTRESCINE BIOSYNTHETIC GENE, speB, ENCODING AGMATINE UREOHYDROLASE IN ESCHERICHIA COLI

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by

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Abstract

Agmatine ureohydrolase (EC.3.5.3.11) was purified 3,300 fold from an *E.coli* strain by ammonium sulphate precipitation, heat treatment, ion-exchange column chromatography, gel permeation column chromatography and chromatofocusing. The active enzyme is probably a dimer of identical subunits with a molecular weight of 38,000, and a pI of 8.3. The K_m for agmatine was 1.3 mM, L-arginine was a competitive inhibitor and ornithine inhibited in a mixed manner. In crude extracts the enzyme was associated with another factor, probably a protein, which reduced the pI to 5.5 and decreased the activity of the enzyme. This factor also blocked reaction with antibodies raised against the purified enzyme in rabbits. In a strain known to express both biosynthetic and biodegradative ornithine decarboxylase and arginine decarboxylase, there was evidence for only the biosynthetic form of agmatine ureohydrolase.

Agmatine ureohydrolase was negatively regulated by cAMP and the cAMP receptor protein, CRP. The specific activity of agmatine ureohydrolase was determined in crude extracts prepared from wild type strains of *E.coli*, from strains carrying a mutation in the structural gene for adenylcyclase (EC.4.6.1.1) (*cya*) or from strains carrying mutations in both *cya* and cAMP receptor protein gene (*crp*). Cyclic AMP when added to a glucose based medium repressed the specific activity of agmatine ureohydrolase, in contrast, cAMP induced the specific activity of β-galactosidase in both the wild type and the *cya* mutant, but not in the *crp* mutant. Addition of 1 mM agmatine to

a glucose based medium induced an the specific activity of agmatine ureohydrolase in wild type, Δ*cya* or Δ*cya*, Δ*crp* strains. Chloramphenicol (150 μg/ml) abolished the inducibility of agmatine ureohydrolase by agmatine. In mutants blocked in the steps leading to the biosynthesis of polyamines, the addition of putrescine repressed the specific activities of arginine decarboxylase and ornithine decarboxylase, but did not affect agmatine ureohydrolase activity. The negative regulation of *speA*, *speB* and *speC* (encode arginine decarboxylase, agmatine ureohydrolase and ornithine decarboxylase respectively) by cAMP was shown not to be mediated by the repressive effect of cAMP on glutamine synthetase (EC.6.3.1.2), as cAMP repressed the expression of all three genes in a strain deleted for *glnA* (encodes glutamine synthetase).

The *speB* gene was cloned into the plasmid vector pBR322 at the *BamHI* site, and was localised by exonuclease digestion of the plasmid pKA5. The extent of digestion of the plasmid was determined and was related to the ability of these religated plasmids to restore agmatine ureohydrolase activity to an *speB*sup- strain of *E.coli*. Using a cell-free transcription and translation system the direction of transcription and the approximate location of the promoter was determined. The direction of transcription was also determined by cloning fragments in both directions into the promoterless genes of *lacZ* (encodes β -galactosidase) and *galK* (encodes galactokinase). The ability of these hybrid genes to confer enzymatic activities (β -galactosidase or galactokinase) to strains deleted in their respective genes confirmed the location of the *speB*-

promoter.

cAMP:CRP was shown to interact with the promoters of *speA* and *speB* using a cell-free transcription and translation system in which cloned copies of the genes served as templates. This was also shown *in vivo*, by demonstrating that cAMP inhibited the expression of β -galactosidase and galactokinase in plasmids carrying fusions of the *speA* and *speB* promoters to the structural genes of these enzymes. In addition, cAMP supplementation of minicells carrying the plasmid pKA5 (carries *speB*) caused repression of agmatine ureohydrolase synthesis. Cyclic AMP decreased and agmatine increased the steady state mRNA concentrations of *speB*, while those of *lacZ* were increased by cAMP in the Δcya strain. In contrast, cAMP did not affect the steady state concentrations of *lacZ* or *speB* mRNAs in the Δcrp strain.

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Publications

Much of the work presented in this thesis have been published or are in preparation. These papers are:

Satishchandran, C. and S.M. Boyle. (1984). Antagonistic transcriptional regulation of agmatine ureohydrolase-a putrescine biosynthetic enzyme by cAMP and agmatine in *Escherichia coli*. *J. Bacteriol.* **157**: 552-559.

Satishchandran, C. and S.M. Boyle. (1985). Purification and properties of agmatine ureohydrolase in *Escherichia coli*. *J. Bacteriol. In press.*

Satishchandran, C., J.M. Wright and S.M. Boyle. (1985). Quick blot: Application to prokaryotic messenger RNA analysis. *Anal. Biochem. Submitted.*

Satishchandran, C., J.K. Buch and S.M. Boyle. (1985). Cyclic AMP and cAMP receptor protein negatively regulates the transcription of *speA* and *speB*. *In preparation.*

Satishchandran, C., J.K. Buch and S.M. Boyle. (1985). Regulation of expression of *speA:galK*, *speB:galK* and *speB:lacZ* gene fusions in *Escherichia coli*. *In preparation.*

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A	=	Adenine;
ACE	=	β-galactoside transacetylase;
ADC	=	arginine decarboxylase;
ADP	=	adenosine 5'-diphosphate;
AdGS	=	adenylated glutamine synthetase;
AMP	=	ampicillin resistance;
5'-AMP	=	adenosine 5'-monophosphate;
ARG	=	arginase;
Arg rep	=	arginine regulon repressor;
ATP	=	adenosine 5'-triphosphate;
AUH	=	agmatine ureohydrolase.
BAP	=	bacterial alkaline phosphatase;
BG	=	β-galactosidase;
BSA	=	Bovine serum albumin.
C	=	cytosine;
cAMP	=	cyclic adenosine-3', 5'-monophosphate;
CPM	=	counts per minute;
CRP	=	cAMP receptor protein;
cAMP(2'-3')	=	cyclic adenosine-2'-3'-monophosphate;
CYA	=	adenylate cyclase.
dead.GS	=	deadenylated glutamine synthetase;
DEAE	=	diethyl amino ethyl;
DNA	=	deoxyribonucleic acid;
DNAase	=	deoxyribonuclease;
DPM	=	disintegrations per minute.
EDTA	=	ethylene diamine tetra acetate;
EGTA	=	ethylene glycol-bis (β-amino-ethyl-ether) N,N',N'-tetra acetate.
g	=	gram;
G	=	guanine;
GDP	=	guanosine 5'-diphosphate;
GK	=	galactokinase;
GMP	=	guanosine 5'-monophosphate;
GS	=	glutamine synthetase;
GTP	=	guanosine 5'-triphosphate.
HEPES	=	N-2-Hydroxyethyl piperazine-N'-2-ethane sulfonic acid;
L	=	liter;
LB	=	Luria broth.

MOPS = 3-(N-morpholino)-propane sulfonic acid;
MES = 2-[N-morpholino] ethane sulfonic acid;
mRNA = messenger ribonucleic acid;
M9 = minimal salts medium.

N = nucleotide.

O = origin of replication;
ODC = ornithine decarboxylase;
ONPG = ortho-nitro phenyl galactoside;
OTC = ornithine transcarbamylase.

PAGE = polyacrylamide gel electrophoresis;
PER = β-galactoside permease;
PIPES = 1,4 - piperazine - diethanesulfonic acid;
PPO = 2,5 - diphenyl oxazole;
ppGpp = guanosine 5'diphosphate 3'-diphosphate;
pppGpp = guanosine 5'triphosphate 3'-diphosphate;
Poly(A) = poly adenylic acid.

RNA = ribonucleic acid;
rRNA = ribosomal ribonucleic acid;
RNAase = ribonuclease.

SDS = sodium dodecyl sulphate;
S-30 extract = supernatant of 30,000xg sedimentation.

T = thymine;
Tet = tetracycline resistance;
TCA = trichloro acetic acid;
Tricine = N-tris (hydroxy methyl) methyl glycine;
Tris = tris (hydroxymethyl) amino methane;
t-RNA = transfer ribonucleic acid.

U = uracil;
UTase = Uridyl transferase;
UV = ultraviolet.

VRC = Vanadium ribonucleoside complex.

W.T. = Wild-type

X-gal = 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

YT = Yeast extract tryptone medium.

Chapter 1

Introduction: A Review

1.1 Scope of the review:

Polyamines (putrescine, spermidine, spermine, cadaverine etc.) have long been considered as biochemical curiosities. The last 30 years of research, an age demanding relevance, have seen a considerable amount of work on polyamines. However, the function and the regulation of synthesis of these ubiquitous molecules remain as elusive as ever. In the 18th century many believed that the poisonous chemicals released upon bacterial infection were either cadaveric alkaloids or ptomaines, both of which were thought to arise from the proteinaceous materials in the body. The idea of disease had long been linked with putrefaction. Studies on these poisonous materials started towards the end of the eighteenth century and culminated one hundred years later in the work of Breiger, who isolated a number of feebly toxic ptomaines (reviewed in Heyningen and Seal, 1982). Since their origins were from either putrefying meat or human cadavers they were termed putrescine and cadaverine respectively. The ptomaine doctrine had powerful adherents, such as Robert Koch who stated that , "We can explain the action of Cholera bacilli only by supposing that they generate poisons belonging to the group of ptomaines" (Heyningen and Seal, 1982). But the history of polyamines dates back about 300 years since Anton von Leeuwenhoek discovered spermine phosphate in human semen

(Leeuwenhoek, 1678; reviewed in Cohen, 1971). Hence polyamines have been referred to as foul smelling indicators of male reproductive function, as poisonous materials derived from bacterial action on putrefying meat or on cadavers, as promoters and regulators of cell growth (Daves, 1978), as a growth industry, as potentially useful markers of human health and disease (Cohen, 1982) and as molecules in search of a function (Hopkins, 1982). The relatively slow development of the area of polyamines changed following the characterisation and elucidation of the structure of spermine phosphate about 60 years ago (Rosenheim, 1924). Although polyamines are ubiquitous and both prokaryotes and eukaryotes have an absolute requirement of polyamines for normal growth and differentiation respectively, the major question as to their biological function still remains unanswered. Polyamines have direct effects on RNA, DNA and protein metabolism, membrane integrity, and intermediary metabolism. The scope of this review is not to focus on the function of polyamines, but will be limited to a summary of some of the functions assigned to polyamines and to the discussion of some of the aspects of regulatory mechanisms in prokaryotic physiology.

1.2 Biosynthesis of simple polyamines:

Putrescine and cadaverine (Table: 1.1) are simple diamines, whereas spermidine and spermine are aminopropyl derivatives of putrescine. Putrescine and spermidine are found in millimolar concentrations in *E.coli* (Bachrach, 1973;

Table 1.1

Structure of simple polyamines

Structure	Name
$H_2N-(CH_2)_3-NH_2$	1,3-Diaminopropane
$H_2N-(CH_2)_4-NH_2$	1,4-Diaminobutane (Putrescine)
$H_2N-(CH_2)_5-NH_2$	1,5-Diaminopentane (Cadaverine)
$H_2N-(CH_2)_3-NH-(CH_2)_3-NH_2$	*Bis*-(3-Aminopropyl) amine
$H_2N-(CH_2)_3-NH-(CH_2)_4-NH_2$	Spermidine
$H_2N-(CH_2)_3-NH-(CH_2)_4-NH-(CH_4)_3-NH_2$	Spermine

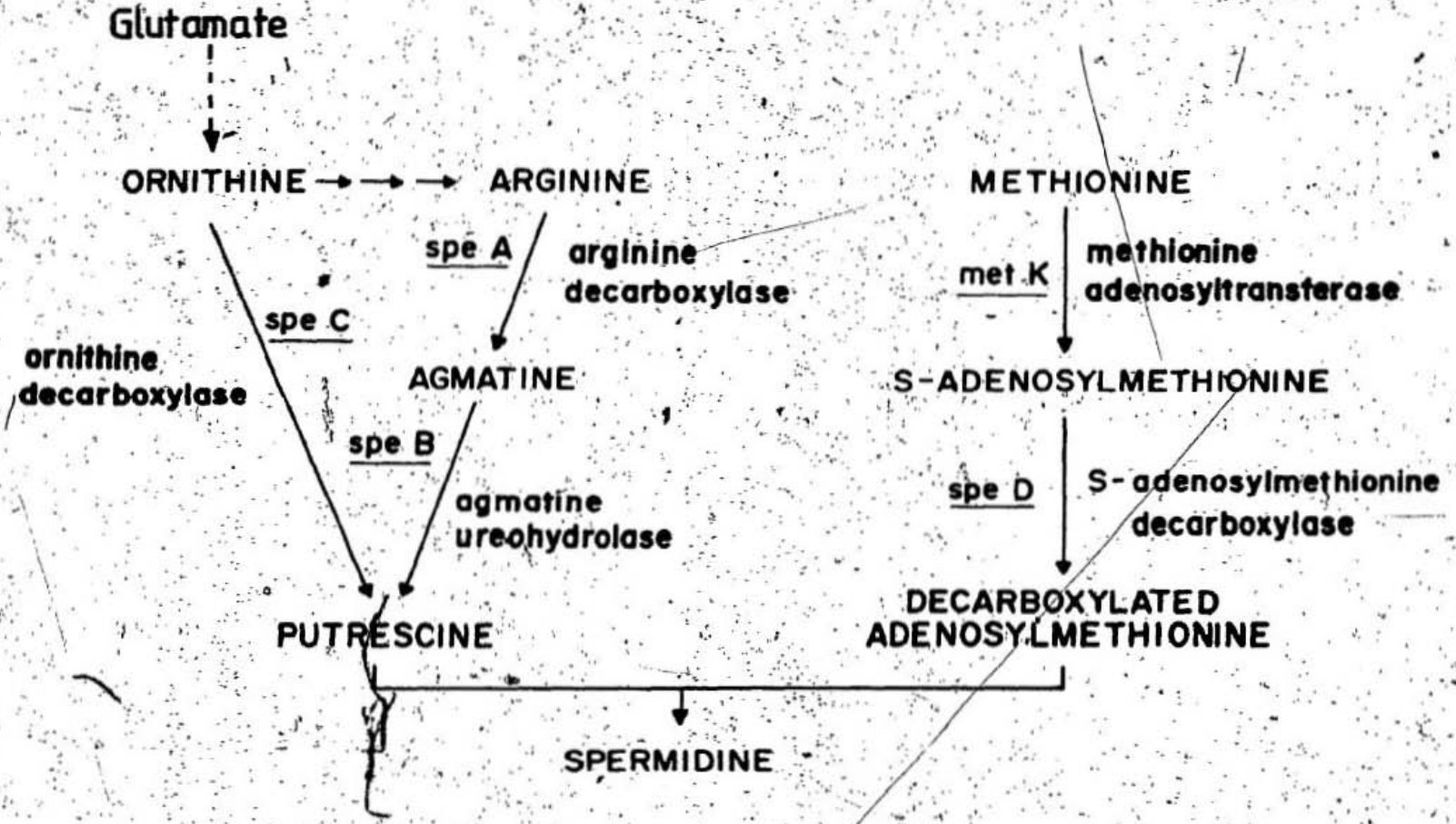
Cohen, 1971). In general, the putrescine content of prokaryotic cells is several fold higher than that of spermidine, whereas, eukaryotic cells have high concentrations of spermidine and little putrescine. Spermine has only been found in eukaryotic cells. The enzymatic degradation of polyamines (via. acetylated and oxidised derivatives) may regulate, in part, the cellular concentrations of polyamines, and hence cell physiology (Pegg and Williams-Ashman, 1979). Despite the potential importance of oxidised and acetylated polyamine derivatives they will not be discussed here.

The pathways for the biosynthesis of putrescine and spermidine are similar in microorganisms and animal cells, but there are certain striking differences (Morris, 1978a; Tabor and Tabor, 1976; 1984) (Figure: 1.1). Polyamine biosynthetic pathways originate from the arginine biosynthetic pathway in prokaryotes and in lower eukaryotes. The arginine biosynthetic pathway is the forerunner of the ornithine cycle in higher eukaryotes, and the polyamine biosynthetic pathway originates from this. Arginine biosynthetic pathway and its relation to polyamine biosynthesis is shown in Figure: 12.1. In *E.coli* there are two biosynthetic pathways leading to putrescine, of which one involves the decarboxylation of ornithine, an intermediate in the arginine biosynthetic pathway (Morris and Pardee, 1965; 1966). This reaction is catalysed by ornithine decarboxylase. The second pathway involves two steps, decarboxylation of arginine into agmatine by arginine decarboxylase and subsequent hydrolysis of agmatine into putrescine and urea (Morris and Koffron, 1969;

Figure 1.1

Biosynthetic pathway of putrescine and spermidine.

speA*, *speB*, *speC*, *speD and ***metK*** are genes that encode enzymes that catalyse the reactions leading to spermidine synthesis.


Glutamate
ORNITHINE
ARGININE
METHIONINE
spe A
arginine decarboxylase
met K
methionine adenosyltransferase
spe C
ornithine decarboxylase
AGMATINE
S-ADENOSYLMETHIONINE
spe B
agmatine ureohydrolase
spe D
S-adenosylmethionine decarboxylase
PUTRESCINE
DECARBOXYLATED ADENOSYLMETHIONINE
SPERMIDINE

Morris *et al.*, 1970) by agmatine ureohydrolase. Agmatine ureohydrolase represents the only pathway for urea biosynthesis in *E.coli* (Morris and Koffron, 1967). No urease is present in *E.coli*, and the urea accumulates in the medium (Morris and Koffron, 1967). In some bacteria and plants agmatine is converted to carbamyl-putrescine (Cohen, 1971; Tabor and Tabor, 1984), which is then converted to putrescine. The putrescine biosynthetic enzymes are found in cells growing in minimal medium at low substrate concentrations, and the enzymes are produced constitutively. Putrescine is synthesised by the decarboxylation of ornithine in *Bacillus subtilis*, *Neurospora crassa*, *Saccharomyces cerevisiae* and in animal cells. These organisms lack the ability to synthesise putrescine from arginine (Tabor and Tabor, 1984).

In certain strains of *E.coli*, biodegradative forms of ornithine decarboxylase and arginine decarboxylase are induced by low pH of the growth medium and high substrate concentrations (Gale, 1946; Morris and Fillingame, 1974). The biosynthetic and biodegradative forms of ornithine decarboxylase and arginine decarboxylase have been purified to near homogeneity and possess many characteristics in common (Wu and Morris, 1973a; b; Applebaum *et al.*, 1975; 1977). The function of biodegradative enzymes may be homeostatic, by a mechanism of proton elimination to neutralise the acidity of the surrounding medium (Morris and Fillingame, 1974). Separate genes for the biodegradative enzymes have been identified and are located at a separate locus on the *E.coli* genome from the cluster of genes for the biosynthetic enzymes (Maas, 1972;

Tabor and Tabor, 1985). Since only one in ten strains of *E.coli* possesses the biodegradative enzymes (Gale, 1946), and since some *E.coli* strains possess DNA sequences homologous to *speC* (gene encoding biosynthetic ornithine decarboxylase) (Wright and Boyle, 1984) although these do not contain biodegradative ornithine decarboxylase enzymic activity, it has been suggested that the biodegradative enzymes may have arisen by divergence from their respective biosynthetic forms by gene duplication (Applebaum *et al.*, 1977; Gale, 1946; Wright and Boyle, 1985).

The pyridoxal phosphate requiring enzymes arginine decarboxylase and ornithine decarboxylase in *E.coli* are considered to be the rate limiting enzymes of polyamine synthesis during growth with L-arginine and D-glucose respectively, (Tabor and Tabor, 1976).

In both bacterial and mammalian cells, an aminopropyl group of decarboxylated adenosyl methionine is transferred to putrescine by aminopropyl transferase to form spermidine (Tabor, 1958; Hannonen *et al.*, 1972). A second aminopropyl transferase that catalyses the transfer of an aminopropyl group of decarboxylated adenosyl methionine to spermidine to form spermine has been purified from animal tissues(Hannonen *et al.*, 1972; Raina and Hannonen, 1971). The conversion of L-methionine to S-adenosyl methionine is catalysed by the enzyme methionine adenosyl transferase, and this is converted to decarboxylated adenosyl methionine by S-adenosyl methionine decarboxylase (Tabor, 1962; Tabor *et al.*, 1958).

1.3 Function of polyamines:

Haemophilus parainfluenzae grew only when putrescine, spermidine or spermine was added to the synthetic medium (Herbst and Snell, 1948). March and Boyle (1982) reported that this strain of *Haemophilus* is unable to synthesise putrescine due to the absence of putrescine synthesising enzymes, ornithine decarboxylase and arginine decarboxylase. Naturally occurring fungal mutants have an absolute requirement for putrescine (Sneath, 1955; Deters *et al.*, 1974). Although the growth of some microorganisms was stimulated by polyamines, this was shown not to be specific, because 0.1 M KCl and NaCl or sucrose had the same effect. Increased supplementation of polyamines inhibited the growth of several Gram positive organisms, and these bacteria were also shown to be more susceptible than gram negative bacteria (Mager, 1955; 1959; Martin *et al.*, 1962). These effects appear to be due to the interaction of polyamines with the bacterial membrane. A strain carrying mutations in *speA*, *speB*, *speC* and *speD* (encoding respectively arginine decarboxylase, agmatine ureohydrolase, ornithine decarboxylase and S-adenosyl methionine decarboxylase) lacked putrescine and spermidine, but grew indefinitely in the absence of polyamines at a rate one third of that in the presence of polyamines (Hafner *et al.*, 1979). These strains appeared to contain cadaverine, which might substitute for the requirement for putrescine and spermidine. However, a strain carrying mutations in *cadA* (biodegradative lysine decarboxylase), *speA*, *speB*, *speC* and *speD*, still grew indefinitely at a reduced growth rate, and addition

of cadaverine to these cells did not cause increased growth rate (Tabor *et al.*, 1980). From these results it might be concluded that polyamines may not be absolutely required for growth, and that cadaverine cannot substitute for putrescine or spermidine to promote growth. Transduction of the *strA* (*rpsL*, streptomycin resistance) gene into a strain of *E.coli* from which the *speA*, *speB*, *speC* and *speD* genes have been deleted produced an absolute requirement for polyamines (Tabor *et al.*, 1981; Tabor, 1981; Tabor and Tabor, 1984). Mutations in the S12 ribosomal protein - a product of *rpsL*, are known to decrease ambiguity in translation (Gorini, 1969). Streptomycin resistance phenotypes have an absolute requirement for polyamines probably because polyamines might interact with this protein and thereby increase the ambiguity in translation.

From the studies on mutants of *speA*, *speB*, *speC* and *speD* Morris and Jorstad (1970) and Tabor (1978) concluded that putrescine can substitute for the growth promoting effect of spermidine and vice versa. Using a homologous series of spermidine analogues, where the distance between the amino groups of a triamine molecule was varied ($NH_2 - (CH_2)_X - NH - (CH_2)_3 - NH_2$, where X was varied), Jorstad *et al.*(1980) concluded that only when X = 3 or 4 (spermidine is X = 4) the analogue supported growth of a polyamine deficient mutant. The nature of this structural constraint remains unknown. Using irreversible inhibitors of the enzymes involved in putrescine and spermidine synthesis an absolute requirement for these molecules could not be shown

(Betonti *et al.*, 1982). Morris (1981), concluded that putrescine concentrations must become undetectable and spermidine concentrations reduced to 25% of the wild type concentrations before an effect on growth rate can be observed. It may be concluded that polyamines are not absolute requirements for growth but may be required for normal growth.

Several studies show that polyamines interact with nucleic acids (reviewed in Bachrach, 1973; Cohen, 1971; Tabor and Tabor, 1976). Positively charged polyamines appear to interact non-covalently with the negatively charged phosphate of DNA, protecting denaturation. Evidence from several laboratories suggests that spermidine may be required for packaging of bacteriophage DNA, as the DNA exists in a compact form in the presence of these polyamines (Gosule and Schellman, 1976; Kaiser *et al.*, 1975). Most studies that attempted to determine the intracellular localisation of polyamines have been plagued by redistribution of these charged molecules among the negatively charged components of the subcellular fractions following cell disruption. The finding by Ames and Dubin (1960) that nearly 40% of the cations associated with the T_4 DNA in mature phage particles are polyamines, and that bacteriophages are impermeable to polyamines, led to the idea of polyamines interacting with DNA and condensing it. In bacteria starved of polyamines several bacteriophages replicate and mature phage particles are produced, albeit at a slower rate and with a smaller burst size (Cohen and McCormick,

1979; Dion and Cohen, 1972). The process appears to be due to some other cation substituting for polyamines (Hafner *et al.*, 1979; Tabor, 1981; Tabor and Tabor, 1984). Excision of bacteriophage lambda *in vivo* and integration *in vitro* had an absolute requirement for spermidine (Gottesman and Gottesman, 1975; Nash, 1975). The work of Kaiser *et al.* (1963) and Flink and Pettijohn (1975) suggests that spermidine influences the stability and the tertiary structure of DNA, as it prevents shearing and thermal denaturation of the DNA. Lipetz *et al.* (1980) and Liu and Wang (1978) reported that polyamines affect DNA supercoiling by modulating the activities of DNA topoisomerases. Although supercoiling of DNA has been shown to affect gene expression (Botchan, 1976; Botchan *et al.*, 1973; De Wyngaert and Hinkle, 1979; Gomez-Eichelman, 1981; Hsieh and Wang, 1975; Kano *et al.*, 1981; Menzel and Gellert, 1983; Smith *et al.*, 1978; Richardson, 1975; Vollenweider *et al.*, 1979; Wang, 1974; Yang *et al.*, 1979), the involvement of polyamines in affecting DNA superhelicity *in vivo* has not been shown.

Russel *et al.* (1983) reported the conversion of B-DNA to Z-DNA in the presence of polyamines at physiological concentrations, unlike this effect caused by divalent cations at higher than physiological concentrations. This appears to be a significant observation because of the increasing importance of Z-DNA in regulation of gene expression (reviewed in Rich *et al.*, 1984). Polyamines have been shown to interact specifically with RNA molecules (Bachrach, 1973). Cohen *et al.* (1971; 1978) showed that spermidine is bound

to t-RNA, the crystallographic data clearly showed that there are two binding sites. Cohen *et al.* (1978) showed that one molecule of spermidine was bound to the major groove near the anticodon loop and the other near the variable loop. The kinetics of binding were co-operative. Cleavage of the t-RNA molecule by enzymes into two or four fragments destroyed the co-operativity of binding of spermidine molecules. It was proposed that spermidine stabilises the helical regions of the t-RNA molecule. RNA bacteriophages R17 and MS2 contain large amounts of spermidine complexed with the genome and infection is greatly enhanced by polyamines (Cohen *et al.*, 1978; Fukuma and Cohen, 1975).

There have been two approaches to the study of the involvement of polyamines in macromolecular synthesis. One involved comparing changes in the concentrations of polyamines and the activities of the enzymes that synthesise them with the rate of synthesis of DNA, RNA and protein. The other involved addition of polyamines into *in vitro* systems to assess the effect of these molecules on DNA synthesis, transcription and translation. Both approaches have limitations. The correlative approach suffers from the difficulty of distinguishing cause-effect from co-incidental relationships, while the *in vitro* approach can hardly be extrapolated to an *in vivo* situation. Many of the observations *in vitro* become inappropriate and insignificant at physiological salt concentrations, and various cations, such as magnesium, can mimic effects of polyamines *in vitro*, although at higher concentrations. Examples of

such discrepancies have been noted by several workers (reviewed in Morris, 1981).

Polyamines stimulate DNA synthesis, RNA synthesis and protein synthesis, and all these effects appear to have one common basis - replacement of magnesium (Abraham *et al.*, 1979; Atkins, 1975; Chakraburrtty *et al.*, 1975; Cohen, 1971; Chiu and Sung, 1972; Bach, 1964; Fuchs *et al.*, 1967; Gumport and Weiss, 1969; Gumport, 1970; Geider and Kornberg, 1974; O'Brien *et al.*, 1966; Igarashi *et al.*, 1972; 1974; Konecki *et al.*, 1975; Takeda and Igarashi, 1969; Tabor and Tabor, 1976; 1984; Teraoko and Tanaka, 1973a; b; Tomizawa *et al.*, 1974; Treadwell *et al.*, 1976; Santi and Webster, 1975; Schwimmer, 1968; Watanabe *et al.* 1981; 1983).

It is interesting to note that spermidine appears to decrease the activity of EF-Tu (elongation factor) specifically during protein synthesis (Teraoko and Tanaka, 1973a; b) and that EF-Tu factor is also associated with the replicating enzyme system of RNA bacteriophages. The viral RNA exists in a folded conformation due to intramolecular hydrogen bonding (Watson, 1976), and is in association with polyamines (Cohen, 1978). The association of EF-Tu both with the replicating enzyme system of RNA phage and with the translational machinery suggests a common basis of removal of hairpin bends in the RNA molecule to facilitate either its replication or translation. Cohen *et al.* (1978) and Fukuma and Cohen (1975) suggested that the large amounts of spermidine associated with the genomes of RNA phages might be to increase the stability

by folding of the RNA molecule. It is therefore possible to speculate that the folding of RNA by spermidine might reduce the activity of EF-Tu factor by stabilising the secondary structure of the RNA molecule. The bacteriophage RNA genome is believed to be in a linear form during translation and replication, and in a folded form which would facilitate storage and transport.

Numerous studies have shown that an increase in the accumulation of polyamines and the activities of enzymes that synthesise them parallel or precede increases in the rates of DNA, RNA and protein syntheses (reviewed in Bachrach, 1973; Cohen, 1971; Tabor and Tabor, 1976). *E.coli* cells have been starved of polyamines either by using mutants blocked in the biosynthetic pathways leading to putrescine or by the use of inhibitors of enzymes that catalyse the conversion of precursors into polyamines. These studies revealed that following addition of polyamine to polyamine depleted cells, protein synthesis is stimulated prior to changes in nucleic acid synthesis, suggesting that the effects on replication and transcription are either indirect or merely a general response to resumption of active protein synthesis.

1.4 Regulation of putrescine biosynthetic enzymes in E.coli:

Most studies have determined biological activities of the enzymes involved in the synthesis of putrescine *in vitro*, and it is often difficult to evaluate the true roles of various effector molecules *in vivo*.

1.4.1 Feedback inhibition and repression:

The polyamines, putrescine and spermidine competitively inhibit ornithine decarboxylase and arginine decarboxylase in crude and purified preparations. Polyamines inhibit the activity of ornithine decarboxylase competitively (Morris *et al.*, 1970; Applebaum, 1977). At concentrations of 7.4 mM ornithine (K_m = 2.0 mM), 20 mM putrescine or 5mM spermidine 50% inhibition of the activity of purified ornithine decarboxylase was observed (Applebaum, 1977). At saturating concentrations of arginine (7.4 mM arginine, K_m = 0.03mM) and 1mM magnesium 50% of the activity of purified arginine decarboxylase was inhibited by 15 mM putrescine or 1.5 mM spermidine (Wu and Morris, 1973a; b). Increasing concentrations of magnesium, a co-factor of arginine decarboxylase activity, fully reversed the inhibition by polyamines. The competition between magnesium and polyamines may be one mechanism which regulates arginine decarboxylase activity *in vivo* (Morris *et al.*, 1970; Wu and Morris, 1973a; b). Feedback inhibition *in vivo* has been demonstrated by Tabor and Tabor (1969a), who observed a rapid reduction in the release of $[^{14}C]CO_2$ from L-[U-^{14}C] arginine and L-[U-^{14}C] ornithine following addition of polyamines to culture medium. The specific activities of ornithine decarboxylase and arginine decarboxylase also decreased following the addition of putrescine or spermidine to the growth medium of *E.coli* strains. Using mutants carrying a partial block in arginine decarboxylase, putrescine deficiency was produced by the addition of arginine to the growth medium. Arginine represses the arginine

biosynthetic pathway (Sercarz and Gorini, 1964; Maas, 1961; Maas, 1964). In the same system Morris *et al.* (1970) showed that ornithine decarboxylase and the mutant arginine decarboxylase were derepressed. These observations suggest that polyamines regulate the synthesis of ornithine decarboxylase and arginine decarboxylase. Putrescine and spermidine have no effect on either the synthesis or the activity of agmatine ureohydrolase in *E.coli* (Morris *et al.*, 1970). The enzymic activity of agmatine ureohydrolase in *Klebsiella aerogenes* and *Proteus vulgaris* is competitively inhibited by the guandio group of several compounds including arginine (Khramov, 1978).

1.4.2 Antizymes and anti-antizymes:

Kyriakidis *et al.* (1978) showed that *E.coli* strains when grown in the presence of putrescine produced a proteinaceous inhibitor of ornithine decarboxylase and arginine decarboxylase. This protein had a molecular weight of 15,000, and inhibited ornithine decarboxylase activity non-competitively. The enzyme and inhibitor form a complex and co-purify, but they can be dissociated by salt to provide active ornithine decarboxylase and inhibitor. This inhibitor, termed antizyme has now been resolved into at least three proteins (Heller *et al.*, 1983a; b), one acidic and two basic. Almost 90% of the total antizyme activity is associated with the basic proteins both of which not only inhibited ornithine decarboxylase activity, but also the activity of arginine decarboxylase. These antizymes appear to have no effect on the biodegradative

forms of arginine decarboxylase and ornithine decarboxylase. Panagiotidis and Canellakis (1984) reported recently, that the two basic antizyme proteins were in fact the ribosomal proteins S20 and L34. The inhibitory activity of these antizymes can be reversed by a protein which has a molecular weight of about 70-78,000 (Heller *et al.*, 1983a). Nucleic acids have also been shown to reverse the inhibitory activity of the antizyme (Kyriakidis *et al.*, 1978). It is speculated that the activity of ornithine decarboxylase is determined by a simple competition between ornithine decarboxylase and anti-antizyme for the antizyme molecule. It is difficult to discern how or why the regulation of ornithine decarboxylase is so paramount to the cell. It is probable that ornithine decarboxylase interacts with ribosomes which might affect translation, especially since polyamines appear to play a role in protein synthesis (*rpsL* mutation and EF-Tu in Section: 1.3). It is also possible to speculate that the ribosome disaggregation by very high concentrations of polyamines observed *in vitro* (Zitomer and Flaks, 1972) might lead to the release of ribosomal proteins which in turn decreases the activity of ornithine decarboxylase and perhaps arginine decarboxylase by forming complexes with these enzymes, although the concentrations (putrescine = 30 mM) required to disrupt ribosome particles are far too high compared to the concentrations (higher estimates of 20 mM putrescine) (Morris *et al.*, 1970) in the cell. These reported cellular levels are of total putrescine concentration and assume none is complexed with cellular sites *in vivo*.

Although the anti-antizyme described by Heller *et al.* (1983a) has no affinity for pyridoxal phosphate, and has a higher affinity for the antizyme as compared to ornithine decarboxylase, it elutes on gel exclusion chromatography immediately following ornithine decarboxylase. It is therefore possible that the anti-antizyme might be a proteolytic product of ornithine decarboxylase itself.

1.4.3 Pathway selection:

E.coli synthesises putrescine by two pathways that branch off the arginine biosynthetic pathway. In glucose grown cells, 75-90% of putrescine is formed by the decarboxylation of ornithine, while arginine supplemented cultures produce putrescine by the arginine decarboxylase - agmatine ureohydrolase pathway, without any change in the net production of putrescine (Morris *et al.*, 1970). Maas (1961) showed that arginine acts through the arginine repressor (*argR* gene product) to repress the transcription of genes that encode the enzymes of the arginine biosynthetic pathway. Arginine also inhibits the activity of the first enzyme of the pathway, acetylglutamate synthetase, allosterically. Thus, as a result of exogenously supplied arginine, the intracellular pool of ornithine is diminished, which would explain the marked decrease in putrescine synthesis from ornithine following supplementation. Although over 60% of the exogenously supplied arginine was converted to putrescine and less than 40% was incorporated into proteins, the specific activity of arginine

decarboxylase did not undergo marked increase (Tabor and Tabor, 1969a; Morris *et al.*, 1970). From studies with radiolabelled arginine and its biosynthetic precursor; citrulline, Tabor and Tabor (1969b) suggested that arginine decarboxylase might be compartmentalised. Buch and Boyle (1985) have shown that arginine decarboxylase is indeed compartmentalised and resides in the inner periplasmic space. These observations support the contention that exogenous arginine can be preferentially channeled into polyamines and accounts for the increased utilisation of arginine decarboxylase - agmatine ureohydrolase pathway.

1.4.4 Nucleotide effects and the stringent response:

The activity of ornithine decarboxylase is influenced by certain nucleoside triphosphates (Applebaum *et al.*, 1977; Holtta *et al.*, 1972). At subsaturating concentrations of ornithine, the mono, di and triphosphates of adenosine, guanosine and their deoxy derivatives stimulated the activity of a partially purified preparation of ornithine decarboxylase (Holtta *et al.*, 1972). 1mM GTP stimulated the enzyme activity, and reduced the K_m for ornithine from 2 mM to 0.2 mM (Applebaum *et al.*, 1977). Since GTP (at 1mM) overcame the inhibitory effect of high ionic strength on ornithine decarboxylase activity, Holtta *et al.* (1972) further suggested that GTP might be involved in subunit interactions. However, the mechanism of activation by nucleotides remain unknown.

cAMP and its receptor protein were shown to negatively regulate the

transcription of *speC* (encodes ornithine decarboxylase) (Wright and Boyle, 1982). Using various mutants blocked either in the synthesis of cAMP or with a lesion in the cAMP receptor protein (CRP) Wright and Boyle (1982) showed that cAMP negatively regulates the transcription of *speC*. They also showed that this effect was independent of growth rate. Boyle *et al.* (1985) confirmed this observation by assessing the effect of cAMP on ornithine decarboxylase synthesis in a minicell producing strain carrying cloned copies of *speC* and also in an *in vitro* transcription and translation system directed by the extracts of a Δ*crp* mutant. The *in vitro* system had to be fortified with CRP in order to obtain repression of synthesis of ornithine decarboxylase by cAMP. Using a plasmid in which the promoter sequence of *speC* was fused to the structural gene for tetracycline resistance, they confirmed the repression of *speC* transcription by cAMP. Wright and Boyle (1982) also showed that arginine decarboxylase was repressed by cAMP and CRP and they suggested that agmatine ureohydrolase might also be similarly regulated.

Several studies have implicated the nucleotide ppGpp in the regulation of ornithine decarboxylase and arginine decarboxylase. During stringent response triggered by amino acid starvation of *E.coli* cells there is a co-ordinate cessation of RNA and protein synthesis with a concomitant increase in ppGpp concentrations (Gallant, 1979). Strains of *E.coli* carrying a lesion in the *relA* gene exhibit relaxed response to amino acid starvation which is evidenced by cessation of protein synthesis without co-ordinate cessation of RNA synthesis.

ppGpp inhibits the transcription of several genes (ribosomal RNA, genes for ribosomal proteins, genes for RNA polymerase proteins etc.), while it stimulates the transcription of genes involved in the catabolic pathways (*lac*, *mal*, *hut* etc.) and of genes involved in amino acid biosynthesis (*trp*, *his*, *ilv* etc.) (Yanofsky, 1981; Cashel and Gallant, 1969; Keller, 1979; Nierlich, 1978; Maaloe, 1979).

Effects of ppGpp on polyamine biosynthesis indicate that ppGpp competes with GTP and decreases the enzymic activity of ornithine decarboxylase activity. Physiological concentrations of ppGpp abolished the activation of ornithine decarboxylase by GTP, and the K_m for ornithine was increased (Holtta *et al.*, 1974). These changes in the activity of ornithine decarboxylase appear to parallel changes in spermidine concentrations in a *relA*$^+$ strain starved for amino acids. The results suggest that the regulation of polyamine synthesis is controlled by changes in the affinity of ornithine decarboxylase for its substrate, and that these changes are in turn mediated by the relative intracellular concentrations of GTP and ppGpp. Studies of Cohen (1967) and Sakai and Cohen (1976) show that there was no consistent correlation between the ratio of ppGpp and GTP, and the activity of ornithine decarboxylase *in vivo*, as measured by putrescine concentrations. Boyle and Adachi (1982) assessed the role of ppGpp on the synthesis of ornithine decarboxylase and arginine decarboxylase in *E.coli* cells during exponential growth and nutritional shift-up. Changes in the activity and the rate of synthesis of ornithine

decarboxylase directly correlated with changes in ppGpp concentrations, whereas the activity and rate of synthesis of arginine decarboxylase were inversely related to ppGpp concentrations. The authors concluded that ppGpp is probably not directly involved in negative transcriptional or post-translational regulation of ornithine decarboxylase.

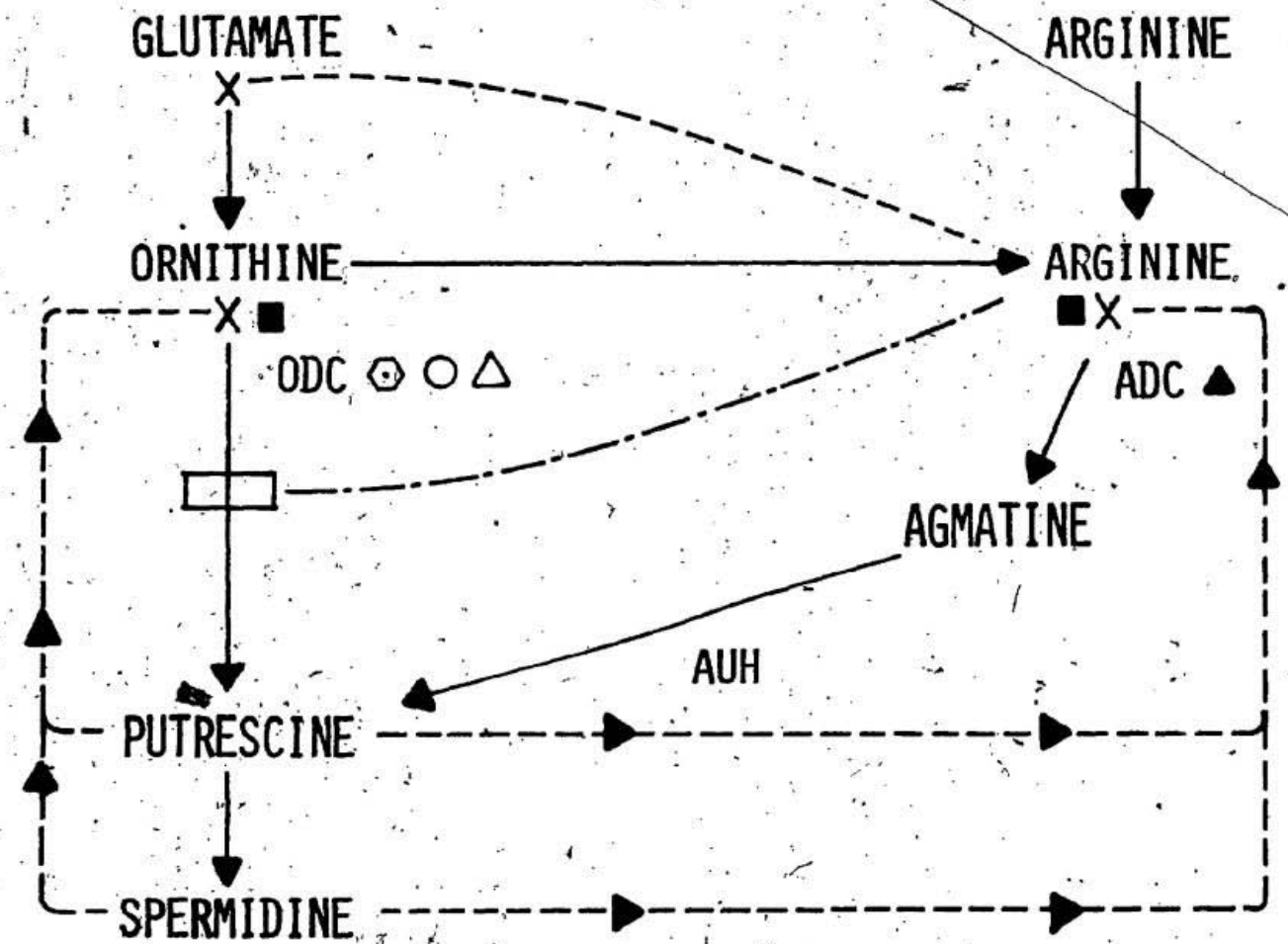
1.4.5 Osmoregulation:

Munro *et al.* (1972;1975) suggested that cellular osmolarity may influence putrescine synthesis in *E.coli*. Peter *et al.* (1978) found an inverse relation between osmolarity of the growth medium and the putrescine content of *E.coli* and various marine bacteria. Following transfer of these bacteria into low ionic strength growth medium there was a rapid and transient increase in the intracellular polyamine concentrations and in the activities of the enzymes that synthesise them. This increase reached a maximum within 30-40 minutes, and by 60 minutes a new lower rate was maintained. The authors suggest that polyamines might stabilise membranes and influence phospholipid synthesis, and the decrease in the rate of synthesis of polyamines following an initial spurt of synthesis was attributed to feed-back inhibition and repression of ornithine decarboxylase and arginine decarboxylase by polyamines. The observations of Munro and Sauerbier (1973) support the above contention, while those of Gunther and Peter (1979) suggest that polyamines are not involved in long term steady state osmoregulation in *E.coli*. Using cells depleted of

Figure 1.2

Regulation of putrescine biosynthetic enzymes in E.coli.

Feedback repression by end products = ■
Feedback inhibition by end products = X
Pathway selection by arginine = ▭
K^+ represses ODC = ⊙
Antizyme to ODC induced by putrescine = ○
GTP activation and ppGpp inhibition of ODC = △
Mg^{+2} activation of ADC = ▲


GLUTAMATE
ARGININE
ORNITHINE
ARGININE
ODC
ADC
AGMATINE
AUH
PUTRESCINE
SPERMIDINE

polyamines by inhibiting the activity of ornithine decarboxylase, they showed that the intracellular concentrations of putrescine and spermidine did not change significantly when subjected to changes in osmolarity. This neither proves that changes in osmolarity do not influence polyamine synthesis nor that transient increases in polyamine concentrations transiently maintain osmotic balance. The relationship between polyamines and osmoregulation, and the mechanism of osmoregulation by polyamines, remain to be elucidated.

The regulation of putrescine biosynthetic enzymes is schematically shown in Figure: 1.2.

1.5 Cyclic AMP and cAMP receptor protein:

The presence of cAMP in *E.coli* cells was demonstrated by Makman and Sutherland (1965). The subsequent finding in the late sixties that cAMP in *E.coli* overcomes different aspects of 'glucose effect', i.e. catabolite repression (Ullmann and Danchin, 1983), transient repression (Perlman and Pastan, 1968) and inducer exclusion (Ullmann *et al.*, 1969) led to a deeper understanding of the role of this nucleotide in the regulation of gene expression in bacteria. Catabolite repression is a recent name (Magasanik, 1961) given to a phenomenon discovered in 1900 (Dienert, 1900), and studied for half a century as 'glucose effect' (Gale, 1943; Monod, 1947); the presence of glucose or any other rapidly metabolisable substrate in the growth medium elicits a severe inhibition of expression of catabolic enzymes. The discovery of the major effect

of cAMP, namely, relieving the inhibitory effect of glucose, strongly linked the process of initiation of transcription of the 'glucose sensitive' genes to a positive control mediated by cAMP via its receptor protein (Schwartz and Beckwith, 1970). There is such a prejudice in favour of this association that cAMP dependent regulation of transcription is usually identified with catabolite repression. This is evident from the recent studies of Cossart and Sanzey (1985), wherein they showed that cAMP:CRP negatively autoregulates its own synthesis although the *crp* gene (encoding CRP) is itself repressed by glucose. These authors showed that cAMP repressed the synthesis of CRP, using a *crp:lacZ* fusion gene, while glucose itself elicited a similar response. This is contradictory to the general belief that cAMP overcomes 'glucose effect'. The complexity of 'glucose effect' (reviewed in Ullmann and Danchin, 1983) and its relation to cAMP is not understood at the molecular level. Two additional lines of evidence appear to suggest that cAMP might not be the exclusive mediator of catabolite repression. 1) There is no detectable cAMP in *Bacillus megaterium*, although it exhibits catabolite repression (Ullmann, 1974). 2) *E.coli* strains deleted for *cya* (gene encoding adenylate cyclase) exhibit catabolite repression of some genes (Dessein *et al.*, 1978). Kline *et al.* (1979; 1980 a;b) showed that imidazole, imidazole derivatives and indole derivatives can circumvent the necessity for cAMP in the induction of the L-arabinose operon in *cya* strains. Kline *et al.* (1984) demonstrated that several benzyl derivatives and indole derivatives stimulated the initiation of transcription at the *ara* and

lac promoters, and can also reverse catabolite repression. This effect has been referred to as 'metabolite gene regulation' (Kline *et al.* ,1979; 1982; 1984). Imposing an amino acid restriction in the presence of cAMP decreased the rate of synthesis of β-galactosidase in *E.coli* strains carrying a wild type *lac* promoter, and in those which carried the catabolite insensitive *lacP5* (*lacUV5*) promoter (Wanner *et al.*, 1978). The authors conclude that permanent catabolite repression is set by catabolites in the growth medium and may not be related to an imbalance between catabolism and anabolism as suggested by Magasanik (1961) and Neidhardt and Magasanik (1956). All that can be inferred at present is that cAMP:CRP contributes only partially (either directly or indirectly) to catabolite repression. The question remains open whether all other forms of this repression share a common mechanism.

Since cAMP does not appear to be the only regulator of catabolite repression two questions arise: 1) what other regulators of catabolite repression are there? 2) is cAMP involved in other regulatory phenomena in bacteria? There are no satisfactory answers for the first question. Since some aspects of the second question are more relevant to the work reported here, the involvement of cAMP in other regulatory phenomena will be discussed.

1.5.1 cAMP and positive control:

From studies on the *lac*, *mal*, *gal* and *ara* operons the currently accepted model for stimulation of gene activity by cAMP was put forth by Pastan and Perlman (1970) and Buettner *et al.*, (1973) and later refined (reviewed in Pastan and Adhya, 1976; Adhya and Garges, 1982; Botsford, 1981; deCrombrugghe *et al.*, 1984; Rickenberg, 1974; Ullmann and Danchin, 1983). The detailed understanding of the mechanism by which cAMP stimulates the synthesis of inducible enzyme systems has been deduced from a variety of genetic, physiological, biochemical and crystallographic studies.

For efficient transcription of the *lac* operon, cAMP and CRP must bind at the promoter region. The CRP is a dimer of two identical subunits (Eilen and Krakow, 1977), each containing 210 amino acids (Anderson *et al.*, 1971; Aiba *et al.*, 1982). Equilibrium dialysis with cAMP indicate that two molecules of cAMP can bind per CRP dimer (Takahashi *et al.*, 1980). CRP is composed of a DNA binding domain and a domain that binds cAMP. Separate domains were identified by assessing the activity of proteolytically cleaved CRP molecules (Krakow and Pastan, 1973; Eilen *et al.*, 1978). The two domain structure was clearly shown by 2.9 Å resolution crystal structure of CRP complexed with cAMP. A larger amino terminal domain extending from residue 1 to 135 (McKay and Steitz, 1981; McKay *et al.*, 1982) is separated by a cleft from a smaller carboxy terminal domain that extends from residue 136-210 (McKay *et al.*, 1982). A segment of the amino terminal domain of CRP (residues 30-89) exhibits significant sequence homology with the cAMP binding

domain of cAMP dependent protein kinases in eukaryotes (Weber *et al.*, 1982). The smaller carboxy terminal domain of each CRP subunit consists of three α-helices connected by short β-sheet structures. In each subunit one of the α-helices (proximal to the carboxy terminal) clearly protrudes from the surface of the CRP dimer. These two α-helices are thought to provide the major interaction sites with the DNA, as their axes run approximately parallel to each other at a distance of 34 Å (McKay *et al.*, 1982). Two *crp* mutations that have been mapped are in the α-helix that is thought to be involved in interacting with DNA (Ebright *et al.*, 1984), implying that this segment provides crucial interactions with the DNA template. Structurally CRP appears to be very similar to Cro and cI proteins of bacteriophage lambda (Anderson *et al.*, 1981; 1982; Pabo and Lewis, 1982; Steitz *et al.*, 1983). A study of the sequence of the *fnr* gene in *E.coli* (essential for anaerobic metabolism) showed that the FNR protein shares a high degree of homology with CRP. Although the sequence for cAMP binding domain is present in this protein, FNR protein does not appear to bind cAMP. Interestingly, FNR protein has functional properties similar to CRP, is a pleiotropic activator of a series of genes which are transcribed during anaerobiosis (Shaw *et al.*, 1983). The authors suggest that *fnr* gene may have derived by duplication of the *crp* gene itself or from a common ancestor.

Binding of cAMP to CRP induces a conformational change in the protein molecule increasing its affinity for specific DNA sequences in the promoter (K_d = 10^{-13}M to 10^{-10}M), relative to non-specific DNA sequences (K_d = 10^{-8}M to

10^{-7}M) (Aiba and Krakow, 1981; Eilen *et al.*, 1978; Kumar *et al.*, 1980). A change in conformation of CRP following cAMP binding has also been demonstrated by a corresponding change in sensitivity of the protein to proteolytic enzymes such as subtilisin, pepsin etc. (Aiba and Krakow, 1981) and by using chemical cross-linking agents (Eilen *et al.*, 1978). The binding of cAMP:CRP to the promoter of the *lac* operon stimulates transcription by RNA polymerase. According to Guiso and Blazy (1980) the amount of CRP does not vary significantly with generation time and carbon source, and they concluded that CRP was in excess (approximately, 3,500 molecules /cell) in *E.coli* cells, and that changes in cAMP concentrations were responsible for the observed changes in the rate of transcription of several genes. Although the regulation of the L-arabinose and D-galactose operons are quite distinct from the regulation of the D-lactose operon, the function of cAMP:CRP in stimulating transcription of all these operons is essentially the same.

Although the specific DNA sequences that bind CRP for a number of genes eg. *lac, ara, cat, deo, gal, ilv,* pBR-P4 (reviewed in Adhya and Garges, 1982; deCrombrugghe *et al.*, 1984; Ullmann and Danchin, 1983), and some aspects of structure-activity correlation, using cAMP analogues, are known (Scholubbers, 1984), it is not clear how cAMP:CRP stimulates transcription. The CRP binding sites have been found to vary not only in DNA sequence and symmetry but also with respect to the distance from the binding site to the transcription initiation site. A variety of techniques have been used to

determine cAMP:CRP binding sites on the promoters of several genes (Schmitz, 1981; Simpson, 1980; Taniguchi *et al.*, 1979). The studies show that cAMP:CRP binding sites vary from 106 base pairs upstream from the transcription start site of *cat*, to 30 base pairs upstream from the *galP1* transcription start site.

All prokaryotic DNA binding proteins appear to act as multimers and have the common feature of alpha helices, which show an axis of two fold symmetry, and are capable of fitting into major grooves of DNA. (Takeda *et al.*, 1983) Since the three dimensional structure of CRP is a mirror image of Cro protein of bacteriophage lambda, McKay and Steitz (1981), suggested that CRP binds to left handed B-DNA as the two important α-helices were unable to bind to any extensive region of successive major grooves of normal right handed B-DNA. Left handed B-DNA has not been demonstrated in prokaryotes or eukaryotes, while the only other left handed form of DNA, Z-DNA, will not fit CRP. If CRP binds to left handed DNA, it must then initially recognise the right handed form of B-DNA, and upon specific binding, alter its conformation to the likeness of a left handed B-DNA. Such a conformational change would affect the local linking number. But, no change in the linkage is observed upon CRP binding to *lac* promoter DNA (Fried and Crothers, 1981; 1983). It is therefore reasonable to assume that CRP does not bind to left handed B-DNA. Salemme (1982) proposed a model that allows CRP to bind to right handed B-DNA, allowing maximum contact between the α-

helices and the major grooves. It involves the DNA forming a left handed solenoid (supercoil) which allows CRP to bind to the major grooves in successive loops of the coil. This would allow CRP to bind in a manner similar to Cro protein, although the Cro protein can bind to successive major grooves of a right handed B-DNA, CRP can bind only to widely separated major grooves (according to the model of Salemme, 1982), brought to close proximity by supercoiling. This model might explain the effect of cAMP:CRP on some promoters where the cAMP:CRP binding site was found to be about 100 base pairs away from the initiation site for transcription, as the RNA polymerase binding site and the transcription initiation site can be brought to close proximity to a CRP binding site 100 base pairs away in a solenoid structure of DNA. Salemme's model was based on the observation that CRP in the absence of cAMP condensed pBR322 molecules four fold, resulting in the formation of long cylindrical rods, probably arranged as tightly wound solenoid supercoils (Chang *et al.*, 1981; Martin *et al.*, 1983; Saxe and Revzin, 1979). The serious objection to this model is that it is based on evidence concerning the non-specific binding of CRP to DNA. If CRP bound to widely separated major grooves brought together by supercoiling, cAMP:CRP protection of widely separated regions of the DNA might be expected. But, this is not the case, CRP binding to DNA appears to involve only a single stretch of 14 base pairs (consensus sequence), deduced from DNA protection studies with cAMP:CRP.

Ebright and Wong (1981) postulated that the adenyl moiety of cAMP directly intercalates with a thymine residue within the CRP binding site causing melting of promoter DNA. In this model CRP functions to position the adenyl moiety of cAMP in an orientation to form hydrogen bonds with the thymine residue. But a class of mutations in the *crp* gene (*crp**) that have CRP activity independent of cAMP have been characterised (Harman and Dobrogosz, 1983), and have been shown to assume a conformation analogous to the conformation of cAMP:CRP, as evidenced by sensitivity to proteolytic enzymes and to certain chemical cross linking agents. Since CRP* enables expression of *lac, mal, ara* operons in a *cya* deletion strain of *E.coli*, cAMP is not necessary for activation of transcription. These observations refute Ebright's model.

Since cAMP:CRP bound tightly to the *lac* promoter DNA fragments, the possibility of solenoidal supercoil formation was also refuted (Fried and Crothers, 1983). Based on the X-ray crystallographic data of Steitz *et al.* (1983), an alternative model was considered (Steitz *et al.*, 1983). This model proposes that the important α-helices at the amino terminal interact in a similar fashion as the intercalation of Cro and cI proteins with lambda DNA (Pabo and Lewis, 1982), but such an interaction between cAMP:CRP and the DNA would allow interactions for only about 8 to 9 base pairs in a region of 14 base pairs, shorter than the known CRP binding sequence (Ebright, 1982). However, a bend in the DNA would provide an opportunity for additional contacts with

the DNA backbone. Structural changes in the DNA are indeed found when cAMP:CRP interacts with specific DNA sites, not involving changes in the linking number. A 1:1 complex between *lac* promoter DNA and cAMP:CRP was found to display an anomalous electrophoretic mobility, probably caused by structural changes in the DNA that are induced by CRP (Fried and Crothers, 1981; 1983; Musso *et al.*, 1977). It is clear that these changes are neither due to transition from right handed to left handed B-DNA (Kolb and Buc, 1982), nor are they due to unwinding of DNA, because binding of cAMP:CRP to its recognition site at the *lac* promoter produces, in fact a stabilisation of the DNA duplex (Unger *et al.*, 1983). However, the significance of the changes in the DNA structure for the activation by cAMP:CRP remains unexplained. The elegant work of Wu and Crothers (1984) indicates that a particular DNA sequence (Trypanosome kinetoplast (K) DNA [CAAAAAAT]) or when *lac* promoter was interacted with cAMP:CRP; can cause the DNA molecule to bend. They generated from the restriction fragments a series of variants of approximately identical lengths, differing only in the relative position of the bending locus to the ends of the fragment i.e. circular permutations. The anomalous electrophoretic and hydrodynamic behaviour of such fragments, was used to locate and to analyse the bending locus on the DNA molecule. The bending locus arrived at from their experiments was identical to the cAMP:CRP binding site deduced by several other workers (reviewed in deCrombrugghe *et al.*, 1984). They proposed models which include smooth curvature along the helix

and localised bends such as kinks or junction bends (McGhee and Felsenfeld, 1980). The bending center appears to be about 5-7 base pairs towards the promoter from the center of the protein binding site. It is possible that RNA polymerase recognises this kinked structure (referred to as the H form) or the region of DNA following the H conformation. It is also possible that the role of DNA bending is primarily to create CRP : polymerase interactions which would not be sterically possible in a linear promoter DNA.

Much of our understanding of transcription initiation is based on a comparison of DNA sequences of wild type and mutant promoters, and various assays designed to measure the functional strength of promoters. The RNA polymerase binding site is characterised by two consensus sequences one at -10 region and another at -35 region. The distance between the -10 and -35 regions is approximately 17 base pairs (Rosenberg and Court, 1979; Siebenlist *et al.*, 1980; Hawley and McClure, 1983; Stefano and Gralla, 1982), but promoters with spacing as little as 15 or as many as 20 base pairs retain partial function. Strong promoters show a higher degree of homology than do weaker promoters, and several promoter mutations fall in one of the two consensus sequences (-35 and -10). Structural features outside this consensus sequences might play a role in determining promoter strength. Walter *et al.* (1967) and Chamberlin (1974) suggested a simple two step model for the interaction between RNA polymerase and promoter to form a closed (inactive) complex (association constant K_B), which is followed by an isomerisation to form an

open complex (active). Transition from a closed to an open complex would include localised unwinding of the DNA over a distance of 12 base pairs near the transcription start site and is irreversible (rate constant K_f) (McClure, 1980). McClure (1980), using the abortive transcription initiation assay, quantitated both K_B and K_f. Strong promoters have high values of both K_B and K_f, whereas weak promoters have lower values for both constants. Most promoter mutations could be explained from their behaviour *in vitro*, as one of the two constants or both were usually affected. Mutations in the -35 region affected K_B, while the value of K_f depended mainly on the degree of homology of the -10 sequence with the consensus sequence, and on the distance between -35 and -10 sequences (Stefano and Gralla, 1982). Using this assay, the role of CRP has been examined with the *lac* promoter (Howley *et al.*, 1982; McClure *et al.*, 1982). Addition of cAMP and CRP to the *lac* promoter first enhanced the rate of open complex formation (K_B) when RNA polymerase was added last to the reaction mixture, while K_f was not affected significantly. This was associated with a decrease in the non-specific binding of RNA polymerase to other regions of the DNA. The increase in K_B increases the probability of forming open complexes and correct initiation of transcription. Several indirect arguments favour the view that cAMP:CRP and RNA polymerase interact, thus increasing K_B. The basis for these arguments is due to several observations. 1) RNA polymerase and CRP co-sediment (Blazy *et al.*, 1980). 2) CRP increases complement fixation response by anti-sigma subunit of RNA polymerase, and

antibodies against α, β, β' subunits elicited no response in the presence of CRP (Stender, 1980). 3) Binding of cAMP to *lac* and *gal* promoters in the presence of RNA polymerase is enhanced. The differences in distance between CRP and RNA polymerase binding sites seen in several CRP stimulated genes, preclude the possibility of a general interaction between cAMP:CRP and RNA polymerase, but in a ternary complex on a supercoiled DNA or a kinked DNA, this might be expected.

1.5.2 Transcription termination and polarity:

cAMP:CRP has been shown to decrease the degree of natural polarity of some polycistronic operons (Ullmann *et al.*, 1979). The role of polarity in the modulation of gene expression appears to be an essential regulatory event controlled by a termination-antitermination mechanism. There are a very few reports on the role of cAMP in the control of polarity and its relation to prokaryotic physiology. Despite the potential importance of the role in transcription termination and polarity, they will not be discussed in detail here. cAMP:CRP may interact with RNA polymerase in an analogous manner as the N protein of bacteriophage lambda to overcome transcription termination (Campbell, 1979). Ullmann *et al.* (1979) suggested that cAMP:CRP might be involved in abolishing the rho dependent termination. Botsford (1981) notes that the interpretation of Ullmann *et al.* (1979) might be ambiguous because

the *rho-15* (*ts*) mutation that they used has pleiotropic effects (Adhya and Gottesman, 1979), and the termination of *lac* operon requires *nusA* gene product (Greenblatt *et al.*, 1980; Greenblatt, 1984) and not rho (Ullmann *et al.*, 1979). Also, it has been argued that the observed polarity can be accounted for by differential decay rates of the *lac* mRNA (Kennel and Reizman, 1977).

1.5.3 Negative control:

Mallick and Herrlich (1979) reported that cAMP exerts co-ordinate negative control on various genes. cAMP represses as many genes as it regulates positively. Based on the observation that the outer membrane protein III is maximally repressed at concentrations of cAMP twenty fold below concentrations required for maximal induction of lambda receptor (*lamB* gene product), it was suggested that two conformational states may exist for CRP or its DNA adduct. One conformation saturates with cAMP at low concentrations and exerts negative control, and the other saturates at higher concentrations of cAMP and mediates positive control. An alternate mechanism was suggested in which cAMP:CRP induces the synthesis of a 'super repressor' that co-ordinates negative regulation of many genes.

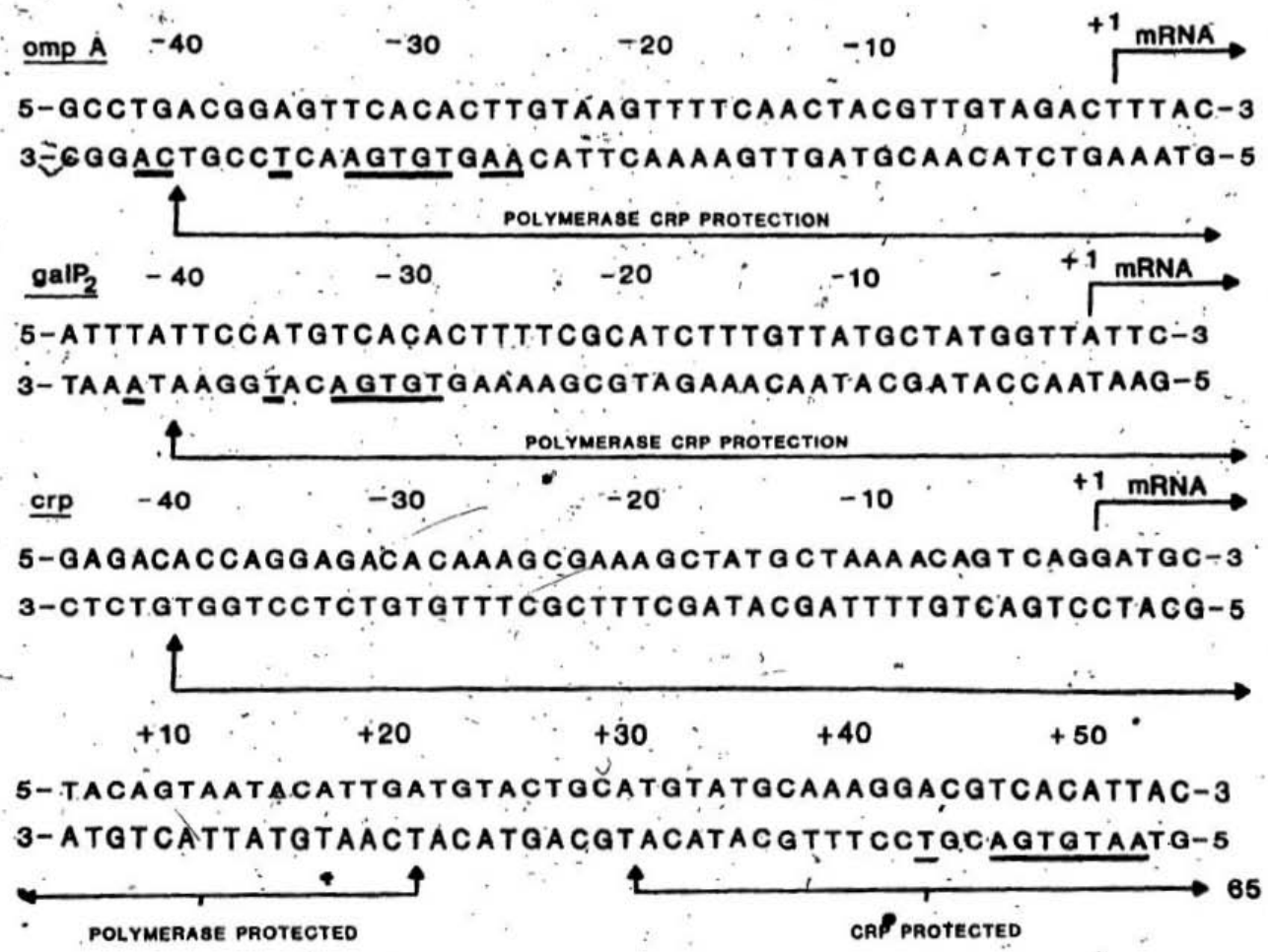
In addition to outer membrane protein III, several other genes have been identified that are negatively regulated by cAMP. These include the genes for glutamine synthetase, glutamate synthase, glutaminase A (Prusiner *et al.*,

1972); adenylate cyclase (Aiba *et al.*, 1983; Majerfeld *et al.*, 1981); CRP (Aiba, 1983; Cossart and Sanzey, 1985); *rrnB* (Glaser *et al.*, 1980); *galP2* (Musso *et al.*, 1977); spot 42 RNA (Sahagan and Dahlberg, 1979); *ompA* (Movva *et al.*, 1981) and *speA, speB, speG* (Wright and Boyle, 1982). Negative control by cAMP of several genes has been examined in detail. Transcription from the *galP2* promoter is inhibited by CRP excluding RNA polymerase from the promoter due to overlapping recognition sites (Musso *et al.*, 1977) (Figure 1,3). Negative control of *ompA* gene *in vitro* appears to be mediated by a similar exclusion mechanism. The *crp* gene is autogenously regulated by cAMP and CRP (Aiba, 1983). A second site weakly protected from DNAase digestion was positioned around -65. Significantly, cAMP:CRP and RNA polymerase bind to the *crp* gene simultaneously, therefore, exclusion of polymerase from the promoter is probably not the mechanism of negative control for this gene. Since CRP binding site is relatively far from the initiation start site, it is assumed that CRP prevents elongation of transcription rather than initiation. It is also possible that there exists a divergent promoter which transcribes the non-coding strand of the *crp* gene and that the internal cAMP:CRP binding site might be a site for the induction of the divergent promoter. This promoter can then potentially initiate an RNA species anti-sense to the *crp* mRNA. This RNA molecule can potentially inhibit translation, examples of such mRNA molecules are known to be formed in *E.coli* (Pestka *et al.*, 1984; Coleman *et al.*, 1984). *speA* (encoding arginine decarboxylase) , *speB* (encoding agmatine

Figure 1.3

CRP binding sites in negatively regulated genes

The figure shows the sequences specifically recognised by cAMP:CRP in the promoter regions of the *ompA*, *galP2* and *crp* genes. Numbers above the sequences indicate distances in base pairs from the corresponding start site of transcription. Bold lines below the nucleotides represent sequences homologous with the consensus cAMP:CRP binding sequence (Ebright, 1982). Horizontal arrows below the sequences indicate the regions protected by cAMP:CRP and RNA polymerase from DNAaseI digestion. Adapted from De Crombrugghe *et al.* (1984).


omp A
-40
-30
-20
-10
+1 mRNA
5-GCCTGACGGAGTTCACACTTGTAAGTTTTCAACTACGTTGTAGACTTTAC-3
3-CGGACTGCCTCAAGTGTGAACATTCAAAAGTTGATGCAACATCTGAAATG-5
POLYMERASE CRP PROTECTION
galP2
-40
-30
-20
-10
+1 mRNA
5-ATTTATTCCATGTCACACTTTTCGCATCTTTGTTATGCTATGGTTATTC-3
3-TAAATAAGGTACAGTGTGAAAAGCGTAGAAACAATACGATACCAATAAG-5
POLYMERASE CRP PROTECTION
crp
-40
-30
-20
-10
+1 mRNA
5-GAGACACCAGGAGACACAAAGCGAAAGCTATGCTAAAACAGTCAGGATGC-3
3-CTCTGTGGTCCTCTGTGTTTCGCTTTCGATACGATTTTGTCAGTCCTACG-5
+10
+20
+30
+40
+50
5-TACAGTAATACATTGATGTACTGCATGTATGCAAAGGACGTCACATTAC-3
3-ATGTCATTATGTAACTACATGACGTACATACGTTTCCTGCAGTGTAATG-5
65
POLYMERASE PROTECTED
CRP PROTECTED

ureohydrolase) and *speC* (encoding ornithine decarboxylase) genes have been shown to be negatively regulated by cAMP:CRP (Wright and Boyle, 1982). They also showed that the specific activity of glucose-6-phosphate dehydrogenase was decreased when cAMP was added to the growth medium of Δ *cya* strains of *E.coli* but not when added to the growth medium of Δ *crp* strains. Boyle *et al.* (1985) further demonstrated that *speC* was indeed regulated at the level of transcription, using cloned copies of the gene in a minicell producing system and in an *in vitro* transcription and translation system, by assessing *in vivo* mRNA concentrations and by assessing the effect of cAMP and CRP using promoter fusion plasmids. Recently, Jovanovich (1985), using *cya:lac* fusions, showed that the *cya* gene in *Salmonella typhimurium* is also regulated negatively by cAMP:CRP.

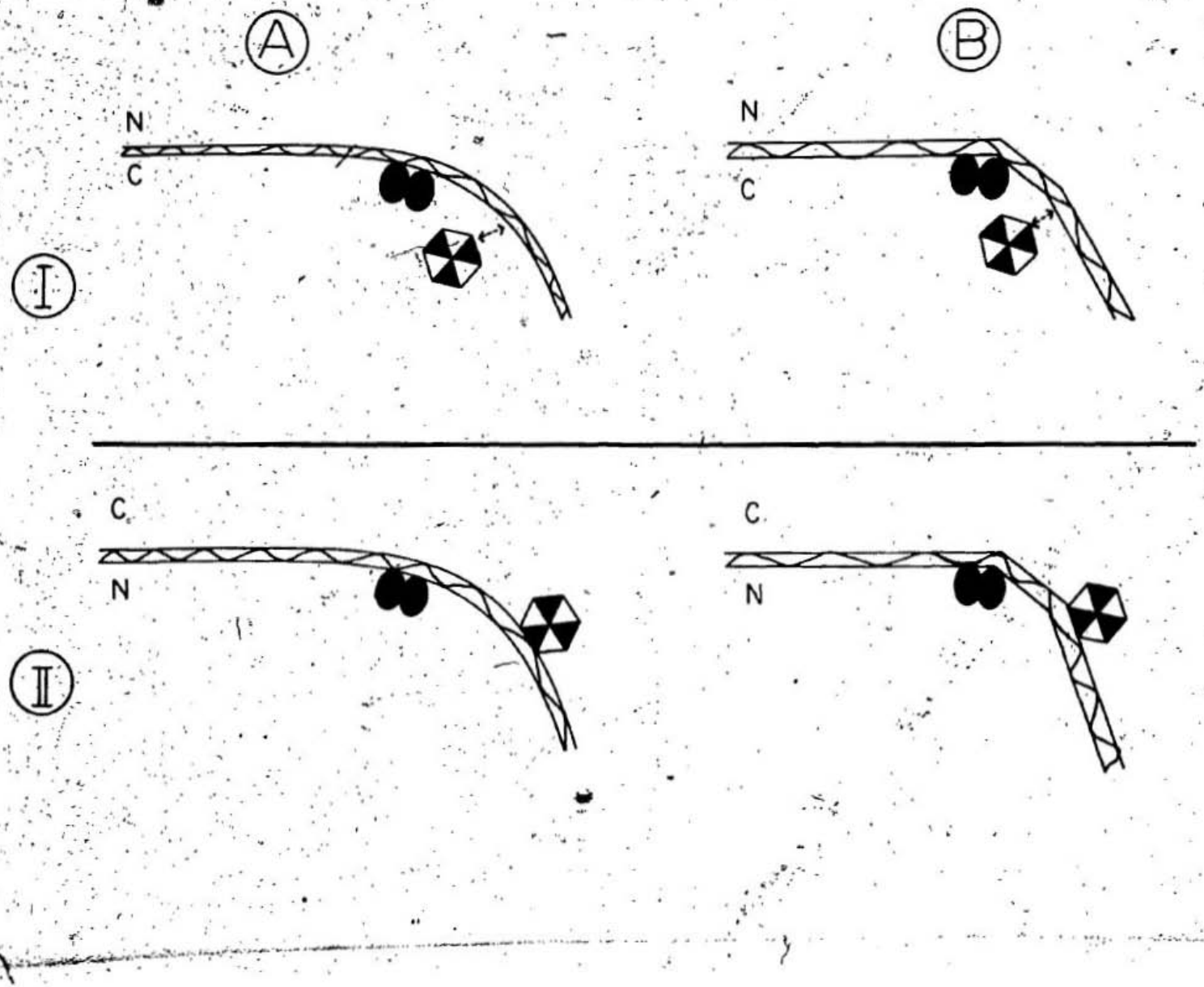
There is a striking homology among the DNA sequences that CRP recognises in negatively and positively controlled genes (Figure: 1.3). The consensus sequence for the CRP binding site in positively regulated genes (*lac, ara, galP1, cat, deoP*) is found on the non-coding strand, whereas, the consensus sequence in negatively regulated genes (*galP2, ompA, crp*) is found on the coding strand. Preliminary data (personal communication, S.M.Boyle) on the promoter sequence of *speC* agree with the above observation. This correlation suggests that some relationship between the orientation of CRP binding site relative to RNA polymerase binding site and CRP function may exist (Aiba, 1983).

The junction bending or kinking of the DNA molecule proposed in Section 1.5.1 (Wu and Crothers, 1984) as a plausible model to explain changes in DNA structure on cAMP:CRP binding fits in with the above contention of Aiba (1983), and is schematically shown in Figure: 1.4. Although binding of cAMP:CRP to the non-coding strand can increase RNA polymerase interaction with promoter DNA, in a manner not clearly understood at the present time, the binding of cAMP:CRP to the coding strand would kink the DNA molecule in a manner shown in Figure: 1.4, probably causing a steric hinderance and thereby decreasing the binding of RNA polymerase to the promoter. Adhya and Garges (1982) suggested that the negative control by cAMP:CRP can best be explained by a competition model, between RNA polymerase and cAMP:CRP on overlapping sites on the DNA, thereby preventing transcription initiation. The results of Aiba (1983), Wu and Crothers (1984) and Boyle (personal communication) do not support the contention of Adhya and Garges (1982), that the negative regulation of a gene by cAMP can best be explained as the result of a simple competition. The dual functional role of cAMP:CRP is not an unique phenomenon, since other examples are known. The *araC* gene product acts both as a positive and negative regulator of *araBAD* operon (Lee *et al.*, 1981; Ogden *et al.*, 1980; Cass *et al.*, 1980). The *araC* gene product appears to exist in two functionally active configurations, that of a repressor or that of an activator (Englesberg, 1971; Englesberg and Wilcox, 1974). Transition from one state to the other is mediated by the inducer molecule,

Figure 1.4

Schematic diagram to illustrate the mechanism of positive and negative regulation by cAMP:CRP by kinking or smooth bending of DNA.

A = Smooth bend DNA
B = Kinked DNA
1 = Negative regulation
2 = Positive regulation
= DNA molecule
= CRP dimer
= RNA polymerase
N = Non-coding strand
C = Coding strand


A
B
N
C
I
C
N
II

arabinose, which when bound to the *araC* gene product transforms the protein into an activator of the *araBAD* operon. Using purified *araC* gene product Wilcox and Meuris (1976) and Lee *et al.* (1981) concluded from DNA protection and competition experiments with RNA polymerase and cAMP:CRP (cAMP:CRP is a positive regulator of the *araBAD* operon), that the *araC* gene product in its activator or repressor form (based on activation or repression of the *araBAD* operon), acts as a repressor for the *araC* promoter, i.e. *araC* is negatively autoregulated by the *araC* gene product in its activator or in its repressor state. This appears to be mediated by the protein (in either state) binding to the same DNA sequence in the *araC* promoter. This is an interesting finding, since the *araC* gene product binds to two separate sites on the *araBAD* promoter, one that activates transcription and the other that represses transcription, while in either state binds to the same sequence on the *araC* promoter to repress transcription.

1.6 Glutamine synthetase:

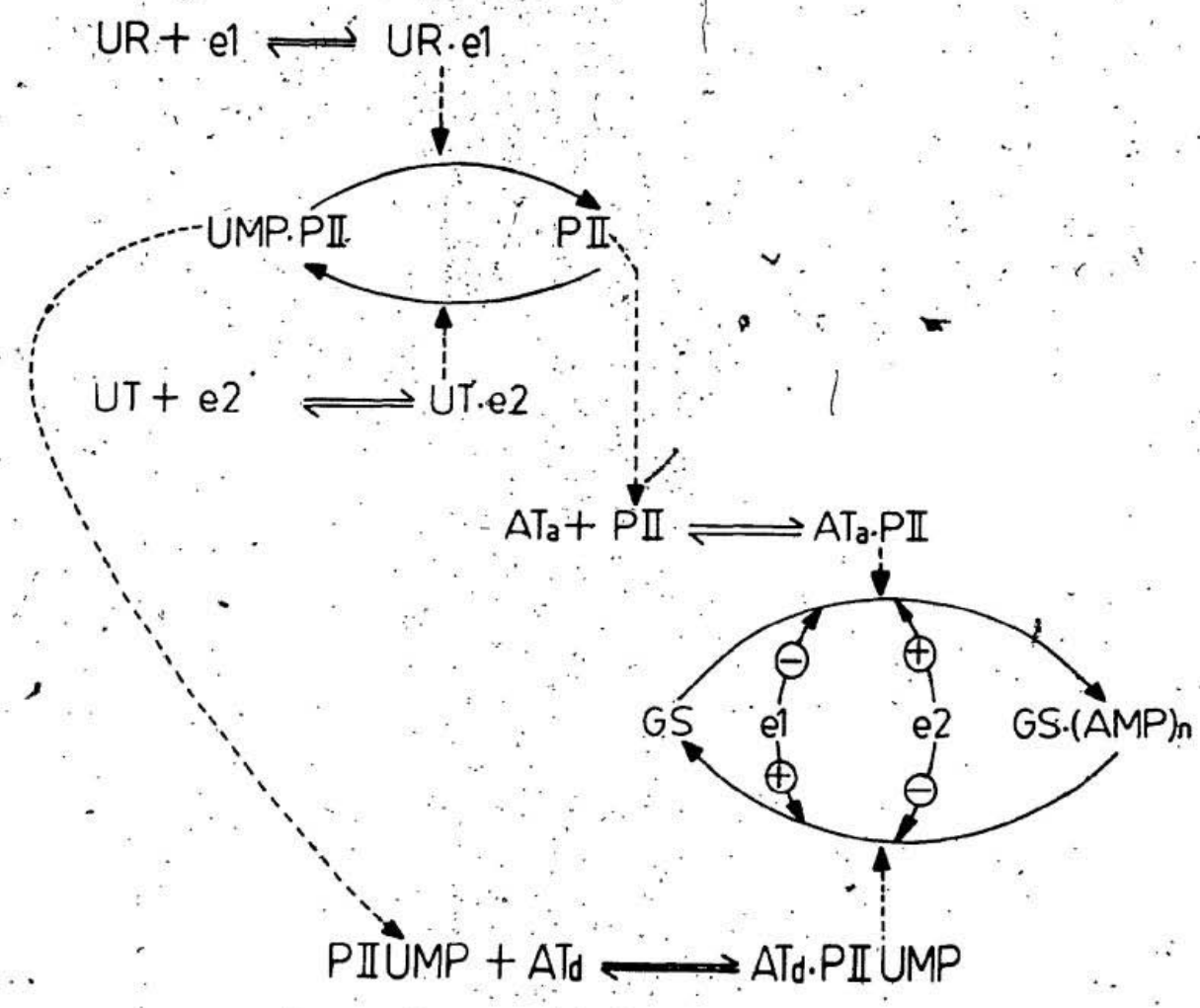
Glutamine is a compound of central importance in metabolism. On the one hand it is a primary intermediate in the assimilation of ammonia, and on the other it serves as a source of nitrogen in the biosynthesis of purine and pyrimidine nucleotides, of most amino acids, glucosamine-6-phosphate, p-aminobenzoic acid and of nicotinic acid derivatives (Stadtman, 1973; Wulff *et*

al., 1967). In order to accommodate these diverse functions, *E.coli* and other Gram negative bacteria have evolved a multi-enzyme cascade system that is able to sense simultaneous changes in the concentrations of many different metabolites and to integrate their combined effects so that the catalytic activity of glutamine synthetase can be adjusted continuously to meet the ever changing demand for glutamine. The cascade system involves the cyclic adenylation and deadenylation of each of the twelve subunits of glutamine synthetase (GS); the other component involves the uridylation and deuridylation of a protein, P_{II}, which regulates the activity of the GS-adenylating enzyme (Stadtman, 1973). For any metabolic condition, a steady state will be established in which the rates of the forward step and regeneration step will be equal. As noted by Stadtman and Chock (1978a; b; Stadtman *et al.*, 1979) and Chock and Stadtman (1978), only slight functional activation of a converter enzyme by its positive allosteric effector is needed to obtain substantial covalent modification of an interconvertible enzyme, and hence a cyclic cascade of this sort would have an appreciable signal amplification potential with respect to the effects of primary allosteric stimuli. The primary allosteric stimuli can be any one of the molecules which derive an amino group from glutamine. The bicyclic cascade is shown in Figure 1.5. This dynamic concept is supported by the *in vitro* and *in vivo* studies (summarised in Stadtman *et al.*, 1979) that show the average state of adenylation of *E.coli* glutamine synthetase varies in response to changes in the concentrations of various metabolites in a manner

Figure 1.5

Regulation of glutamine synthetase through a bicyclic cascade.

UR is the uridyl removing enzyme and UT is the uridyl transferase, GS = glutamine synthetase. ATa is the adenyltransferase enzyme that adenylates GS in its adenylating mode, and ATd is the same enzyme in its deadenylating mode. e1 and e2 are effector molecules e.g. glutamine (e1) and α-ketoglutarate (e2), the concentrations of which which modulate the uridylation of the P_{II} protein, which in turn regulates the GS adenylating system.


UR + e1
UR·e1
UMP·PII
PII
UT + e2
UT·e2
ATa + PII
ATa·PII
GS
e1
e2
GS·(AMP)n
PIIUMP + ATd
ATd·PIIUMP

predicted from a theoretical-analysis of the steady state model (Chock and Stadtman, 1978).

The genetic studies from the laboratory of Boris Magasanik can be summarised as follows: 1) glutamine synthetase is autoregulated negatively by the adenylated form of the enzyme, 2) glutamine synthetase stimulates the synthesis (in its deadenylated form) of enzymes responsible for the degradation of amino acids to glutamate, 3) in its deadenylated form glutamine synthetase is a repressor of genes that use glutamine and glutamate as an amino group donor (Magasanik *et al.*, 1973; 1974; Magasanik and Stadtman, 1980; Magasanik and Rothstein, 1980).

The regulatory activities of glutamine synthetase are an elegant example of a cascade effect. Starvation for ammonia brings about deadenylation of glutamine synthetase (Ginsburg and Stadtman, 1973; Wohlhueter *et al.*, 1973). This starvation also converts glutamine synthetase into a form which is enzymatically active but can no longer repress the synthesis of glutamine synthetase. The increase in the level of this enzyme brings about repression of the synthesis of glutamate dehydrogenase, the induction of amino acid degrading enzyme systems (*hut*, *put*, *sut* etc.), and of transaminases that are capable of producing glutamate (Magasanik, *et al.*, 1973; 1974; Magasanik and Rothstein, 1980).

The results from the laboratories of R.C.Valentine, F. Ausubel and

W.Brill clearly demonstrate that nitrogen fixing genes (*nif*), in nitrogen fixing strains of *Clostridium, Rhizobium* and *Klebsiella* are regulated by glutamine synthetase (McFarland *et al.*, 1981; Tuli *et al.*, 1982). Thus it may be that, in a sense, glutamine synthetase is ultimately responsible for all nitrogen assimilation since the nitrogen cycle begins with nitrogen fixation.

1.7 Objectives:

Cloned copies of genes encoding ornithine decarboxylase, arginine decarboxylase and agmatine ureohydrolase were selected by the ability of the plasmids that carry them to confer elevated levels of enzymatic activity to the host cells (Boyle *et al.*, 1984; Tabor *et al.*, 1983). Using minicells, they identified the proteins encoded by these plasmids, including a 38,000 dalton protein, which was deduced to be agmatine ureohydrolase. Although ornithine decarboxylase (Applebaum *et al.*, 1977) and arginine decarboxylase (Wu and Morris, 1973a;b) have been purified from *E.coli* cells and studied extensively, almost nothing is known about agmatine ureohydrolase. Therefore, it was proposed to purify agmatine ureohydrolase and to assess some of its physical and biochemical properties.

Wright and Boyle (1982) showed that the synthesis of ornithine decarboxylase and arginine decarboxylase are negatively regulated by cAMP, and suggested that agmatine ureohydrolase synthesis might also be negatively regulated by cAMP. It was therefore proposed to assess the role of cAMP in the

regulation of agmatine ureohydrolase. It was also proposed to assess the roles of precursors and products of agmatine metabolism in the regulation of ornithine decarboxylase, arginine decarboxylase and agmatine ureohydrolase synthesis. Prusiner *et al.* (1972) demonstrated that glutamine synthetase and other enzymes of glutamine biosynthesis were negatively regulated by cAMP. Polyamines contain amino groups, and agmatine and putrescine have been shown to provide substrates for the enzymes of the nitrogen assimilatory pathways in *Klebsiella pneumoniae* and *Klebsiella aerogenes*. (Friedrich and Magasanik, 1978; 1979). Therefore, it was proposed to examine the possibility that glutamine synthetase itself might mediate the negative regulation by cAMP of polyamine biosynthetic enzyme synthesis.

Following the demonstration that cAMP:CRP appeared to act directly at the level of transcription of the genes, various *in vitro* studies were designed to assess the sites of action of agmatine, putrescine and cAMP:CRP. Promoter location and the direction of transcription were also studied. An *in vitro* transcription and translation system and the minicell system were employed to assess whether the roles of the regulatory elements were direct or indirect in the synthesis of agmatine ureohydrolase. Messenger RNA concentrations in whole cells, treated appropriately, were analysed to confirm that the regulation was indeed at the level of transcription, this was extended further by assessing the effects of cAMP:CRP, agmatine and putrescine using fused genes of *speB* and either *lacZ* or *galK*.

Chapter 2

Materials and Methods

2.1 Bacterial strains and plasmids used:

The bacterial strains and plasmids used in this study are listed in Table: 2.1 and Table: 2.2, respectively.

2.2 Bacterial growth and preparation of bacterial extracts:

All cultures were started from a single colony which had been phenotypically characterized on appropriate media. The latter were made as follows: MOPS - minimal plates (Neidhardt *et al.*, 1974); Luria agar plates (Miller, 1972); Yeast extract-tryptone plates (Miller, 1972), MacConkey agar plates (Miller, 1972), M-9 minimal agar plates (Miller, 1972). Glucose, lactose, maltose, galactose or glycerol were added at 0.2%. Amino acids were added at 50 μg/ml, and antibiotics at 50 μg/ml. LS853 (Δcya) and LS854-1 (Δcya, Δcrp) were further characterized on MacConkey agar plates containing 1 mM cAMP and 0.2% lactose or maltose.

Cultures in liquid media were grown overnight at 37 ° C in a reciprocating water-bath using either MOPS-minimal medium or M9-minimal medium with glucose as the sole carbon and energy source. These were used to inoculate experimental cultures (50-100ml in 250ml flasks) to an absorbance of 0.025 - 0.050 at 575 nm. Growth was followed spectrophotometrically (Lawrence and Maier, 1977). For the determination of arginine decarboxylase, agmatine ureohydrolase and ornithine decarboxylase the cultures were grown to an

Table: 2.1

Bacterial strains used:

Organism	Genotype	Relevant Phenotype	Source or Reference
A. Escherichia coli			
CA84-85	Δ*crp,thi,rpsL*	CRP⁻,*Str*ʳ	Fraser and Yamazaki (1980)
EC145	Δ*argR*,Δ*(pro-lac)*, *(argA::lacZ)* λ	Arg.rep⁻	Dr. Thomas Eckhardt, Smith Kline and French Laboratories, Philadelphia.
ET8000	*rbs,lacZ:IS1,hut*ᶜ, *nalA*		Dr. Douglas MacNeil, Merck, Sharp and Dohme,Rahway, NJ. (Bachman, 1972)
ET8053	*rbs,lacZ,nalA, hut*ᶜ,*glnD:Tn10*	UTase⁻, V.Low Ad.GS	"
ET8348	Δ*glnA,rbs,lacZ: IS1,hut*ᶜ,*nalA.*	GS⁻	"
ET8411	*glnG,rbs,lacZ: IS1,hut*ᶜ,*nalA.*	High dead.GS	"
ET8689	*rbs,lacZ:IS1, hut*ᶜ,*nalA,glnL.*	low ad.GS.	"
ET10643	*rbs,lacZ,nalA, hut*ᶜ,*glnL*	V.high Ad.GS	"
HB101	*pro,leu,thi, rpsL,hsdR,hsdM, ara,galK,xyl,mtl, lac,supE,recA.*	Lac⁻	Boyer *et al.* (1969)
HT289	*leu,thr*,Δ*speA,* Δ*speB,* Δ*speC,*	ODC⁻ ADC⁻	Hafner *et al.* (1979)

	Δglc,hsd,thi.	AUH⁻	
HT322	*thi,leu,thr,thyA, hsd,ΔspeA,ΔspeB, ΔspeC,Δglc*	ODC⁻,ADC⁻, AUH⁻	"
HT328	*thr,leu,hsd, recA,strA,ΔspeA, ΔspeB,ΔspeC,Δglc.*	ODC⁻,ADC⁻, AUH⁻,RecA⁻	"
JM103	*Δlac-pro,thi,strA, endA,sbcB15,hsdR4, supE,F'traD36, proAB,lacIqZΔM15.*	Lac⁻,Pro⁺	P.L. Biochemicals
LS340	*trpA,his-85,metE, trpR*		Brinkman *et al.* (1973).
LS853	*trpA,his-85,Δcya, trpR*	CYA⁻	"
LS854-1	*trpA,his-85,Δcya, trpR,Δcrp,rpsL*	CYA⁻, CRP⁻	This work
MA17	*thi,thr,leu,his,serA, rpsL,mal*		Maas (1972)
MA135	*thi,argE,his,trp,pro, rpsL,speB,mal*	AUH⁻	" "
MA163	*thi,thr,his,rpsL, speA,mal*	ADC⁻	"
MA255	*thi,thr,leu,speC, speB,mal*	ODC⁻AUH⁻	"
MRE600	*glu,rna*	RNAase⁻	Pratt *et al.* (1981).
N100	*pro,recA ΔgalK*	Gal⁻	Dr. M. Rosenberg, Smith Kline and French Philadelphia.
P678-54	*thrL,leuB6,minA,*	Minicell	Adler *et al.*

	thi, ara, lac Y, gal, malA, xyl, mtl, minB rpsL, tonA, azi	producer	(1967)
UW44		Biodegradative ODC and ADC	Applebaum *et al.* (1975).
K-12	Reference strain from center for Disease Control, Atlanta, Georgia.		Dr. D. Brenner, Center for Disease Control, Atlanta, Georgia.
B. Bacillus subtilis		This laboratory	

Table: 2.2

Plasmids and viruses used

Plasmid/virus	Genotype	Source or Reference
pBR322	*amp,tet*	P.L Biochemicals
pEC1492	*kan,argR*	Dr. Eckhardt, Smith Kline and French Laboratories, Philadelphia.
pKA5	*tet,amp,speA,speB,metK*	Boyle *et al.* (1984). Tabor *et al.* (1983).
pKA10	*amp*	Dr. Douglas Markham, Fox Chase Cancer Center, Philadelphia.
pKA12	*amp,speB,metK*	Boyle *et al.* (1984). Tabor *et al.* (1983).
pKO11	*amp*	Dr. M Rosenberg, Smith, Kline and French Laboratories, Philadelphia.
pMC1403	*amp*	Casadaban *et al.* (1980)
pUC8/9	*amp,lacZM15*	P. L. Biochemicals (Vieira and Messing, 1982)
pLC(29-36)	*ColE1,glnA*	Dr. D. MacNeil, Merck, Sharp and Dohme, Rahway, N[illegible]larke and Carbon (1976)
pKA14	*amp, speB*	This work

pKA15	*tet*	This work
pKA16	*tet, speA*	Buch and Boyle, Memorial Univ., St.John's, Newfoundland
pADG	*amp,speA::galK*	"
pAUG	*amp,speB::galK*	This work
pAUL	*amp,speB::lacZ*	This work
M13 mp7	*lacZ*	P.L. Biochemicals (Vieira and Messing, 1982)
P1 *vir*		This laboratory.

absorbance of 0.75 - 0.80 at 575 nm. For the sodium iodide mediated immobilisation of RNA and DNA from whole cells, and for the determination of β-galactosidase, galactokinase and β-lactamase activities, the cultures were grown to an absorbance of 0.45 - 0.50 at 575 nm. Where indicated cAMP (1-10 mM) and IPTG (0.50 mM) were included in the growth medium. β-galactosidase activities reported in Chapter: 4 and in Sections 10.2.8 and 10.2.9 were determined from the crude extracts prepared to determine the activity of agmatine ureohydrolase. The cells were harvested by centrifugation at 4 °C, resuspended in about 25 ml of cold MOPS-minimal medium and centrifuged again. The washing was repeated, and the final pellet was resuspended in breakage buffer (0.1M HEPES, pH 7.4; 5 mM $MgCl_2$; 5 mM dithiothreitol; 0.04 mM pyridoxal phosphate), which was one fiftieth to one twenty fifth of original culture volume. All further manipulations were carried out on ice. For the measurement of agmatine ureohydrolase, arginine decarboxylase and ornithine decarboxylase cells were broken by passing the suspension twice through a chilled Aminco pressure cell, at 10,000 pounds per square inch. The resulting suspension was sonicated with a Virsonic Cell Disruptor (Model 16-850) using a fine tip probe. There were three sonications each of 20 seconds, interspersed with a period of one minute cooling on ice. The cell debris and unbroken cells were removed by two successive centrifugations at 12,000xg for 10 minutes.

For the measurement of β-galactosidase, galactokinase and β-lactamase cells were resuspended in breakage buffer (Miller, 1972), toluene (0.1%) was

added and the suspension was vortexed vigorously for 30-40 seconds. The toluenised cells were incubated for one hour at 37 °C in a fume hood, and the extract was returned to an ice bath. For the immobilisation of nucleic acids from whole cells mediated by sodium iodide, the cells were grown and then chilled quickly by the addition of an equal volume of crushed, frozen MOPS medium containing 300 μg/ml of chloramphenicol and 20 mM vanadium ribonucleoside complex (VRC). The suspension was centrifuged for 5 minutes in an IEC centrifuge at 4 ° C. The washing procedures for the pellet were identical to that described earlier, but the MOPS medium contained 150 μg/ml of chloramphenicol and 10 mM vanadium ribonucleoside complex.

2.3 Enzyme assays:

2.3.1 Ornithine decarboxylase and arginine decarboxylase:

The arginine decarboxylase assay was that of Wu and Morris (1973a), and the ornithine decarboxylase assay was that of Applebaum *et al.* (1977), as modified by Wright and Boyle (1982). (Figure: 2.1.)

2.3.2 The colorimetric measurement of agmatine ureohydrolase:

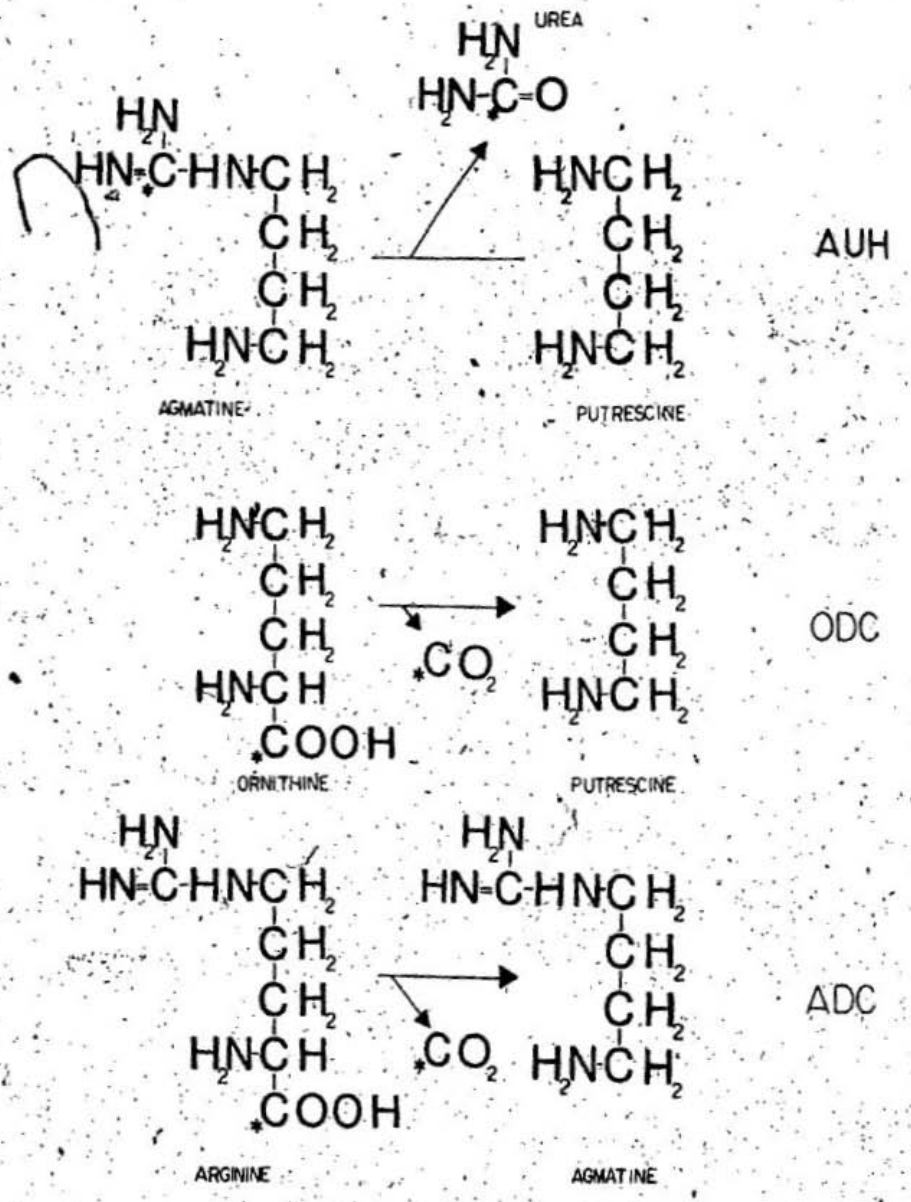
The method of Morris and Pardee (1966) with the following modifications was used. The reactions were stopped by placing the reaction vessels in a boiling water-bath for three minutes. The coagulated proteins were removed by centrifugation, and urea was estimated in the supernatant by conversion of the

Figure 2.1

The reactions catalysed by agmatine ureohydrolase, ornithine decarboxylase and arginine decarboxylase.

• denotes the ^{14}C - label in the molecule , used to monitor the reactions.

ODC = ornithine decarboxylase
ADC = arginine decarboxylase
AUH = agmatine ureohydrolase


UREA
AGMATINE
PUTRESCINE
AUH
ORNITHINE
PUTRESCINE
ODC
ARGININE
AGMATINE
ADC

urea to ammonia catalysed by urease, and determination of the ammonia by the phenol hypochlorite method (Weatherburn, 1967). More than 96% of the enzymic activity of agmatine ureohydrolase was destroyed by this denaturation process. To check if inactivation by heating itself caused a significant reaction before denaturation was complete, a control reaction mixture was placed in the boiling water-bath immediately after the enzyme had been added. To check if urea was lost during heating, known concentrations of urea were added to the reaction mixtures (without the enzyme), and heated for periods up to 10 minutes, and the urea concentration was determined. No urea hydrolysis was observed upon heating the tubes up to 7 minutes (Figure 3.1.).

2.3.3 The radiochemical measurement of agmatine ureohydrolase:

A sensitive radioactive assay described by Morris and Koffron (1967) was modified to determine agmatine ureohydrolase activity during purification, and to assess the kinetic parameters of the enzyme, reported in Chapter 3. The release of [^{14}C] CO_2 from [guanido-^{14}C] agmatine was measured by trapping the [^{14}C] CO_2 in filter paper wicks saturated with Protosol (New England Nuclear), and carried in the center wells suspended from the rubber stopper of the reaction flask.

2.3.3.1 Preparation of L-[guanido-^{14}C] agmatine:

A reaction mixture was prepared as follows: 5 ml of reaction mixture contained 133 mM HEPES, pH 7.5; 0.053 mM pyridoxal phosphate; 5.3 mM

$MgCl_2$; 1.33 mM dithiothreitol; 2.66 mM L-arginine, 125 μci L-[guanido-^{14}C] arginine and 1250 units of arginine decarboxylase. The mixture was made up in an ice-bath, and the reaction was started by incubating the reaction vessels at 37 °C in a water-bath. The incubation was continued for 4 hours and the reaction was terminated by placing the reaction vessels in a boiling water-bath for 10 minutes. Denatured proteins were removed by centrifugation at 12,000xg for 15 minutes, and the supernatant was used as the source of [guanido-^{14}C] agmatine. The source of arginine decarboxylase for this preparation was a dialysed crude extract of an *E.coli* strain HT328 (Δ*speA*, Δ*speB*, Δ*speC*) which was transformed with plasmid PKA13 which carried only *speA* (the gene for arginine decarboxylase). This transformed strain when grown in LB with 0.2% glucose exhibited a 12-15 fold higher activity of arginine decarboxylase compared with other *E.coli* K-12 strains carrying a single gene for arginine decarboxylase. The crude extract prepared according to the method of Section 2.2 from these transformed cells was concentrated to 7-10 mg protein/ml by filtration through a collodion bag (Schleicher and Schuell) which has a cut off limit of 25 kilodaltons.

In the preparation of [guanido-^{14}C] agmatine nearly 80% of the arginine was decarboxylated. This was determined as follows: Arginine decarboxylase activity was monitored in a similar reaction mixture (0.2 ml), but containing L-[U-^{14}C] arginine instead of L-[guanido-^{14}C] arginine. After incubating the reaction vessels at 37 °C for 4 hours, the amount of [^{14}C] CO_2 formed was

determined, and was used to calculate the concentration of decarboxylated arginine. It was assumed that the same amount of L-[guanido-^{14}C] agmatine was produced in the parallel reaction.

2.3.3.2 Assay of agmatine ureohydrolase:

To 0.2 ml of the preparation of [guanido-^{14}C] agmatine, 0.01 ml of urease (2000 units/ml) (Sigma Chemical Company) and 0.01 ml of agmatine sulphate (250 mM) were added. The reactions were started by adding up to 0.08 ml of the enzyme preparation and placing the reaction vessels at 37 ° C. The reactions were stopped by injecting an equal volume of 10% trichloro acetic acid (TCA) through the stopper into the reaction mixture. The vessels were incubated for an hour at 37 ° C, and the center wells containing the filter paper wick and the protosol were cut, and placed in scintillation vials containing 10 ml of scintillent [4g of PPO (Sigma Chemical Company) in 1000 ml of toluene (British Drug House)]. The radioactivity was measured using a Beckman LS9000 liquid scintillation spectrometer. The reaction catalysed by agmatine ureohydrolase is shown in Figure: 2.1.

2.3.4 Preparation of $MgCl_2$ depleted reaction mixtures:

In order to assess the effect of various metal ions on agmatine ureohydrolase activity, the radioactive assay described in 2.3.3 was modified by preparing [guanido-^{14}C] agmatine in an extract lacking added $MgCl_2$. This was possible because arginine decarboxylase still possessed 30% of its enzymic activity

in the absence of added Mg^{2+}. Therefore to achieve 75% decarboxylation of arginine the reaction mixtures without $MgCl_2$ were incubated for 6 hours at 37 °C. This mixture was deproteinised and used for the determination of agmatine ureohydrolase activity as described in Section: 2.3.3.1.

2.3.5 Detection of agmatine ureohydrolase activity in fractions from column chromatography:

In order to screen for agmatine ureohydrolase activity in fractions obtained from column chromatography, a qualitative radiochemical method was used. A reaction mixture containing 133 mM HEPES, pH 7.5; 0.053 mM pyridoxal phosphate; 5.3 mM $MgCl_2$; 1.33 mM dithiothreitol; 2.66 mM L-arginine, 5 μci L-[guanido-^{14}C] arginine and 50 units of arginine decarboxylase, was incubated at 37 °C for 15 minutes. To 0.2 ml lots of solution, 0.02 ml containing urease (20 units) and agmatine sulphate (125 mM) was added, and these were placed in an ice-bath. A sample of chromatographic fraction (0.08 ml) was added, the reaction vessels were stoppered. The stoppers carried paper wicks soaked in 0.2 ml of Protosol (New England Nuclear) in a hanging center well. A set of reactions were started by placing the vessel in a water-bath at 37 °C, and these were incubated for 60 minutes, and then the reactions were simultaneously terminated by placing the reaction vessels at 100 °C for 90 seconds. The reactions were quickly chilled by placing the vessels in an ice-bath. An equal volume of 10% TCA was injected and the vessels were incu-

bated at 37 °C in a water-bath for 60 minutes, and the radioactivity in the center wells was measured as described in Section: 2.3.3.

This method was not quantitative because the initial reaction catalysed by arginine decarboxylase results in the production of [guanido-^{14}C] agmatine, but this reaction is not complete and it continues during the reaction catalysed by agmatine ureohydrolase. Consequently the concentration of agmatine and the specific activity of the radiolabelled agmatine in the reaction mixture may actually increase during the second incubation. Therefore, in order to determine the recovery and purification, the fractions containing maximum agmatine ureohydrolase activity were combined, and concentrated using a collodion bag, and the enzymic activity was redetermined according to the more accurate and sensitive assay described in Section: 2.3.3. Control incubations, lacking in turn each of the enzymes arginine decarboxylase, urease or agmatine ureohydrolase were made (Table: 3.1). The enzymic activity was linear for at least 90 minutes (Figure 3.2) and the activities were routinely determined over a 30 minute incubation period. The enzymic activity was also linear with the various protein concentrations in the assay mixture (Figure: 3.3). The maximum activity was obtained at pH 7.2-7.5 (Figure: 3.4) at 42 °C.

2,3.6 Definition of enzymic and specific activities:

One unit of enzymic activity is that which, in the standard assay produces 1 μmole of product in one minute. The specific activity is defined as the

units per mg protein. Protein concentrations were determined according to the method of Bradford (1976).

2.3.7 Enzymic assays of β-galactosidase, β-lactamase and galactokinase:

β-Galactosidase activity was measured in crude extracts or toluenised cells according to the method of Miller (1972). Galactokinase activity was determined by the method described by Adhya and Miller (1979), and β-lactamase activity was determined according to the method of O'Callaghan *et al.* (1972). Nitrocefin, a chromogenic cephalosporin used to determine β-lactamase activity was a gift of Dr. Paul Fardy, Clinical Microbiology, Health Science Center, Memorial University of Newfoundland. The results of all these activities are expressed as units (μmoles/minute) per unit absorbancy of the bacterial cells at 575 nm in the reaction mixture.

2.4 The construction of Δ*crp* mutant (LS854-1):

Δ*cya*, Δ*crp* mutant LS854-1 was constructed according to the method described by Miller (1972). P1 *vir* lysates were prepared from CA84-45 (Δ*crp*, *str*), and were used to infect LS853 (Δ*cya*). Δ*crp* mutants (LS854-1) were identified as white colonies on MacConkey agar plates containing 1 mM cAMP and 100μg/ml of streptomycin (*str* is used as a marker for co-transduction to increase the probability of selecting a transductant with a *crp* deletion rather than a point mutation). In order to characterise the nature of the mutation,

the white colonies were retested on MacConkey agar plates containing cAMP, streptomycin and either maltose or lactose. The pleiotropic effect (inability to ferment lactose or maltose) in the presence of cAMP and the co-transduction of *str* with the pleiotropic effect suggests that the Δ*crp* mutation has been introduced into LS853.

2.5 Isolation and manipulation of plasmid DNA:

Plasmid DNA was amplified in cultures growing in LB by the addition of chloramphenicol (Sigma Chemical Company) to a final concentration of 170 μg/ml as described by Clewell (1972). The extraction and purification of plasmid DNA was achieved by the alkaline extraction procedure described by Birnboim and Doly (1979), modified as described by Maniatis *et al.* (1982). Further purification of the supercoiled DNA was done by bouyant density centrifugation in CsCl gradients containing ethidium bromide (Maniatis *et al.*, 1982).

2.6 Transformation:

E.coli cells were prepared for transformation by the method of Cohen *et al.* (1972), as modified by Dagert and Ehrlich (1979).

2.7 Purification of chromosomal DNA and total RNA:

Total RNA was purified from *E.coli* MRE600 (Gopalkrishna *et al.*, 1981), while chromosomal DNA was purified from an *E.coli* K12 isolate and

from *B.subtilis* by the method of Marmur (1961).

2.8 Endonuclease digestion and ligation:

Restriction endonuclease digestion of plasmid DNA and the ligation of DNA fragments were performed as recommended by the manufacturer of the enzymes (Bethesda Research Laboratories, P. L. Biochemicals, and Boehringer Mannheim Canada).

2.9 Bal 31 exonuclease digestion:

Bal 31 (Bethesda Research Laboratories) was used to exonucleolytically digest the plasmid DNA, according to the method of the manufacturer.

2.10 Ribonuclease A and deoxyribonuclease I digestion:

Ribonuclease A (Sigma Chemical Company) and deoxyribonuclease I (RNAase free) (Worthington Chemicals) were prepared, stored and used according to the method of Maniatis *et al.* (1982).

2.11 Removal of single stranded ends from duplex DNA:

Digestion of restricted or *Bal 31* treated plasmid DNA using S1 (single strand nuclease) (P. L. Biochemicals) was carried out according to the method of Maniatis *et al.* (1982) at 25 °C with 0.5 units of enzyme and 5μg DNA (e.g *PstI* digested pBR322) in a 0.1 ml of reaction mixture. S1 digestion of restricted DNA was carried out only when the restriction digestion caused a 3'

single stranded end.

The Klenow fragment of DNA polymerase I (P.L. Biochemicals) was also used to remove single stranded ends by filling in the recessed 3' ends. A typical reaction mixture contained 2 units of the enzyme, 10 μg of *EcoRI* restricted pBR322, 20 mM Tris-HCl (pH 7.8), 0.5 mM EDTA, 10 mM $MgCl_2$, 10 mM dNTPs and 1 mM dithiothreitol, and was incubated at 20 °C for 30 minutes.

2.12 Digestion of restricted DNA with alkaline phosphatase:

The 5' phosphate group was removed from the restricted DNA with either calf intestinal phosphatase (Bethesda Research Laboratories) or BAP-MATE (bacterial alkaline phosphatase immobilised on Sepharose beads) (Bethesda Research Laboratories). The reactions were conducted according to the recommendation of the manufacturer. The dephosphorylated DNA was heated at 70 °C for 20 minutes to destroy the calf intestinal phosphatase activity and was extracted with phenol as described by Maniatis *et al.* (1982), to remove proteins. BAP-MATE treated samples were centrifuged and the supernatant was extracted with phenol.

2.13 Agarose gel electrophoresis:

Agarose gel electrophoresis was carried out in 0.8 or 1.0% (w/v) agarose or in low melt agarose (Bio-Rad), in Tris-Borate-EDTA buffer (Maniatis *et al.*, 1982). The gels were run horizontally at room temperature, stained with ethi-

dium bromide as described by Maniatis *et al.*, (1982), and photographed with a red filter using a polaroid camera with Kodak 660 film. The *HindIII* restriction endonuclease digests of lambda (P. L. Biochemicals) and the *HaeIII* digests of ϕx174 (Boehringer Mannheim Canada) were used as fragment length standards. The distances migrated by the restriction fragments of the bacteriophage DNA were plotted against the logarithm of their size (base pairs). This was used to determine the sizes of the uncharacterised fragments from their distances of migration. The lengths determined were confirmed by using the NA2 Nucleic Acid Analyser (Bethesda Research Laboratories). The restriction sites determined from the analysis of the sizes of the fragments generated by single and multiple digestion of the DNA were confirmed by using the MAPC/MAPL restriction mapping programs in Fortran available on VMS (in the ICR sequence program package) compiled and integrated by Holly Cael and Peter Young of the Fox Chase Cancer Center, Philadelphia; adapted for use at Memorial University of Newfoundland by Donna Green of Computer Services, Faculty of Medicine, Memorial University of Newfoundland.

2.14 Polyacrylamide gel electrophoresis (PAGE):

The fractionation of proteins by PAGE in 10-15% acrylamide was performed according to the method of Laemmli (1970) at 18 °C with 0.1% SDS. Radioactively labelled proteins were detected by autofluorography (Bonner and Laskey, 1974). PAGE under non-denaturing conditions was carried out accord-

ing to the method of Davis (1984) at 4 ° C. To detect non-radioactive proteins the polyacrylamide gels were stained with Coomassie Blue according to the method of Laemmli (1970). The denaturing gels were also stained with silver nitrate according the method described by Switzer *et al.* (1979). Radioactive proteins for use as molecular weight standards were obtained from Amersham Corporation, and the non-radioactive standards were obtained from Pharmacia Fine Chemicals.

2.15 Recovery of DNA fragments from low-melt agarose:

Restricted DNA was separated on low-melt agarose (Sigma Chemical Company), and a section of the agarose gel was stained with ethidium bromide. The desired fragment was cut from the unstained section of the gel, and isolated by the method recommended by the manufacturer of Elutip-D columns (Schleicher and Schuell). The DNA was precipitated with ethanol at room teperature, the supernatant was decanted and the pellet was redissolved in DNAase free water. A sample was electrophoresed on an agarose gel, stained and the intensity compared visually with that given on the same gel by standard quantities of pBR322.

2.16 Purification and radiolabelling of proteins in minicells:

The purification of minicells was done according to the method of Schoemaker and Markovitz (1981), as modified by Boyle *et al.* (1984). The minicells were pre-incubated for 15 hours to allow the degradation of

endogenous mRNA and were labelled according to the method described by Boyle *et al.* (1984) using [^{35}S] methionine as schematically diagramed in Figure: 9.1.. The incorporation was linear in minicells carrying pBR322 or pKA5 for at least a period of 45 minutes. (Figure: 9.1)

2.17 Cell - free transcription and translation:

The coupled transcription and translation system used was that described by Zubay (1973), as modified by Collins (1979), Pratt *et al.* (1981) and Pratt (1984). With 3 μg pKA5, 4μg pKA10 or 2.0 μg pBR322 as supercoiled plasmids (Figure 8.1), or with 5.0 μg pKA5 or 6.0 μg pKA10 as linear fragments (Figure: 8.1), the incorporation was linear for a period of up to 35 minutes (Figure: 8.1). Radioactively labelled proteins were analysed on 10% PAGE containing 0.1% SDS. In experiments where the S-30 extract was prepared from a *crp* deletion mutant LS854-1, cAMP receptor protein (0.5-1.0 μg) (gift from J. Krakow, Hunter College, NY.) was added to assess the effect of cAMP on transcription of *speA* and *speB*.

2.18 Sodium Iodide mediated immobilisation of nucleic acids:

The *speA*, *speB* and *lacZ* mRNA concentrations were determined by the method of Bresser *et al.* (1983 a;b) and Gillespie and Bresser (1983), as modified by Boyle *et al.* (1985). A ninety six well manifold (Bio-Rad) was used to support the nitrocellulose (mRN^{TC}) filter. Cell extracts were prepared separately for RNA and DNA immobilisation, were diluted serially, and

immobilised alongside each other on the same filter. The filters were processed according to Gillespie and Bresser (1983) and hybridised with the plasmid DNA probe (Section: 2.20). The hybridised probe DNA was removed following autoradiography by placing the filters in boiling 0.01xSSPE (0.0015 M NaCl; 0.00015 M sodium citrate; 1 mM sodium phosphate buffer, pH 7.0; and 1 mM EDTA) for 30 seconds (Gillespie and Bresser, 1983), and dried for storage. These filters could then be rehybridised with another plasmid DNA.

2.19 ^{32}P-Labelling of plasmid DNA:

Plasmid DNA was radioactively labelled by nick translation, using a kit purchased from Amersham Inc. The method was that given by the manufacturer. Unincorporated nucleotides were removed by column chromatography on Sephadex G-50 using 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. The fractions (0.2 ml), containing 10^8 DPMs per μg DNA were used for molecular hybridisation.

2.20 Molecular hybridisation:

Non-specific binding sites on the nitrocellulose filters were saturated with salmon sperm DNA and BSA at 45 °C for 24 hours in a sealed plastic bag as described by Riley and Anilionis (1980) and modified by Wright and Boyle (1984). The solution in the bag was replaced with one containing the radioactive probe DNA (Boyle *et al.*, 1985; Wright and Boyle, 1984). Hybridisation was carried out at 45 °C for 24 hours. The filters were prepared for

autoradiography (Wright and Boyle, 1984) and exposed to Kodak X-ray film, XRP-1 at -70 °C. The autoradiograms were scanned as described in Section: 2.26.

2.21 Ammonium sulphate fractionation:

Crude cell extract was diluted with the breakage buffer to a final protein concentration of approximately 5 mg/ml. A ninety percent saturated ammonium sulphate solution in 0.1 M HEPES-NaOH, pH 7.4 (% saturation at 25 °C) was added to the crude extract at a rate of 35 ml per hour with constant slow stirring to achieve 33% ammonium sulphate saturation. The mixture was allowed to stand for four hours at 4 °C. The precipitate formed was removed by centrifugation at 12,000xg for 20 minutes. Further addition of the 90% saturated solution of ammonium sulphate to the supernatant to give 48% saturation, followed by standing at 4 °C for 4 hours, gave a further precipitate which was collected by centrifugation at 12,000 xg for 20 minutes. This sediment was resupended in 48% ammonium sulphate solution in 0.1 M HEPES, pH 7.4, and recentrifuged at 12,000xg for 20 minutes.

The precipitate collected between 33 and 48% saturation was resuspended in 100 ml of 40% saturated ammonium sulphate solution in 0.1 M HEPES, pH 7.4 containing 1 mM agmatine sulphate, and was stirred overnight at 4 °C. The precipitate was again separated from the supernatant by centrifugation at 12,000 xg for 20 minutes at 4 °C. The sediment was resuspended in 0.1 M

Tris-HCl, pH 7.5, and transferred to a dialysis sac. The contents were dialysed for 16 hours against two four liter changes of 0.1 M Tris-HCl, pH 7.5, containing 1 mM agmatine sulphate. The contents of the dialysing bag were centrifuged at 12,000xg for 20 minutes at 4 °C to remove insoluble material. (Table: 3.3).

2.22 Heat treatment and precipitation with 1M Tris-HCl:

The dialysed material obtained by ammonium sulphate fractionation (2.21) was heated at 65 °C for 10 minutes. The precipitates were removed by centrifugation at 12,000xg for 20 minutes at 4 °C (Figure: 3.13).

The supernatant was mixed with an equal volume of 2M Tris-HCl, pH 7.5. The solution was allowed to stand at 4 °C for 1-2 hours, and the precipitate was removed by centrifugation at 12,000xg for 20 minutes at 4 °C. This step was necessary to rid the solution of proteins which otherwise precipitated in the column during subsequent DEAE-Sephacel column chromatography (Table: 3.2).

2.23 Column chromatography:

2.23.1 Ion exchange chromatography:

Ion exchange chromatography was performed at 4 °C in columns (2.6 cm x 40.0 cm) packed with 130 ml. (packed volume) of DEAE Sephacel. The column was equilibrated with 10 mM Tris-HCl, pH 7.5, and the sample after

Tris-HCl precipitation, following dialysis for 16 hours against two four liter changes of 10 mM Tris-HCl, pH 7.5, and was then applied to the column. This was washed with 150 ml of Tris-HCl (10 mM, pH 7.5), followed by 130 ml of 200 mM Tris-HCl, pH 7.5, at a flow rate of 0.8 ml per minute. The proteins were then eluted with a linear gradient of Tris-HCl, pH 7.5. The concentration of Tris-HCl changed from 200 mM to 600 mM in a volume of 500 ml. Fractions (5 ml) were collected in tubes containing 0.1 ml of 60 mM agmatine sulphate. The elution was monitored with a UV monitor (LKB), and was recorded with a strip chart recorder (LKB), in the absorbance mode.

2.23.2 Molecular sieving

2.23.2.1 Molecular sieving using Sephadex G-100:

Molecular sieving was carried out in columns (2.6 cm x 70 cm) packed with Sephadex G-100 (packed volume of 210 ml). The column was equilibrated with 100 mM Tris-HCl, pH 7.5, containing 1 mM agmatine sulphate. The sample prior to loading was concentrated and equilibrated by collodion bag filtration (cut off limit of 25 Kdaltons) (Schleicher and Schuell), with 100 mM Tris-HCl, pH 7.5 containing 1 mM agmatine sulphate. The sample volume was approximately 1% of the column bed volume. The sample was applied and eluted at 4 °C using flow adaptors and a peristaltic pump at a rate of 25 ml per hour. Fractions of 5 ml were collected, and the elution was monitored using an UV-monitor with a strip chart recorder in an absorbance mode. The

column was calibrated prior to use and after the sample run, using blue dextran (Pharmacia Fine Chemicals) to determine the void volume and proteins of known molecular weight (Pharmacia Fine Chemicals).

2.23.2.2 Molecular sieving using Sephacryl S-200:

Molecular sieving using Sephacryl S-200 was carried out in a column (2.6 cm x 70 cm) (packed volume of 250 ml) equilibrated in 200 mM Tris-HCl, pH 7.5 containing 1 mM agmatine. Samples of purified agmatine ureohydrolase (from chromatofocusing, Section: 3.2.7, stage-8) were dialysed against 200 mM Tris-HCl, pH 7.5 containing 1 mM agmatine, and then concentrated to about 1% of the gel bed volume by collodion bag filtration. The column was eluted with 200 mM Tris-HCl, pH 7.5, containing 1 mM agmatine sulphate at a flow rate of 40 ml per hour, and fractions were collected (2.5 ml). The elution was monitored as described for Sephadex G-100 (2.23.2.1). The column was calibrated prior to use and after the sample run, using blue dextran and standard proteins of known molecular weight (Pharmacia Fine Chemicals).

2.23.3 Chromatofocusing:

Chromatofocusing was carried out in a column (1.0 cm x 40 cm) packed with 20.0 ml of Poly Buffer Exchanger (PBE) (Pharmacia Fine Chemicals), and was equilibrated with either 0.025 M ethanolamine-HCl, pH 9.4 or 0.025 M imidazole-HCl, pH 7.4. The samples were equilibrated with the loading buffer (ethanolamine-HCl, pH 9.4 or imidazole-HCl, pH 7.4) prior to loading and

washed with 100 ml of the same buffer. The proteins were eluted with 300 ml of either polybuffer 96, pH 7.0 or polybuffer 74, pH-4.0 (Pharmacia Fine Chemicals), and 2.5 ml fractions were collected. The elution was monitored using an UV monitor and a strip chart recorder. The pH of each of the fractions was determined, and 2.5 M Tris-HCl, pH 7.5 and 100 mM agmatine sulphate were added to the fractions to a final concentration of 100 mM and 1 mM respectively.

2.24 Antiserum production and Ouchterlony Analysis:

Antibodies to purified agmatine ureohydrolase were prepared in New Zealand white male rabbits, and purified by ammonium sulphate precipitation according to the method of Clausen (1969). Ouchterlony analyses were performed as described by Clausen (1969) in 1% agarose, 0.05 M Tris-HCl, (pH 7.5), 0.1 M NaCl and 0.01% sodium azide.

2.25 Estimation of agmatine ureohydrolase enzymic activity and radioactivity from non-denaturing PAGE:

The 12 cm, 5% acrylamide gels were sliced into 1mm sections, each section was soaked overnight at 4 °C in breakage buffer containing 1 mM agmatine, and the extracts were assayed for enzymic activity. Radioactivity in the radiolabelled proteins in gel slices was determined by the alkaline digestion method as described by Long (1976). The gel slices were soaked in protosol overnight in scintillation vials, neutralised with acetic acid and the

radioactivity measured.

2.26 Densitometry:

Autoradiograms were traced on a Corning scanning densitometer (Model 750) using the log inverse mode. The peaks were cut out and weighed. The peak weights determined from the same autoradiograph were taken as a measure of the relative radioactivity incorporated into different proteins.

2.27 Quantitative analysis of the ratio of mRNA to DNA:

The autoradiograms were scanned using a densitometer, and the peak weights for mRNA and DNA samples hybridised at each dilution was plotted to give corrected mRNA and DNA values (Gillespie and Bresser, 1983). The corrected value obtained for mRNA from a given number of cells was divided by the corrected value obtained for DNA from the same number of cells to give the relative mRNA level.

2.28 Spectrophotometry:

Spectrophotometric readings were made with a Beckman UV - Visibile spectrophotometer, model 24, using cuvettes with a 1 cm. path length.

Chapter 3

Purification and Characterisation of Agmatine Ureohydrolase

3.1 Introduction:

Agmatine ureohydrolase has been purified about 50 fold from *Proteus vulgaris* grown in the presence of agmatine. Some properties of this purified protein have been reviewed by Pegg and Williams-Ashman (1979). Although ornithine decarboxylase (Applebaum *et al.* , 1977) and arginine decarboxylase (Wu and Morris 1973a; 1973b) have been purified from *E.coli* and studied extensively, almost nothing is known about agmatine ureohydrolase.

In this chapter the purification of agmatine ureohydrolase from a strain of *E.coli* carrying the *speB* gene on a plasmid is reported, together with some properties of the enzyme. The *speB* gene encodes the biosynthetic form of agmatine ureohydrolase. The host *E.coli* HT328 (Δ*speA*, Δ*speB*, Δ*speC*) did not produce the biodegradative enzyme under any conditions of growth. *E.coli* bearing a gene cloned on a high copy number plasmid vector had an activity about ten fold higher than that observed in strains carrying *speB* on the chromosome only.

The purification procedures included ammonium sulphate precipitation, heat treatment, DEAE Sephacel column chromatography, G-100 gel exclusion column chromatography and chromatofocusing. The molecular mass of the protein was determined by Sephacryl S-200 column chromatography and by SDS polyacrylamide gel electrophoresis.

3.2 Results:

3.2.1 Assay of agmatine ureohydrolase activity and the determination of optimum conditions for activity measurements:

The stability of agmatine ureohydrolase and urea at 100°C was determined by incubating aliquots of the enzyme or known quantities of urea, and estimating enzymic activity or urea concentration at various time intervals. The enzymic activity was measured by the colorimetric assay described in Section 2.3.2. The results are shown in Figure: 3.1. Approximately 90% of the enzymic activity was lost following 4 minutes of incubation at 100° C, while urea hydrolysis was minimal for up to 7 minutes at same temperature. Therefore, the enzyme was inactivated in the reaction mixtures routinely by placing the vessels at 100° C for five minutes and the urea concentration was determined in the supernatant according to the method described in Section 2.3.2.

To determine if the enzymic activity was linear during the period of incubation, the reaction mixtures (Section 2.3.3.2) were incubated up to 2 hours at 37° C. Duplicate reaction vessels were removed at various time intervals, the reactions were stopped and the release of [^{14}C] CO_2 was determined (Section 2.3.3.2). The results are shown in Figure 3.2. The enzymic activity was linear up to 60 minutes and therefore the enzyme assays were routinely carried out for 30 minutes. The enzymic activity was linear for a period of 90 minutes, when the colorimetric assay system was used (data not shown), and the reac-

Figure 3.1

Stability of agmatine ureohydrolase and urea at 100 °C.

○ = Urea, ● is the enzymic activity of agmatine ureohydrolase, determined according to the colorimetric method described in Section: 2.3.2. Source of the enzyme was a partially purified preparation from stage: 7 (Table: 3.2).

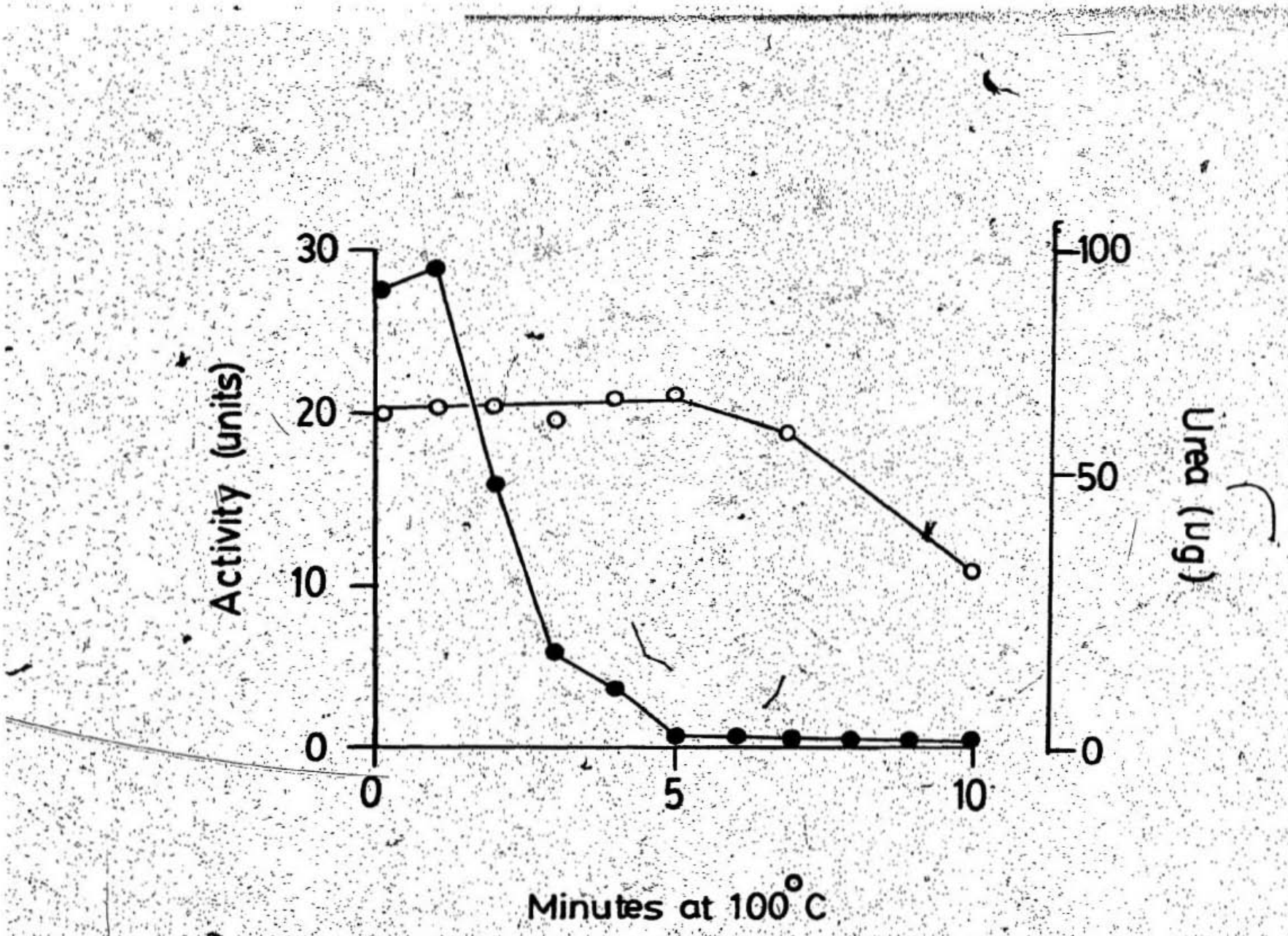
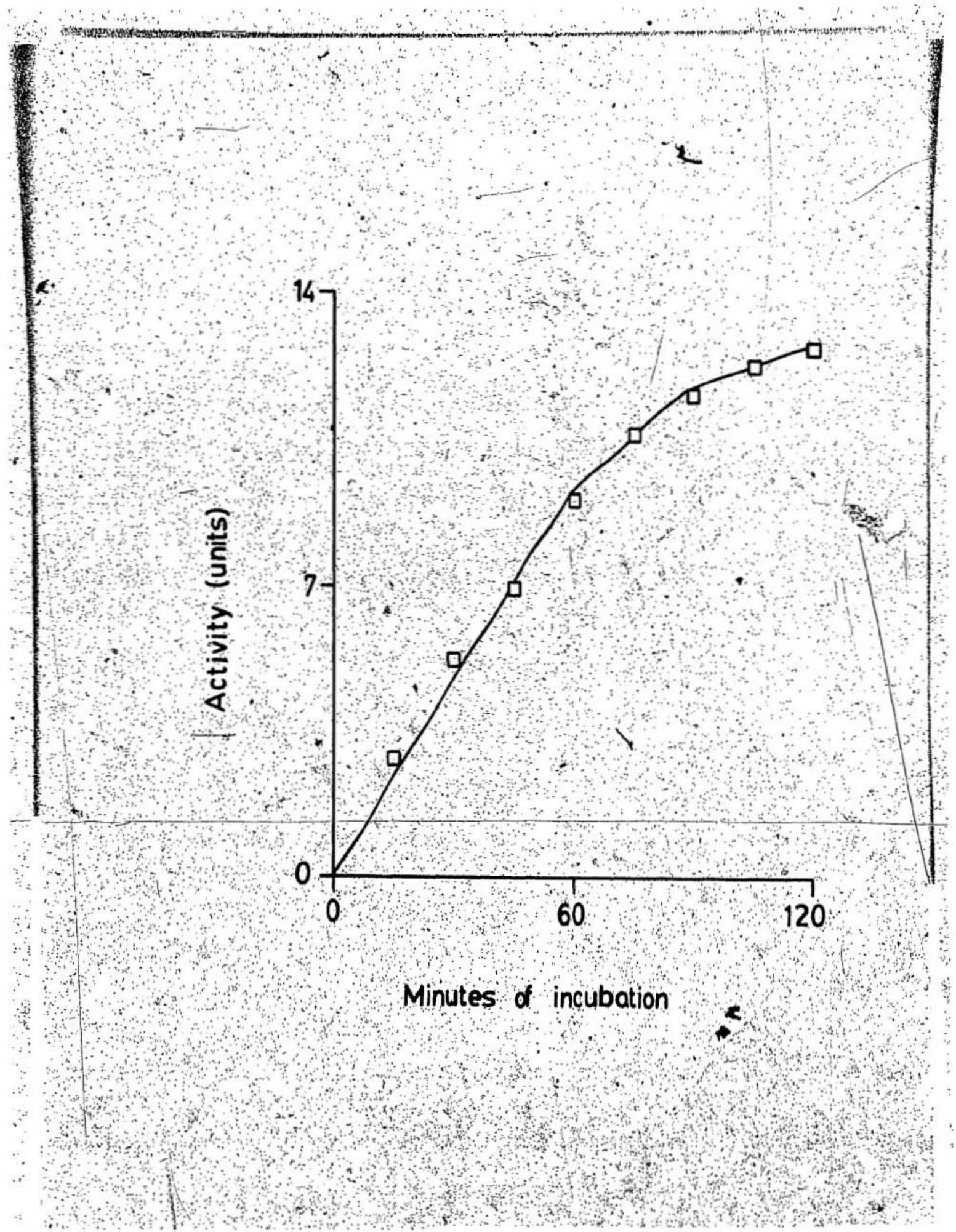

30
20
10
0
Activity (units)
0
5
10
Minutes at 100°C
100
50
0
Urea (µg)

Figure 3.2

The activity of agmatine ureohydrolase as a function of time of incubation.

The enzyme activity was determined according to the method described in Section: 2.3.3.2. The source of the enzyme was a stage: 7 preparation (Table: 3.2).


14
7
0
Activity (units)
0
60
120
Minutes of incubation

tions were routinely performed by incubating the reaction vessels at 37 °C for 30 minutes.

In order to determine if the activity of agmatine ureohydrolase was linear over a range of protein concentrations of purified enzyme and crude extracts using the radioactive assay (Section: 2.3.3.2) or using the colorimetric assay (Section: 2.3.2), the reaction mixtures were fortified with increasing concentrations of protein from crude extracts of purified enzyme preparation (stage-8, Table: 3.2). The enzymic activities were determined and are shown in Figure: 3.3. The enzymic activity was linear over the concentrations of protein tested in the reaction mixtures. 100-200μg of crude extract or 1-2 μg of purified enzyme were routinely used in the assay mixture.

To determine the optimum pH for the activity of enzyme, the purified preparation and the crude extract (stage 8 and stage 1, respectively) were used in the reaction mixtures. The reaction mixtures of desired pH were prepared by substituting the set of buffers shown in Figure: 3.4 legend for the HEPES-NaOH in the standard reaction mixture. The reactions were stopped by placing the vessels at 100 °C for five minutes; 2 M Tris-HCl (pH 7.5) to a final concentration of 0.3 M and urease were added, and the concentration of urea in the reaction mixture was determined according to the method described in Section: 2.3.3. The enzymic preparations (crude extract or the purified enzyme) were equilibrated against 0.025 M HEPES-NaOH (pH 7.5), MOPS-KOH (pH 7.2), K_2HPO_4 - KH_2PO_4 (pH 7.0), Tricine-NaOH (pH 8.2), Tris-HCl (pH 8.0) or

Figure 3.3

The effect of increasing concentrations of protein on the activity of agmatine ureohydrolase.

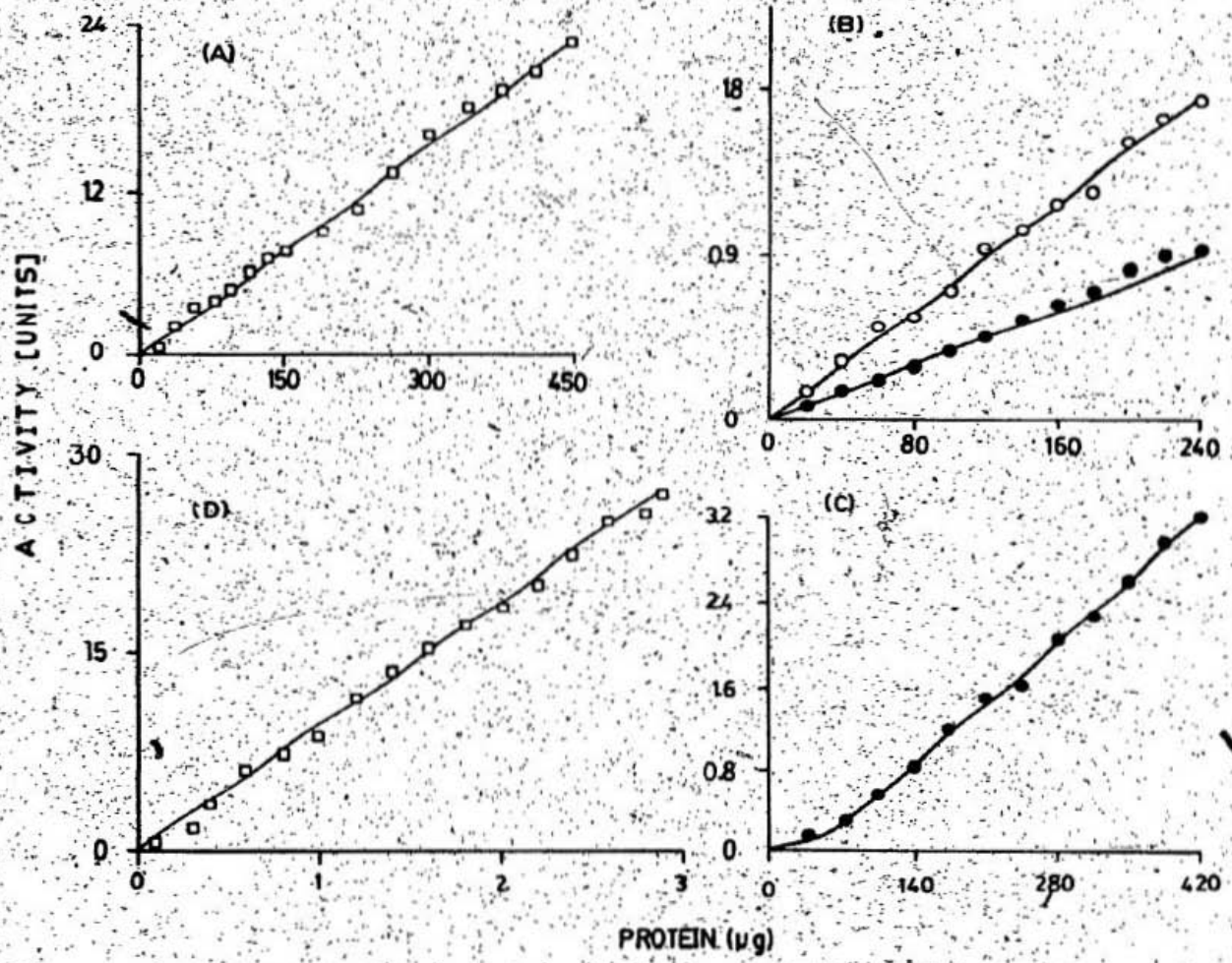
(A) = Crude extract of LS853 grown in MOPS minimal medium.

(B) = Crude extracts of LS340 grown in MOPS minimal medium with (○) and without (●) agmatine.

(C) = Crude extract of LS853 grown in LB medium.

(D) = Purified agmatine ureohydrolase (stage: 8, Table: 3.2).

Enzymic activities in **(A)** and **(B)** were determined according to the colorimetric method described in Section: 2.3.2, and those in **(C)** and **(D)** were according to the radiochemicl method described in section 2.3.3.2.


(A)
(B)
(C)
(D)
ACTIVITY [UNITS]
PROTEIN (µg)
24
12
0
150
300
450
18
0.9
80
160
240
30
15
1
2
3
32
16
0.8
140
280
420

MES-NaOH (pH 6.2) prior to use in the appropriate reaction mixtures. The results are shown in Figure: 3.4. Maximum activity was obtained between pH 7.2 and 7.5 when HEPES-NaOH or Tris-HCl was used. Therefore, reactions were routinely performed in HEPES-NaOH at pH 7.5.

To determine the specificity and the reliability of the radioactive assay (Section: 2.3.3.2), the assay was performed as described in Section: 2.3.3.2. In replicate reaction mixtures one of the enzymes (arginine decarboxylase, agmatine ureohydrolase or urease) was omitted. In the -ADC system, arginine decarboxylase was omitted from the mixture in the preparation of L-[guanido-^{14}C] agmatine; the crude extract (stage 1 Table: 3.2) and urease were added and [^{14}C] CO_2 released form L-[guanido-^{14}C] arginine was measured. In the -urease system, urease was omitted and in the -enzyme system, the partially purified enzyme (stage-5; Table: 3.2) was omitted. The results are shown in Table: 3.1. The release of [^{14}C] CO_2 was negligible in the reaction mixtures where any of the enzymes was omitted. The results are consistent with the predicted reaction arginine ----> agmatine -----> urea ----> CO_2

3.2.2 Preparation of crude extract containing agmatine ureohydrolase:

E.coli (HT328) containing plasmid pKA5 was grown in LB as described in Section 2.2, but with the addition of 2 mM agmatine to the medium. Cells were grown in batches of 500 ml. in 2 liter flasks, and the harvested cells

Figure 3.4

The effect of pH on the enzymic activity of agmatine ureohydrolase.

(✕) = HEPES-NaOH
(⊙) = Tris-HCl
(▲) = MOPS-KOH
(○) = MES
(●) = Potassium phosphate
(△) = Tricine

The enzymic activities were determined in reaction mixtures adjusted to appropriate pH, according to the method described in Section: 2.3.2. The source of the enzyme was **(A)**- crude extract or **(B)**- Purified preparation (stage: 8, Table: 3.2).

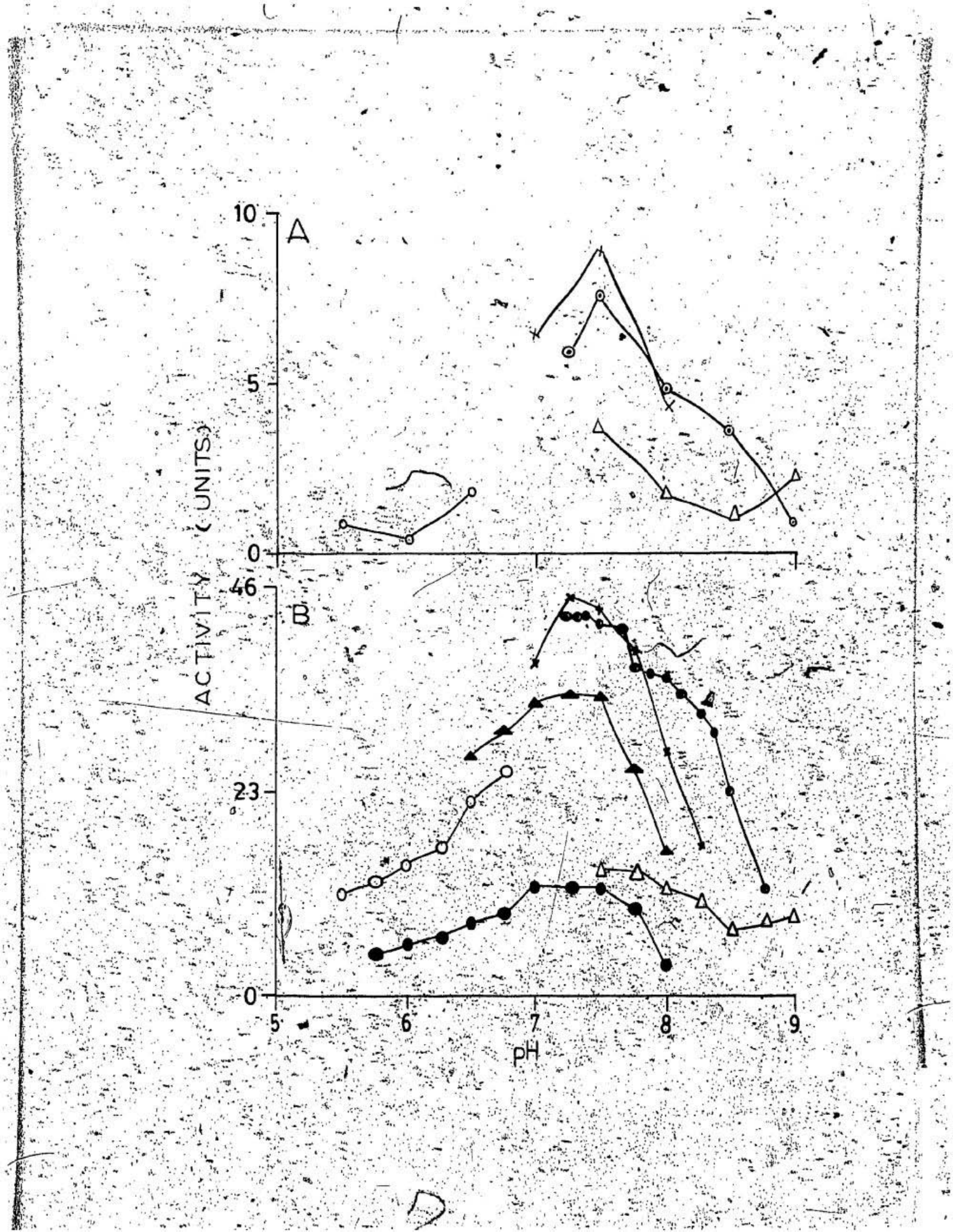

A
B
10
5
0
46
23
0
ACTIVITY (UNITS)
5
6
7
8
9
pH

Table 3.1

The determination of agmatine ureohydrolase activity.

Assay condition	% activity of agmatine ureohydrolase
Control(ADC+urease+enzyme)	100.00
-ADC	0.08
-Urease	0.03
-enzyme	1.26

Control incubation is as described in Section 2.3.3.2. 100% represents 180 units/mg protein. ADC (arginine decarboxylase), urease and enzyme were separately omitted for each of the reaction mixtures. Source of enzyme was a partially purified one from stage-5, Table 3.2.

packed by centrifugation (Section: 2.1) were often stored at -20° C for 3-4 weeks. The enzymic activity and the protein concentration of extracts (prepared as described in Section: 2.2) were determined as described in Sections: 2.3.3.2 and 2.3.6 respectively, and the results are given in Table 3.2 (stage-1).

3.2.3 Ammonium sulphate precipitation of agmatine ureohydrolase activity:

Ammonium sulphate precipitation was carried out as described in Section 2.21. The enzymic activity and protein concentration were determined, after dialysis, as described in Sections: 2.3.3.2 and 2.3.6, respectively. The fraction precipitating between 33 and 48% saturation with ammonium sulphate contained 74% of the activity of the crude extract. The specific activity was not significantly increased (Table: 3.2, stage-2; Table: 3.3). The material from stage-2 was resuspended in 40% saturated ammonium sulphate solution as described in Section: 2.21. The insoluble material was recovered by centrifugation, resuspended in 0.1 M Tris-HCl, pH 7.5 containing 1 mM agmatine, and was dialysed. Following centrifugation to remove insoluble material the enzymic activity and protein concentration were determined as described in Sections 2.3.3.2 and 2.3.6, respectively. This fraction contained 73% of the activity of the crude extract and was purified 3 fold. (Table: 3.2, stage-3; Table: 3.3).

Table: 3.2

Steps in the Purification of agmatine ureohydrolase

Stage	Step	Total Units	Total Protein (mg.)	Specific Activity	Purification	Yield %
1.	Crude Extract	11,130	1,085	10.3	1	100
2.	Ammonium Sulph. (33-48%)	8,230	307	26.8	2.6	74
3.	Ammonium Sulph. (33-40%)	8,070	270	30	3	73
4.	Heat Treatment	7,670	26	290	28	69
5.	1M Tris. Adjustment	7,240	15	512	50	65
6.	DEAE-Sephacel Chromatography	22,100	3.1	7,200	700	200
7.	Sephadex G-100 Chromatography	27,900	2.1	13,000	1,270	250
8.	Chromatofocusing (Polybuffer96/PBE)	23,600	0.7	34,700	3,390	220

Units of activity are expressed as μmoles of urea formed per minute. The enzymic activities were measured according to the method described in Section: 2.3.3.2.

Table: 3.3.

Activity of agmatine ureohydrolase in ammonium sulphate fractions

	Total Units	Total Protein (mg.)	Spe. Act.	Yield(%)	Purification
Crude Extract	896.53	80.72	11.11	100	1
0-33%$(NH_4)_2 SO_4$	13.10	33.4	0.39	1.5	0.04
33-48%$(NH_4)_2 SO_4$	716.20	27.92	25.65	80	2.3
33-40%$(NH_4)_2 SO_4$	669.40	21.46	31.19	75	2.8

The activity in the precipitates was determined following dialysis against 0.1M Tris-HCl, containing 1mM agmatine (Section: 2.21). The enzymic activity was determined according to the method described in Section: 2.3.3.2. Spe. Act. = Specific activity.

3.2.4 Heat treatment and Tris-HCl treatment:

The material from stage-3 was heat treated as described in Section 2.22 and the enzymic activity and protein concentration were determined (stage-4, Table: 3.2). The supernatant contained 69% of the activity of the crude extract and was purified 28 fold.

The supernatant of the heat treated fraction (stage-4) was adjusted to 1 M Tris-HCl, pH 7.5, in order to remove proteins precipitable by high salt concentrations during DEAE Sephacel column chromatography. After dialysis to remove excess Tris-HCl enzymic activity and protein concentrations were determined. The recovery of activity was 65% and there was a 50 fold purification (Table: 3.2, stage-5).

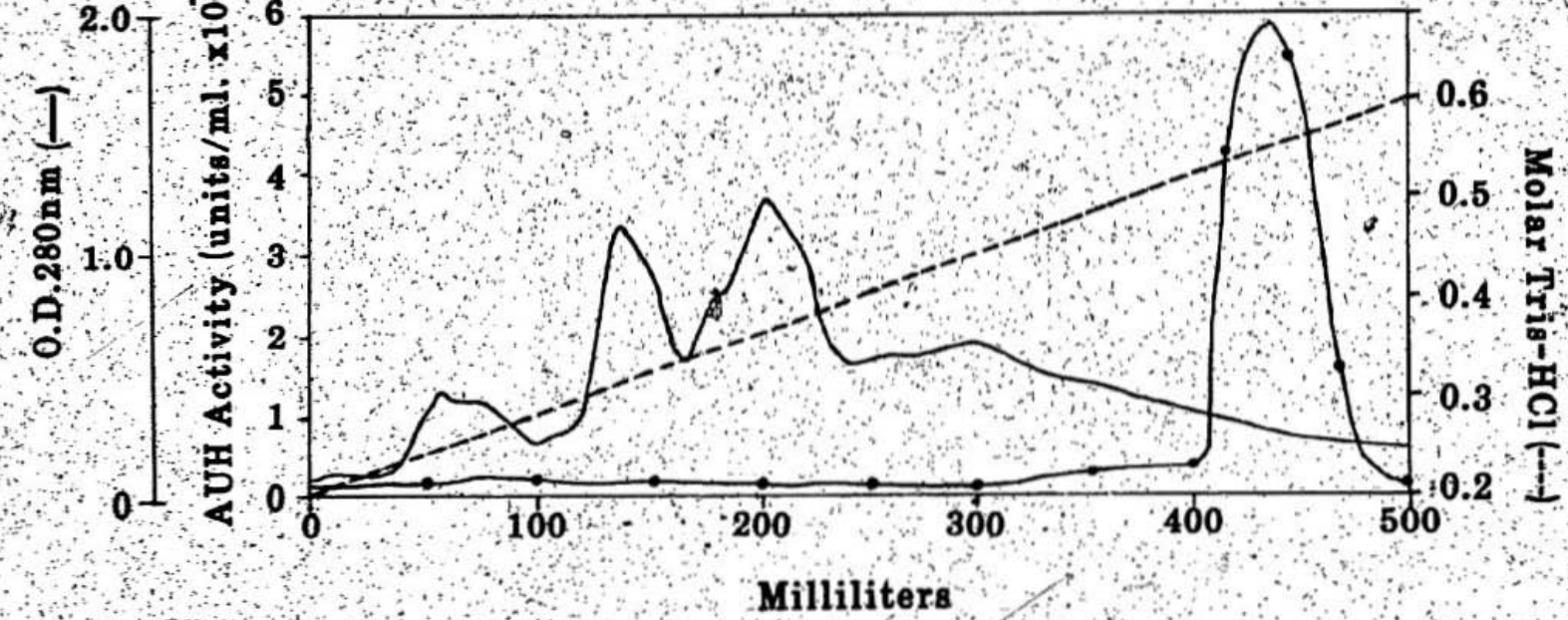
3.2.5 Ion exchange column chromatography:

The dialysed material (stage-5) was loaded on to the DEAE Sephacel column and eluted as described in the Section 2.23.1. Agmatine ureohydrolase activity was detected in the elution volume of 420-470 ml (Figure: 3.5). The fractions containing maximum activity were pooled, concentrated and equilibrated by collodion bag filtration as described in Section: 2.23.1, and the enzymic activity and protein concentration were determined, as described in Sections: 2.3.3.2 and 2.3.6 respectively. The recovery of activity was 200%, and the preparation was purified 700 fold (Table: 3.2, stage-6). The large increase in activity compared to stage-1 suggests the elimination of an

Figure 3.5

Elution profile of agmatine ureohydrolase during DEAE Sephacel column chromatography.

The stage: 5 material was applied over the DEAE Sephacel column (stage: 6), the figure shows the enzymic activity (●—●) determined according to the method described in Section: 2.3.5, the protein profile (——) by a UV flow through cell, and the linear gradient of Tris-HCl (pH 7.5) (- - - -).


O.D.280nm (—)
AUH Activity (units/ml. x10^{-3})(•—•)
Molar Tris-HCl (---)
Milliliters
2.0
1.0
0
6
5
4
3
2
1
0
0.6
0.5
0.4
0.3
0.2
0
100
200
300
400
500

inhibitor.

3.2.6 Gel exclusion column chromatography:

The fractions in the eluate volume between 421 and 470 ml from the DEAE Sephacel column were combined and concentrated. They contained about 88% of the activity eluted from the column. This material was applied to the Sephadex G-100 column, and the proteins were eluted as described in Section: 2.23.2.1. Activity was detected by the method described in Section: 2.3.5. The results are presented in Figure: 3.6. The fractions with maximum activity (eluate volume: 50 - 85 ml) were pooled and concentrated by collodion bag filtration using 0.025 M ethanolamine-HCl (pH 9.4), and the enzymic activity and protein concentrations were determined as described in Sections: 2.3.3.2 and 2.3.6, respectively. The agmatine ureohydrolase activity eluted in a volume corresponding to a molecular weight of 85,000 - 90,000. The recovery was 250% and the preparation was purified 1270 fold (Table: 3.2, stage-7).

3.2.7 Chromatofocusing:

The material from stage-7 was applied to a column of Poly Buffer Exchanger and eluted as described in Section: 2.23.3. Fractions containing agmatine ureohydrolase activity were identified by the method described in Section: 2.3.5. (Figure: 3.7c). Fractions with volumes 120 - 135 ml were combined and concentrated by collodion bag filtration, and the enzymic activity and protein concentrations were determined. The final recovery was 220% of

Figure 3.6

Elution profile of agmatine ureohydrolase during gel exclusion chromatography on Sephadex G-100.

Stage: 6 material was applied over Sephadex G-100 column (stage: 7), the figure shows protein profile (——) as determined by an UV flow through cell, and the enzymic activity (●—●) determined according to the method given in Section: 2.3.5.

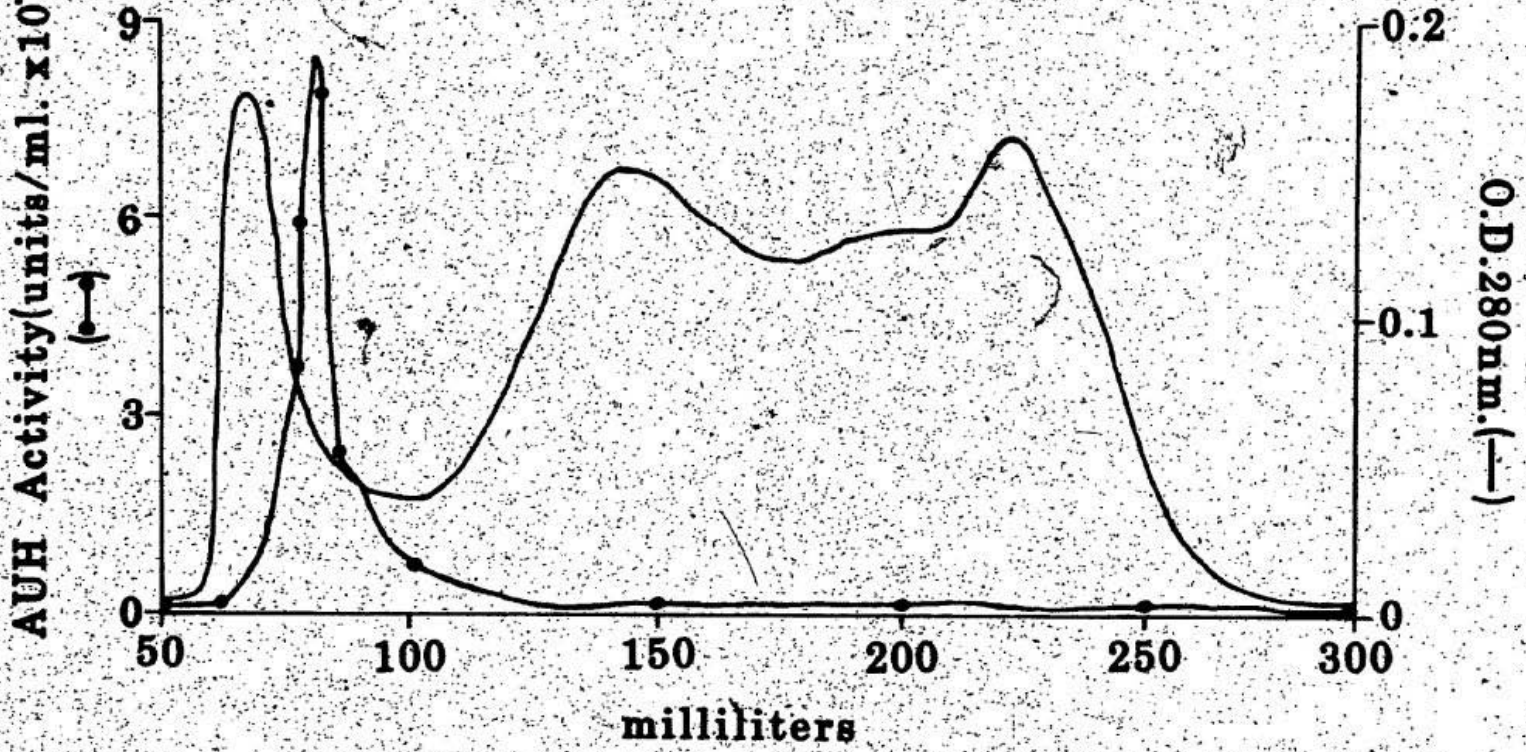
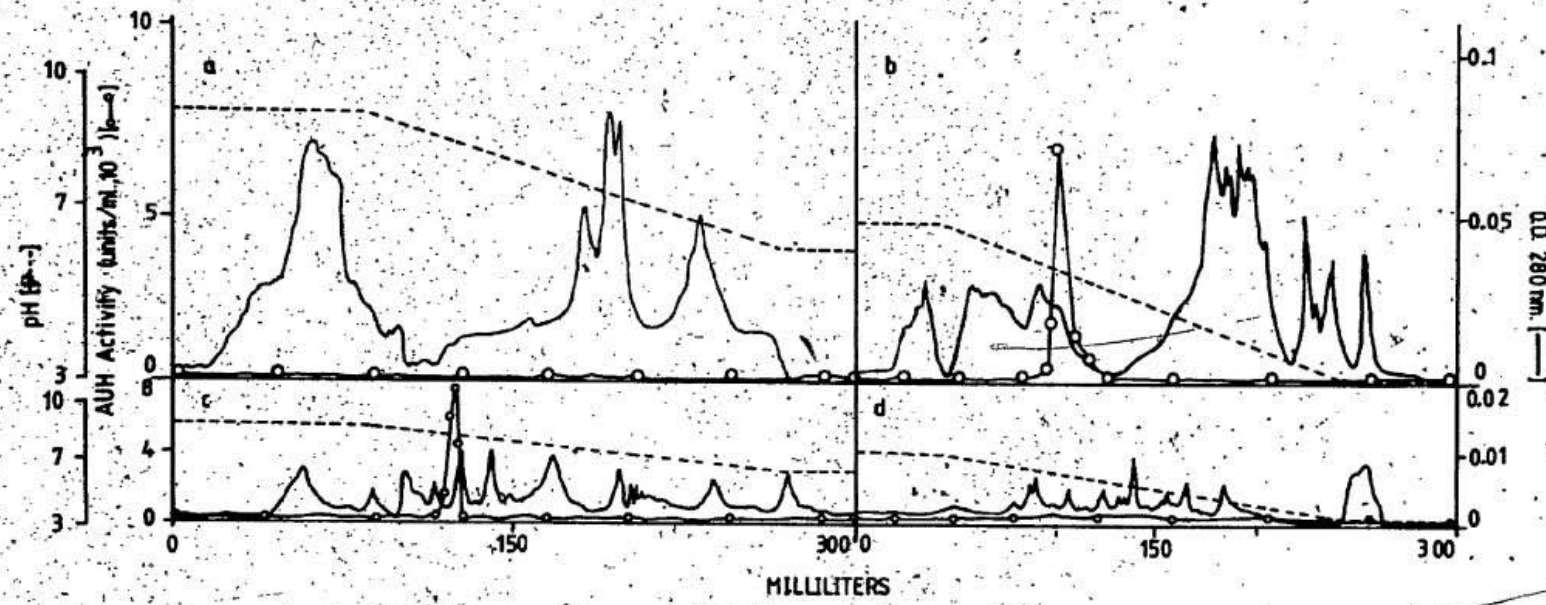

AUH Activity(units/ml. x10⁻³)
9
6
3
0
0.2
0.1
0
O.D.280nm.(—)
50
100
150
200
250
300
milliliters

Figure 3.7

Elution profile of agmatine ureohydrolase during column chromatography on Polybuffer Exchanger.

The figure shows the protein profile (——) as determined by a flow through UV cell, the pH gradient (----) as determined from each fraction, and the enzymic activity (o—o) determined according to the method described in Section: 2.3.5. The samples applied and eluted were: **(a)**- stage: 5 material with poly buffer 96 (Section: 3.2.8), **(b)**- stage: 5 material with polybuffer 74 (Section: 3.2.8), **(c)**- stage: 7 material with polybuffer 96 (described in Section: 3.2.7, stage: 8, Table: 3.2) and **(d)**- stage: 7 material with polybuffer 74 (Section: 3.2.8).


a
b
c
d
pH (—•—)
AUH Activity (units/ml.x10^{-3}) (o—o)
O.D. 280 nm. (——)
MILLILITERS
0
150
300

that in stage-1, and the purification was 3390 fold (Table: 3.2, stage-8).

3.2.8 The activation of agmatine ureohydrolase during purification:

In a search for an abbreviated method of purification, it was noted that the chromatography of material from stage-5 (Table: 3.8) gave rise to agmatine ureohydrolase with a pI of 5.5. The stage 5 fraction was equilibrated with 0.025 M ethanolamine-HCl (pH 9.4) and applied to the Poly, Buffer Exchanger column, and eluted with Polybuffer 96 (pH 7.0), as described in Section: 2.23.3. No activity was eluted (Figure 3.7a). The column was therefore equilibrated with 0.025 M imidazole-HCl (pH 7.4), and then eluted with Polybuffer 74 (pH 4.0). Agmatine ureohydrolase was eluted in fractions with eluate volumes between 95 and 110, and had a pI of 5.5 (Figure: 3.7b). When the complete purification method had been used (Table: 3.2; Figure: 3.7c), and the column from the final chromatofocusing (stage-8) was re-equilibrated and eluted with polybuffer 74 (pH 4.0), there was no further elution of agmatine ureohydrolase (Figure: 3.7d), indicating that there were not two forms of the enzyme in the material obtained at stage-7.

3.2.9 The molecular weight of agmatine ureohydrolase by chromatography on Sephacryl S-200:

A sample was applied to a Sephacryl S-200 column to determine the molecular weight of the purified preparation of agmatine ureohydrolase obtained from chromatofocusing (stage 8, Table: 3.2). The loading, elution

and fraction collection are described in 2.23.2.2. The fractions were assayed for enzymic activity as described in Section: 2.3.3.2. The results are presented in Figure: 3.8. The elution volumes of standard proteins are plotted against the logarithms of their molecular weight, and are compared to that of agmatine ureohydrolase in Figure: 3.9. Agmatine ureohydrolase activity was associated with a fraction with a molecular weight of 75-80,000, but material with a lower molecular weight was also present.

3.2.10 Electrophoresis on non-denaturing polyacrylamide gels:

The native purified enzyme obtained at stage-8 (Table: 3.2) was examined by electrophoresis in polyacrylamide gels. It was also of interest to determine which of the proteins labelled with [^{35}S]-methionine in minicell preparations carrying plasmid pKA5 was in fact agmatine ureohydrolase. The purified enzyme was electrophoresed in duplicate non-denaturing polyacrylamide gels. In addition, a mixture of the purified agmatine ureohydrolase and ^{35}S-labelled proteins from minicells was also electrophoresed on duplicate non-denaturing polyacrylamide gels (Section: 2.14). One of the tube gels with the purified preparation, after electrophoresis, was stained with Coomassie Blue and the other was sliced into 0.5mm slices. After soaking each slice in breakage buffer, the enzymic activity was determined in the extract (Section 2.25). The gels of the mixture of purified agmatine ureohydrolase and ^{35}S-labelled proteins were also sliced. The slices of one were used for determining radioactivity (Section

Figure 3.8

Elution profile of the purified preparation of agmatine ureohydrolase gel exclusion chromatography on Sephacryl S-200.

Stage 8 material (Table 3.2) was to applied and eluted from a Sephacryl S-200 column. The figure shows the protein profile (——) obtained from an UV flow through cell and the enzymic activity (●—●) determined according to the method described in Section 2.3.3.2.

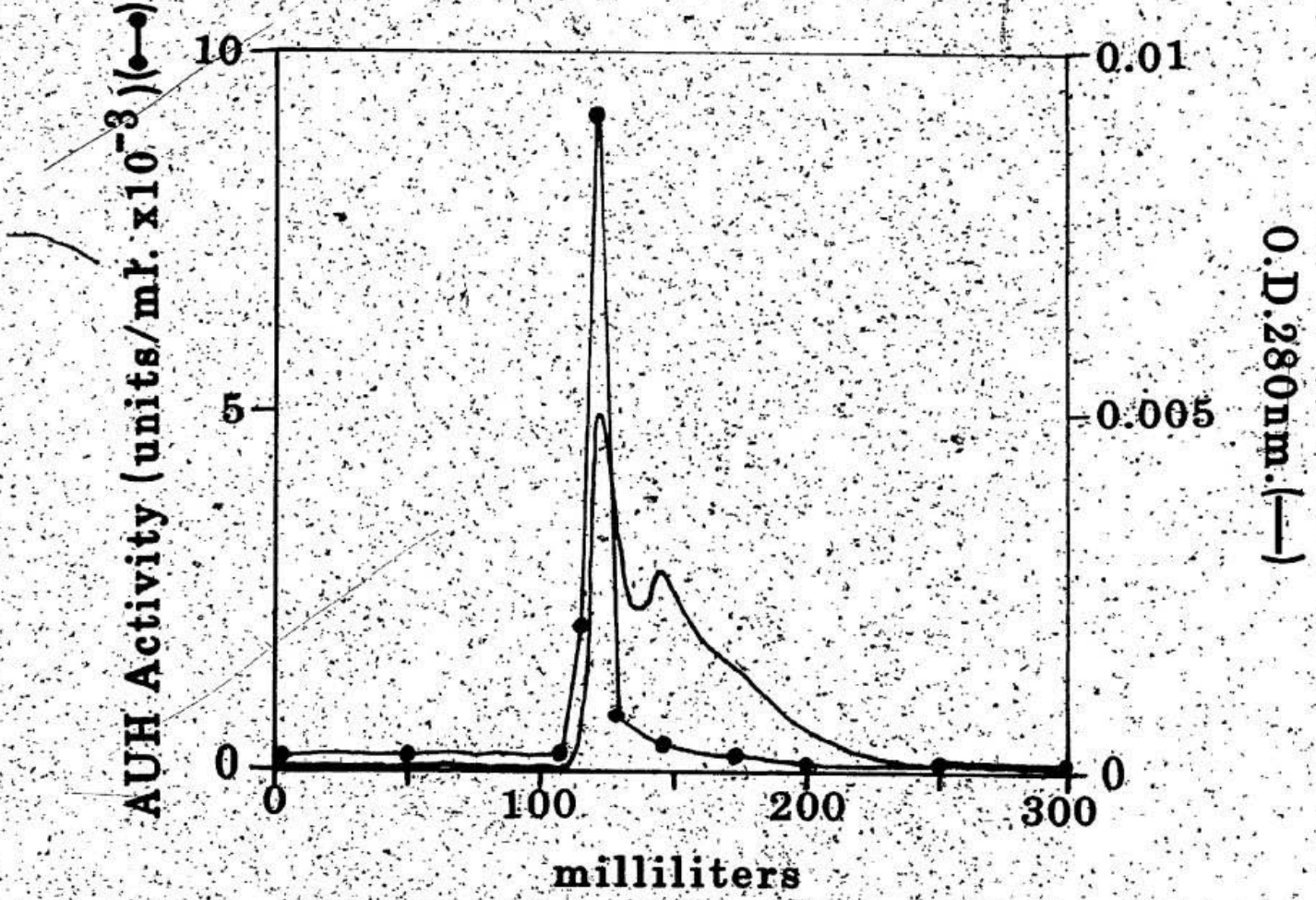
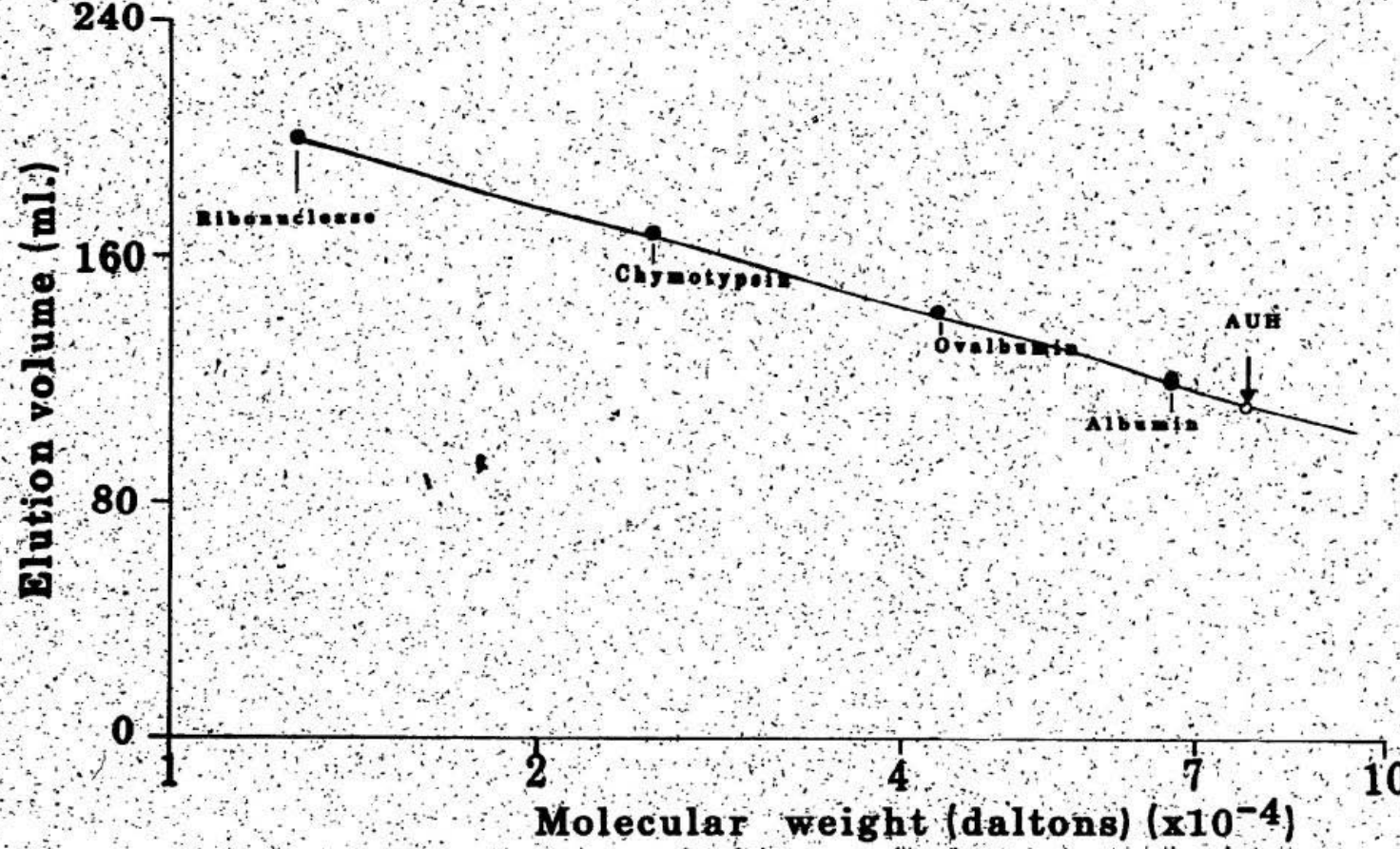

AUH Activity (units/ml. x10^{-3})(—●—)
10
5
0
0
100
200
300
milliliters
0.01
0.005
0
O.D.280nm.(——)

Figure 3.9

Molecular weight determination of agmatine ureohydrolase on Sephacryl S-200.

Standard proteins of known molecular weights were used to calibrate the column and to assess the molecular weight of agmatine ureohydrolase. Molecular weight of the fraction that eluted with the protein peak in Figure 3.8 which had the enzyme activity is shown in the figure.


240
160
80
0
Elution volume (ml.)
Ribonuclease
Chymotrypsin
Ovalbumin
Albumin
AUH
1
2
4
7
10
Molecular weight (daltons) (x10^{-4})

2.25), and those of the other for determining enzymic activity. The enzymic activity and the major band of radioactivity corresponded to the major protein stained with Coomassie Blue. (Figure: 3.10).

3.2.11 Electrophoresis in SDS-polyacrylamide gels:

During the purification of agmatine ureohydrolase, samples were examined by SDS-gel electrophoresis (Section: 2.14). ^{35}S-labelled proteins from minicells carrying pKA5 were also examined by this method. Gels were stained with Coomassie Blue, and then prepared for fluorography (Section: 2.14). Radioactive and non-radioactive molecular weight markers of proteins were also electrophoresed in the same gel. During purification, there was an enrichment of a protein with a molecular weight of 38,000, and this was also observed by fluorography of proteins from the labelled minicells. (Figure: 3.11).

3.2.12 Ouchterlony analysis of the antibodies prepared against agmatine ureohydrolase:

Antibody preparation and Ouchterlony analysis of the antibody were carried out as described in Section 2.24. The fractions obtained from various steps of purification were analysed against the antibody. The Ouchterlony plates were incubated at 37° C overnight. Precipitin bands were observed, but could not be photographed. The plate was washed with 0.15 M NaCl for 16 hours, stained with Coomassie Blue, and the stained bands were photographed. Stage 5 material did not give a precipitin band, but the material from later stages

Figure 3.10a

The radioactivity and the enzyme activity in gel slices of non-denaturing PAGE of pKA5 encoded [^{35}S]-methionine labelled proteins in minicells and the purified enzyme.

The figure shows the activity of agmatine ureohydrolase activity (●—●) in gel slices determined by the method described in Section: 2.3.3.2 and the radioactivity (-----) associated with the gel slices according to the method described in Section: 2.25.

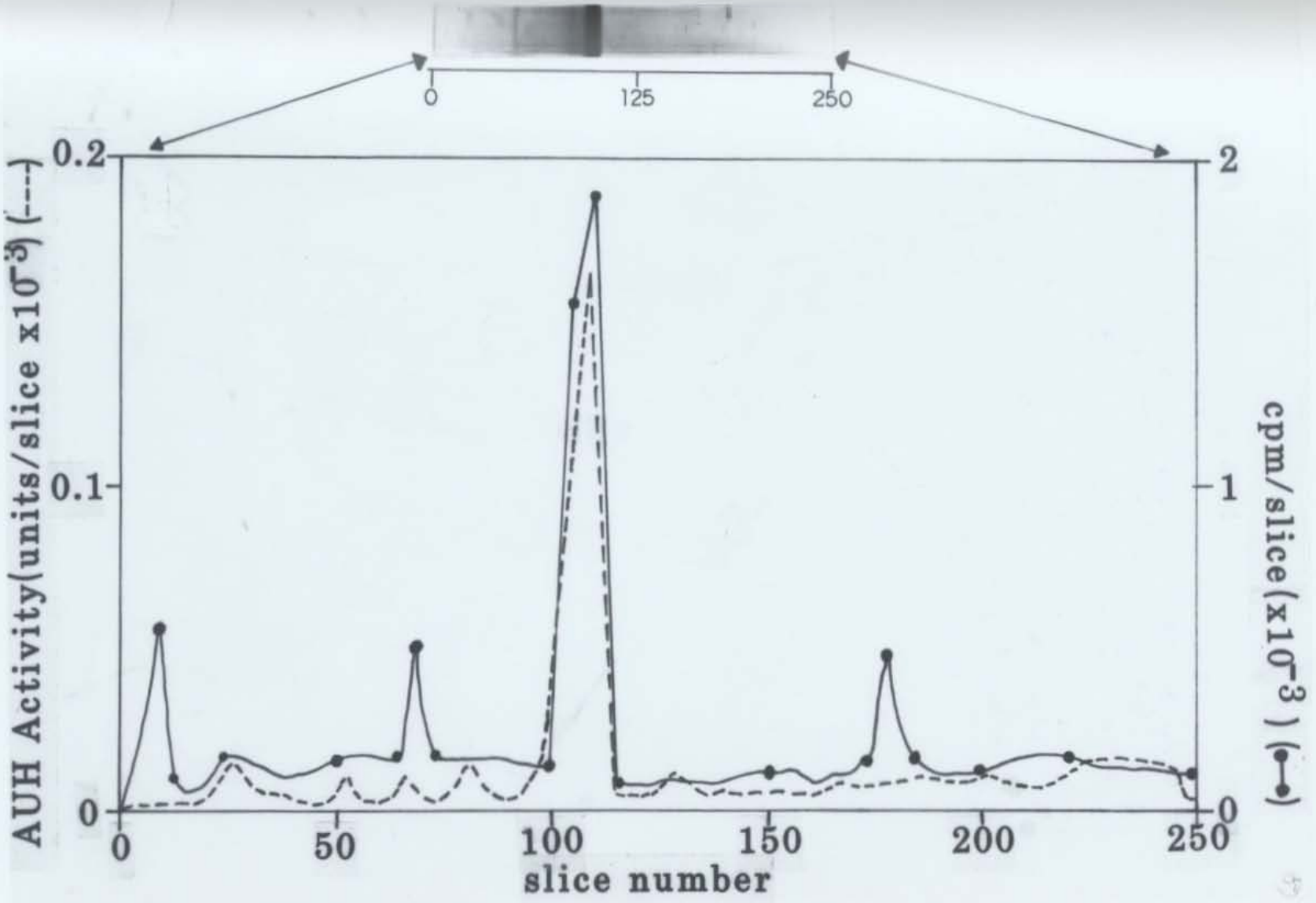

0
125
250
0.2
0.1
0
2
1
0
0
50
100
150
200
250
slice number
AUH Activity(units/slice x10⁻³) (---)
cpm/slice(x10⁻³) (—●—)

Figure 3.10b

Non-denaturing PAGE of agmatine ureohydrolase stained with Coomassie Blue.

The purified enzyme (stage: 8, Table: 3.2) was electrophoresed on non-denaturing PAGE, and the figure shows a Coomassie Blue stained gel. The unstained gel was sliced to determine the enzymic activity and is shown in Figure: 3.10a.


Origin
AUH

Figure 3.11.

SDS PAGE of proteins from the various steps of purification containing agmatine ureohydrolase activity and pKA5 encoded $[^{35}S]$-methionine labelled proteins from minicells

(A)= Coomassie Blue stained (lanes a-i),Lane**(k)**= Autofluorograph.

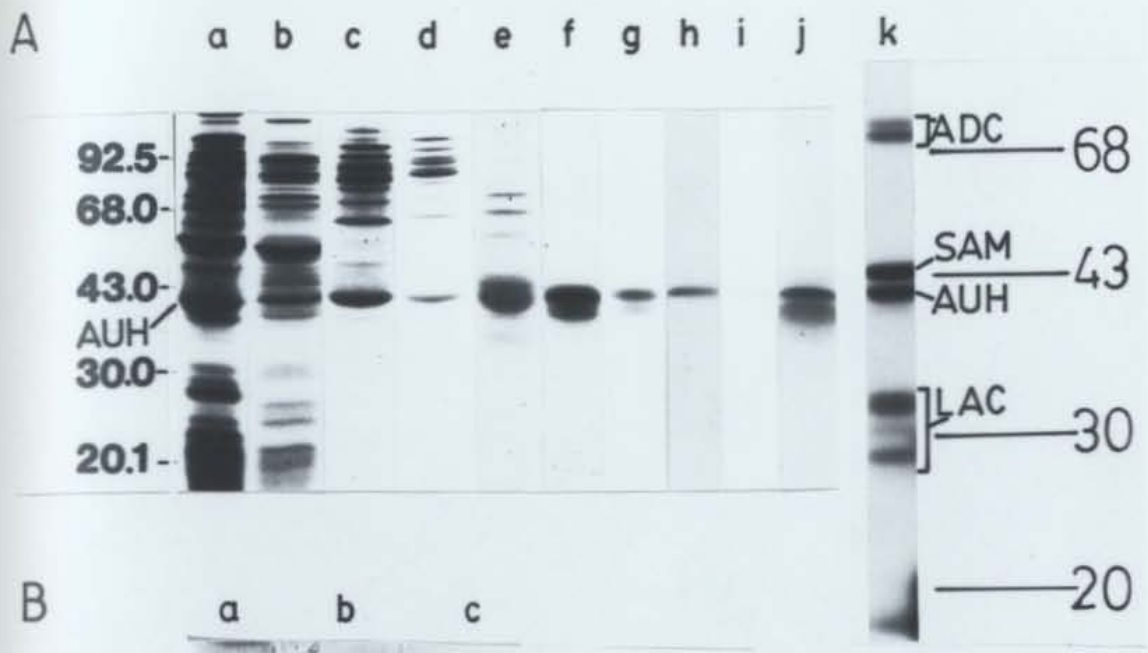
(B)= Silver nitrate stained.

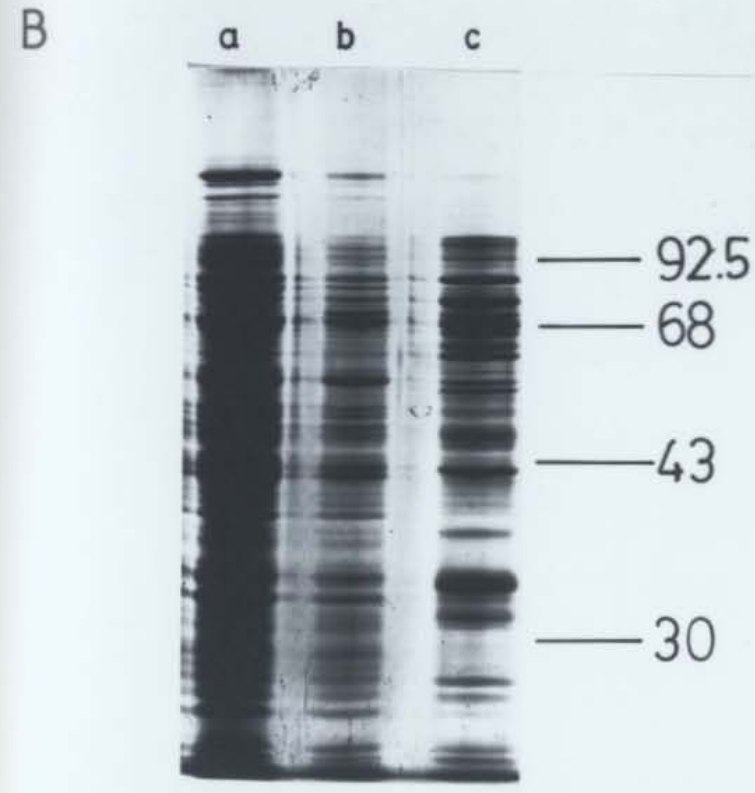
Figure: **(A)**: **(a)**=crude extract, **(b)**=ammonium sulphate, **(c)**=DEAE Sephacel, **(d, e)**=Sephadex G-100, **(f, g, j)**= Chromatofocusing of Stage: 5 material (Table: 3.2), preparations 1, 2 and 3 respectively, **(h, i)**=Purified enzyme (Stage: 8, Table: 3.2), preparations 1 and 2 respectively.

Figure: **(A)**, lane**(k)**= Radiolabelled proteins from minicells carrying plasmid pKA5.

Figure: **(B)**: **(a)**=Crude extract, **(b)**=ammonium sulphate, **(c)**=heat treatment.

AUH=agmatine ureohydrolase
ADC=arginine decarboxylase
LAC=β-lactamase
SAM=S-adenosyl methionine transferase


A
a b c d e f g h i j k
92.5-
68.0-
43.0-
AUH
30.0-
20.1-
ADC
68
SAM
AUH
43
LAC
30
20


B
a b c
92.5
68
43
30

did (Figure: 3.12).

3.2.13 The stability of agmatine ureohydrolase:

3.2.13.1 Stability following storage:

The purified enzyme obtained at stage-8 was stored either at -20°C or at -70°C in the presence or absence of glycerol and/or agmatine. The activity of the enzyme was determined at the various times indicated with the results as presented in Table: 3.4. The enzyme was stable for several weeks at both -20°C and -70°C in the presence of agmatine and to a lesser extent in the presence of glycerol.

3.2.13.2 Stability following dialysis against β-mercaptoethanol:

The Sephadex G-100 fraction (stage-7) (Table: 3.2) was dialysed at 4°C against 0.1 M Tris-HCl (pH 7.5), with or without 1 mM β-mercaptoethanol. Replicate preparations contained agmatine. Dialysis was continued for either 24 hours or 48 hours. The enzyme activities are shown in Table: 3.5. Agmatine ureohydrolase was stabilised by agmatine, but inactivated by β-mercaptoethanol, even in the presence of agmatine.

3.2.13.3 Stability at elevated temperatures:

The stability of the enzyme at elevated temperatures in the presence of agmatine was assessed using the material from stage 7 (Table: 3.2). The

Figure 3.12

Analysis of the specificity of antiserum against purified agmatine ureohydrolase by Ouchterlony.

The antiserum prepared against the purified agmatine ureohydrolase (stage: 8, Table: 3.2) was reacted against the preparations from various stages of purification.

(A): The center well contained **(a)**=Pre-immune serum, **(1, 2)** and **(3)** carried three different purified preparations of agmatine ureohydrolase (stage: 8), **(4)**=Sephadex G100 fraction (stage: 7) and **(5)**=DEAE Sephacel fraction (stage: 6).

(B): The wells carried identical samples as the one described for **(A)** except that the antiserum against the purified enzyme (stage: 8) was used instead of the pre-immune serum, and to observe the precipitin bands clearly, the agarose gel was washed extensively in physiological saline, fixed and stained with Coomassie Blue.

(C): The center well **(a)** carried antiserum raised against the purified enzyme as in **(B)** and the samples from various stages were the similar in wells **(1, 2, 3)** and **(4)** *as in* , while the sample in well **(5)** was the material from stage: 5 (after heat treatment).

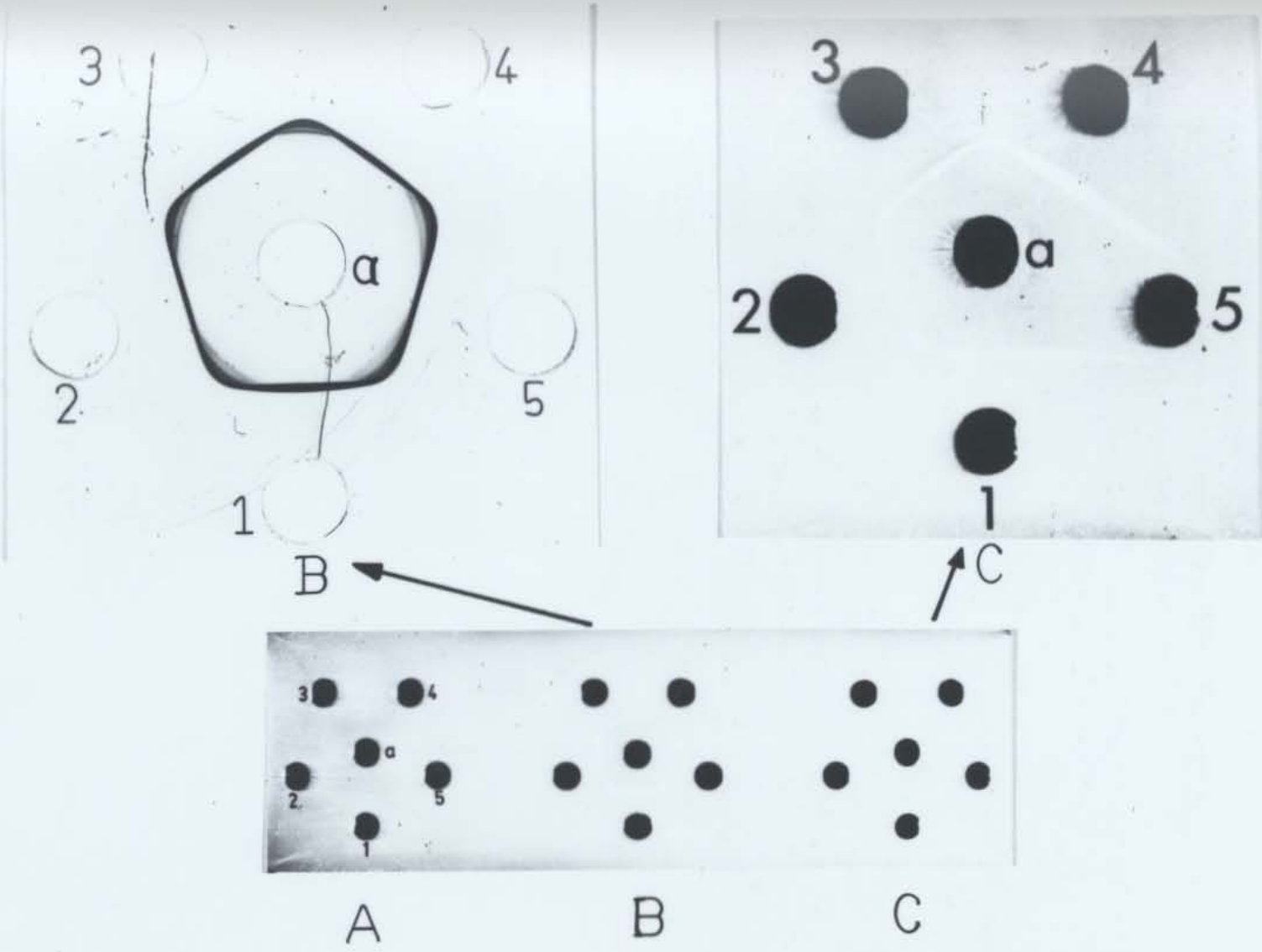

3
4
a
2
5
1
B
3
4
a
2
5
1
C
3
4
a
2
5
1
A
B
C

Table 3.4

Stability of AUH to storage in the presence or in the absence agmatine and/or glycerol.

Storage	Condition	-Glycerol /Agmatine	+Glycerol	+Agmatine	+Glycerol +Agmatine
-20 °C	4 Weeks	92	93	101	98
-20 °C	5 Weeks	85	96	104	94
-20 °C	6 Weeks	70	88	97	103
-20 °C	10 Weeks	52	73	98	94
-70 °C	12 Weeks	89	96	98	107
-70 °C	16 Weeks	69	87	98	96
-70 °C	20 Weeks	44	83	93	88
-70 °C	25 Weeks	40	71	94	93

AUH = agmatine ureohydrolase

The activity shown is the remaining activity after storage for the indicated period of time. 300 units of enzyme was stored in aliquots and samples were withdrawn at various times indicated. 100% activity represents 300 units of enzyme activity determined by the method given in Section 2.3.3.2, prior to storage.

Table 3.5

Stability of AUH on dialysis, and the effect of β-mercaptoethanol on its activity.

Period of dialysis	Condition ±agmatine	AUH activity
A.		
24 hours	–	36
48 hours	–	16
24 hours	+	42
48 hours	+	14
B.		
24 hours	–	69
48 hours	–	53
24 hours	+	104
48 hours	+	97

AUH = agmatine ureohydrolase

The activity shown is the remaining activity after dialysis for the indicated period of time against 0.1M Tris-HCl (pH 7.4), 1mM β-mercaptoethanol **(A)** or 0.1mM Tris-HCl (pH 7.4)**(B)**. Agmatine where indicated **(+)** was included in the dialysing medium at a concentration of 1mM. 100% corresponds to 830 units of enzymic activity determined according to the method described in Section: 2.3.3.2 prior to dialysis.

preparation containing 3500 units/ml (600 μl) was incubated in the presence or in the absence of 1 mM agmatine, in stoppered vessels at 30 °C, 40 °C, 50 °C, 65 °C or 100 °C, and aliquots of 0.1ml were removed at intervals up to 120 minutes. The aliquots were chilled on ice, and the remaining enzymic activity was determined. The results are presented in Figure: 3.13. The enzyme was quite stable at 30 °C and 40 °C, but unstable at 50 °C, 65 °C and 100 °C. At 50 °C and 65 °C agmatine gave some protection.

3.2.14 The effect of metal ions, chelating agents, nucleotides, amino acids and polyamines on the activity of agmatine ureohydrolase:

Nucleotides, chelating agents, metal ions, polyamines or amino acids (listed in Table: 3.6) were added to the reaction mixtures used to determine agmatine ureohydrolase activity at concentrations of 1 or 5 mM. The results are given in Table: 3.6. Chelating agents inhibited at 1mM, and arginine and ornithine inhibited at 5 and 1 mM respectively.

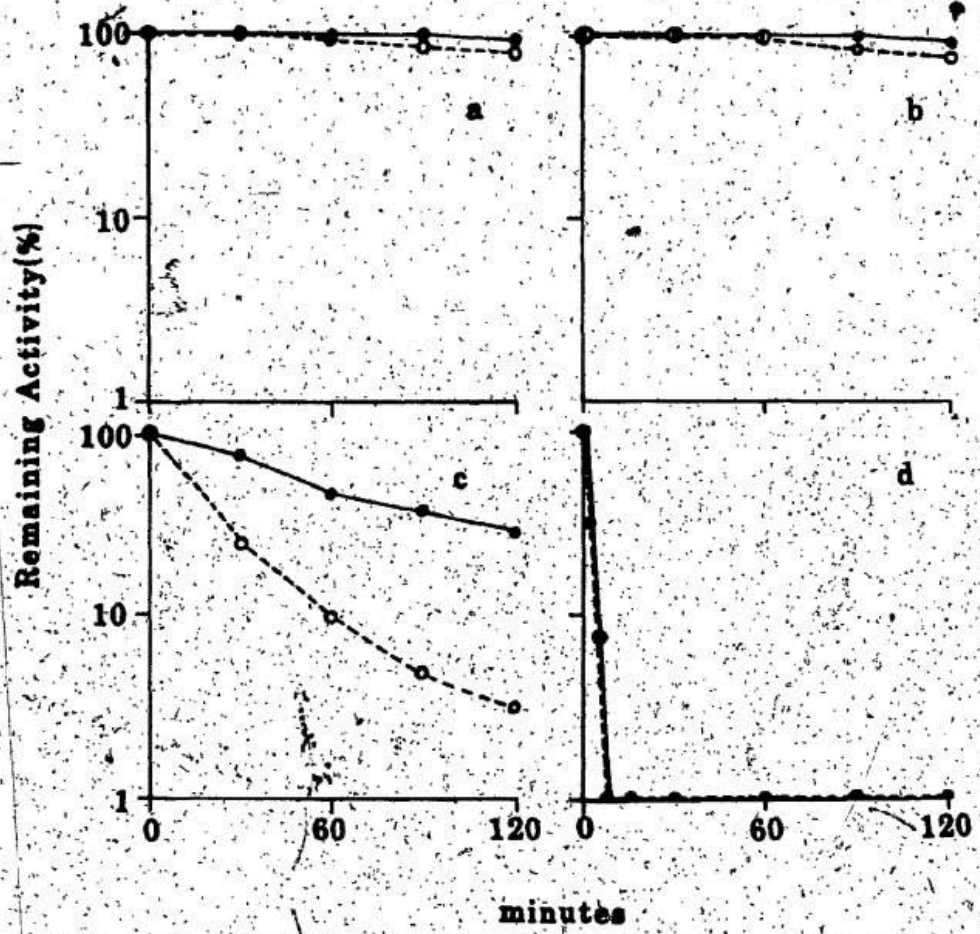
3.2.15 The effect of metal ions on the activity of agmatine ureohydrolase inactivated by chelating agents:

EDTA and EGTA both caused inhibition of the enzyme activity (50% and 74% respectively) at a concentration of 1 mM. In order to assess the possible restoration of enzymic activity, compounds of divalent metal ions (magnesium chloride, manganese chloride, calcium chloride, zinc chloride, barium chloride, or copper chloride) were added to the reaction mixtures. The purified

Figure 3.13

The stability of agmatine ureohydrolase at various temperatures.

The enzyme was incubated at (a)=30 °C, (b)=40 °C, (c)=50 °C, (e)=65 °C or (d)=100 °C. In Figures (a, b, c) and (d), in the presence (●) or in the absence (○) of 1 mM agmatine. In Figure (e), (○) = in the presence of agmatine and (●) = in the absence of agmatine. The enzymic activity was determined as described in Section: 2.3.3.2.


Remaining Activity(%)
100
10
1
a
b
c
d
0
60
120
minutes

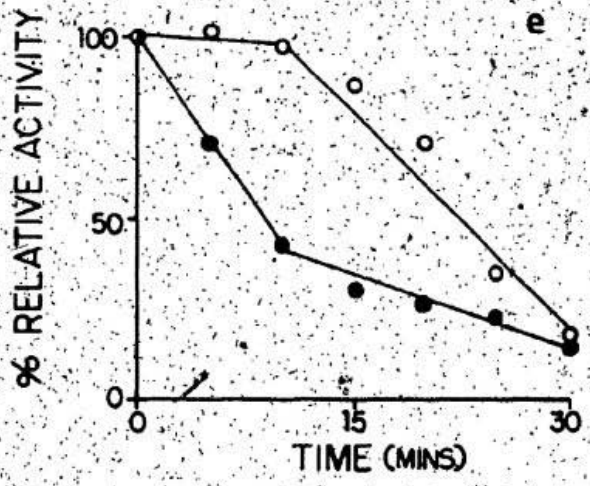

% RELATIVE ACTIVITY
100
50
0
e
0
15
30
TIME (MINS)

Table 3.6

The effect of metal ions, polyamines, amino acids and nucleotides on the activity of agmatine ureohydrolase.

Compound	AUH activity: 1 mM	AUH activity: 5mM
Control-none added	100	100
Sodium acetate	98	104
Sodium sulphate	98	102
Sodium chloride	103	103
Pottasium chloride	100	96
Ammonium chloride	102	104
Magnesium sulphate	96	100
Magnesium acetate	97	100
Magnesium chloride	97	99
Manganese sulphate	98	103
Manganese acetate	103	98
Manganese chloride	101	98
Calcium chloride	95	100
Calcium acetate	98	97
Barium chloride	98	103
Copper chloride	99	104
Copper sulphate	103	97
Glutamate	104	98
Glutamine	103	103
Methionine	103	103
Arginine	93	87
Ornithine	61	53
Putrescine	96	102
Spermidine	96	98
Spermine	105	99
ATP	103	104
AMP	N.D.	101
GTP	N.D.	99
GMP	N.D.	103
ppGpp	N.D.	95
cAMP(3'-5')	98	95
cAMP(2'-3')	96	95
EDTA	48	26
EGTA	26	19

Table 3.6

AUH = agmatine ureohydrolase
N.D. = Not determined

The compounds were added to the reaction mixture at 1 or 5 mM as indicated. The activities are presented as the remaining AUH activity. 100% represents the activity (180 units) in the absence of any added compounds into the reaction mixture.

enzyme (stage-8, Table: 3.2) (350 units) in 0.5 ml was treated with EDTA or EGTA (final concentrations of 5 mM), and dialysed against a salt solution. Each solution contained 100 mM Tris-HCl, pH 7.5, 1 mM agmatine, and 10 mM divalent cation. The results are presented in Table: 3.7 as a percentage of the activity obtained from a sample dialysed similarly without pretreatment with the chelating agents. No reactivation of the enzyme activity was observed.

3.2.16 Determination of kinetic parameters:

3.2.16.1 K_m for agmatine:

In order to determine the K_m for agmatine, the concentration of agmatine was varied from 0.1 mM to 10 mM in a set of reaction mixtures containing the purified enzyme (stage-8, Table: 3.2). The enzymic activities measured by the colorimetric assay (Section: 2.3.2) are plotted against the substrate concentration (inset Figure: 3.14) A Lineweaver-Burke plot of the same is presented in Figure: 3.14. The K_m determined by the least squares weighted regression was 1.3 ± 0.15 and the V_{max} was $36,300 \pm 1,200$ units/mg protein.

3.2.16.2 Inhibition by arginine:

To assess the nature of inhibition of agmatine ureohydrolase by arginine and to determine the kinetic constant, experiments similar to those described above (3.2.16.1) were set up. Different concentrations of arginine were added to

Table 3.7

The effect of chelating agents on the activity of agmatine ureohydrolase.

	Treatment	% Remaining activity:
A.	Control	100
	EDTA treated	24
	Magnesium chloride	21
	Manganese chloride	26
	Calcium chloride	28
	Barium chloride	29
	Copper chloride	26
	Zinc chloride	21
B.	Control	100
	EDTA treated & dialysed	22
	Magnesium chloride	25
	Manganese chloride	28
	Calcium chloride	19
	Barium chloride	22
	Copper chloride	17
	Zinc chloride	19

AUH = agmatine ureohydrolase

The activities shown are the activities of AUH after the treatments described.

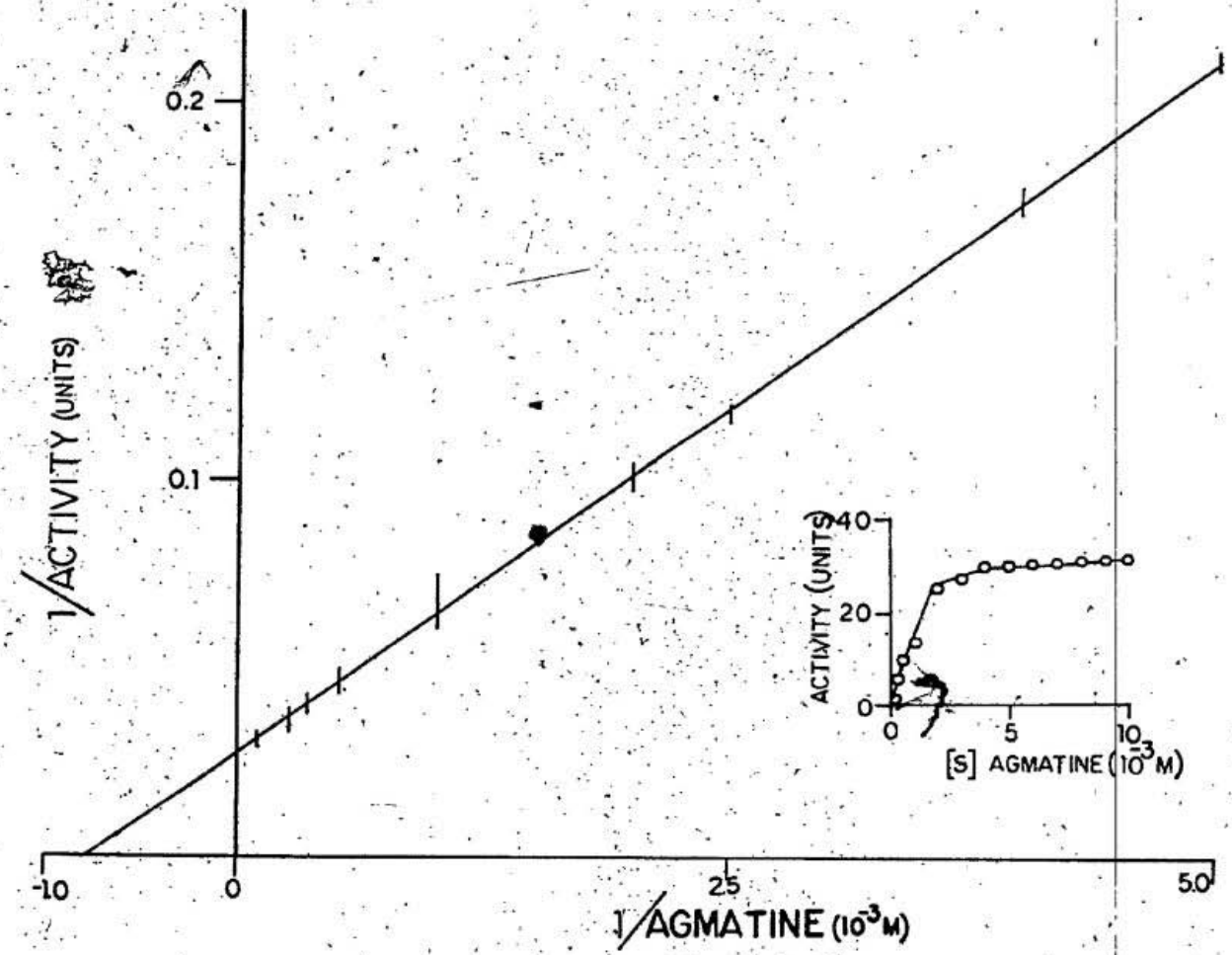
A. = Enzyme was treated with EDTA and various compounds were supplemented in the reaction mixture at 10mM. 100% represents control without EDTA treatment (350 units).

B. = Enzyme was treated with EDTA and was dialysed against 0.1mM Tris-HCl (pH 7.5), 1mM agmatine and 10mM of salt. 100% is the control activity, without EDTA treatment and dialysed against 0.1mM Tris-HCl (pH 7.5)containing 1mM agmatine, and represents 342 units of enzyme activity.

Figure 3.14

Lineweaver Burk plot of the activity of agmatine ureohydrolase.

The activities of the enzyme for various substrate concentrations were determined according to the method described in Section: 2.3.2. The inverse of the velocities are plotted against the inverse of the substrate concentrations. The bars represent the range of activities obtained from four sets of readings. The lines were fitted for all experimental points in one set by a weighted least square method (Wilkinson, 1961). In the inset of the figure the activity of the enzyme is plotted against the substrate concentration.


0.2
0.1
1/ACTIVITY (UNITS)
-10
0
2.5
5.0
1/AGMATINE (10^{-3}M)
40
20
0
ACTIVITY (UNITS)
0
5
10
[S] AGMATINE (10^{-3}M)

a set of reaction mixtures containing increasing concentrations of agmatine. The arginine concentrations were varied from 5 to 25 mM. Lineweaver-Burk plots for each of a set of reaction. (constant arginine concentration) are plotted in Figure: 3.15. In order to determine the K_i of inhibition by arginine, the apparent K_m for agmatine determined for each of the arginine concentrations is plotted against the respective concentrations of the inhibitor (Figure: 3.15 inset). The inhibition was of the competitive type, and the K_i was 8.4 mM.

3.2.16.3 Inhibition by ornithine:

To assess the nature of inhibition of agmatine ureohydrolase by ornithine and to determine the kinetic constant, an experiment similar to the one described above except that ornithine (1-10 mM) was used in place of arginine, was carried out. The results are shown in Figure: 3.16. Ornithine inhibited agmatine ureohydrolase activity in a mixed manner.

3.3 Discussion:

The results presented in this chapter describe the purification of agmatine ureohydrolase from an AUH⁻ strain of *E.coli* (HT328), transformed with a plasmid (pKA5) carrying *speB*. Agmatine ureohydrolase was associated with the 32-48% saturated ammonium sulphate fraction (Table: 3.3). The enzymic activity was preserved during heat treatment or dialysis (Figure: 3.13 and Table: 3.5), when agmatine sulphate was present. This suggests that agmatine stabilises the conformation of the enzyme.

Figure 3.15

Lineweaver Burk plot of the activity of agmatine ureohydrolase in the presence of various concentrations of arginine.

The inverse of velocities of the enzyme activity for various substrate concentrations at different concentrations of the inhibitor is plotted against the inverse of substrate concentrations. Each result obtained is in tetraplicate, and the bars represent the extremes of each set of parameters. The lines were fitted for all experimental points in one set by a weighted least square method (Wilkinson, 1961). The apparent K_m for agmatine at each of the arginine (ARG) concentrations is plotted against the corresponding inhibitor (arginine) concentration (**inset**), and the intercept on the X- axis gives K_i. The mM concentration of arginine is shown next to the plot of the particular concentration.

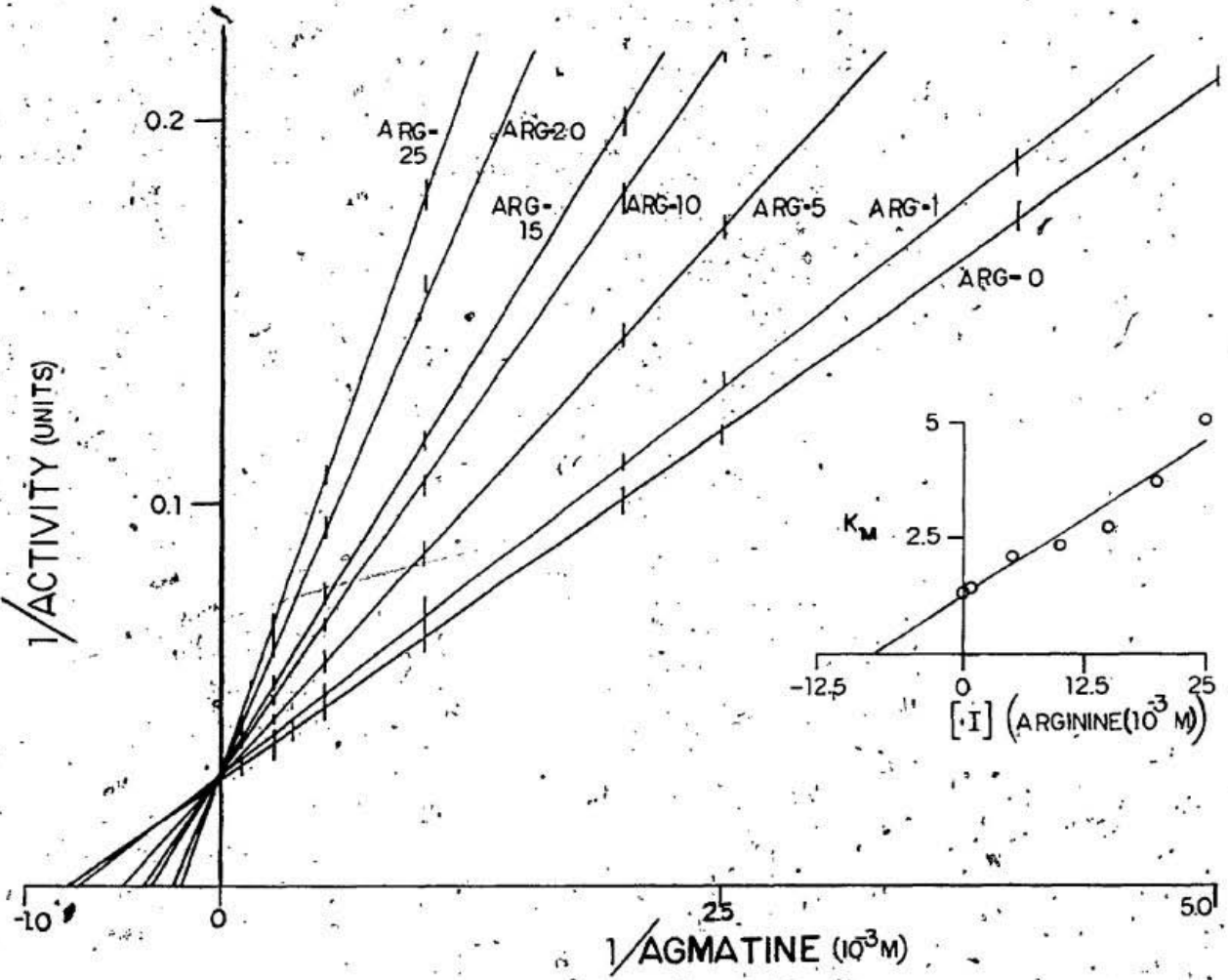
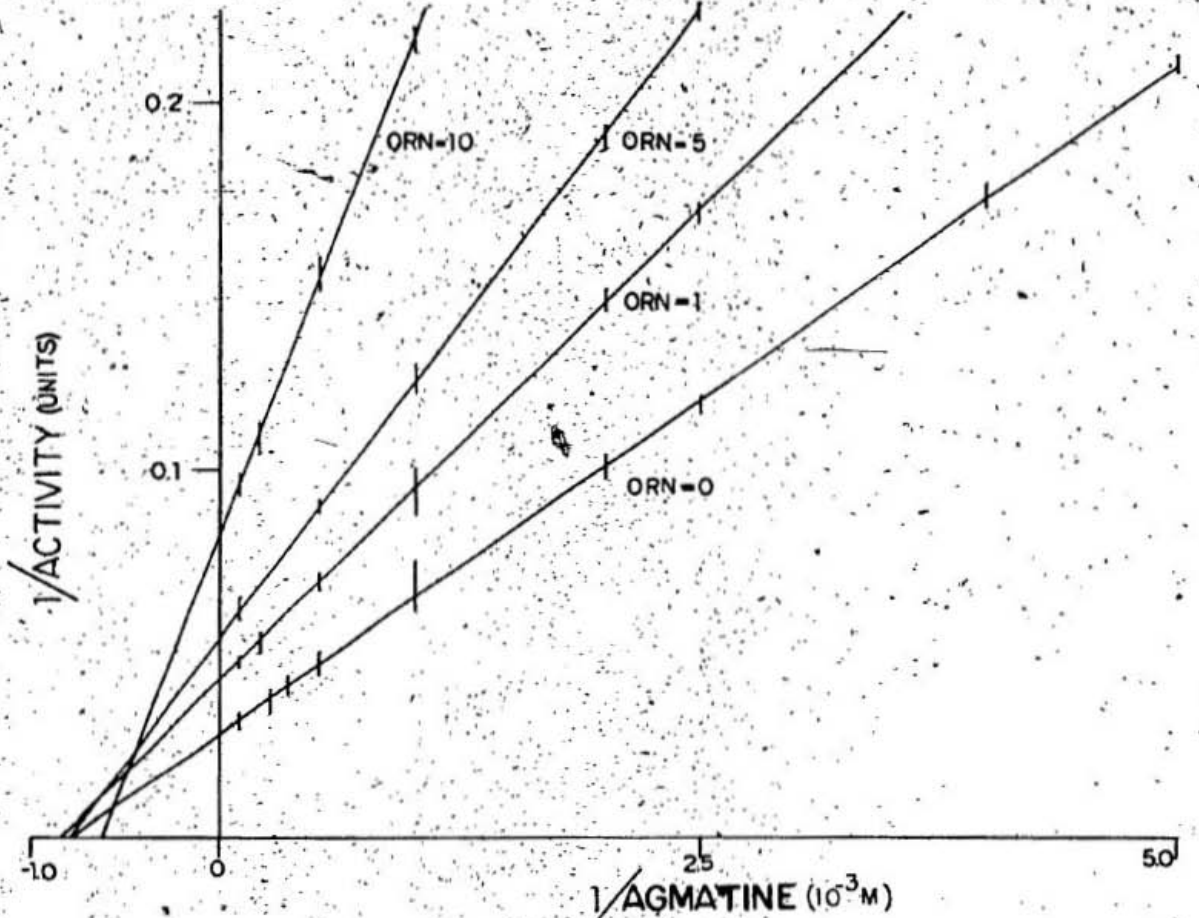

ARG-25
ARG-20
ARG-15
ARG-10
ARG-5
ARG-1
ARG-0
0.2
0.1
1/ACTIVITY (UNITS)
-10
0
25
5.0
1/AGMATINE (10³M)
5
2.5
Km
-12.5
0
12.5
25
[I] (ARGININE(10³ M))

Figure 3.16

Lineweaver Burk plot of the activity of agmatine ureohydrolase in the presence of various concentrations of ornithine.

The inverse of velocities of the enzyme activity for various substrate concentrations at different concentrations of the inhibitor is plotted against the inverse of substrate concentrations. Each result obtained is in triplicate, and the bars represent the extremes of each set of parameters. The lines were fitted for all experimental points in one set by a weighted least square method (Wilkinson, 1961). The concentration of the inhibitor (ornithine) (mM) used to determine the apparent K_m is shown next to the plot obtained for that particular concentration of ornithine (ORN).


0.2
0.1
ORN-10
ORN-5
ORN-1
ORN-0
1/ACTIVITY (UNITS)
-1.0
0
2.5
5.0
1/AGMATINE (10^{-3}M)

The enzymic activity eluted from a DEAE-Sephacel column at a Tris-HCl, pH 7.5 concentration of approximately 0.55 M (Figure: 3.5). There was a three fold increase in the total amount of enzymic activity during DEAE Sephacel column chromatography, suggesting the removal of an inhibitory substance (Table: 3.2). In the gel exclusion chromatography on Sephadex G-100 (Figure: 3.6) the enzymic activity was associated with an elution volume that corresponds to a molecular weight of approximately 85,000 (data not shown).

The enzymic activity eluted from the chromatofocusing column at a pH of 8.3 (Figure: 3.7C). The separation of proteins on a chromatofocusing column is based on the isoelectric pH of the proteins. At its isoelectric pH a protein is not bound to the column and is eluted. The pI thus determined is in agreement with the pI of 8.5 for the 38 Kdalton protein (putative agmatine ureohydrolase determined from the isoelectric focusing of the labelled proteins from minicells carrying pKA5) (Buch and Boyle, personal communication).

When the stage-5 material was passed directly through a chromatofocusing column, however, the agmatine ureohydrolase activity eluted at pH 5.5 (Figure: 3.7b). Although it has not been demonstrated that the increase in pI from 5.5 to 8.3 occurs during the purification step on DEAE Sephacel (stage 6, Table: 3.2), it may be that the increase in activity observed at this stage is associated with the removal of an inhibitor which also decreases its pI.

The results in Figures: 3.8 and 3.9 suggest that the purified enzyme

(stage-8) has a molecular mass of about 80,000 daltons, but this estimate depends on an extrapolation of calibration, and furthermore even an error of one fraction in determining the peak volume would lead to a change in the estimated molecular weight of ± 10%.

After SDS-PAGE, the stage-8 material gave a single band on staining with Coomassie Blue (Figure: 3.11). The more sensitive silver staining revealed small proportions of material with molecular weight 24,000 and 70,000 (Data not shown). The Sephacryl S-200 chromatography gave a peak of activity corresponding to a molecular weight of 80,000 but also showed the presence of enzymatically inactive protein (about 50% of total protein) (Figure: 3.8). The component with a molecular weight of about 42,000 daltons observed on Sephacryl S-200 chromatography could be an enzymatically inactive monomer of agmatine ureohydrolase, or some other uncharacterised protein (an impurity).

The SDS-PAGE (Figure: 3.11) of preparations from various stages of purification shows an enrichment of 38,000 dalton protein, and it co-migrates with the putative agmatine ureohydrolase (Boyle *et al.*, 1984) encoded by the plasmid pKA5 in minicells (Figure: 3.11). The fraction containing the enzymic activity obtained from the chromatofocusing of stage-5 material which elutes at a pH of 5.5, was electrophoresed on SDS polyacrylamide gels (Figure: 3.11). Two protein bands of approximately equal intensities were observed after staining with Coomassie Blue. They had molecular weights of 38,000 and

35,000 respectively. This observation was reproducible with different preparations. The 35 Kdalton protein may well be an inhibitor which binds to agmatine ureohydrolase (monomer 38 Kdaltons) to lower the pI and to reduce enzymic activity.

The observation of preparations of agmatine ureohydrolase with pI 5.5 and 8.3 probably does not indicate two separate enzymes. DEAE Sephacel chromatography was stopped shortly after the material with pI 8.3 was obtained, and it is possible that a protein with pI 5.5 would have been bound more tightly and required a higher salt concentration to displace it. On the other hand, the chromatofocusing of stage-5 material did not reveal an enzyme with a pI 8.3 (Figure: 3.7a). Furthermore, the activation observed on chromatography on DEAE Sephacel is not consistent with the separation of two enzymes. Direct chromatofocusing of stage-5 material did not lead to the marked increase in activity observed on chromatography on DEAE Sephacel (Table: 3.8). It therefore seems most probable that the increase in activity and pI are due to the removal of an inhibitor on DEAE Sephacel chromatography. This employs high concentrations of salt relative to that used in chromatofocusing, and might promote the dissociation (and separation) of the inhibiting protein.

In non-denaturing PAGE a radiochemically labelled protein from minicells carrying pKA5 co-migrated with the enzymic activity and the major Coomassie Blue stainable protein band (Figure: 3.10). These results together with

Table: 3.8

Activation of AUH during ion-exchange chromatography.

Stage	Step	Total Units	Total Protein (mg.)	Specific Activity	Purification	Yield %
5.	1M Tris. Adjustment	5,280	9.9	536	1.0	100
6.	DEAE-Sephacel Chromatography	1,6430	2.3	7,143	13.3	311
6.	Chromatofocusing (Polybuffer74/PBE)	3,592	1.7	2,112	3.9	68

Stage: 5 material was chromatographed either on polybuffer exchanger (PBE) using polybuffer74 (pH 4.0) (the agmatine ureohydrolase activity eluted from the column at an eluant pH of 5.5) or on a DEAE- Sephacel ion exchanger.

these of SDS-PAGE show that the 80,000 dalton dimer encoded by pKA5 in minicells is indeed agmatine ureohydrolase.

Analysis by the method of Ouchterlony of the antibody produced against agmatine ureohydrolase showed a single and continuous precipitin band for all preparations beyond stage 5, but not for stage 5 material (Figure: 3.12). This suggests that the antigen is pure and homogeneous. No precipitin bands were seen when the antibody was reacted against stage-5 fraction, and the antibody did not precipitate any labelled protein from the ^{35}S-labelled extracts of minicells carrying pKA5. This anomalous behaviour is consistent with the view that the antibody does not recognize the enzyme when the latter is complexed with the inhibitor. Presumably all antigenic determinants are blocked by binding the inhibitor, or altered by conformational changes consequent to binding. It is also possible that the antibody species which react with the antigenic determinants present in both stage-5 and stage-8 preparations might be rare in the antibody preparations used in this experiment. When the purified enzyme (stage-8) was mixed with the antibody, the enzymic activity measured after 3 hours of incubation at 37° C was decreased by 83% relative to controls incubated without antibody.

The results presented in Table: 3.4 suggests that agmatine ureohydrolase is stable on storage for several weeks if agmatine (1 mM) is present and is stable to a lesser degree if 10% glycerol is present. β-Mercaptoethanol at 1 mM inactivated the enzyme (Table: 3.5) in the presence or in the absence of 1 mM

agmatine. This may be due to the reducing environment enhancing the formation and stabilisation of monomeric units of agmatine ureohydrolase which are enzymatically inactive. Dialysis of the enzyme against 0.1 M Tris-HCl (pH 7.5), without β-mercaptoethanol also resulted in the partial inactivation of the enzyme, although this was prevented when 1 mM agmatine was included in the dialysing medium (Table: 3.5). Addition of 1 mM agmatine to the purified enzyme (stage-8) conferred significant stability to the enzymic activity at elevated temperatures. The enzymic activity at 50 °C and 65 °C was lost at about half the rate of a control when 1 mM agmatine was present in the reaction mixture (Figure: 3.13). No significant differences in the stability was observed at 30 °C, 40 °C, and 100 °C with or without agmatine included in the preparation.

Although ornithine decarboxylase and arginine decarboxylase were activated by GTP and Mg^{2+} respectively, and inhibited by both ppGpp and putrescine, agmatine ureohydrolase was neither significantly activated nor inhibited by the metal ions, nucleotides, amino acids (except ornithine and arginine) and polyamines (Table: 3.6).

Both of the chelating agents EDTA and EGTA inhibited enzyme activity when included in the reaction mixture (Table: 3.6). Dialysis of the enzyme treated with EDTA or EGTA against 0.1 M Tris-HCl (pH 7.5) containing 10 mM concentrations of different divalent cations (Table: 3.7) did not restore the enzymic activity. This suggests that agmatine ureohydrolase is a

metaloprotein which requires a metal ion for its structural stability (as well as activity) rather than an enzyme which requires a metal for its activity alone. Alternatively perhaps the enzyme required another metal ion not tested.

The K_m for agmatine was 1.3 mM. (Figure: 3.14). Arginine and ornithine were inhibitors. Arginine appeared to be a competitive inhibitor, with K_i 8.4 mM (Figure: 3.15). The inhibition by ornithine was quite different from that of arginine, and may be consistent with mixed inhibition. (Figure: 3.16).

Chapter 4

Transcriptional Regulation of Agmatine Ureohydrolase by Cyclic AMP.

4.1 Introduction:

Different carbon sources permit different growth rates, and intracellular concentrations of cAMP (Buettner *et al.*, 1973; Epstein *et al.*, 1975; Pastan and Adhya, 1976) and polyamines (Boyle *et al.*, 1977) vary with these. An inverse correlation was shown to exist between cAMP and polyamine concentrations in *E.coli* cells. Wright and Boyle (1982) showed that ornithine decarboxylase and arginine decarboxylase were negatively regulated by cAMP, and suggested that agmatine ureohydrolase might also be negatively regulated by cAMP. Therefore the role of cAMP in the regulation of agmatine ureohydrolase was examined further using strains bearing mutations in the gene encoding adenylate cyclase (EC.4.6.1.1), *cya* and in both *cya* and the gene for cAMP receptor protein (CRP), *crp*. The cells were grown in the presence or absence of cAMP, and enzymic activities were measured in crude extracts. The activities of ornithine decarboxylase, arginine decarboxylase and β-galactosidase were measured in the same strain as controls.

4.2 Results:

4.2.1 The effect of carbon sources on the growth rate of LS340, and the activities of agmatine ureohydrolase, ornithine decarboxylase and arginine decarboxylase:

Lactose and glycerol were used as carbon sources for the growth of LS340, which has functional *cya* and *crp* genes. The results are shown in Table: 4.2, and the specific activities of the enzymes in LS340 are shown in Table: 4.1. The activities of agmatine ureohydrolase, arginine decarboxylase and ornithine decarboxylase were decreased in LS340, when grown in lactose or glycerol, as compared to cells grown in glucose.

4.2.2 The growth rate of LS853 and the activities of agmatine ureohydrolase, ornithine decarboxylase, arginine decarboxylase and β-galactosidase:

Two strains of *E.coli*, LS340 and LS853, were examined. LS853 is a *cya* deletion mutant derived from LS340. Cultures were grown with glucose as a sole carbon source and replicate cultures were supplemented with different concentrations of cAMP. The results of the growth rate and the enzymic activities are shown in Tables: 4.3 and 4.4 and in Figure: 4.1. The specific activities of the enzymes are shown in Table: 4.1. With increasing concentrations of cAMP the activities of agmatine ureohydrolase, arginine decarboxylase and ornithine decarboxylase were decreased, and that of β-galactosidase increased.

4.2.3 The growth rate of LS854-1 and the activities of agmatine ureohydrolase, ornithine decarboxylase, arginine decarboxylase, and β-galactosidase:

Table 4.1

Specific activities of AUH, ODC and ADC in LS340, LS853 and LS854-1.

Strain	Phenotype	AUH	ODC	ADC
LS340	Wild type	1.1	11.3	1.0
LS853	CYA^-	2.0	19.1	1.6
LS854-1	CYA^-,CRP^-	1.8	16.7	1.5

ODC = ornithine decarboxylase
ADC = arginine decarboxylase
AUH = agmatine ureohydrolase

Cells were grown in minimal medium containing glucose (0.2%) as the sole source of carbon and energy. Specific activities are expressed as μmoles of product formed per minute per milligram protein (Section: 2.3.2).

Table 4.2

Effect of carbon sources on the growth rate and activities of ODC, ADC and AUH in LS340.

Carbon source	Growth rate	ODC	ADC	AUH
Glucose	0.71	1.0	1.0	1.0
Lactose	0.50	0.7	0.7	0.5
Glycerol	0.48	0.7	0.6	0.5

ODC = ornithine decarboxylase
ADC = arginine decarboxylase
AUH = agmatine ureohydrolase

All carbon sources were added at 0.2%, in MOPS minimal medium. Growth rate is expressed as generations per hour. Activities are expressed as specific activities relative to the specific activity in glucose grown cells. The specific activities are given in Table: 4.1.

Table 4.3

The effect of cAMP on the activity of β-galactosidase in LS340, LS853 and LS854-1.

cAMP(mM)	LS340	LS853	LS854-1
0.0	530	270	260
1.0	5,500	5,600	270
2.5	8,400	8,100	270
5.0	13,600	13,000	270

β-galactosidase activity was measured in crude extracts of the strains grown in minimal glucose medium in the presence of 0.5mM IPTG. The specific activity is presented as units of activity per milligram protein (Section: 2.3.7). No β-galactosidase activity was detected when IPTG was not included in the growth medium even in the presence of 1mM cAMP.

Table 4.4

The effect of cAMP on the activities of ODC and ADC in LS340, LS853 and LS854-1.

cAMP (mM)	LS340 ODC	LS340 ADC	LS853 ODC	LS853 ADC	LS854-1 ODC	LS854-1 ADC
0.0	1.0	1.0	1.0	1.0	1.0	1.0
1.0	0.7	0.8	0.7	0.6	1.0	1.1
2.5	0.4	0.5	0.4	0.4	0.9	1.0
5.0	0.4	0.4	0.4	0.4	0.9	0.9

ODC = ornithine decarboxylase
ADC = arginine decarboxylase

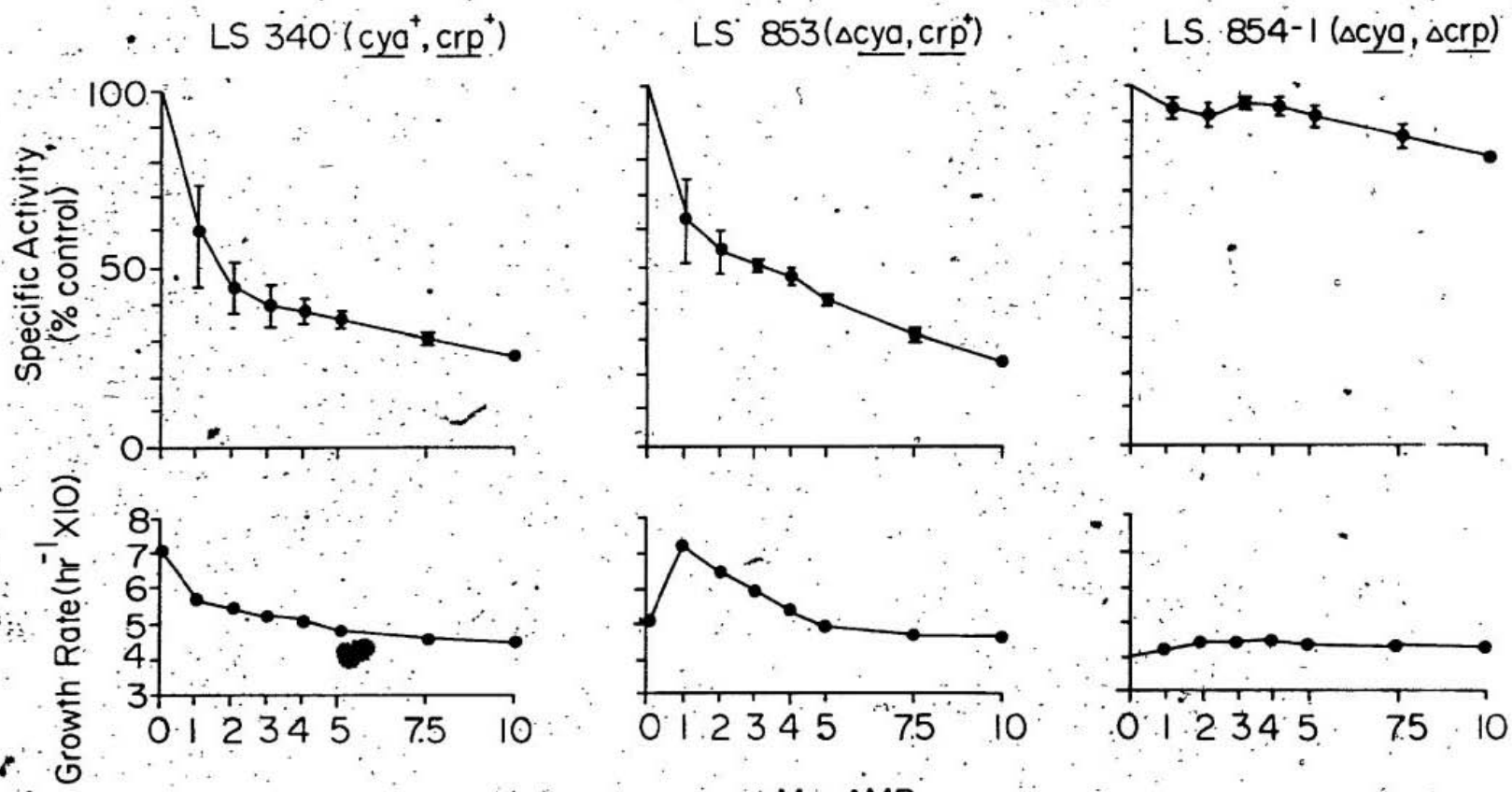
Activities are expressed as specific activities relative to the specific activity in glucose grown cells without added cAMP (Table 4.1).

Figure 4.1

The effect of cAMP on the growth rates and the activity of agmatine ureohydrolase in LS340, LS853 and LS854-1.

100% activity represents the specific activity of agmatine ureohydrolase without added agmatine (Table. 4.1). The bars represent 95% confidence values for each set from five separate experiments.

Effect of cAMP on AUH and growth rate



E.coli LS854-1 is a *crp* deletion strain of LS853 (Section 2.4). Cultures of LS854-1 and LS340 were grown with glucose as carbon source, and replicate cultures were supplemented with different concentrations of cAMP. The results of the growth rate and of the enzymic activities are given in Tables: 4.3 and 4.4 and in Figure 4.1. The specific activities of the enzymes are given in Table: 4.1. Increasing concentrations of cAMP neither decreased nor increased the growth rate and the activities of the enzymes.

4.2.4 Does cAMP induce an inhibitor of agmatine ureohydrolase?:

To determine if the repression of agmatine ureohydrolase observed in cultures grown in the presence of cAMP was a direct effect or due to the induction of an inhibitor, LS853 was grown with and without added cAMP (5 mM), and enzyme activities were measured in the individual extracts and in mixtures of these (Table: 4.5).

The possibility of a direct effect of cAMP on the enzymic activity was examined in two ways. Extracts of LS853 grown on glucose with and without cAMP, were dialysed, and the enzyme activities in individual and mixed extracts of cultures measured. The activity of agmatine ureohydrolase was also examined in non-dialysed extracts of LS853 grown without added cAMP. To these reactions different concentrations of cAMP were added. The results are in Table: 4.5 and in Figure: 4.2. Cyclic AMP did not appear to induce an inhibitor of the enzyme, and did not affect the enzymic activity by acting directly

Table 4.5

Effect of mixing of extracts of cAMP treated and untreated cells on agmatine ureohydrolase activity in LS853

Ratio of mixing C:R	Before dialysis units/ml	Predicted value units/ml	After dialysis units/ml	Predicted value units/ml
0:1	68	–	45	–
1:9	74	72	51	52
3:7	101	102	71	71
1:1	124	126	90	91
7:3	147	149	112	110
9:1	169	172	N.D.	N.D.
1:0	189	–	141	–

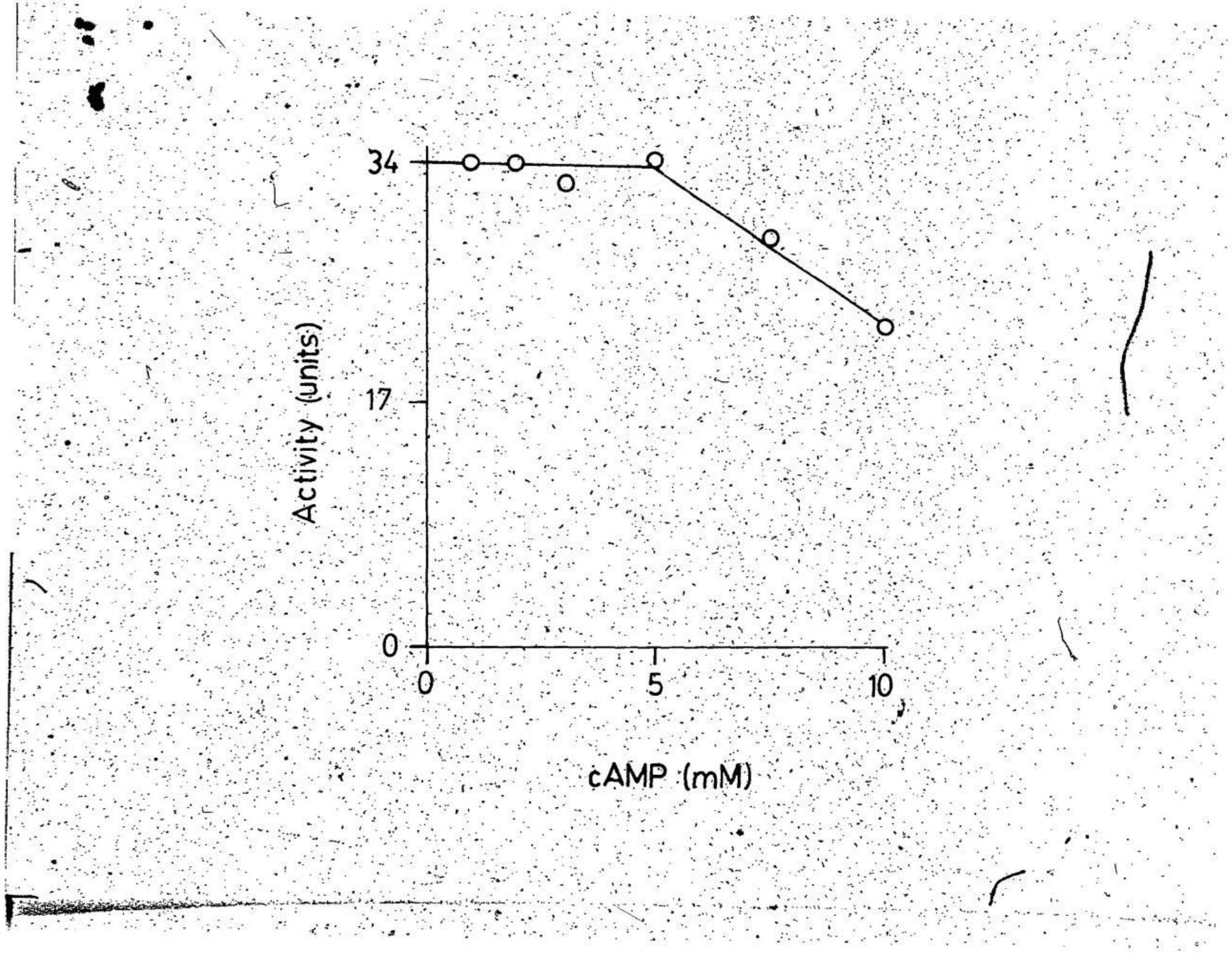
AUH = agmatine ureohydrolase
C = Control
R = Repressed (with cAMP)
N.D. = Not determined

Predicted values were obtained by averaging the values obtained of individual extracts diluted in various proportions with breakage buffer containing 1mg/ml of bovine serum albumin. 3 ml of extracts were dialysed for 18 hours against 1L of breakage buffer.

Figure 4.2

The effect of cAMP on the activity of agmatine ureohydrolase *in vitro*.

The activity is expressed as μmoles of urea formed per minute per ml. cAMP was added to reaction mixtures at concentrations indicated. The source of the enzyme was a crude extract of LS853.


34
17
0
Activity (units)
0
5
10
cAMP (mM)

on agmatine ureohydrolase.

4.2.5 Temporal repression of agmatine ureohydrolase following the addition of cAMP:

A culture of LS853 was grown for three generations with glucose as the sole carbon source, to an absorbance of 0.1 at 575 nm. Cyclic AMP (5 mM) was then added, samples of cultures were removed at intervals, and the activity of agmatine ureohydrolase was measured. The results are shown in Figure 4.3. Agmatine ureohydrolase activity was repressed by cAMP, and a new steady state level was reached 2.5 generations after the addition of cAMP.

4.3 Discussion:

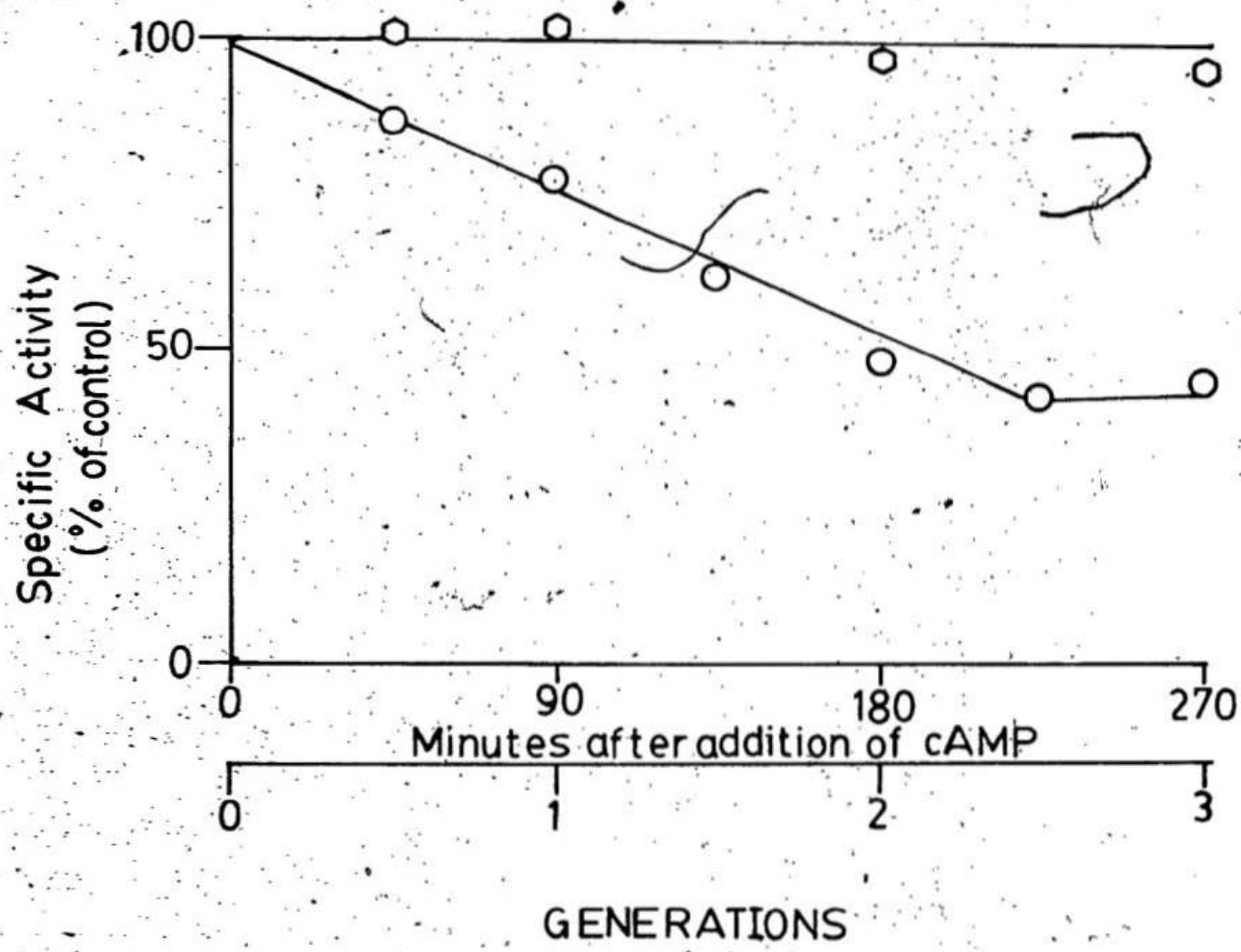
The experiments described in this chapter demonstrate that agmatine ureohydrolase is negatively controlled in *E.coli* by cAMP at the transcriptional level and the regulation is mediated by the cAMP receptor protein.

Assessment of the intracellular concentrations of cAMP was avoided in this study because of technical difficulties of a direct assay (Pastan and Adhya, 1976), which have at best a fifty percent standard deviation (Primakoff, 1981). It was assumed that if added cAMP restored the growth rate of a culture of a Δ*cya* mutant, the intracellular concentration had been restored to its normal physiological value. The growth rate of LS853 (Δ*cya*) was restored to that of the *cya*$^+$ strain (LS340) by a concentration of 1 mM cAMP, but a concentra-

Figure 4.3

Temporal repression of agmatine ureohydrolase activity following the addition of cAMP to growing cultures of LS853.

Enzyme activity was measured in crude extracts of aliquots withdrawn at various time intervals indicated. 100% represents the specific activity of the enzyme prior to the addition of cAMP, and is given in Table: 4.1. (○)=cAMP added and (○)=without the addition of cAMP.


100
50
0
Specific Activity
(% of control)
0
90
180
270
Minutes after addition of cAMP
0
1
2
3
GENERATIONS

tion of 2 mM or higher decreased it. The growth rate of LS340 decreased progressively as cAMP was added. On the other hand, cAMP had no effect on the growth rate of the Δ*crp* mutant LS854-1, presumably because cAMP normally functions together with the cAMP receptor protein to regulate transcription positively (Pastan and Adhya, 1976) or negatively (Aiba, 1983), depending on the genes concerned (Figure: 4.1).

In accord with this interpretation it was observed that β-galactosidase was induced in *E.coli* LS340 and LS853 when these strains were grown on glucose in the presence of added cAMP. In contrast, the specific activities of agmatine ureohydrolase, ornithine decarboxylase and arginine decarboxylase decreased progressively as the concentration of added cAMP was increased in cultures of the same strains. In cultures of LS854-1 (Δ*cya*,Δ*crp*), cAMP did not increase the activity of β-galactosidase and did not decrease the activities of agmatine ureohydrolase ornithine decarboxylase and arginine decarboxylase (Figure: 4.1, Tables 4.3 and 4.4).

It was possible that the effect of cAMP was either to induce the production of an inhibitor of agmatine ureohydrolase or to inhibit the enzyme directly. To test the first possibility mixing experiments with extracts of LS853 Δ*cya*, grown with (5 mM) cAMP and without cAMP, were carried out (Table: 4.5). The results show activities expected from the proportions of extracts mixed, and do not indicate the presence of an excess of inhibitor in the extract from the culture grown with 5 mM cAMP. A direct effect of cAMP

was sought by adding the compound to the reaction mixtures used to measure agmatine ureohydrolase (Table: 4.2). With 10 mM cAMP the enzyme was inhibited, but this concentration of cAMP is greater than that required to repress the activities *in vivo* (Figure: 4.1). The preparation of the extract and its eventual use in the reaction mixtures resulted in a thousand fold dilution, and therefore carry over of cAMP would be insignificant unless it was tightly bound to agmatine ureohydrolase. The addition of up to 5 mM of cAMP to agmatine ureohydrolase did not decrease activity, and it is therefore most unlikely that repression *in vivo* is in fact due to a carry over of cAMP into the reaction mixtures for measuring agmatine ureohydrolase.

The effects of cAMP on β-galactosidase activity are those expected from the known mechanism of regulation of *lac* operon. Moreover, 1 mM cAMP increased the growth rate of LS853 but decreased the activities of agmatine ureohydrolase, ornithine decarboxylase and arginine decarboxylase. Therefore it was concluded that this effect of cAMP might be at the level of the regulation of these enzymes and not merely a general lowering of activity associated with decreased growth rates observed with higher concentrations of cAMP.

The observations reported in this chapter confirm the suggestion made by Wright and Boyle (1982) that agmatine ureohydrolase might be regulated similarly to ornithine decarboxylase and arginine decarboxylase. From the observations made in this chapter, it can also be concluded that the negative transcriptional regulation of agmatine ureohydrolase is either due to a direct

interaction between *speB* (gene for agmatine ureohydrolase) and the cAMP : cAMP receptor protein (CRP), or an indirect one by stimulating transcription of an uncharacterised transcriptional repressor of agmatine ureohydrolase. Alternatively, an inhibitor of agmatine ureohydrolase activity might be produced in limiting amounts which associate with the enzyme in an irreversible manner. Hence it would not be detected in the mixing experiments or by dialysis.

Antizymes of ornithine decarboxylase and arginine decarboxylase are induced very rapidly (Kyriakidis *et al.*, 1978). The observation made here that 5 mM cAMP causes maximal repression only after as long a period as 2.5 generations (Figure: 4.3) suggests that cAMP is not inducing an antizyme of agmatine ureohydrolase, and is consistent with a dilution after repression.

Chapter 5

Antagonistic Transcriptional Regulation of Agmatine Ureohydrolase by cAMP and Agmatine

5.1 Introduction:

The specific activities of agmatine ureohydrolase and arginine decarboxylase in crude extracts of *E.coli* cells grown on glucose are very similar and are about one tenth the specific activity of ornithine decarboxylase. (Table: 4.1). When *E.coli* is grown on a minimal medium with glucose as the sole carbon source the primary reaction for the production of putrescine is the decarboxylation of ornithine (Boyle *et al.*, 1977; Morris and Pardee, 1966). Ornithine is also an intermediate in the biosynthesis or arginine.

The transcription of enzymes for the biosynthesis of arginine is repressed by the arginine repressor (*argR* gene product) when *E.coli* cells are grown either in a rich medium, or in a minimal medium supplemented with arginine and glucose as the sole carbon source (Maas *et al.*, 1964). Transcriptional repression of these enzymes, coupled with the allosteric inhibition by arginine of the first enzyme of the arginine biosynthetic pathway, N-acetyl-glutamate synthetase (EC. 2.3.1.1), depletes the cell of metabolic intermediates involved in arginine biosynthesis. (Morris *et al.*, 1970; Sercarz and Gorini, 1964; Vogel *et al.*, 1963). Therefore arginine decarboxylase and agmatine ureohydrolase would become the primary enzymes for putrescine biosynthesis in *E.coli* growing in the presence of arginine.

Tabor *et al.* (1969), showed that arginine when supplied exogenously is

preferentially routed into putrescine biosynthesis and only a small fraction is directed into protein synthesis. Buch and Boyle (1985) have shown that arginine decarboxylase is localised in the inner periplasm of *E.coli*. Therefore, a large proportion of exogenously added arginine might enter the cell in the form of agmatine.

Consequently, the effect of adding agmatine to cultures of *E.coli* strains LS340, LS853 (Δ*cya*) and LS854-1 (Δ*cya*, Δ*crp*) growing with glucose as the sole carbon source was examined. Cells were grown for 4-5 generations to an absorbance at 575 nm of 0.8, harvested, lysed and the activity of agmatine ureohydrolase in crude extracts determined.

5.2 Results:

5.2.1 The effect of agmatine on the growth rates of LS340, LS853 and LS854-1, and on the activity of agmatine ureohydrolase:

The three strains of *E.coli*, LS340, LS853 (Δ*cya*) and LS854-1 (Δ*cya*,Δ*crp*) were examined. Cultures were grown in minimal medium with glucose as the sole carbon source. Replicate cultures were supplemented with different concentrations of agmatine. The results are shown in Figure: 5.1. The specific activity of agmatine ureohydrolase in LS340, LS853 and LS854-1 is shown in Table: 5.1. Agmatine increased the specific activity of agmatine ureohydrolase, while growth rate was unaffected.

Table 5.1

Specific activities of AUH in *E.coli* strains grown in MOPS glucose medium.

Strain	Phenotype	AUH
LS340	W.T.	1.1
LS853	CYA^-	2.1
LS854-1	CYA^-,CRP^-	1.8
HT328	ODC^-,ADC^-,AUH^-	<0.1
HT289	ODC^-,ADC^-,AUH^-	<0.1
HT328 /pKA5	ODC^-,ADC^+,AUH^+	11.4
HT289 /pKA5	ODC^-,ADC^+,AUH^+	10.9

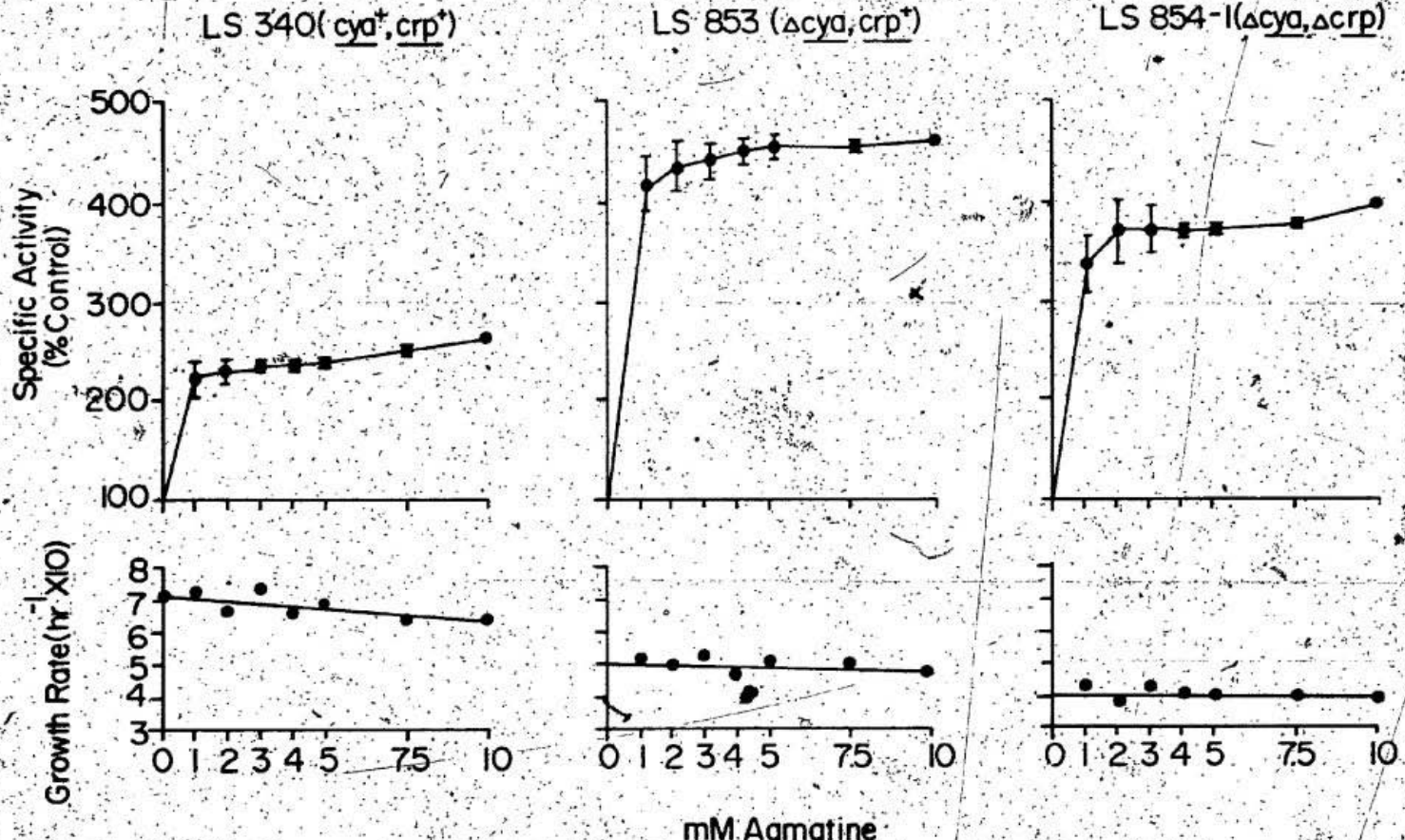
AUH = agmatine ureohydrolase

Activities are expressed as μmoles of product formed per minute per milligram protein (Section: 2.3.2).

Figure 5.1

The effect of agmatine on the growth rates and the activity of agmatine ureohydrolase in LS340, LS853 and LS854-1.

100% activity represents the specific activity of agmatine ureohydrolase without added agmatine (Table: 5.1). The bars represent 95% confidence values for each set from five separate experiments.


LS 340(cya+, crp+)
LS 853 (Δcya, crp+)
LS 854-1(Δcya, Δcrp)
Specific Activity (% Control)
500
400
300
200
100
Growth Rate(hr-1 X10)
8
7
6
5
4
3
0 1 2 3 4 5 7.5 10
mM Agmatine

5.2.2 The effect of increasing concentrations of agmatine in the presence of a fixed concentration of cAMP on the activities of agmatine ureohydrolase in LS340, LS853 and LS854-1:

The induction of agmatine ureohydrolase by agmatine and the greater induction observed in the Δ*cya* and Δ*cya*, Δ*crp* strains suggested that agmatine itself might be an inducer. To determine whether agmatine could derepress agmatine ureohydrolase activity after repression with cAMP, LS340, LS853 (Δ*cya*) and LS854-1 (Δ*cya*, Δ*crp*) were grown in the presence or in the absence of 1 mM cAMP. The concentration of added agmatine (up to 5 mM) was varied in each case. The results are shown in Figure: 5.2. Agmatine at higher concentrations relieved the decrease in activity of agmatine ureohydrolase caused by cAMP, in LS340 and LS853 and increased the activity of the enzyme in the Δ*cya*, Δ*crp* mutant (LS854-1).

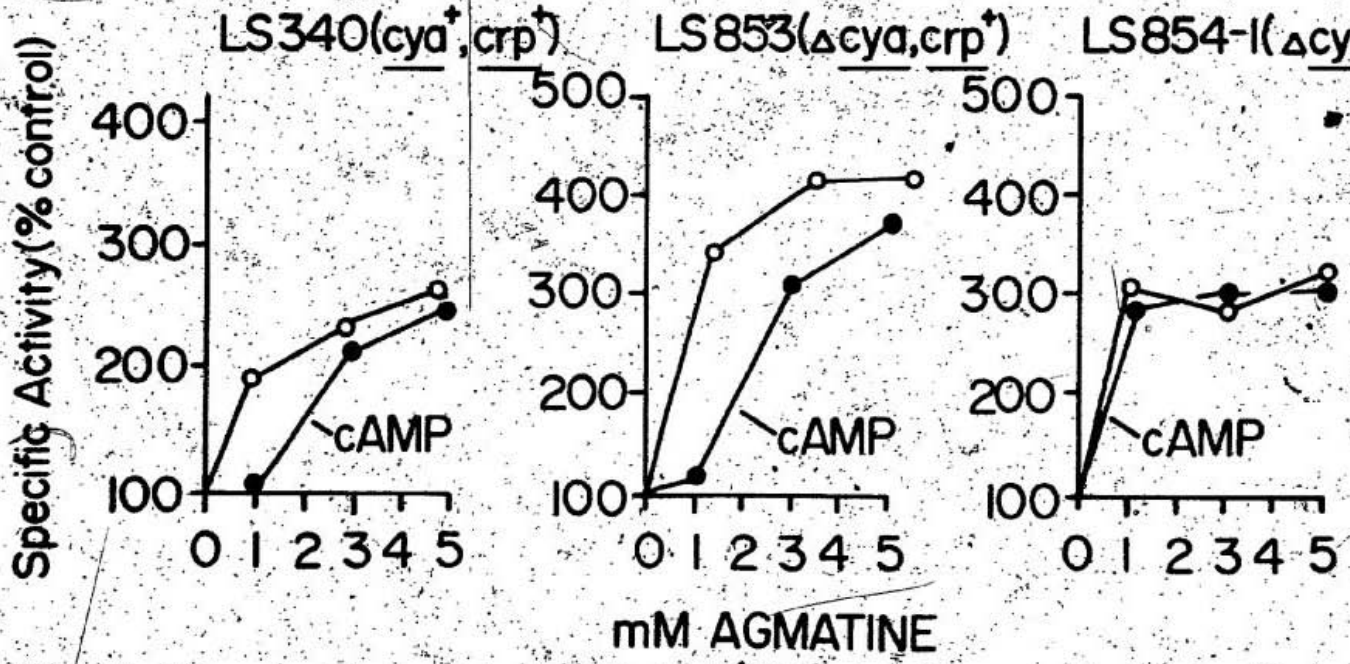
5.2.3 The effect of increasing concentrations of cAMP in the presence of a fixed concentration of agmatine on the activities of agmatine ureohydrolase in LS340, LS853 and LS854-1:

Experiments similar to those described in section 5.2.2 were performed to determine the effect of cAMP on agmatine ureohydrolase in cultures growing in the presence of a fixed concentration of added agmatine. *E.coli* strains LS340, LS853 (Δ*cya*) and LS854-1 (Δ*cya*, Δ*crp*) were grown with glucose as the sole source of carbon in the presence or absence of 1 mM agmatine, and

Figure 5.2

The effect of increasing concentrations of agmatine on agmatine ureohydrolase activity in the presence of 1mM cAMP.

100% activity represents the specific activity of agmatine ureohydrolase in the absence of cAMP and agmatine (Table: 5.1). (●)= with cAMP and (○) = without cAMP.


LS340(cya+, crp+)
LS853(Δcya, crp+)
LS854-1(Δcya; Δcrp)
Specific Activity (% control)
400
300
200
100
500
400
300
200
100
cAMP
0 1 2 3 4 5
mM AGMATINE

cAMP was added in concentrations up to 5 mM. (Figure: 5.3). cAMP at higher concentrations decreased the activity of agmatine ureohydrolase, even in the presence of agmatine in LS340 and LS853, but not in LS854-1.

5.2.4 The pH optimum, temperature optimum, temperature stability and K_m of induced and uninduced agmatine ureohydrolase in LS853; wild type strain, UW44 and in HT328 and HT289:

Ornithine decarboxylase and arginine decarboxylase each occur in two distinct forms in some strains of *E.coli*. Either a low pH or the presence of substrate of the enzyme in the growth medium induces the production of the 'biodegradative' form. The 'biosynthetic' form of each enzyme is produced constitutively. (Morris and Koffron, 1969). Agmatine ureohydrolase was not studied by earlier workers and so various strains of *E.coli* were examined to see if such forms of this enzyme could be found.

5.2.4.1 The pH optimum, temperature optimum, temperature stability and K_m of agmatine ureohydrolase in LS853 grown in minimal medium:

The pH optimum, temperature optimum, stability at 60° and K_m of agmatine ureohydrolase was determined is crude extracts of the Δ*cya* strain LS853, grown on glucose-minimal medium at pH 7.2 in the presence or absence of 5 mM agmatine. The results are shown in Table: 5.2 and Figure: 5.4. Two forms of agmatine ureohydrolase were not distinguishable.

Figure 5.3

The effect of increasing concentrations of cAMP on agmatine ureohydrolase activity in the presence of 1mM agmatine.

100% activity represents the specific activity of agmatine ureohydrolase in the absence of cAMP and agmatine (Table: 5.1). (●)= with agmatine and (○) = without agmatine.

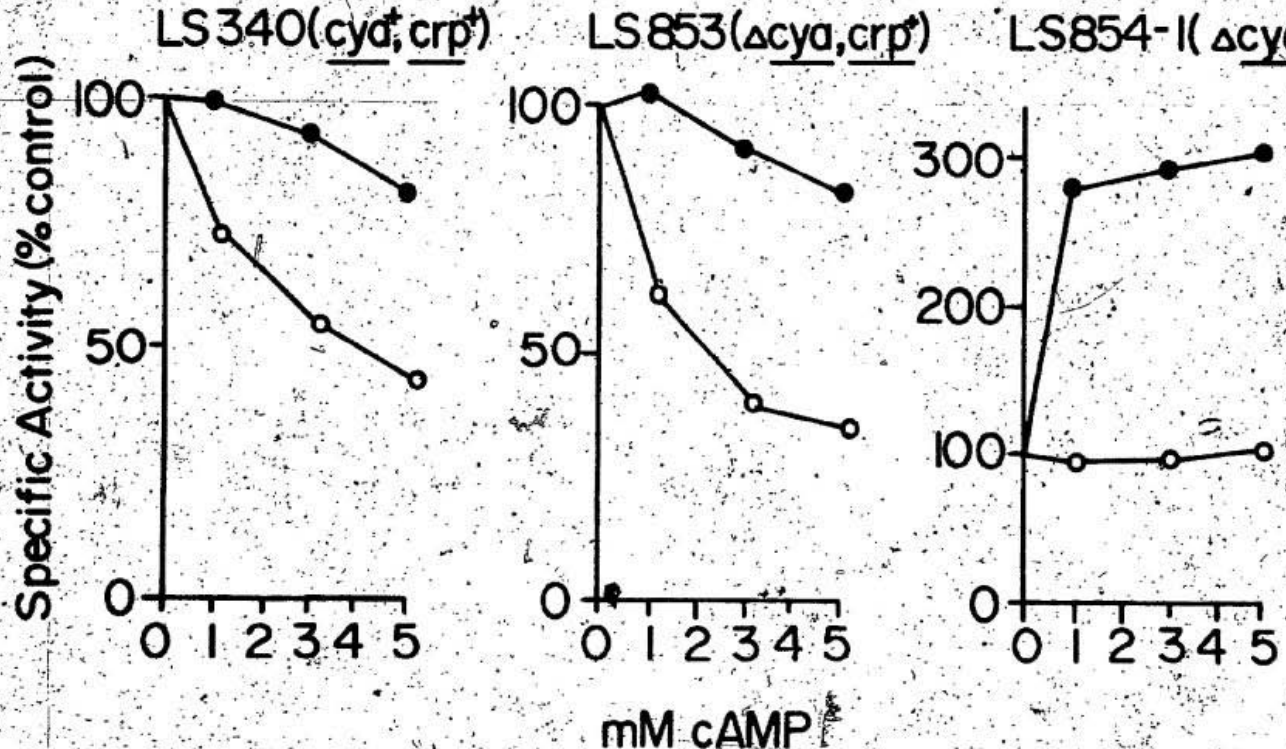

LS340(cya+, crp+)
LS853(Δcya, crp+)
LS854-1(Δcya, Δcrp)
Specific Activity (% control)
100
50
0
300
200
100
0 1 2 3 4 5
mM cAMP

Figure 5.4a

The effect of pH on the activity of agmatine ureohydrolase in crude extracts of LS853 and UW44 grown under different conditions.

A: LS853 grown in MOPS minimal medium (pH 7.2) containing agmatine, **B:** LS853 grown in MOPS minimal medium (pH 7.2), **C:** UW44 grown in LB- glucose medium (pH 5.5) or at pH 7.2 without agmatine, **D:** LS853 grown in LB-glucose medium (pH 5.5) containing agmatine and **E:** LS853 grown in LB- glucose medium (pH 5.5). Activities are expressed as units/ml. **A, B, D** and **E** the buffers used to obtain the desired pH were: (○)= HEPES, (●)= Tris, (◎)= Tricine and (◒)= MES.

In **C:** (○, ◎) represents pH 7.2 of the medium and (●, ◒) represents pH 7.2 of the medium. (○—○) and (●—●) = HEPES, (◎—◎) and (◒—◒) = Tris, and (●---●) and (○---○) = Tricine.

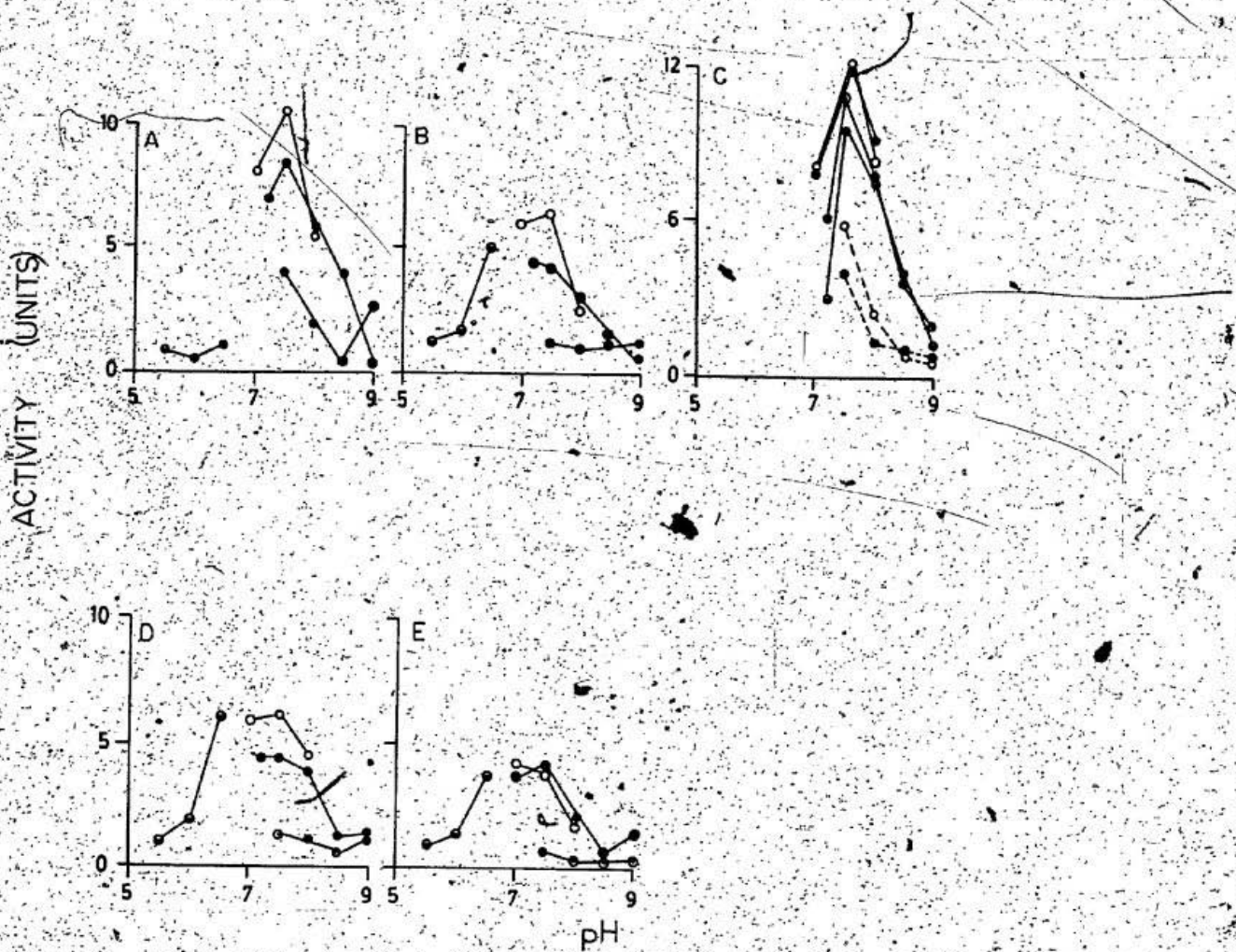

A
B
C
D
E
10
5
0
12
6
5
7
9
ACTIVITY (UNITS)
pH

Figure 5.4b

The stability at 60 ° C of agmatine ureohydrolase activity.

Aliquots of the crude extracts of strains grown under various conditions were removed at various time intervals indicated, from vessels incubated at 60° C, and the enzymic activity was measured according to the method described in Section 2.3.2. The extracts were from: (□)=LS853 grown in MOPS minimal medium, (○)=LS853 grown in MOPS minimal medium containing agmatine, (●)=LS853 grown in LB- glucose medium (pH 5.5) containing agmatine, and (◯)=UW44 grown in LB- glucose medium (pH 5.5) containing arginine, agmatine and ornithine. 100% activity represents 10 units /ml in crude extracts not incubated at 60 ° C. The results are summarised in Table 5.2.

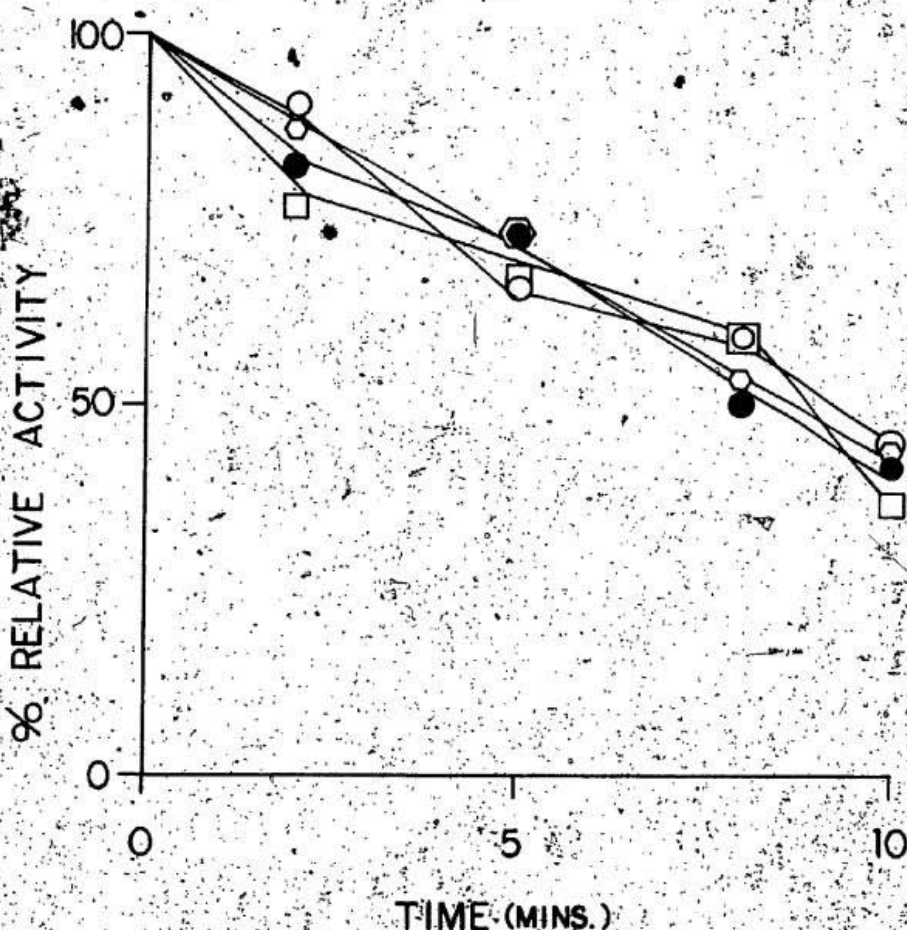

100
50
0
% RELATIVE ACTIVITY
0
5
10
TIME (MINS.)

Table 5.2

The pH optimum, temperature optimum, stability at 60 °C and K_m of agmatine ureohydrolase in crude extracts of *E.coli* strains under various growth conditions.

	LS853[1]	LS853[2]	LS853[3]	LS853[4]	UW44[5]
pH optimum	7.0-7.5	7.0-7.5	7.0-7.5	7.0-7.5	7.0-7.5
[a]Temperature optimum(°C)	42	42	42	42	42
[b]K_m(mM)	2.9	2.8	2.7	2.8	3.2
[c]T_{50} at 60 °C(mins.)	8.5	8.2	N.D	8.5	8.0

1 = MOPS minimal medium pH 7.2.
2 = 1 + agmatine.
3 = LB medium pH 5.5.
4 = 3 + agmatine.
5 = 3 + arginine, +ornithine, +agmatine.
a = Incubation temperatures were 20, 30, 37, 42, 50 and 60 °C.
b = K_m for agmatine is as determined from Lineweaver-Burk plot.
c = T_{50} at 60 °C (time required to decrease activity by 50%).
N.D = Not determined

5.2.4.2 The pH optimum, temperature optimum, temperature stability and K_m of agmatine ureohydrolase in LS853 grown in a complex and acidic medium:

The *cya* deletion strain, LS853, was grown in Luria broth, pH 5.5 with or without added 5 mM agmatine. The results of the pH optimum, temperature optimum, stability at 60° C and K_m of agmatine ureohydrolase are presented in Table: 5.2 and Figure: 5.4. Only one form of agmatine ureohydrolase was detected.

5.2.4.3 The pH optimum, temperature optimum, temperature stability and K_m of agmatine ureohydrolase in UW44 grown in a complex and acidic medium:

For comparison with the results obtained with LS853, similar experiments were carried out with a strain (UW44) known to produce two forms of ornithine decarboxylase and of arginine decarboxylase (Applebaum *et al.*, 1975). Strain UW44 was grown in Luria broth, pH 5.5, supplemented with 5 mM agmatine. The pH optimum, temperature optimum, stability at 60° C and the K_m of agmatine ureohydrolase in a crude extract were determined. The results are shown in Table: 5.2 and Figure: 5.4. *E.coli* strain UW44 did not exhibit distinguishable forms of agmatine ureohydrolase.

5.2.4.4 Does a separate gene encode an inducible, biodegradative form of agmatine ureohydrolase in a strain deleted for the gene encoding the biosynthetic enzyme?:

The previous results (Sections: 5.2.4.1, 5.2.4.2 and 5.2.4.3) provided no evidence for the existence of more than one form of agmatine ureohydrolase, but did not rule out the possibility that different genes subject to different forms of regulation produced essentially identical enzymes. Therefore mutants with the *speA*, *speB* and *speC* genes deleted were grown on minimal medium with glucose as the sole carbon source in the presence or absence of agmatine, and the activity of agmatine ureohydrolase was measured in cell extracts. For comparison the same strains transformed with plasmid pKA5 which carries the *speA* and *speB* genes was also examined (Table: 5.3). Agmatine ureohydrolase was induced only in the strain carrying the plasmid pKA5.

5.2.5 The time course of induction of agmatine ureohydrolase by agmatine, and the effect of chloramphenicol on induction:

E.coli strains LS340 and the *cya* deletion mutant LS853 were grown in the presence of glucose as the sole carbon source in minimal medium. Replicate cultures were grown for 3 generations to an absorbance at 575 nm of 0.2. Chloramphenicol (150 μg/ml), agmatine (1 mM) or chloramphenicol (150 μg/ml) and agmatine (1 mM) together were added to the cultures. Samples were withdrawn at various time intervals, and enzymic activity was deter-

Table 5.3

The effect of agmatine on the induction of agmatine ureohydrolase in HT328 and HT328 transformed with pKA5.

Strain	Phenotype	Condition	AUH
HT328	AUH^-	-	Nil
HT289	AUH^-	-	Nil
HT328	AUH^-	+	Nil
HT289	AUH^-	+	Nil
HT328 /pKA5	AUH^+	-	1.00
HT289 /pKA5	AUH^+	-	1.00
HT328 /pKA5	AUH^+	+	2.1
HT289 /pKA5	AUH^+	+	1.9

+ = +1mM agmatine
- = -agmatine
AUH = agmatine ureohydrolase

Activities are given relative to the activity of AUH in the transformed strain grown in the absence of agmatine (Table: 5.1). The genotypes of the strains are listed in Table: 2.1.

mined in crude extracts. The results are shown in Figure: 5.5. Chloramphenicol abolished the induction of agmatine ureohydrolase by agmatine.

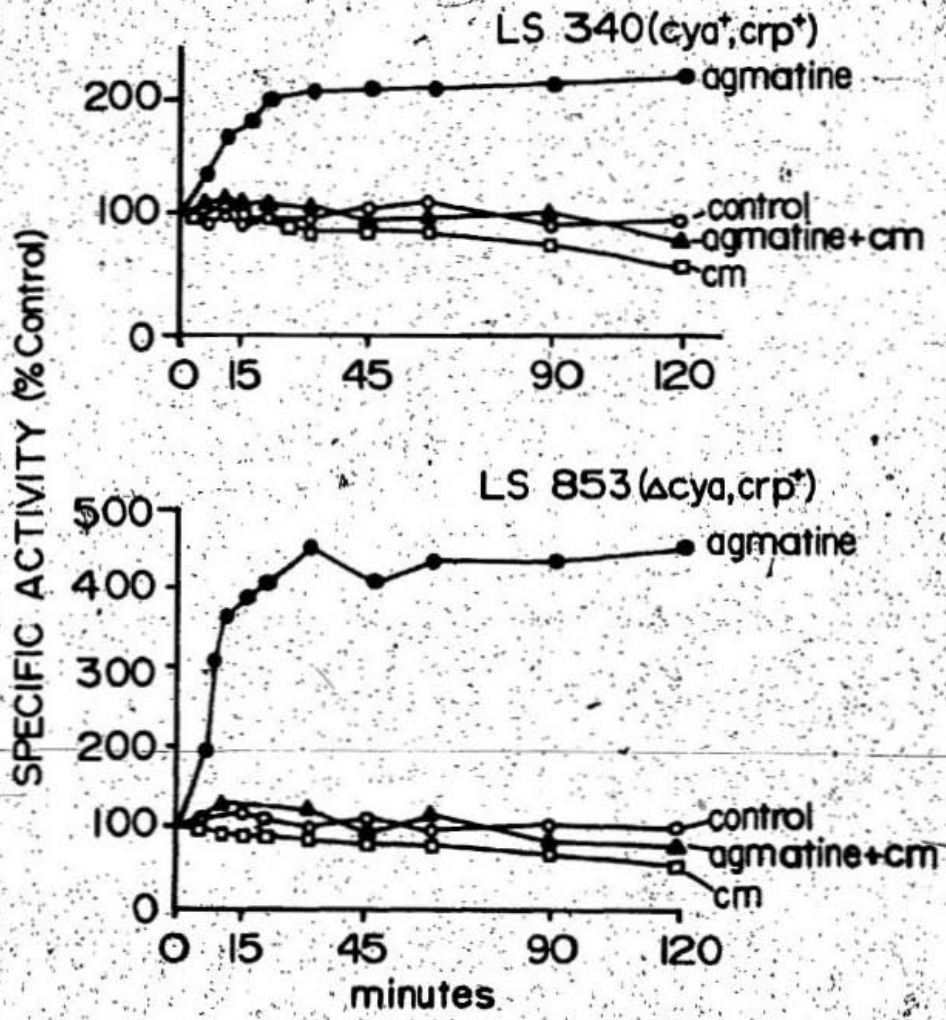
5.3 Discussion:

The results presented in this chapter demonstrate that agmatine ureohydrolase is induced by agmatine, and that agmatine and cAMP have antagonistic effects. Because the activities measured in induced and non-induced cultures had similar pH optima, temperature optima, stability at 60 °C and K_m values, it is probable that there was only one form of the enzyme. Neither was there evidence that more than one gene was involved in the production of this enzyme. Mutants deleted for the *speB* gene contained no detectable agmatine ureohydrolase activity and the enzyme could not be induced. It was inducible in the same strain carrying a plasmid bearing the *speB* gene. The induction of agmatine ureohydrolase was blocked by chloramphenicol, thereby indicating that the induction involved translation rather than activation of the enzyme. However, the possibility of chloramphenicol inhibiting the synthesis of an activator peptide of agmatine ureohydrolase cannot be ruled out. Synthesis of a peptide that inactivates the antizyme of ornithine decarboxylase has been reported. This is referred to as an anti-antizyme. (Heller *et al.*, 1983a; b).

Figure 5.5

The effect of chloramphenicol on the induction of agmatine ureohydrolase in LS340 and LS853 by agmatine.

Agmatine and chloramphenicol were added to exponentially growing cultures (zero time = 100%), and samples were removed for analysis at intervals indicated in the figure. (○)=without added agmatine, (●)=with added agmatine, (□)=with chloramphenicol and (▲)=with chloramphenicol and agmatine.


LS 340(cya+,crp+)
agmatine
control
agmatine+cm
cm
200
100
0
0 15 45 90 120
LS 853(Δcya,crp+)
agmatine
control
agmatine+cm
cm
500
400
300
200
100
0
0 15 45 90 120
minutes
SPECIFIC ACTIVITY (% Control)

Chapter 6

The Roles of Precursors and Products of Agmatine Metabolism, Arginine Repressor and Glutamine Synthetase in the Regulation of Agmatine Ureohydrolase, Arginine Decarboxylase and Ornithine Decarboxylase.

6.1 Introduction:

Tabor *et al.* (1969, 1976) demonstrated that putrescine and spermidine both repressed and inhibited ornithine decarboxylase and arginine decarboxylase. They also suggested that putrescine and spermidine might be co-repressors of ornithine decarboxylase, arginine decarboxylase and agmatine ureohydrolase. Morris *et al.* (1970), using a mutant carrying a partial block in arginine decarboxylase found that agmatine ureohydrolase may not be regulated by putrescine. Arginine decarboxylase and ornithine decarboxylase were derepressed when this mutant was grown in the presence of arginine. Addition of putrescine to the growth medium repressed both enzymes. Under both conditions, agmatine ureohydrolase activity was unchanged. Interpretation of these experiments was ambiguous due to the partial block in arginine decarboxylase. Therefore, a more extensive study was made of the inhibition of agmatine ureohydrolase in crude extracts and of the regulation of synthesis of agmatine ureohydrolase, arginine decarboxylase and ornithine decarboxylase by products and precursors of agmatine metabolism. Mutants blocked in various steps of putrescine biosynthesis were grown in minimal medium plus glucose, with or without the addition of ornithine, arginine, agmatine or putrescine. Prusiner *et al.* (1972), demonstrated that a number of enzymes involved in the biosynthesis of glutamine were negatively regulated by cAMP in *E.coli*. The

genes encoding the enzymes for the pathways of nitrogen assimilation and dissimilation are also regulated by glutamine synthetase (EC6.3.1.2) at the level of transcription (Magasanik and Stadtman, 1980). The regulation by glutamine synthetase appears to be dependent on the extent of adenylation of the enzyme. Since, agmatine could provide substrates for the enzymes in the nitrogen assimilatory pathways (Friedrich and Magasanik, 1978; Mercenier *et al.*, 1980; Roon and Barker, 1972) in *Streptococcus faecalis, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Klebsiella aerogenes*, the possibility exists that the regulation of agmatine ureohydrolase in *E.coli* is mediated by glutamine synthetase, and that the negative regulation of agmatine ureohydrolase by cAMP might be mediated via glutamine synthetase which itself is negatively regulated by cAMP.

To test these possibilities *E.coli* LS853 was grown under different conditions that have been shown to induce or repress glutamine synthetase and alter its states of adenylation and deadenylation, and the activities of agmatine ureohydrolase, ornithine decarboxylase and arginine decarboxylase were measured. Mutants of *E.coli* that either lack glutamine synthetase (ET8348) or that have fixed states of adenylation of glutamine synthetase (ET10643, ET8689, ET8411, and ET8053) were grown in minimal medium with glucose, and the activities of agmatine ureohydrolase, arginine decarboxylase and ornithine decarboxylase were measured.

The arginine repressor (*argR* gene product) regulates the enzymes of arginine biosynthesis at the level of transcription. Arginine is a co-repressor and therefore arginine in the growth medium might repress the enzymes of arginine biosynthesis, and itself be the source of putrescine. The hypothesis was made that arginine bound to the arginine repressor might be an inducer of enzymes for the conversion of arginine to putrescine and a repressor of the biosynthesis of putrescine from ornithine. To assess the possible role of the arginine repressor in the regulation of arginine decarboxylase, agmatine ureohydrolase and ornithine decarboxylase for pathway selection, *E.coli* strains EC145 (Δ*argR*) and EC146 (EC145 transformed with a pBR322 derived plasmid pEC1492 which carries the gene for *argR*), were grown in minimal medium with glucose. Replicate cultures were supplemented with arginine, and the activities of agmatine ureohydrolase, arginine decarboxylase and ornithine decarboxylase were measured in crude extracts.

6.2 Results:

6.2.1 Regulatory effects of arginine, agmatine, ornithine and putrescine on agmatine ureohydrolase, ornithine decarboxylase and arginine decarboxylase in LS340, LS853, and LS854-1:

The three strains of *E.coli*, LS340, LS853 (Δ*cya*) and LS854-1 (Δ*cya*, Δ*crp*), was examined. Cultures were grown in minimal medium with glucose, and replicate cultures were supplemented with 1 mM of arginine, ornithine,

agmatine or putrescine, and enzymic activities were measured. The specific activities of agmatine ureohydrolase, arginine decarboxylase and ornithine decarboxylase in various strains are given in Table: 6.1. The results of the effects of added polyamines and amino acids are shown in Table: 6.2. Ornithine, arginine and agmatine increased the specific activity of agmatine ureohydrolase and decreased the activities of ornithine decarboxylase and arginine decarboxylase.

6.2.2 Regulatory effects of some combinations of arginine, ornithine, agmatine and putrescine on agmatine ureohydrolase in LS340, LS853 and MA17:

In order to determine if arginine and ornithine induced the activity of agmatine ureohydrolase directly, the three strains of *E.coli* LS340 (wild type), LS853 (Δ*cya*), and MA17 (wild type) were grown in minimal medium with glucose. Replicate cultures were supplemented with various combinations of 1 mM arginine, ornithine, agmatine and putrescine, and agmatine ureohydrolase activity was measured in crude extracts. The results are shown in Tables: 6.2 and 6.3. Putrescine did not affect the activity of agmatine ureohydrolase. Agmatine increased activity of the enzyme to the same extent in the presence of putrescine. Arginine and ornithine did not increase the activity of agmatine ureohydrolase beyond the increase observed with agmatine alone.

Table 6.1

Specific activities of ODC, ADC and AUH in *E. coli* strains grown in MOPS glucose medium.

Strain	Phenotype	ODC	ADC	AUH
LS340	W.T.	11.3	1.1	1.0
LS853	CYA⁻	19.0	1.5	2.0
LS854-1	CYA⁻ CRP⁻	16.7	1.4	1.7
MA17	W.T.	7.8	0.7	0.8
MA135	AUH⁻	9.2	0.8	<0.1
MA163	ADC⁻	9.5	<0.1	1.0
MA255	ODC⁻ AUH⁻	<0.1	1.7	<0.1
ET8000	W.T.	10.9	1.4	1.5
ET8348	GS⁻	9.6	1.2	1.2

AUH = agmatine ureohydrolase
ADC = arginine decarboxylase
ODC = ornithine decarboxylase

Activities are expressed as μmoles of product formed per minute per milligram protein (Section: 2.3.2).

Table 6.2

The effects of agmatine, ornithine, arginine and putrescine on the activities of ODC, ADC and AUH in LS340, LS853 and LS854-1

Strain	Phenotype	+Agm ODC	+Agm ADC	+Agm AUH	+Orn ODC	+Orn ADC	+Orn AUH
LS340	W.T.	0.6	0.5	2.0	0.7	0.6	1.3
LS853	CYA⁻	0.5	0.6	3.8	0.6	0.6	1.3
LS854-1	CYA⁻,CRP⁻	0.6	0.5	2.9	0.6	0.6	1.3

Strain	Phenotype	+Arg ODC	+Arg ADC	+Arg AUH	+Put ODC	+Put ADC	+Put AUH
LS340	W.T.	0.6	0.7	1.7	0.5	0.4	0.9
LS853	CYA⁻	0.7	0.7	1.6	0.5	0.5	1.0
LS854-1	CYA⁻,CRP⁻	0.7	0.7	1.5	0.5	0.6	1.0

AUH = agmatine ureohydrolase
ADC = arginine decarboxylase
ODC = ornithine decarboxylase
Agm = Agmatine
Orn = Ornithine
Arg = Arginine
Put = Putrescine

The specific activities of ODC, ADC and AUH are given relative to that observed in MOPS minimal medium without added amino acids or polyamines (1mM) (=1.0)(Table 6.1).

Table 6.3

The effects of some combinations of agmatine, ornithine, arginine and putrescine on the activity of AUH in LS340, LS853 and MA17.

Strain	+Putrescine +Agmatine	+Ornithine +Arginine	+Agmatine +Ornithine +Arginine
LS340	1.7	1.7	1.8
LS853	3.3	2.6	3.3
MA17	1.7	1.4	1.7

The specific activity of AUH is given relative to that observed in the same strain in MOPS minimal medium without added amino acids or polyamines (1mM) (=1.0)(Table: 6.1).

6.2.3 Regulatory effects of arginine, agmatine, ornithine and putrescine on agmatine ureohydrolase, ornithine decarboxylase and arginine decarboxylase in MA17, MA135, MA163 and MA255:

Agmatine, ornithine and arginine added individually to cultures induced agmatine ureohydrolase, and all repressed ornithine decarboxylase and arginine decarboxylase. Putrescine repressed ornithine decarboxylase and arginine decarboxylase but did not affect agmatine ureohydrolase. Since induction of agmatine ureohydrolase by agmatine, ornithine and arginine was not additive when supplied together, it was thought that their effects may not be direct. To determine if the effects were direct or indirect, mutants blocked in the metabolism of arginine, ornithine and agmatine were examined. MA135 (*speB*, AUH^-), and MA163 (speA; ADC^-) were derived from the parent strain (MA17), by chemical mutagenisis, and MA255 (*speB*, *speC*; AUH^-, ODC^-) was derived from MA135 (*speB*; AUH^-) (Maas, 1972). Replicate cultures of these strains were supplemented with 1 mM concentrations of ornithine, arginine, agmatine or putrescine, and enzymic activities were measured in crude extracts. The results are presented in Tables 6.4 and 6.5. Arginine and ornithine did not increase the activity of agmatine ureohydrolase or decrease the activities of ornithine decarboxylase and arginine decarboxylase in mutants which were unable to convert these compounds to agmatine and putrescine respectively.

Table 6.4

The effects of agmatine, ornithine, arginine and putrescine on the activities of ODC, ADC and AUH in MA17, MA135, MA163 and MA255.

Strain	Genotype	+Agm ODC	+Agm ADC	+Agm AUH	+Orn ODC	+Orn ADC	+Orn AUH
MA17	W.T.	0.5	0.5	1.8	0.5	0.5	1.5
MA135	*speB*	0.9	1.0	–	0.5	0.7	–
MA163	*speA*	0.5	–	2.1	0.5	–	1.0
MA255	*speB,speC*	–	0.9	–	–	1.8	–

Strain	Genotype	+Arg ODC	+Arg ADC	+Arg AUH	+Put ODC	+Put ADC	+Put AUH
MA17	W.T.	0.6	0.6	1.5	0.5	0.5	0.9
MA135	*speB*	1.6	1.7	–	0.4	0.5	–
MA163	*speA*	1.5	–	1.1	0.5	–	1.0
MA255	*speB,speC*	–	0.9	–	–	0.5	–

AUH = agmatine ureohydrolase (encoded by *speB*)
ADC = arginine decarboxylase (encoded by *speA*)
ODC = ornithine decarboxylase (encoded by *speC*)
Agm = Agmatine
Orn = Ornithine
Arg = Arginine
Put = Putrescine

The specific activities of ODC, ADC and AUH are given relative to that observed in MOPS minimal medium without added amino acids or polyamines (1mM) (=1.0)(Table 6.1).

Table 6.5

The activities of ODC, ADC and AUH in *E.coli* polyamine auxotrophs cultured in MOPS glucose medium in the presence and absence of arginine.

Strain	Genotype	-Arg ODC	-Arg ADC	-Arg AUH	+Arg ODC	+Arg ADC	+Arg AUH
MA17	W.T.	1.0	1.0	1.0	1.0	1.0	1.0
MA135	*speB*	1.2	1.2	–	3.8	3.5	–
MA163	*speA*	1.2	–	1.0	3.3	–	0.8
MA255	*speB,speC*	–	2.5	–	–	4.1	–

AUH = agmatine ureohydrolase (encoded by *speB*)
ADC = arginine decarboxylase (encoded by *speA*)
ODC = ornithine decarboxylase (encoded by *speC*)
Arg = Arginine

Each activity is given as a fraction of that in the wild type under the same conditions. The actual specific activities in the absence of arginine are given in Table: 6.1 and in the presence of arginine the activities of AUH, ADC and ODC in the wild type strain are 1.1, 0.5 and 4.7 respectively.

6.2.4 The effect of arginine, ornithine and putrescine on the enzymic activity of agmatine ureohydrolase:

The concentrations of arginine, ornithine and putrescine were varied up to 10 mM in the reaction mixtures for measuring the activity of agmatine ureohydrolase. The results are presented in Figure 6.1. Ornithine increasingly inhibited the activity of agmatine ureohydrolase.

6.2.5 The role of glutamine synthetase in the regulation of polyamine biosynthesis:

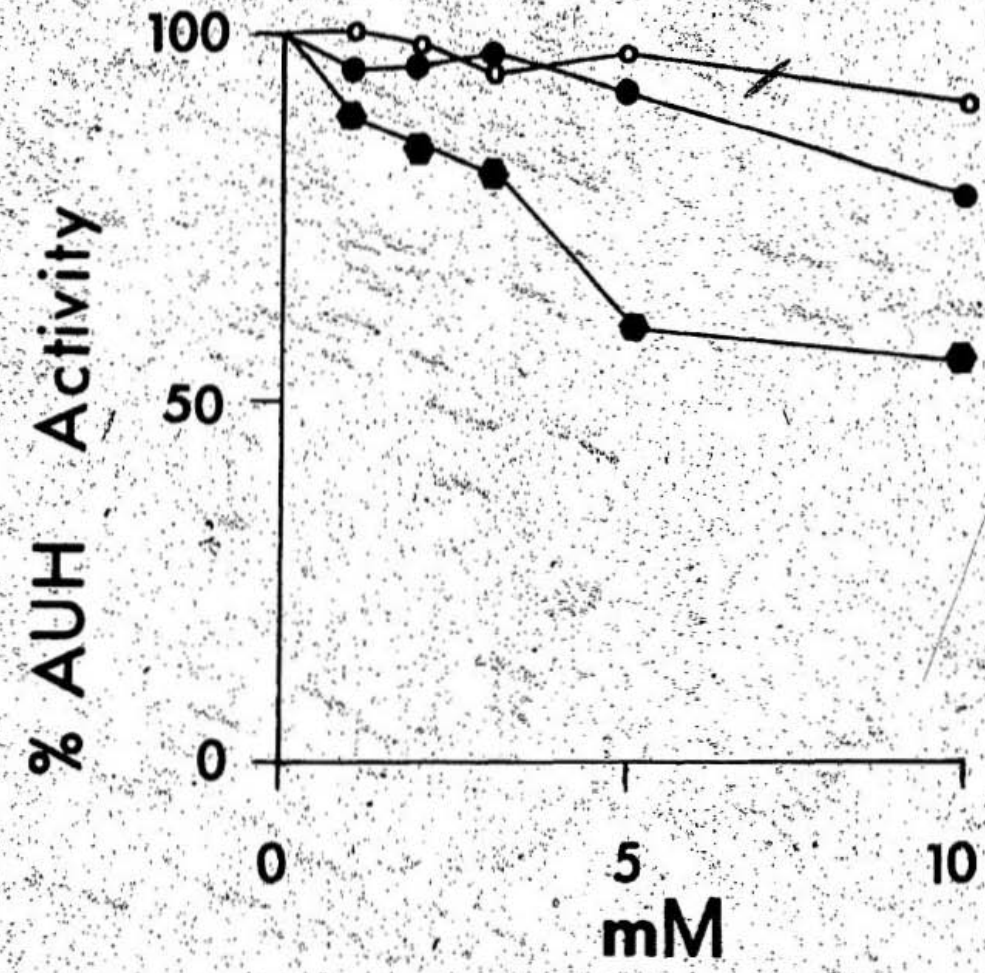
6.2.5.1 The effect of varying concentrations of ammonium chloride on the enzymic activities of ornithine decarboxylase, arginine decarboxylase and agmatine ureohydrolase:

In order to determine if glutamine synthetase was involved in the regulation of the transcription of enzymes for polyamine synthesis, and if the cAMP-CRP mediated repression of the putrescine biosynthetic enzymes is itself mediated through glutamine synthetase, the *cya* deletion strain LS853 was grown in minimal medium with glucose and with high (40 mM) or low (1 mM) concentrations of ammonium chloride. Stadtman *et al.* (1976), have shown that the adenylated form of glutamine synthetase is predominant in cultures grown in the presence of more than 20 mM ammonium chloride, while the non-adenylated form of glutamine synthetase is predominant in *E.coli* grown in the presence of less than 2 mM ammonium chloride. The activities of

Figure 6.1

The effects of ornithine, arginine and putrescine on the activities of agmatine ureohydrolase *in vitro*.

Putrescine, arginine and ornithine were added separately to the reaction mixtures for the measurement of enzymic activity at the concentrations indicated. 100% activity represents 18 units/ml of enzyme activity in crude extracts, in the absence of any added compound. (○)=putrescine, (●)=arginine and (●)=ornithine.


100
50
0
% AUH Activity
0
5
10
mM

agmatine ureohydrolase, ornithine decarboxylase and arginine decarboxylase and growth rates were measured. These are presented in Table: 6.6. The activities of agmatine ureohydrolase, ornithine decarboxylase and arginine decarboxylase were increased in strains grown in the presence of high concentrations of ammonium chloride

6.2.5.2 The activities of ornithine decarboxylase, arginine decarboxylase and agmatine ureohydrolase in strains mutant for the glutamine synthetase adenylating system:

The activities of ornithine decarboxylase, arginine decarboxylase, and agmatine ureohydrolase all increased during growth in the presence of a high concentration of NH_4^+, under which conditions glutamine synthetase is expected to be adenylated. In the presence of a low concentration of NH_4^+ when glutamine synthetase is deadenylated, cultures contained low activities of the enzymes. To determine if these were direct relationships, enzyme activities in strain ET8348 ($\Delta glnA$) was compared with the same strain transformed with the plasmid pLC(29-36) which carries *glnA*, and with the strains ET8000, ET10643, ET8689, ET8411 and ET8053. The latter are mutants in the system determining the adenylation of glutamine synthetase, and each has a fixed degree of adenylation which is independent of the concentration of NH_4^+ in the growth medium. Cultures were grown on minimal medium plus glucose, and the enzymic activities were measured in crude extracts. The results are

Table 6.6

The effects of varying concentrations of ammonium chloride on the activities of ODC, ADC and AUH in LS853.

State of GS (Ad/Dead)	Amm.Chl. (mM)	cAMP (mM)	ODC	ADC	AUH	Gen.Time (mins.)
Ad/Dead	20	-	19.1	1.6	2.0	120
Ad/Dead	20	2.5	11.7	1.1	1.2	75
Ad	40	-	25.6	2.1	3.2	110
Ad	40	2.5	16.1	1.4	1.9	70
Dead	1	-	12.9	1.1	1.3	165
Dead	1	2.5	8.7	0.8	0.8	120

The experiment was carried out in a minimal medium (M9), in which ammonium chloride was omitted. Ad/Dead represents Adenylated (Ad) and Deadenylated (Dead) Glutamine synthetase (GS) are in equal proportions. The activities of ODC, ADC and AUH are expressed as specific activities.

Amm.Chl. = Ammonium chloride
ODC = Ornithine decarboxylase
ADC = Arginine decarboxylase
AUH = Agmatine ureohydrolase
Gen.Time = Generation time

shown in Table: 6.7. Activities of the enzymes were increased in strains containing high levels of adenylated glutamine synthetase.

6.2.5.3 Does glutamine synthetase mediate repression of ornithine decarboxylase, arginine decarboxylase and agmatine ureohydrolase by cAMP?:

In order to establish if cAMP mediates repression of agmatine ureohydrolase, arginine decarboxylase and ornithine decarboxylase by repressing glutamine synthetase, the parent strain ET8000 and the Δ*glnA* strain ET8348 were examined for the activities of the enzymes. Cultures were grown on minimal medium containing glucose. Replicate cultures were supplemented with 2.5 mM cAMP or 2 mM agmatine. The results are given in Table: 6.8. cAMP decreased the activities of agmatine ureohydrolase, arginine decarboxylase, and ornithine decarboxylase to the same extent in the parent strain and in Δ*glnA* strain.

6.2.6 The role of the arginine repressor in the regulation of putrescine biosynthetic enzymes:

To determine if the arginine repressor was involved in the repression of ornithine decarboxylase and the derepression of arginine decarboxylase and agmatine ureohydrolase, cultures of *E.coli* strains EC145 (Δ*argR*) and EC146 (EC145 transformed with a plasmid pEC1492 carrying *argR*) were grown in minimal medium containing glucose. Replicate cultures contained arginine (1

Table 6.7

The growth rate and activities of ODC, ADC and AUH in *E.coli* strains mutant in GS or in the GS adenylating system.

Strain	Relevant phenotype	ODC	ADC	AUH	Growth rate (gen./hr.)
ET8000	Wild type	100	100	100	0.82
ET10643	High level aden.GS	122	156	168	1.15
ET8348	GS	90	87	88	0.75
ET8689	Low level fully aden.GS	127	142	145	1.08
ET8411	High level dead.GS	98	92	89	0.87
ET8053	V.low level aden.GS	101	104	100	0.81
ET8348/ pLC29-36	V.high level aden. & dead.GS	123	154	201	1.18

AUH = agmatine ureohydrolase
ADC = arginine decarboxylase
ODC = ornithine decarboxylase
GS = glutamine synthetase
aden. = adenylated
dead. = deadenylated
gen. = generations

50μg/ml of glutamine was supplied in the medium. 100% represents the specific activities of ODC, ADC and AUH in the wild type strain ET8000 (Table: 6.1).

Table 6.8

The effects of cAMP and agmatine on the activities of ODC, ADC and AUH in ET8000 and ET8348.

Strain	Growth condition	Phenotype	ODC	ADC	AUH
ET8000	–	W.T.	100	100	100
ET8000	2.5mM cAMP	W.T.	58	54	62
ET8000	2mM agmatine	W.T.	43	59	208
ET8348	–	GS⁻	100	100	100
ET8348	2.5mM cAMP	GS⁻	61	59	64
ET8348	2mM agmatine	GS⁻	54	58	181

AUH = agmatine ureohydrolase
ADC = arginine decarboxylase
ODC = ornithine decarboxylase
GS = glutamine synthetase
W.T. = wild type

100% represents the specific activities of ODC, ADC and AUH in ET8000 and ET8348 in the absence of cAMP or agmatine (Table 6.1). The strains were grown in MOPS- minimal medium containing 50μg/ml of glutamine.

mM). The enzymic activities of agmatine ureohydrolase, arginine decarboxylase and ornithine decarboxylase were measured. The results are shown in Table: 6.9. Activities of the enzymes did not show significant differences in the $ArgR^+$ and $ArgR^-$ strains. The enzymic activities of ornithine decarboxylase and arginine decarboxylase were decreased and that of agmatine ureohydrolase increased to approximately the same extent in both strains, when arginine was added to the growth medium.

6.3 Discussion:

In the parent strain LS340 agmatine ureohydrolase is induced by growth in the presence of ornithine, arginine and agmatine. In the same strain arginine decarboxylase and ornithine decarboxylase are repressed under the same conditions. Putrescine did not affect agmatine ureohydrolase, but repressed arginine decarboxylase and ornithine decarboxylase. In a *cya* deletion mutant (LS853) and a *cya*, *crp* deletion mutant (LS854-1) similar observations were made, except that in these strains agmatine ureohydrolase was more induced by agmatine. (Tables: 5.1 and 6.2). If arginine, ornithine and agmatine acted independently at sites on the promoter of the gene for agmatine ureohydrolase (*speB*), alone or in conjunction with regulatory proteins, then additive effects of combinations of these compounds might be expected. The observations reported in Table: 6.3 do not, however, provide evidence for independent activity.

Table 6.9

The effect of arginine on the activities of ODC, ADC and AUH in EC145 and EC146.

Strain	Growth condition	Phenotype	ODC	ADC	AUH
EC145		ArgR⁻	9.4	1.9	1.7
EC145	+1mM arginine	ArgR⁻	6.3	1.1	2.4
EC146		ArgR⁺	10.1	1.8	1.4
EC146	+1mM arginine	ArgR⁺	6.2	1.0	2.4

AUH = agmatine ureohydrolase
ADC = arginine decarboxylase
ODC = ornithine decarboxylase

The specific activities of ODC, ADC and AUH were determined in crude extracts of strains grown in MOPS minimal medium in the presence or in the absence of arginine.

In order to determine if the effects of arginine, ornithine or agmatine were direct, studies were made with strains carrying mutations for steps in putrescine biosynthesis. These were MA135 (*speB*; AUH^-), MA163 (*speA*; ADC^-) MA255 (*speB*, *speC*; AUH^-, ODC^-), and MA17 (wild type with respect to *speA*, *speB*, and *speC*). MA17 gave results identical to those obtained with LS340. (Table: 6.4). In the *speA* (ADC^-) mutant agmatine ureohydrolase was induced by agmatine but not by ornithine, arginine or putrescine, indicating that agmatine has a direct effect on the synthesis of agmatine ureohydrolase. Arginine decarboxylase and ornithine decarboxylase were repressed by putrescine, and the effects of agmatine, arginine and ornithine are consequent to their conversion to putrescine. Thus in the *speB* (AUH^-) mutant agmatine did not repress ornithine decarboxylase and arginine decarboxylase. In the *speA* (ADC^-) mutant arginine did not repress ornithine decarboxylase and in the *speB*, *speC* (AUH^-, ODC^-) mutant ornithine, arginine and agmatine did not repress arginine decarboxylase.

In these experiments it was noted that ornithine decarboxylase was elevated in the *speA* (ADC^-) mutant grown in the presence of arginine. Ornithine decarboxylase and arginine decarboxylase were elevated in the *speB* (AUH^-) mutant grown in the presence of arginine. In the *speB*, *speC* (AUH^-, ODC^-) mutant arginine decarboxylase activities were elevated. These observations may have a common basis, limitation of putrescine. Arginine represses enzymes for the synthesis of ornithine (Maas *et al.* 1964; Secarz and Gorini,

1964), and is a feedback inhibitor of these enzymes (Maas, 1961), and so growth in the presence of arginine would be expected to lower the intracellular concentrations of putrescine in *speA* (ADC^-) and *speB* (AUH^-) mutants. Putrescine would be extremely low or absent in the *speB*, *speC* (AUH^-, ODC^-) mutant. This conclusion is supported by the observation that addition of putrescine to the *speB*, *speC* mutant repressed arginine decarboxylase to the same extent as in the parent strain (MA17) (Table 6.5). The activities reported in Tables 6.2, 6.3 and 6.4 were not subject either to activation or inhibition due to direct effects of putrescine, ornithine or arginine carried into the reaction mixtures with the cell extract. These compounds were added separately to reaction mixtures in concentrations up to 10 mM. The activities of agmatine ureohydrolase are given in Figure 6.1. Ornithine caused a 40% inhibition at 5 mM, but at a 1 mM concentration none of the compounds caused a significant inhibition or activities. The estimated upper concentration of ornithine, arginine or putrescine carried from extracts of washed cells was 0.4 μM.

The strains carrying mutations in agmatine ureohydrolase, arginine decarboxylase and ornithine decarboxylase were obtained by chemical mutagenisis (Maas, 1972). These mutations have neither been mapped nor transferred to non-mutagenised strains, and it is possible that these mutants strains carry other lesions besides those indicated by the phenotypes expressed. Thus, it is possible that the *speB* mutant (MA135) might have lesions in the *speA* and

speC promoters or in the gene for a regulatory protein, which then would affect the regulation of these genes by putrescine, ornithine, arginine and agmatine. To test this possibility the effect of cAMP on arginine decarboxylase, ornithine decarboxylase and agmatine ureohydrolase in the parent strain MA17 and in the mutants MA135, MA163 and MA255 were examined. Cyclic AMP repressed the enzymes in the mutants as in the parent strain (Table 6.10). Cyclic AMP has been shown to negatively regulate the transcription of *speA* and *speC* (Wright and Boyle, 1982), and *speB* (Chapter 4). Furthermore, in the case of *speC*, the effect is due to interaction with the promoter (Boyle *et al.*, 1985). Although it is tempting to conclude that there are no lesion in the promoters of the genes of the mutants examined in this study, this possibility cannot be ruled out.

The effect of of ornithine, arginine, agmatine and putrescine on ornithine decarboxylase activity reported in this chapter (summarised in Figure 6.2) are in good agreement with the reported regulatory effects of these compounds on tetracycline resistance of the fused gene *speC:tet*^{R}, in which the *speC* promoter was fused to the structural gene for tetracycline resistance. (Boyle *et al.*, 1985). In *Klebsiella* agmatine ureohydrolase (called agmatinase) is positively regulated by deadenylated glutamine synthetase. Agmatine, and arginine, can be used in this species as the sole source of nitrogen and carbon (Friedrich and Magasanik, 1978). Although *E.coli* cannot use agmatine or arginine in this way (data not shown), it was of interest to see if agmatine

Table 6.10

Effect of cAMP on the activities of ODC, ADC and AUH in MA17, MA135, MA163 and MA255.

Strain	ODC +cAMP	ODC -cAMP	ADC +cAMP	ADC -cAMP	AUH +cAMP	AUH -cAMP
MA17	0.44	1.00	0.47	1.00	0.41	1.00
MA135	0.42	1.00	0.45	1.00	–	–
MA163	0.45	1.00	–	–	0.39	1.00
MA255	–	–	0.44	1.00	–	–

AUH = agmatine ureohydrolase
ADC = arginine decarboxylase
ODC = ornithine decarboxylase

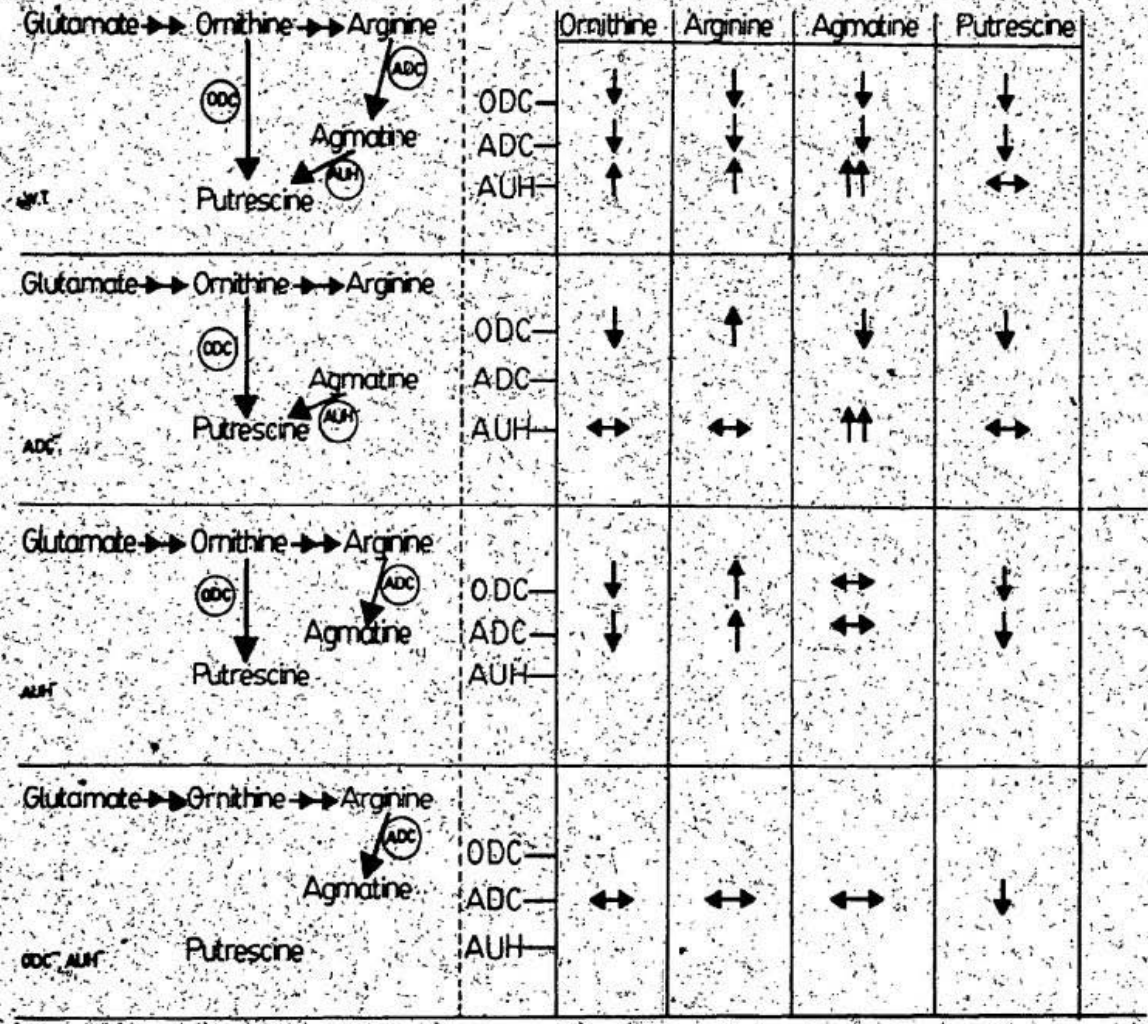
2.5 mM cAMP was added to the growth medium. 1.00 represents the relative specific activity in the absence of cAMP. The specific activities are given in Table 6.1.

Figure 6.2

Schematic diagram of the regulation of ODC, ADC and AUH by ornithine, arginine, agmatine and putrescine

ODC = ornithine decarboxylase
ADC = arginine decarboxylase
AUH = agmatine ureohydrolase

The figure shows the effects of ornithine, arginine agmatine and putrescine on the activities of ODC, ADC and AUH in mutants blocked in different steps in the biosynthesis of putrescine. The phenotype of the mutants is shown on the left of the figure. (↔)=No effect, (↑)=Induced, (↓)=Repressed.


Glutamate
Ornithine
Arginine
ODC
ADC
Agmatine
AUH
Putrescine
wt
ADC
ODC
AUH
Ornithine
Arginine
Agmatine
Putrescine

ureohydrolase, ornithine decarboxylase and arginine decarboxylase were subject to a similar mode of regulation, and if glutamine synthetase mediated the repression observed with cAMP:CRP. Cultures of the *cya* mutant LS853 were grown in media containing high or low concentrations of NH_4Cl which respectively cause adenylation and deadenylation of glutamine synthetase (Stadtman *et al.*, 1976, Magasanik and Stadtman, 1980). The activities of arginine decarboxylase, ornithine decarboxylase and agmatine ureohydrolase were measured and are shown in Table: 6.6. High NH_4^+ concentrations (adenylated glutamine synthetase) elevated the activity of all three enzymes, and cAMP repressed the activities in cells with either adenylated or deadenylated glutamine synthetase. The set of mutants which, when grown on a standard medium, have different states of adenylation of glutamine synthetase was also examined. The growth rate of these strains, and their activities of ornithine decarboxylase, arginine decarboxylase and agmatine ureohydrolase are given in Table: 6.7. The specific activities of all three enzymes increased with the reported levels of adenylation of glutamine synthetase in these mutants. A *glnA* (glutamine synthetase) deletion mutant and one having deadenylated glutamine synthetase had the lowest enzymic activities. The same *glnA* deletion mutant transformed with the plasmid pLC(29-36) (which carries *glnA*) had the highest activities of these enzymes. All of these results are consistent with adenylated glutamine synthetase acting as a positive regulator of the *speA*, *speB* and *speC* genes. Since cAMP repressed agmatine ureohydrolase, arginine decarboxylase and ornithine

decarboxylase to the same extent in the Δ*glnA* strain (ET8348) and in the parent strain (ET8000), it can be concluded that cAMP does not mediate repression through glutamine synthetase (Table: 6.8).

The arginine repressor does not appear to have any marked effect on the activities of arginine decarboxylase, ornithine decarboxylase and agmatine ureohydrolase (Table: 6.9). Although it represses enzymes involved in the conversion of glutamate to ornithine, it does not repress ornithine decarboxylase, and it does not induce arginine decarboxylase and agmatine ureohydrolase. Although the intracellular concentration of arginine determines the pathway of putrescine synthesis (ornithine decarboxylase versus arginine decarboxylase and agmatine ureohydrolase) (Morris *et al.*, 1970), the pathway selection probably is not mediated through the arginine repressor.

Chapter 7

Localisation of the *speB* Gene in a Chromosomal Fragment Cloned in a Plasmid Vector, and its Sub-Cloning, and Direction of Transcription

7.1 Introduction:

Plasmid pKA5, derived from pBR322 and plasmids of the Clarke-Carbon collection (Clarke and Carbon, 1976; Hafner *et al.*, 1979), was known to contain the *speA*, *speB* and *metK* genes, which respectively specify arginine decarboxylase, agmatine ureohydrolase, and S-adenosyl methionine transferase (Tabor *et al.*, 1983; Boyle *et al.*, 1984). Plasmid pKA12, also derived from pBR322 and carrying a smaller insert than pKA5, contains *speA* and *metK* (Boyle *et al.*, 1984) (Figure: 7.1). Plasmid pKA10, another derivative of pKA5, carries a smaller insert than pKA5 and pKA12, and does not carry *speA*, *speB* or *metK* (D. Markham, personal communication) (Figure: 7.1). These three plasmids were used as the starting point for the localisation of the *speB* gene. The two plasmids pKA12 and pKA10 carried between themselves the entire 7.8 kb insert of the parent plasmid pKA5 (Figure: 7.1). The plasmid pKA10 carried the *EcoRI-BalI* segment of the insert in pKA5 which is proximal to the amp^R gene of the vector (pBR322) DNA. The plasmid pKA12 carried the rest of the insert (*BalI-EcoRI*) from the parent plasmid pKA5 (Figure: 7.1). Since agmatine ureohydrolase was not expressed in Δ*speB* strains of *E.coli* containing either pKA10 or pKA12, it can be concluded that the *BalI* restriction site lies within the *speB* gene (Figure: 7.1). Using the restriction enzyme *BamHI* the 1.8 kb segment of the insert was cloned into the *BamHI* site of the phage vec-

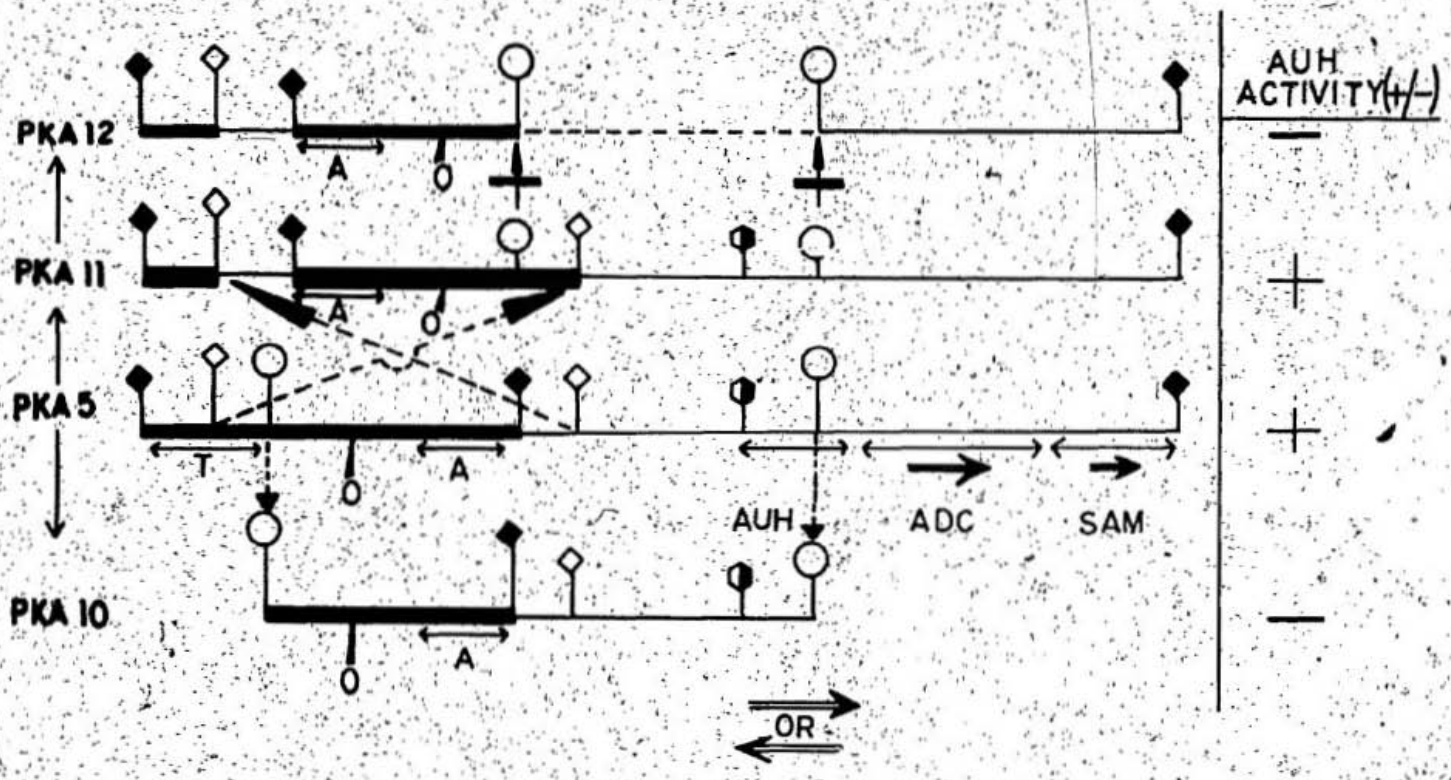
Figure 7.1

The restriction maps and the construction of pKA10, pKA11 and pKA12 from pKA5 and the complementation of AUH activity in *speB* deletion mutants.

Direction of cloning = ---→
Direction of transcription= ⇒
Extent of the gene= ⟷
Origin of replication = O
Tetracycline resistance = T
Ampicillin resistance = A
1 Kilo base pair = 1K (scale)

AUH = agmatine ureohydrolase activity (encoded by *speB*), ADC = arginine decarboxylase (encoded by *speA*) and ODC = ornithine decarboxylase (encoded by *speC*). (+) represents ability to confer AUH activity to *speB* deletion mutants and (−) represents inability to confer AUH activity to *speB* deletion mutants. (◇)=*SalI*, (◆)= *EcoRI*, (◨)=*HindIII*, (○)=*BalI*.

(Adapted from Boyle *et al.*, 1984)


PKA 12
PKA 11
PKA 5
PKA 10
A
O
T
AUH
ADC
SAM
OR
1K
AUH ACTIVITY (+/-)
+
+
-

tor M13mp7. A *BalI* restriction site was contained within this *BamHI* segment. This segment was also cloned into the *BamHI* site of pBR322. These recombinant plasmids elevated agmatine ureohydrolase activity in the cells that contained them, and hence carried *speB*.

In order to determine where the *speB* gene began in the *BamHI* insert the plasmid pKA5 was cleaved with *SmaI* and then digested with exonuclease *Bal31* and religated. The family of deleted and ligated plasmids were used to transform *E.coli* HT328 ($\Delta speA$, $\Delta speB$, $\Delta speC$). tetracycline resistant transformants were selected, and these were screened for clones containing agmatine ureohydrolase activity. The latter were then screened to determine the extent of each deletion.

It was concluded that the *HincII* segment within the *BamHI* segment carried a transcribed portion of the *speB* gene in plasmid pKA5, and this was cloned into pBR322, at the *XorII* site. This construct was later used in the hybridisation experiments in order to assess *speB* messenger RNA concentrations after repression by cAMP or induction by agmatine.

In order to determine the direction of transcription, the two fragments *SalI-BalI* and *BamHI-BalI* (Figure 7.3) were cloned separately into the promoter cloning site of the promoter cloning vectors pMC1403 and and pKO11. These vectors carry structural genes for *lacZ* (β-galactosidase) and *galK* (galactokinase) respectively. Since the *BalI* site of the insert in plasmid pKA5 lies

within *speB*, the direction of transcription is either *SalI*----->*BalI* or *BamHI*---->*BalI*. The cloning strategy was to ligate the *BalI* side proximal to the structural genes in the cloning vector.

7.2 Results:

7.2.1 Sub-cloning of the DNA segment carrying speB into pBR322 and M13mp7:

7.2.1.1 Sub-cloning into M13mp7:

Plasmid pKA5 was digested with the restriction enzyme *BamHI* which generated 5 fragments (Table: 7.1a). These fragments were cloned into the vector M13mp7 which had been previously hydrolysed with the same restriction enzyme. The 5' end phosphate of the vector was removed by digesting the linearised vector with bacterial alkaline phosphatase. The ligated mixture was introduced into JM103 by transformation and the transformants were mixed with soft agar containing X-gal and IPTG and spread on YT agar plates. White plaques, indicating insertional inactivation of the *lacZ* gene carried on the phage by DNA fragments generated by the *BamHI* restriction of pKA5, were screened for elevated levels of agmatine ureohydrolase activity (Figure: 7.2 and Table: 7.2). The insert size in the replicative form of the recombinant phage, which conferred elevated levels of agmatine ureohydrolase to the host strain, was determined.

Table 7.1
Lengths of the restriction fragments of plasmids pKA5, pKA10, pKA14, pKA15, pAUL and pAUG.

7.1a

Plasmid	*BamHI*	*HincII*
pKA5	6,241	3,239
	1,836	3,064
	1,590	1,959
	1,476	1,478
	693	1,381
		608

7.1b

Res.Enz.	pKA10	pKA14	pKA15	pAUL	pAUG
HindIII					4,665
					1,687
PstI	2,947	4,235	4,212		4,106
	2,223	1,970	677		2,229
	967				
BamHI		4,281		~12,200	
		1,847		1,130	
BalI		4,075	2,564		
		1,996	2,333		
PvuII	3,207				
	2,848				
BglI	3,186				
	2,869				
PvuII/	1,674				
BglI	1,491				
	1,414				
	1,086				
PstI/	2,947				
EcoRI	2,223				
	541				
	456				
PstI/	2,947				
BalI	1,983				
	967				
	286				

Table 7.1

The length of the fragments are in base pairs, and were determined using the NA2 nucleic acid analyser (BRL). The combination of restriction enzymes used to digest the plasmid DNA are shown.

Figure 7.2

Schematic diagram of shot-gun cloning of the *BamHI* fragments of plasmid pKA5 into the *BamHI* site of M13mp7.

The recombinants (white plaques) were selected on YT agar plates containing IPTG and X-gal. The replicative form of the bacteriophage DNA was isolated from infected host strains that had elevated AUH activity and the insert size was determined. (O)= origin of replication, *lacZ* encodes β-galactosidase and *speB* encodes agmatine ureohydrolase (AUH).

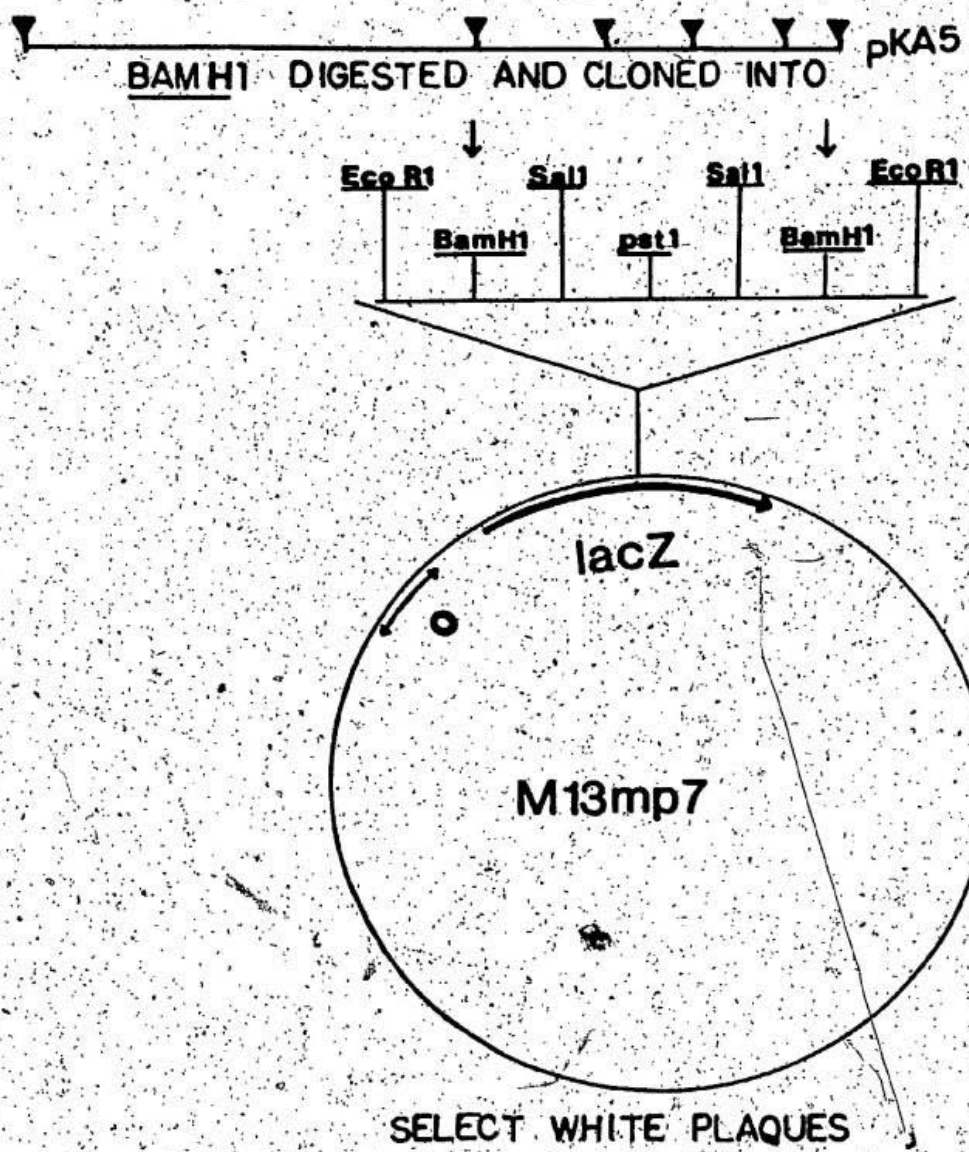

pKA5
BAMH1 DIGESTED AND CLONED INTO
Eco R1
Sal1
Sal1
Eco R1
BamH1
pst1
BamH1
lacZ
M13mp7
SELECT WHITE PLAQUES

Table 7.2

The activity of agmatine ureohydrolase, and *BamHI* fragment sizes of the RF forms isolated from recombinant M13mp7, white plaques.

Strain	AUH specific activity	*BamHI* fragment sizes (base pairs)
JM103/M13mp7 (Blue plaque)	0.8	7495
JM103/M13mp7/AUH1 (White plaque)	4.0	7550/1739
JM103/M13mp7/AUH2 (White plaque)	4.3	7461/1743

AUH = agmatine ureohydrolase

Phages from white or blue plaques were used to infect JM103, and agmatine ureohydrolase activity was measured in crude extracts. The replicative form (RF), was isolated from the phage infected cells according to Section: 2.5 and was digested with *BamHI*.

7.2.1.2 Sub-cloning into pBR322:

The 1.8 kbp *BamHI* fragment which carried the *speB* gene, as determined from Section: 7.2.1.2, was isolated from low melting agarose gels (Section: 2.15), in which the *BamHI* digested plasmid pKA5 was previously separated, and ligated into the *BamHI* site of the plasmid vector pBR322. The ligated mixture was used to transform the *speA, speB, speC* deletion strain (HT328) (Section: 2.6). The transformants were screened for ampicillin resistance, tetracycline sensitivity and the presence of agmatine ureohydrolase activity. (Table: 7.3 and Figure: 7.3). One clone pKA14 was selected for further work. Clones containing this plasmid did not express arginine decarboxylase and ornithine decarboxylase activities. This plasmid was further analysed by digesting it with *BalI, BamHI* or *PstI*. The results are given in Table: 7.1b. The orientation of the insert thus determined is shown in Figure: 7.3.

7.2.2 The localisation of the speB gene:

The plasmid pKA5 was restricted at a unique site *SmaI* (proximal to the amp^R gene of the vector DNA). The linearised plasmid was digested with 0.5 units of exonuclease *Bal31* (Section: 2.9). Samples were withdrawn at intervals, and the reaction was stopped by adding EDTA to a final concentration of 15 mM. The DNA was then precipitated and digested with S1 nuclease to remove single stranded ends (Section: 2.11), and was then reprecipitated and ligated. The ligated mixtures obtained after digestion with *Bal31* for various

Table 7.3

The *BamHI* fragment sizes of plasmids, and the activities of ODC, ADC and AUH in ampicillin resistant clones of HT328 transformed with the ligated mixture.

Strain	ODC	ADC	AUH	*BamHI* fragment sizes (base pairs)
HT328(Tet^s,Amp^s)	<0.1	<0.1	<0.1	-
HT328/ pBR322(Tet^r,Amp^r)	<0.1	<0.1	<0.1	4348
HT328/ pKA14(Tet^s,Amp^r)	<0.1	<0.1	13.1	4348/1763
HT328/ pKA14-1(Tet^s,Amp^r)	<0.1	<0.1	9.0	4348/1763
HT328/ pKA14-2(Tet^s,Amp^r)	<0.1	<0.1	14.6	4348/1763
HT328/ pKA14-3(Tet^s,Amp^r)	<0.1	<0.1	9.3	4348/1763
HT328/ pKA14-4(Tet^s,Amp^r)	<0.1	<0.1	<0.1	4348

ODC = ornithine decarboxylase
ADC = arginine decarboxylase
AUH = agmatine ureohydrolase

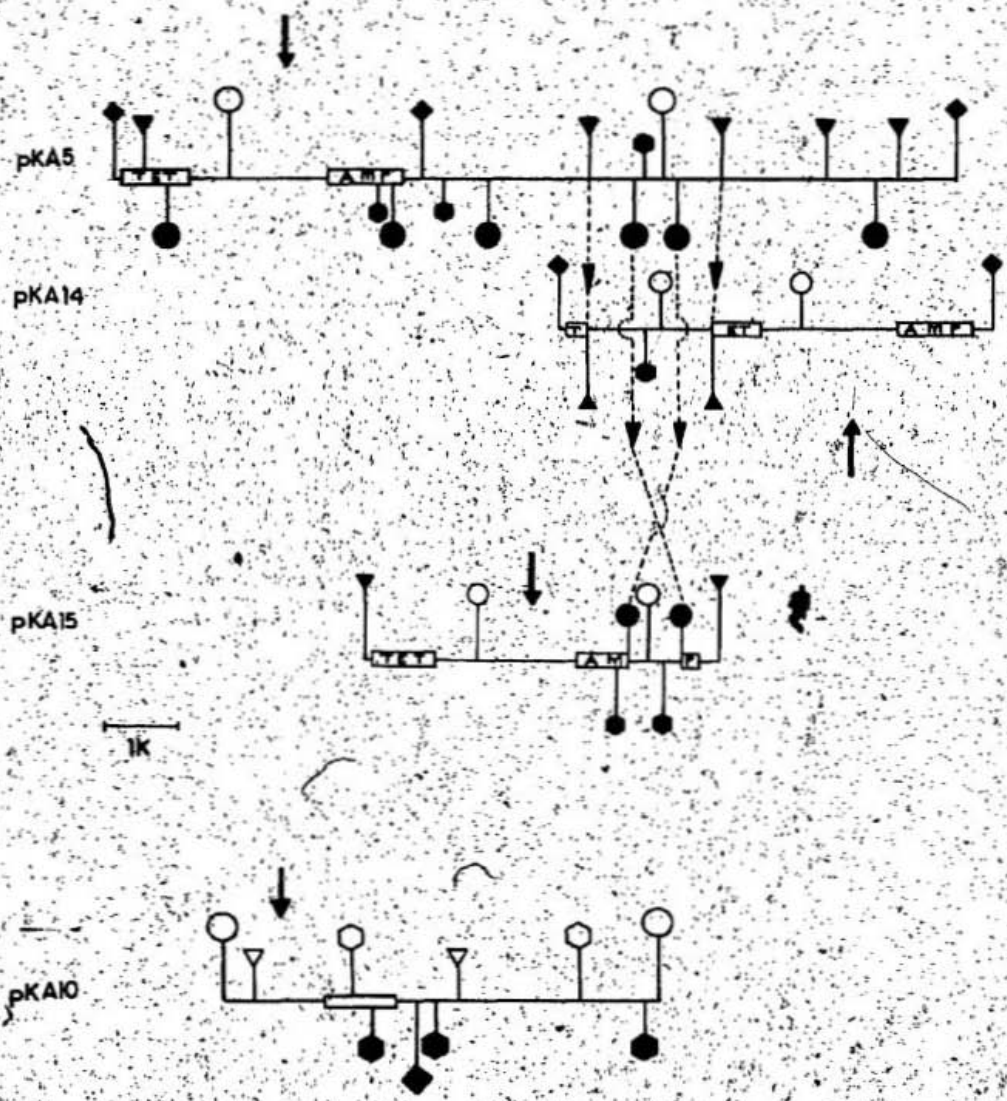
The 1.8 k base pair fragment of plasmid pKA5 was extracted from agarose gels and cloned into the *BamHI* site in pBR322. Tet^s colonies were screened for elevated AUH activity in Δ*speB* strain (HT328). Specific activities were determined in crude extracts.

Figure 7.3

The restriction maps of pKA5, pKA10, pKA14 and pKA15 and the construction of pKA14 and pKA15.

Direction of cloning = ----►
Origin of replication = ➡
Tetracycline resistance gene = TET
Ampicillin resistance gene = AMP
1 Kilo base pair = 1K

(◆)=*EcoRI*, (◇)=*SalI*, (▽)=*PvuII*, (○)=*BalI*, (●)=*HincII*, (▼)=*BamHI*, (⬡)=*BglI* and (⬢)=*PstI*. The restriction fragment sizes are given in Table: 7.1.


pKA5
TET
AMP
pKA14
TET
AMP
pKA15
TET
AMP
1K
pKA10

times were used separately to transform the *speB* deletion strain of *E.coli* (HT328). The transformants were selected for tetracycline resistance and then tested for ampicillin resistance. Twenty randomly selected tetracycline resistant clones were also analysed for agmatine ureohydrolase activity. The percentage of tetracycline resistant colonies which had ampicillin resistance and which had agmatine ureohydrolase activity is shown in Figure: 7.4. The rate of digestion of the DNA by *Bal31* was monitored, in a parallel assay set up simultaneously, by analysing the rate of change in the molecular weight of the 2.0 kb fragment of the HindIII digested lambda DNA (Figure: 7.5). A schematic diagram showing the extent of digestion of pKA5 with *Bal31* exonuclease is given in Figure: 7.6. The ampicillin resistant gene of the vector was completely inactivated following 8.5 minutes of digestion, while no agmatine ureohydrolase activity was detected in strains transformed with the digested plasmid following 6 minutes of exonuclease digestion. One transformant from each time period was selected, the plasmid was extracted and restricted with *BalI*. The rate of change in the molecular weight of the 6.15 kbp *BalI* fragment is shown, as a function of time in Figure: 7.6.

7.2.3 Cloning of the coding region of *speB* into pBR322:

Boyle *et al.* (1984) deduced from their experiments with minicells that the 38,000 dalton protein expressed by the plasmid pKA5 is agmatine ureohydrolase. In Chapter: 3 the molecular weight of the monomeric agmatine ureohy-

Figure 7.4

The percentage of tetracycline resistant clones carrying ampicillin resistance and agmatine ureohydrolase activity as a function of the times of digestion of plasmid pKA5 with *Bal31*.

Bal31 digested linear plasmid pKA5 was used to transform a *speB* deletion strain of *E.coli* and were selected on tetracycline containing LB-glucose agar plates. At each time point indicated, ampicillin resistance was determined in 84 tetracycline resistant transformants, and agmatine ureohydrolase activity was determined in 20 randomly selected tetracycline resistant clones. (○)=agmatine ureohydrolase activity containing clones and (●)= ampicillin resistant clones.

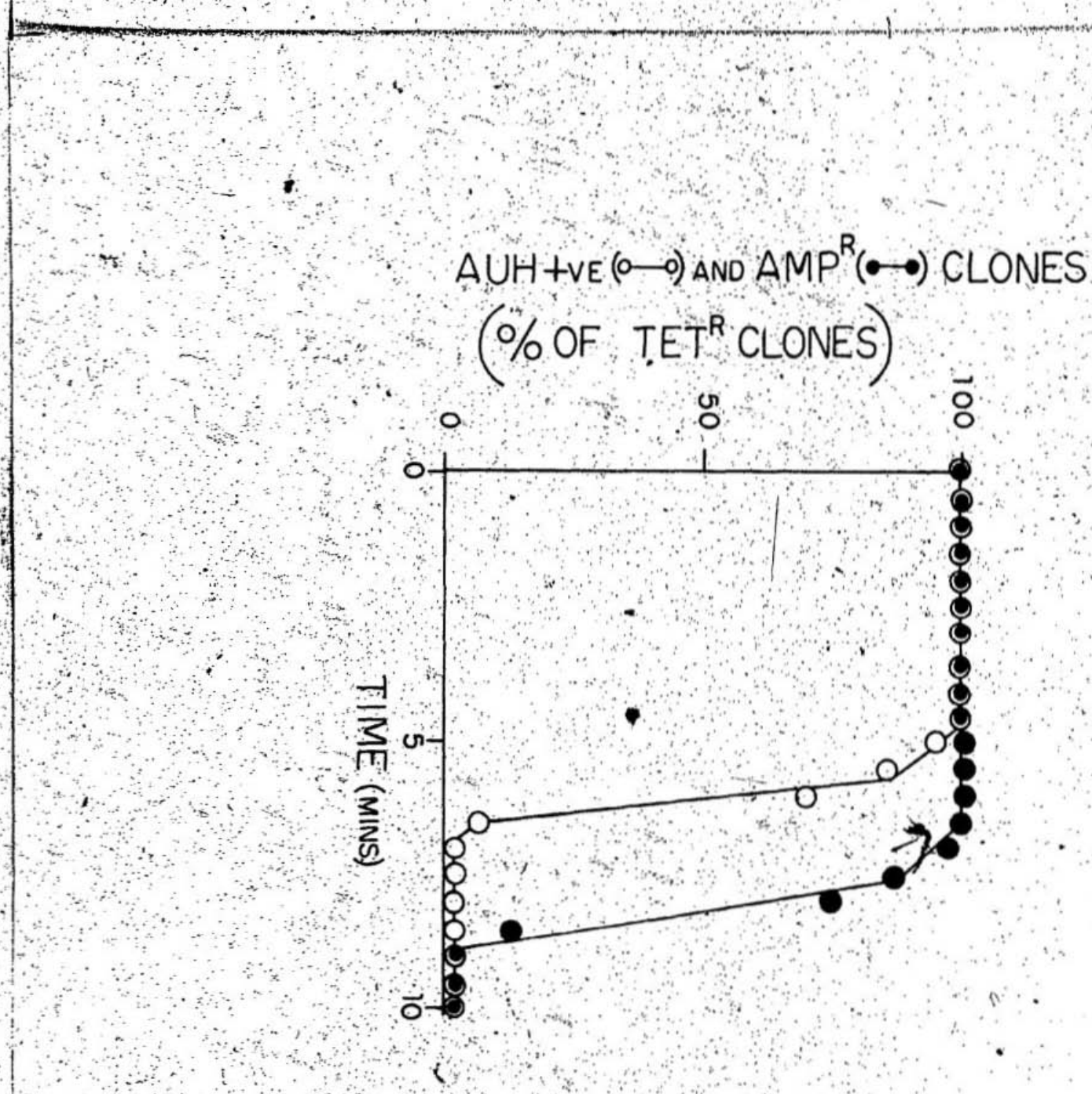

AUH +VE (o—o) AND AMP^R (•—•) CLONES
(% OF TET^R CLONES)
0
50
100
0
5
10
TIME (MINS)

Figure 7.5

Rate of change in the length of the 2.2 Kbp *HindIII* fragment of lambda DNA, following *Bal31* exonuclease digestion.

The *Bal31* exonuclease digest of the lambda *HindIII* digested DNA was electrophoresed on an agarose gel. Following staining with ethidium bromide the range of sizes was estimated from the length over which the fragment was spread, and this is indicated by bars in the figure.

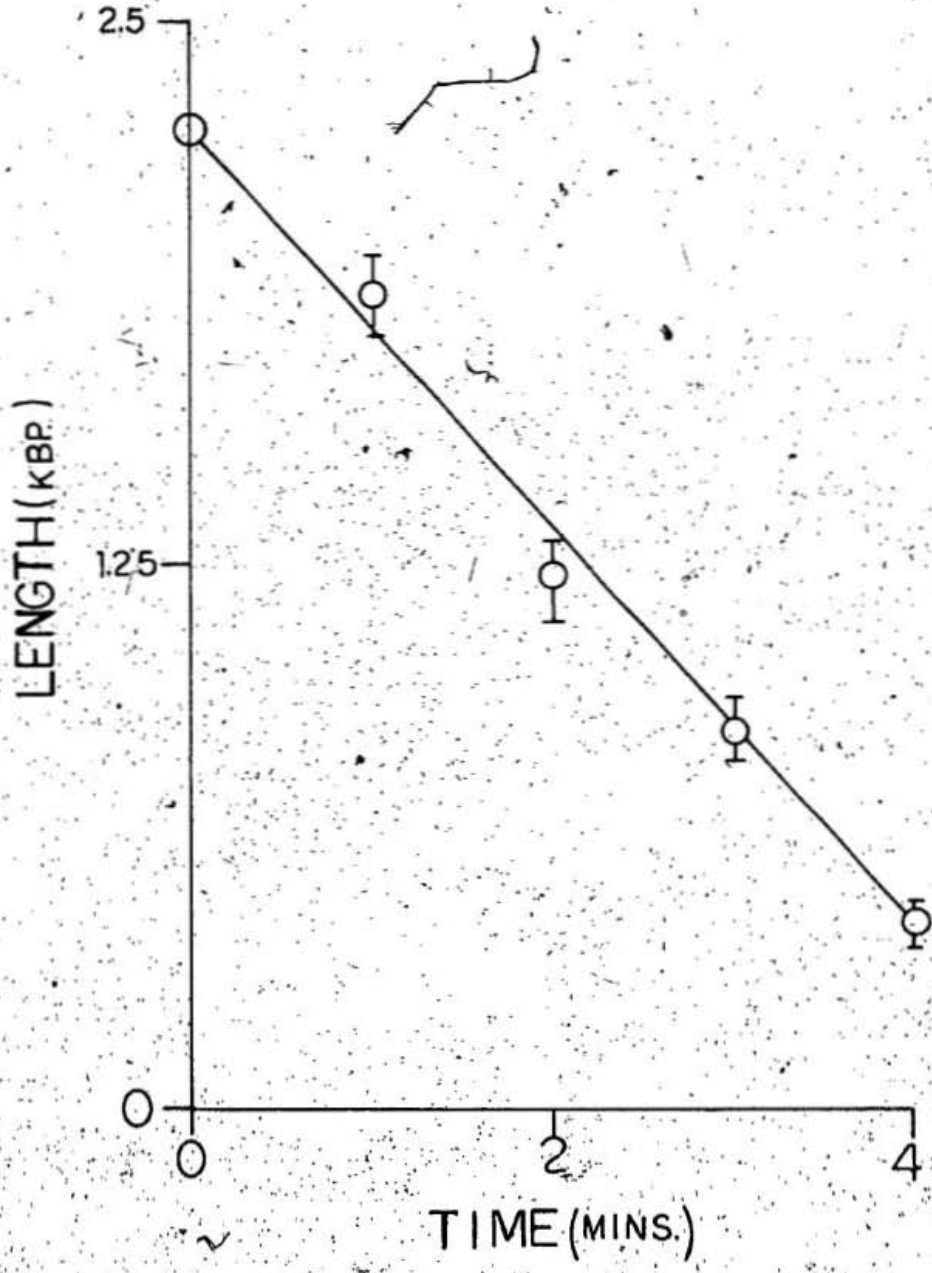
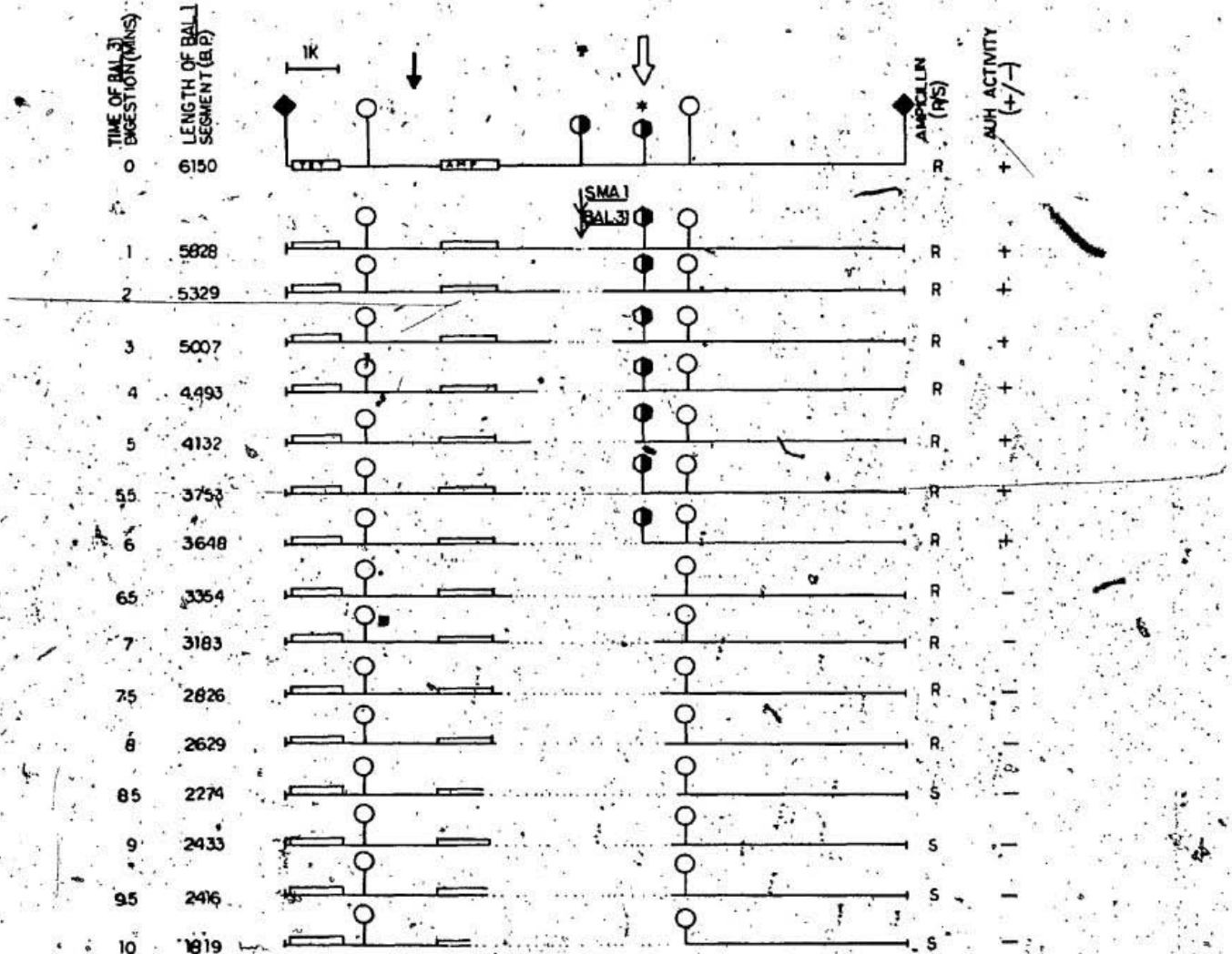

2.5
1.25
0
LENGTH (KBP.)
0
2
4
TIME (MINS.)

Figure 7.6

The rate of change of the length of the *BalI* fragment of plasmids from the tetracycline resistant clones, derived by transforming HT328 (Δ*speB*) with pKA5 DNA digested for various times by *Bal31*.

The DNA (pKA5) was linearised with *SmaI* and exonucleolytically digested with *Bal31*, religated and the ability of these reconstructed plasmids to confer ampicillin resistance and AUH activity to the host cells is shown. The size of the *BalI* fragment which reduces in length with increasing time of digestion is also shown. In the schematic diagram the digestion by *Bal31* is assumed to be equal in both directions on the same molecule. Ampicillin resistance (**R**) and sensitivity (**S**), inability (−) and ability (+) to confer agmatine ureohydrolase activity, the various times of exonucleolytic digestion (minutes), and the length of the *BalI* fragment (in base pairs=B.P) along with the schematic diagram of the *Bal31* digested plasmid are shown. 1K=1Kilo base pair (to scale), (•)= *HindIII* site (the approximate position of the promoter), (◇)=*EcoRI*, (◐)=*HindIII*, (◑)=*SmaI* and (○)=*BalI*.


TIME OF BAL 31 DIGESTION (MINS)
LENGTH OF BAL1 SEGMENT (B.P.)
1K
AMP
SMA1
BAL31
AMPICILLIN (R/S)
AUH ACTIVITY (+/-)
0 6150 R +
1 5828 R +
2 5329 R +
3 5007 R +
4 4493 R +
5 4132 R +
5.5 3753 R +
6 3648 R +
6.5 3354 R -
7 3183 R -
7.5 2826 R -
8 2629 R -
8.5 2274 S -
9 2433 S -
9.5 2416 S -
10 1819 S -

drolase was shown to be approximately 38,000 daltons. Approximately one thousand bases of DNA are required to encode a 38,000 dalton protein. Agmatine ureohydrolase activity was not expressed in clones obtained after 6 minutes of digestion with *Bal31* (Section: 7.2.2). Since the rate of digestion by *Bal31* was approximately 400 bases per minute (approximately 200 bases in one direction), it can be concluded that *speB* is approximately 1200 bases from the *SmaI* site in plasmid pKA5, ie. at or close to the *HindIII* site. Based on the approximations presented above it can be deduced that *speB* extends one kilobase from the *HindIII* site towards the *BalI* site in plasmid pKA5 and pKA14. This is also consistent with the *BalI* site being within the *speB* gene.

The restriction of plasmid pKA5 with *HincII* generates 6 fragments (Table: 7.1a). The 0.6kb *HincII* fragment, which is associated with the insert, was isolated from agarose gels and cloned into the *XorII* site of the vector pBR322. The ligated mixture was transformed into the *speB* deletion strain HT328. The transformants were selected for tetracycline resistance and ampicillin sensitivity. They did not confer agmatine ureohydrolase, arginine decarboxylase or ornithine decarboxylase activity to the host strain. The recombinant plasmid thus constructed, pKA15, is shown in Figure: 7.3. This recombinant plasmid was digested with *BalI* or *PstI* to detect the *BalI* and *PstI* sites in the insert in plasmid pKA15 (Table: 7.1b). The recombinant plasmid contained the 0.6 kb segment derived from within the *speB* gene. The orientation of the insert in the vector is shown in Figure: 7.3.

7.2.4 Analysis of the direction of transcription of speB:

SalI-BalI (or *EcoRI-BalI*) and *BamHI-BalI* are DNA segments from either side of *BalI*. The direction of transcription is either *SalI* (or *EcoRI*) towards *BalI* or *BamHI* towards *BalI* (Figure: 7.1 and 7.3). Plasmid pKA5 was digested with *BalI* and following extraction with phenol saturated with 10 mM Tris-HCl (pH 7.5); 1 mM EDTA buffer, was precipitated with ethanol. The precipitated DNA was redissolved in 10 mM Tris-HCl, 1 mM EDTA buffer (pH 7.5), and digested with *SalI*, *EcoRI* or *BamHI*. The desired fragments (*SalI-BalI*, *EcoRI-BalI* or *BamHI-BalI*) were isolated from agarose gels (Section: 2.19). The fragments *SalI-BalI* and *BamHI-BalI* were separately cloned into plasmid pKO11. The vector was restricted with *SmaI* and either *SalI* (for cloning *SalI-BalI*) or *BamHI* (for cloning *BamHI-BalI*). The fragment *EcoRI-BalI* was cloned into plasmid pMC1403 restricted with *SmaI* and *EcoRI* (for cloning *EcoRI-BalI*). The fragment *BamHI-BalI* isolated from the agarose gel was "blunt ended" using the Klenow fragment (Section: 2.11), and was cloned into plasmid pMC1403 restricted with *SmaI*. The *SmaI* site in the vectors lies between the *SalI* (*EcoRI* or *BamHI*) and the structural genes *lacZ* (in pMC1403) and *galK* (in pk011). The *SmaI* sites were linked by blunt end ligation to the *BalI* sites of the inserts, and the *BamHI* (or *SalI* or *EcoRI*) sites into their complementary site in the insert. The cloning strategy is schematically diagramed in Figure: 7.7. The ligation mixtures from pMC1403 were used to transform HB101 (*lacZ*$^-$), and that from pKO11 to transform N100 (Δ*galK*). Lac$^+$ clones were

selected on MacConkey agar containing lactose and ampicillin, and gal$^+$ clones were selected on minimal agar plates containing ampicillin, and galactose as the sole carbon source. Only the *BalI-SalI* and *BalI-EcoRI* fragments, representing the same section of the DNA segment (proximal to *amp*^{R} gene of the vector DNA in pKA5), provided a cloned promoter in the vectors pKO11 and pMC1403 respectively. A clone containing pMC1403 (pAUL) and one containing pKO11 (pAUG) were selected for further study. The orientation of the insert DNA was also determined by restricting plasmid pAUL with *BamHI*, and pAUG with *PstI* or *HindIII*. The results are shown in Table: 7.1b. The inserts in plasmids pAUG and pAUL were in the predicted orientation, with their *BalI* sites proximal to the structural genes of *lacZ* and *galK*.

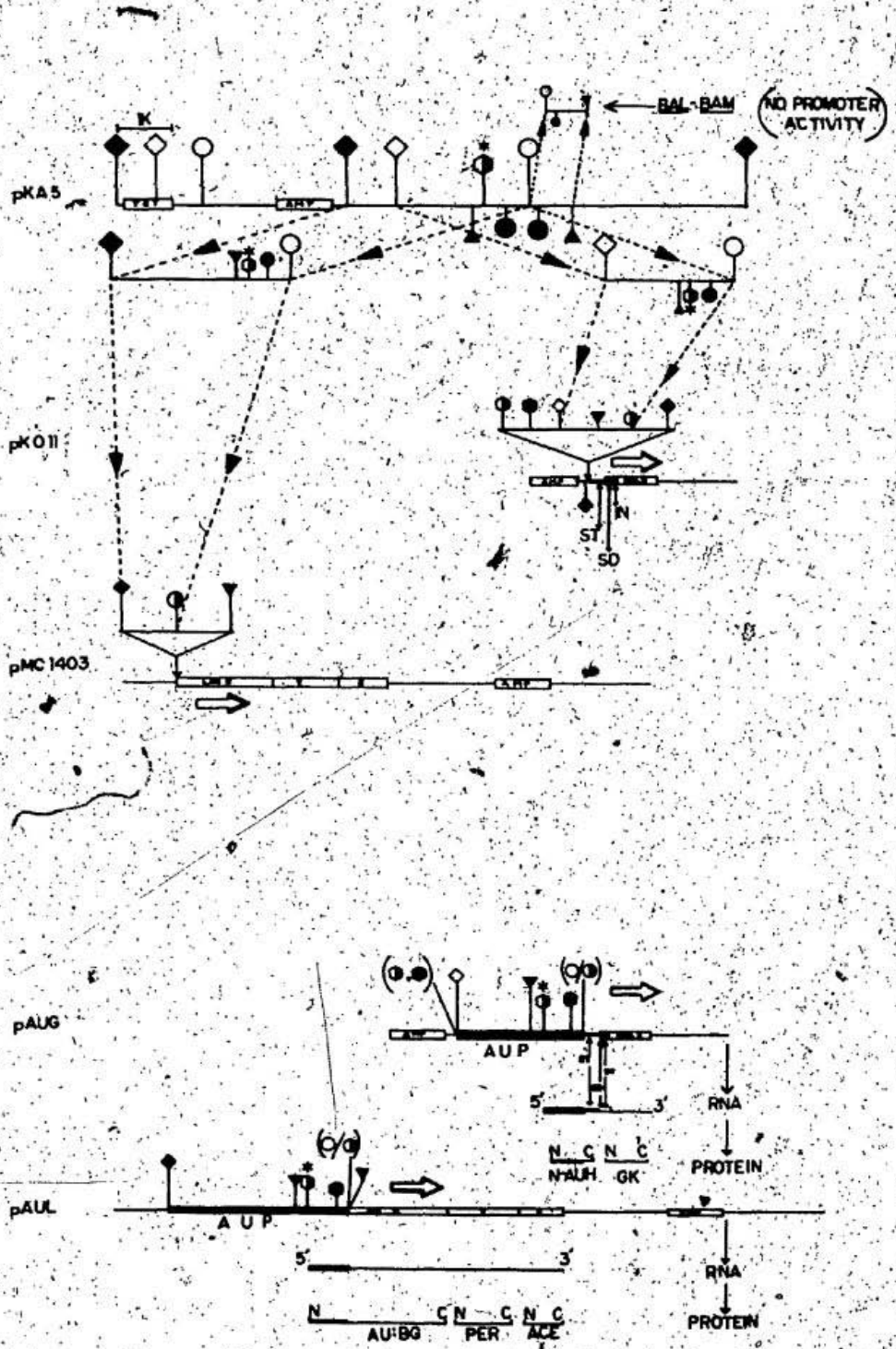
7.2.5 The restriction map of plasmid pKA10:

Restriction maps of the plasmids pKA10, pKA12 and pKA5 have been described (Boyle *et al.*, 1984; Tabor *et al.*, 1983). In order to find the sites for the restriction enzymes that restrict the plasmid pKA10 within or near the *speB* region, the plasmid pKA10 was hydrolysed with *BglI*, *PvuII* and *PstI* separately and in combinations. The restriction fragments were separated either on 0.8% or 1.0% agarose gels. The gels were stained with ethidium bromide (Section: 2.13) and photographed (Section: 2.13). The size of the restriction fragments was determined as described in Section: 2.14. The results are shown in Table: 7.1b. To construct a map for these restriction sites, the

Figure 7.7

Construction and restriction maps of pAUG and pAUL.

(/)=Fusion by ligation of non complementary restriction ends. (,)=Adjacent restriction sites. (➡)=Direction of transcription.(----➡)=Cloning of the fragment (direction of cloning).*EcoRI (SalI)–BalI* and *BalI–BamHI* represents the two sections of the *speB* on either side *BalI* site which is within the *speB* gene. **ST**=stop codons in all three reading frames.**IN**=Initiation codon (ATG). AMP= Ampicillin resistance gene. TET=Tetracycline resistance gene. **(5')** and **(3')** are the ends of the mRNA molecule.**N**= Amino terminal of the polypeptide. **C**=Carboxy terminal of the polypeptide. **AUP**=*speB* promoter (AUH promoter). **N-AUH**=Amino-terminal of AUH. AUH=agmatine ureohydrolase. FBGK=galactokinase. **AU:BG**=Agmatine ureohydrolase:β-galactosidase fusion protein. PER=Galactoside permease. ACE=galactoside transacetylase. **1K**=1 Kilo base pair (to scale). (•)=The approximate position of the *speB* promoter determined from *Bal31* digestion experiment (Section: 7.2.2). (◇)=*SalI*, (◆)=*EcoRI*, (○)= *BalI*, (●)=*HincII*, (▲)=*PvuII*, (▲)=*BamHI*, (⬡)=*BglI*, (⬢)= *PstI*, (◑)=*HindIII* and (◑)=*SmaI*.


BAL - BAM
NO PROMOTER ACTIVITY
pKA5
pKO11
ST
SD
pMC1403
pAUG
AUP
5'
3'
RNA
PROTEIN
N-AUH
GK
pAUL
AUP
AU-BG
PER
ACE

known restriction sites in the vector (Maniatis *et al.*, 1982) and the known restriction sites for *EcoRI, SalI, SmaI, BamHI, BalI* and *HindIII* (Boyle *et al.*, 1984) were used as reference points. The results are presented in Table: 7.1b and in Figure: 7.3.

7.3 Discussion:

speB is contained within the *BamHI* segment cloned into pBR322 in the construction of pKA14. *speB* is expressed in the cloned pKA14, but *speA* and *speC* are not (Table: 7.2). The latter genes have been cloned separately (Buch and Boyle personal communication; Boyle *et al.*, 1984). Digestion of linearised plasmid pKA5 with *Bal31* demonstrate that the *speB* gene is approximately 1200 base pairs away from the *SmaI* site in plasmid pKA5 (Figures: 7.4, 7.5 and 7.6). This places the *speB* gene at about the *HindIII* site (Figure: 7.6). The distance of the amp^R gene of the vector from the *SmaI* site of the insert, calculated from the rate of digestion, was in agreement with the location deduced from the preliminary deletion mapping of pKA5 (Boyle *et al.*, 1984; Tabor *et al.*, 1983).

The cloning of the two fragments, *SalI-BalI* (or *EcoRI-BalI*) and *BamHI-BalI*, flanking the *BalI* site of the insert in pKA5 into the promoter cloning vectors demonstrate that the *speB* promoter is located in the *SalI-BalI* fragment (Figure: 7.7). Since the *BalI* end of the segment is proximal to *lacZ* and *galK* structural genes in pAUL and pAUG, and since the *BalI* site is within

speB, it can be concluded that the direction of transcription is from the *HindIII* site towards *BalI*. The ligation of the *BamHI-BalI* fragment into the promoter cloning vectors did not activate the transcription of either the *lacZ* or the *galK* structural genes. No attempt was made to demonstrate that the *BamHI-BalI* fragment had been inserted, or inserted with the correct orientation. It was assumed that only one fragment contained a promoter. The recombinants pAUL and pAUG were used in the study of regulation of *speB* by assessing the activities of β-galactosidase and galactokinase.

The recombinant plasmid pKA15 carries the *HincII* insert (Figure: 7.3). This insert carries the *BalI* site which is within *speB*. Approximately 1kbp of DNA is required to encode agmatine ureohydrolase. The promoter for *speB* lies close to the *HindIII* site and is transcribed toward the *BalI* site (Figure: 7.7). Consequently the *HincII* insert must carry a large part of the structural gene. This plasmid was used in later experiments to assess mRNA concentrations during repression by cAMP and induction by agmatine.

Further details of the restriction map of pKA10 have been elucidated (Figure: 7.3). Digestion with *PstI* gave three fragments, of which one fragment of 1.0 kbp was further restricted on digestion with *EcoRI* into two fragments of 0.55 and 0.45 kbp. Digestion of the *PstI* digested pKA10 DNA with *BalI* resulted in the formation of 4 fragments and the 2.0 kbp PstI fragment disappeared. Since the positions of *EcoRI* and *BalI* in this plasmid are known the positions of the *PstI* sites were determined. This was also confirmed by

restricting the plasmid pKA10 with *PstI* and *BamHI* which resulted in the largest *PstI* fragment (3.0 kbp) being digested into two smaller fragments of 2.5 kbp and 0.5 kbp (Table: 7.1b). The positions of the *PstI* restriction sites are shown in Figure: 7.3.

Digestion of plasmid pKA10 DNA with *BglI* or *PvuII* resulted in two fragments of approximately equal sizes (3.0 kbp). Since there was only one site for each of these restriction enzymes in the vector DNA of the plasmid pKA10, the 6.0 kbp plasmid DNA was presumably cleaved by these two enzymes at approximately 3.0 kbp from the restriction sites in the vector. The restriction sites were mapped as shown in Figure: 7.2. These sites determined from the above deduction were confirmed by digesting the plasmid pKA10 with both *PvuII* and *BglI*. This resulted in the cleavage of the plasmid into four fragments of predicted length (Table: 7.1b).

Chapter 8

Cell - Free Transcription and Translation

8.1 Introduction:

Cell-free protein synthesising systems have been used to determine the influence of various effector molecules on gene expression at the levels of transcription and translation (Zubay, 1973). The roles of protein factors in gene regulation can be studied by using S-30 extracts prepared from mutants (e.g. *crp*) (Boyle *et al.*, 1985; Collins, 1979). The S-30 extract can then be reconstituted with the purified protein and the protein's effect examined. Molecules of low molecular weight, such as cAMP, can be readily removed from the S-30 extract by dialysis and added to the reaction systems in defined amounts.

The DNA directed synthesis of various truncated protein products encoded by different restriction fragments, can be used to deduce the direction of transcription. Genes on specialised transducing phages (Mackie, 1979) and plasmids (Chen and Zubay, 1983) have been located using this approach.

The direction of *speB* transcription on pKA5 was shown by the gene fusion studies reported in Chapter 7 to be from the *HindIII* restriction site towards *BalI*, and the approximate location of the *speB* promoter was deduced by exonuclease digestion of the plasmid pKA5 using *Bal31*. In order to confirm the direction of transcription and the approximate transcription start point, plasmid pKA10 DNA, which carries the promoter proximal part of the *speB* gene, and pKA14, which carries the *speB* gene were used. These plasmids were linearised using restriction sites within the *speB* gene. S-30 extracts of

MRE600 and LS854-1 (Δ*crp*, Δ*cya*) were prepared, fortified with nucleoside triphosphates, amino acids, [^{35}S]-methionine, and ATP regenerating system, and transcription and translation were initiated by adding the plasmid. After incubation, proteins were denatured and electrophoresed on SDS-polyacrylamide gels. Radiochemically labelled proteins were detected by fluorography (Section 2.14). The effects of CRP and cAMP on the transcription of *speA* and *speB*, were examined using pKA5 and pKA14 which carry the genes for arginine decarboxylase and and agmatine ureohydrolase, respectively.

8.2 Results:

8.2.1 Optimisation of conditions for the incorporation of [^{35}S]-methionine into proteins in a cell-free transcription and translation system:

In order to determine the optimum concentration of plasmid DNA to direct protein synthesis in an *in vitro* transcription and translation system, replicate reaction mixtures containing the S-30 extracts of MRE600 or LS854-1 (Δ*cya*, Δ*crp*) were prepared and the concentration of plasmid DNA (supercoiled or linearised) was varied. The linearised plasmids were obtained by digesting the plasmids (pKA5, pKA10, pBR322 or pKA14) with *BalI*. A sample of the digested DNA was checked by agarose gel electrophoresis. The reactions were incubated for 15 minutes, and terminated by the addition of 0.25 ml of 15% TCA. Radioactivity associated with the proteins was determined, and the

results are presented in Figure: 8.1. The incorporation into proteins was maximum with 3μg pKA5, 4μg pKA14, 4μg pKA10 or 2μg pBR322 as supercoiled plasmids or 2μg pBR322 as linear DNA to direct the synthesis of proteins in reaction systems containing MRE600 extract. The incorporation was maximum with 5μg pKA5, 4μg pBR322 or 6μg pKA10 as supercoiled plasmids in reaction systems containing LS854-1 extract. The optimum plasmid DNA concentrations determined here were used in the following experiments.

In order to determine if the incorporation of [^{35}S]-methionine was linear with time of incubation, the S-30 extracts of MRE600 and LS854-1 were separately directed to synthesise proteins in the presence or in the absence of supercoiled or linearised pBR322 DNA. The results are presented in Figure: 8.1. The incorporation was linear for a period of up to 35 minutes and reactions were routinely carried out for 20 minutes.

8.2.2 The effect of cAMP on the cell-free synthesis of agmatine ureohydrolase and arginine decarboxylase:

In order to determine the effect of cAMP on the synthesis of agmatine ureohydrolase *in vitro*, the reaction system using an S-30 extract derived from *E.coli* MRE600 was directed to transcribe and translate pKA14, pKA5 or pBR322 DNA (Section: 2.17), in the presence or absence of 1 x 10^{-4}M cAMP. The proteins were analysed on a 10% polyacrylamide gels containing 0.1% SDS, fluorograms were prepared and are presented in Figure: 8.2. The addition

Figure: 8.1

The Effect of Increasing Concentrations of Supercoiled and Linear DNA and Time of Incubation on the Incorporation of [^{35}S]- Methionine into Proteins Synthesised in a Cell-Free Transcription and Translation System:

In reaction mixtures containing the S-30 extracts of MRE600 **(B and D)** and LS854-1 **(F)**, the concentration of linearised **(D)** and supercoiled **(B, F)** DNA were varied. The plasmids were linearised by digestion with *BalI*. (○) = pBR322, (●) = pKA10, (⊙) = pKA14 and (▫) = pKA5.

In reaction mixtures containing the S-30 extracts of MRE600 **(A and C)** and LS854-1 **(E)**, supercoiled **(A and E)** and linearised (C) pBR322 DNA (○) was used to direct the synthesis of proteins. The plasmid was linearised with *BalI*. (●) = no DNA added to the reaction system.

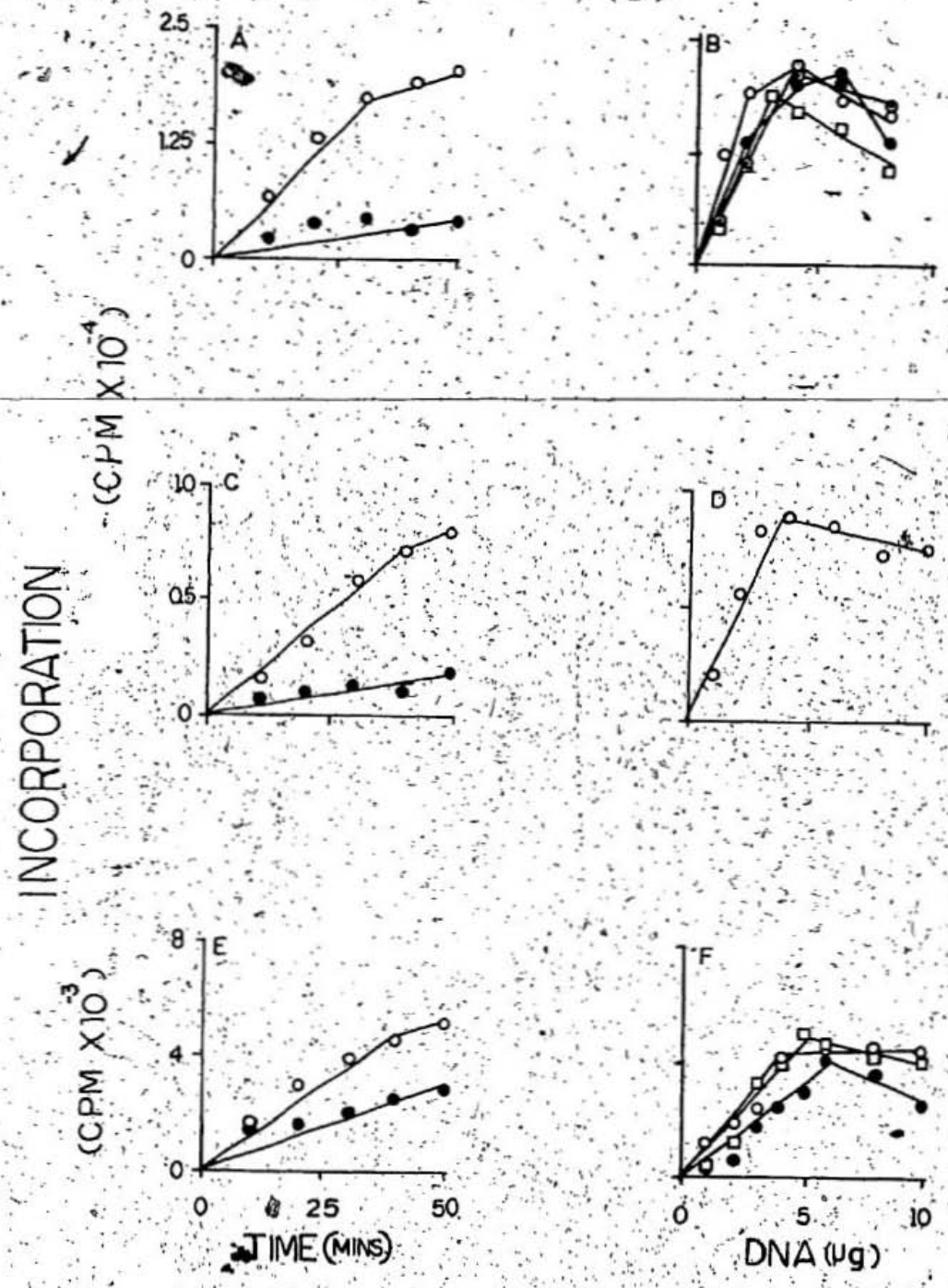

A
B
C
D
E
F
2.5
1.25
0
10
0.5
8
4
0
25
50
5
10
INCORPORATION
(CPM X 10⁻⁴)
(CPM X 10⁻³)
TIME (MINS)
DNA (µg)

Figure: 8.2.

The effect of cAMP on the synthesis of arginine decarboxylase and agmatine ureohydrolase in a cell-free transcription and translation system using an S-30 extract of MRE600 directed by plasmids pKA5 and pKA14.

Figure(**A**): (**a**) = pKA5, (**b**) = pKA5 + 0.1mM cAMP, (**c**) = pBR322, (**d**) = pBR322 + 0.1mM cAMP.

Figure(**B**): (**a**) = No DNA, (**b**) = pKA5, (**c**) = pKA5, (**d**) = pKA5 + 0.1mM cAMP, (**e**) = pKA5, (**f**) = pKA14, (**g**) = pKA14 + 0.1mM cAMP, (**h**) = pBR322.

Figure(**C**): (**a**) = pKA5- 8h exposure, (**b**) = pKA5- 36 hour exposure, (**c**) = pKA5 + 0.1mM cAMP- 3h exposure, (**d**) = pKA5 + 0.1mM cAMP- 6h exposure, (**e and f**) = pKA5 + 0.1mM cAMP- 36h exposure, (**g**) = No DNA- 36h exposure, (**h and i**) = pKA5 + 0.1mM cAMP- 72h exposure, (**j**) = No DNA- 72h exposure, (**k**) = pBR322- 8h exposure, (**l**) = pBR322 + 0.1mM cAMP- 6h exposure, (**m**) = pBR322- 36h exposure, (**n**) = pBR322 + 0.1mM cAMP- 36h exposure, (**o**) = pBR322, (**p**) = pBR322 + 0.1mM cAMP.

Figure(**D**): (**a**) = pKA14, (**b**) = pKA14 + 0.1mM cAMP, (**c and d**)=No DNA.

ADC = arginine decarboxylase, SAM = S-adenosyl methionine synthetase, AUH = agmatine ureohydrolase, LAC = β-lactamase.

Molecular weights are expressed as daltons X 10^{-3}.


A
a b c d
93
68
43
30
ADC
SAM
AUH
LAC
B
a b c d e f g h
93
68
43
30
ADC
SAM
AUH
LAC
C
a b c d e f g h i j k l m n o p
93
68
43
30
ADC
SAM
AUH
LAC
D
a b c d
93
68
43
30
20
AUH
LAC

of 10^{-4}M cAMP to the MRE600 S-30 extract completely repressed the synthesis of agmatine ureohydrolase and arginine decarboxylase, whereas β-lactamase encoded by the same plasmid appeared unaffected.

8.2.3 The role of CRP in the negative regulation of agmatine ureohydrolase and arginine decarboxylase synthesis by cAMP in a cell-free transcription and translation system:

In order to assess the role of CRP, the S-30 extracts of MRE600 or LS854-1 (Δcya, Δcrp) were used. pKA5 DNA was used to direct the synthesis of arginine decarboxylase, agmatine ureohydrolase, S-adenosyl methionine synthetase and β-lactamase in the S-30 extracts of each strain. Replicate reaction mixtures contained various concentrations of cAMP (10^{-7}M to 10^{-3}M) and the proteins synthesised were analysed on 10% polyacrylamide gels containing 0.1% SDS.

In an identical experiment an S-30 extract of LS854-1 (Δcya, Δcrp) was directed to synthesise proteins using pKA5 DNA. Replicate reaction mixtures were supplemented with cAMP (10^{-7}M to 10^{-3}M) and/or 1μg CRP. The proteins were analysed as described above and the fluorograms prepared. When S-30 extract of MRE600 was used, cAMP at 10^{-4}M abolished the synthesis of agmatine ureohydrolase, while 10^{-3}M did not affect its synthesis, unless CRP was added to the S-30 extracts of LS854-1. β-lactamase was not affected even when 10^{-3}M cAMP was added to the cell-free transcription and translation sys-

tem in extracts of MRE600 and LS854-1 (data not shown).

8.2.4 Direction of transcription and localisation of the *speB* promoter

pKA10 which carries the promoter proximal region of the *speB* gene and pKA14 which carries the entire *speB* gene were used to determine the location of the *speB* promoter and the direction of transcription. The plasmid pKA10 was digested either with *BalI*, *PstI* or with *HindIII* , and plasmid pKA14 was digested with *BamHI*. These digests were used separately to direct the synthesis of proteins in an S-30 extract of MRE600. The proteins were analysed on 10% polyacrylamide gels containing 0.1% SDS and the fluorograms are shown in Figure: 8.3. After digestion of pKA14 with *BamHI*, the proteins which were observed on the final fluorogram (Figure: 8.3) corresponded to β-lactamase, its precursor, and agmatine ureohydrolase (monomeric form). After digestion of pKA10 with *BalI* or *HindIII* the β-lactamase and its precursor appeared unchanged, whereas after digestion with *PstI* both β-lactamase and its precursor were reduced in size by about 7000 daltons, consistent with the known presence of a *PstI* site within the *amp* gene. In these latter reactions the agmatine ureohydrolase monomer disappeared, and was replaced with a 35 Kdalton fragment after *BalI* digestion, a 32 Kdalton fragment after *PstI* digestion, and nothing appeared after *HindIII* digestion. The digestion of the DNAs and the protein products are schematically shown in Figure: 8.4.

Figure: 8.3.

Synthesis of agmatine ureohydrolase in a cell-free transcription and translation system using restricted DNA to determine direction of transcription in an S-30 extract of MRE600

Figure(**A**): **(a)** = pBR322, **(b)** = No DNA, **(c)** = pKA14 (*BamHI*), **(d)** = pKA14, **(e)** = pKA10 (*BalI*), **(f and g)** = pBR322 (*PstI*), **(h and i)** = pKA10 (*PstI*), **(j)** = No DNA.

Figure(**B**): **(a, b and d)** = pKA14 (*HindIII*), **(c)** = No DNA.
AUH = agmatine ureohydrolase, LAC = β-lactamase.

Molecular weights are expressed as daltons X 10^{-3}

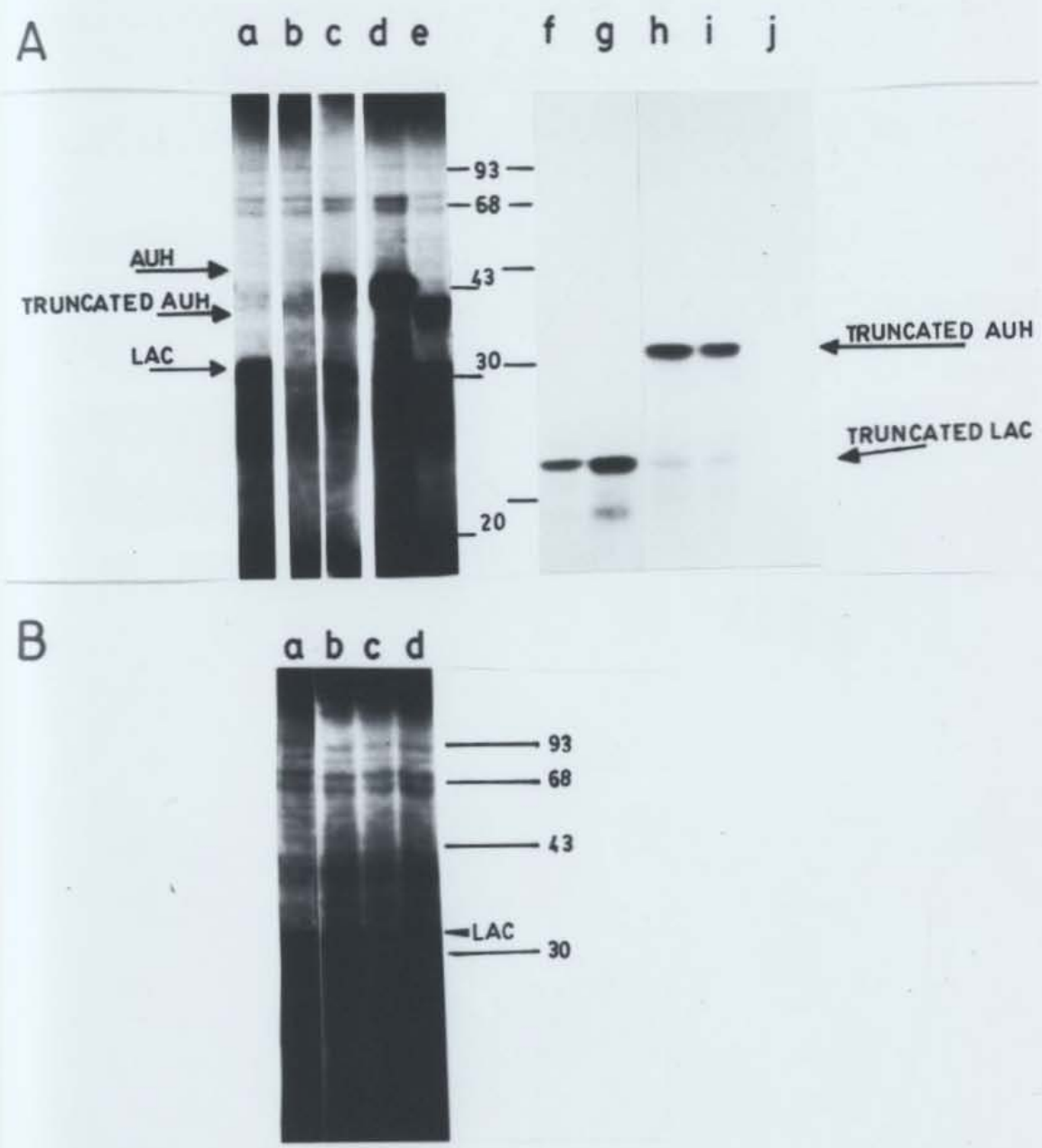
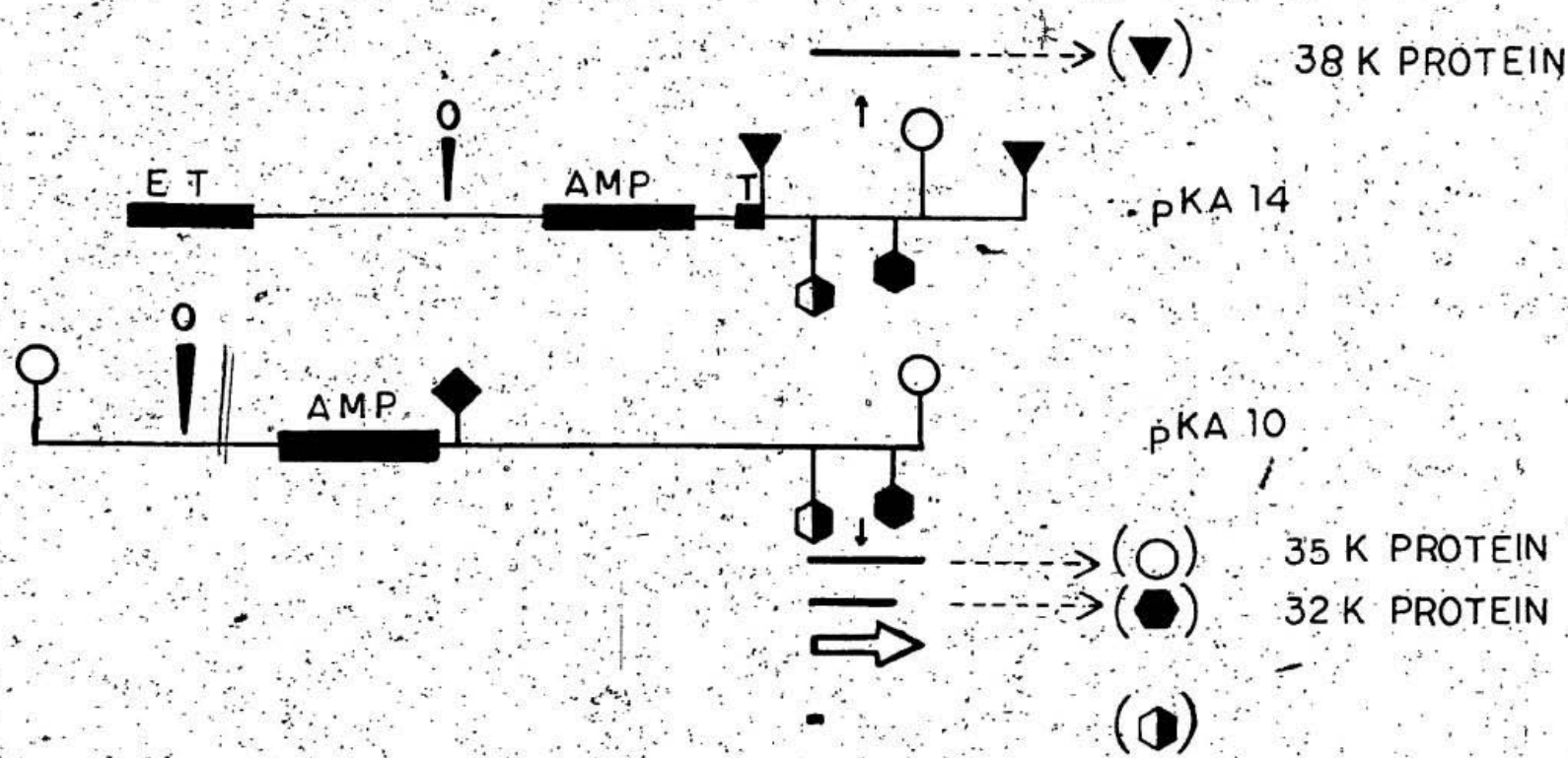

A
a b c d e
f g h i j
93
68
43
30
20
AUH
TRUNCATED AUH
LAC
TRUNCATED AUH
TRUNCATED LAC
B
a b c d
93
68
43
LAC
30

Figure 8.4

Schematic diagram of plasmids pKA14 and pKA10 and the products of cell-free transcription and translation of whole and restricted plasmids.

The restriction enzymes used to digest the DNA to direct the synthesis of truncated polypeptides are shown in parentheses.

(O) represents the origin of replication, and (⟹) represents the direction of transcription. **TET**=Tetracycline resistance and **AMP**=Ampicillin resistance. The restriction enzymes are represented by (○)=*BalI*, (◆)=*EcoRI*, (◐)=*HindIII*, (▼)= *BamHI* and (⬡)=*PstI*.


38 K PROTEIN
E T
O
AMP
T
pKA 14
O
AMP
pKA 10
35 K PROTEIN
32 K PROTEIN

8.3 Discussion:

cAMP and CRP have been shown not to repress the synthesis of β-lactamase (Aiba, 1983; Boyle *et al*, 1985). The results in Figure: 8.2 are in accord with this in that cAMP repressed the synthesis of agmatine ureohydrolase but the synthesis of β-lactamase protein was not affected. This suggests that cAMP:CRP negatively regulates the transcription of the *speB* gene. pKA5 and pKA14 produce a limited number of proteins in the *in vitro* transcription and translation system (and in minicell systems, Chapter: 9), and they can be identified with known proteins. An unidentified protein which might have a regulatory function has not been observed, and the small size of the insert in pKA14 suggests there there would not be one. Therefore, the effect of cAMP and of cAMP:CRP observed here suggest a direct effect on transcription. On longer exposures, the autoradiograms (Figure: 8.2) showed an unidentified minor protein species of approximately 35 Kdaltons in the cAMP treated reaction system, using pKA5 DNA in MRE600 extract. This protein was not detected either in the minicell system (Chapter: 9 and Boyle *et al.*, 1985) or in the reaction systems conducted in the absence of cAMP (Figure: 8.2). It is possible that this protein, which cAMP appears to induce, represses the *speB* and *speA* genes, but repression of *speB* was also observed in a reaction system directed by pKA14 DNA (Figure: 8.2), in which the insert does not have the coding potential for both a 35 Kdalton protein and a 38 Kdalton protein (AUH). This protein is not detected, although *speB* is repressed by cAMP in a

pKA14 directed reaction system. It is possible that the 35 Kdalton protein is a truncated arginine decarboxylase or agmatine ureohydrolase. cAMP:CRP has been implicated in transcription termination and polarity (Ullmann and Danchin, 1983), and therefore it may be that cAMP:CRP represses *speA* and *speB* expression by increasing transcription termination of these genes.

In the experiments described in Section: 8.2.3 the S-30 extract was prepared using various methods of cell breakage (Omnimixer, alumina grinding, freeze breakage or sonication), as a French pressure cell was unavailable. These methods of cell breakage were not as efficient, and the incorporation of the radiolabel was lower, in extracts prepared in this manner, as compared to extracts obtained by breaking cells using a French pressure cell. In the experiments reported in Section: 8.2.3 several other proteins were detected, which often masked the detection of plasmid encoded proteins. Arginine decarboxylase was almost never detected, and agmatine ureohydrolase, S-adenosyl methionine synthetase and β-lactamase were seen at times. Despite longer preincubation to degrade endogenous mRNA or decreasing the amount of S-30 extract added to the reaction system (as recommended by Zubay, 1983), the plasmid directed proteins were not detected. Therefore, the autoradiograms could not be scanned for quantitation, and scanned values thus obtained would be meaningless in a system that appears to be limiting in some factors for transcription and/or translation. Although, treatment of the S-30 extract with S1 nuclease (10 units/ml), for one hour prior to dialysis improved the specific

labelling of plasmid directed proteins approximately two fold, the addition of 1 mM zinc sulphate to the extracts (required for S1 activity) resulted in the precipitation of proteins. It appears that all available methods of cell breakage other than the French pressure cell cause large fragments of DNA to be present in the extracts. These DNA fragments would compete with the plasmid DNA for available transcriptional and translational factors. Addition of purified RNA polymerase (1 unit) to the reaction mixtures did not improve the synthesis of plasmid directed proteins. Although, quantitation of these results was not possible, it can be concluded from the results presented in Section: 8.2.1 (Figure: 8.2) that cAMP negatively regulates the synthesis of arginine decarboxylase and agmatine ureohydrolase. The autoradiograms (Section: 8.2.3) showed that the repression of agmatine ureohydrolase by cAMP required a functional CRP (data not shown).

The results of using restriction digests of pKA10 to direct protein synthesis in the *in vitro* system are consistent with the results reported in Chapter 7. The position and orientation of *speB* is as shown in Figure: 8.3. The *HindIII* site is within the promoter or an early section of the structural gene, (the method of electrophoresis would not detect a protein with molecular weight less than 10,000), and the *PstI* and *BalI* sites are progressively further along the structural gene.

Chapter 9

Regulation of *speA* and *speB* Genes in Minicells

9.1 Introduction:

Minicells of *E.coli* offer an ideal system to analyse gene expression and regulation (Inselburg, 1970; 1971; Fraser and Curtiss, 1975). The expression of the genes segregated in minicells on plasmids can be studied without the presence of chromosomal genes. Using [^{35}S]-methionine to label the proteins encoded by the plasmids segregated in minicells (schematically diagrammed in Figure: 9.1a), Boyle *et al.*, (1984) identified arginine decarboxylase and methionine adenosyl transferase among the plasmid pKA5 encoded proteins. The identification of these proteins was based on their migration after SDS-polyacrylamide gel electrophoresis. Based on the ability of various subclones of plasmid pKA5 to confer enzymatic activities to the *speA*, *speB*, *speC* deleted strain of *E.coli* (HT328), the 38,000 dalton protein was deduced to be agmatine ureohydrolase (Boyle *et al.*, 1984).

The minicell system was used to discriminate between the two possibilities that either cAMP-CRP represses *speB* directly or cAMP-CRP induces the formation of a protein which in turn regulates the expression of *speB*. Because minicells lack chromosomal DNA, cAMP-CRP would not be able to effect the repression of *speB* by inducing repressor formation (unless a gene for such is cloned in the plasmid). If cAMP-CRP is involved in the repression of *speB* directly, the synthesis of the 70 kilodalton, 74 kilodalton and 38 kilodalton

proteins would be diminished relative to the β-lactamase synthesis. The minicells carrying plasmid pKA5 were labelled in the presence of cAMP, and the minicell proteins were analysed on polyacrylamide gels. The fluorographs were scanned using a densitometric scanner, and the relative synthesis of the insert encoded proteins to the β-lactamase synthesised by the vector was determined.

9.2 Results:

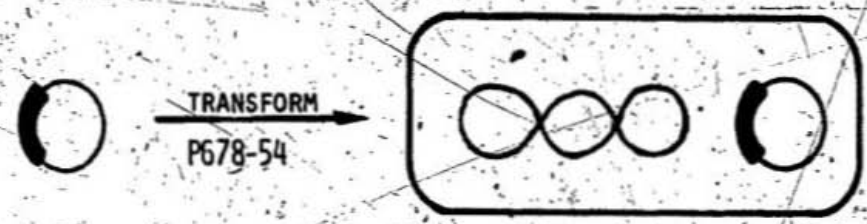
9.2.1 The effect of increasing time of incubation on the incorporation of [^{35}S]-methionine into proteins of minicells carrying pBR322:

In order to determine if the incorporation of [^{35}S]-methionine into minicells carrying pBR322 was linear with respect to time of incubation, the purified minicells were incubated at 37°C in the presence of amino acids, nucleoside triphosphates and [^{35}S]- methionine for various time intervals. The reactions were terminated in samples withdrawn at various times by precipitating the proteins with 5% TCA, and the radioactivity associated with the precipitates was determined as described in 2.16. Incorporation of [^{35}S]-methionine into proteins of minicells carrying pBR322 was compared with that of minicells which do not carry any plasmid DNA (Figure: 9.1b). The incorporation of [^{35}S]-methionine was linear for at least 45 minutes. The reactions were routinely carried out for 30 minutes.

Figure 9.1a

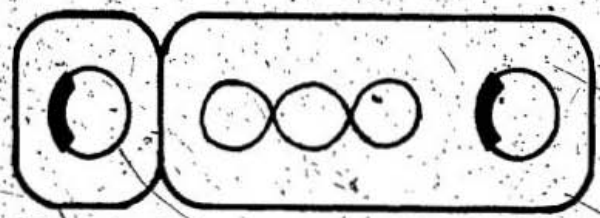
Schematic diagram of the labelling of minicell proteins.

The figure shows the labelling of minicell proteins by ^{14}C labelled amino acids. In the experiments described in this chapter the minicells were labelled with [^{35}S]-methionine instead of [^{14}C]-amino acids as shown in the figure.



pKA 5

INCUBATE AT 37 C
IN L-BROTH.
HARVEST IN STATIONARY
PHASE.



PURIFY MINICELLS THROUGH
TWO SUCROSE DENSITY-GRADIENTS.

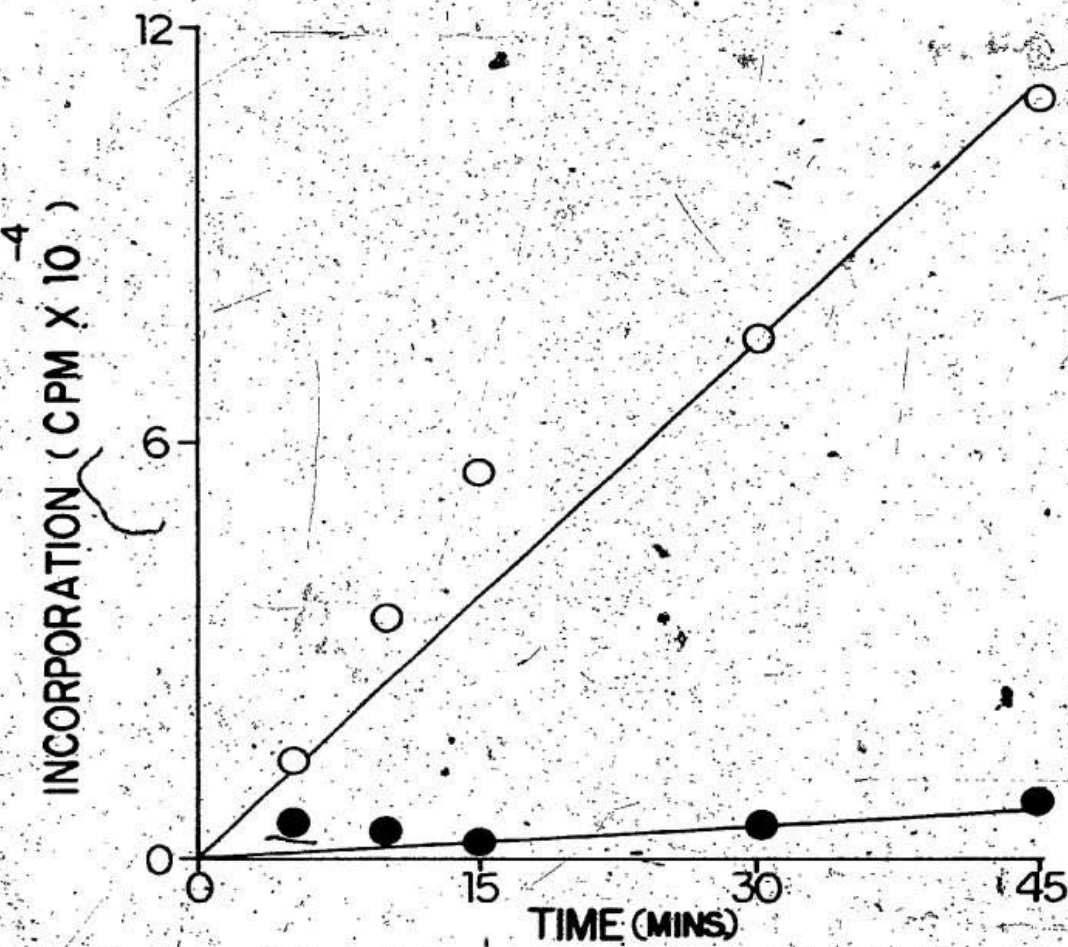
INCUBATE AT 37 C TO
DEGRADE ENDOGENOUS mRNA.
ADD ^{14}C-AMINO ACIDS

^{14}C-PROTEINS
ANALYZED BY GEL ELECTROPHORESIS
AND FLUOROGRAPHY

Figure 9.1b

Incorporation of [^{35}S]-methionine into TCA precipitates of minicells carrying plasmid pBR322.

Samples of pBR322 containing minicells (○) and minicells that carry no plasmid (●) were withdrawn following various times of incubation, and the radioactivity associated with the TCA precipitates was determined.


12
6
0
INCORPORATION (CPM X 10 -4)
0
15
30
45
TIME (MINS)

9.2.2 The effect of cAMP on the synthesis of arginine decarboxylase and agmatine ureohydrolase in minicells carrying plasmid pKA5:

The minicells were isolated as described in Section: 2.16, and were pre-incubated for 15 hours at 37°C to allow for the degradation of endogenous mRNA. The minicells were further incubated for 60 minutes at 37°C in the presence or absence of added cAMP. The cAMP concentrations used in the incubation medium was between 1 and 5 mM. Nucleoside triphosphates and amino acids were added along with [^{35}S]-methionine. The mixture was further incubated for 20 minutes at 37°C , and non-labelled methionine (final concentration 5 mM) was then added and the incubation continued for a further 5 minutes. This allowed the completion of synthesis of partially synthesised ^{35}S-labelled proteins. The extracts of the minicells were prepared as described in Section: 2.16, and subjected to SDS polyacrylamide gel electrophoresis. The results are presented in Figure: 9.2, and the relative band intensities are given in Figure: 9.3. As a function of cAMP concentration, cAMP decreased the synthesis of agmatine ureohydrolase and increased the synthesis of arginine decarboxylase.

9.2.3 The effect of agmatine on the synthesis of arginine decarboxylase and agmatine ureohydrolase in minicells carrying plasmid pKA5:

This experiment is similar to the one described in Section: 9.2.2 but cAMP was replaced by agmatine. The minicells were labelled, and the proteins

Figure 9.2

The effect of cAMP and agmatine on the synthesis of ADC and AUH by pKA5 in minicells.

AUH = agmatine ureohydrolase , ADC = arginine decarboxylase , SAM = S-adenosyl methionine transferase and LAC = β- lactamase, (a)=1mM agmatine, (b)=2.5mM agmatine, (c)=5mM agmatine, (d)=10mM agmatine, (e)=control (none), (f)=1mM cAMP, (g)=2.5mM cAMP, (h)=5mM cAMP,(i)=10mM cAMP. The molecular weights determined from the migration of proteins of known molecular weight are shown as daltons X 10^{-3}, and are shown on the right of the figure. The relative peak area determined from the autoradiogram, shown in Figure: 9.3 is the peak area of the scanned band in an autoradiogram relative to the peak area of β-lactamase band in the same lane, in the same autoradiogram.


a b c d e f g h i
ADC
SAM
AUH
LAC
68
43
30

Figure 9.3

The effect of cAMP and agmatine on the synthesis of ADC, AUH and SAM, and on the total incorporation of [^{35}S]-methionine into minicell proteins.

Figure: **A:** shows the effect of cAMP, and Figure: **B:** shows the effect of agmatine. AUH = agmatine ureohydrolase (⊙), ADC = arginine decarboxylase (●), SAM = S- adenosyl methionine transferase (◒) and (○) = incorporation of [^{35}S]- methionine into total protein of the minicells that carry the plasmid pKA5. The relative peak area is the peak area of the scanned band in an autoradiogram relative to the peak area of β-lactamase band in the same lane, in the same autoradiogram.

1.0
0.5
0
0
5
10
12
8
4
0
mM cAMP
RELATIVE PEAK AREA
TOTAL INCORPORATION (CPM X 10⁻⁴)
1.0
0
0
5
10
12
6
0
mM AGMATINE

were separated on 10% polyacrylamide gels as described in Section: 2.14, and the results are presented in Figure: 9.2, and the densitometric scans of the fluorograms are presented as band intensities relative to the band intensity of β-lactamase precursor protein in Figure: 9.3. Agmatine increased the synthesis of agmatine ureohydrolase and decreased the synthesis of arginine decarboxylase and S-adenosyl methionine transferase.

9.2.4 The effect of cAMP and agmatine on the incorporation of ^{35}S methionine into total protein of minicells carrying pBR322:

The major protein expressed by pBR322 in minicells appears to be β-lactamase. In order to assess whether cAMP or agmatine affected β-lactamase synthesis, the minicells carrying pBR322, following 15 hours of pre-incubation, were incubated at 37 °C for one hour in the presence or absence of either 5 mM cAMP or 5 mM agmatine. The proteins encoded by the plasmid were labelled by starting the reaction by the addition of nucleoside triphosphates, amino acids and ^{35}[S]-methionine, and the incubation was continued for 20 minutes at 37 °C. Cold methionine was added, and the mixture was incubated for a further 5 minutes to complete the protein chains initiated. The extracts of minicells were prepared, and aliquots were treated with an equal volume of 10% TCA. The mixture was kept in a boiling water bath for 10 minutes to remove any [^{35}S]-methionine from charged t-RNAs. These samples were then incubated on ice for one hour, and were filtered through 0.4 micron millipore

Table 9.1

Effects of cAMP and agmatine on the incorporation of [^{35}S]-methionine into proteins of minicells carrying pBR322.

Minicells carrying pBR322	Total incorporation (cpm)
None	6.8×10^4 ($\pm 0.3 \times 10^4$)
+5mM cAMP	6.3×10^4 ($\pm 0.1 \times 10^4$)
+5mM Agmatine	6.6×10^4 ($\pm 0.1 \times 10^4$)

The results are an average of two experiments.

filters. The filters were dried and the radioactivity counted. The results are presented as total incorporation of the [^{35}S]-methionine into minicell proteins in Table: 9.1. Neither cAMP nor agmatine affected the total incorporation of [^{35}S]-methionine into pBR322 encoded proteins.

9.3 Discussion:

Cyclic AMP and agmatine do not affect the synthesis of proteins encoded by pB322 in minicells (Table: 9.1). Boyle *et al.* (1985), showed that cAMP does not affect the synthesis of β-lactamase encoded by pBR322 in minicells. Therefore, in these experiments results were expressed as band intensities relative to that of the β-lactamase precursor encoded in pKA5.

The results demonstrate that cAMP represses the synthesis of the 38 kilodalton protein (agmatine ureohydrolase) and the 43 kilodalton protein (S-adenosyl methionine synthetase) while the proteins 74 kilodalton and 70 kilodalton (arginine decarboxylase - precursor and product respectively) were induced. (Figure: 9.3). The repression of S-adenosyl methionine synthetase by cAMP has not been reported previously. The induction of the precursor and product forms of arginine decarboxylase is surprising in view of the repression of enzyme activities *in vivo* (Chapter 3; Wright and Boyle, 1982). It is not clear whether the induction of arginine decarboxylase seen in this experiment is an artifact of the minicell system, or if cAMP induces a repressor which in turn represses the *speA* gene (arginine decarboxylase) in normal cells.

Although the reason for this discrepancy is not clear at the moment, minicells may be limited in their ability to regulate gene expression (Adler *et al.*, 1967; Roozen *et al.*, 1971; Stoker *et al.*, 1984).

Agmatine induced the synthesis of agmatine ureohydrolase protein while it repressed the synthesis of S-adenosyl methionine synthetase and arginine decarboxylase (Figures: 9.2 and 9.3). It can be concluded that cAMP mediated repression and agmatine mediated induction of agmatine ureohydrolase do not require the synthesis of other gene products. Because cAMP mediates repression through CRP (Chapter 3; Wright and Boyle, 1982) it can be concluded that cAMP mediates the repression of agmatine ureohydrolase at the level of transcription. Agmatine being a charged molecule may interact with nucleic acids and proteins as does putrescine. Therefore, the effect of agmatine on agmatine ureohydrolase could be at the level of transcription, mRNA stability, or at the level of translation. If minicells are responding normally to agmatine (unlike the response proposed for arginine decarboxylase to cAMP) the slight repression of arginine decarboxylase by agmatine, which is small compared with the repression observed in normal cells, suggests that in normal cells the synthesis of arginine decarboxylase is regulated by putrescine derived from agmatine.

The extent of repression of cAMP and induction by agmatine of agmatine ureohydrolase is much less in minicells than in normal *E.coli* cells. The aberrant regulation of genes expressed in minicells has been reported previously

(Boyle *et al.*, 1985; Stoker *et al.*,1984).

Plasmid copy number is not likely to affect the results presented in this chapter as the minicells can support only 1-2 rounds of replication of plasmid contained in them (Inselburg, 1970; 1971), and this would be completed during the 15 hour pre-incubation prior to the commencement of labelling. No attempt was made in this work to understand the aberrant behaviour of arginine decarboxylase encoded by pKA5 in minicells. It is possible to speculate that minicells may not be abundant in CRP molecules, and therefore, cAMP:CRP would be limiting in these cells. If the *speB* promoter has greater affinity for cAMP:CRP binding than the *speA* promoter, then the subsaturating levels of cAMP-CRP would be titrated away, and would thus derepress *speA*.

Although pKA5 carries functional tet^R and amp^R genes, the proteins specified by the tet^R gene were not always detected in the fluorograms (Backman and Boyer, 1983; Curiale *et al.*, 1984).

Chapter 10

Regulation of *speB:lacZ*, *speB:galK* and *speA:galK* Gene Fusions

10.1 Introduction:

The specific nucleotide sequences that reside in the promoter determine the action of various regulatory factors of transcription and the rate of transcription by RNA polymerase. The promoter is characterised by 2 conserved sequences arrived at by analysing several prokaryotic promoters. These conserved sequences reside around the -10 and -35 base pairs upstream from the transcription start site (Rosenberg *et al.*, 1983 and Rosenberg and Court, 1979). Recombinant DNA techniques have made it possible to place the transcription of genes for easily assayable gene products under the control of different promoters. These techniques have proved to be extremely useful to analyse the role and mode of regulation by various factors involved in the regulation of the initiation of transcription. (McKenny *et al.*, 1981).

In order to analyse the precise roles and sites of action of cAMP and agmatine, and the roles of other putative regulatory elements, expression of the β-galactosidase or galactokinase genes fused to the promoter of the *speB* gene was examined. In order to analyse the expression and regulation of the *speA* gene (arginine decarboxylase), expression of galactosidase encoded by the *speA:galK* fusion plasmid was examined. The construction of the plasmids pAUL (*speB:lacZ* fusion plasmid) and pAUG (*speB:galK* fusion plasmid) is described in Chapter 7. The *speA:galK* fusion plasmid (pADG) which carries

the promoter of *speA* fused to the structural gene for galactokinase was constructed by Buch and Boyle (unpublished results).

10.2 Results:

10.2.1 The effect of cAMP, agmatine, ornithine, arginine and putrescine on the expression of galactokinase encoded by pAUG in N100:

The *E.coli* strain N100 (ΔgalK) carrying pAUG (*speB:galK* fusion plasmid) was grown in the presence of glucose as sole carbon source in minimal medium. Replicate cultures were supplemented with increasing concentrations of cAMP, agmatine, ornithine, arginine or putrescine. The cells were harvested and the activity of galactokinase in toluenised cells was determined as described in Section: 2.3.7. (Figure: 10.1). The specific activity of the enzyme is given in Table: 10.1. While cAMP repressed, arginine, ornithine and agmatine induced galactokinase activity, putrescine did not significantly affect the activity of galactokinase.

10.2.2 The effect of cAMP, agmatine, ornithine, arginine and putrescine on the expression of β-galactosidase encoded by pAUL in HB101:

E.coli HB101 carrying pAUL was grown in minimal medium containing glucose. Replicate cultures were supplemented with increasing concentrations of cAMP, arginine, ornithine, agmatine or putrescine. The enzymic activity of β-galactosidase was determined in toluenised cells and the results are shown in

Table 10.1

Specific activities of β-galactosidase β-lactamase and galactokinase in strains carrying fusion genes.

Strain	Plasmid	β-galactosidase	galactokinase	β-lactamase
N100	pAUG	N.D	568	69
N100	pADG	N.D	337	66
HB101	pAUL	1110	N.D	53
HT328	pAUL	1080	N.D	72
LS340	pAUL	1380	N.D	63
LS853	pAUL	2010	N.D	61
LS854-1	pAUL	1860	N.D	61

N.D = Not Determined

The activities of the enzymes were determined as described in Section 2.3.7 in toluenised cells.

Figure 10.1

The effects of cAMP, amino acids and polyamines on the expression of galactokinase encoded by plasmid pAUG in N100.

Relative activity is that relative to the activity in the absence of any added compounds (Table: 10.1) (=1.0). Each result obtained is the average of three experiments, and the bars represent the extreme values for each set of parameters. Figure: (A): shows the effect of cAMP and Figure: (B): show the effects of agmatine, ornithine, arginine, putrescine and spermidine on the expression of galactokinase. (⬢)=agmatine, (▲)=ornithine, (●)=arginine, (△)=putrescine, (○)=spermidine and (○)=cAMP.

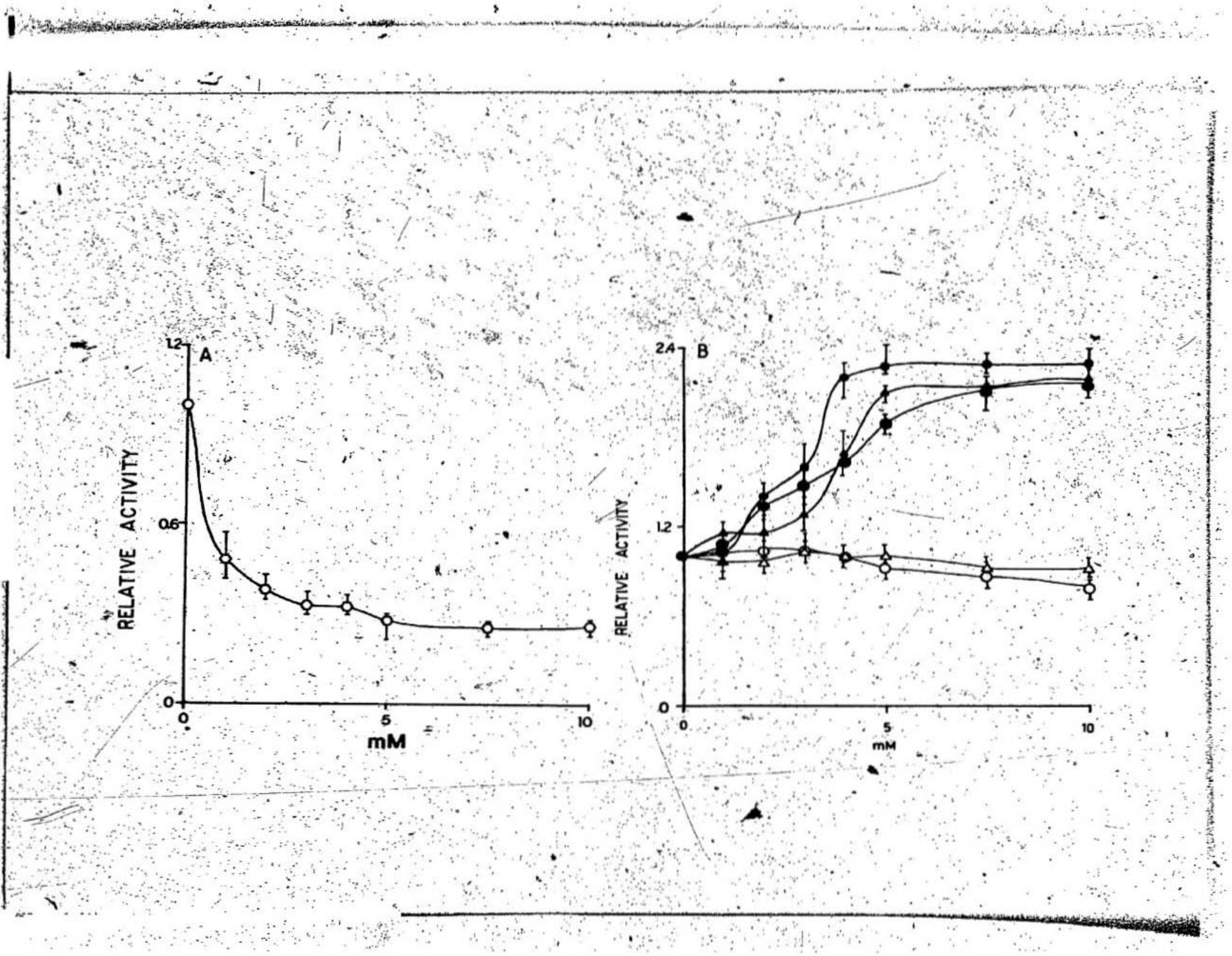

A
RELATIVE ACTIVITY
1.2
0.6
0
0
5
10
mM
B
RELATIVE ACTIVITY
2.4
1.2
0
0
5
10
mM

Figure: 10.2. The specific activity of the enzyme is given in Table: 10.1. Cyclic AMP repressed, while agmatine, arginine and ornithine induced the β-galactosidase activity. Putrescine neither induced nor repressed the activity of β-galactosidase.

10.2.3 The effect of agmatine, ornithine, arginine, putrescine, spermidine and cAMP on the expression of chromosomally encoded β-galactosidase in LS340, LS853, LS854-1 and HT328:

In order to demonstrate the role of CRP in the negative regulation of the *speB:lacZ* gene fusion in plasmid pAUL, it was necessary to analyse the effect of cAMP in Δ*cya* and Δ*crp* strains of *E.coli*. These strains have a chromosomal *lacZ* gene, whose expression might mask the expression of the gene in the fusion plasmid pAUL. The strains LS340, LS853 and LS854-1 were grown in minimal medium containing glucose. Two sets of cultures were grown, one with and one without IPTG (0.5mM). The replicate cultures in each set were then supplemented with cAMP. The results for the β-galactosidase activity in toluenised cells are presented in Table: 10.2. The chromosomal *lacZ* expression in negligible in the absence of IPTG.

From the results of Section 10.2.2 it was not clear whether the effect of ornithine and arginine on the expression of the fusion gene in pAUL was direct or indirect. In order to clarify this, the effect of these compounds had to be examined in HT328, a strain deleted for *speA*, *speB* and *speC* genes and which

Figure 10.2

The effects of cAMP, amino acids and polyamines on the expression of β-galactosidase encoded by plasmid pAUL in HB101.

Relative activity is that relative to the activity in the absence of any added compounds (Table: 10.1) (=1,0). IPTG was not included in the medium. Each result obtained is the average of 5 experiments, and the bars represent the extreme values for each set of parameters. Figure: **(A)**: shows the effect of cAMP and Figure: **(B)**: show the effects of agmatine, arginine, ornithine, putrescine and spermidine on the expression of β-galactosidase. (⬢)=agmatine, (▼)=ornithine, (●)=arginine, (▽)=putrescine, (○)=spermidine and (○)=cAMP.

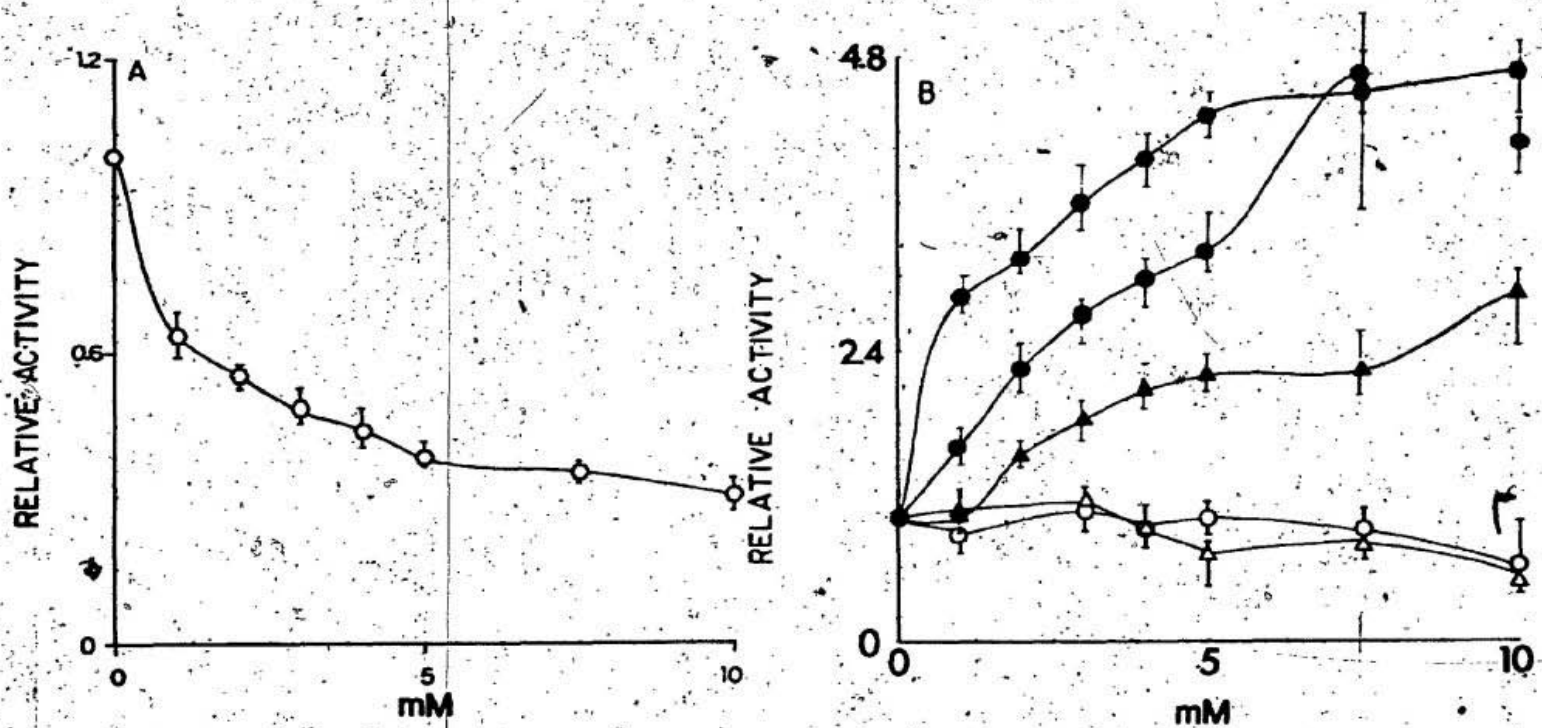

A
1.2
0.6
0
RELATIVE ACTIVITY
0
5
10
mM
B
4.8
2.4
0
RELATIVE ACTIVITY
0
5
10
mM

Table 10.2

The effects of cAMP, amino acids and polyamines on the expression of chromosomally located *lacZ*.

Strain	Condition	β-gal
HT328	-	<1
HT328	+1mM cAMP	<1
HT328	+0.5mM IPTG	120
HT328	+0.5mM IPTG +1mM cAMP	740
HT328	+5mM Putrescine	4
HT328	+5mM Spermidine	4
HT328	+5mM Agmatine	2
HT328	+5mM Arginine	<1
HT328	+5mM Ornithine	<1
LS340	-	<1
LS340	+1mM cAMP	<1
LS340	+5mM cAMP	90
LS340	+IPTG	120
LS853	-	<1
LS853	+1mM cAMP	<1
LS853	+5mM cAMP	90
LS853	+IPTG	70
LS854-1	-	<1
LS854-1	+1mM cAMP	<1
LS854-1	+5mM cAMP	<1
LS854-1	+IPTG	70

N.D = Not Determined
agmatine = 2 mM
cAMP = 1 mM
IPTG = 0.5 mM
β-gal = β-galactosidase

The strains were grown in minimal media and the compounds were added into the medium at the concentrations indicated. The activities were determined in toluenised cells as described in Section: 2.3.7.

therefore could not convert the compounds to putrescine. *E.coli* strain HT328 carries an intact *lacZ* gene on the chromosome. In order to determine if the expression of the chromosomally encoded β-galactosidase would mask the expression of the fusion gene in pAUL, replicate cultures of HT328 were grown in minimal media containing glucose. One set of cultures were supplemented with 0.5mM IPTG. The replicate cultures in one set (those without IPTG) were grown with agmatine, putrescine, spermidine, arginine or ornithine and replicate cultures of the other set were cultured in the presence or in the absence of cAMP. The enzymic activity of β-galactosidase was determined and the results are presented in Table: 10.2. The expression of *lacZ* is negligible in the absence of IPTG or in the presence of amino acids and polyamines.

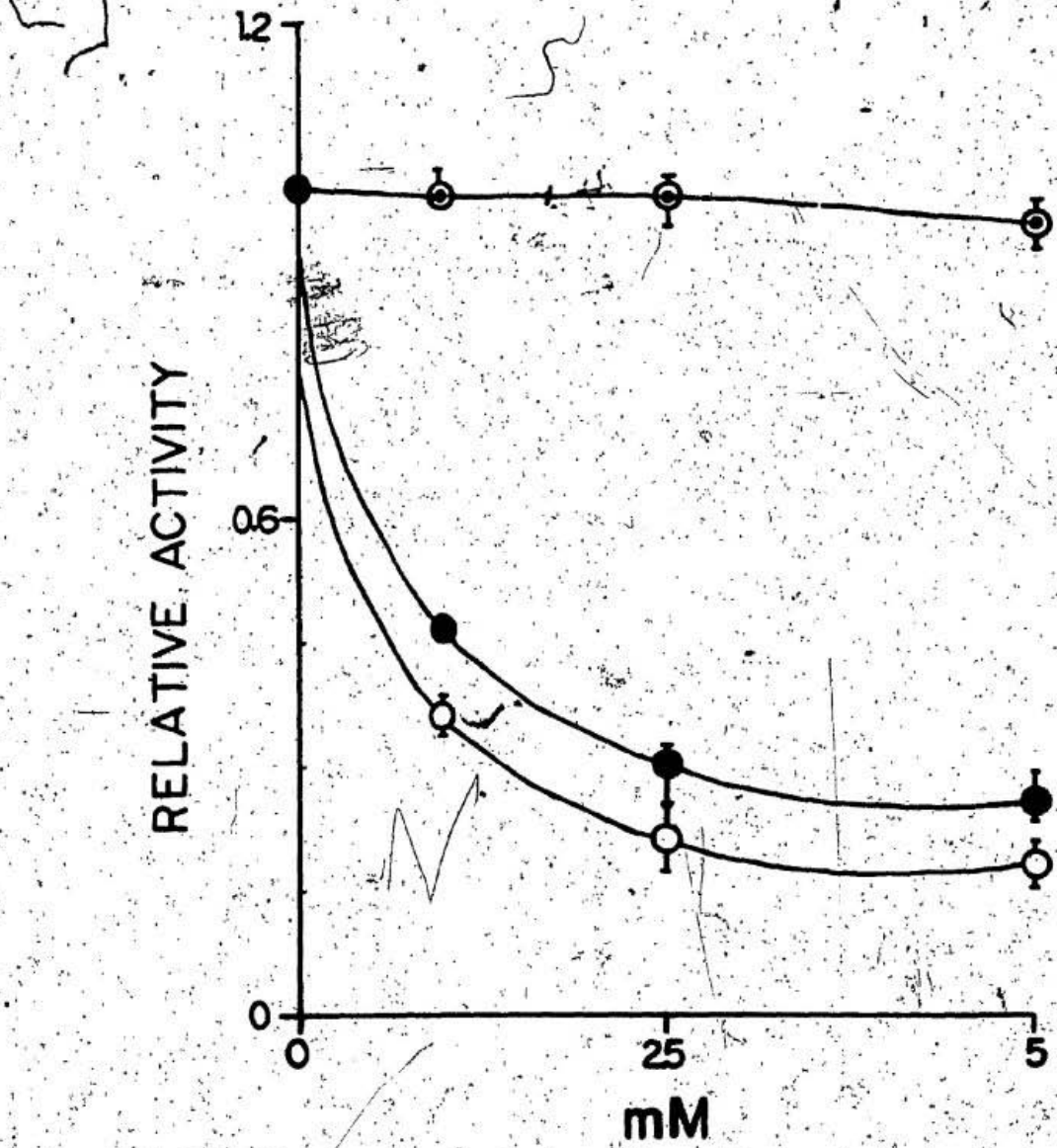
10.2.4 The effect cAMP on the expression of β-galactosidase encoded by pAUL in LS340, LS853 and LS854-1:

E.coli strains LS340, LS853 (Δ*cya*) and LS854-1 (Δ*cya*,Δ*crp*) carrying plasmid pAUL (carrying *speB:lacZ* gene fusion) were grown in the presence of glucose in minimal medium. Replicate cultures were supplemented with increasing concentrations of cAMP (1-5 mM). The enzymic activity of β-galactosidase was determined and is shown in Figure: 10.3. The absolute activities of the enzyme in LS340, LS853 and LS854-1 are given in Table: 10.1. cAMP repressed β-galactosidase activity in LS340 and LS853 but not in LS854-1.

Figure 10.3

The effect of cAMP on the expression of β-galactosidase encoded by plasmid pAUL in LS340, LS853 and LS854-1.

Relative activity is that relative to the activity in the absence of any added compounds (Table: 10.1) (=1.0). IPTG was not included in the medium. Each result obtained is the average of three experiments, and the bars represent the extreme values for each set of parameters. The figure shows the effect of cAMP on the expression of β-galactosidase in LS340=(●), LS853=(○) and LS854-1(◎).


1.2
0.6
0
RELATIVE ACTIVITY
0
25
5
mM

10.2.5 The effect of ornithine, arginine, putrescine spermidine and agmatine on the expression of β-galactosidase encoded by pAUL in HT328:

HT328 (Δ*speA*, Δ*speB*, Δ*speC*) carrying pAUL (carrying *speB:lacZ* gene fusion) was grown in minimal medium containing glucose as the sole carbon source. Replicate cultures were supplemented with increasing concentrations (up to 5mM) of ornithine, arginine, agmatine, putrescine or spermidine and the activity of β-galactosidase was determined. The absolute activity of the enzyme is given in Table: 10.1. The results are presented in Figure: 10.4. Agmatine induced, while putrescine, spermidine, arginine and ornithine neither induced nor repressed the activity of β-galactosidase.

10.2.6 The effect of cAMP, agmatine, ornithine, arginine, putrescine and spermidine on the expression of galactokinase encoded by pADG in N100:

N100 (Δ*galK*) carrying the fusion gene *speA:galK* in plasmid pADG was grown in the presence of glucose in minimal medium. Increasing concentrations of cAMP, agmatine, arginine, ornithine, putrescine or spermidine were added to the growth medium, and galactokinase activities were determined, and are shown in Figure: 10.5. Cyclic AMP, agmatine, arginine, ornithine, putrescine and spermidine repressed the activity of galactokinase.

Figure 10.4

The effects of amino acids and polyamines on the expression of β-galactosidase encoded by plasmid pAUL in HT328.

Relative activity is that relative to the activity in the absence of any added compounds (Table: 10.1) (=1.0). IPTG was not included in the medium: Each result obtained is the average of 3 experiments, and the bars represent the extreme values for each set of parameters. The figure show the effects of (⬢)=agmatine, (▲)=ornithine, (●)=arginine, (△)=putrescine, and (○)=spermidine on the expression of β-galactosidase.

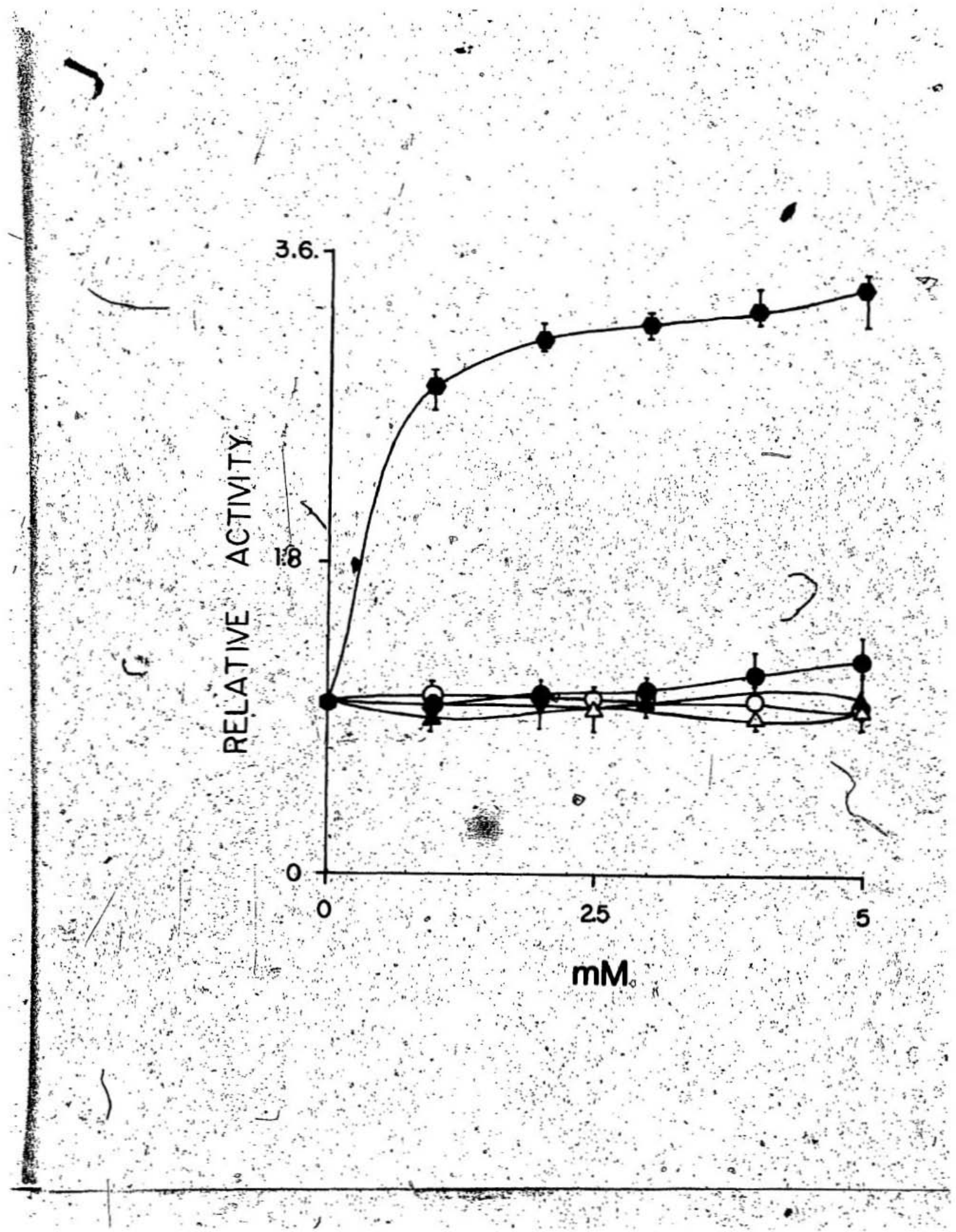
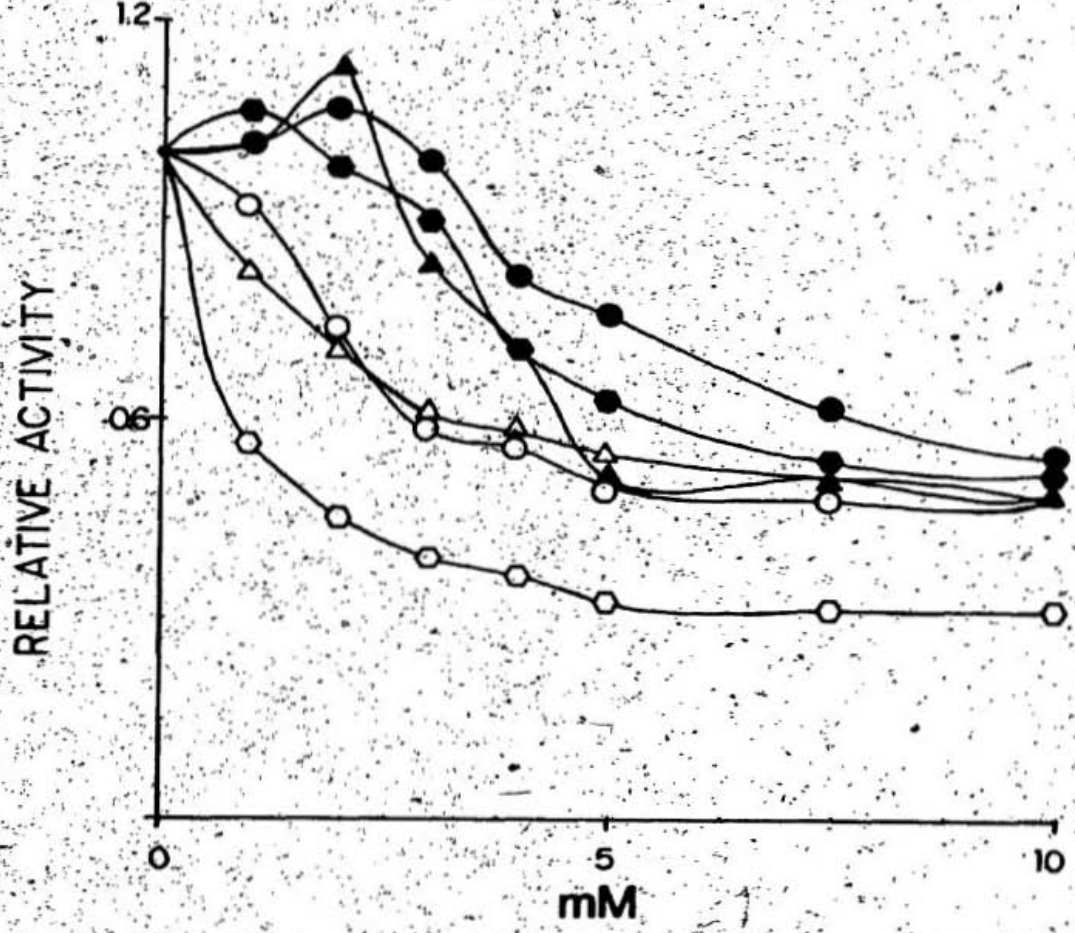

3.6
1.8
0
RELATIVE ACTIVITY
0
25
5
mM

Figure 10.5

The effects of cAMP, amino acids and polyamines on the expression of galactokinase encoded by plasmid pADG in N100.

Relative activity is that relative to the activity in the absence of any added compounds (Table 10.1) (=1.0). Each result obtained is the average of two experiments. The figure show the effects of cAMP, agmatine, ornithine, arginine, putrescine and spermidine on the expression of galactokinase. (⬢)=agmatine, (▼)=ornithine, (●)=arginine, (▽)=putrescine, (○)=spermidine and (⬡)=cAMP.


1.2
.6
RELATIVE ACTIVITY
0
5
10
mM

10.2.7 The effect of cAMP, agmatine, putrescine, spermidine, arginine and ornithine on the expression of β-lactamase encoded by plasmids pADG, pAUG and pAUL in HB101, N100, HT328, LS340, LS853 and LS854-1:

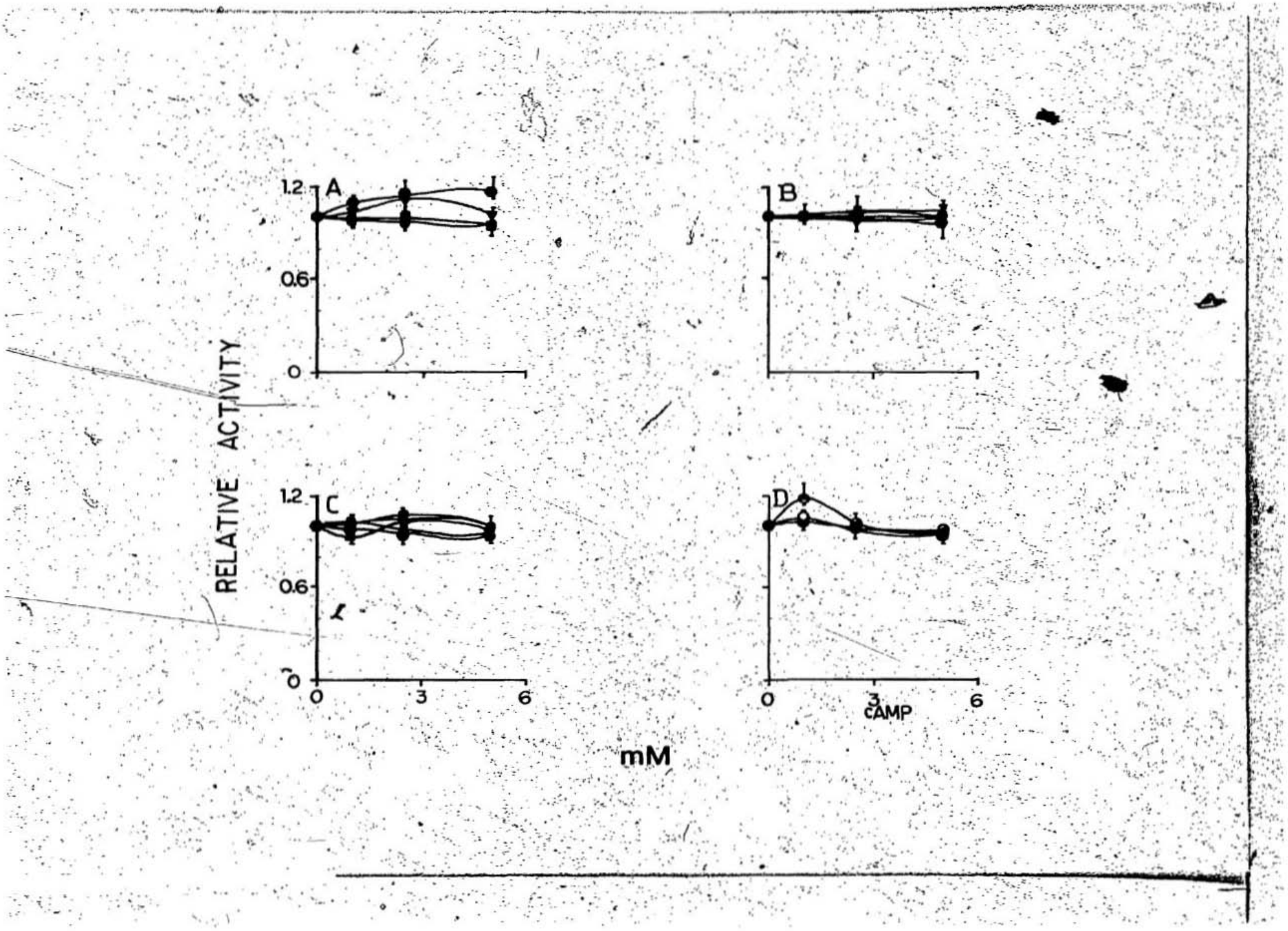
Enzyme activity has been shown to be proportional to the number of gene copies in a cell (Clewell and Helinski, 1972). Since these experiments were performed with multicopy plasmids it is possible that the effects of various compounds on the expression of the fusion genes were due to changes in the plasmid copy numbers caused by these compounds in the growth medium. Putrescine and cAMP cause marked change in growth rate (Tabor and Tabor, 1969; Wright and Boyle, 1982; Chapter-4). Although Clewell and Helsinki (1972) have shown that the copy number of ColE1 derived plasmids does not vary significantly with growth rate, the ColE1 derived plasmids used in this work are significantly different, and may not behave similarly.

To address this problem, the strains carrying various plasmids (pAUG and pADG, in N100, and pAUL in HB101, HT328, LS340, LS853 and LS854-1) were grown under the conditions described earlier for each combination of plasmid plus host. Replicate cultures were supplemented with increasing concentrations of cAMP (up to 5mM), agmatine, arginine, ornithine, putrescine or spermidine, and enzymic activity of β-lactamase was determined in toluenised cells. The results are presented in Figure 10.6, and the specific activity of the enzyme in various strains is shown in Table 10.1. β-lactamase activity

Figure: 10.6

The effects of cAMP, polyamines and amino acids on the expression of β-lactamase encoded by pAUL and pAUG in N100, HB101, HT328, LS340, LS853 and LS854-1.

1.0 represents the activity in the absence of any added compounds (Table 10.1). Each result is an average of 3 experiments and the bars represent the extreme values for each set of experiments. The enzymic activity was determined in cultures of HT328 carrying pAUL (**A**), N100 carrying pAUG (**B**) and HB101 carrying pAUL (**C**) grown in minimal glucose media containing agmatine (●), ornithine (▼), arginine (○), cAMP (●) and putrescine (■). In Figure: 10.6D, the effect of cAMP on the expression of β-lactamase is shown in LS340 (○), LS853(⊙) and LS854-1 (●) carrying pAUL.


A
B
C
D
1.2
0.6
0
RELATIVE ACTIVITY
0
3
6
cAMP
mM

(encoded by the plasmids) was unaffected by increasing supplementation of cAMP, amino acids or polyamines in the growth medium.

10.2.8 cAMP:CRP binds to the speB promoter:

In order to substantiate that cAMP:CRP indeed regulated the experession of the *speB* gene by interacting with the *speB* promoter, the following experiment was performed. The assumption was that cAMP and therefore cAMP:CRP is limiting in glucose grown cultures, although CRP has been shown to be present in large excess in cultures grown similarly (Cossart and Sanzey, 1985). Limiting concentrations of cAMP:CRP could then be titrated away by a high copy number plasmid; carrying a cAMP:CRP binding site. Plasmid pKA10 lacks a functional *speB* gene, as it does not confer agmatine ureohydrolase activity to strains deleted for the *speB* gene when these strains are transformed with the plasmid. The plasmid pKA10 has been shown to carry the *speB* promoter and about one half the coding region for agmatine ureohydrolase (Chapter-7) (Figure: 10.7). It would therefore be a suitable plasmid to test this hypothesis. The segment of chromosomal DNA that pKA10 carries, when transferred to pMC1403 and pKO11, repressed β-galactosidase (Section: 10.2.2) and galactokinase (Section: 10.2.1) activities in the presence of cAMP, suggesting that cAMP:CRP binding site is associated with this segment of DNA. Introduction of this plasmid (pKA10) into strains of *E.coli* growing on glucose (cAMP is limiting and therefore cAMP:CRP

Figure 10.7

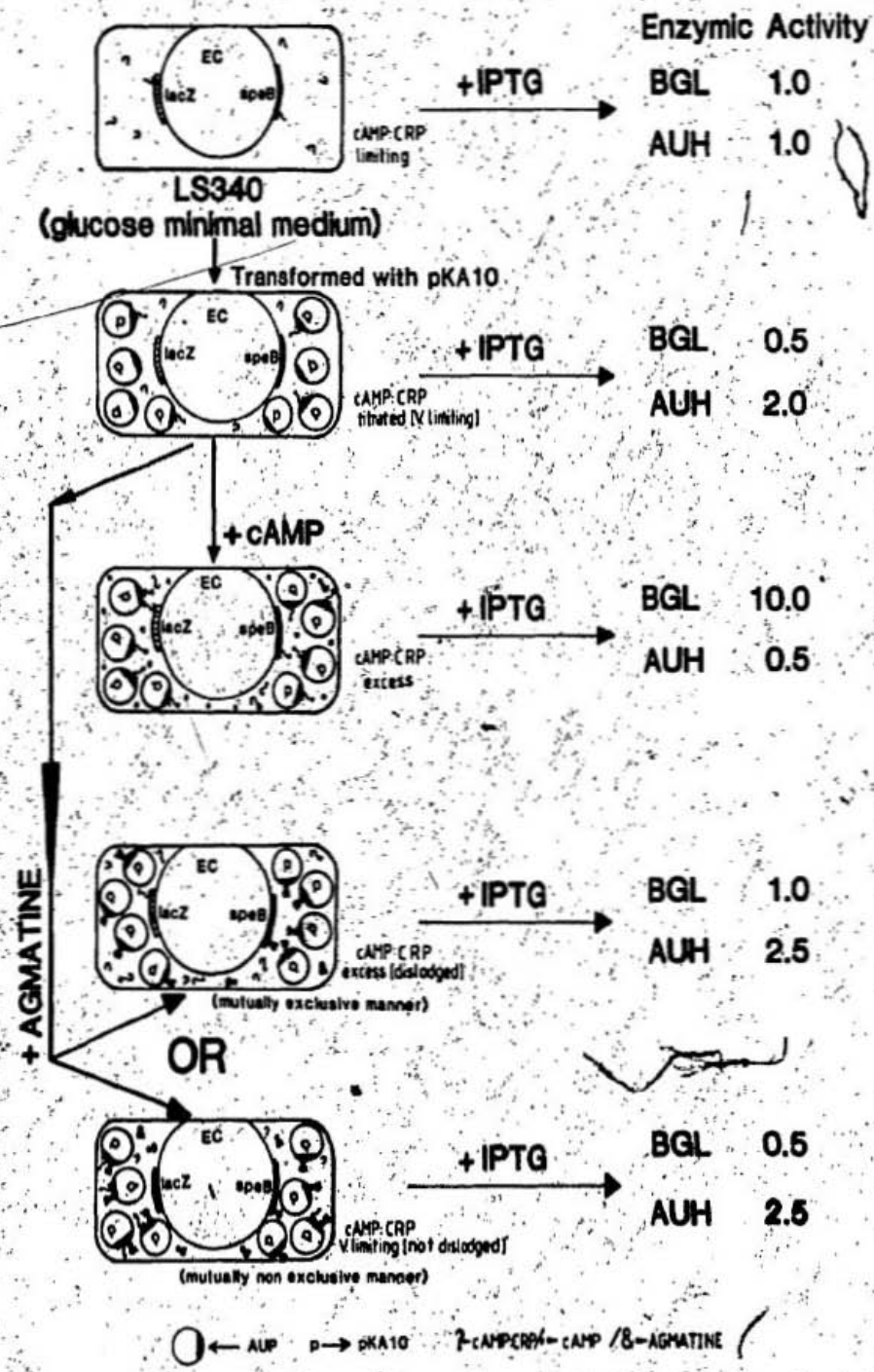
Schematic diagram illustrating 'mutually exclusive' and 'mutually non-exclusive' mechanisms of action by cAMP and agmatine on the *speB* gene.

Mutually exclusive manner: Agmatine displaces cAMP:CRP from the *speB* promoters on the chromosome and the plasmid pKA10 and thus restores the activity of β-galactosidase encoded by the chromosomal *lacZ*, to the level of that observed in cells without the *speB* promoter containing plasmid (more cAMP would be available for the increased transcription of *lacZ*).

Mutually non-exclusive manner: Agmatine and cAMP:CRP act at separate sites and therefore cAMP:CRP is not dislodged from the *speB* promoters on the chromosome and on the plasmid that carries the *speB* promoter, and therefore β-galactosidase activity would not be increased beyond the activity observed in cells that carry plasmids bearing the *speB* promoter (pKA10) when agmatine is added in the growth medium.

The activities of **BGL** (β-galactosidase) and of**AUH** (agmatine ureohydrolase) are examples of what might be expected, and are given relative to the activity in strains not carrying plasmid pKA10 **(P)**. The results of the experiment are given in Table: 10.3.

(EC) = *E.coli* chromosome, **(AUP)** = AUH promoter, and the *lacZ* and *speB* genes on the *E.coli* chromosome are shown as (▭) and (▬) respectively.


Enzymic Activity
EC
lacZ
speB
+IPTG
BGL 1.0
AUH 1.0
cAMP:CRP limiting
LS340
(glucose minimal medium)
Transformed with pKA10
BGL 0.5
AUH 2.0
cAMP:CRP titrated [V limiting]
+cAMP
BGL 10.0
AUH 0.5
cAMP:CRP excess
+AGMATINE
BGL 1.0
AUH 2.5
cAMP:CRP excess [dislodged]
(mutually exclusive manner)
OR
BGL 0.5
AUH 2.5
cAMP:CRP V limiting [not dislodged]
(mutually non exclusive manner)
← AUP
→ pKA10
?-cAMPCRP- cAMP /&-AGMATINE

would also be limiting) would be expected to relieve the repression of chromosomally encoded genes that are repressed by cAMP, and conversely to repress the genes that are induced by cAMP:CRP.

An *E.coil* strain LS340 which carries chromosomally encoded functional *speB* and *lacZ* genes was employed. This strain was transformed either with plasmid pKA10 or with the plasmid pBR322. The transformed and the untransformed strains were grown separately in M9-minimal medium containing glucose as the sole carbon source. Replicate cultures were supplemented with 0.5 mM IPTG. The enzymic activities of the chromosomally encoded β-galactosidase and agmatine ureohydrolase were determined in crude extracts. The results are presented in Table: 10.3. In control experiments cAMP (1mM) was included in the growth medium of cultures growing in the presence or in the absence of IPTG. IPTG induced β-galactosidase activity in LS340 and LS340/pBR322, but did so to a lesser extent in LS340/pKA10. At the same time agmatine ureohydrolase activity was increased in LS340/pKA10 as compared to the activities in LS340 and LS340/pBR322. These observations are consistent with the binding of cAMP:CRP to the copies of pKA10 (at their *speB* promoter sites).

10.2.9 Does agmatine induce speB expression by excluding cAMP:CRP binding to the speB promoter?:

From the experiment described above (10.2.8) it may be concluded that

Table 10.3
The effects of agmatine and titration of cAMP: CRP by the *speB* promoter in LS340 and LS853 on the expression of chromosomally located *lacZ* and *speB* genes.

Table 10.3(a)

Strain	Condition	β-gal	AUH
LS340	-	< 1	1.1
LS340/pBR322	-	< 1	1.0
LS340/pKA10	-	< 1	1.0
LS340	+IPTG	540	1.1
LS340/pBR322	+IPTG	520	1.0
LS340/pKA10	+IPTG	260	1.9
LS340	+IPTG+agmatine	530	2.1
LS340/pBR322	+IPTG+agmatine	550	2.5
LS340/pKA10	+IPTG+agmatine	260	2.2
LS340	+cAMP	< 1	0.6
LS340/pBR322	+cAMP	< 1	0.6
LS340/pKA10	+cAMP	< 1	0.6
LS340	+IPTG+cAMP	5700	0.7
LS340/pBR322	+IPTG+cAMP	5300	0.6
LS340/pKA10	+IPTG+cAMP	5300	0.7
LS340	+agmatine	< 1	2.4
LS340/pBR322	+agmatine	< 1	2.4
LS340/pKA10	+agmatine	< 1	2.4

Table 10.3(b)

Strain	Condition	β-gal	AUH
LS853	-	< 1	2.2
LS853/pBR322	-	< 1	2.1
LS853/pKA10	-	< 1	2.1
LS853	+IPTG	280	2.1
LS853/pBR322	+IPTG	260	2.2
LS853/pKA10	+IPTG	260	2.1
LS853	+IPTG+agmatine	230	6.7
LS853/pBR322	+IPTG+agmatine	250	6.0
LS853/pKA10	+IPTG+agmatine	240	6.3
LS853	+IPTG+cAMP	5700	N.D
LS853/pBR322	+IPTG+cAMP	5400	N.D
LS853/pKA10	+IPTG+cAMP	5500	N.D

Table 10.3

N.D = Not Determined agmatine = 2 mM cAMP = 1 mM IPTG = 0.5 mM - = M9 glucose minimal medium β-gal = β-galactosidase AUH = agmatine ureohydrolase

The activities of the enzymes were determined in crude extracts and are expressed as units of activity per milligram protein. The activities were determined in the host strains and in strains transformed with plasmids grown in M9 minimal medium, under conditions described in the table.

cAMP:CRP binds to the *speB* promoter region. Whereas cAMP:CRP repressed the expression of agmatine ureohydrolase the enzyme is induced by agmatine, and sufficiently high concentrations of agmatine can overcome the action of cAMP:CRP (Chapter 5). To determine if cAMP:CRP and agmatine act at the same or different sites, the effect of agmatine on chromosomally encoded β-galactosidase in a strain carrying pKA10 was tested.

In an identical experiment to that described in Section: 10.2.8, 2mM agmatine was included in the growth media of transformed and untransformed strains supplemented with or without 0.5mM IPTG. The enzymic activities of β-galactosidase and agmatine ureohydrolase were determined and are shown in Table: 10.3. β-Galactosidase activity in LS340 growing on glucose was not induced by added agmatine. Hence there is no direct effect of agmatine on the induction of β-galactosidase. Agmatine did not relieve the decreased induction of β-galactosidase in IPTG supplemented cultures of LS340/pKA10. A schematic diagram of cAMP:CRP and agmatine interaction is shown in Figure: 10.7.

10.3 Discussion:

In this chapter, it is clearly demonstrated that cAMP represses *speB* as a function of CRP by recognising its promoter to regulate its transcription (Figures: 10.1, 10.2). Cyclic AMP also regulates *speA* promoter expression (Figure: 10.5). It is apparent from the experiments described in this chapter that the

information for the negative transcriptional regulation of *speA* and *speB* is carried on the promoter.

The chromosomal *lacZ* expression in *E.coli* strains HT328, LS340, LS853 and LS854-1 is negligible in the absence of IPTG and therefore does not mask the expression of the fusion genes introduced into these strains (Table: 10.2). From the results presented in Figure: 10.6 it is clear that β-lactamase activities expressed by the genes carried on pAUL, pADG, and pAUG were not significantly affected when the strains that carry them were subjected to various growth conditions. Variations in copy number of these plasmids would have affected β-lactamase enzyme activity as has been shown for galactokinase activity encoded by the *galK* gene in a derivative of pBR322 (Duester *et al.*, 1982).

The results presented in Figures 10.1 and 10.2 are consistent with those reported in Chapters 4, 5 and 6. Ornithine, arginine and agmatine induced, cAMP repressed, and putrescine and sperimidine did not affect the activities of β-galactosidase or galactokinase when added to the growth medium of strains carrying pAUL and pAUG. When the *speB:galK* and *speB:lacZ* promoter fusion plasmids are present in a normal host (ADC^+, AUH^+ and ODC^+), *galK* and *lacZ* are expressed at elevated levels when ornithine, arginine or agmatine is present. In the deletion strain HT328 (*speA, speB, speC*), however, the enzymes encoded by the fusion plasmids are not induced by arginine or ornithine. Galactokinase and β-galactosidase are induced, however, by

agmatine (Figure: 10.4). Consequently, it is concluded that agmatine acts directly on the expression of *speB*, whereas the induction of *speB* by ornithine and arginine appears to be due to their metabolic conversion to agmatine. The observations reported above are also consistent with those reported in Chapter 6. Cyclic AMP repressed the activity of β-galactosidase in both LS340 and LS853 (Δ*cya*) but not in LS854-1 (Δ*crp*). The results are consistent with those reported in Chapter 4, and it can be concluded that a functional CRP is required for cAMP mediated repression of *speB* promoter activity (Figure: 10.3).

The repression of *speA* promoter activity of cAMP, agmatine, ornithine arginine, putrescine and spermidine (Figure: 10.5) is consistent with the results presented in Chapters 4 and 6. The results are also consistent with the observations of Buch and Boyle (personal communication). The *speA* promoter was fused to the structural gene for tetracycline resistance on a plasmid, and the sensitivity to the the drug of the host strain carrying this plasmid was determined, when grown in the presence of these compounds. Cyclic AMP, agmatine, arginine, ornithine and putrescine decreased the resistance of the strain that carried the fusion plasmid (*speA:tet*), to tetracycline. However, it is not clear in these experiments whether arginine, ornithine and agmatine repress *speA* directly or upon their conversion to putrescine as had been shown in Chapter 6.

The induction by agmatine of β-galactosidase activity encoded by pAUL in HB101 and HT328 (Figures: 10.2 and 10.4) was about twice that of the galactokinase activity encoded by pAUG in N100 (Figure: 10.1). The reasons for this are not known. The expression of *lacZ* in the promoter cloning vector pMC1403 not only requires the promoter activity of the inserted DNA but also the translation initiation codon and the Shine-Dalgarno sequence (for ribosome binding). In this plasmid the sequences upstream from the seventh codon of the β-galactosidase structural gene are deleted. In pK011, the galactokinase gene is constructed in such a way that only the promoter of the *gal* operon is deleted. The galactokinase structural gene carries both the Shine-Dalgarno sequence and the initiation codon for its translation. This promoter cloning vector was constructed with translational stop codons in all three reading frames upstream from the Shine-Dalgarno sequence. Therefore, the inserted fragment imparts only the promoter activity required for the expression of galactokinase in this plasmid. Any proteins initiated at the initiation codons in the insert DNA would be terminated prior to the translation initiation of galactokinase. Although a multicistronic mRNA is produced in this fusion, a fusion protein (with galactokinase) is not produced, unlike the *lacZ* fusion in which both a fusion RNA and a fusion protein result. (Schematically shown in Figure: 10.7). Hence, in the *speB:lacZ* fusion, the expression of β-galactosidase activity is dependent upon the *speB* promoter, the Shine-Dalgarno sequences, and the initiation codon specified by the *speB* DNA. The galactokinase

activity in the *speB:galK* fusion is dependent upon the *speB* promoter only. It should also be noted that in the *speB:lacZ* fusion, the agmatine ureohydrolase : β-galactosidase fusion protein is translated from multicistronic *lac* mRNA from the first cistron; while in the *speB:galK* fusion galactokinase is translated from the second cistron of the multicistronic mRNA (schematically shown in Figure 10.7). It is possible to speculate that the differential induction of *speB:lacZ* versus *speB:galK* expression by agmatine is due to polarity effects, whereas the translation of the second cistron from the *speB:galK* fusion mRNA is reduced due to the specific effect of agmatine on *speB* mRNA translation. These fusions carry substantial amount of the 5' coding region of *speB*, and since polyamines are known to specifically associate with t-RNA and stabilise its structure (Cohen, 1978), a possible interaction between *speB* mRNA and agmatine resulting in an increased stability of the fusion mRNA might be possible. Increased activity of agmatine ureohydrolase might either be due to increased stability of the *speB* mRNA or due to agmatine interactions with the ribosomes resulting in a specific increase in initiation and/or elongation on the *speB* mRNA. On the other hand, agmatine might associate with RNA polymerase to specifically increase the expression of the fusion genes, probably by affecting the rate of elongation of transcription. The results presented in Table 10.3a show that cAMP:CRP is indeed titrated away by the plasmid pKA10, and not by pBR322. Therefore the cAMP:CRP binding site must reside in the chromosomal segment carried on the plasmid pKA10. The possibility of pKA10

encoding an inducer molecule for *speB* expression is unlikely, because *lacZ* expression is concomitantly reduced. Agmatine induced the derepressed *speB* gene but did not induce the expression of *lacZ* gene (Table: 10.3a). This is consistent with one of the possibilities mentioned earlier, that is, agmatine did not displace the cAMP:CRP molecules bound to pKA10, and therefore does not compete for the same site. Agmatine may exert its effect directly or indirectly at a separate site. Similar experiments using the Δ*cya* strain, LS853, did not distinguish between the two possibilities because *lacZ* expression was minimal in the presence of 0.5mM IPTG and *speB* expression was derepressed, as this strain lacks cAMP and therefore cAMP:CRP. Transformation of this strain with either pKA10 or pBR322 did not change the observation. Addition of 1mM cAMP resulted in the expression of *lacZ* and *speB* (Table: 10.3b) at the level identical to those described for LS340 and LS340 transformed with either pBR322 or pKA10 treated with cAMP (Table: 10.3a).

Chapter 11

Relative Concentrations of *speA*, *speB* and *lacZ* Messenger RNA in *E.coli*: Sodium Iodide Mediated Immobilisation of Nucleic Acids.

11.1 Introduction:

Our understanding of gene structure has been greatly enhanced by DNA hybridisation techniques. In contrast our understanding of gene expression has been less, due to the lack of corresponding RNA hybridisation techniques. Until recently the RNA hybridisation techniques on solid supports have been done primarily in concentrated solutions of sodium chloride (Thomas, 1980). White and Bancroft (1982), showed that when a cytoplasmic fraction from whole cells had been treated with formaldehyde, the RNA could be bound to a nitrocellulose paper by filtration. This method requires a cytoplasmic extract free from DNA because the latter can also be bound. Such a technique would not be useful to analyse extracts of bacteria because they contain DNA and basic proteins which also bind to the nitrocellulose filter. Recently, Bresser *et al.* (1983 a;b) and Gillespie and Bresser (1983) have shown that eukaryotic mRNA in whole cell lysates can be selectively bound to nitrocellulose in the presence of sodium iodide (a chaotropic salt) and detergents. DNA can also be selectively bound to nitrocellulose by filtering samples at elevated temperatures in the absence of detergents. Although the precise mechanism of nucleic acid adsorption to nitrocellulose is less well understood, this method has the advantage of being selective, rapid and quantitative. The binding of mRNA to nitrocellulose is not dependent upon a 5' cap structure, a 3' poly (A) tail, and it

does not appear that the secondary structure or G+C content are involved in the selective binding of mRNA over r-RNA and t-RNA (Bresser *et al.*, 1983 a;b).

Although several eukaryotic mRNAs selectively bind to nitrocellulose using this method (called the 'quick blot' method), there are only a few reports to show that prokaryotic mRNAs from whole cells can be selectively immobilised on nitrocellulose (Boyle *et al.*, 1985).

In this chapter *lacZ*, *speA* and *speB* genes are analysed. *E.coli* strains LS853 (Δ*cya*) and LS854-1 (Δ*cya*, Δ*crp*), were grown in minimal medium containing glucose as the sole carbon source, and to replicate cultures cAMP, agmatine or putrescine were added. Extracts were prepared, and the nucleic acids were immobilised on the nitrocellulose filters as described in Section 2.12. In order to compare the concentrations of mRNA in cells grown in the presence or absence of cAMP, agmatine or putrescine, the filters were hybridised with radiolabelled DNA of plasmid pKA15 (which carries the *HincII* fragment from within the transcribed region of the *speB* gene) (Section: 6.2.3), plasmid pKA16 (which carries the *speA* gene) (Buch and Boyle, unpublished results), or pUC8 (which carries the *lacZ* gene), and the amounts of probe (plasmid DNA) bound were analysed.

The 'quick-blots' were hybridised with plasmid DNA, and could be re-used after washing to remove the previously hybridised DNA. In order to

determine the specificity of hybridisations, *B.subtilis* and *E.coli* (strain HT328 (Δ*speA*, Δ*speB*)) which do not have homologous genes for *speA* and *speB* were grown and 'quick blots' were made from cell extracts. The filters were hybridised with either pKA15 or pKA16. To demonstrate the specificity of this technique further, the *speA*, *speB* deletion strain of *E.coli* (HT328) was transformed with plasmid pKA5 which carries *speA* and *speB* genes and was analysed to detect the expression of these genes. In order to determine the selectively of this technique RNAase-A treated samples were quick blotted to bind RNA or DNA. DNAase treated samples were similarly processed. Purified DNA and RNA samples were each quick blotted under conditions for binding RNA and for binding DNA.

Following hybridisation, the nitrocellulose filters were exposed to X-ray films. After developing the autoradiograms were analysed by densitometric scanning. The ratio of the result for immobilised mRNA to the result for immobilised DNA, each from a given number of cells, yielded a relative mRNA concentration for a given gene.

11.2 Results:

11.2.1 The effect of IPTG and cAMP on the steady state concentrations of β-galactosidase mRNA in LS853 and LS854-1:

RNA and DNA from whole cells of *E.coli* strains LS853 and LS854-1 grown in the presence or absence of 0.5 mM IPTG and 2 mM cAMP, in

minimal medium with glucose as the sole carbon source, were selectively immobilised on a nitrocellulose filter, and hybridised to the ^{32}P-labelled plasmid pUC8 (*lacZ*) DNA (Figure: 11.1). The dot intensities were analysed and the ratio of the intensity of RNA to DNA (Section: 2.27) is given in Table: 11.1. The relative concentration of *lacZ* mRNA increased when LS853 was grown in the presence of IPTG and was further increased when cAMP was added to the growth medium. The further increase in *lacZ* mRNA concentrations was not detected in LS854-1 following the addition of cAMP.

11.2.2 The effect of cAMP, agmatine and putrescine on the steady state concentration of speB mRNA in LS853 and LS854-1:

E.coli strains LS853 and LS854-1 were grown in minimal medium containing glucose and replicate cultures were supplemented with cAMP, agmatine or putrescine. The DNA and RNA samples prepared from these cultures were immobilised on nitrocellulose filters and hybridised to ^{32}P-labelled plasmid pKA15 DNA (Figure: 11.2). The autoradiograms were analysed to determine the relative concentrations of mRNA, and the results are given in Table: 11.1. Cyclic AMP decreased the *speB* mRNA concentration in LS853 but not in LS854-1. Agmatine increased the concentrations of *speB* mRNA in both the strains, while putrescine did not.

Figure 11.1

The effect of cAMP on the concentration of *lacZ* mRNA from whole cells of *E.coli* strains LS853 and LS854-1.

RNA **(R)** and DNA **(D)** from whole cells of *E.coli* strains LS853 **(A)** and LS854-1 **(B)** grown in the presence of IPTG, IPTG + cAMP or in their absence were immobilised on the same nitrocellulose filters and the filters were probed with radiolabelled pUC8 plasmid to detect *lacZ* mRNA and DNA sequences. In **(A)**, **(1)**= control, **(2)**= +IPTG and **(3)**= +IPTG+2.5mM cAMP. In **(B)**, **(1)**= +IPTG, **(2)**= +IPTG+1mMcAMP and **(3)**= +IPTG+5mM cAMP.

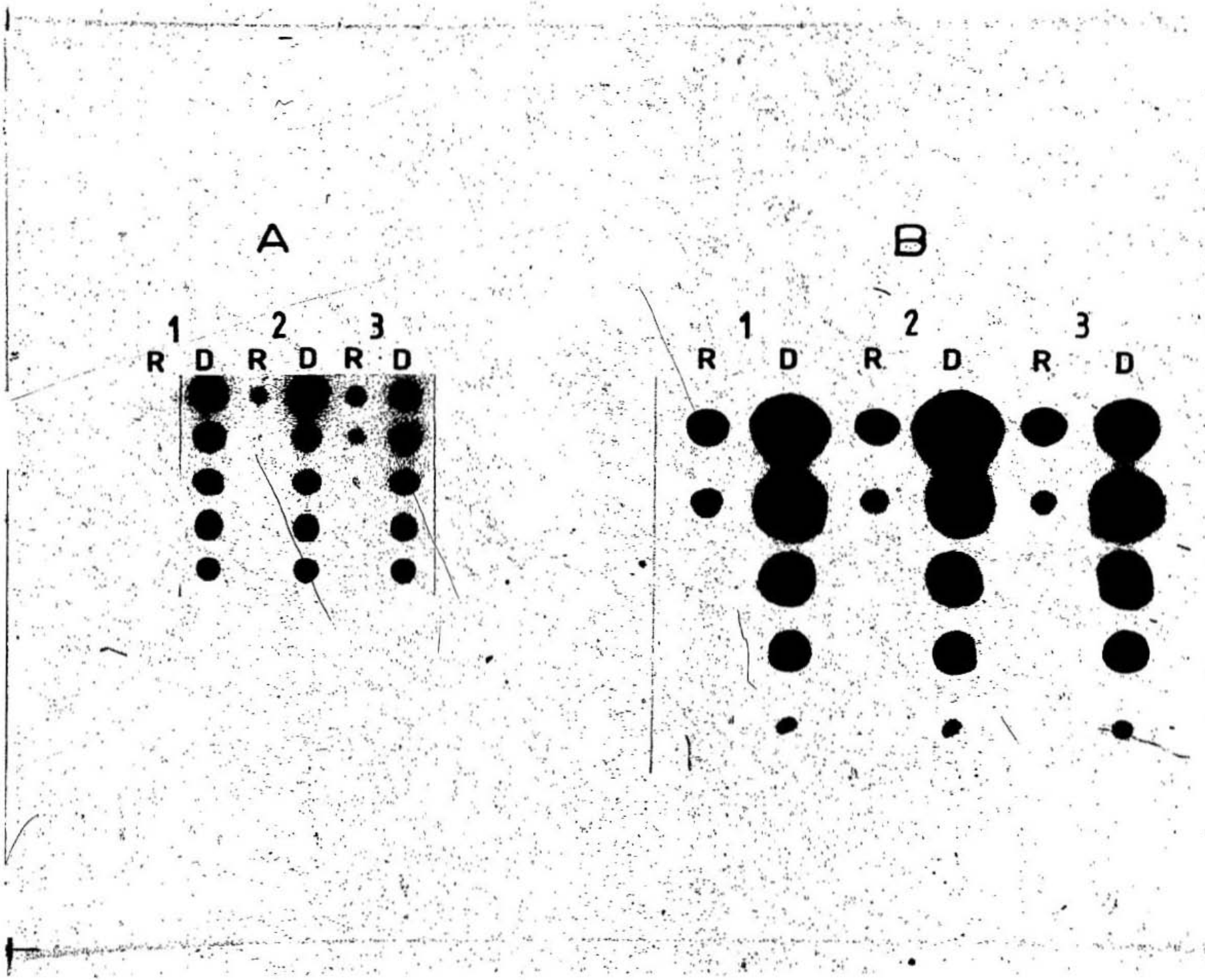

A
1
2
3
R
D
R
D
R
D
B
1
2
3
R
D
R
D
R
D

Figure 11.2

The effects of cAMP, agmatine and putrescine on the concentration of *speB* mRNA in whole cells of *E.coli* strains LS853 and LS854-1.

mRNA **(R)** and DNA **(D)** from whole cells of *E.coli* strains LS853 *(Δcya)* and LS854-1 *(Δcya, Δcrp)* were immobilised alongside on nitrocellulose filters and hybridised with radiolabelled pKA15 DNA. The strains were grown in the presence or in the absence of cAMP, IPTG, agmatine or putrescine.

A, B, C, F, G are filters carrying nucleic acids from LS853, and filters **D, E** carried nucleic acids from LS854-1. **(1)** represents control (untreated), **(2)** represents culture treated with 1mM cAMP, **(3)** represents those treated with 5mM cAMP, **(4)** corresponds to cultures grown in the presence of IPTG, **(5)** are those grown in the presence of IPTG and 2.5mM cAMP, **(6)** and **(7)** were from cultures treated with 0.5mM and 1mM agmatine respectively, and **(8)** is from cultures treated with 1mM putrescine.

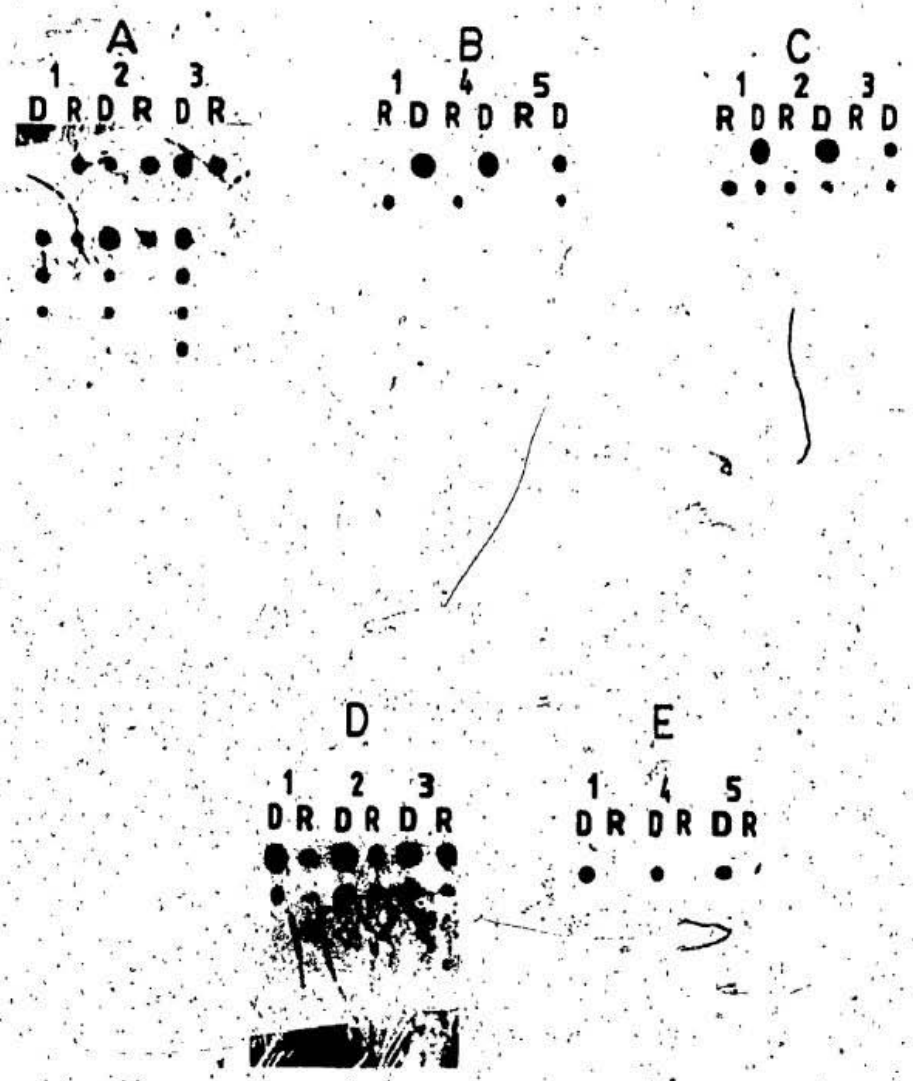

A
1 2 3
D R D R D R
B
1 4 5
R D R D R D
C
1 2 3
R D R D R D
D
1 2 3
D R D R D R
E
1 4 5
D R D R D R
F
1 6 7
D R D R D R
G
1 8
R D R D

Table 11.1

Effects of cAMP, agmatine and putrescine on *lacZ* and *speB* mRNA levels in *E.coli cya* and *crp* strains.

Strain---->	LS853	LS853	LS854-1	LS854-1
Condition	*lacZ*	*speB*	*lacZ*	*speB*
None	N.D	1.00	N.D	1.00
+IPTG	1.00	0.92(±0.05)	1.00	0.99(±0.04)
+IPTG+cAMP(1mM)	1.85(±0.08)	0.37(±0.06)	0.95(±0.02)	***
+IPTG+cAMP(2.5mM)	3.93(±0.22)	0.18(±0.12)	1.19(±0.10)	***
+IPTG+cAMP(5mM)	***	0.092(±0.02)	1.05(±0.11)	1.02(±0.06)
None	***	1.00	***	1.00
+Agmatine(0.5mM)	***	3.15(±1.14)	***	1.98(±0.13)
+Agmatine(1mM)	***	2.16(±0.06)	***	2.05(±0.04)
+Agmatine(2mM)	***	2.70(±0.18)	***	***
None	***	1.00	***	1.00
+Putrescine(1mM)	***	0.90 (±0.01)	***	***

*** = Not Determined
N.D = Not Detected

The strains from which DNA and RNA were 'quick-blotted' and the radiolabelled genes (pUC8 for *lacZ* and pKA15 for *speB*) used to probe these blots are shown in the table. *speB* mRNA concentrations are expressed as % of control (without any additions). *lacZ* mRNA concentrations are expressed as % of IPTG induced concentrations. The results are presented as an average of three experiments and the extremes of each set of values are shown in parenthesis.

11.2.3 The effect of cAMP, agmatine and putrescine on the steady state levels of speA mRNA in LS853 and LS854-1:

The blots prepared in Section 11.2.2 were washed and re-used as described in Section: 2.18, or fresh 'quick blots' were made. The nitrocellulose filters were hybridised to ^{32}P-labelled plasmid pKA16 DNA, which carries the gene encoding arginine decarboxylase (*speA* , Buch and Boyle, personal communication). The autoradiograms were analysed and the results are given in Table: 11.2. Cyclic AMP, agmatine and putrescine decreased the concentration of *speA* mRNA in both the strains of *E.coli*.

11.2.4 The selectivity of RNA and DNA immobilisation and the specificity of hybridisation:

E.coli strain HT328 (Δ*speA*; Δ*speB*) does not share homologous sequences with *speA* and *speB* genes. *Bacillus subtilis*, a Gram positive bacillus, does not express agmatine ureohydrolase and arginine decarboxylase activities, and does not show any homology to the *speC* gene (gene encoding ornithine decarboxylase) (Boyle *et al.*, 1985; Wright and Boyle, 1984). Therefore, to assess the specificity of hybridisation and to determine if the basic proteins co-immobilised on the nitrocellulose filters along with nucleic acids, bound the radioactively labelled plasmid probes, *B.subtilis* and *E.coli* HT328 (Δ*speA*, Δ*speB*) were grown in LB medium containing glucose, the DNA and RNA samples were prepared and immobilised on to nitrocellulose filters. These filters

Table 11.2

Effects of cAMP, agmatine and putrescine on *speA* mRNA levels in *E.coli cya* and *crp* strains.

Strain---->	LS853	LS854-1
Condition	*speA*	*speA*
None	1.00	1.00
+IPTG	0.97(±0.13)	1.12(±0.08)
+IPTG+cAMP(1mM)	0.41(±0.18)	1.09(±0.11)
+IPTG+cAMP(2.5mM)	0.20(±0.03)	1.02(±0.06)
+IPTG+cAMP(5mM)	0.07(±0.05)	0.89(±0.09)
None	1.00	1.00
+Agmatine(0.5mM)	0.62(±0.03)	0.51(±0.13)
+Agmatine(1mM)	0.31(±0.08)	0.33(±0.02)
+Agmatine(2mM)	0.11(±0.08)	***
None	1.00	1.00
+Putrescine(1mM)	0.22(±0.10)	***

*** = Not Determined

DNA and RNA were 'quick-blotted' from LS853 and LS854-1 and radiolabelled pKA16 for *speA*) was used to probe these blots. *speA* mRNA concentrations are expressed as % of control (without any additions). The results are presented as an average of two experiments.

were hybridised to either *speA* (pKA16) or *speB* (pKA15) genes (Figure: 11.3). HT328 transformed with plasmid pKA5 which carries the *speB* and *speA* genes was also analysed similarly. HT328 and *B.subtilis* did not show detectable hybridisation to the *speA* and *speB genes*.

In order to assess the selectivity of immobilisation, the extract prepared from LS853 was treated with either RNAase-A or DNAaseI. These samples were then prepared for DNA and RNA quick blotting. To further demonstrate the selectivity of immobilisation the purified chromosomal DNA of *E.coli* K12 strain was treated as a DNA or as an RNA sample, and then immobilised on the nitrocellulose filter. The total *E.coli* RNA from MRE600 (a strain of *E.coli*) was isolated and treated as a DNA or as an RNA sample, and immobilised on the nitrocellulose filter. These filters were hybridised to either pKA15 or pKA16. The results are shown in Figure: 11.3. RNAase and DNAase treatment of the extracts abolished hybridisation of the labelled DNA to the RNA and DNA samples respectively. Purified DNA and RNA did not hybridise to the plasmid probe when treated as RNA or DNA respectively.

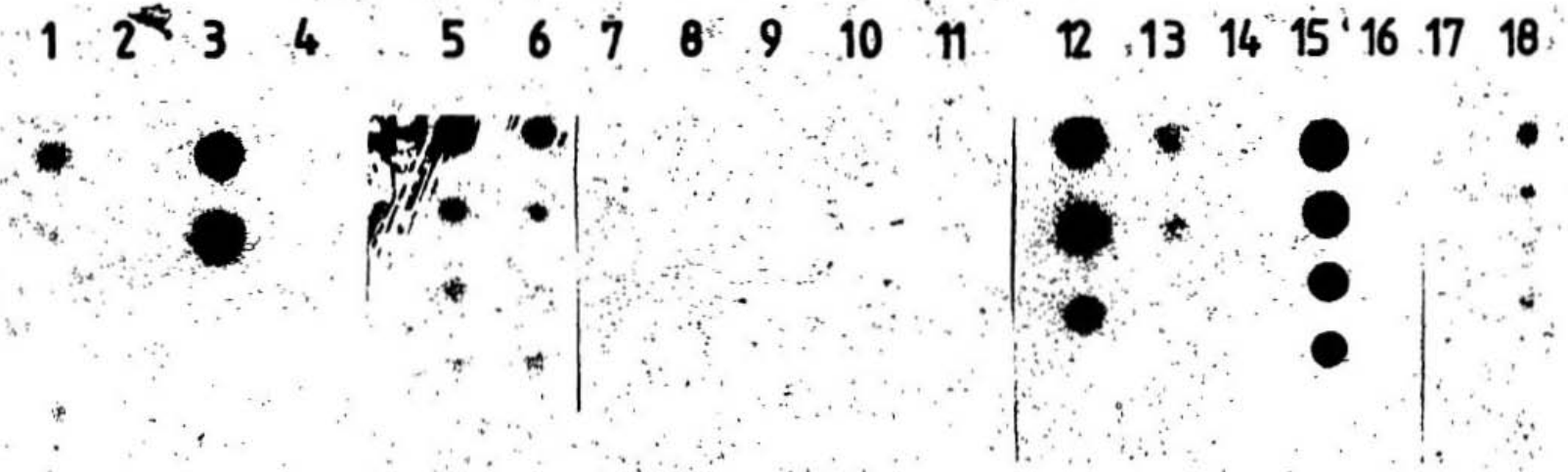
In order to assess the nature of the mutation in MA135 (*speB*; AUH^-) (used in Chapter 6), the strain was grown in LB medium containing glucose and extracts were prepared for DNA and RNA blotting. The filters were hybridised to ^{32}P-labelled pKA15. The results are shown in Figure: 11.3. The AUH^- mutant MA135 appears to have a normal concentration of *speB* mRNA.

Figure 11.3

The specificity and selectivity of hybridisation

The DNA and RNA samples from different strains of *E.coli* and from *B.subtilis* were analysed to determine the specificity of hybridisation. The selectivity of the technique was determined by treating purified RNA or DNA samples with RNAase or DNAase respectively and analysed by the 'quick-blot' method. A strain of *E.coli* MA135 (AUH^-) was also used to immobilise RNA and DNA to analyse the nature of mutation in this chemically mutagenised strain.

(1)=RNA sample (LS853), **(2)**=RNAase treated RNA sample (LS853), **(3)**=DNA sample (LS853), **(4)**=DNAase treated DNA sample (LS853), **(5, 6)**=DNA and RNA respectively from MA135, **(7, 8)**=*B.subtilis* DNA and RNA respectively, **(9, 10)**=HT328 DNA and RNA respectively, **(11)**=HT289 DNA, **(12, 13)**=HT328 carrying pKA5 plasmid DNA and RNA respectively, **(14)**=purified DNA immobilised as an RNA, **(15)**=purified DNA, **(16)**=DNAase treated purified DNA, **(17)**=RNAase treated purified RNA, and **(18)**=purified RNA.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

11.3 Discussion:

The selectivity and specificity of this technique is demonstrated in Figure: 11.3. RNAase-A digested extract immobilised as an RNA sample and DNAase-I digested extract immobilised as DNA did not hybridise to the labelled plasmid DNAs, while the plasmid DNAs did hybridise to the former when treated as a DNA sample and to the latter when treated as an RNA sample (Figure: 11.3). The DNA and RNA samples of *Bacillus subtilis* HT289 (Δ*speB*, Δ*speA*, Δ*speC*) and HT328 (Δ*speA*, Δ*speB*) did not, and HT328 transformed with pKA5 did hybridise to the labelled plasmid DNAs (Figure: 11.3). Purified DNA and purified RNA from *E.coli* hybridised to the labelled probe DNA only when immobilised as a DNA and as an RNA sample respectively. These results indicate that the technique employed to assess relative concentrations of mRNA is selective and specific.

The results presented in Figure: 11.1 and Table: 11.1, demonstrate that IPTG increased *lacZ* mRNA concentrations in both LS853 and LS854-1, and cAMP increased the *lacZ* mRNA concentrations further in LS853 but not in LS854-1. This parallels the increase in enzyme activity detected in this strain grown in the presence of 0.5 mM IPTG with or without added cAMP (1 mM) in minimal medium containing glucose (Chapter: 4) (Wright and Boyle, 1982; Boyle *et al.*, 1985). The specificity of hybridisation is also evident from the results presented in Figure: 11.1 where no β-galactosidase mRNA was detected when extracts were prepared from cells grown in the absence of IPTG and

cAMP. The inability of cAMP to increase the *lacZ* mRNA concentrations in the Δ*crp* strain (LS854-1) further demonstrates the specificity of hybridisation and the involvement of CRP in the action of cAMP.

The results presented in Figure: 11.2 and Tables 11.1 and 11.2 demonstrate that cAMP decreases *speB* and *speA* mRNA concentrations in the Δ*cya* strain (LS853), but not in the Δ*crp*, Δ*cya* strain (LS854-1). 1 mM and 2.5 mM cAMP decreased the concentration of *speB* mRNA by 70 and 85% respectively and the *speA* mRNA concentration by 60 and 80% respectively in LS853, while cAMP at 5 mM did not decrease the concentration of *speB* and *speA* mRNAs in LS854-1. The results indicate that the cAMP mediated decrease in *speB* and *speA* mRNA concentrations require a functional CRP, and therefore it may be concluded that cAMP and CRP together regulate the transcription of *speA* and *speB* (there are no reports on the involvement of cAMP:CRP in translational control, or in the control of stability of mRNA). These observations are consistent with those reported in Chapters 4, 8, 9 and 10.

Agmatine increased *speB* mRNA concentrations (2-3 fold) in LS853 and LS854-1 when 1 mM agmatine was included in the growth medium. Agmatine at 1 mM maximally increased the *speB* mRNA concentrations, as 2 mM agmatine did not increase *speB* mRNA concentrations further in both strains, when agmatine was supplied in the growth medium (Figure: 11.2). Agmatine at 2.5 mM in the growth medium decreased the *speA* mRNA concentrations by about 70% in both strains (Table: 11.2).

Putrescine at 2 mM in the growth medium decreased the concentration of *speA* mRNA but not of *speB* (Figure: 11.2 and Tables: 11.1 and 11.2). These observations are consistent with those reported in Chapters 5, 6, 9 and 10. The decrease in *speA* mRNA concentration by agmatine is consistent with the observations made in strains wild type with respect to *speB* (Chapter 6). Agmatine was shown to repress *speA* expression only upon its metabolic conversion to putrescine.

Although the circumstantial evidence coupled with the involvement of CRP suggest that cAMP negatively regulates *speB* transcription, the effect of agmatine on *speB* expression appears to be at the level of transcription, but its role in the stabilisation of *speB* mRNA cannot be ruled out. The decrease in *speA* and *speB* mRNA concentrations by cAMP appears to be more severe, relative to the decrease in the enzymic activities of arginine decarboxylase and agmatine ureohydrolase. This may be due to other factors influencing enzymic activity and/or translation which are not understood at present. This difference may reflect the relative stabilities of mRNAs and the proteins.

E.coli MA135, (*speB*, AUH$^-$), a strain employed in the regulatory studies reported in Chapter 6 appears to contain *speB* mRNA at concentrations similar to those in LS853 and LS854-1 (Figure: 11.3). The nature of the mutation in *speB* in this strain is not known, but based on these results it can be surmised that the mutation causes the synthesis of a nonfunctional protein.

Chapter 12

Concluding Remarks and General Discussion

12.1 Purification of agmatine ureohydrolase:

Agmatine ureohydrolase was purified 3300 fold by steps involving ammonium sulphate fractionation, heat treatment, ion exchange chromatography on DEAE Sephacel, gel filtration on Sephadex G-100, and chromatofocusing (Chapter 3). The material obtained was substantially but not completely pure. Its molecular weight by gel filtration was approximately 80,000 (Section: 3.2.8), and SDS-PAGE gave a major component with a molecular weight of 38.,000 (Section: 3.2.10). The purified enzyme had a pI of 8.3 (Section: 3.2.6), but in crude extracts was associated with a protein which reduced its catalytic activity, and the pI to 5.5. (Section: 3.2.7). The optimum pH for the purified enzyme was 7.5. It was stabilised by agmatine (Section: 3.2.12.1 and 3.2.12.3) and inactivated by β-mercaptoethanol (Section: 3.2.12.2), EDTA and EGTA (Section: 3.2.14). Antibodies prepared in rabbit against the purified enzyme did not react with the material with pI of 5.5 (Section: 3.2.11). The K_m for agmatine was 1.3 mM. Arginine was a competitive inhibitor with a K_i of 8.4 mM. Ornithine caused mixed inhibition (Section: 3.2.15).

12.2 Antizymes of ornithine decarboxylase, arginine decarboxylase and agmatine ureohydrolase:

Antizymes (proteins that block enzymic activity) have been reported for arginine decarboxylase and ornithine decarboxylase in *E.coli* cells grown in a

rich medium in the presence of large quantities of putrescine (Kyriakidis *et al.*, 1978). A protein inhibitor of agmatine ureohydrolase was detected during purification (Chapter: 3). The occurrence of such an inhibitor is analogous to the occurrence of antizymes for arginine decarboxylase and ornithine decarboxylase (Kyriakidis *et al.*, 1978), and the co-purification of this inhibitor (Chapter: 3) is analogous to the property of the ornithine decarboxylase antizymes (Kyriakidis *et al.*, 1978). Whereas, the antizymes for ornithine decarboxylase and arginine decarboxylase virtually block the enzymic activity, the inhibition of agmatine ureohydrolase by its co-purified inhibitor is not as dramatic. Experiments with purified inhibitor and agmatine ureohydrolase have not been carried out, but in crude extracts the inhibitor appears to be in excess because only enzyme bound to the inhibitor is detected. The activity of this form of the enzyme is probably lower than that of purified enzyme, and its K_m is higher (1.1 mM for purified enzyme; 3.0 mM for crude extracts). The inhibitor has only been characterised indirectly, as a protein with a molecular weight of 35,000. It co-purifies with agmatine ureohydrolase activity obtained by chromatofocusing of a partially purified preparation (stage: 5, Tables: 3.2 and 3.3; Figure: 3.11), but is absent in the purified preparation obtained by chromatofocusing after chromatography on DEAE Sephacel.

If the inhibitor is analogous to the antizymes of arginine decarboxylase and ornithine decarboxylase, its presence in crude extracts may have been due to agmatine which was added to induce agmatine ureohydrolase, because

putrescine is known to induce antizymes for arginine decarboxylase and ornithine decarboxylase (Kyriakidis *et al.*, 1978). If the protein inhibitor of agmatine ureohydrolase is to be viewed as an antizyme, although it is not as effective as are the antizymes of ornithine decarboxylase and arginine decarboxylase. It is not clear why an antizyme for agmatine ureohydrolase should be present, particularly when agmatine ureohydrolase may not be a rate limiting enzyme in the pathway of putrescine synthesis from arginine (Morris *et al.*, 1970; Tabor and Tabor, 1969). A comparison of *E.coli* and *Bacillus subtilis* arginine and putrescine biosynthetic pathways (Figure: 12.1) suggests that an antizyme for agmatine ureohydrolase might be an evolutionary relic. In *B.subtilis* there is only one pathway to putrescine, the decarboxylation of ornithine. Arginine induces arginase which catalyses the conversion of arginine to ornithine. Arginase binds to ornithine transcarbamylase to block the resynthesis of arginine from ornithine, thus avoiding a futile cycle (Paulus, 1983). The arginase : ornithine transcarbamylase complex retains arginase activity, but the K_m for arginine is increased 2-3 fold.

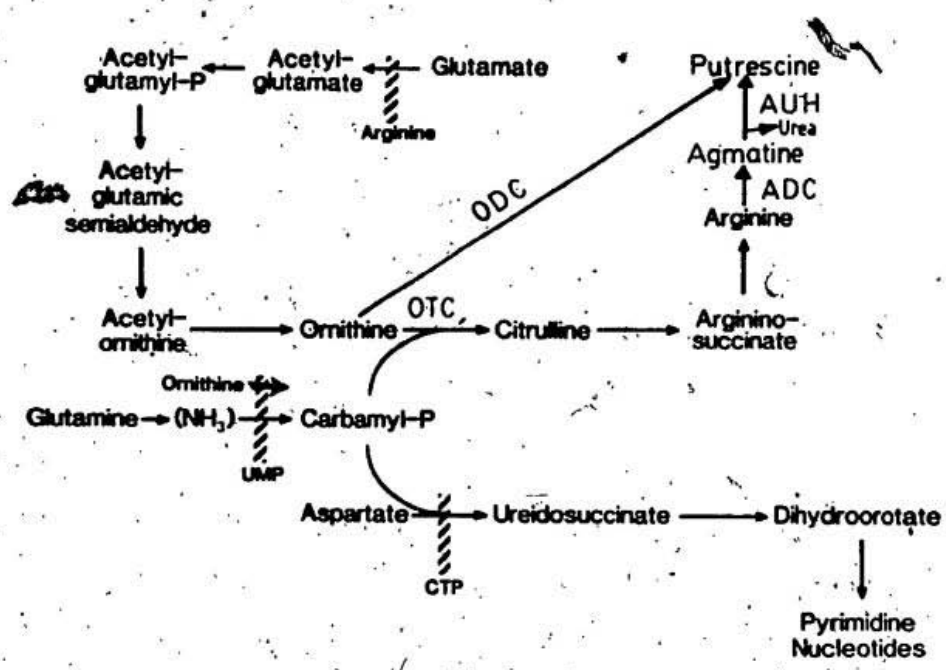
In *E.coli* there is a chemically analogous reaction to that catalysed by arginase in *B.subtilis*, i.e. the reaction catalysed by agmatine ureohydrolase. The enzymes for the reactions share the property of being induced by substrate, bind a protein that inactivates it partially and have almost similar monomeric molecular weight (agmatine ureohydrolase appears to be dimer with a monomeric molecular weight of 38,000 and arginase in *B.subtilis* is a

Figure 12.1

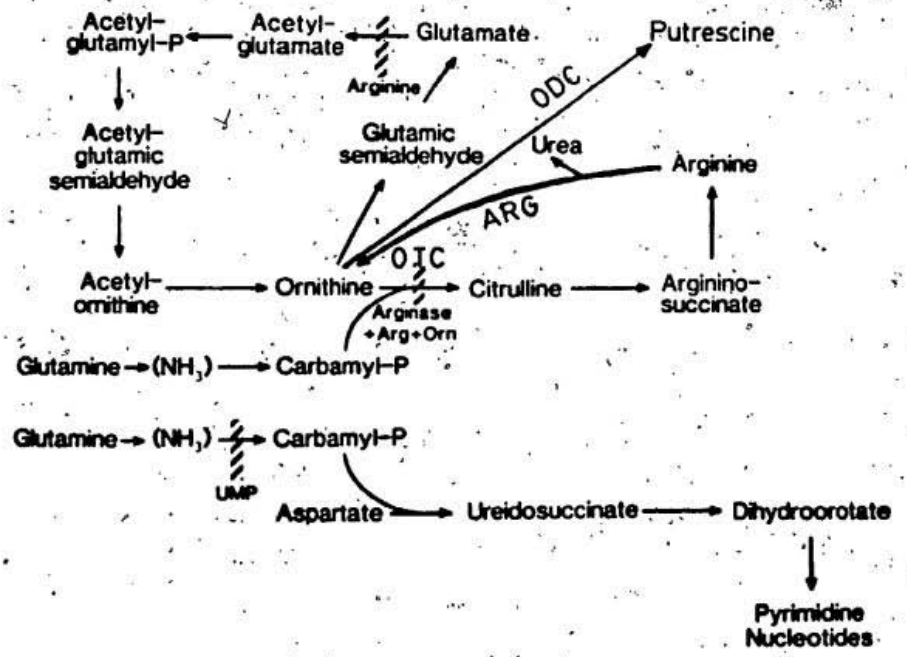
Arginine and putrescine biosynthetic pathways in *E.coli* and *B.subtilis*.

(ODC)=ornithine decarboxylase, **(ADC)**=arginine decarboxylase, **(AUH)**=agmatine ureohydrolase, **(OTC)**=ornithine transcarbamylase and **(ARG)**=arginase.

(➡) represents activation and (▭) represents inhibition.



Arginine and putrescine biosynthetic pathways in E.coli



Arginine and putrescine biosynthetic pathways in B.subtilis

trimer with a monomeric molecular weight of 37,000). Could the genes for agmatine ureohydrolase and arginase have evolved from a common ancestral gene? Could this inhibitor of agmatine ureohydrolase in *E.coli* be also ornithine transcarbamylase? Ornithine transcarbamylase in *E.coli* has a monomeric molecular weight of 35,000 (Gigot *et al.*, 1977; Issaly and Issaly, 1974; Legrain *et al.*, 1976; 1977; Legrain and Stalon, 1976; Rosenbusch and Weber, 1971), similar to that observed for the probable inihibitor in the SDS-PAGE of the inhibited agmatine ureohydrolase from chromatofocusing (Figure: 3.11). This possibility has not been tested directly.

cAMP-CRP mediated repression and agmatine mediated induction of agmatine ureohydrolase are probably not due to the modulation in the synthesis of this inhibitor protein, as the effect of cAMP and agmatine are observed even in the *in vitro* transcription and translation system (Chapter: 8) and in the gene fusion studies (Chapter: 10). Besides, cAMP decreased and agmatine increased the *speB* mRNA concentration (Chapter: 11).

12.3 Evolution of two forms of arginine decarboxylase and ornithine decarboxylase:

A few *E.coli* strains contain both biosynthetic and biodegradative ornithine decarboxylases (Applebaum *et al.*, 1977; Gale, 1946) and arginine decarboxylases (Wu and Morris, 1973). Of those which do not, some may contain a second gene which is not expressed (Wright and Boyle, 1984). The

biosynthetic enzymes are repressed by putrescine, whereas the biodegradative form is induced by acid conditions (Morris and Fillingame, 1974; Tabor and Tabor, 1984). If the role of the biodegradative enzyme is to neutralise environmental acid, its regulation by putrescine would be disadvantageous, and therefore the evolution of a second enzyme with a separate regulatory mechanism would be advantageous. This proposition does not appear to be true for agmatine ureohydrolase since *speB* is induced by agmatine (Section: 5.2.1), and not repressed by putrescine (Section: 6.2.1), independently of whether it has a biosynthetic or biodegradative role. Therefore two forms of agmatine ureohydrolase in *E.coli* might not be expected and in fact are not found.

12.4 Effect of cAMP on agmatine ureohydrolase in vivo:

Modulation of agmatine ureohydrolase activity by cAMP in related strains was compared with that of β-galactosidase, ornithine decarboxylase and arginine decarboxylase. In a strain wild type with respect to *crp* and *cya*, (LS340), cAMP progressively reduced growth rate, increased activity of β-galactosidase, and decreased the activities of agmatine ureohydrolase, arginine decarboxylase and ornithine decarboxylase (Section: 4.2.1). This repression depended on a functional *crp* gene, as the inhibition was abolished in LS854-1 (Δ*cya*, Δ*crp*) (Section: 4.2.3). In a Δ*cya* mutant (LS853) cAMP had the same effect as in LS340, except that the lowest added concentration (1 mM) caused an increase in growth rate (Section: 4.2.2). It was concluded that agmatine

ureohydrolase might be regulated similarly to arginine decarboxylase and ornithine decarboxylase (Wright and Boyle, 1982), being repressed by cAMP:CRP.

12.5 Effect of agmatine on agmatine ureohydrolase in vivo:

The growth rate of *E.coli* was not altered in the presence of agmatine, but there was an increase in the specific activity of agmatine ureohydrolase (Section: 5.2.1). This effect of agmatine probably required *de novo* synthesis of agmatine ureohydrolase, and it did not activate a preformed agmatine ureohydrolase (Section: 5.2.5). Although in some strains of *E.coli* 'biodegradative forms' of arginine decarboxylase and ornithine decarboxylase are known (Applebaum *et al.*, 1975; 1977), these have not been detected in the strains used in this work, and there was no evidence for the induction of a second form of agmatine ureohydrolase by agmatine (Section: 5.2.4). The ability of agmatine to induce agmatine ureohydrolase was counteracted by the ability of cAMP to repress its transcription; i.e., antagonistic transcriptional regulation (Sections: 5.2.2 and 5.2.3). In Δ*crp* and Δ*cya* mutants agmatine induced higher activities of agmatine ureohydrolase (Section: 5.2.1). These results suggest that agmatine affects agmatine ureohydrolase activity at the level of transcription. However, the results did not determine whether the same or different sites of interaction are involved. These conclusions were pursued further in the studies reported in Chapter: 10.

12.6 The effect of amino acids and polyamines on the activities of ornithine decarboxylase, arginine decarboxylase and agmatine ureohydrolase, and the modulation of the enzymic activities by glutamine synthetase and the repressor of the arginine regulon:

Arginine, ornithine, agmatine and putrescine are linked by metabolic pathways. (Figures: 1.1 and 12.1), and the effects of one of these on the activity of enzymes in these metabolic pathways might be due to its conversion to another. This was studied (Chapter: 6) using various mutants of *E.coli*. Repression of arginine decarboxylase and ornithine decarboxylase by arginine was not observed in *speA* and *speB* mutants, repression by agmatine was not observed in *speB* mutants, and repression of arginine decarboxylase by ornithine was not observed in a *speB* mutant (Section: 6.2.3). Consequently these repressions by ornithine, arginine and agmatine are due to their conversion to putrescine. This conclusion is in accord with the observation that conditions that limit putrescine production increase activities of ornithine decarboxylase and arginine decarboxylase. In *speA* and *speB* mutants added arginine is not converted to putrescine, and results in decreased putrescine synthesis from ornithine by feedback inhibition of the enzymes of ornithine synthesis (Section: 6.2.3). In the same mutants, the addition of putrescine to a culture decreased ornithine decarboxylase to its normal activity (and arginine decarboxylase to its normal activity in the *speB* mutant) (Section: 6.2.3). Induction of agmatine ureohydrolase by ornithine and arginine was blocked by a

mutation in *speA* (Section: 6.2.3).Therefore, induction of agmatine ureohydrolase was due to agmatine itself.

Because glutamine synthetase is known to induce agmatine ureohydrolase (agmatinase) in *Klebsiella pneumoniae* (Friedrich and Magasanik, 1978), arginine utilisation in *Pseudomonas aeruginosa* (Mercenier *et al.*, 1980) and agmatine fermentation in *Streptococcus faecalis* (Roon and Barker, 1972), its possible role with ornithine decarboxylase, arginine decarboxylase and agmatine ureohydrolase in *E.coli* was also examined. In mutants lacking glutamine synthetase or having de-adenylated glutamine synthetase, the activities of all three enzymes were at their lowest, and transformation of the Δ*glnA* strain with a plasmid carrying *glnA* restored the activities of ornithine decarboxylase, arginine decarboxylase and agmatine ureohydrolase (Sections: 6.2.5.1 and 6.2.5.2). In a Δ*glnA* mutant the normal repression by cAMP was observed, and so the effect of this compound is not mediated by glutamine synthetase (Section: 6.2.5.3). Although the arginine repressor also represses the synthesis of enzymes for ornithine synthesis, the regulation of agmatine ureohydrolase, arginine decarboxylase and ornithine decarboxylase in an arginine repressor mutant was normal (Section: 6.2.6). Arginine repressed arginine decarboxylase and ornithine decarboxylase and induced agmatine ureohydrolase independently of the presence or absence of the arginine repressor (Section 6.2.6).

12.7 Sub-cloning and localisation of speB:

The molecular basis for induction of agmatine ureohydrolase by agmatine and its repression by cAMP was examined using cloned genes in different experimental systems. Minicells (Chapter: 9) and in an *in vitro* transcription and translation system (Chapter: 8) were used to examine the modulation of agmatine ureohydrolase synthesis by cAMP and agmatine, and to demonstrate that these compounds have direct effects at the level of transcription that do not involve the induction of other proteins. The *in vitro* transcription and translation system was also used to determine the direction of transcription (Chapter 8) and the approximate location of the promoter (Chapter 8). The modulation of promoter activity by cAMP, arginine, ornithine and polyamines was examined by measuring concentrations of mRNAs (by hybridisation)(Chapter: 11), and by observing the expression of other genes (*lacZ* and *galK*) fused to the *speB* promoter (Chapter: 10).

pKA14 (bearing *speB*) was prepared by sub-cloning from pKA5 (bearing *speA*, *speB* and *metK*) (Section: 7.2.1). pKA15 was prepared from pKA5, and contained a fragment from within the *speB* gene (Section: 7.2.3).

12.8 Effect of cAMP and agmatine on agmatine ureohydrolase synthesis in minicells:

The synthesis of agmatine ureohydrolase directed by pKA5 in minicells was compared with the synthesis of β-lactamase encoded by the vector.

Synthesis of agmatine ureohydrolase was repressed by cAMP. However, a concentration of 5 mM was required to obtain the same effect as observed with 1 mM in normal cells (Section: 9.2.1). Agmatine induced agmatine ureohydrolase but only 20-25% of the effect of the same concentration in normal cells was observed (Section: 9.2.2). The minicells used in these experiments had been pre-incubated for 15 hours (Boyle *et al.*, 1985), a period longer than is usually reported (Inselburg, 1970; 1971; Pratt, 1984), in order to reduce the synthesis of proteins due to endogenous mRNA segregated from the parent cells. If CRP were preferentially degraded in this period, the requirement for a higher cAMP concentration might be explained. If agmatine induced agmatine ureohydrolase indirectly through a protein which uses agmatine as a ligand (either positively or negatively), and if this protein was preferentially degraded in the period of 15 hours of pre-incubation, the requirement for higher concentrations of agmatine and lower levels of induction might also be explained. The longer time of incubation to degrade the endogenous messenger RNA might reflect an inherent stability of mRNA in minicells, and if agmatine increased agmatine ureohydrolase synthesis by increasing the stability of *speB* mRNA specifically, a lower level of induction of agmatine ureohydrolase by agmatine might be explained.

12.9 The roles of cAMP and CRP in the negative regulation of agmatine ureohydrolase by cell-free transcription and translation:

The synthesis of agmatine ureohydrolase was compared with the synthesis of β-lactamase in a cell-free transcription-translation system directed by pKA5 DNA. The synthesis of agmatine ureohydrolase was decreased (>90%) in an S-30 extract (prepared from normal cells), when 10^{-4}M cAMP was included in the reaction mixture (Section: 8.2.2). The synthesis of agmatine ureohydrolase was not repressed when an S-30 extract prepared from a Δ*crp* strain of *E.coli* (LS854-1) was used to direct the synthesis of proteins encoded by plasmid pKA5 in the presence of 10^{-4}M cAMP (Section: 8.2.3). On addition of purified cAMP receptor protein to an S-30 extract of LS854-1 (Δ*crp*) synthesising proteins directed by pKA5, cAMP (10^{-4}M) repressed the synthesis of agmatine ureohydrolase (Section: 8.2.3). This demonstrates that cAMP:CRP directly represses the expression of *speB* gene and does not act by the induction of a receptor protein.

12.10 Effect of cAMP on the speB mRNA concentration in vivo:

In order to establish the molecular effects of cAMP and agmatine *in vivo*, mRNA concentrations were determined in a Δ*cya* strain (LS853) and in a Δ*cya*, Δ*crp* strain (LS854-1), grown in the presence of cAMP or agmatine. Messenger RNA and DNA from whole cells grown in the presence or absence of cAMP or agmatine were separately immobilised on nitrocellulose filters in the presence of sodium iodide. Following hybridisation of the filter bound nucleic acids with radiolabelled pKA15 (which carries a DNA fragment from within

the *speB* gene), the filters were subjected to autoradiography. The signal intensities obtained for pKA15 hybridised to mRNA were compared to those hybridised to DNA on the same nitrocellulose filter, from the same number of cells. Cyclic AMP decreased the *speB* mRNA concentrations in the Δ*cya* mutant (Section: 11.2.2), but not in the Δ*crp* mutant (Section : 11.2.2), while agmatine increased the *speB* mRNA concentration in both the strains (Section 11.2.2). These results demonstrate that cAMP:CRP either decreases transcription of *speB* or stabilises the *speB* mRNA in a specific manner. No conclusions can be drawn as to whether agmatine acts directly, or indirectly, e.g., through a protein that uses agmatine as a ligand.

12.11 Effect of cAMP, amino acids and polyamines on the expression of speB:lacZ and speB:galK fusion genes:

To determine if the repression by cAMP and induction by agmatine of agmatine ureohydrolase was indeed through the interaction of these compounds with the *speB* promoter, the expression of gene fusions of the *speB* promoter to the structural genes of β-galactosidase or galactokinase was measured. The effect of ornithine and arginine on the induction of agmatine ureohydrolase was also tested by assessing the expression of these fused genes in a strain carrying a deletion in the genes for *speA*, *speB* and *speC*. This strain would be unable to metabolise these compounds into polyamines. Agmatine, but not ornithine and arginine, induced the activity of β-galactosidase encoded

by the fusion plasmid (Section: 10.2.5). Putrescine neither decreased nor increased the activity of β-galactosidase (Section: 10.2.5). In the strains LS853 (Δ*cya*) and LS854-1 (Δ*cya*,Δ*crp*) carrying pAUL (*speB:lacZ* fusion plasmid), cAMP repressed the synthesis of β-galactosidase in the Δ*cya* mutant, but not in the *crp* mutant (Section: 10.2.4). These results demonstrate that cAMP:CRP interacts directly with the *speB* promoter to alter its expression. The nature of the effect of agmatine could not be established. Agmatine might interact either with the promoter or with the 5' end of the mRNA, thus stabilising it.

12.12 Constitutive production of ornithine decarboxylase, arginine decarboxylase and agmatine ureohydrolase:

Ornithine decarboxylase, arginine decarboxylase and agmatine ureohydrolase, have been referred to as constitutive enzymes (Morris and Koffron, 1969). Constitutive enzymes are defined as those which are not regulated (i.e., they are neither inducible nor repressible). The genes for these enzymes are transcribed as a function of growth rate (availability of RNA polymerase, availability of precursors of RNA and protein synthesis), and promoter strength (affinity of RNA polymerase for the promoter) (Clarke, 1979; Maaløe, 1979). But, the activities of ornithine decarboxylase and arginine decarboxylase have been shown to be modulated independently of growth rate. For example, putrescine represses arginine decarboxylase and ornithine decarboxylase at various growth rates (Tabor and Tabor, 1969), as was demonstrated by using

cultures grown in a chemostat. Wright and Boyle (1982) showed that ornithine decarboxylase and arginine decarboxylase are repressed by cAMP:CRP independently of growth rate. In the present studies it has been confirmed that ornithine decarboxylase and arginine decarboxylase are indeed regulated by the putrescine concentration (Chapters 4, 5 and 6). Furthermore, agmatine induces and cAMP represses agmatine ureohydrolase.

The modulation of these three enzymes is not as marked as is often observed with catabolic enzymes. Under different conditions ornithine decarboxylase, arginine decarboxylase and agmatine ureohydrolase were subject to an eight fold changes in expression. However, despite the smaller magnitude of the regulation, these genes may not be considered to be constitutive.

12.13 The effect of cAMP:

Boyle *et al.* (1977) showed that the activities of arginine decarboxylase and ornithine decarboxylase at various growth rates correlate well with corresponding changes in intracellular putrescine concentrations. Cyclic AMP was shown to repress *speC* (Wright and Boyle, 1982), *speA* (Wright and Boyle, 1982; Chapter: 10; Chapter: 11) and *speB* (Chapter: 10, Chapter: 11). Hence the high activities of ornithine decarboxylase, arginine decarboxylase and agmatine ureohydrolase at high growth rate, when the concentration of cAMP is low. Cyclic AMP concentrations have been shown to vary in inverse proportion to growth rate (Pastan and Adhya, 1976). Therefore, the apparent growth

rate dependence of the activities of the three enzymes may be due to changes in the intracellular concentrations of cAMP. Although *speA*, *speB* and *speC* do not form an operon (Chapter: 7), the expression of these three genes appear to be co-ordinately regulated by cAMP, for the changes in activity are approximately proportional and co-incident in time. This mode of regulation of separate genetic units is reminiscent of a regulon.

The finding that cAMP:CRP reduces the transcription of the *speA*, *speB* and *speC* genes as of *gal* P_2 (Musso *et al.*, 1979), spot 42 RNA (Sahagan and Dahlberg, 1979), *ompA* (Movva *et al.*, 1981), *crp* (Aiba, 1983), *cya* (Majerfeld *et al.*, 1981), and possibly of *glnA* (Prusiner *et al.*, 1972) further extends the role of cAMP:CRP in prokaryotes as a negative regulator. The mechanism by which cAMP:CRP exerts positive or negative regulation is not well understood.

12.14 The effect of agmatine:

Agmatine induces agmatine ureohydrolase, independently of a functional CRP (Section: 5.2.1). It is not clear whether the induction by agmatine is a direct or an indirect effect, but it is independent of the effect of cAMP:CRP (Section: 11.2.9). Cyclic AMP:CRP and agmatine must act at separate sites in a mutually non-exclusive manner (Section: 11.2.9). There are four possible modes of action of agmatine in the induction of agmatine ureohydrolase. i) Agmatine might interact directly with the DNA and promote initiation of transcription. ii) Agmatine might interact with a regulatory protein (not

encoded by the cloned DNA, unless agmatine ureohydrolase is itself an autogenous regulator). iii) Agmatine might interact with RNA polymerase and increase the rate of initiation or the rate of elongation of transcripts of *speB*. iv) Agmatine might stabilise the *speB* mRNA.

12.15 Effect of putrescine:

The inhibition of ornithine decarboxylase and arginine decarboxylase by putrescine is also not understood but might occur by one of the following mechanisms. i)Putrescine *in vitro* bind to t-RNA in a specific manner (Cohen, 1971; 1978), and polyamines have been reported to bind to alternating G and C residues in the DNA causing a transition under physiological conditions *in vitro* of the B form of DNA to the Z form (Russel *et al.*, 1983; Rich *et al.*, 1984). This might repress the transcription of *speA* and *speC* genes, although it is difficult to attribute specificity in the putrescine:*speC* or *speA* DNA interactions. ii) Putrescine might bind to a protein, and repress transcription of *speA* and *speC* genes. iii) Putrescine might interact with RNA polymerase and thus decrease the selectively of RNA polymerase for *speA* and *speC* promoters affecting initiation. iv) The regulation of *speA* and *speC* might be mediated by arginine decarboxylase and ornithine decarboxylase respectively in an autogenous manner, putrescine acting as a co-repressor. v) Putrescine has been shown to bind to nucleic acids, and has also been shown to activate the DNA supercoiling topoisomerase (Lipetz *et al.*, 1980). If supercoiling decreases tran-

scription of *speA* and *speC*, this would account for the effects of putrescine. This is in fact observed in the *in vitro* transcription and translation of cloned *gyrA* and *gyrB* genes. Transcription was greater with relaxed DNA (Menzel and Gellert, 1983). However, observations in Chapter-8 and those of Boyle *et al.* (1985) are just the opposite. Transcription and translation *in vitro* was lower with relaxed DNA.

12.16 General regulation by cAMP, glutamine synthetase and guanosine tetra-phosphate:

The activities of ornithine decarboxylase, arginine decarboxylase and agmatine ureohydrolase, and the concentrations of polyamines vary proportionately with growth rate (Chapter: 4; Wright and Boyle, 1982; Boyle and Adachi, 1982). The growth rate in polyamine deficient cells is proportional to the concentration of polyamine added (Tabor and Tabor, 1969; Morris, 1981). The synthesis of arginine decarboxylase, ornithine decarboxylase and agmatine ureohydrolase, besides being regulated by specific regulatory molecules, viz. agmatine and putrescine (Chapters: 5 and 6), is also regulated by general effectors (regulators) [cAMP, glutamine synthetase (Chapters: 4 and 6) and ppGpp (Boyle and Adachi, 1982)]. Nutritional changes lead to changes in the growth rate of *E.coli*, which are accompanied by an increase or decrease in the concentrations of general regulatory molecules (Boyle and Adachi, 1982; Pastan and Adhya, 1976; Gallant, 1979; Yanofsky, 1981). Amino acid deficiency

(uncharged t-RNA) induces the synthesis of ppGpp (Galland, 1979; Yanofsky, 1981; Primakoff, 1981). A deficiency of a readily utilisable carbon source causes an increase in the cAMP concentration (Pastan and Adhya, 1976; Calcott, 1982), both of which negatively regulate *speA*, *speB* and *speC*. A deficiency in the availability of ammonium ions leads to de-adenylation of glutamine synthetase (Magasanik and Stadtman, 1980), which would then prevent the induction of *speA*, *speB* and *speC*.

It therefore appears that arginine decarboxylase, ornithine decarboxylase and agmatine ureohydrolase have a particular role in metabolism not observed in any other group of enzymes. Therefore, polyamines may play a critical role in mediating the effects of the environment and levels of general regulators on the growth rate.

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