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POSITIVE GENE REGULATION BY *relA* IN *E. coli*

by

© Christopher R. Somerville

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Positive gene regulation by *relA* in *E. coli* submitted by Christopher R. Somerville in partial fulfilment of the requirements for the degree of Master of Science.

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Date August 4, 1976

ABSTRACT

Several new methionyl-tRNA synthetase (*metG*) mutants of *E. coli*, which require methionine for growth, were isolated. The methionine requirement of these mutants was shown to be due to a decrease in the affinity of the methionyl-tRNA synthetase for methionine. These mutants undergo spontaneous reversions which relieve the growth requirement for methionine. A detailed analysis of the mechanisms by which one of these *metG* mutants reverts to a met^+ phenotype revealed two classes of revertants: (i) Revertants of the first class exhibit constitutive synthesis of the methionine biosynthetic enzymes due to a *metJ* regulatory mutation; (ii) Revertants of the second class have undergone a reversion which results in partial or complete restoration of methionyl-tRNA synthetase activity.

Several of the revertants of the second class were shown to require the presence of a wild type *relA* gene for maintenance of the met^+ phenotype. The analysis of this effect resulted in the discovery that the synthesis of the methionine biosynthetic enzymes is under a positive form of regulatory control involving the *relA* gene.

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I wish to thank my supervisor, Doctor A. Ahmed, for his guidance and generous material support during the course of this study.

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INTRODUCTION

Genetic analysis of the regulation of metabolic pathways in bacteria has generally been directed towards the elucidation of the pathway-specific regulatory mechanisms governing the synthesis or degradation of a particular metabolite. In most cases it has been found that one of the substrates or the product of the pathway interacts with a regulatory macromolecule, generally a protein, to effect transcriptional control of the genes involved in the pathway. The specific mechanisms, which are very diverse and include both positive and negative control systems, are described in detail in recent reviews by Beckwith and Rossow (1974), and Gots and Benson (1974). The recently elucidated mechanism of activation-attenuation is described by Bertrand *et al.* (1975), and Artz and Broach (1975) for the *trp* and *his* operons respectively.

The first evidence for an integrated form of control, in which unrelated pathways are subject to control by a common regulatory element, was the elucidation of the role of cyclic 3':5'-adenosine monophosphate (cAMP) as a positive regulator of the catabolite sensitive genes. In this regulatory system, cAMP (which is not a substrate or a product of any of the pathways involved) interacts with the catabolite gene activator protein (CAP) to stimulate transcription of the catabolite sensitive genes (Zubay *et al.*, 1970). The stimulatory effect of cAMP and CAP is proportional to the degree to which a particular gene or operon is available for transcription as determined by the appropriate pathway-specific regulatory system. The two forms of regulation therefore act in a complementary fashion. The regulatory

effects of cAMP and CAP are described in detail in a recent review by Rickenberg (1974).

Recently, evidence has been presented which suggests that the amino acid biosynthetic pathways may also be subject to a similar form of integrated control. Stephens *et al.* (1975) presented *in vivo* and *in vitro* evidence supporting the conclusion that the synthesis of the histidine biosynthetic enzymes in *Salmonella* is stimulated by guanosine 5'-diphosphate-3'-diphosphate (ppGpp), which is synthesized by the product of the *relA* gene (Sy and Lipman, 1973). Although the *relA* gene has not as yet been shown to be involved in the regulation of other biosynthetic pathways, the implication is that the analysis of the regulation of amino acid biosynthesis must henceforth include an examination of the possible effects of supra-regulatory systems. The present study is concerned in this respect with a regulatory phenomenon involving methionyl-tRNA synthetase, the product of the *relA* gene, and the genes of the methionine biosynthetic pathway.

The aminoacyl-tRNA synthetases

In bacteria, the aminoacyl-tRNA synthetases are recovered from the non-sedimentable supernatant of cell extracts. With one possible exception, there is a unique aminoacyl-tRNA synthetase for each amino acid. The complex reaction catalyzed by this family of enzymes is generally represented as a two step reaction by the following equations:

$$AA + ATP + E \rightleftarrows E.AA\text{-}AMP + PPi \qquad \text{Eq. 1}$$

$$E.AA\text{-}AMP + tRNA \rightleftarrows AA\text{-}tRNA + E + AMP \qquad \text{Eq. 2}$$

The activation reaction (Eq. 1), which is generally unaffected by the presence of tRNA, is measured by the amino acid-dependent exchange of radioactive pyrophosphate into ATP. The overall reaction, which occurs 10-100 fold more slowly that the activation reaction, is monitored by measuring the esterification of radioactive amino acid to tRNA. The rate-limiting step for the overall reaction appears to be the release of AA-tRNA from the enzyme (Eldred and Schimmel, 1972).

Since the synthetase catalyzed reaction is an essential function, mutants have been sought which are only conditionally expressed or only partially defective. The first synthetase mutant of *E. coli* was isolated by Fangman and Neidhardt (1964a) as a *p*-fluorophenylalanine resistant mutant. The analog resistance of this mutant is due to an impaired ability to aminoacylate $tRNA^{Phe}$ with *p*-fluorophenylalanine, but an almost normal ability to aminoacylate $tRNA^{Phe}$ with phenylalanine. Several other amino acid analogs (*i.e.*, thiosine, canavanine) have subsequently been used to isolate similar mutants for other synthetases. The first temperature sensitive synthetase mutant was obtained for the valyl-tRNA synthetase by Eidlic and Neidhardt (1965). Since then, temperature sensitive mutants have been described for several other synthetases. By screening amino acid auxotrophs, mutants have been obtained for at least nine of the synthetases. These mutants generally have an aminoacyl-tRNA synthetase which has an increased *Km* for the amino acid. This type of alteration in the synthetase results in a requirement for a higher endogenous concentration of the appropriate amino acid than that which is normally available.

The regulation of the synthesis of the aminoacyl-tRNA synthetases is poorly understood, but several studies have suggested that a repression-derepression phenomenon may be in effect for at least some of the synthetases. Culture conditions which limit the supply of an amino acid may specifically derepress the synthesis of a particular synthetase, although the derepression is often difficult to observe since many of the synthetases are rapidly inactivated under conditions which limit the availability of their cognate aminoacyl-tRNA (Williams and Neidhardt, 1969). In some instances, the cognate tRNA, or a derivative thereof, has been suggested as an end product effector in the repression mechanism. There is also a suggestion that the synthetases are under some form of "metabolic regulation" since in *E. coli* the rate of synthesis of the synthetases is coupled to the growth rate of the organism (Parker and Neidhardt, 1972). The implication seems to be, that as part of the translational apparatus, the synthetases may be regulated in concert with or by other parts of the protein synthesis system. Clark *et al.* (1973) have recently isolated the first regulatory mutant for a synthetase. Their analysis of the mutant suggests that it is an operator constitutive mutant of seryl-tRNA synthetase.

The relatively vast body of literature dealing with the aminoacyl-tRNA synthetases has been reviewed recently by Söll and Schimmel (1974), and Kiesselev and Favorova (1975).

Methionyl-tRNA synthetase

Methionine is incorporated into protein via two distinct

methionyl-tRNA species, $tRNA_M^{Met}$ and $tRNA_F^{Met}$, the latter of which may be formylated subsequent to methionylation (Marcker and Sanger, 1964). The $tRNA_F^{Met}$ species has been shown to incorporate methionine only in the *N*-terminal position of *E. coli* proteins (Clark and Marcker, 1966), and has been ascribed a role in polypeptide chain initiation in bacteria (Adams and Capecchi, 1966). Both species of $tRNA^{Met}$ are aminoacylated by a single methionyl-tRNA synthetase (Henrikkson and Hartley, 1967), which in its native form is a dimer composed of two identical subunits of molecular weight 90,000 (Fayat and Waller, 1974). The subunit, which appears to contain a high degree of sequence duplication (Bruton *et al.*, 1974), can be cleaved by trypsin to a fully active fragment with a molecular weight of 65,000 (Cassio and Waller, 1971). Both monomeric forms of the enzyme have distinct binding sites for methionine, tRNA, and ATP (Fayat and Waller, 1974). The native monomer has in addition, a second non-catalytic binding site for ATP, the function of which is not known.

The first methionyl-tRNA synthetase (*metG*) mutant reported for *E. coli* was isolated by Calendar and Lindahl (1969) in *E. coli* C. Blumenthal (1972), and Armstrong and Fairfield (1975) have subsequently reported similar mutants for *E. coli* K12. All of these mutants require exogenous methionine for growth due to an increase in the Km^{Met} of the synthetase (Ahmed, 1973). An unusual *metG* mutant was isolated by Archibold and Williams (1973) on the basis of an increased resistance to the methionine analog ethionine. The methionyl-tRNA synthetase of this strain has an increased *Km* for $tRNA^{Met}$ but the Km^{Met} and Km^{ATP} are apparently unaffected. A disturbing aspect of this study is that no mechanism is proposed to account for the ethionine resistance of

this mutant.

The map position of *metG* has not yet been accurately determined. A location near the *his* operon was suggested by Cassio *et al.* (1970) who showed that the introduction of the F32 F' plasmid into an F⁻ strain caused an additive increase in methionyl-tRNA synthetase activity. Blumenthal (1972) showed cotransduction of a *metG* mutation with one of the P2 attachment sites, and Ahmed (1973) established the clockwise gene sequence *metG,his, rspL* by a conjugal cross. The orientation of *metG* with respect to other loci has not been reported.

Methionyl-tRNA synthetase is specifically inhibited *in vivo* and *in vitro* by L-methioninyl-adenylate (Cassio and Mathien, 1974; Cassio *et al.*, 1973). By supplementing the medium with methioninyl-adenylate it is therefore possible to modulate the degree of amino-acylation of $tRNA^{Met}$ *in vivo*. Cassio (1975) observed that when more than 25% of $tRNA^{Met}$ is deacylated, the level of derepression of methionyl-tRNA synthetase is proportional to the amount of $tRNA^{Met}$ deacylated. She interpreted this result to mean that methionyl-tRNA is involved as a repressor or as a corepressor of the synthesis of the synthetase. Cassio also observed that the synthetase is subject to a specific inactivation in the presence of high levels of $tRNA^{Met}$. As noted previously, inactivation of the other aminoacyl-tRNA synthetases has been observed under conditions which limit the supply of the cognate aminoacyl-tRNA (Williams and Neidhardt, 1969).

By taking advantage of the proximity of the *metG* locus to one of the phage P2 attachment sites, Cassio *et al.* (1975) have isolated a class of mutants in which the regulation of methionyl-tRNA synthetase

appears to have been altered by phage eduction deletions. In these strains, the amount of th[redacted]nthetase is increased several-fold as compared to the parental strain, but the enzyme does not appear to be structurally altered. Although genetic evidence is not presented, the authors have suggested that the eductants might represent alterations in the *cis*-acting regulatory locus of the *metG* gene. However, since the rate of synthesis of the enzyme remains coupled to the growth rate of the organism, they concluded that there are at least two dissociable or independent processes which regulate the intracellular level of methionyl-tRNA synthetase.

Methionine biosynthesis: regulation and utilization

The genetic and biochemical aspects of methionine biosynthesis have been reviewed by Smith (1971) and more recently by Flavin (1975). The essential features of this biosynthesis are outlined below.

Six structural genes participate in the conversion of homoserine to methionine (Fig. 1). These are clustered in non-contiguous segments of the *E. coli* chromosome (Fig. 2), *metA* and *metH* being located at 89 min, *metB* and *metF* at 87 min, and *metE* at 84 min.

The first specific precursor of methionine *O*-succinylhomoserine is formed from succinyl-CoA and homoserine by the enzyme *O*-succinylhomoserine synthetase (*metA*). The next enzyme, cystathionine-γ-synthetase (*metB*), catalyzes the replacement of the succinyl group to give cystathionine. Cystathionine is then hydrolyzed to homocysteine by the enzyme β-cystathionase (*metC*). The methyl group donors for the methylation of homocysteine to methionine are synthesized by

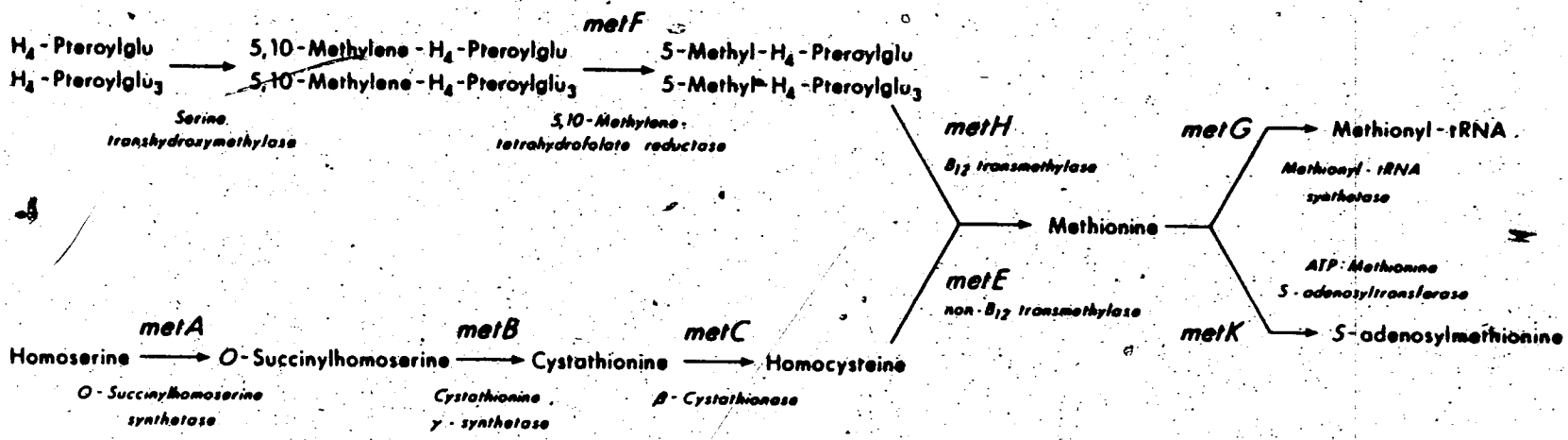

FIGURE 1 - The pathway for biosynthesis and utilization of methionine in *E. coli*.

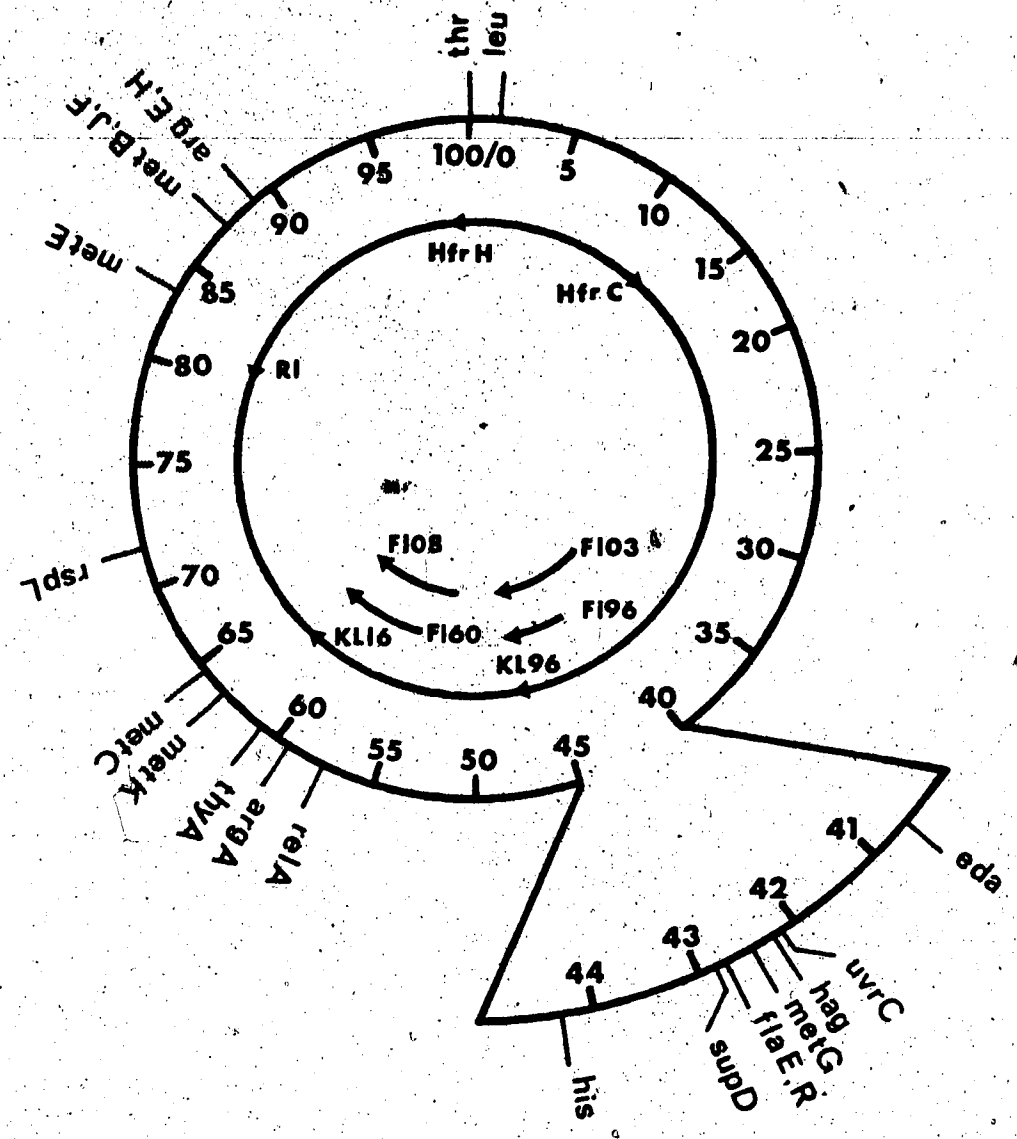

Figure 2 - Genetic map of *E. coli* K12 showing the location of some of the genes relevant to this study. The point of origin and direction of transfer of several Hfr strains are indicated by arrows on the interior circle. The extent of the F episomes used in this study are indicated by the interior line fragments. The map is derived from Bachmann *et al.* (1976).

the enzyme N^5,N^{10}-methylenetetrahydrofolate reductase (*metF*) and the actual methylation of homocysteine is carried out by either the vitamin B12-independent transmethylase (*metE*), or by the B12-dependent enzyme (*metH*).

Once methionine is formed in the cell it is utilized as a substrate for protein synthesis or in the formation of *S*-adenosylmethionine. The formation of *S*-adenosylmethionine from methionine and ATP is catalyzed by the enzyme ATP:methionine *S*-adenosyl transferase (*metK*) which is specific for the L-isomer. The principal metabolic functions of *S*-adenosylmethionine (SAM) are participation in polyamine biosynthesis via decarboxylated SAM (Tabor *et al.*, 1961), and in various transmethylation reactions (Cantoni, 1965).

As in many other biosynthetic pathways, the synthesis of the methionine biosynthetic enzymes is subject to non coordinate repression by addition of the end product of the pathway. Regulation of methionine biosynthesis is affected by the allelic condition of at least three genes.

The *metA* enzyme catalyzes the first reaction of the biosynthetic pathway and is subject to strong cooperative feedback inhibition by methionine and SAM (Lee *et al.*, 1966). Analog resistant mutants which have normal levels of the biosynthetic enzymes have been mapped within the *metA* gene. These mutants have a *metA* enzyme which is not sensitive to feedback inhibition (Chater and Rowbury, 1970).

Another class of regulatory mutants (*metJ*) have been mapped near the *metB* locus. Mutations at the *metJ* locus confer analog resistance and exhibit constitutive synthesis of all the methionine biosynthetic enzymes and of the *metK* enzyme. Several complementation studies

(Chater, 1970; Su and Greene, 1971; Holowachuck, 1976), have established that the *metJ* gene exerts its effects in a negative manner through the formation of a cytoplasmic repressor, rather than by the alteration or modification of methionyl-tRNA^{Met} (Ahmed, 1973). The isolation of *amber metJ* mutants of *E. coli* by Morowicz (1975) confirmed that the repressor is a protein.

The third class of regulatory mutants map in the *metK* gene, and also confer resistance to several methionine analogs. Many of these mutants show very low levels of ATP:methionine *S*-adenosyltransferase activity and are derepressed for the synthesis of the methionine biosynthetic enzymes (Hobson and Smith, 1973; Greene *et al.*, 1970). The *metK* enzyme is apparently not essential for growth since Morowicz (1975) has isolated several *amber metK* mutants of *E. coli*. The analysis of the *metK* phenotype is complicated by the recent isolation of a *metK* mutant which has normal ATP:methionine *S*-adenosyltransferase activity and which complements with other *metK* mutants (Hobson, 1974). The implication is that SAM, or a derivative of SAM, could act as the corepressor. Hobson (1974) has suggested that the *metK* enzyme itself could be involved in the repression mechanism.

The interaction of methionyl-tRNA synthetase with the *met* biosynthetic enzymes has been examined by several authors. Chater *et al.* (1970) examined the effect of *metJ* and *metK* mutations in a methionine requiring *metG* mutant of *Salmonella*. They observed that some of the *metK* or *metJ* mutants were able to suppress the met$^-$ phenotype of the *metG* mutant by causing an increase in the endogenous methionine level to the point that the K_m^{Met} defect of the synthetase is compensated for. The *metJ* and *metK* mutations had no observable

effect on the rate of synthesis of methionyl-tRNA synthetase. Ahmed (1973) confirmed that the methionine biosynthetic enzymes are regulated independently of methionyl-tRNA synthetase.

In summary, the general model for the regulation of methionine biosynthesis involves a *metJ* coded repressor protein which controls methionine biosynthesis in a negative manner. Presumably, the repressor binds one or more corepressors and modulates transcription by interacting with operator sequences adjacent to the *met* structural genes. However, the precise nature of the corepressor has not been established, and no direct evidence for operator sequences is available.

The rel phenomenon

In many strains of *E. coli*, stable RNA synthesis is abruptly cutrailed upon starvation for an essential amino acid. Stent and Brenner (1961) called this phenomenon the stringent response and showed that a mutation at a single locus could alleviate the effect. A spontaneous mutation, which they designated as RC^{rel}, caused the relaxation of RNA synthesis in that RNA continued to accumulate following amino acid starvation. The site of this mutation has subsequently been redesignated as the *relA* locus (Bachmann *et al.*, 1976), since mutations at several other sites which confer the same phenotype have recently been reported (Cashel and Gallant, 1974; Parker *et al.*, 1976).

Fangman and Neidhardt (1964) demonstrated that the inactivation of any of the aminoacyl-tRNA synthetases invoked the stringent response even in the presence of a full complement of amino acids.

This suggested that the phenomenon is a response to depletion of at least one of the aminoacyl-tRNA pools rather than to the concentration of the amino acids themselves. Cashel and Gallant (1969) and Cashel (1969) subsequently reported that amino acid starvation or inactivation of an amionacyl-tRNA synthetase resulted in the rapid accumulation of guanosine 3'-diphosphate-5'-diphosphate (ppGpp) and guanosine 3'-diphosphate-5'-triphosphate (pppGpp) in stringent but not relaxed strains. They postulated that one or both of the compounds could be a causitive factor in the cessation of RNA accumulation and the other characteristics of the stringent response.

By employing an *in vitro* translation assay, Haseltine and Block (1973, 1974) demonstrated that ppGpp is synthesized from ATP and GTP in response to an uncharged tRNA in the acceptor site of the translating ribosome. This work was extended by Sy and Lipmann (1973) who obtained and purified to near homogeneity a small protein from the high salt wash of rel^+ ribosomes, which catalyzes the *in vitro* formation of ppGpp from ATP and GTP. They concluded that this protein is the product of the *relA* gene.

Although several attempts to demonstrate the *in vitro* regulation of RNA synthesis by ppGpp have been unsuccesful, Reiness *et al.* (1975) have recently provided convincing evidence that ppGpp selectively modifies the rate of transcription of different classes of genes *in vitro*. Using purified DNA from a variety of sources, they demonstrated that the addition of ppGpp to an *in vitro* transcription system causes a selective inhibition of rRNA synthesis but a stimulation of transcription of the *trp* and *lac* operons. They postulated that ppGpp interacts with RNA polymerase in such a way that the affinity for

different promoters is differentially altered.

This hypothesis was extended by Stephens *et al.* (1975) who presented *in vivo* and *in vitro* evidence that ppGpp acts to positively stimulate transcription of the *his* operon of *Salmonella*. The ppGpp mediated stimulation of *his* operon expression is distinct from the *his* operon specific regulatory mechanism, and appears to act by increasing the rate of initiation of transcription. On the basis of this result and several indirect lines of evidence, Stephens *et al.* (1975) have suggested that the *relA* gene is generally important in the regulation of all amino acid biosynthetic pathways. On the basis of their own results and those of Reiness *et al.* (1975), they have suggested that ppGpp acts as a general signal molecule or alarmone which acts in a manner analogous to the cAMP alarmone to redirect the cells economy in response to a change in enviornmental conditions. Cashel (1975) has recently presented a comprehensive review of the other pleiotropic effects associated with ppGpp and pppGpp accumulation.

The present study represents a detailed analysis of the mechanisms by which a methionine requiring methionyl-tRNA synthetase (*metG*) mutant reverts to a met^+ phenotype. This approach has resulted in the discovery that the synthesis of the methionine biosynthetic enzymes is subject to a form of positive regulatory control involving the *relA* gene. Also, a genetic system has been developed in which the *rel* phenotype can be recognized by a methionine requirement. The potential usefullness of this system for the analysis of the *rel* phenomenon is discussed.

MATERIALS AND METHODS

Bacterial and bacteriophage strains

The genotypes and sources of the strains used in this study, all of which were derived from *E. coli* K12, are described in Table 1. The bacteriophages T4$^+$, an *amber* Mutant of T4 (T4*am*), and the generalized transducing phage P1*vir* were obtained from Dr. A. Ahmed's collection. Phage ϕ80p*su3*$^+$*su3*$^-$*h*$^-$ was supplied by Dr. N. Franklin. The male specific phage R17 was obtained from Dr. W. Paranchych.

Media

The medium of Davis and Mingioli (as described by Roth, 1970) containing 0.2% glucose, lactose or xylose, was used as the minimal medium. This medium was supplemented, when necessary, with 20 ug/ml of the required L-amino acid, 10 ug/ml of thiamine.HCL, 0.1 ug/ml of vitamin B12, and thymine or uracil at 50 ug/ml. D,L-ethionine was used at 3 mg/ml for scoring ethionine resistance. L broth or L agar was generally used as the complete medium. On several occasions a modified L broth (LP broth) was used. The composition of LP broth is the same as L broth except that the NaCl buffer is replaced by the phosphate buffer of Davis and Mingioli (Roth, 1970).

Motility agar was prepared as described by Silverman and Simon (1974) except that the tryptone motility agar was modified by the addition of 0.8 g/litre of sodium citrate.

Table 1 - Bacterial strains

Strain	Genotype	Source
AB1111	F⁻ *thr leu ara proA lac supE gal hisC rspL xyl mtl thi*	A. Ahmed, University of Alberta
AB3059	Hfr *leu lacZ supE thyA rspL ilvD thi deo*	" " "
B36	Hfr H *proB relA1 metB36 thi*	" " "
CA274	Hfr C lac_{am} trp_{am} *thi* su^{o}_{am}	" " "
KL16	Hfr *relA1 thi*	" " "
KL96	Hfr *relA1 thi*	" " "
KLF3/JC1552	F103 his^{+} $metG^{+}$/ *leu tonA supE gal trp his argG rspL xyl mtl metB thi*	" " "
MA220	F⁻ *trp his argA serA rspL thi*	" " "
SB1803	F⁻ *thr leu ara proA lac supE gal hisC metG rspL xyl mtl thi*	" " "
X407	Hfr H *proB relA1 thi*	" " "
DF71	Hfr H *lacI eda relA1 thi*	B. Bachmann, Yale University
KL96-B	Hfr *supD32 relA1 thi*	" " "
KLF8/MA50	F108 $argA^{+}$ $relA^{+}$/ *thr leu lac csy lysA mtl mal thi*	" " "
MX383	F196 his^{+} *supD/ lacZ trp his nalA recA relA1 arg*	" " "
R1	Hfr *relA1 metB thi* su^{o}_{am}	" " "
CSH4	F⁻ $lacZ_{am}$ trp_{am} *rspL thi* su^{o}_{am}	Cold Spring Harbor Strain Kit
CP78	F⁻ *thr leu ara tonA gal his xyl mtl argH mal*	J. Friesen, York University

Table 1 - continued

Strain	Genotype	Source
CP79	F⁻ *thr leu ara tonA gal his xyl relA1 mtl argH mal*	J. Friesen, York University
NF306	F160 *argA*$^+$ *relA*$^+$/ *leu his recA argG rspE** *rspL metB pyrB*	" "
MS72	F⁻ *galU uvrC flaB his thyA rspL argE su*$^+_{am}$	M. Simon, University of California
MS1338	F1829 *his*$^+$ *metG*$^+$ *fla1829*/ *thr leu ara pro lac gal hag his recA rspL xyl mtl argE thi*	" " "
MS1977	F1977 *his*$^+$ *fla1829 Δ(hag-flaE)*/ *thr leu ara pro lac gal hag his recA rspL xyl mtl argE thi*	" " "
AM29	F⁻ *thr leu ara lac*$_{am}$ *metG46 his rspL xyl mtl metJ thi*	Spontaneous *metJ* mutant of CS57
CS45	F⁻ *thr leu ara proA lac supE gal metG24 his rspL xyl mtl thi*	*MetG* mutant of AB1111
CS46	F⁻ " " " " " " " *metG44* " " "	" " "
CS47	F⁻ " " " " " " " *metG41* " " "	" " "
CS48	F⁻ " " " " " " " *metG3* " " "	" " "
CS49	F⁻ " " " " " " " *metG17* " " "	" " "
CS50	F⁻ " " " " " " " *metG46* " " "	" " "
CS54	F⁻ *thr leu ara proA lac supE gal metG46 his rspL xyl mtl ilv thi*	ilv⁻ mutant of CS50

Table 1 - continued

Strain	Genotype	Source
CS56	F⁻ *thr leu ara pro metG46 his rspL xyl mtl thi* su^{o}_{am}	Mating between Hfr H X407 and CS50
CS57	F⁻ *thr leu ara* lac_{am} *metG46 his rspL xyl mtl thi* su^{o}_{am}	Mating between Hfr C CA274 and CS56
CS60	F⁻ *thr leu ara* lac_{am} *metG46 supD rspL xyl mtl thi*	Transduction of CS57 to his^{+} $supD^{-}$
CS62	F⁻ *thr leu ara proA lac supE gal metG46 his rspL xyl mtl ilv metB metJ thi*	*metB metJ* mutant of CS54
CS63	F⁻ *leu ara* lac_{am} *metG46 his rspL xyl mtl argE thi*	Mating between Hfr 318 and CS57
CS62-C	Hfr *relA1 metB metJ thi*	Transduction of CS92 to arg^{+} $metB^{-}$
CS68	F⁻ *thr leu ara* lac_{am} *metG46 his rspL xyl mtl thi*	Uncharacterized revertant of CS57
CS92	Hfr *relA1 glpK argH* $rpoB^{*}$ *thi*	rif^{r} mutant of Hfr 316
CS122	F⁻ *thr leu ara proA lac supE gal eda metG rspL xyl mtl thi*	Mating between Hfr DF71 and SB1803
CS125	Hfr *relA1 thyA thi*	Spontaneous thy^{-} mutant of KL16
CS126	Hfr *relA1 argA thi*	Transduction of CS125 to thy^{+} arg^{-}
CS127	F⁻ *thr leu ara proA lac supE gal metG46 his thyA rspL xyl mtl thi*	Spontaneous thy^{-} mutant of CS50
CS130	F⁻ *thr leu ara* lac_{am} *metG46-31 his relA1 argA rspL thi*	Transduction of SU31-A to thy^{+} rel^{-}
CS156	F⁻ *thr leu ara tonA gal his xyl mtl metB mal thi*	Transduction of CP78 to arg^{+} met^{-}
CS157	F⁻ *thr leu ara tonA gal his relA1 xyl mtl metB mal thi*	Transduction of CP79 to arg^{+} met^{-}

Table 1 - continued

Strain	Genotype	Source
CS164	F^- *uvrC flaB his thyA rspL argE thi*	Mating between Hfr KL96 and MS72
Hfr 305	Hfr *thi*	Transduction of CS126 to arg^+rel^+
Hfr 312	Hfr *thr leu ara proA lac supE gal metG46 rspL xyl mtl thi*	Mating between Hfr KL96 and CS127
Hfr 313	Hfr H *proB relA1 thyA metB36 thi*	Spontaneous thy^- mutant of B36
Hfr 314	Hfr H *proB relA1 argA metB36 thi*	Transduction of Hfr 313 to thy^+arg^-
Hfr 315	Hfr H *proB metB36 thi*	Transduction of Hfr 314 to arg^+rel^+
Hfr 316	Hfr *relA1 glpK argH thi*	Transduction of R1 to $met^+arg^-glpK^-$
Hfr 318	Hfr C *argE thi* su^+_{am}	H. Morowicz strain
SU3-47	F^- *thr leu ara* lac_{am} *metG his rspL xyl mtl thi*	met^+ revertants of CS57
SU24-A	F^- *thr leu ara* lac_{am} *metG46-24 his thyA rspL xyl mtl thi*	Spontaneous thy^- mutant of SU24
SU31-A	F^- *thr leu ara* lac_{am} *metG46-31 his thyA rspL thi*	Mating between AB3059 and SU31
SU31-C	F^- *thr leu ara* lac_{am} *metG46-31 his argA rspL thi*	Transduction of SU31-A to thy^+arg^-
SU31-F	F^- *thr leu ara* lac_{am} *metG46-31 his rspL thi*	Transduction of SU31-C to arg^+
SU31-I	F^- lac_{am} *metG46-31 his rspL glpK argH thi*	Mating between Hfr 316 and SU31-F
SU32-A	F^- *thr leu ara* lac_{am} *metG46-32 his thyA rspL thi*	Mating between AB3059 and SU32

Table 1 - continued

Strain	Genotype	Source
SU43-A	F⁻ *thr leu ara* lac_{am} *metG46-43 his thyA rspL xyl mtl thi*	Spontaneous thy⁻ mutant of SU43

Those symbols noted with an asterisk have recently been redesignated by Bachmann *et al.* (1976). The relevant alternate symbols are as follows: *rspL (strA), rspE (spcA), rpoB (rif).*

Isolation of methionyl-tRNA synthetase (metG) mutants

The strain AB1111 (F⁻ *thr leu ara proA lac supE gal his rspL xyl mtl thi*) was used as the parent strain for the isolation of methionyl-tRNA synthetase mutants which required methionine for growth. A culture of the strain AB1111 was grown to late log phase in L broth, then *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine was added to a final concentration of 50 ug/ml, and the culture was incubated without aeration for 30 min at 37°. The mutagenized culture was then washed twice with saline , resuspended in an equal volume of minimal medium, and incubated for 90 min at 37° to ensure methionine depletion. At this time the culture was supplemented with vitamin B12, homocysteine, and Penicillin-G (10,000 I.U./ml). After incubation for 90 min, the culture was shifted to an equal volume of fresh medium, and incubated for an additional 90 min. The culture was then washed with saline, concentrated 30-fold, and dilutions were plated on methionine supplemented minimal agar. Those colonies which showed retarded growth after 48 hrs of incubation at 37°, were transferred to master plates containing the same medium, then replica plated onto minimal agar supplemented with homocysteine and vitamin B12. Those strains which failed to grow on this combination of supplements were assayed for methionyl-tRNA synthetase activity by the aminoacylation assay. Strains which showed low levels of synthetase activity were tentatively designated as *metG* mutants. The presumptive *metG* mutants were further characterized by determination of the Km^{Met} by the pyrophosphate exchange assay.

Construction of strain CS57

The strain CS57 (F⁻ *thr leu ara* lac_{am} *metG46 his rspL xyl mtl thi*) was derived from the strain AB1111 (F⁻ *thr leu ara proA lac supE gal his rspL xyl mtl thi*) by the three step procedure described below:

(i) The strain CS50 (F⁻ *thr leu ara proA lac supE gal metG46 his rspL xyl mtl thi*) was derived from AB1111 by mutagenesis as described, and is assumed to be isogenic except for the presence of an altered methionyl-tRNA synthetase due to the *metG46* mutation.

(ii) The su^{o}_{am} strain CS54 (F⁻ *thr leu ara pro metG46 his rspL xyl mtl thi*) was obtained from a conjugal cross between Hfr H X407 (*proB relA1 thi*) and CS50. Fifty lac^{+} gal^{+} str^{r} recombinants selected from this cross were tested for su^{o}_{am} on L broth plates seeded with $T4^{+}$ or T4*am* phage. A su^{o}_{am} recombinant was retained and designated CS54.

(iii) A lac_{am} derivative of CS54 was obtained from a conjugal cross in which Hfr C CA274 (lac_{am} trp_{am}) was the donor. Fifty pro^{+} str^{r} recombinants, selected from this cross, were tested for the inheritance of the lac_{am} allele by streaking on lactose minimal agar. A lac^{-} met^{-} recombinant was retained and designated CS57. The presence of the lac_{am} allele is indicated by the ability of $\phi 80psu3^{+}$ lysogens of CS57 to grow on lactose minimal agar.

Isolation of methionine independent revertants of CS57

Spontaneous methionine independent revertants, designated as the SU3-47 series, were isolated from the met^{-} parental strain CS57 (F⁻ *thr leu ara* lac_{am} *metG46 his rspL xyl mtl thi*). Single colonies of

CS57 were innoculated into L broth, grown to saturation, then washed and resuspended in saline to 1/5 of the original volume. Minimal agar plates supplemented with homocysteine were spread with 0.1 ml of the cell suspension and incubated for 48 hrs at 37°. The number of colonies appearing by this time was generally about 100 per plate. Whenever possible, 25 colonies from each plate were transferred to a master plate containing the same medium. Those colonies which were surrounded by a halo of background growth were not picked since this phenotype corresponds to that observed for the methionine excreting regulatory mutants observed by Chater *et al.* (1970) in a similar study of *Salmonella*.

All of the revertants were tested for the ability to grow on unsupplemented minimal agar at 30° and 37°. This combination of selection and screening procedures was designed to allow the recovery of revertants which either require homocysteine for growth, or have a temperature conditional met^+ phenotype. For example, a reversion mutation resulting in a temperature sensitive *metJ* repressor, might be expected to give rise to a met^+ phenotype at the inactivating temperature (37°), wheras at the permissive temperature (30°) the met^- phenotype of the parental strain would be expressed.

The revertants from those cultures which failed to produce either a homocysteine-dependent or a temperature-dependent phenotype, were then examined for the presence of an *amber* reversion-mutation as follows. Replicas of the revertants were lysogenized with $\phi 80 psu3^+$ by printing on lactose minimal agar which was seeded with the phage and supplemented with methionine. Phage $\phi 80 psu3^+$ carries suppressor $tRNA_1^{Tyr}$ which suppresses the lac_{am} mutation in CS57 thereby allowing

the growth of lysogens under these conditions. After partial purification of the lysogens on the same medium, they were tested for growth on methionine-free lactose minimal agar. A large proportion of the lysogens showed very reduced growth in the absence of methionine. The revertant from each culture which showed the greatest differential growth response when lysogenized and tested on the methionine-free lactose medium, was retained for further characterization. This class of revertants is represented by the SU3-47 series and by the exceptional revertant CS68.

One unusual revertant designated CS62 (F^- *thr leu ara proA lac supE gal metG46 his rspL xyl mtl ilv metB metJ thi*), was obtained by a similar procedure from the parental strain CS54 (F^- *thr leu ara proA lac supE gal metG46 his rspL xyl mtl ilv thi*).

Construction of isogenic relA$^+$/relA$^-$ strains

The rel$^+$ strain Hfr 315 (*proB metB36 thi*) was obtained from Hfr H B36 (*proB relA1 metB36 thi*) by a three step procedure outlined below:

(i) The strain Hfr 313 (*proB relA1 thyA metB36 thi*) was obtained as a spontaneous trimethoprim (40 ug/ml) resistant mutant of B36.

(ii) The strain Hfr 314 (*proB relA1 argA metB36 thi*) was obtained by transducing Hfr 313 to thy$^+$ arg$^-$ with a P1*vir* lysate of MA220 (F^- *trp his argA serA rspL*).

(iii) The strain Hfr 315 was obtained by transducing Hfr 314 to arg$^+$ rel$^-$ with a P1*vir* lysate prepared on AB1111 (F^- *thr leu ara proA lac supE gal his rspL xyl mtl thi*).

The rel$^+$ strain Hfr 305 (*thi*) was obtained from Hfr KL16 (*relA*

thi) by an identical procedure.

The strain CS156 (F$^-$ *thr leu ara tonA gal his mtl metB thi mal*) and the otherwise isogenic rel$^-$ strain CS157 were obtained by P1 transduction from the strains CP78 (F$^-$ *thr leu ara tonA gal his mtl argH thi mal*) and CP79 (F$^-$ *thr leu ara tonA gal his relA1 mtl argH thi mal*). A P1*vir* lysate of Hfr R1 (*relA1 metB thi*) was used to transduce CP78 and CP79 to arg$^+$ met$^-$.

The rel$^-$ strain CS130 (F$^-$ *thr leu ara lac*$_{am}$ *metG46-31 his relA1 argA rspL thi*) and the otherwise isogenic rel$^+$ strain SU31-C were obtained from the met$^+$ revertant strain SU31 (F$^-$ *thr leu ara lac*$_{am}$ *metG46-31 his rspL xyl mtl thi*) by the procedure described below:

(i) The strain SU31-A (F$^-$ *thr leu ara lac*$_{am}$ *metG46-31 his thyA rspL thi*) was obtained from a conjugal cross between Hfr AB3059 (*leu lacI supE thyA rspL ilvD thi deo*) and SU31. Nutritional selection was applied against both donor and recipient by plating the mating mixture on xylose minimal agar supplemented with threonine, leucine, histidine, and thymine. Several xyl$^+$ thyA$^-$ recombinants of SU31 were recovered, and one of these was designated SU31-A.

(ii) A P1*vir* lysate of Hfr CS126 (*relA1 argA thi*) was used to transduce SU31-A. ThyA$^+$ transductants were selected on minimal agar supplemented with arginine and methionine. Of 312 thy$^+$ transductants, 3 were arg$^-$ met$^-$ rel$^-$. One of these transductants was designated as CS130. An arg$^-$ met$^+$ rel$^+$ transductant from this cross was also retained and was designated SU31-C.

Genetic techniques

Where nutritional requirements were available as selective

markers, transductions were performed with the generalized transducing phage P1*vir* according to the procedure described by Lennox and Yanofsky (1959). In those instances where flagellar mutations were used as the selected marker, the washed transduction mixture was placed in a trough in the surface of a motility agar plate. Under these circumstances, fla^+ transductants swarm outward from the trough and can be separated from the nonmotile bacteria.

F' plasmid transfers and Hfr mating were performed according to the procedures described by Miller (1972).

Construction of strain Hfr 312

The strain Hfr 312 (*thr leu ara proA lac supE gal metG46 rspL xyl mtl thi*) was obtained from a three hour non-interrupted mating between Hfr KL96 (*relA1 thi*) and CS127 (F^- *thr leu ara proA lac supE gal metG46 his thyA rspL xyl mtl thi*). Several hundred thy^+ his^+ str^r recombinants were recovered from this mating. Since the point of origin of Hfr KL96 is interior to the *thyA-his* interval, this selection scheme was designed to enrich for recombinants in which F had become integrated into the recipient chromosome in the same position as in KL96. Four of the recombinants, obtained from this cross, retained the methionine requirement of the F^- parent and gained the ability to support the growth of the male-specific phage R17. The ability of one of these recombinants to effect transfer of the *metG46* allele was determined by mating it with the F^- strain MA220 (F^- *trp his argA serA rspL*). Nutritional selection was applied against the donor by omitting threonine, leucine, and proline from the medium. Fifty his^+ or trp^+ recombinants were tested for a methionine

requirement. Twenty-one of the trp^+ recombinants were met^- and twenty-nine of the his^+ recombinants were met^-. Similar results were obtained in a cross in which the strain MS827 (F^- *galU uvrC flaR his thyA rspL argE*) served as the recipient. The recovery of met^- recombinants is considered as evidence that Hfr 312 can donate the *metG46* allele. It appears however that the frequency of transfer is relatively low.

Growth of bacteria for enzyme assays

For the assay of the methionine biosynthetic enzymes and ATP:methionine *S*-adenosyltransferase, cultures were grown under non-repressing conditions. Cells were first grown to stationary phase in 10-20 ml of minimal medium. The bacteria were then collected by centrifugation, resuspended in 250 ml of fresh minimal medium, and grown with aeration for 5-6 hrs at 30°. The cultures were centrifuged, washed with 50 mM potassium phosphate (pH 7.3), and stored as a cell pellet at -40°.

For the assay of methionyl-tRNA synthetase, cells were grown to mid log phase in 50 ml LP broth, centrifuged, washed with 20 mM potassium phosphate (pH 7.3)/ 4% glycerol (v/v), and stored as a cell pellet at -40°.

Preparation of cell free extracts

For extract preparations, cell pellets were resuspended in the appropriate buffer (depending on the enzyme assay to be performed), sonicated for 45 sec in ice, and then centrifuged at 31000 x *g* for

30 min in a Sorvall RC2-B centrifuge.

For the methionine biosynthetic enzymes and for ATP:methionine *S*-adenosyltransferase, a 50 mM potassium phosphate (pH 7.3) extraction buffer was used. Assays were performed immediately afterwards on undialyzed extracts.

Two different buffer systems were used for the preparation of extracts for the methionyl-tRNA synthetase assay. Initially, extracts were prepared and dialyzed in a buffer consisitng of 10 mM Tris.HCL (pH 7.6), 10 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 50 mM KCL (Nirenberg and Matthaei, 1961). In several instances, as an alternative to dialysis, extracts were desalted on a 1 x 5 cm column of Sephadex G-25 equilibrated with Nirenberg buffer. During the latter stages of this study, the Nirenberg buffer was replaced with a buffer containing 20 mM potassium phosphate (pH 7.3), 10% glycerol (v/v), and 10 mM 2-mercaptoethanol. The effect of this change in buffers is described in detail elsewhere.

Enzyme assays

Cystathionine-γ-synthetase was assayed by measuring the amount of α-ketobutyrate formed from *O*-succinyl-L-homoserine as described by Kaplan and Flavin (1966). The specific activity is expressed as the decrease in absorbance at 340 nm per 20 min per mg of protein.

β-cystathionase was assayed by the procedure of Flavin (1962). The specific activity is described as increase in absorbance at 412 nm per mg protein at room temperature.

N^5,N^{10}-methylenetetrahydrofolate reductase was measured by the menadione-dependent oxidation of ^{14}C-N^5-methyltetrahydrofolate to tetrahydrofolate and ^{14}C-formaldehyde, as described by Dickerman and Weissbach (1964). The specific activity is expressed as nmoles formaldehyde formed in 30 min per mg of protein at 37°.

ATP:methionine S-adenosyltransferase was assayed by a modification of the procedure described by Tabor and Tabor (1971). The reaction mixture contained 100 umoles triethanolamine.SO_4 (pH 8.4), 20 umoles $MgSO_4$, 2 umoles ATP, 1 umole NaCN, 0.1 umole ^{14}C-L-methionine (5 uCi/mmole), 2 umoles potassium phosphate (pH 7.3), and approximately 100 ug of S-30 protein, in a final volume of 200 ul. The radioactive product ^{14}C-*S*-adenosylmethionine (SAM) was separated from the unreacted substrate by applying the chilled reaction mixture to a 0.5 x 3 cm column of BioRex-70 (50-100 mesh) which was prepared as described by Holcomb and Shapiro (1975). The unreacted methionine was eluted from the column by applying 4 mls of water, then the SAM was eluted directly into scintillation vials with 2 mls of 0.1 N H_2SO_4 and counted by adding 10 mls of toluene/Triton-X (1:1), 0.4% Omnifluor scintillation mixture.

The specific activity is defined as umoles SAM formed in 20 min per mg protein at 37°.

Aminoacylation assay

For routine determinations, methionyl-tRNA synthetase activity was measured by a modification of the assay described by Calendar and Berg (1966). The reaction mixture contained 15 umoles sodium cacodylate (pH 6.9), 0.3 umoles ATP, 2 umoles magnesium acetate, 1.5 umoles

2-mercaptoethanol, 0.195 mg crude tRNA, 500 ug bovine serum albumin, and 3.2 nmoles ^{3}H-L-methionine (20 mCi/mmole), in a final volume of 150 ul. The assay was performed as described by Calendar and Berg except that 5% trichloroacetic acid was used instead of 2N HCL, and GF/A filters were used instead of GF/C filters. Specific activity is defined as nmoles methionyl-tRNA formed in 15 min per mg protein at 37°.

Pyrophosphate exchange assay

The amino acid activation reaction was measured by the pyrophosphate exchange assay described by Calendar and Berg (1966). Specific activity is defined as umoles ^{32}P-ATP formed in 15 min per mg protein at 37°.

Thermal inactivation of methionyl-tRNA synthetase

Cell extracts were prepared in the phosphate/glycerol buffer as described elsewhere. Aliquots of the extract were incubated for various time intervals at 42°, then stored in ice until use. Residual methionyl-tRNA synthetase activity was measured by the aminoacylation assay.

Kinetics of enzyme synthesis following methionine deprivation

For those strains which were capable of normal methionine biosynthesis the conditions were as follows.

Cells were grown to mid log phase in 250 ml of minimal medium supplemented with 50 ug/ml of all amino acids except methionine which was included at a concentration of 10 mM (1.49 mg/ml). At this

time the cells were collected by centrifugation, washed in saline, and resuspended in an equal volume of minimal medium supplemented with 50 ug/ml of all amino acids except methionine. A 40 ml zero-time sample was taken and the remaining cells were allowed to continue growth. At timed intervals, additional 40 ml samples were withdrawn from the culture, washed, and frozen as a cell pellet at -40°

A modification of this procedure was used for those strains which were unable to synthesize methionine due to a mutation in one of the methionine biosynthetic enzymes. Cells were grown to mid log phase in 250 mls of minimal medium supplemented with 20 ug/ml of all amino acids except methionine which was included at a concentration of 1 mM (0.15 mg/ml). The cells were collected by centrifugation, washed in saline, and resuspended in an equal volume of minimal medium supplemented with 20 ug/ml of all amino acids except methionine which was included at a concentration of 2.5 uM (0.375 ug/ml). Forty ml samples were withdrawn at timed intervals, washed, and frozen as cell pellets at -40°.

Scoring the rel phenotype

For those strains which have an amino acid requirement, the allelic condition of the *relA* gene was determined by the ^{3}H-uracil uptake assay described by Fiil and Friesen (1968). Those strains which had no amino acid requirement were starved for isoleucine by adding valine (400 ug/ml) to the medium 45 min prior to the addition of the ^{3}H-uracil. Under these conditions, valine causes repression of the enzymes required for isoleucine biosynthesis and thereby causes effective isoleucine starvation.

Measurement of in vivo levels of aminoacyl-tRNA

Cells were grown to mid log phase in minimal medium then harvested and extracted by the modified procedure of Folk and Berg (1971) as described by Lewis and Ames (1972). As a control, the strain CS50 (F⁻ *thr leu ara lac supE gal metG46 his rspL xyl mtl thi*), which requires methionine for growth due to the *metG46* mutation, was grown in the presence of methionine then starved for one hour prior to harvesting by transferring the culture to a medium lacking this amino acid.

The cells were killed by adding 25 ml of 55% trichloroacetic acid to a 250 ml culture. The culture was shaken for one minute, then 2.5 ml of 1% sodium dodecyl sulfate (SDS) was added. The culture was shaken for an additional 1 min, then chilled in an ice water bath. After 15 min, the precipitate was collected by centrifugation, then suspended in 4 ml of sodium acetate (0.25 M, pH 6.5) containing 0.05% SDS and 0.001 M EDTA. An equal volume of phenol (saturated with 0.25 M sodium acetate, pH 5.0 and containing 0.001 M EDTA) was added, and the mixture was sonicated for 60 sec. Following centrifugation of the suspension, the aqueous layer was removed and the phenol layer was washed with an equal volume of sodium acetate EDTA buffer. The two aqueous supernatant fluids were combined and precipitated by adding 4 vol. of ethanol (-20°) and 0.2 vol. of 5 M NaCl. After 20 min at -20°, the RNA was collected by centrifugation (26,000 x *g*, 20 min). The pellet was resuspended in 1.5 ml sodium acetate (0.1 M, pH 4.6) and divided into two equal portions. Sodium periodate (0.25 ml of a fresh 0.01 M solution in 0.1 M sodium acetate, pH 4.6) was added to one portion, and only buffer was added to the other. After 30 min

at room temperature in the dark, the RNA was precipitated with ethanol and NaCl and collected by centrifugation. The precipitated RNA was dissolved in 1 ml of 0.1 M sodium acetate (pH 4.6) containing 0.1 M ethylene glycol, incubated for 10 min in the dark at room temperature, then precipitated with ethanol and collected by centrifugation. The RNA was resuspended in 1 ml 1.8 M Tris-acetate (pH 8.2), and incubated for two hours at 37° to deacylate the tRNA. The RNA was collected by ethanol precipitation and centrifugation, and resuspended in 1 ml water.

The tRNA obtained by this procedure was charged to completion with a tRNA-free extract from AB1111. The substrate ^{3}H-L-methionine was included in the reaction mixture at a final concentration of 0.67 uM (10.5 Ci/mmole). The other 19 non-radioactive amino acids were included in the reaction mixture at a concentration of 0.1 mM in order to avoid mischarging.

Protein concentration was estimated by the Folin-Ciocalteau reagent or by the Biruet reaction as described by Layne (1957).

RESULTS

isolation and characterization of methionyl-tRNA synthetase (metG) mutants

Mutants were sought which, although competent to synthesize normal levels of methionine, have acquired a growth requirement for methionine due to an increase in the Km^{Met} of the methionyl-tRNA synthetase. This approach is identical to the method which Blumenthal (1972) has previously used for the isolation of *metG* mutants of *E. coli*.

From a single mutagenized culture of AB1111 (F⁻ *thr leu ara proA lac supE gal his rspL xyl mtl thi*), 1300 colonies which exhibited poor growth on methionine supplemented minimal agar, were transfered to master plates, then tested for a methionine requirement by replica plating. Forty-six of these failed to grow on minimal agar supplemented with homocysteine and vitamin B12. This combination of supplements will support the growth of strains harboring a mutation in any one of the methionine biosynthetic genes except *metF*. Since it is not feasible to supplement the medium with N^5-methyltetrahydropteroyltriglutamate (the product of the *metF*-catalyzed reaction), the 46 unclassified mutants were assayed directly for methionyl-tRNA synthetase activity by the aminoacylation reaction. Six of these strains were found to have markedly reduced synthetase activity and were therefore classified as *metG* mutants. The mutations in these strains were designated *metG3*, *17*, *24*, *41*, *43*, and *46* respectively.

As expected, these six mutants show an increase in the Km^{Met} of the methionine activation reaction as determined by the pyrophosphate exchange assay. The Km^{Met} values obtained for the six mutants are

presented in Table 2. From the results presented in this table it appears that there are four non-identical *metG* alleles represented among the six mutants. The alleles *metG17* and *metG24* are not distinguishable from one another and may represent identical mutations. Similarly, *metG3* and *metG43* appear to be identical.

The Km^{ATP} of the methionine activation reaction (2 mM L-methionine), was determined for the wild type and the *metG46* allele. Km^{ATP} values of 0.46 mM and 1.25 mM were obtained for the wild type and the *metG46* allele respectively. The slight difference in Km^{ATP} values may be attributed to the fact that the methionine concentration used for the determination is below the Km^{Met} value of the *metG46* allele. These results suggest relative independence of the ATP binding site from the methionine binding site of the enzyme and are consistent in this regard with biochemical studies of the wild type enzyme by Fayat and Waller (1974).

All six of the *metG* mutations appear to be located in the 40-44 min interval of the Bachmann *et al.* (1976) linkage map. This is inferred from the results of an experiment in which the F103 F' plasmid from KLF3/Jc1552 (F103 *his*$^+$ *metG*$^+$/ *leu lac supE gal trp his argG rspL xyl mtl metB mal*) was introduced into each of the mutants by a short mating. Nutritional selection was applied against both donor and recipient by plating the mating mixture on minimal agar supplemented with threonine, leucine, proline, and methionine. Fifty his$^+$ merodiploids, recovered from each mating, were tested for the methionine requirement. In each case, the presence of the F103 F' plasmid resulted in restoration of methionine independence. It is therefore concluded that each of the mutations is recessive to the wild type *metG* allele present on the F103

Table 2

Kinetic constants for the activation reaction catalyzed by extracts of *metG* mutants.

Strain	Allele	K_m^{Met}	*Vmax*	Relative K_m^{Met}
AB1111	*metG*$^+$	0.033	1.36	1.0
CS48	*metG3*	7.590	0.27	228.0
CS49	*metG17*	0.882	2.83	26.5
CS45	*metG24*	0.799	1.84	24.0
CS47	*metG41*	5.890	0.27	177.0
CS46	*metG43*	7.590	0.46	228.0
CS50	*metG46*	8.850	0.39	266.0

The K_m^{Met} is expressed as a mM concentration. *Vmax* is expressed in units of activity as defined under "Materials and Methods". The relative K_m^{Met} represents the ratio of the K_m^{Met} of the mutant strain and the wild type strain (AB1111).

Extracts for this series of assays were prepared and dialyzed in Nirenberg buffer.

plasmid. This result agrees with the previous report by Ahmed (1973) that the F103 plasmid carries the *metG* locus. More detailed mapping experiments involving one of the *metG* mutants are presented elsewhere in "Results".

Mapping the metG locus

In a previous series of mapping experiments, Ahmed (1973) established that the *metG* locus was located near the *his* operon in the clockwise gene sequence *metG,his,rspL*. Ahmed also noted that *metG* is not cotransducible with *his* by phage P1 mediated transduction. Since Hoffman and Wilhelm (1970) have reported that *supD* is 8% cotransducible with *his*, the sequence can be inferred to be *metG, supD, his*.

The above orientation of the *metG* locus with respect to *supD* was confirmed by introducing the F196 plasmid from MX383 (F196 *his*$^+$ *supD /lac trp his/nalA recA relA1 arg*) into CS57 (F$^-$ *thr leu ara* lac_{am} *metG46 his rspL xyl mtl thi*) by selecting for his$^+$ str^r merodiploids of CS57. The F196 plasmid has the same point of origin as the F103 plasmid but extends for a shorter interval counterclockwise from *supD*. The *supD* allele on the plasmid effectively suppresses the lac_{am} mutation in CS57 and thereby provides a convenient test for the presence of the F196 plasmid. Of fifty his$^+$ str^r merodiploids obtained in this manner, all were met$^-$ lac$^+$(supD$^-$). This result implies that the F196 plasmid does not carry the *metG* locus, and substantiates the proposed sequence.

Since it was desirable to find an easily selected marker with which *metG* could be cotransduced, the linkage of *metG* to the *eda* locus was examined. This locus is carried by the F103 plasmid but not by the

F196 plasmid and is the closest counterclockwise marker from *his* which confers a nutritional requirement. A P1 lysate of AB1111 (F⁻ *thr leu ara proA lac supE gal his rspL xyl mtl thi*) was used to transduce CS122 (F⁻ *thr leu ara proA lac supE gal eda metG rspL xyl mtl thi*). Of 250 eda⁺ transductants, none were met⁺. These results are considered as sufficent evidence that *metG* is not cotransducible with the *eda* locus.

A relatively precise location for the *metG* locus was obtained by using F' plasmids for the *his* region, which are deleted for various intervals of the chromosome. The secondary F' strains MS1338 (F1829 *his⁺ metG⁺ fla1829/leu lac his recA argG rspL xyl mtl met malA*) and MS1977 (F1977 *his⁺ fla1829 Δ(hag-flaE)/ leu lac his recA argG rspL xyl mtl met malA*) were used as donors in a mating with CS57. Fifty his⁺ merodiploids were retained from each cross and tested for a methionine requirement. It was observed that the presence of the F1829 plasmid restores methionine independence whereas the deleted F1977 plasmid does not. The only reported difference between these episomes is a deletion which extends from *hag* to *flaE* (Silverman and Simon, 1974). It therefore appears that the *metG* gene is located in the *hag-flaE* interval.

On the basis of the previous results which suggest a location for *metG* within the cluster of genes coding for flagella synthesis, it seemed probable that *metG* would be cotransducible with these loci. This was verified by a transduction experiment in which a P1*vir* lysate of MS827 (F⁻ *galU uvrC flaR his thyA rspL argE* su^{+}_{am}) was used to transduce CS60 (F⁻ *thr leu ara* lac_{am} *metG46 supD rspL xyl mtl thi*). Three hundred and sixty-one met⁺ transductants, recovered from this cross, were scored

for the allelic condition of the *uvrC*, *flaR*, and *supD* loci. The results of this transduction are presented in Table 3. Under these circumstances, the cotransduction frequency between *metG* and *flaR*, *uvrC*, and *supD*, were 50.1%, 42.4%, and 32.1% respectively.

The position of *metG* with respect to these three loci is inferred to be *uvrC*, *metG*, *flaR*, *supD*. The inference is based on the following considerations. If the correct order is *metG*, *flaR*, *supD* then the frequency of met^+ fla^- $supD^+$ transductants will greatly exceed the frequency of met^+ fla^+ $supD^+$ transductants. In contrast, if the correct sequence is *flaR*, *metG*, *supD*, one expects a substantial frequency of met^+ fla^+ $supD^+$ transductants. From the results in Table 3, the frequency of met^+ fla^- $supD^+$ transductants is 32% whereas the frequency of met^+ fla^+ $supD^+$ transductants is 0.3%. This result is, therefore, in close agreement with the results predicted for the sequence *metG*, *flaR*, *supD*. This sequence is also consistent with the result obtained from deletion analysis.

By a similar argument, the order with respect to *uvrC* can be deduced from these results. If the correct gene order was *metG*, *uvrC*, *flaR* then the frequency of met^+ uvr^- fla^- transductants will greatly exceed the frequency of met^+ uvr^+ fla^- transductants. In contrast, if the gene order were *uvrC*, *metG*, *flaB*, a substantial frequency of both classes of transductant would be obtained. From the results in Table 3, the frequency of met^+ uvr^+ fla^- is 35% and the frequency of met^+ uvr^- fla^- is 14.6%. This result clearly contradicts the expectation for the *metG*, *uvrC*, *flaR* gene order, but is consistent with the sequence *uvrC*, *metG*, *flaR*. This sequence is also supported by results obtained from deletion analysis.

Table 3

Ordering *metG* with respect to adjacent loci by a four-point transduction cross.

Selected phenotype	Recombinant phenotype	number of recombinants
met^+	fla^- sup^o uvr^-	37
"	fla^- sup^o uvr^+	78
"	fla^- sup^+ uvr^-	16
"	fla^- sup^+ uvr^+	50
"	fla^+ sup^o uvr^-	0
"	fla^+ sup^o uvr^+	1
"	fla^+ sup^+ uvr^-	100
"	fla^+ sup^+ uvr^+	79

A P1*vir* lysate prepared on a *uvrC flaR* donor strain was used to transduce a *metG supD* recipient. $MetG^+$ transductants were selected and scored for the *flaR*, *supD*, and *uvrC* markers.

In theory, sup$^+$, fla$^+$, and uvr$^+$ are all phenotypes which can be selected. However, in practice, each of these phenotypes present technical difficulties when used as selective markers in transduction experiments. Several attempts to demonstrate cotransduction of *metG* with these loci by selecting for sup$^+$ or fla$^+$ transductants are described below.

A P1*vir* lysate of CS60 (F$^-$ *thr leu ara* lac_{am} *metG46 supD rspL xyl mtl thi*) was used to transduce CSH4 (F$^-$ lac_{am} trp_{am} *rspL*) to lac$^+$ trp$^+$ on methionine supplemented minimal lactose agar. This approach is based on the assumption that supD$^-$ transductants (lac$^+$ trp$^+$ because of *amber* suppression) should be far more frequent than double transductional events in which functional *trp* and *lac* genes are introduced into CSH4. Of 300 lac$^+$ trp$^+$ transductants, recovered from this cross, none were met$^-$. Since it was not verified that the lac$^+$ trp$^+$ transductants were actually supD$^-$, the only thing which can be concluded from this result is that the approach is not a useful one for mapping *metG*.

Several attempts to demonstrate cotransduction of *metG* with flaB$^+$ as the selected phenotype also failed. A P1*vir* lysate of CS60 was used to transduce CS164 (F$^-$ *uvrC flaB his thyA rspL argE* su^+_{am}) on tryptone motility agar. Of 100 fla$^+$ transductants, obtained from this cross, none were met$^-$. This inconsistent result may be due to the fact that strains harboring a *metG* mutation swarm more slowly than do metG$^+$ strains. The result is that metG$^+$ transductants quickly overtake the entire plate and might therefore prevent the recovery of metG$^-$ transductants. In order to overcome this technical difficulty, a *metG, flaB* strain has been constructed so that the approach can be reinvestigated.

In summary, the *metG* locus appears to be located near 43 min on the linkage map of *E. coli* K12 (Bachmann *et al.*, 1976). Cotransduction of *metG* with several loci in this region has been demonstrated, but the precise gene sequence has not been confirmed by the appropriate crosses in which *metG* is not the selected marker. The proposed location of *metG* with respect to several other loci is illustrated in Figure 3. This location has been confirmed by a more detailed linkage analysis using deletion episomes (M. Simon, personal communication), but differs from the position proposed by Bachmann *et al.* (1976). The position suggested by Bachmann *et al.* is not consistent with the reported position (Blumenthal, 1972; Ahmed, 1973) and appears to be derived by analogy with the position of the *metG* locus in *Salmonella*.

Isolation of methionine-independent revertants of CS57

From 100 independent cultures of CS57 (F^- *thr leu ara* lac_{am} *metG46 his rspL xyl mtl thi*), approximately 2000 revertants were selected by their ability to grow on minimal agar supplemented with homocysteine at 37°. The parent strain CS57 does not grow under these circumstances since the enzymes responsible for converting homocysteine to methionine are not sufficently derepressed to bring about production of methionine at the levels required to overcome the Km^{Met} defect of the mutant synthetase. This selection procedure was specifically designed to allow the recovery of mutants which are normally regulated for the early steps in methionine biosynthesis, but have become derepressed for the synthesis of one or more of the terminal enzymes of the biosynthetic pathway.

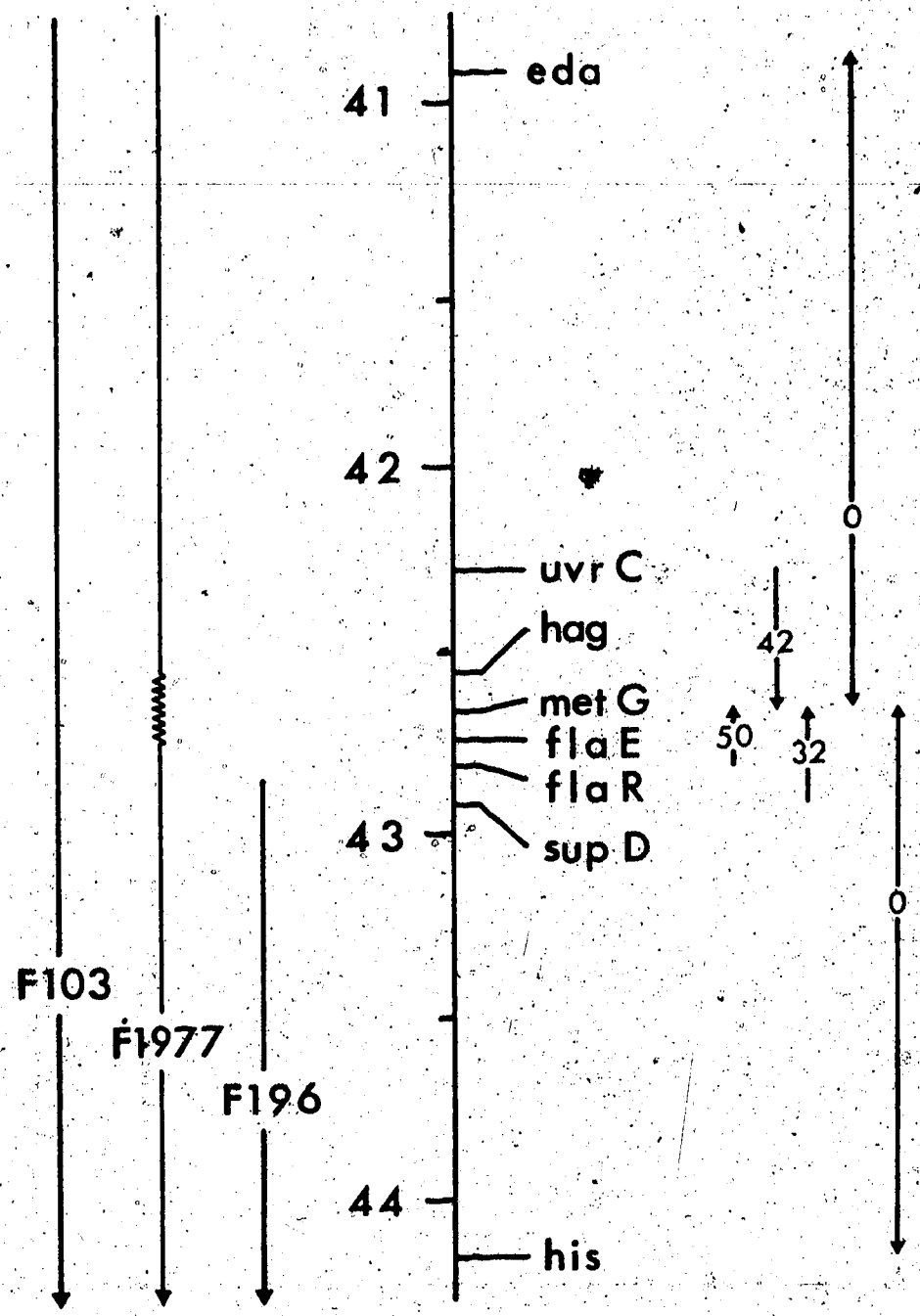

Figure 3 - Proposed map position for the *metG* gene.
The numbered arrows to the right of the map represent cotransduction frequencies. The direction of the arrow indicates the selected marker. The arrows to the left of the map represent the F episomes used for this study. The direction of transfer and point of origin of the episomes are indicated by the arrow. The positions of all the genes except *metG* are from Bachmann *et al.* (1976).

When tested on minimal agar at 37° or 30°, all but one of the revertants were able to grow a either temperature without exogenous homocysteine. The single exceptional mutant, designated CS68, is discussed in a later section of "Results".

All of the remaining revertants were then subjected to a screening procedure designed to facilitiate the identification of strains in which an *amber* mutation was responsible for the met^+ phenotype. Forty independent revertants, which initially appeared as met^- when lysogenized with $\phi 80 psu3^+$, were retained for further characterization. However, after purification and retesting under more rigorous circumstances, it was concluded that the presence of the prophage had no pronounced effect on the met^+ phenotype of these strains. These forty revertants, designated SU3-47, were subsequently used for a detailed analysis of the mechanisms by which CS57 reverta to a met^+ phenotype.

Preliminary classification of revertant strains

It was expected that some of the reversions of the SU3-47 series would be caused by mutations resulting in partial or complete restoration of methionyl-tRNA synthetase activity. To examine this possibility, all of the revertants were initially screened for levels of synthetase activity by the aminoacylation assay. For this assay, extracts were prepared in Nirenberg buffer and desalted by passage through Sephadex G-25. Under these conditions, 37 of the 40 revertants gave approximately the same low level of synthetase activity as the *metG* parent strain CS57. Therefore it appeared that some other mechanism was responsible for the restoration of methionine independence in the

Table 4 - Enzyme activities in *class-1* revertant strains

Strain	Classification	Relative specific activity[a]	
		N^5,N^{10}-methylenetetrahydrofolate reductase (*metF*)	methionyl-tRNA synthetase (*metG*)
AB1111	*wild type*	-	1.000
CS57	*metG mutant*	1.0	0.027
SU2	*revertant*	16.06	0.024
SU3	"	16.88	0.022
SU4	"	20.40	0.035
SU6	"	21.71	0.016
SU8	"	23.55	0.022
SU10	"	8.75	0.015
SU12	"	12.22	0.030
SU18	"	8.31	0.027
SU19	"	33.45	0.022
SU20	"	15.92	0.036
SU21	"	33.47	0.038
SU22	"	20.38	0.041
SU25	"	19.02	0.018
SU26	"	15.04	0.036
SU27	"	9.09	0.042
SU34	"	14.11	0.047
SU35	"	19.64	0.024
SU36	"	19.66	0.031
SU39	"	19.64	0.039
SU40	"	13.07	0.046
SU42	"	15.19	0.032
SU46	"	23.00	0.045

[a] The enzyme activities of the mutants are expressed relative to the specific activity of the wild type strain (AB1111) or the *metG* parent strain (CS57). In each case, the activity of the relevant parent strain is taken as 1.0. For the strain CS57, the specific activity of N^5,N^{10}-methylenetetrahydrofolate reductase was 7.50. For the strain AB1111, the specific activity of methionyl-tRNA synthetase as determined by the aminoacylation reaction was 1.77. The units of specific activity are defined under "Materials and Methods".

Table 5 - Enzyme activities in *class-2* revertant strains.

Strain	Class	Relative specific activity[a]		
		N^5,N^{10}-methylene-tetrahydrofolate reductase *(metF)*	β-cystathionase *(metC)*	ATP:methionine *S*-adenosyl-transferase *(metK)*
AB1111	*wild type*	1.00	1.00	1.00
AM29	*metJ mutant*	10.77	14.00	3.12
SU5	*revertant*	2.40	0.70	0.87
SU7	"	1.03	0.74	0.95
SU9	"	2.55	1.19	1.02
SU11	"	1.63	1.04	1.20
SU13	"	2.30	0.88	0.89
SU23	"	1.78	1.36	0.98
SU24	"	1.02	1.45	1.13
SU28	"	0.93	1.18	1.23
SU31	"	1.25	0.96	0.85
SU32	"	1.22	1.30	0.95
SU33	"	1.62	0.52	0.92
SU37	"	2.07	1.20	1.06
SU38	"	0.92	1.12	1.03
SU41	"	1.10	1.29	1.07
SU43	"	2.72	0.35	1.21
SU44	"	1.74	1.26	1.09
SU45	"	1.90	1.11	1.41
SU47	"	0.69	1.31	1.11

[a] The enzyme activities of the revertant strains are expressed as relative to the specific activity of the wild type strain (AB1111) which is taken as 1.0 in each case. The actual specific activities were: (i) N^5,N^{10}-methylenetetrahydrofolate reductase, 36.0; (ii) β-cystathionase, 0.11; and (iii) ATP:methionine *S*-adenosyltransferase, 9.08. The units of specific activity are defined under "Materials and Methods". The strain AM29 was included as a control to ensure that the assay would reveal high levels of activity.

majority of the revertant strains. However, as noted in a later section of "Results", it was eventually discovered that this mechanism could account for eighteen of the forty revertants.

Since Chater *et al.* (1970) have previously shown that *metJ* and *metK* mutations are frequently able to suppress the methionine requirement of *metC* mutations in *Salmonella*, it was expected that a substantial number of the revertants isolated for this study would have a mutation in one of the regulatory genes affecting methionine biosynthesis. To examine this possibility, all of the revertants of the SU3-47 series were screened for levels of N^5,N^{10}-methylenetetrahydrofolate reductase (*metF*) activity. This enzyme was chosen as an indicator of the state of regulation of the methionine biosynthetic enzymes since Ahmed (1973) has reported that the synthesis of this enzyme is particularly responsive to conditions leading to derepression of the enzymes involved in methionine biosynthesis. The results of this assay are presented in Tables 4 and 5. Twenty-two of the revertants showed non-repressible levels of *metF* activity when grown in the presence of methionine (10 ug/ml), and had the same low level of *metC* activity as the parental strain CS57. The remaining eighteen revertants have more or less normal levels of N^5,N^{10}-methylenetetrahydrofolate reductase (*metF*), β-cystathionase (*metC*), and ATP:methionine *S*-adenosyltransferase (*metK*) activity when grown under non-repressing conditions.

On the basis of these results the revertants were tentatively divided into two categories. *Class-1* revertants (Table 4) are those which showed non-repressible levels of the *metF*-enzyme, and *class-2* revertants are those which appeared to be normally regulated for the methionine biosynthetic enzymes.

Characterization of metJ revertant strains

Mutations in either the *metJ* or *metK* genes are known to lead to generalized derepression of the methionine biosynthetic enzymes. Since these are the only loci known to be involved in the regulation of all the methionine biosynthetic enzymes, it seemed probable that the *class-1* revertants would be characterized by a mutation in one of these genes.

All *metJ* mutations which have been isolated to date confer ethionine resistance. The presumed mechanism of resistance involves metJ$^-$ mediated derepression of the methionine biosynthetic enzymes and a concomitant increase in the endogenous methionine pool to the extent that ethionine is outcompeted by methionine as a substrate for protein synthesis. Since the *metJ* locus is approximately 90% cotransducible with the *metB* locus, *metJ* mutations are easily identified by demonstrating close linkage to the *metB* locus of the gene responsible for the ethionine resistance phenotype.

In order to test for the presence of a *metJ* mutation in the *class-1* revertants, a transducing lysate of P1*vir* was prepared on each of the revertants, and subsequently used to transduce the ethionine sensitive strain Hfr R1 (*relA1 metB thi*). Twenty-five met$^+$ transductants from each cross were tested for ethionine resistance at 37°. It was found that all of the *class-1* revertants give rise to ethionine resistant transductants of Hfr R1 with an average frequency of 85%. This is approximately the cotransduction frequency expected for mutations at the *metJ* locus (Holowachuk, 1976). In contrast, under identical conditions, none of the *class-2* revertants give rise to ethionine resistant transductants of Hfr R1.

On the basis of these transduction experiments, and the non-repressible phenotype (indicated by high levels of *metF* activity), all of the *class-1* revertants have been designated as *metJ* mutants. The met^+ phenotype of these revertants is therefore attributed to an increase in the endogenous methionine levels to the point that exogenous methionine is no longer required for adequate charging of $tRNA^{Met}$ by the defective synthetase. The absence of any *metK* mutants among the revertants is attributed to the fact that methionine excreting strains were not retained for further study.

In vivo levels of aminoacylation of $tRNA^{Met}$ in class-2 revertants

The initial difficulty encountered in demonstrating the ability of extracts of the *class-2* revertants to aminoacylate $tRNA^{Met}$ *in vitro*, raised the possibility that $tRNA^{Met}$ was also not being charged efficently *in vivo*. In order to clarify this situation, the *in vivo* level of aminoacylation of $tRNA^{Met}$ was estimated for several of the *class-2* revertants by the procedure described by Lewis and Ames (1972). The results of this experiment, presented in Table 6, indicate that the *class-2* revertants have essentially wild type levels of charged $tRNA^{Met}$. The simplest explanation of these observations is that these strains are competent to effect high levels of aminoacylation of $tRNA^{Met}$ *in vivo*. The resolution of this discrepancy between the *in vivo* and *in vitro* situations is noted elsewhere in "Results".

Methionyl-tRNA synthetase activity in class-2 revertants

A major difficulty encountered in the preliminary analysis of

Table 6

The percentage of $tRNA^{Met}$ which is aminoacylated *in vivo* in several *class-2* revertants.

Source of tRNA	Class	Percentage of $tRNA^{Met}$ charged *in vivo*	Relative $tRNA^{Met}$ content per A_{260} unit
AB1111	*wild type*	87	1.00
CS50	*metG mutant*	26	1.00
SU31	*revertant*	76	0.76
SU32	*revertant*	87	1.19

The amount of methionine accepted per A_{260} unit of tRNA is expressed relative to the value obtained for the wild type strain (AB1111), which is taken as 1.0. The actual value obtained for AB1111 was 0.34 pmoles of L-methionine accepted per A_{260} unit of tRNA.

the *class-2* revertants, was the inability to recreate *in vitro*, the conditions responsible for the met^+ phenotype. It was subsequently observed that if the aminoacylation assay was performed on undialyzed extracts, these revertants show levels of methionyl-tRNA synthetase activity which are comparable to wild type. However, when the extracts were dialyzed against Nirenberg buffer or desalted on Sephadex G-25 equilibrated with this buffer, the activity was drastically reduced. The discrepancy observed between the *in vivo* and *in vitro* situations was therefore ascribed to inappropriate conditions attending the preparation of extracts. A series of experiments was undertaken to determine conditions more amenable to the recovery of synthetase activity in extracts of *class-2* revertants. ATP, tRNA, $MgCl_2$, and L-methionine, either alone or in combination, had no pronounced stabilizing effect on synthetase activity during dialysis or passage through Sephadex G-25. However, the replacement of Nirenberg buffer with a buffer containing 20 mM potassium phosphate (pH 7.3), 10% glycerol (v/v), and 10 mM 2-mercaptoethanol, resulted in apparently complete stabilization of synthetase activity in *class-2* revertant extracts during dialysis or passage through Sephadex G-25.

The results of a series of experiments in which various procedures were employed in the preparation of extracts of the *class-2* revertants are presented in Table 7. It can be seen that when extracts are prepared and dialyzed in the phosphate/gylcerol buffer (Method A) all of the revertants show a signifigantly higher level of synthetase activity than the *metG* parental strain CS57. This suggests that the mechanism by which these revertants regain methionine independence is by a mutation resulting in an increase in synthetase activity. Also; On the

Table 7 - The effect of various preparative procedures on methionyl-tRNA synthetase activity in extracts of *class-2* revertants.

Strain	Class	Relative specific activity of methionyl-tRNA synthetase[a]			
		Method A	Method B	Method C	Method D
AB1111	*wild type*	1.00	0.81	1.08	1.96
CS57	*metG mutant*	0.13	0.02	0.02	0.02
SU5	*revertant*	0.86	0.69	0.23	0.01
SU7	"	0.99	0.65	0.81	1.20
SU9	"	1.02	0.56	0.46	0.03
SU11	"	0.70	0.56	0.32	0.68
SU13	"	0.97	0.40	0.36	0.01
SU23	"	0.66	0.40	0.48	0.03
SU24	"	0.83	0.78	0.85	0.10
SU28	"	0.71	0.55	0.52	0.02
SU31	"	0.86	0.78	0.36	0.03
SU32	"	0.99	0.67	0.66	0.02
SU33	"	0.89	0.42	0.32	0.01
SU37	"	0.51	0.44	0.22	0.01
SU41	"	1.17	0.39	0.34	0.01
SU43	"	0.60	0.60	0.72	0.14
SU44	"	0.78	0.70	0.30	0.01
SU45	"	0.69	0.42	0.20	0.03
SU47	"	0.82	0.61	0.81	1.14

[a] The enzyme activities are expressed relative to the specific activity of the wild type strain (AB1111), which is taken as 1.0 for extracts prepared by "Method A". The actual specific activity is 2.33. The units of activity are defined under "Materials and Methods".

The extracts were prepared by a variety of procedures as follows: (A) dialysis for 8 hr in 20 mM potassium phosphate (pH 7.3), 10% glycerol, and 10 mM 2-mercaptoethanol; (B) fresh undialyzed extract in Nirenberg buffer; (C) undialyzed extract in Nirenberg buffer incubated for 8 hr at 4° prior to assay; and (D) extract dialyzed in Nirenberg buffer for 8 hr at 4° prior to assay.

basis of the data in Table 7, a preliminary sub-classification of the revertants can be made. Those revertants which show an almost complete loss of activity during dialysis in Nirenberg buffer, represent the major subclass. The strains SU7 and SU47, which show no loss of activity under these circumstances, represent another class. The strains SU11, SU24, and SU43, which show an intermediate loss of activity, represent a third subclass. As noted in the next section of "Results", a similar but more complete subclassification can be made on the basis of other characteristic of the synthetase activity in these strains.

Km^{Met} determinations for the class-2 revertants

In the absence of other information, two simple hypotheses can be proposed to account for the restoration of synthetase activity observed in extracts of *class-2* revertants. One possibility is that an increase in the amount of defective synthetase results in the observed increase in specific activity. The other possibility is that an alteration in the structure of the synthetase has resulted in partial or complete restoration of synthetase function.

In order to distinguish between these two possibilities, the Km^{Met} of the methionyl-tRNA synthetase of the *class-2* revertants was determined by the pyrophosphate exchange assay. For this assay, extracts were prepared and dialyzed in a buffer containing 20 mM potassium phosphate (pH 7.3), 10% glycerol (v/v), and 10 mM 2-mercaptoethanol. The Lineweaver-Burke plots for this series of experiments are presented in Appendix II. The Km^{Met} and *Vmax* values obtained from these plots are included in Table 8. From these results it can be seen that all of the *class-2* revertants have undergone a mutation which results in a

Table 8 - A summary of the characteristics of methionyl-tRNA synthetase activity in *class-2* revertants.

Strain	Relevant genotype	K_m^{Met} (mM)	*Vmax*	Relative K_m^{Met}	Relative stability during dialysis	Relative thermal stability	Subclass
AB1111	*metG*$^+$	0.033	0.444	1	1.00	1.00	-
CS57	*metG46*	5.260	0.664	158	0.01	-	-
CS130	*metG46-31 relA1*	0.666	0.432	20	-	-	-
CS148	*metG46-32 relA1*	0.666	0.536	20	-	-	-
SU2	*metG46 metJ*	5.888	0.530	158	-	-	-
SU5	*metG46-5*	0.434	0.573	13	0.01	0.06	*2.1*
SU7	*metG46-7*	0.666	1.100	20	0.61	1.05	*2.2*
SU9	*metG46-9*	0.666	0.485	20	0.01	0.06	*2.3*
SU11	*metG46-11*	0.033	1.102	1	0.34	1.02	*2.4*
SU13	*metG46-13*	0.666	0.357	20	0.01	0.06	*2.3*
SU23	*metG46-23*	0.666	0.292	20	0.01	0.04	*2.3*
SU24	*metG46-24*	0.147	0.614	4	0.05	0.82	*2.5*
SU28	*metG46-28*	0.417	0.588	13	0.01	0.08	*2.1*
SU31	*metG46-31*	0.666	0.292	20	0.01	0.08	*2.3*
SU32	*metG46-32*	0.666	0.349	20	0.01	0.03	*2.3*
SU33	*metG46-33*	0.666	0.433	20	0.01	0.04	*2.3*
SU37	*metG46-37*	0.666	0.489	20	0.01	0.20	*2.3*
SU41	*metG46-41*	0.666	0.550	20	0.01	0.04	*2.3*
SU43	*metG46-43*	0.154	0.852	4	0.07	0.71	*2.5*
SU44	*metG46-44*	0.769	0.539	23	0.01	0.04	*2.3*
SU45	*metG46-45*	0.833	0.634	25	0.01	0.12	*2.3*
SU47	*metG46-47*	0.666	0.912	20	0.58	0.99	*2.2*

Vmax is expressed in units of activity as defined under "Materials and Methods". All "relative" values are expressed in relation to the value obtained for the wild type strain (AB1111) which is taken as 1.0 in each case. The actual values for stability during dialysis (in Nirenberg buffer) are presented in Table 7. The actual values for thermal stability are presented in Table 9.

substantial decrease in the K_m^{Met} of the activation reaction. One of the revertant strains (SU11) is indistinguishable from the wild type strain by this criterion. Also, at least two of the other revertants (SU24 and SU43), which are not distinguishable from one another, are readily distinguished from the remaining revertants by a K_m^{Met} which is only four-fold higher than that of the wild type strain (AB1111). It should be noted that these three strains are among those in which the synthetase activity is relatively stable during dialysis in Nirenberg buffer. The two strains (SU7 and SU47) which show no loss of activity during dialysis in Nirenberg buffer (Table 7), cannot be distinguished from the major class of revertants on the basis of K_m^{Met}. It also appears that SU5 and SU28 represent another distinct class of revertants.

From the distinctions afforded by the K_m^{Met} determinations, the instability during dialysis, and the thermal lability of the synthetase, five sub-classes can be distinguished among the *class-2* revertants. These sub-classes are designated *class-2.1, 2.2, 2.3, 2.4, 2.5* respectively. A summary of the criteria by which these classifications were made is presented in Table 8.

The interpretation of these results was that in each of the *class-2* revertants, a mutation had occured which resulted in a structural modification of the synthetase. By this reasoning, and as a matter of notational convenience, the *metG* allele designations of the *class-2* revertants have been adjusted by the addition of a numerical suffix corresponding to the number of the revertant strain (see Table 8).

From the data presented in Table 8, it can also be concluded that the presence of a *metJ* or *relA* mutation has no appreciable effect on the kind or amount of methionyl-tRNA synthetase produced.

Thermal inactivation studies

In an attempt to further distinguish among the *class-2* revertants, and in order to substantiate the classifications already made, the rate and extent of thermal inactivation of synthetase activity was determined. Undialyzed extracts prepared in Nirenberg buffer were subjected to a five min[illegible]ion at 42°, then assayed by the aminoacylation assay. The re[illegible] experiment are presented in Table 9. The synthe[illegible] in extracts of those revertants previously classified as *class-2.1* or *class-2.3* is seen to be very rapidly inactivated at this temperature, whereas the synthetase activity in extracts of the remaining revertants is relatively resistant to thermal inactivation under these circumstances. It should also be noted that although *class-2.2* revertants cannot be distinguished from *class-2.3* revertants on the basis of K_m^{Met} determinations, they are readily distinguished by a differential stability during heat treatment or dialysis in Nirenberg buffer. The results of this experiment therefore corroborate the classifications already made by other criteria.

In order to examine the thermal inactivation of synthetase activity more rigorously, the kinetics of thermal inactivation were determined for representatives of several of the subclasses. The extracts were prepared in the phosphate/glycerol buffer and incubated at 42° for various times. The results of these experiments are presented in Figures 4 and 5. The rate of inactivation of SU11 is seen to be indistinguishable from the wild type strain AB1111. Therefore, by all available criteria this strain appears to be a true revertant in which the reversion mutation has restored the synthetase to its wild type

Table 9 - Thermal inactivation of methionyl-tRNA synthetase activity at 42° in *class-2* revertants.

Strain	% residual activity*	Classification
AB1111	98	*wild type*
SU5	6	*class-2.1*
SU7	103	" 2.2
SU9	6	" 2.3
SU11	100	" 2.4
SU13	6	" 2.3
SU23	4	" 2.3
SU24	80	" 2.5
SU28	8	" 2.1
SU31	8	" 2.3
SU32	3	" "
SU33	4	" "
SU37	20	" "
SU38	9	" "
SU41	4	" "
SU43	70	" 2.5
SU44	4	" 2.3
SU45	12	" 2.3
SU47	97	" 2.2

* Extracts were incubated at 42° for 5 min prior to being assayed by the aminoacylation assay.

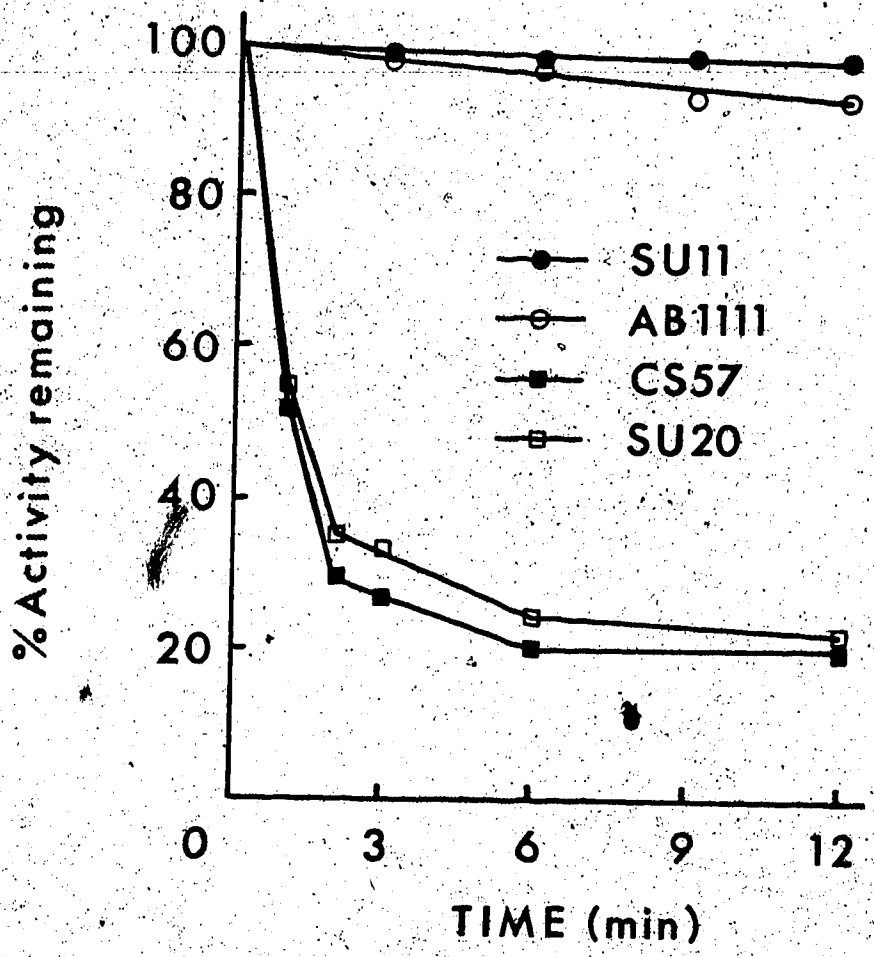

Figure 4 - Thermal inactivation of methionyl-tRNA synthetase activity in wild type and mutant strains at 42°.

The relevant genotypes of the strains are as follows: AB1111 *(metG$^+$)*, CS57 *(metG46)*, SU20 *(metG46 metJ)*, SU11 *(metG46-11)*.

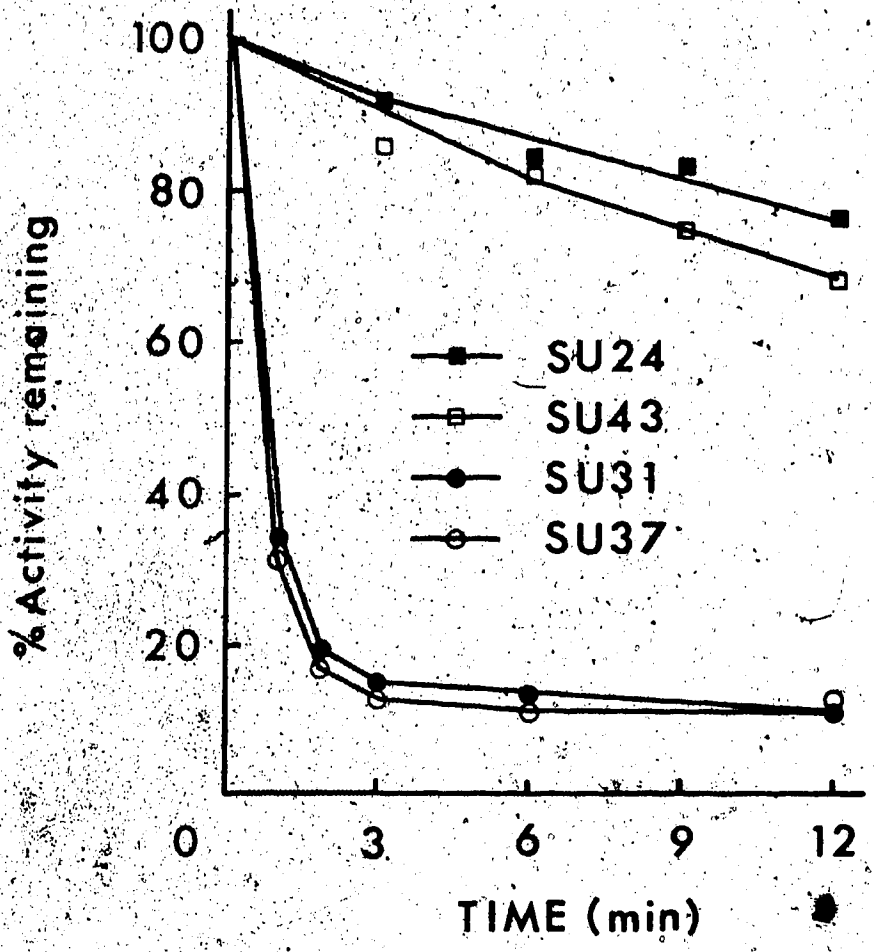

Figure 5 - Thermal inactivation of methionyl-tRNA synthetase activity in *class-2* revertant strains at 42°.

The strains SU24 and SU43 are *class-2.5* revertants. The strains SU31 and SU37 are *class-2.3* revertants.

form. The two strains SU24 and SU43 previously classified as *class-2.5*, are not distinguishable from one another by this criteria, and may therefore represent identical mutations. The *class-2.3* strains SU31 and SU37 represent a particularly interesting case. The initial rate of inactivation of synthetase activity is remarkably fast in that approximately 85% of the synthetase activity is lost during the first two minutes of exposure to the elevated temperature. This is followed by a very gradual decline in activity until an apparent plateau is reached at approximately 12% residual activity. This thermal lability is also expressed *in vivo* since *class-2.3* revertants require exogenous methionine for growth at temperatures above 37°.

Mapping the reversion mutation in class-2.3 revertants

Since all of the revertants were obtained as spontaneous mutants, it is assumed that a single mutation is responsible for the met^+ phenotype. In the *class-2* revertants, the mutation has either occured within the structural gene for the synthetase or at some other site resulting in the creation of an external suppressor. The two hypotheses can generally be distinguished by a variety of mapping experiments in which, for example, the original mutation is recovered by transferring it to a strain lacking the suppressor mutation, or the reversion mutation is introduced into the parent strain by cotransduction with a suitable marker. In the present case, the absence of a suitable selective marker which is cotransducible with the *metC* locus has made this a technically difficult problem.

The approach used for mapping the reversion-mutation is based on the assumption that introduction of the wild type site by recombination

should result in restoration of the met$^-$ phenotype. This approach requires that the site defining the *metG46* mutation must not be removed by recombination and, therefore, precludes the use of metG$^+$ donor strains for mapping experiments involving the *metG* region of the chromosome. In order to satisfy this technical requirement, an Hfr strain (Hfr 312) which carries the *metG46* mutation, and which has a point of origin near the *metG* locus, was constructed.

A conjugal cross was performed between Hfr 312 (*thr leu ara proA lac supE gal metG46 rspL xyl mtl thi*) and SU31-I (F$^-$ *lac*$_{am}$ *metG46-31 his rspL glpK argH thi*). Nutritional selection was applied against both donor and recipient by selecting for growth of recombinants on minimal medium supplemented with arginine and methionine. Of 40 his$^+$ recombinants recovered from this cross, 29 were met$^-$. This result therefore suggests that the mutation responsible for the met$^+$ phenotype in SU31 is closely linked to the *his* operon. Since this is the region of the chromosome where *metG* is located, this result does not distinguish between a reversion mutation within the *metG* locus and a closely linked mutation outside the locus. Since Hfr 312 acts as a donor with a relatively low efficiency, an interrupted mating experiment was not feasable. A more detailed mapping of the reversion-mutation must await the construction of strains with selective markers which are cotransducible with the *metG* locus.

A rel-dependent methionine requirement in class-2.3 revertants

During the course of mapping experiments designed to elucidate the location of the mutation responsible for the met$^+$ phenotype of

the *class-2.3* revertants, it was observed that met$^-$ recombinants were obtained at a high frequency from a conjugal cross between the met$^+$ strains Hfr KL16 (*relA1 thi*) and SU31-A (F$^-$ *thr leu ara lac*$_{am}$ *metG46-31 his thyA rspL thi*) Of 104 thy$^+$ str^r recombinants, obtained from this cross, 34% were met$^-$. The results of this experiment, included in Table 10, suggested that a locus which is in some way responsible for the met$^+$ phenotype of SU31-A, might be cotransduced with *thyA*. This was confirmed by transducing SU31-A with P1 prepared on X407 (Hfr H *proB relA1 thi*). Of 312 thyA$^+$ transductants recovered from this cross, 1% were met$^-$ (table 10). A similar transduction experiment revealed that the locus responsible for the met$^+$ phenotype is cotransducible with *argA*. A P1*vir* lysate of X407 was used to transduce SU31-C (F$^-$ *thr leu ara lac*$_{am}$ *metG46-31 his argA rspL thi*). Of the 203 arg$^+$ transductants selected from this cross, 18% were met$^-$ (Table 10). Since the *argA* gene is approximately 20% cotransducible with *thyA*, these results indicate the clockwise gene sequence to be "met",*argA*, *thyA*.

In contrast to the above results, when rel$^+$ strains were used as donors under identical conditions, no met$^-$ recombinants were recovered. For example, all of the 40 arg$^+$ str^r recombinants recovered from a conjugal cross between Hfr 305 (*thi*) and SU31-C, remained met$^+$. Similarly; When a P1*vir* lysate of the ancestral strain AB1111 (F$^-$ *thr leu ara proA lac supE gal his rspL xyl mtl thi*) was used to transduce SU31-C, none of the 152 arg$^+$ transductants were met$^-$ (Table 10). Similar crosses in which two other unrelated rel$^+$ strains were used as donors produced the same results.

The interpretation of these results is that the introduction of the *relA1* mutation into SU31 results in the met$^-$ phenotype. The

inferred orientation of the locus responsible for the met$^-$ phenotype is consistent with the *relA,argA,thyA* sequence reported by Fiil and Friesen (1968). Also, the 24% cotransduction frequency reported by Fiil and Friesen for the *relA* and *argA* loci is similar to the 18% cotransduction frequency observed for the *argA* locus and the locus responsible for the met$^-$ phenotype.

A specific prediction of the hypothesis is that from a cross between SU31 and a *relA* donor strain, only rel$^+$ met$^+$ and rel$^-$ met$^-$ recombinants should be obtained. This was confirmed by a transduction experiment in which a P1 lysate of Hfr R1 (*relA1 metB thi*) was used to transduce SU31-C. One hundred and four arg$^+$ transductants were scored for the rel phenotype and for the met phenotype. The results obtained from this cross were in perfect agreement with the prediction. Seventy-four of the transductants were rel$^+$ met$^+$ and thirty were rel$^-$ met$^-$.

The recessive nature of the mutation responsible for the met requirement was determined by introducing several rel$^+$ F$'$ plasmids into the rel$^-$ met$^-$ strain CS130 (F$^-$ *thr leu ara lac metG46-31 his relA1 argA rspL thi*) and the isogenic rel$^+$ strain SU31-C. The F$'$ strains KLF8 /MA50 (F108 *argA*$^+$ *relA*$^+$/ *thr leu lac cys lysA mtl mal thi*) and NF306 (F160 *argA*$^+$ *relA*$^+$/ *leu his recA argG rspE rspL metB pyr*), were mated with CS130 and SU31-C, then plated on an appropriate selective medium supplemented with methionine. Fifty arg$^+$ merodiploids were retained from each cross and tested for a methionine requirement. In each case, all of the merodiploids were met$^+$. In view of the previous report by Fiil (1969) that the *relA1* mutation is recessive, these results are consistent with the hypothesis that it is the *relA1* mutation which

is responsible for the met$^-$ phenotype. The reciprocal experiment in which an F$'$ plasmid carrying the *relA1* mutation is introduced into CS130 and SU31-C was not performed since such an F$'$ plasmid is not available. Several attempts in this and several other laboratories (J.D. Friesen, personal communication) failed to produce such a plasmid. As might be expected, the met$^-$ phenotype is also recessive to a gene carried on the F103 plasmid. This was demonstrated by introducing the F103 plasmid from KLF3/JC1552 (F103 *his*$^+$ *metG*$^+$/ *leu lac supE gal trp his argG rspL mtl metB mal*) into CS130. Of 40 his$^+$ merodiploids recovered from this mating, all were met$^+$. Since the F103 plasmid carries the *metG* locus but not the *relA* locus, this result suggests that the met$^-$ phenotype is dependent on the allelic condition of the *metG* locus.

In order to demonstrate the the mutation responsible for the met$^-$ phenotype was not present in the parental strain, a P1 lysate of CS57 was used to transduce the rel$^-$ met$^-$ strain CS130. Of 156 arg$^+$ transductants recovered from this cross, 84% were met$^+$ (Table 10). This cotransduction frequency between *argA* and the locus responsible for the met$^-$ phenotype is substantially higher than that observed for the previously noted transduction experiments. This discrepancy in transduction frequency, although unexplained, is consistent with previous observations by other workers (Ryan and Borek, 1971), who have noted that rel$^+$ transductants or recombinants are preferentially recovered under similar circumstances.

The effect of the *relA1* mutation has been examined in several other *class-2* revertants. Of 52 thy$^+$ str^r recombinants obtained from a conjugal cross between the *class-2.3* revertant SU32B (F$^-$ *thr leu ara lac*$_{am}$ *metG46-32 his thyA rspL xyl mtl thi*) and Hfr CS126 (*relA1 argA thi*),

Table 10 - Mapping a locus responsible for the met^- phenotype in *class-2* revertants.

Donor	Recipient	Recipient phenotype	Method[a]	Selected phenotype	Number of recombinants	% met^- recombinants
X407 *relA1*	SU31-A *metG46-31 thyA*	met^+	1	thy^+	312	1%
X407 *relA1*	SU31-C *metG46-31 argA*	met^+	1	arg^+	203	18%
AB1111	SU31-C *metG46-31 argA*	met^+	1	arg^+	152	0%
KL16 *relA1*	SU31-A *metG46-31 thyA*	met^+	2	thy^+ str^r	104	34%
Hfr 305	SU31-C *metG46-31 argA*	met^+	2	arg^+ str^r	40	0%
CS57	CS130 *metG46-31 argA relA1*	met^-	1	arg^+	156	16%
CS126 *relA1*	SU32-B *metG46-32 thyA*	met^+	2	thy^+ str^r	53	45%
CS126 *relA1*	SU24-A *metG46-24 thyA*	met^+	2	thy^+ str^r	52	0%
CS126 *relA1*	SU43-A *metG46-43 thyA*	met^+	2	thy^+ str^r	52	0%

[a] The methods used in performing the crosses were: (i) Method-1, P1*vir* transduction; (ii) Method-2, conjugation.

The complete genotypes of the strains used is presented in Table 1.

45% were met$^-$. In contrast, when Hfr CS126 was crossed with the *class-2.5* revertants SU24-A (F$^-$ *thr leu ara* lac_{am} *metG46-24 his thyA rspL xyl mtl thi*) or SU43-A (F$^-$ *thr leu ara* lac_{am} *metG46-43 his thyA rsp xyl mtl thi*), no met$^-$ recombinants were recovered (Table 10). The *class-2.5* revertants are therefore distinguished by yet another criteria. This result also provides additional evidence that it is the allelic state of the *metG* gene which is the primary determinant of the met$^-$ phenotype.

Under the assumption that CS130 is met$^-$ because of the *relA1* mutation, it follows that one of the ways in which this strain can revert to become met$^+$ is by reversion to rel$^+$. Unfortunately, reversion to rel$^+$ is expected to be much less frequent than forward mutation to *metJ* or *metK*, so that rel$^+$ revertants might not be readily recovered. Forty spontaneous met$^+$ revertants of CS130 were scored for their rel phenotype and found to be rel$^-$. It therefore appears that another class of mutants is preferentially recovered under such circumstances.

In summary, it seems probable that the *relA1* mutation is responsible for a methionine requirement in at least two of the revertants. A presentation of possible mechanisms for the *rel*-dependent methionine requirement is outlined elsewhere in "Results".

The involvement of the relA gene in the regulation of methionine biosynthesis

In view of the well established role of the *relA* gene as a regulator of stable RNA synthesis, and the recently elucidated role in the regulation of histidine biosynthesis in *Salmonella* (Stephens *et al.*,

1975), it seemed likely that the *rel*-dependent methionine requirement of *class-2.3* revertants represented a regulatory phenomenon. Within this context, it was considered possible that the synthesis of methionyl-tRNA synthetase and/or the methionine biosynthetic enzymes were under a form of regulatory control involving the *relA* gene.

A series of experiments were designed to test this hypothesis. In the first set of experiments, the strain SU31-C (F^- *thr leu ara* lac_{am} *metG46-31 his argA rspL thi*) and the otherwise isogenic rel^- strain CS130 were used to study the effect of the *relA1* mutation on the synthesis of methionyl-tRNA synthetase and β-cystathionase (*metC*). Cultures of these strains were grown to mid log phase in minimal medium supplemented with twenty amino acids, then shifted to a medium containing all amino acids except methionine. The shift from repressing to non-repressing conditions (with respect to the methionine biosynthetic enzymes), when combined with the defective synthetase of these strains, is expected to induce the synthesis and accumulation of relatively high levels of ppGpp in the rel^+ strain but not in the rel^- strain. The 19 amino acids were included in the growth medium since Stephens *et al.* (1975) have reported that these conditions cause a reduction in the basal level of ppGpp in a leaky rel^- strain (such as the *relA1* strains). The level of methionyl-tRNA synthetase activity and β-cystathionase activity was determined from samples of the cultures taken at timed intervals following the shift to methionine-free medium.

The results of this experiment are presented in Figures 6 and 7. From the results presented in Figure 6, it is apparent that the rate of synthesis of β-cystathionase is dramatically increased in the presence of a functional *relA* allele. From the results presented in Figure 7, it

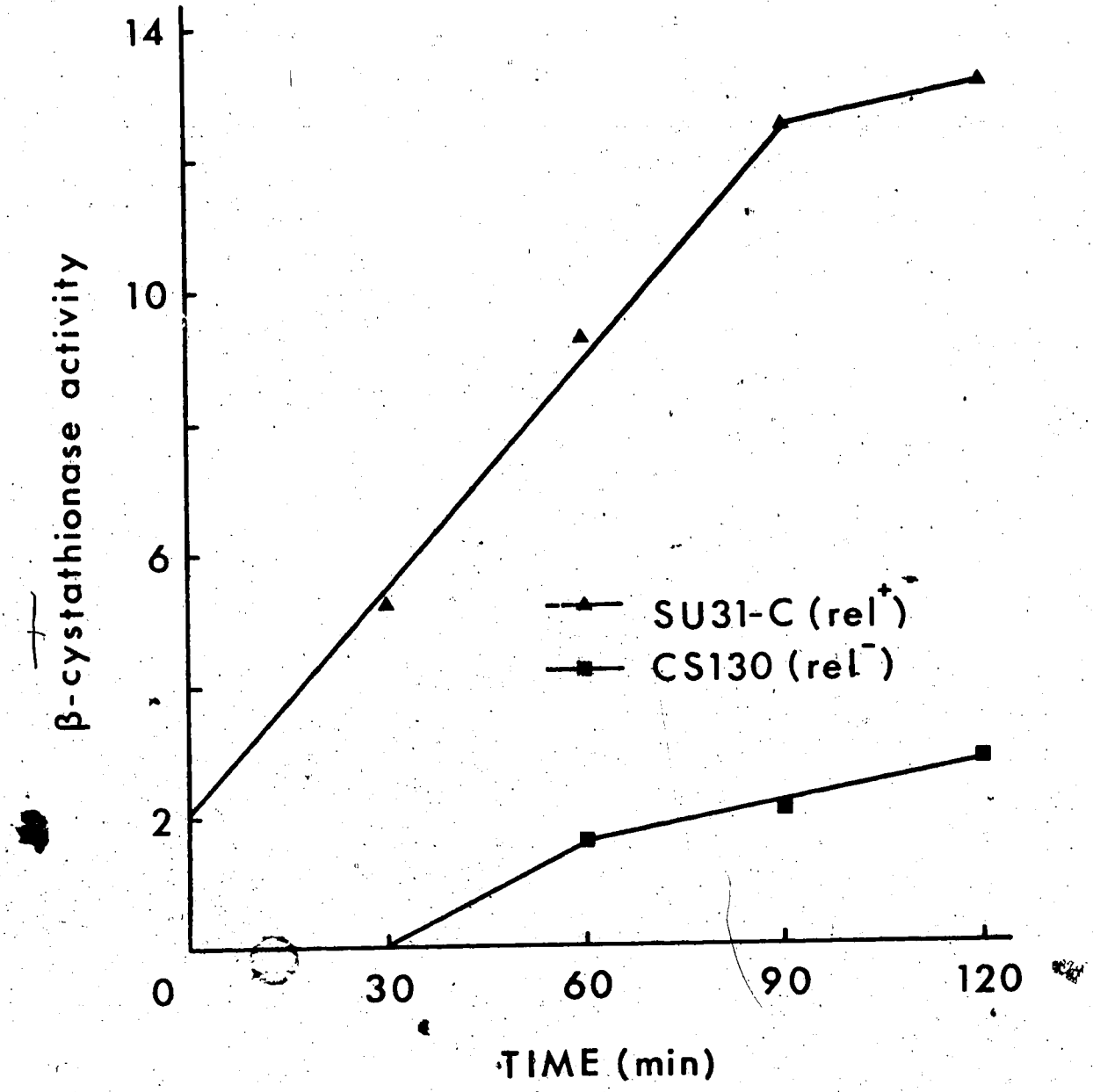

Figure 6 - The kinetics of appearence of β-cystathionase activity in a rel^+ and a rel^- strain following a shift to methionine-free medium. Enzyme activity is defined in "Materials and Methods".

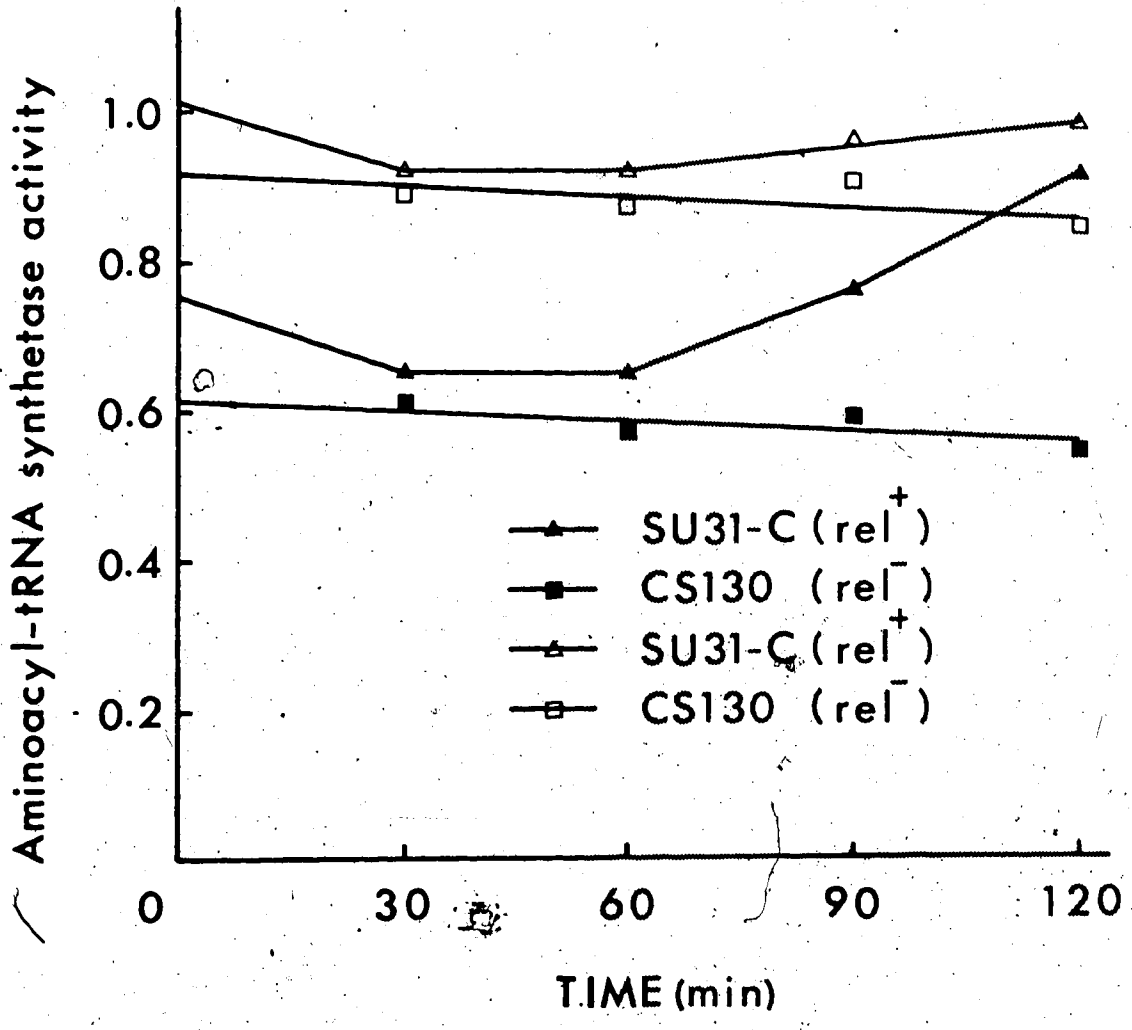

Figure 7 - Aminoacyl-tRNA synthetase activity in a rel^+ and a rel^- strain following a shift to methionine-free medium. The closed symbols represent methionyl-tRNA synthetase activity. The open symbols represent isoleucyl-tRNA synthetase activity. The units of enzyme activity are defined in "Materials and Methods".

appears that the level of methionyl-tRNA synthetase activity is slightly higher in a rel$^+$ strain than in a rel$^-$ strain. Moreover, the difference in the activity increases substantially following methionine deprivation. This is in contrast to isoleucyl-tRNA synthetase activity which does not undergo a differential increase in a rel$^+$ strain following methionine starvation. Considered by itself, this result might be interpreted as evidence that the synthesis or degradation of methionyl-tRNA synthetase is under some form of regulatory control by the *relA* gene. However, this conclusion is confounded by the evidence that at least two of the substrates for this enzyme, tRNA and methionine, also appear to be under some form of regulatory control by the *relA* gene. Since these substrates are believed to be involved in the regulation of synthetase production, the influence of the *relA* gene can not be ascribed to a primary effect under these circumstances. Also, since the synthesis of methionine in the rel$^-$ strain is occuring at a very low level, it may simply be that the rel$^-$ strain is not able to carry out protein synthesis at a sufficiently high rate.

The potentially equivocal nature of the above results necessitated the construction of a pair of strains in which the endogenous level of methionine could be more precisely controlled. The strains B36 (Hfr H *proB relA1 metB36 thi*) and Hfr 312 (*proB metB36 thi*) satisfy this requirement in that they have an auxotrophic methionine requirement which facilitates experimental control of the endogenous level of free methionine. These strains were grown to mid log phase in minimal medium containing all twenty amino acids, then shifted to a medium in which the methionine concentration was reduced to 2.5 uM. The culture was sampled at timed intervals and the specific activities of methionyl-

tRNA synthetase, β-cystathionase, and ATP:methionine *S*-adenosyltransferase were determined. The results of this experiment are presented in Figures 8-10. From the results presented in Figure 8, it appears that the amount of methionyl-tRNA synthetase is independent of the allelic condition of the *relA* gene. This suggests that the differential level of synthetase activity observed for SU31-C and CS130 is a secondary effect due to methionine limitation in CS130, or is somehow due to the fact that these strains have an altered synthetase.

In contrast, from the results presented in Figures 9 and 10, it seems that the rate of synthesis of β-cystathionase (*metC*) and ATP:methionine *S*-adenosyltransferase (*metK*) is dramatically influenced by the allelic condition of the *relA* gene. These results are therefore consistent with those previously obtained for β-cystathionase in the SU31-C, CS130 strain pair. The slight initial increase in *metK* activity following methionine limitation in the rel$^-$ strain (Fig. 10) is believed to represent experimental error.

An identical experiment was also acrried out on the strains CS156 (F$^-$ *thr leu ara tonA gal his xyl mtl metB thi mal*) and the isogenic rel$^-$ strain CS157. The results obtained for these strains with respect to *metC* and *metK* activity were similar to those obtained with other pairs of strains. In addition, the activity of N^5,N^{10}-methylenetetrahydrofolate reductase (*metF*) was measured (Fig. 11). From these results it is clear that the synthesis of this enzyme is also under a similar form of control involving the *relA* gene.

It should be noted that the four strains B36, Hfr 315, CS156, and CS157 are not repressible for the synthesis of the methionine biosynthetic enzymes. That is, even in the presence of 1 mM L-methionine

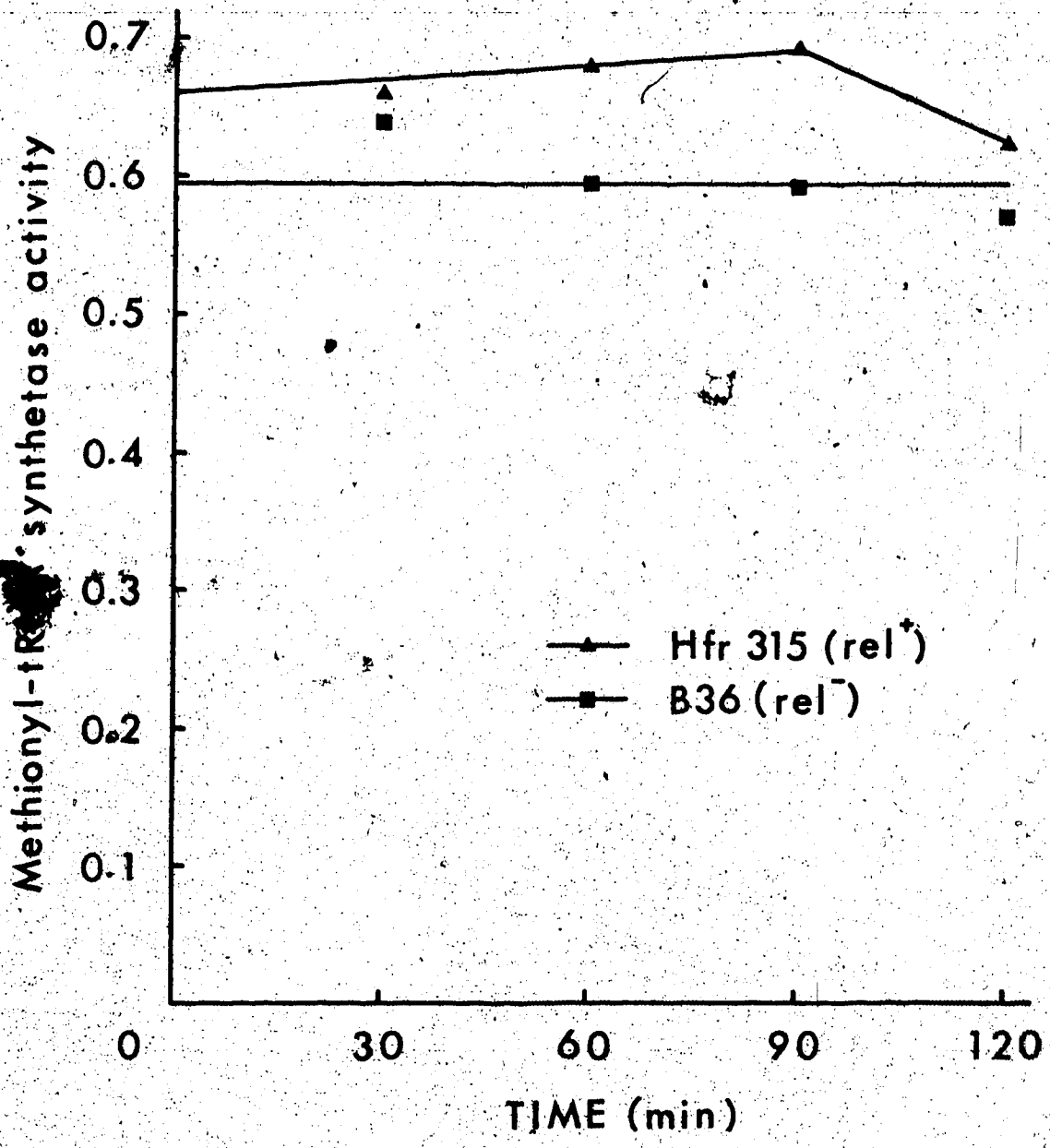

Figure 8 - Methionyl-tRNA synthetase activity following methionine deprivation in a rel^+ and a rel^- strain. The units of enzyme activity are defined in "Materials and Methods".

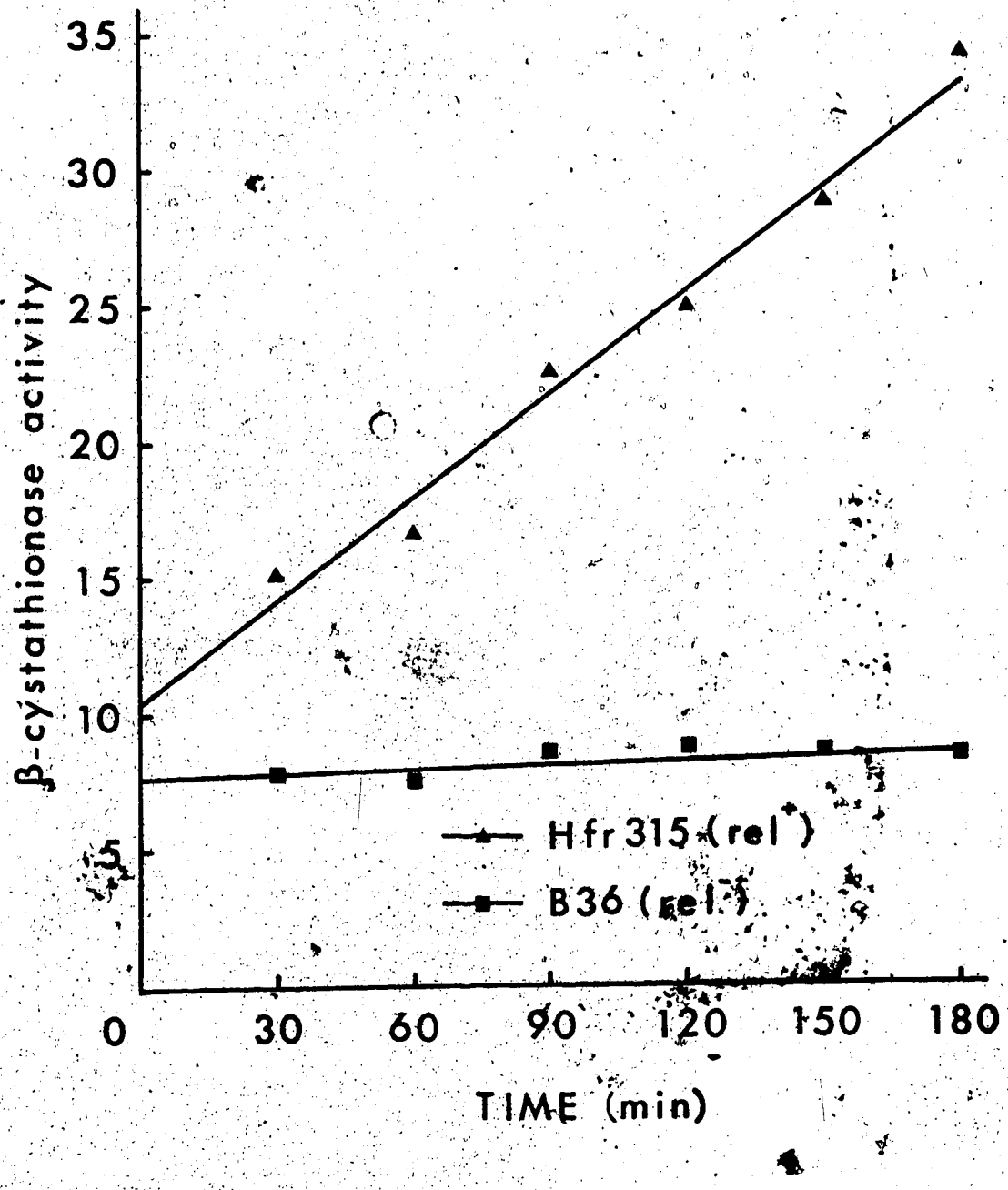

Figure 9 - β-Cystathionase activity in a rel$^+$ and a rel$^-$ strain following methionine deprivation. The units of enzyme activity are defined under "Materials and Methods".

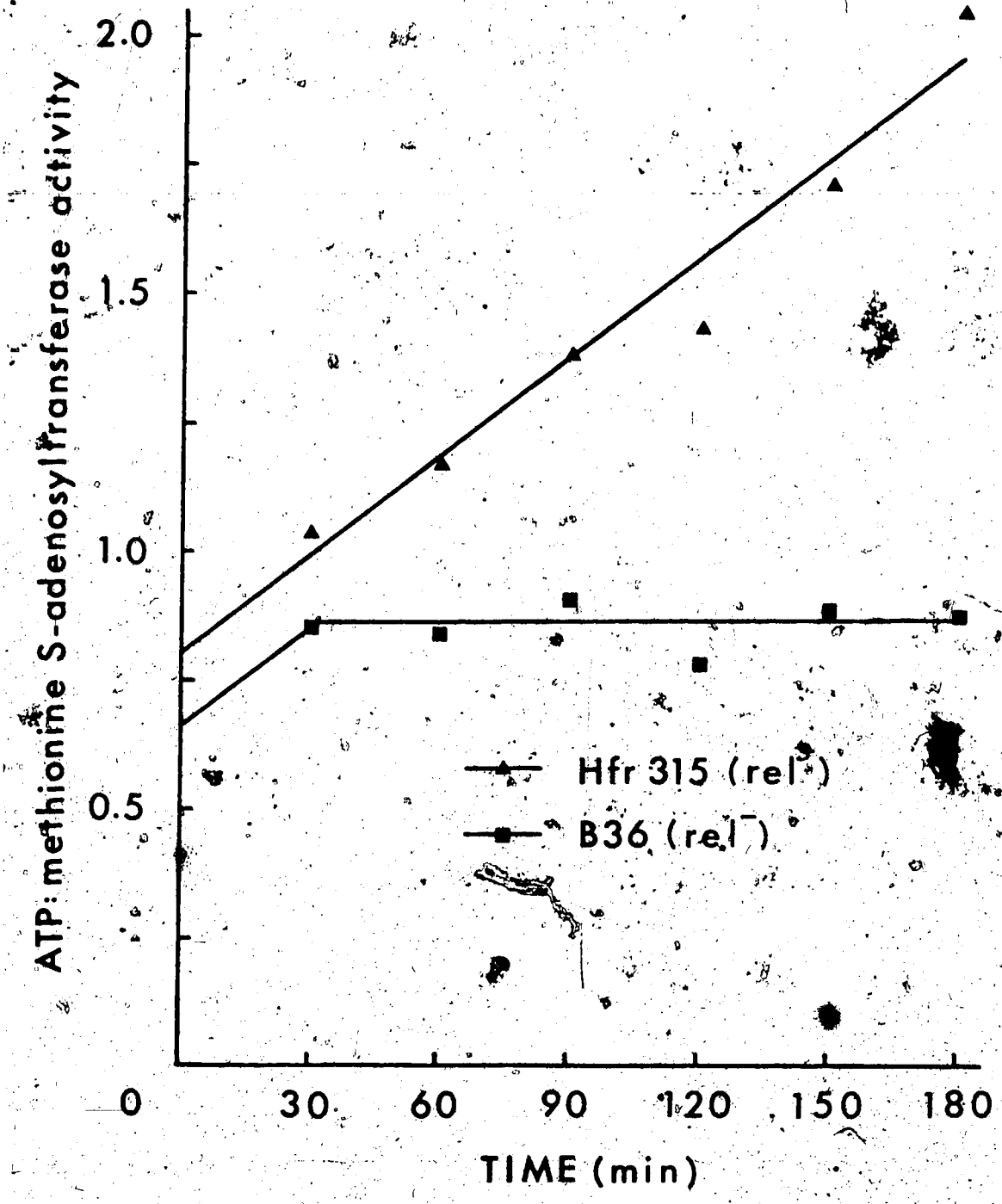

Figure 10 - ATP:methionine *S*-adenosyltransferase activity in a rel^+ and a rel^- strain following methionine deprivation. The units of enzyme activity are defined under "Materials and Methods".

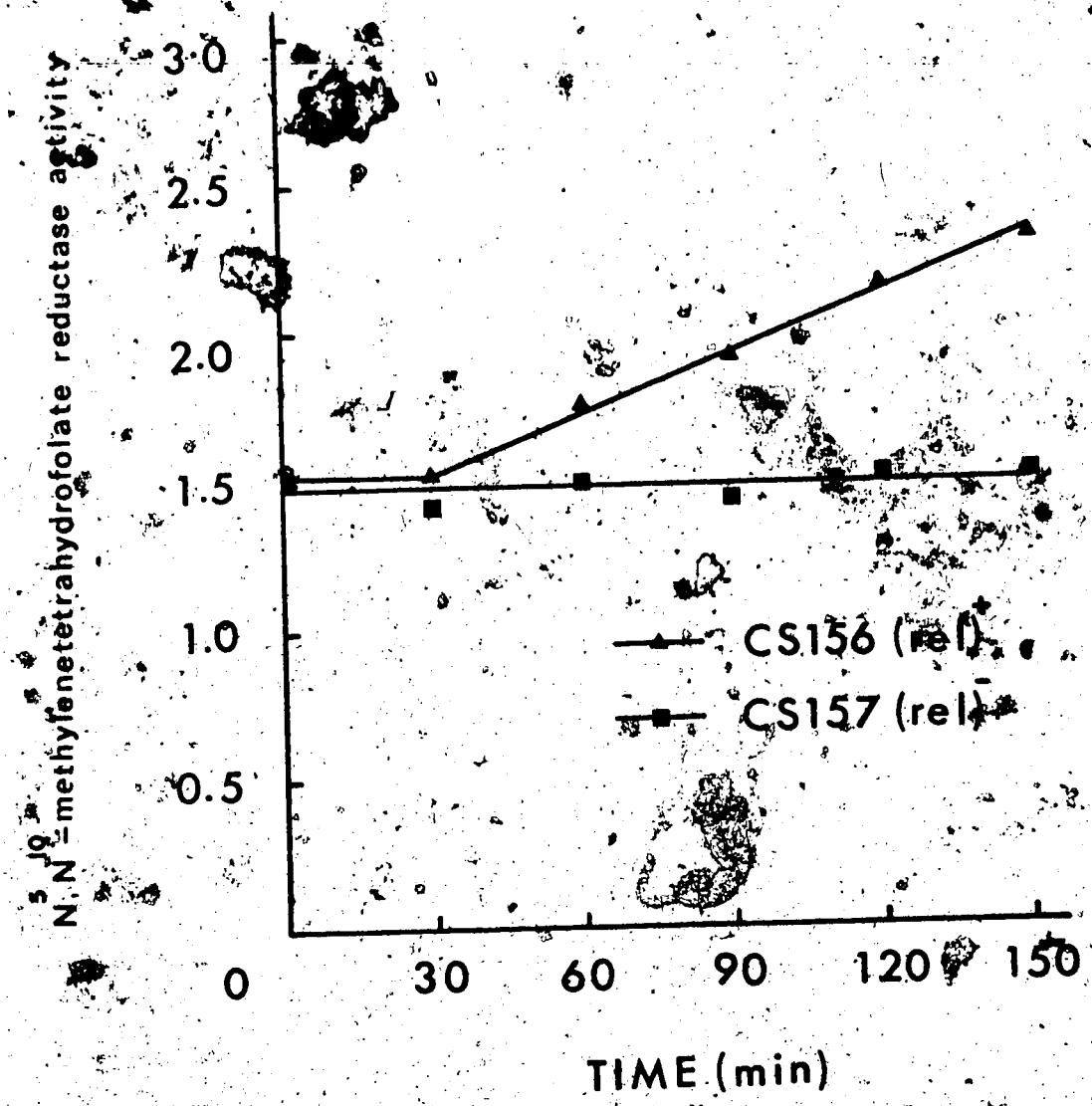

Figure 11 - N^5,N^{10}-methylenetetrahydrofolate reductase activity in a rel^+ and a rel^- strain following methionine deprivation. The units of enzyme activity are defined under "Materials and Methods".

these strains show relatively high levels of activity for the biosynthetic enzymes. The mutation responsible for this effect has not been identified, but in view of the current model for the regulation of methionine biosynthesis, it seems likely that these strains have an altered *metJ* product. This hypothesized alteration in *metJ* might have arisen during the original isolation of the closely linked *metB* mutations, or might conceivably be due to a polar effect of the *metB* mutations on the *metJ* locus.

In view of these observations, it appears that the synthesis of the methionine biosynthetic enzymes is stimulated by the presence of a functional *relA* allele under conditions which allow partial expression of the stringent response. In view of recent evidence that the *relA* locus codes for a ribosomally bound enzyme which catalyzes the formation of ppGpp from GTP and ATP (Sy and Lipmann, 1973), it seems unlikely that the *relA* product is directly involved in this phenomenon. By analogy with the recent results of Stephens *et al.* (1975) it seems probable that the effect is mediated by ppGpp which is able to stimulate transcription of the methionine biosynthetic enzymes.

The fortuitous occurence of a non-repressible phenotype in four of the strains used for this study, indicates that the stimulation of synthesis of the biosynthetic enzymes is a regulatory control which is exerted in addition to the repression-derepression mechanism of control specific for this amino acid.

In view of these observations, a specific mechanism can be proposed to account for the *rel*-dependent methionine requirement of *class-2.3* revertants. Since this class of revertants has a defective methionyl-tRNA synthetase, the cell has a higher level of uncharged

$tRNA^{Met}$ than the wild type strain. In a rel^+ strain, the presence of a signifigant proportion of uncharged tRNA results in the production of ppGpp by the product of the *relA* gene. The accumulation of ppGpp stimulates the synthesis of the methionine biosynthetic enzymes and eventually results in an increase in the rate of methionine production to a level which is sufficent to overcome the *Km* defect of the synthetase. A rel^- strain is not able to respond in this manner, and is therefore unable to maintain the level of methionyl-$tRNA^{Met}$ required for continued growth.

Indirect evidence for a threshold effect is derived from the observation that the *metG17* and *metG24* mutations, which have a Km^{Met} which is only slightly higher than that of the *metG46-31* allele, require exogenous methionine for growth regardless of the *rel* phenotype. The implication is that at the level of aminoacylation in the *class-2.3* revertants, a relatively slight decrease in the rate of aminoacylation will result in a met^- phenotype. It is relevant to note at this point that the *relA* gene has no observable effect on the kind or amount of methionyl-tRNA synthetase produced in *class-2.3* revertants (Table 8).

Other revertants

The strain CS62 was isolated as a spontaneous revertant of the strain CS54 (F^- *thr leu ara proA lac supE gal metG46 his rspL xyl mtl thi*). This revertant is exceptional in that it grows on minimal agar supplemented with homocysteine or cystathionine, but not homoserine. This strain has highly derepressed levels of the *metC* and *metK* enzymes, but no detectable cystathionine-γ-synthetase (*metB*) activity

when grown in minimal medium supplemented with homocysteine. The results of these enzyme assays are included in Table 11.

The mutation responsible for this effect was localized by a conjugal cross between Hfr CS92 (*relA1 glpK argH rpoB thi*) and CS62. Of 42 ilv^+ leu^+ str^r recombinants obtained from this cross, all had lost the ability to grow on homocysteine supplemented minimal agar. A transduction experiment was conducted in order to specify the map position more precisely. A P1*vir* lysate of CS62 was used to transduce CS92. One hundred arg^+ transductants, recovered from this cross on minimal agar supplemented with methionine, were scored for their *met* phenotype, and for the ability to use glycerol as a carbon source ($glpK^+$). The results of this transduction, presented in Table 12, indicate that CS62 has a *metB* mutation. In addition, one of the transductants, retained for further study and designated CS62-C (Hfr *relA1 glpK metB metJ thi*), was subsequently found to be resistant to ethionine on minimal agar supplemented with homocysteine. This result suggests that CS62 also had a *metJ* mutation. This observation provides a convenient explanation for the highly derepressed levels of activity observed for the biosynthetic enzymes in CS62.

In view of the fact that CS62 was isolated as a spontaneous revertant it seems possible that the *metB metJ* mutations in this strain represent a deletion for these two loci. This hypothesis demands that the *metB* mutation should not revert. To test this prediction, spontaneous or induced revertants of CS62-C were sought. A concentrated culture (10^{10} cells/ml) was spread on a minimal agar plate and crystals of 2-aminopurine, ICR-191, and Nitrosoguanidine were placed on the agar.

This procedure repeatedly failed to produce any met^+ revertants. This evidence, although suggestive, is not compelling evidence for a *metB-metJ* deletion. Fine structure mapping experiments are required to provide unequivocal evidence for a deletion. The recent fine structure map of the *metJ* locus (Holowachuk, 1976) might prove useful in this regard. An alternative hypothesis is that the *metB* mutation is a polar mutation which prevents the synthesis of the methionine repressor form the closely linked *metJ* gene. In this event, the introduction of a polarity suppressor into CS62 should result in the disappearence of one of the two phenotypes.

The only unclassified revertant CS68 (F^- *thr leu ara lac*$_{am}$ *metG46 his rspL xyl mtl thi*) was isolated from the parental strain CS57. This exceptional revertant grows on minimal agar supplemented with homoserine, cystathionine, or homocysteine, but not on minimal agar. Enzyme assays performed on extracts of this strain suggest that it is not derepressed for the synthesis of the methionine biosynthetic enzymes in general (Table 11).

The mutation responsible for this phenotype was first localized by a conjugal cross between Hfr CS92 and CS68. Of 50 leu^+ str^r recombinants, 10 were $glpK^-$ and were no longer able to grow on homocysteine supplemented minimal agar. Since this result suggested a location in the *metJ* region, a transduction was performed in which a P1 lysate of CS68 was used to transduce CS63 (F^- *leu ara lac metG46 his rspL xyl mtl argE thi*). Of 90 arg^+ transductants, 24 were able to grow on homocysteine supplemented minimal agar. This cotransduction frequency is approximately that expected for a metJ mutation.

A further characterization of this mutant was not attempted. It was also observed that this mutant has become phenotypically thr^+. Since it is difficult to reconcile this observation with other aspects of the phenotype of this strain, no hypothesis is proffered to account for the suppression of the *met046* mutation.

Table 11

Enzyme assays for two unusual revertants.

Strain	Relative specific activity[a]		
	β-cystathionase (*metC*)	cystathionine-γ-synthetase (*metB*)	ATP:methionine *S*-adenosyltransferase (*metK*)
AB1111	1.00	1.00	1.00
CS62	12.22	0	3.29
CS68	0.45	–	1.00

[a] The enzyme activities of the revertant strains are expressed relative to the specific activity of the wild type strain (AB1111), which is taken as 1.0 in each case. The actual specific activities were: (i) β-cystathionase, 0.11; (ii) cystathionine-γ-synthetase, 0.12; (iii) ATP:methionine *S*-adenosyltransferase, 9.08. The units of activity are described under "Materials and Methods".

Table 12

A transduction experiment demonstrating linkage between *argH* and a mutation responsible for homocysteine auxotrophy in CS62.

Selected phenotype	recombinant phenotype	number of recombinants
$argH^+$	$glpK^-$ met^+	75
"	$glpK^-$ met^-	15
"	$glpK^+$ met^+	1
"	$glpK^+$ met^-	4

The donor in this cross was CS62 and the recipient was Hfr CS92 (*glpK argH*).

DISCUSSION

Six new methionyl-tRNA synthetase (*metG*) mutants have been isolated and partially characterized. These mutants are similar to the *metG* mutants previously described (Calendar and Lindahl, 1969; Blumenthal, 1972; Armstrong and Fairfield, 1975) in that they require exogenous methionine for growth. The methionine requirement of these mutants appears to be due to an increase in the Km^{Met} of the methionine activation reaction catalyzed by methionyl-tRNA synthetase (Ahmed, 1973). At least two, and possibly four, non-identical alleles can be distinguished among the six mutants on the basis of differences in the Km^{Met}. The Km^{ATP} for one of these mutants was also determined, and was found to be essentially unaffected by the mutation. This result suggests relative independence of the methionine binding site from the ATP binding sites of the enzyme, and is consistent with biochemical analysis of the wild type enzyme by Fayat and Waller (1974). A previous analysis of a similar *metG* mutant revealed no alteration in the *Km* for $tRNA^{Met}$ (T. Clandinin; cited in Ahmed, 1973).

All of the *metG* mutations are recessive to the $metG^+$ gene which is present on the F103 or F1829 plasmids but not on the F196 or F197Z plasmids. These observations suggest a location for the *metG* locus in the *hag-flaE* interval of the chromosome. The results of a transduction experiment in which several loci in this region were cotransduced with *metG*, are consistent with the proposed location. In addition, the previous mapping experiments by Ahmed (1973) are consistent with this location. The proposed location in the cluster of *fla* genes varies by several minutes from

the location suggested in the recent linkage map of Bachmann *et al.* (1976). The map position suggested by Bachmann *et al.* is derived from the data of Blumenthal (1972) in which the map position of *metG* appears to have been assumed rather than demonstrated by any mapping experiments

A previous analysis of several met$^+$ revertants of a *metG* mutant of *E. coli* revealed a class of revertants in which no satisfactory mechanism could be proposed to account for the restoration of methionine independance (Ahmed, 1973). In contrast to the results of a similar study in *Salmonella* (Chater *et al.*, 1970), these revertants were not derepressed for the synthesis of the methionine biosynthetic enzymes, and did not show any restoration of aminoacyl-tRNA synthetase activity. Since trivial mechanisms could not be invoked to explain these revertants, a detailed analysis of the reversion pattern of one of the newly isolated *metG* mutants (*metG46*) was undertaken.

Forty spontaneous revertants of a strain harboring the *metG46* mutation were obtained as mutants which no longer required exogenous methionine for growth. The choice of these forty revertants from among the several thousand which were isolated, was made in such a way that methionine excreting *metK* mutants were discriminated against. In addition, an unsuccesful attempt to recover a revertant in which an *amber* mutation was responsible for the met$^+$ phenotype effectively resulted in the selection of revertants which grow poorly on lactose minimal agar. The procedure by which revertants were selected for further study was therefore biased, so the analysis may not accurately reflect the relative frequency with which various classes of revertants arise.

The revertants obtained in this manner were first characterized on the basis of a repressible versus a non-repressible phenotype with respect to the levels of one or more methionine biosynthetic enzymes. All of the members of the non-repressible category (*class-1*) were characterized as *metJ* mutants by the demonstration that these strains have a mutation which is located close to the *metB* locus and which, when transduced into an otherwise ethionine sensitive strain, conferred an ethionine resistant phenotype. The met^+ phenotype of the *class-1* revertants is therefore ascribed to a *metJ*-mediated increase in the synthesis of the methionine biosynthetic enzymes. The increased synthesis of the biosynthetic enzymes results in an increase in the endogenous methionine pool to a concentration which is sufficent to allow adequate aminoacylation by the altered synthetase. This class of revertants is therefore similar to those characterized by Chater *et al.* (1970).

The other class of revertants (*class-2*) appears to be normally regulated with respect to the synthesis of the methionine biosynthetic enzymes. These mutants presumably correspond to the unexplained revertant class noted by Ahmed in that, when extracts of these mutants were prepared according to the procedure described, most of these revertants showed very low levels of synthetase activity. It was subsequently observed that the low level of methionyl-tRNA synthetase activity in these revertant strains was due to the inactivation of the mutant synthetase during dialysis or desalting on Sephadex. Similar instability of mutant synthetases has been noted previously for other aminoacyl-tRNA synthetases (for example, Milkulka *et al.*, 1972).

Although glycerol is routinely used to stabilize many of the aminoacyl-tRNA synthetases, it has no effect on the stability of the wild type methionyl tRNA synthetase. However, when extracts of the *class-2* revertants are prepared in the presence of 10% glycerol, the synthetase activity is not lost during dialysis. Under these conditions all of the *class-2* revertants show a partial or complete restoration of synthetase activity to a level which is sufficent to account for the met$^+$ phenotype of these strains. Also, all of the *class-2* revertants show a substantial decrease in the Km^{Met} for the methionine activation reaction when extracts are prepared in the presence of glycerol. It is therefore concluded that these revertant strains have undergone a mutation either within the structural gene for the synthetase, or at some other location, which results in an alteration of the enzyme function.

Four distinct classes of revertants were distinguished on the basis of Km^{Met} determinations and a fifth class was distinguished by a differential stability of synthetase activity during dialysis or exposure of extracts to elevated temperatures. There are therefore at least five different mutations which can result in partial or complete restoration of synthetase activity. Two subclasses of the *class-2* revertants have a methionyl-tRNA synthetase which was very rapidly inactivated when extracts were incubated at 42° prior to assay. This property corresponds with the *in vivo* phenotype in that these strains are met$^+$ at 30-37° but met$^-$ at temperatures above 37°.

An approximate location for the reversion mutation in one of the temperature sensitive revertants was obtained by a conjugal cross. The mutation appears to be closely linked to the *his*-operon and close

linkage to the *metG* locus is therefore inferred. Because of technical difficulties it has not been possible to define the exact map location of the reversion mutation.

Two other revertants which were able to grow on methionine intermediates were also isolated and partially characterized. One of these unusual revertants exhibits the characteristics of a double (*metB metJ*) mutant. In view of the fact that this revertant was isolated as a spontaneous mutant, it seems likely that a single mutational event had given rise to the two phenotypes. For example, a deletion extending from the *metB* gene into the closely linked *metJ* gene could account for this effect. Since the *metB* mutation does not revert either spontaneously or by treatment with several different mutagens, the evidence is consistent with this hypothesis. An alternate, but less likely possibility, is that *metB* and *metJ* are part of a common transcriptional unit. In this event, a polar mutation in one of these loci could prevent expression of the adjacent locus. Fine structure mapping experiments are required in order to resolve these hypotheses.

Another revertant which requires methionine intermediates for growth was also recovered. The reversion mutation in this strain has been localized in the region of the chromosome where *metJ* and several of the methionine biosynthetic genes are located, but this strain does not appear to be derepressed for the synthesis of the methionine biosynthetic enzymes. Since the available evidence suggests that normal levels of the biosynthetic enzymes are not sufficent to convert exogenous precursors to methionine at the level required by the defective synthetase, this mutant is anomalous. The analysis of this revertant is further confounded by the observation that the *thr*

mutation (threonine requirement) of this strain is also suppressed in this revertant. This revertant remains unexplained.

During the course of mapping experiments involving the *class-2.3* revertants, two widely separated loci were found to be involved in the maintenance of the met^+ phenotype of these strains. One of these loci, subsequently identified as the site of the reversion mutation, is closely linked to the *his*-operon and is believed to be within the *metG* gene. The other locus is closely linked to the *argA* gene. When *argA* is the selected marker in a cross in which a strain with the *relA1* mutation serves as the donor, met^- recombinants of the *class-2.3* revertants are recovered at a high frequency. In contrast, when an otherwise isogenic rel^+ strain is used as the donor, met^- recombinants are not recovered. This result was interpreted to mean that the introduction of the *relA1* mutation into these strains was responsible for the met^- phenotype. The results of a transduction cross confirmed that the mutation responsible for the met^- phenotype was identical with the *relA1* mutation. In particular, a transductional cross in which a rel^- strain was used as a donor and a met^+ rel^+ *class-2.3* revertant was used as the recipient, produced only met^+ rel^+ and met^- rel^- transductants.

Since the K_m^{Met} of the methionine activation reaction catalyzed by extracts of *class-2.3* revertants is not affected by the allelic condition of the *relA* locus, it was concluded that the *rel*-dependent met^- phenotype was not due to an alteration of the synthetase function. In view of the well-established involvement of the *relA* locus in the regulation of rRNA synthesis (Lazzarini and Dahlberg, 1971) and tRNA synthesis (Ikemura and Dahlberg, 1973), it was

postulated that the *rel*-dependent methionine requirement also represents a regulatory phenomenon. Within this context it was considered possible that the synthesis of either methionyl-tRNA synthetase and/or the methionine biosynthetic enzymes were under a form of regulatory control involving the *relA* locus. This was examined by observing the rate and degree of synthesis of these enzymes in several pairs of strains under conditions in which partial expression of the stringent response was induced by limiting the availability of methionine . It was observed that under conditions in which the endogenous methionine concentration was low, the *relA1* mutation had no pronounced effect on the level of wild type methionyl-tRNA synthetase activity. It therefore appears that the regulation of the synthetase is not affected by the allelic condition of the *relA* locus. It should be noted however that the synthetases are in general subject to rapid inactivation under conditions which limit the supply of the cognate aminoacyl-tRNA (Williams and Neidhardt, 1969). This fact has thwarted several previous attempts to study the regulation of the synthetases and has resulted in the development of special techniques, not employed in this study, which partially ameliorate these difficulties. The conclusion from such studies has generally been that the synthetases are under a repression-like mechanism of regulation in which the charged cognate tRNA acts as the repressor or corepressor. Since these experiments have consistently ignored the *rel* phenotype, they might be considered suspect since the presence of a large proportion of uncharged tRNA will, in a rel^+ strain, be accompanied by a high level of ppGpp production. Therefore, the increased synthesis of aminoacyl-tRNA synthetase observed in these experiments could conceivably be due

to the stimulation of transcription by ppGpp or some derivative thereof

In contrast to the negative results obtained for the methionyl-tRNA synthetase, it was observed that the synthesis of the methionine biosynthetic enzymes is dramatically affected by the allelic condition of the *relA* gene. Under conditions of methionine limitation, the rate of synthesis of two of the biosynthetic enzymes was observed to be much higher in a rel^+ strain than in a rel^- strain. In addition, the synthesis of one of the enzymes involved in methionine utilization (ATP:methionine *S*-adenosyltransferase) was affected in a similar manner. The increased rate of synthesis of these enzymes in a rel^+ strain appears to be due to a mechanism which is distinct from the *metJ*-mediated repression regulation of these enzymes. This is inferred from the results of several experiments in which non-repressible strains were used. Under these conditions a rel^- strain showed no increase in the rate of synthesis of the biosynthetic enzymes following methionine limitation, wheras an otherwise isogenic rel^+ strain showed a dramatic increase in the rate of synthesis of these enzymes. Furthermore, even under conditions in which methionine was present in the growth medium at high concentrations (1mM), the level of β-cystathionase (*metC*) was at a substantially higher level in a repressible rel^+ strain than in an otherwise isogenic rel^- strain. This result suggests that the product of the *relA* gene is able to stimulate synthesis of the biosynthetic enzymes even under conditions in which repression of these genes is in effect.

These observations are superficially similar to the *in vivo* results of Stephens *et al.* (1975) who reported that under comparable conditions, the rate of synthesis of the histidine biosynthetic enzymes

of *Salmonella* is at a substantially higher level in a rel$^+$ strain than in a rel$^-$ strain. They also presented evidence that in an *in vitro* transcription system, ppGpp (guanosine 5'-diphosphate 3'-diphosphate) provokes a specific increase in the rate of transcription of *his*-operon DNA. Since ppGpp has been shown to be the primary product of the *relA* gene (Sy and Lipmann, 1973; Haseltine and Block, 1973), these observations provide a convincing cause and effect relationship between the allelic condition of the *relA* gene and the rate of synthesis of the histidine biosynthetic enzymes. These results also lend credence to the previous report by Reiness *et al.* (1975) that, in an *in vitro* transcription system, ppGpp inhibits the transcription of rRNA but stimulates the transcription of *trp*-operon DNA. Therefore, it appears that ppGpp is in some way responsible for both the inhibition of rRNA synthesis and the stimulation of transcription of the amino acid (*i.e.*, histidine and tryptophan) biosynthetic enzymes in a rel$^+$ strain following amino acid limitation. The precise mechanism by which ppGpp mediates these effects is unknown, but it has been suggested by Reiness *et al.* (1975) that ppGpp may interact with RNA polymerase in such a way that different classes of promoters are recognized with different efficencies.

From results presented here, and by analogy with the results of Stephens *et al.* (1975), it seems apparent that the synthesis of the methionine biosynthetic enzymes is under two complementary forms of regulatory controls. One level of control is pathway specific and presumably responds to the absolute concentration of methionine in the cell. Regulation at this level is mediated by the *metJ* repressor, which in the presence of a wild type *metK* gene, regulates the synthesis

of the biosynthetic enzymes in a negative manner (Ahmed, 1973; Holowachuk, 1976). The role of the *metK* gene in this repression mechanism is poorly understood but may reflect the involvement of *S*-adenosylmethionine as a corepressor (Hobson, 1974; Morowicz, 1975).

In addition to this pathway specific form of negative control, it appears that methionine biosynthesis may also be subject to a general form of positive control. This regulatory system requires the presence of a functional *relA* gene. The protein specified by this gene recognizes an inadequate supply of any amino acid at the level of translation, and responds by producing a non-specific signal (or alarmone, Stephens *et al.*, 1975) which is presumably ppGpp. It appears that even under conditions in which the synthesis of the methionine biosynthetic genes is partially repressed, the alarmone can provoke an increase in the rate of transcription of the biosynthetic genes. Presumably, the degree of stimulation is contingent upon the availability, as determined by the pathway specific regulatory mechanism, of a particular gene or operon for transcription, and the level of ppGpp within the cell. In this way the interaction of this relatively non-specific signal with the pathway specific regulatory mechanism can result in a relatively specific form of positive control. For example, under conditions in which methionine is the only limiting amino acid, it will be primarily methionine biosynthesis which is stimulated by the accumulation of the *rel*-alarmone.

This form of control is therefore analogous to the regulation of the catabolite sensitive genes. In this system, cAMP (adenosine 3':5' cyclic monophosphate) serves as the alarmone which signals an inadequate level of glucose. cAMP interacts with CAP (catabolite gene

activator protein) which binds to DNA at promoter sites and stimulates transcription of catabolite repressible genes (Zubay *et al.*, 1970; Riggs *et al.*, 1971). This relatively non-specific signal stimulates the synthesis of only those genes which are inducible and therefore results in a specffic stimulation of synthesis of the gene products which are appropriate to the prevailing environmental conditions (for example, availability of a particular carbon source)

It is apparent that whatever the precise mechanism by which the *relA* gene exerts a stimulatory effect on amino acid biosynthesis, it must be a relatively flexible system so that it can interact with very divergent forms of pathway specific regulatory systems. For example, the scattered genes of the methionine biosynthetic pathway are regulated in a negative manner by a protein repressor, wheras the *his* operon appears to be regulated in a positive manner by an activator-attenuator mechanism involving $tRNA^{His}$ (Artz and Broach, 1975). The observation that methionine biosynthesis is subject to control by the *relA* gene supports the suggestion of Stephens *et al.* (1975) that the synthesis of all amino acids is under this form of control. The advantage of such a regulatory system is that, in conjunction with the pathway specific regulatory mechanisms, the *relA* gene provides a mechanism for coordinating the synthesis of all amino acids with respect to a demand at the level of protein synthesis.

The observation that the synthesis of ATP:methionine S-adenosyl transferase is also under positive control by the *relA* gene suggests that a class of enzymes other than amino acid biosynthetic enzymes are under some form of control by the *relA* gene. It is not immediately obvious why *metK* is under this form of control. Since

metK can be considered as a structural gene in several biosynthetic pathways (*i.e.*, as an isopropylamine donor in polyamine biosynthesis, Tabor *et al.*, 1961), it seems possible that one or more of these pathways is also regulated by *relA*.

In view of the conclusions regarding the regulation of methionine biosynthesis, a specific mechanism can be proposed to account for the *rel*-dependent methionine requirement of the *class-2.3* revertants. These strains have a 20-fold increase in the Km^{Met} of the methionine activation reaction. It is therefore suggested that the defect in the methionyl-tRNA synthetase is so severe in these strains that they require a relatively high level of methionine production in order to effect adequate aminoacylation of $tRNA^{Met}$. This is substantiated by the observation that the rel^+ *metG* strain CS49, which has a 25-fold increase in Km^{Met}, requires exogenous methionine for growth. The pathway specific regulatory mechanism recognizes only the absolute methionine concentration and therefore does not respond to the demand for methionine at the level of translation. However, in the presence of a functional *relA* gene, the cell responds to a deficiency of methionyl-$tRNA^{Met}$ by stimulating amino acid biosynthesis even under conditions of partial repression. The introduction of a *relA* mutation into these strains renders the cell insensitive to the presence of uncharged tRNA and results in a reduction in the rate of synthesis of methionine to the point that adequate charging is not effected by the defective synthetase. It should be noted that this *rel*-dependent amino acid requirement must be at the level of translation. That is, a leaky auxotrophic mutation would not be expected to behave in this manner

since such a mutation would be expected to lead to derepression of the biosynthetic pathway due to a reduction in the absolute concentration of the amino acid.

The identification of the strains with a *rel*-dependent amino acid requirement provides an *in vivo* confirmation of the regulatory role of the *relA* gene in amino acid biosynthesis. In addition, it is now possible to recognize the allelic condition of the *relA* gene by a nutritional requirement. This should facilitate the genetic analysis of the components of the *rel*-dependent regulatory system. The genetic analysis can proceed in two ways. By using a rel$^-$ met$^-$ parental strain, and selecting for met$^+$ revertants, it should be possible to recover new classes of mutants in which methionine biosynthesis is independent of the *relA* gene, or in which the loss or alteration in some other function results in a rel$^+$ phenocopy. For example, in the presence of a leaky *relA* mutation (*i.e.*, *relA1*), a *spoT* mutant (Laffler and Gallant, 1974) which is defective in the turnover of ppGpp, might be expected to give rise to met$^+$ revertants. This is due to the fact that under these conditions ppGpp will accumulate to levels approaching that observed in a rel$^+$ strain. It also appears that other classes of revertants will be obtained. A preliminary investigation of the feasibility of this approach resulted in the recovery of a class of temperature sensitive mutants in which the reversion mutation has not occured in the *relA* gene or in the *metG* gene (Appendix 1).

Alternatively, it should be possible to obtain single step rel$^-$ met$^-$ mutants of a rel$^+$ met$^+$ *class-2.3* strain by forward mutation. Two classes of mutations are expected in this case, those affecting the production of ppGpp, and those which render the transcription apparatus

insensitive to ppGpp. The first class of mutants is expected to include new *relA* mutants or mutations in ribosomal genes affecting *rel* function (for example, the *relC* mutants of Parker *et al.*, 1976). Since the precise function of the *relA* gene as a component of the translational apparatus is poorly understood, a temperature sensitive or *amber relA* mutation would be particularly interesting. New *rel* mutations in ribosomal proteins might prove useful as a means of mapping the topography of the ribosome.

The other, as yet unidentified class of *rel* mutants, is that in which the cell does not respond to the production of ppGpp. Following the suggestion by Reiness *et al.* (1975) that ppGpp may interact with RNA polymerase to modify the recognition of different classes of promoters, it may be possible to obtain a mutant form of RNA polymerase which is insensitive to ppGpp. Such a mutant would be expected to be a dominant *rel* mutant with respect to RNA synthesis, but recessive with respect to the regulation of amino acid biosynthesis. Another possibility is suggested by the analogy between the *rel* dependent form of control and the catabolite sensitive gene system. In this system, the alarmone (cAMP) does not interact directly with RNA polymerase. Instead, cAMP interacts with a non-essential protein which binds to specific promoter sites and enhances the rate of transcription from these promoters. The implication is that one or more proteins similar to CAP may exist, which interact with ppGpp to bring about a modification of transcriptional specificity. Assuming the existence of a single protein with this function, a mutation in the corresponding gene should be expressed as a recessive *rel* mutation.

In summary, a positive component of the regulatory system for methionine biosynthesis has been discovered. The regulatory network incorporates a pathway specific form of negative control and a non-specific form of positive control involving the *relA* gene. In addition, a system has been developed in which the allelic condition of the *relA* gene can be recognized by an amino acid requirement. The use of this system should facilitate the analysis of the components of the *rel* phenomenon.

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APPENDICES

APPENDIX 1

An increased phosphate requirement in class-2.3 revertants

The *class-2.3* revertant SU31 (F⁻ *thr leu ara* lac_{am} *metG46-31 his rspL xyl mtl thi*) has a doubling time of approximately 130 min in L broth at 30°. Under identical conditions, the parental strain CS50 (F⁻ *thr leu ara proA lac supE gal metG46 his rspL xyl mtl thi*) has a doubling time of only 70 min. As a result of experiments designed to determine optimal growth conditions for SU31, it was discovered that the doubling time of this strain could be reduced to 65 min at 30° by increasing the phosphate concentration of the medium. A satisfactory medium for the growth of these strains is the LP broth noted in the "Materials and Methods" section. The form of the phosphate does not seem to be important since the growth of SU31 is identical in either sodium or potassium phosphate. Addition of 1% NaCl to the LP broth does not cause any inhibition of growth rate. The reason for this effect is not known.

Serine sensitivity in SU31

The growth of the *class-2.3* revertant strain SU31 is completely inhibited by high levels of serine. This was observed by spreading 0.1 ml of a saturated L broth culture on a minimal agar plate, then placing a crystal of L-serine in the center of the plate. After 36 hr of incubation at 30°, a clear zone of inhibition was observed in the center of the plate. Under identical conditions, serine has no effect on the growth of the wild type ancestral strain

AB1111 (F⁻ *thr leu ara proA lac supE gal his repL xyl mtl thi*). Under similar circumstances, none of the other 19 amino acids had any inhibitory effect on the growth of SU31.

This effect may be related to the observation that serine reverses the trimethoprim induced stringent response in rel⁺ strains (noted in: Ikemura and Dahlberg, 1973).

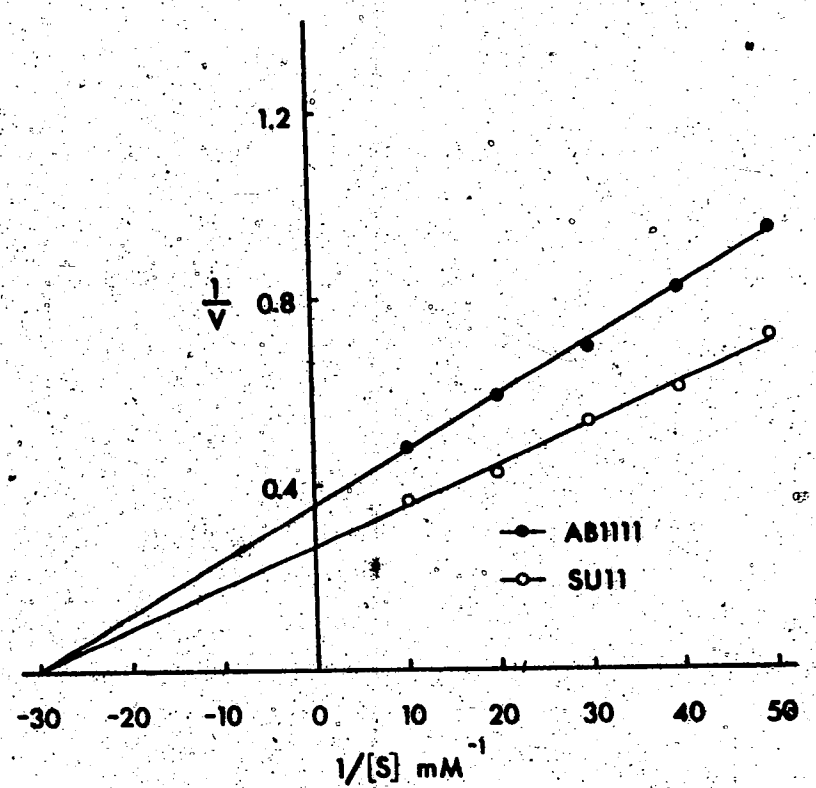
Partial characterization of a met⁺ revertant of CS130

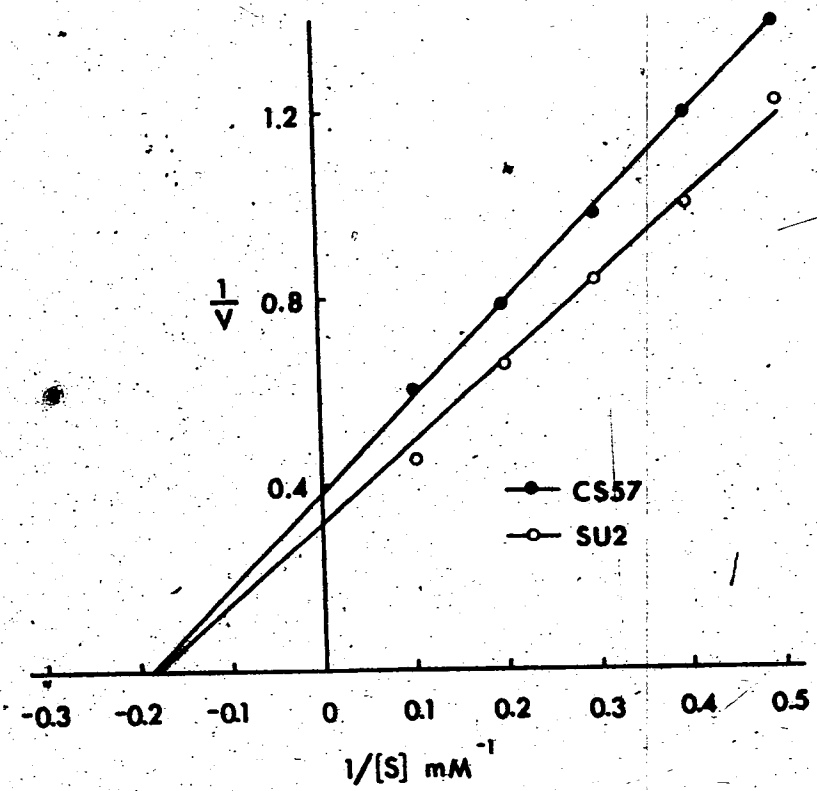
Twenty spontaneous met⁺ revertants of the strain CS130 (F⁻ *thr leu ara lac*$_{am}$ *metG46-31 his relA1 argA repL thi*) were selected on minimal agar at 30°. One of these revertants, tentatively designated RV3, was found to be temperature sensitive on L broth agar at 38°. Several mapping experiments suggest that the reversion mutation in this strain has not occured within the *relA* gene or the *metG* gene. A conjugal cross was performed between Hfr KL16 (*relA1 thi*) and RV3. Thirty-five argA⁺ str^r recombinants were recovered from this cross and all were temperature sensitive and met⁺. These results imply that the reversion has not occured within the *relA* gene. A conjugal cross was also performed between Hfr KL96 (*relA1 thi*) and RV3. All of the 50 his⁺ str^r recombinants recovered from this cross were temperature sensitive. This result implies that the reversion has not occured within the *metG* gene.

Although these results are merely exploratory, they suggest that a third gene, an indispensible product, is interacting with either the product of the *relA* gene or the product of the *metG* gene. In view of the fact that at least one ribosomal protein has been

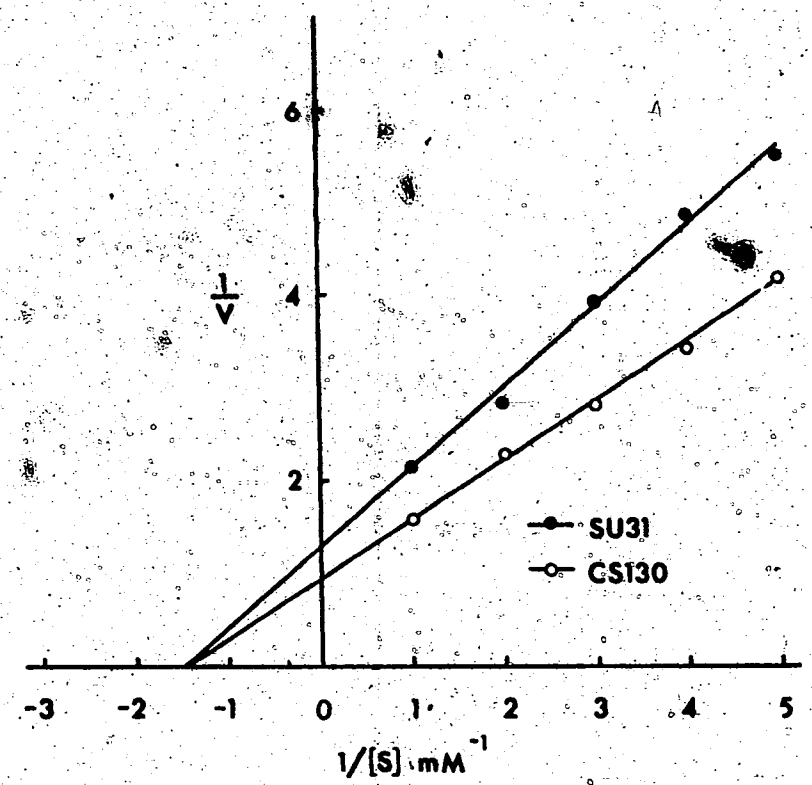
implicated in the stringent response (Parker *et al.*, 1976), it seems likely that the reversion mutation in RV3 is in one of the ribosomal proteins affecting the function of the *relA* product.

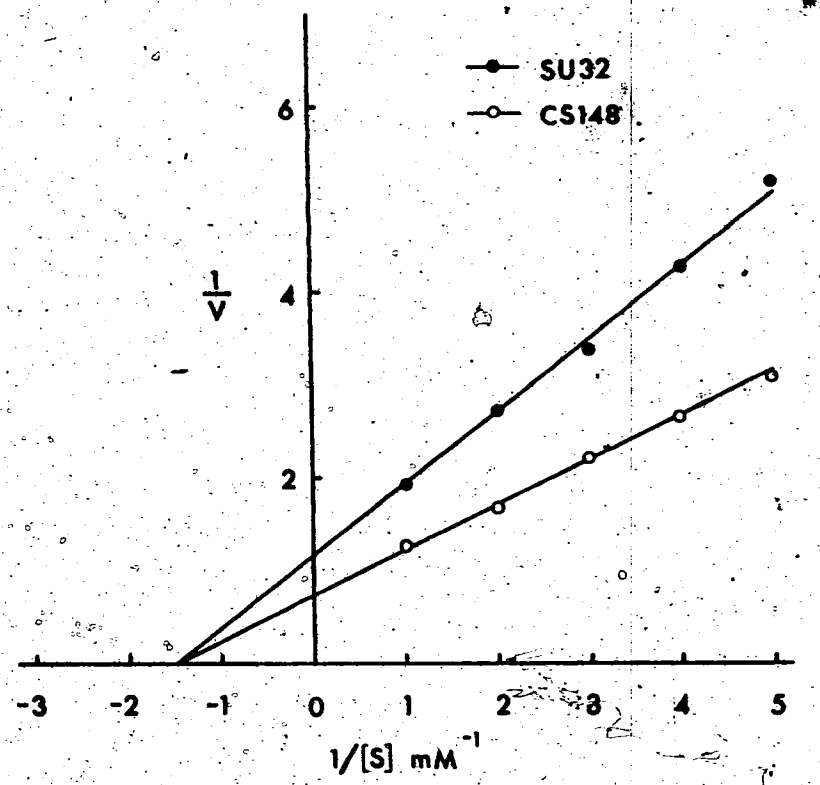
APPENDIX II



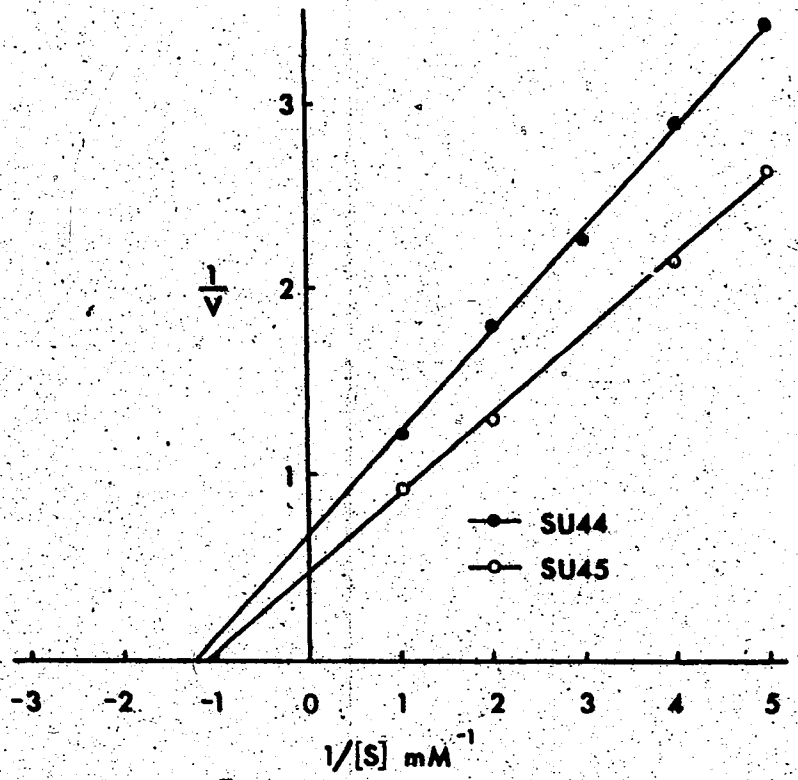


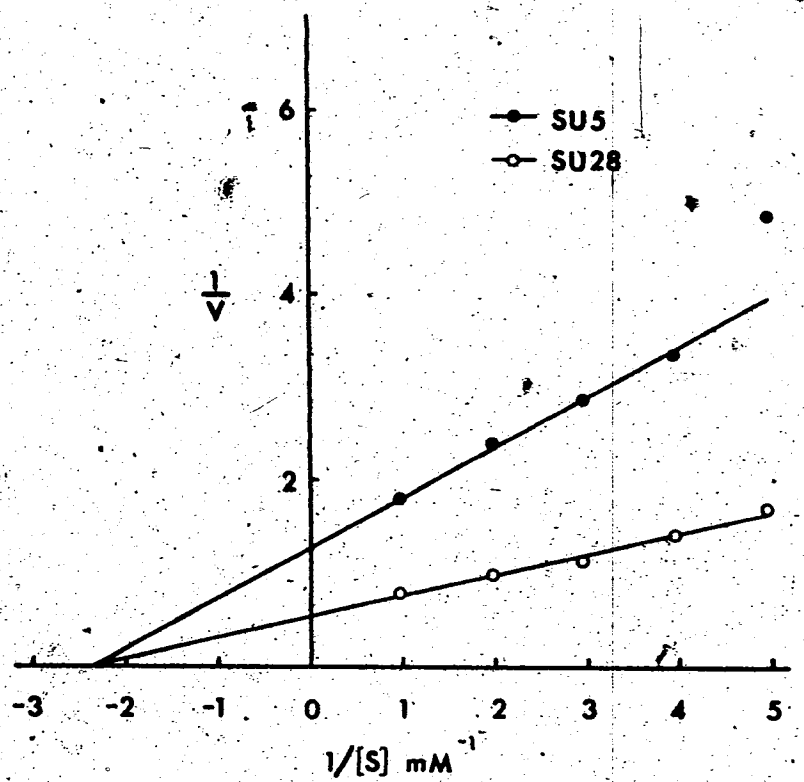
APPENDIX II - Lineweaver-Burke plots of the methionine activation reaction catalyzed by parental and revertant strains. The substrate [S] was L-methionine. The velocity (V) represents CPM x 10^{-5}. The conditions for the assay are presented in "Materials and Methods". The Km and Vmax values are presented in Table 8.



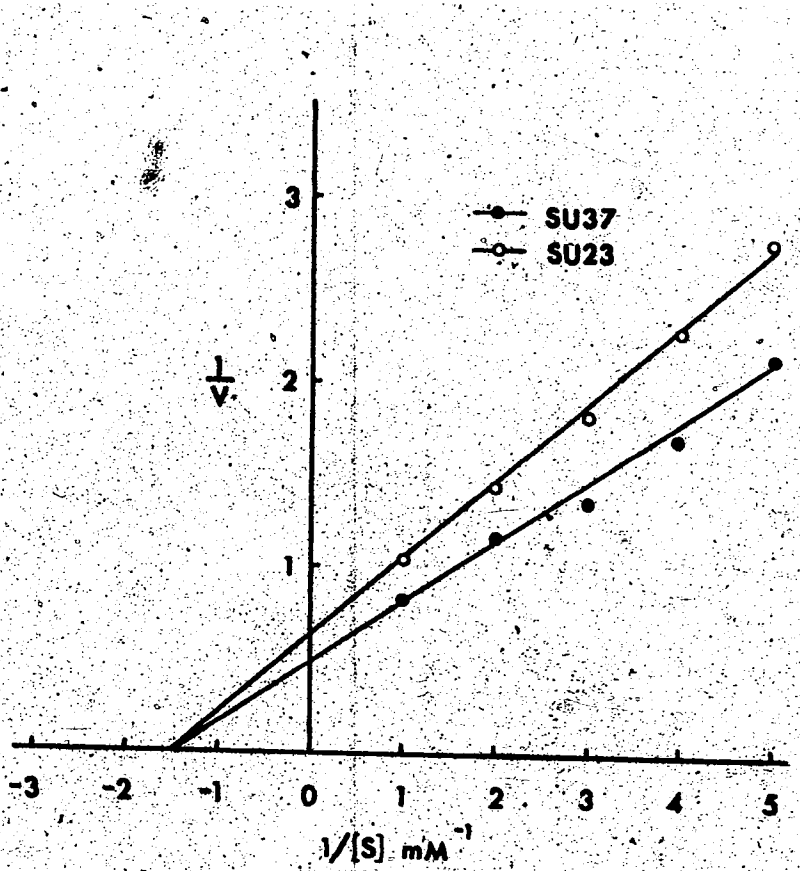


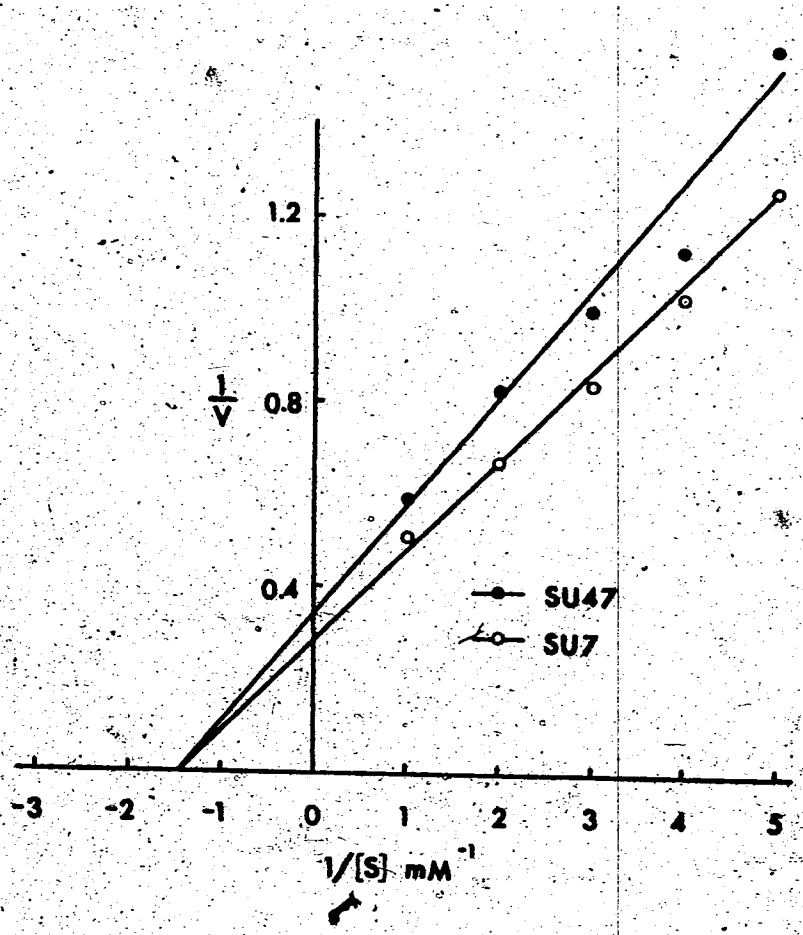
APPENDIX II - continued





APPENDIX II - continued





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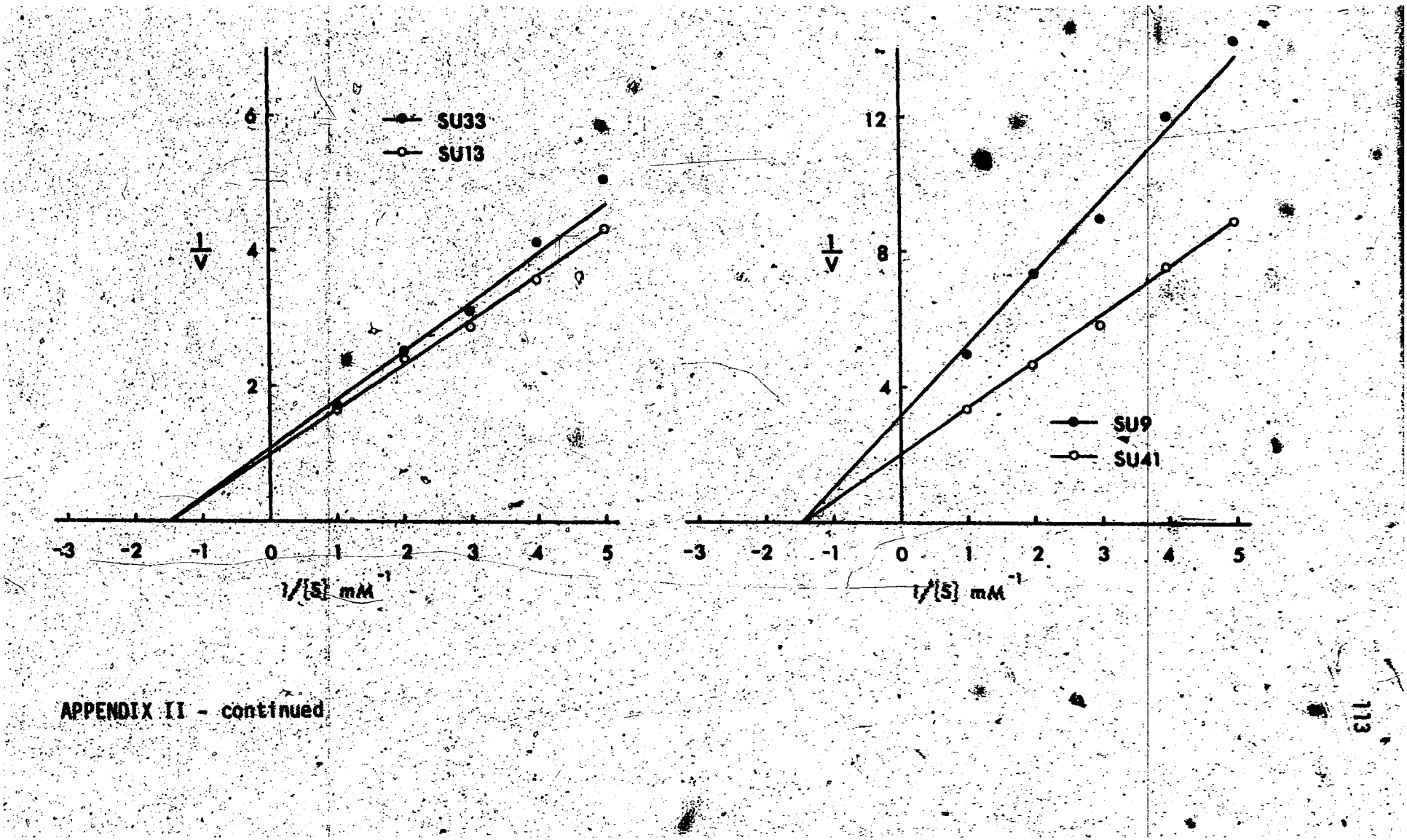

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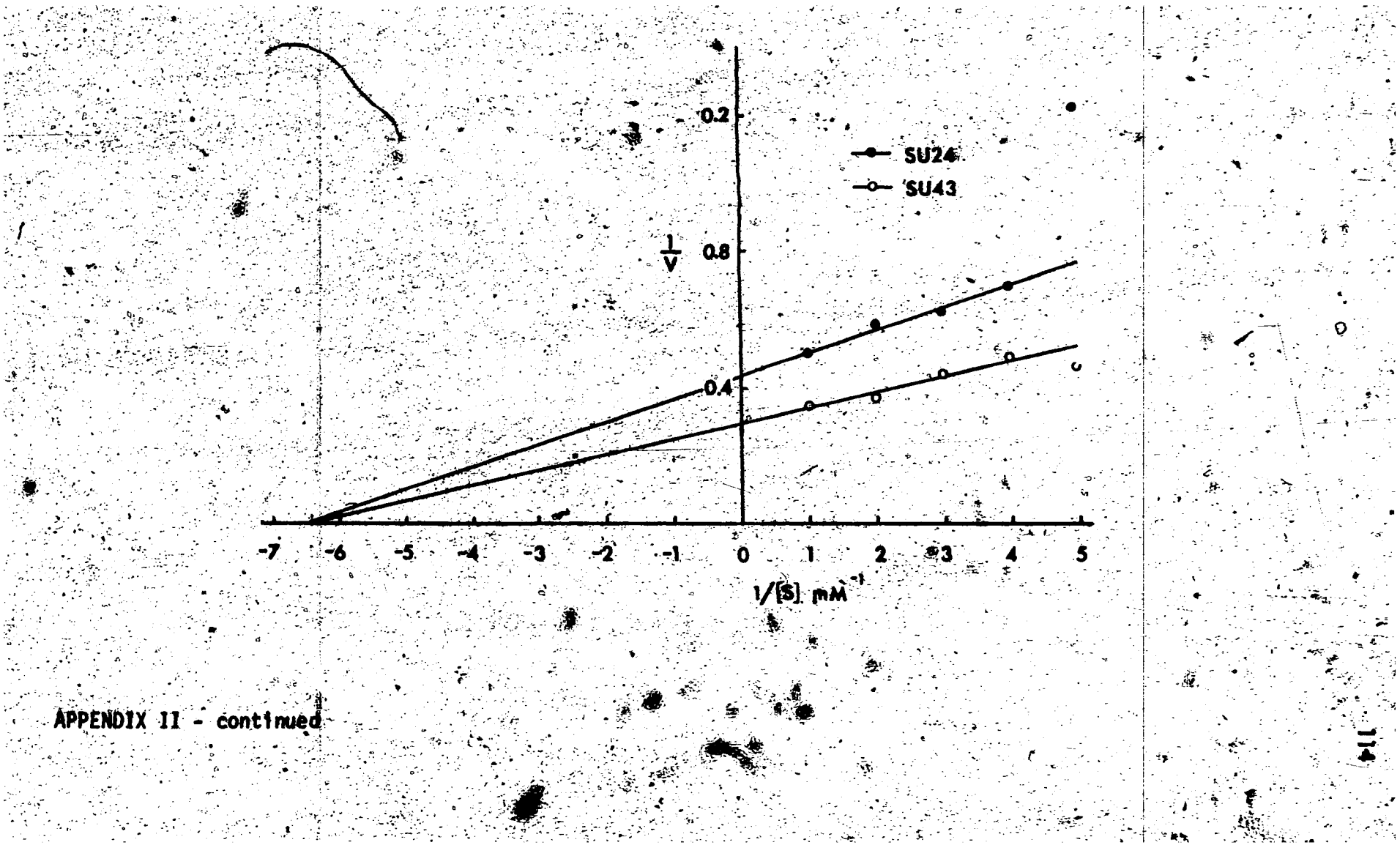

APPENDIX II - continued