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Suppression by Thy- strains of *Escherichia coli* as a Function of Folate Levels

Evangelos Tiganos

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ABSTRACT

Suppression by Thy- strains of *Escherichia coli* as a Function of Folate Levels

Evangelos Tiganos

Suppression is the phenomenon whereby a secondary mutation is in some way able to mask, or suppress, the phenotypic expression of the first mutation.

Suppression of nonsense mutants in infecting bacteriophage T4 by *Escherichia coli* mutants lacking thymidylate synthase was correlated to intracellular folate levels. All sulphonamides tested inhibited suppression by Thy- strains but did not affect the plating efficiency of wild type T4 phage. High concentrations of thymidine in the medium also inhibited the suppression additively with sulphonamides.

Genetic mutations causing blocks in the purine biosynthetic pathway which utilizes 10-formyl-tetrahydrofolate and in the histidine biosynthetic pathway, which can complement purine mutants upstream of AICAR, in some cases, made *thyA* suppression resistant to inhibition by thymidine and/or sulfisoxazole.

Total folate concentrations were determined and found to be significantly different in Thy- and Thy+ strains. Thy- strains were shown to have 20% higher folates than Thy+ strains under low thymidine (20.0μg/ml) conditions. The distribution of folates was also found to be different under

low thymidine (suppressing) conditions as compared to high thymidine (non-suppressing). Both Thy- and Thy+ strains had significantly more methyl folates under suppressing conditions.

ACKNOWLEDGEMENTS

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INTRODUCTION

Suppression is the phenomenon whereby a secondary mutation occurs at a site in the chromosome different from the first mutation site, and is in some way able to mask, or suppress, the phenotypic expression of the first mutation. In this case suppression will produce an organism that appears to be reverted but is in actual fact a double mutant.

There are two main classes of suppression (Hartman and Roth, 1973): (1) intragenic suppression, and (2) intergenic suppression.

Intragenic suppression occurs when a second mutation lies within the gene or carrying the first mutation. This must not be confused with reversion to the wild type, because even though the wild type phenotype has been restored, the original mutation is still present in the gene. An example of intragenic suppression is a double frameshift mutation in the rIIB cistron (Crick et al, 1961).

In intergenic suppression, the secondary mutation is in a different gene (cistron) from that of the first mutation. Intergenic suppression can originate in two different ways; one way is by direct or informational suppressors (Hawthorne and Leopold, 1974), and the other way is by indirect or functional suppressors. A functional suppressor overcomes the initial mutation without restoring the gene function. Therefore, the mutant phenotype is altered to that of the

parent by offering an alternative pathway to bypass the block caused by the initial mutation. For instance, if the primary mutation resulted in a less active enzyme, a secondary mutation affecting another biosynthetic pathway could make this pathway more active, and this could now compensate for the less active enzyme which resulted from the initial mutation. Functional suppressors are usually gene specific (Hartman and Roth, 1973). That is, they can suppress mutations affecting only a single gene or a group of related genes.

An informational or direct suppressor mutation affects genes whose products function in the translation of mRNA into protein. An informational suppressor therefore works at the translational level rectifying the mutation by inserting an amino acid in response to a premature termination codon, by substituting an amino acid for another one creating a functional protein or by correcting the reading frame. The suppressor strain permits a mutated codon to be translated incorrectly but beneficially. In Escherichia coli nine different tRNA genes have been observed to mutate so that their tRNA product now inserts an amino acid in response to a nonsense triplet (Goodenough, 1984).

Informational suppressors are allele specific (Hartman and Roth, 1973). That is they are able to suppress mutations affecting a variety of unrelated genes.

Suppression by Thymidine Requiring Strains:

E.coli cells that lack thymidylate synthase activity cannot catalyze the formation of thymidylate from uridylate and hence become auxotrophic for thymine or thymidine. In order to grow Thy-strains must be exogenously supplied with thymidine or thymine which they can convert to thymidylate and hence satisfy their pyrimidine requirements. If thymine or thymidine become limited these cells undergo extensive mutagenesis, and increased recombination, followed by death, (thymineless death), (Barclay et al, 1982).

Thy- strains also have a suppressor phenotype. Thymine auxotrophs suppress all three nonsense types of mutations and some frameshift mutations of T4 phage (Cheung and Herrington 1982; Herrington, Kholi, and Faraci, 1986). Although suppression occurs in media containing sufficient thymine or thymidine to support cell growth, it may require thymidylate limitation since it is inhibited by the addition of high concentrations of thymine or thymidine (Cheung and Herrington, 1982; Herrington, Kohli, and Lapchak, 1984). Many Thy- derivatives of E.coli K-12 acquire the ability to suppress (M.B. Herrington , Kohli and Lapchak, 1984). This suggests that the suppression phenotype is a consequence of the thyA mutation.

Suppression by Thy- strains is not the result of enhanced mutation frequency and consequently reversion, since phage produced under suppressing conditions retain their mutant phenotype; indicating that suppression rather than

reversion is occurring (Herrington, Lapchack, and Kohli, 1983).

Several observations indicate that Thy- strains are informational suppressors. First suppression by Thy- strains has been observed for mutations affecting a great variety of unrelated T4 functions including DNA polymerase, head components, lysozyme, and tail fibres, (Cheung and Herrington, 1982). This evidence strongly favors informational suppression since informational suppression affects a variety of unrelated genes. Secondly, suppression by thymine auxotrophs occurs during translation. When cells are made streptomycin resistant by the introduction of a mutant rpsL allele they no longer suppress nonsense mutations implying that the suppression event occurs at the ribosomal level (Herrington, Kholi, and Lapchak, 1984). Furthermore, the aminoglycoside antibiotic kasugamycin which reduces translational errors in vitro (Van Buul, Visser, and Knippenberg, 1984) inhibits suppression (E. Tiganos and M.B. Herrington, unpublished result). Thus, thyA mutations which prevent the biosynthesis of a DNA precursor appear to affect the accuracy of translation to produce a suppressor phenotype.

A model has been proposed to explain the suppression by Thy- strains (Cheung and Herrington, 1982). The thymidylate synthase reaction is the only known tetrahydrofolate dependent reaction, where tetrahydrofolate is stoichiometrically oxidized to dihydrofolate (O'Donovan and

Neuhard, 1970), suggesting that this reaction may regulate folate biosynthesis or the distribution of folate cofactors. Thymidylate synthase catalyzes the conversion of dUMP to dTMP (Fig 1.). In this reaction a methylene group and two reducing equivalents are transferred from 5,10-methylene tetrahydrofolate to dUMP, producing dTMP and dihydrofolate (Mollgaard and Neuhard, 1983). Regeneration of tetrahydrofolate subsequently occurs in a specific reaction catalyzed by dihydrofolate reductase with NADPH as the hydrogen donor (Neuhard and Nygaard, 1987). The model for suppression proposes that thymine requiring strains have different levels or distribution of folates than wild type cells. This folate imbalance may then lead to changes in tRNA modification patterns so that thymine requiring strains produce tRNA's that are more susceptible to misreading and frameshifting than those in wild type cells.

Alternatively, the modification of rRNA or proteins may be altered such that the translational fidelity is reduced. This would also result in suppression of nonsense codons.

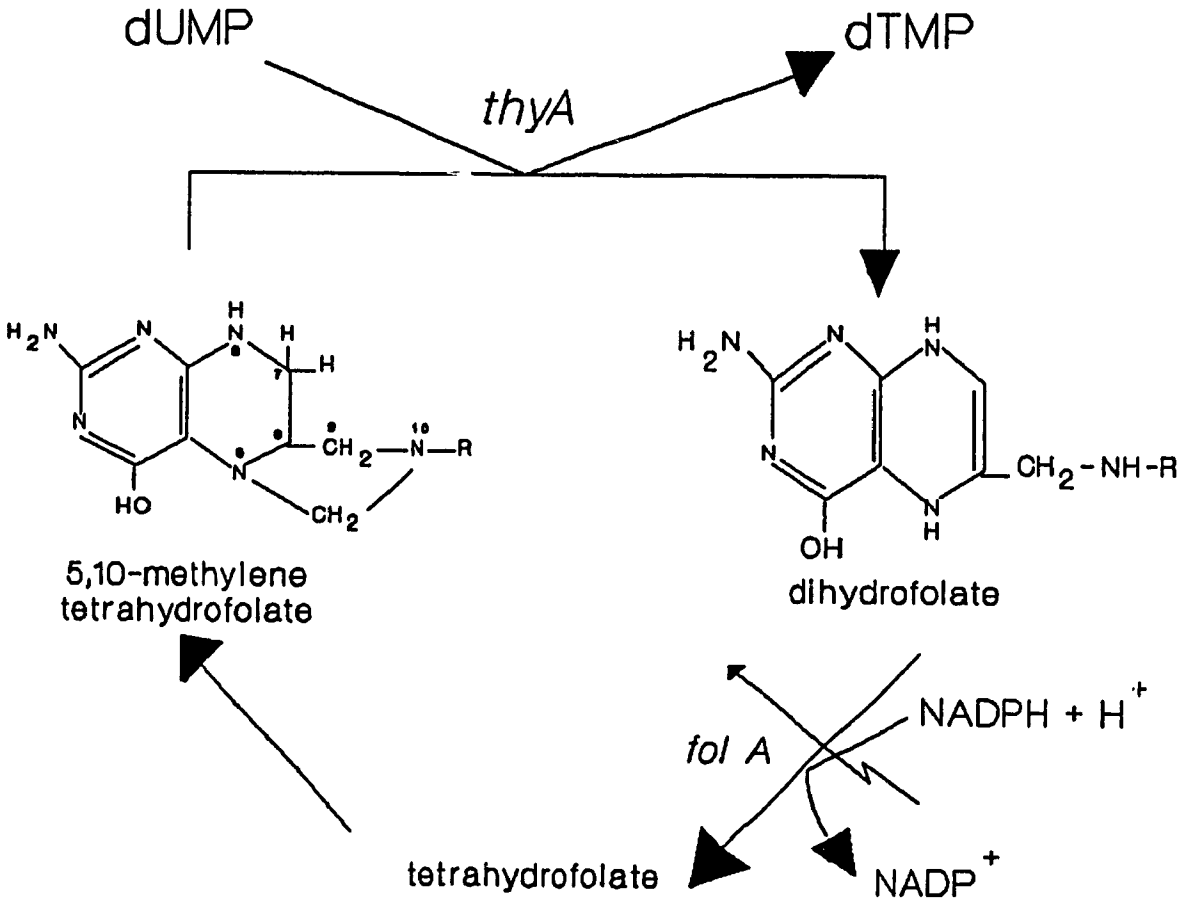
An alteration in tRNA modification resulting in a suppressor phenotype has been demonstrated in Salmonella typhimurium. S.typhimurium supK mutants have a defect in tRNA methylation resulting in the undermethylation of several types of tRNAs. Some of these undermodified tRNAs are capable of suppressing both UGA and frameshift mutations (Pope et al, 1978).

Figure 1

The Thymidylate Synthase Reaction

The thymidylate synthase reaction catalyzes the conversion of dUMP to dTMP. This is the only reaction in E. coli whereby N^5-N^{10}-methylenetetrahydrofolate is stoichiometrically oxidixed to dihydrofolate, suggesting that this reaction may regulate folate biosynthesis or the distribution of folate cofactors. In this reaction a methylene group and two reducing equivalents are transferred from 5,10-methylenetetrahydrofolate to dUMP, producing dTMP and dihydrofolate. Regeneration of tetrahydrofolate subsequently occurs in a specific reaction catalyzed by dihydrofolate reductase with NADPH as the hydrogen donor.

(Mollgaard and Neuhard 1983)


dUMP
dTMP
thyA
5,10-methylene
tetrahydrofolate
dihydrofolate
NADPH + H+
fol A
tetrahydrofolate
NADP+

The phenomenon of Thy- suppression is media dependent (Cheung and Herrington, 1982; M.B. Herrington, A. Kohli, and J. Basso, unpublished results). Suppression has only been observed in some rich media, such as the broth medium (AB medium) described by Apirion (Apirion, 1966). This suggests that AB medium somehow alters the metabolism in such a way as to favor suppression. For instance the media composition may directly influence the distibution of intracellular folates, rendering the conditions favorable for the observed suppression. AB medium contains many of the folate end-products, such as glycine, methionine, purines, and thymidine. Since these products are readily available to E.coli, one would assume that they are quickly internalized and utilized for growth. This may result in a sparing effect for folates which may be necessary to support suppression.

In an attempt to determine if folate metabolism was involved in suppression I looked at the effect of perturbing folate levels. Folate metabolism is tightly linked to a number of metabolic pathways, such as the purine pathway and may have a regulatory role in histidine biosynthesis. Consequently by genetically modifying the flow through these pathways, folate levels may either increase or decrease. Furthermore environmentally induced changes may alter folate levels. Such environmental stimuli include anti-folate drugs as well as the presence of folate end-products.

Folates:

Folic acid or folates are the general terms for a group of compounds which consist of a substituted pteridine, p-aminobenzoate (p-ABA), and one or more L-glutamic acid units (Keagy, 1980)(Fig.2).

The enzymatic reactions required for the synthesis of folates, in E.coli, from p-aminobenzoate, glutamate, and a pteridine derived from guanylate (GMP), are illustrated in Fig.3 . The first enzyme in folate biosynthesis catalyzes the formation of the pyrophosphate ester of H_2-pterin-CH_2OH. Once this intermediate is synthesized then the enzyme H_2-pteroate synthase utilizes it along with p-ABA and forms H_2-pteroate. A glutamate is then added to H_2-pteroate to form H_2-folate (Brown and Williamson, 1987).

Tetrahydrofolates (THF) vary in one-carbon units (Stryer, 1981). These units can be interconverted (Fig.4), to form the biologically important coenzymes. For instance N-5,N-10-methylene-tetrahydrofolate can be reduced to N-5-methyltetrahydrofolate by a methylene-THF reductase. In a similar manner N-5,N-10-methenyltetrahydrofolate, can be oxidized by methylene-THF dehydrogenase to N-10-formyltetrahydrofolate (Stryer, 1981).

Within the cell folates act as coenzymes and the tetrahydrofolate derivatives are essential for the biosynthesis of thymidylate, purines, glycine, methionine, formyl methionine, and pantothenate (Bognar et al., 1987).

Figure 2

Structure of a Folic Acid Molecule

Folic acid molecules consist of a substituted pteridine, a p-aminobenzoate, and one or more L-glutamic acid units. (Keagy ,1980)

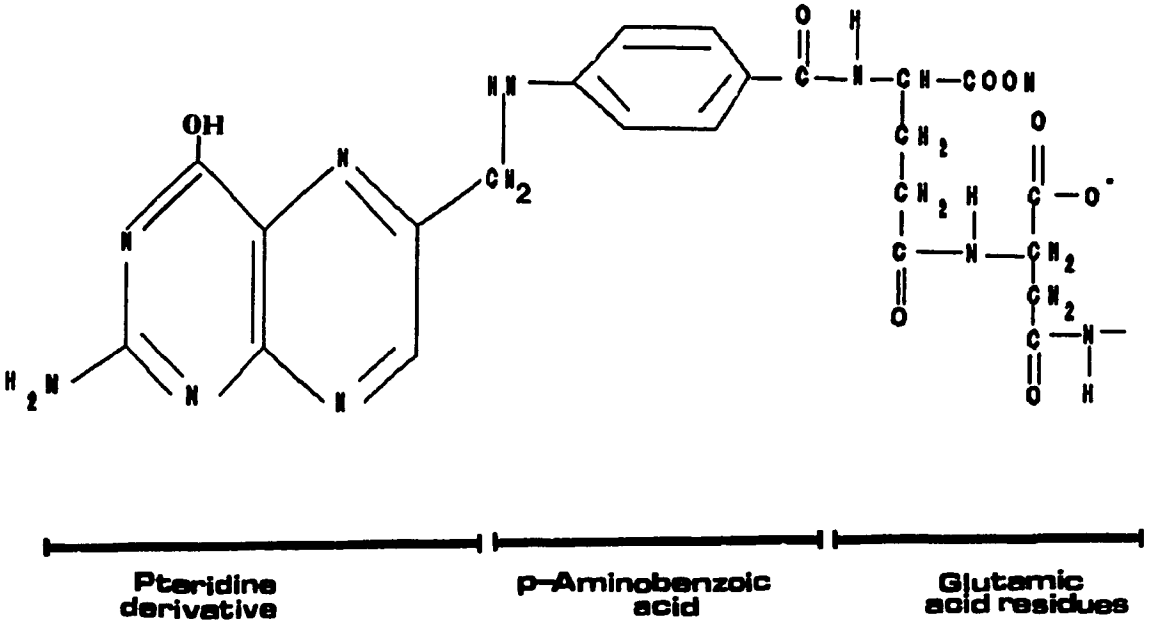

Pteridine derivative
p-Aminobenzoic acid
Glutamic acid residues

Figure 3

The Reactions Involved in Folate Acid Biosynthesis

Folates are synthesized from p-aminobenzoate, (P-ABA), glutamate, and a pteridine derived from guanylate,(GMP). The first enzyme in this reaction catalyzes formation of the pyrophosphate ester of H_2-pterin-CH2OH. This intermediate along with P-ABA are further metabolized to form H_2-pteroate, to which a glutamate is added to form H_2-folate.

(Brown and Williamson 1987).

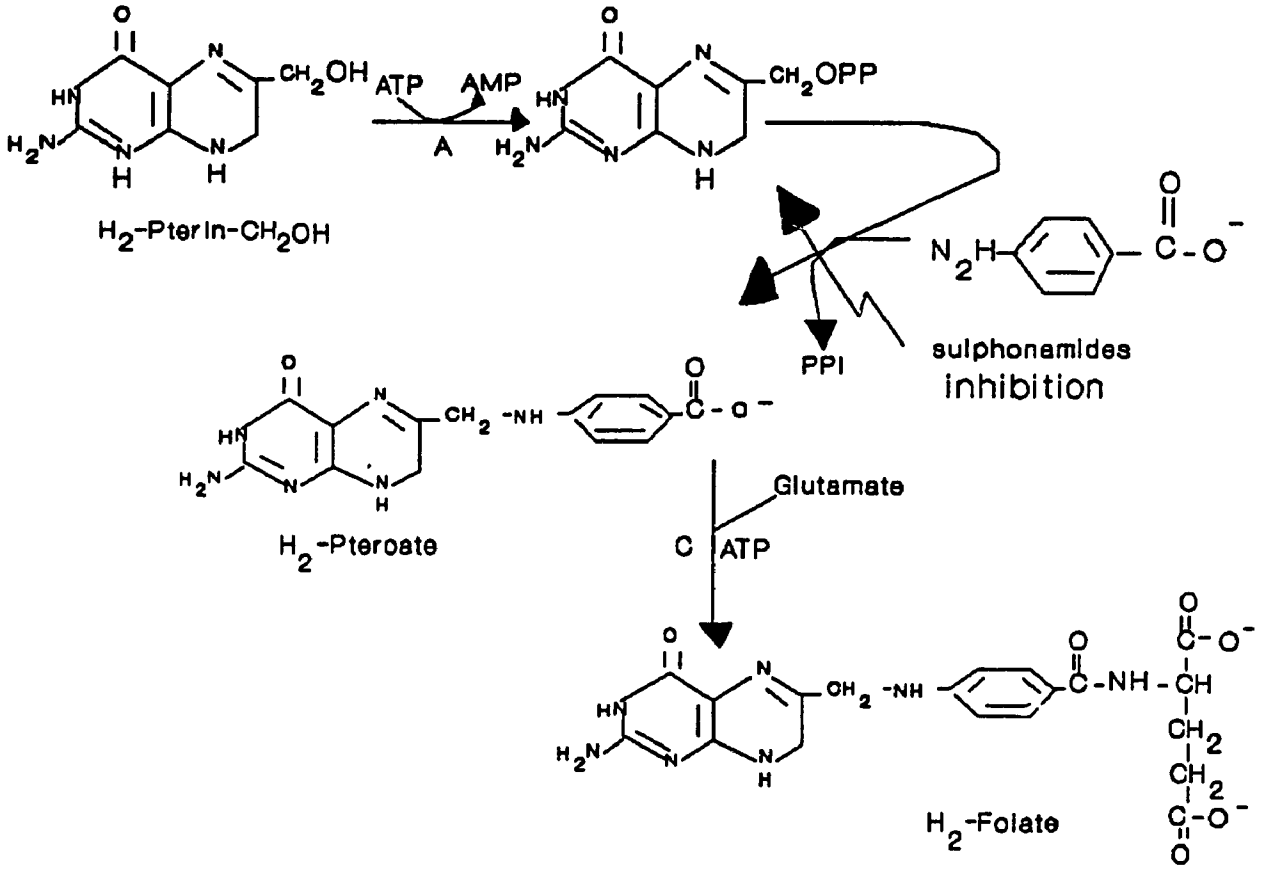
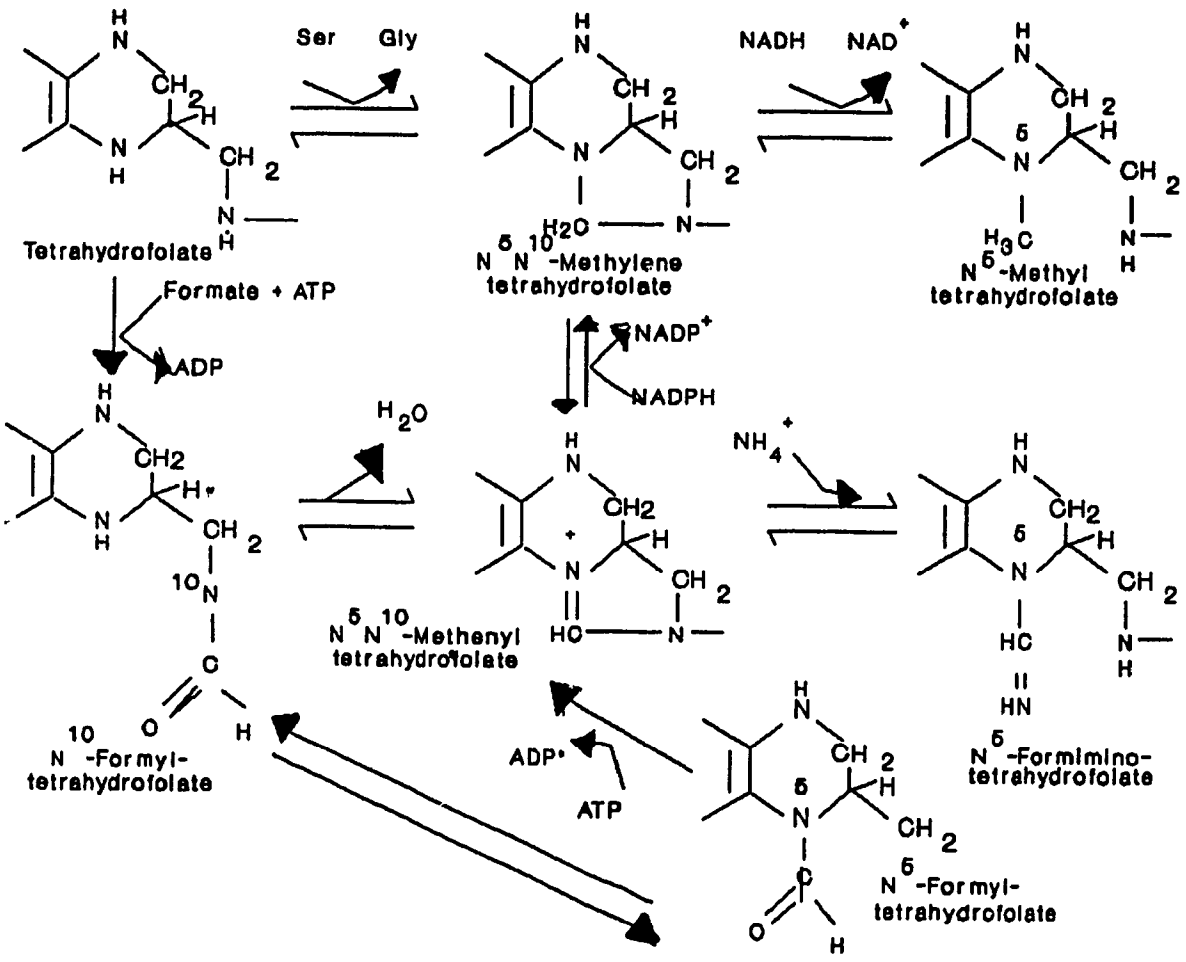

ATP
AMP
A
CH_2OH
CH_2OPP
H_2-Pterin-CH_2OH
PPi
sulphonamides
inhibition
H_2-Pteroate
Glutamate
C
ATP
H_2-Folate

Figure 4

The Conversion of One Carbon Units Attached to Tetrahydrofolate

Tetrahydrofolates are unique in that they may vary in one carbon units. These units can be reduced or oxidized to form the biologically active coenzyme

(Stryer, 1981).


Ser
Gly
NADH
NAD+
Tetrahydrofolate
N5N10-Methylene tetrahydrofolate
N5-Methyl tetrahydrofolate
Formate + ATP
ADP
NADP+
NADPH
H2O
NH4+
N5N10-Methenyl tetrahydrofolate
10 N-Formyl-tetrahydrofolate
N5-Formimino-tetrahydrofolate
ADP+
ATP
N5-Formyl-tetrahydrofolate

During methionine biosynthesis, methionine is synthesized via homocysteine by the transfer of the methyl group of N-5-methyl-tetrahydrofolate. In purine biosynthesis, some of the carbon atoms incorporated into the purine ring are derived from the N-10-formyl derivatives of tetrahydrofolate. Similarly the methyl group of thymine, a pyrimidine, is donated by N-5,N-10-methylenetetrahydrofolate. Tetrahydrofolate and serine are required for the synthesis of glycine. Furthermore, folate coenzymes are also required for the synthesis of formylmethionine and pantothenate (Stryer, 1981).

In addition to donating one-carbon units during anabolic reactions, tetrahydrofolates can also serve as acceptors of one-carbon units in some catabolic reactions. For instance in S.typhimurium the breakdown of histidine results in the formation of N-formiminoglutamate, which in turn transfers its formimino group to tetrahydrofolate to form the N-5-formimino derivative (Stryer, 1981).

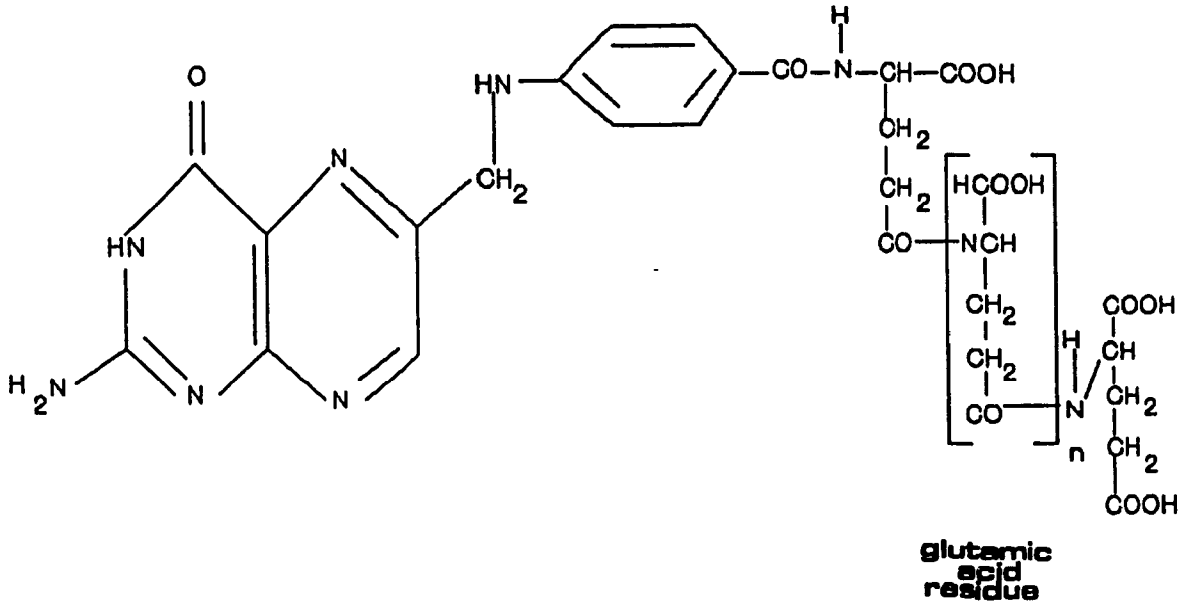
Polyglutamation:

Folate coenzymes can be found in two forms, either as pteroylmonoglutamates or as poly-γ-glutamates (McGuire et al, 1980) (Fig.5). Almost without exception whenever folates are isolated from natural sources they are found predominantly as poly-γ-glutamates, containing anywhere from two to twelve glutamates (Baugh and Krumdieck, 1971).

Figure 5

The Structure of a Polyglutamate

Folate coenzymes may be found in two forms, either as pteroylmonoglutamates or as poly-γ-glutamates. These poly-γ-glutamates contain anywhere from two to twelve glutamates. (Covey, 1980)


glutamic
acid
residue

Until recently the physiological roles of folyl - polyglutamates in one-carbon metabolism were not understood. It was originally believed that folypolyglutamates served only as storage forms of the vitamin and that the small amounts of monoglutamates present in cells were the metabolically active cofactors. As the pathways of one-carbon metabolism became better understood, polyglutamates were shown to possess equal or greater coenzyme activity than the corresponding monoglutamates, in most cases (Krumdieck and Baugh 1969). One of the earliest examples of this was the increased coenzyme activity of 10-formyl-H_4PteGlu$_n$ over its monoglutamate counter-part 10-formyl-H_4PteGlu, in Clostridium sticklanddii (Covey, 1980).

Therefore one way of perturbing folate distribution was by introducing environmental stimuli or mutations which might alter the polyglutamation state of the intracellular folates

Changes in folate distribution may be environmentally induced (Foo and Shane, 1980) in Chinese hamster ovary cells (CHO). For example, it has been demonstrated that variations in methionine levels can modulate the 5-methyl-H_4PteGlu$_n$ pool via SAM inhibition of methylenetetrahydrofolate reductase (Krebs et al, 1976), or by the inhibition of methionine synthase (Cheng et al, 1975). It has been shown that suboptimal or high concentrations of methionine in the medium favor the formation of pteroylmonoglutamate derivatives of 5-methyl-H_4PteGlu (Foo and Shane, 1982). Since these pteroylmonoglutamates are used less efficiently by such

enzymes as methionine synthases, this would cause the accumulation of 5-methyl-H_4PteGlu (Foo and Shane, 1982). Furthermore, since this folate coenzyme is a poor substrate for folylpolyglutamate synthetases (McGuire and Bertino, 1981), (Cichowicz et al, 1981), this would undoubtedly change the rate of folypolyglutamate synthesis. This would lead to the alteration in the distribution of intacellular folates.

Similarly, it been observed that bacteria cultured in medium that contains high levels of adenine display large changes in their distribution of their folypolyglutamates. This results due to an adenine induced redistribution of folate one-carbon forms favoring formyl derivatives, which are poor substrates for bacterial folylpolyglutamate synthetases (Cichowicz et al, 1981). This however is not true for the E coli folypolyglutamate synthetase which prefers formylmonoglutamate derivatives as substrates (Brown and Williamson, 1987).

Genetic modifications have also been shown to alter intracellular folate pools and distribution. Chinese hamster ovary (CHO) cell mutants which lack folylpolyglutamate synthase, have reduced folate levels due to the inability to synthesize folylpolyglutamates (McBurney and Whitmore, 1974), (Taylor and Hanna, 1977), (Taylor and Hanna, 1979). The low folate levels in these mutants are due to the failure of these mutants to intracellularly retain their folates. Thus, polyglutamation is important for the retention of intracellular folates, and for their compartmentalization.

Retention of polyglutamates however would not be important in E.coli as it is in CHO mutants, since this bacteria does not transport folates and depends on biosynthesis for its folate pools (Brown and Williamson, 1987).

Folate distribution may also be altered by overproduction of the folC gene product. The folC gene codes for the above mentioned folypolyglutamate synthase enzyme (FPGS), which catalyzes the addition of glutamic residues to folates.

FPGS has been purified from a number of bacteria, including Lactobacillus casei (Bognar and Shane 1983), Corynbacterium sp. (Shane, 1980), and E.coli (Bognar et al, 1985). In Corynbacterium sp., and in E.coli, this enzyme is also able to catalyze the reaction by which dihydropteroate is converted to dihydrofolate, and therefore is required for the de novo biosynthesis of folate (Bognar et al, 1985), (Shane, 1980), (Ferone and Warskow, 1983).

In E.coli overexpression of the folC gene is unlikely to lead to any increase in polyglutamylation. In E.coli the folC product has been shown to only synthesize triglutamates and almost all of the cellular folate pools are already triglutamated (Brown and Williamson, 1987). Since folate compounds with three or more glutamate residues occur in E.coli, the possibility thus exists that more than one enzyme is needed for the formation of these higher polyglutamates (Brown and Williamson, 1987). Some evidence does exist for

the presence of such an additional enzyme that adds several more glutamates but in α-linkages (Brown and Williamson, 1987). Therefore, the only likely result of overproduction of the folC gene is the accumulation of intracellular dihydrofolate. This increase in one type of folate may alter the distribution of the folate cofactors, if dihydrofolate is no longer efficiently converted to the tetrahydrofolate forms. The lack of conversion amongst the different folate species might then fail to produce the required form of folate for suppression to occur.

Inhibitors of Folate Biosynthesis:

A number of drugs and metabolites have been identified which are postulated to either perturb directly folate biosynthesis or distribution.

Sulphanilamides are competive inhibitors of the enzyme dihydropteroate synthase, competing for the same active site as p-aminobenzoic acid (p-ABA), resulting in a decrease in folate biosynthesis. This enzyme links p-ABA and 2-amino-4-hydroxy-6-pyrophosphorylmethyl-7-8-dihydropteridine, (H_2pteridine-CH_2O-PP), to give rise to the dihydropteroate precursor -7-8-dihydropteroic acid, (H_2-pteroate), (Fig. 3) (Swedberg and Skold, 1980). It also has been postulated that sulphanilamides become incorporated in place of p-ABA by the enzymatic reaction (Swedberg and Skold, 1980). The resultant analogs of dihydropteroate have been shown to inhibit another step in the folate biosynthetic pathway (Lopez et al, 1987).

Folate biosynthesis can also be perturbed drastically by limiting p-ABA, the substrate for dihydropteroate synthase (Gots and Chu 1952), or by inhibiting dihydrofolate reductase by the addition of trimethoprim (Bochner and Ames, 1982).

Folate Alarmone:

When folate biosynthesis is inhibted in S.typhimurium, there is a characteristic accumulation in a unique ribonucleotide, 5-amino-4-imidazole-carboxamide-riboside-5'-triphosphate (ZTP). Starving the cell for other molecules not related to folates does not result in the accumulation of ZTP. Therefore it has been proposed that ZTP is an alarmone for folate deficiency and that it is synthesized via ZMP (AICAR), which is an intermediate in purine biosynthesis (see Fig.6) (Bochner and Ames, 1982). Studies have demonstrated that whenever 10-formyl-THF, a substrate for ZMP-transformylase is depleted, there is a characteristic increase in AICAR (Bochner and Ames, 1982). Therefore perturbing AICAR levels may perturb folates and affect suppression.

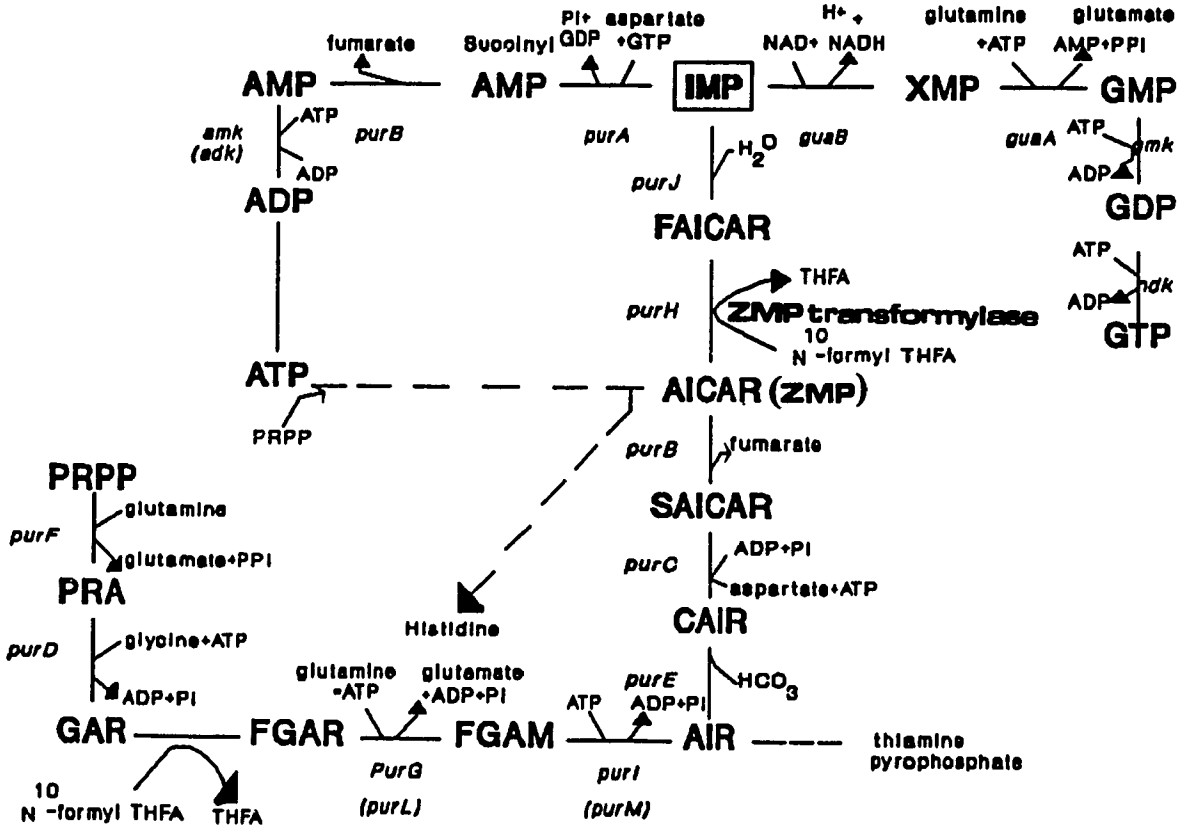
AICAR is made via two biosynthetic pathways: the purine biosynthetic pathway and the histidine pathway. Therefore, I attempted to alter AICAR levels by perturbing purine and histidine metabolism. These pathways were manipulated both genetically and metabolically.

Figure 6

De novo synthesis of ATP and GTP

The individual enzymes are identified by their gene symbols as used in S.typhimurium. E.coli gene symbols are in most cases identical; exceptions are given in parentheses. THFA (tetrahydrofolic acid), PurF (PRPP-amidotransferase), PurC (SAICAR-synthetase), PurE (AIR-carboxylase), PurD (Gar-synthetase), PurH (AIR-transformylase), FGAM (5'-phosphoribosyl-N-formylglycinamide), AIR (5--phosphoribosyl-5-aminoimidazole).

(Neuhard and Nygaard 1987)


AMP
fumarate
Succinyl AMP
Pi+ GDP
aspartate +GTP
IMP
H+ + NAD+ NADH
XMP
glutamine +ATP
glutamate AMP+PPi
GMP
amk (adk)
ATP
ADP
purB
purA
purJ
H2O
guaB
guaA
ATP
gmk
ADP
GDP
ADP
FAICAR
ATP
ndk
ADP
GTP
purH
THFA
ZMP transformylase
10 N -formyl THFA
ATP
AICAR (ZMP)
PRPP
purB
fumarate
PRPP
SAICAR
purF
glutamine
glutamate+PPi
PRA
purC
ADP+Pi
aspartate+ATP
CAIR
Histidine
purD
glycine+ATP
ADP+Pi
glutamine +ATP
glutamate +ADP+Pi
purE
HCO3
ATP
ADP+Pi
GAR
FGAR
FGAM
AIR
thiamine pyrophosphate
10 N -formyl THFA
THFA
PurG (purL)
purI (purM)

Alteration in Purine Biosynthesis:

Folate coenzymes are essential for the biosynthesis of purines. Purines specifically require two formyl-THF molecules for each purine synthesized. One folate is used at a step preceding the synthesis of the folate alarmone AICAR and one is used to convert AICAR to FAICAR (Fig. 6). Therefore either AICAR levels or the amount of 10-formyl-THF used for purine biosynthesis might be altered by the introduction of various mutations in purine genes. These changes might then affect folate levels and suppression.

Alteration in Histidine Biosynthesis:

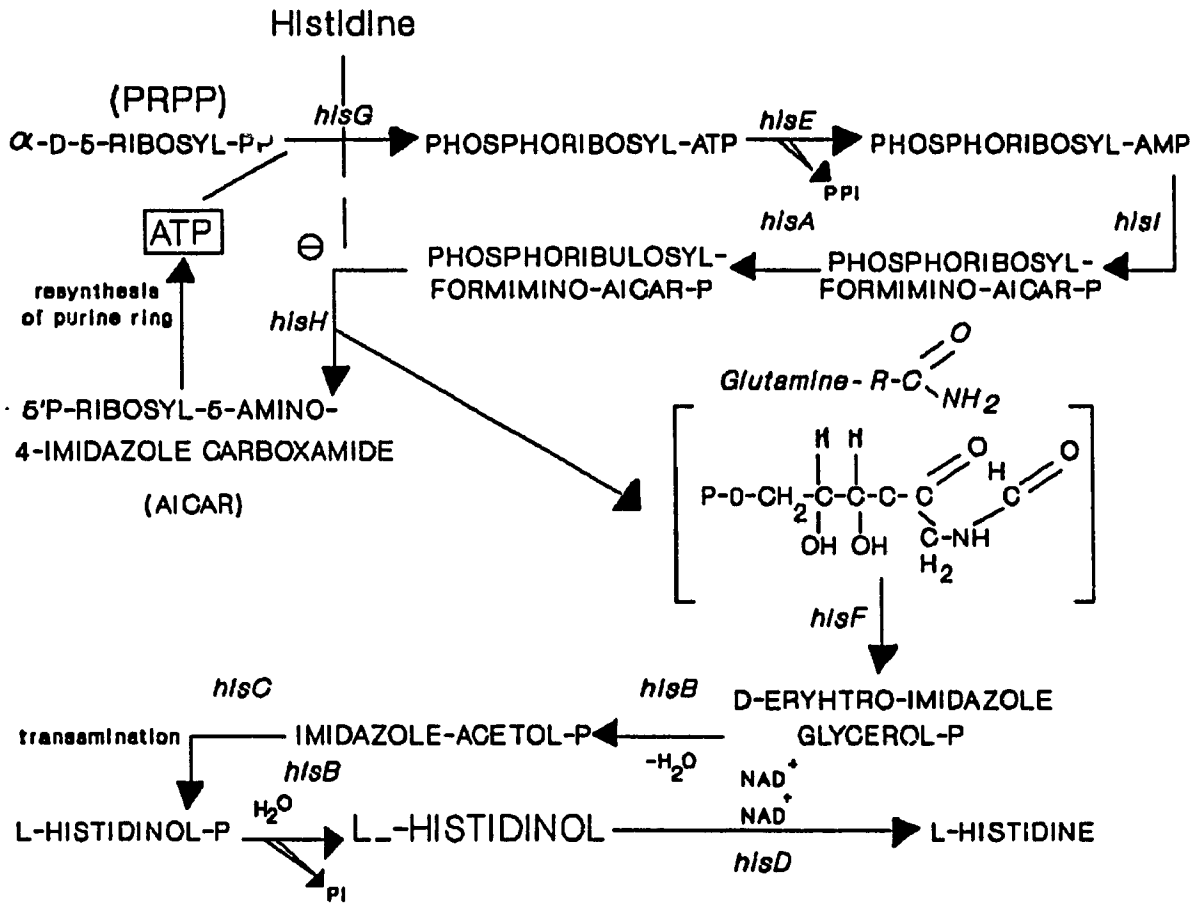
Changes in histidine biosynthesis might also perturb folate metabolism, since one formyl-THF molecule is utilized to regenerate the adenine moeity required for histidine biosynthesis. (Fig. 6). As in the case of the purine pathway, it is possible to construct histidine auxotrophs blocked at various steps along the pathway (Fig 7), and see if these mutations affect suppression.

This study was undertaken to determine if <u>thyA</u> dependent suppression is a function of altered folate levels. In support of this model, I will demonstrate that the above mentioned environmental or genetic factors which have been reported to affect folate distribution result in altered suppression patterns. In addition, I will demonstrate that the abolishment of suppression by conditions expected to

Figure 7

The Histidine Biosynthetic Pathway

The enzymes are identified by their gene symbols. HisG (ATP-phosphoribosyltransferase), hisD (Histidinol dehydrogenase, hisC (Histidinol-phosphateaminotransferase), hisB (Imidazoleglycerol-phosphate dehydratase, hisH (glutamine-amidotransferase), hisA phosphoribosylformimino-5-amino-1-phosphoribosyl-4-Imidazole carboxamide isomerase, hisF (cyclase), hisIE (phosphoribosyl-ATP pyrophosphohydrolase). (Winkler, 1987)


Histidine
(PRPP)
α-D-5-RIBOSYL-PP
hisG
PHOSPHORIBOSYL-ATP
hisE
PPI
PHOSPHORIBOSYL-AMP
ATP
hisA
hisI
PHOSPHORIBULOSYL-FORMIMINO-AICAR-P
PHOSPHORIBOSYL-FORMIMINO-AICAR-P
resynthesis of purine ring
hisH
5'P-RIBOSYL-5-AMINO-4-IMIDAZOLE CARBOXAMIDE (AICAR)
Glutamine-R-C
NH2
hisF
hisC
hisB
transamination
IMIDAZOLE-ACETOL-P
D-ERYHTRO-IMIDAZOLE GLYCEROL-P
-H2O
NAD+
hisB
H2O
L-HISTIDINOL-P
L-HISTIDINOL
NAD+
L-HISTIDINE
hisD
PI

lower intracellular folate levels, such as anti-folate drugs, may be overcome by a number of biosynthetic alterations in the purine and histidine pathway. I will also quantify folate levels in order to show a correlation between the suppression ability of thymine auxotrophs and the quantity of THF present.

MATERIALS AND METHODS:

BACTERIA

The E.coli K-12 derivatives used are described in Table 1. Strains were maintained on AB agar plates (see "media"). Lactobacillus casei ATCC 7469 and Enterococcus hirae ATCC 8043 were obtained from the American Type Culture Collection. These bacteria were maintained by preparing two agar stabs, (see "media") and were incubated at 37°C for 24hr. One stab was reserved unopened and was stored at 4°C. The other stab was used to prepare cultures for experiments. Fresh stab vials were prepared each month from the previously unopened culture.

BACTERIOPHAGE STRAINS

Bacteriophage T4 mutants are listed in Table 2.

P1CM phage was used for transductions.

PLASMIDS

Plasmids used during this study are described in Table 3.

MEDIA:

The AB medium consists of 10.0 g of Difco Nutrient Broth and 10.0 g of Difco Vitamin free casamino acids in one liter of distilled water. The AB medium is the broth medium described by Apirion, (1966). It was solidified with the use

Table 1. List of *Escherichia coli* strains

Strain[a]	Genotype[b]	Source and Reference[c]
CA165	*supB lacI lacZ*	M.C. Ganoza[(1)]
CAJ64	*lacZ* Su2	J. Friesen[(2)]
CR63	*supD60*	M.C. Ganoza[(3)]
MH128	*metB lysA24 rna*	M.B. Herrington[(4)]
MH294	*metB leuB*	M.B. Herrington
	hisB::Tn10	unpublished results
	thyA723	
MH429	*metB thyA723 rna*	M.B. Herrington[(4)]
MH540	*metB thyA723 rna*	this study **
	purF91::Tn10	
MH540a	*metB thyA723 rna*	this study **
	purF91	
MH542	*metB thyA723 rna*	J. Basso
	ArgE86::Tn10	unpublished results
MH543	*metB thyA723 rna*	J. Basso
	ArgE86::Tn10 purH	unpublished results
MH570	*metB thyA723 rna*	this study **
	purE79::Tn10	
MH575	*metB thyA723 rna*	this study **
	hisG::Tn10	

TABLE 1 CONT'

MH578	metB thyA723 rna purC::Tn10	this study **
MH581	metB rna	J. Basso unpublished results
MH616	metB thyA723 rna purF91 hisG::Tn10	this study **
MH652	metB thyA723 rna purD81::Tn10	this study **
MH653	metB thyA723 rna purB fadR613::Tn10	this study **
MH654a	hisI903::Tn10 malA1	this study **
MH654	metB thyA723 rna hisI903::Tn10	this study **
MH655a	hisA323::Tn10	this study **
MH655	metB thyA723 rna hisA323::Tn10	this study **
MH656	metB thyA723 rna hisB::Tn10	this study **
MH657	metB thA723 rna purFΔ91	this study **
MH658	metB thyA723 rna purFΔ91	this study **

TABLE 1 CONT'D

MH659	metB thyA723 rna purF▲91	this study **
MH660	metB thyA723 rna purF▲91	this study **
MH667	metB thyA723 rna hisG▲	this study **
MH668	metB thyA723 rna hisG▲	this study **
MH669	metB thyA723 rna hisG▲	this study **
UTH046	hisI903 malA1 lambda^{R}	CGCS[d]
HfrG6	hisA323 lambda^{s}	Ibid
NK6051	(gpt-lac)5 purE79::Tn10 lambda^{s}	CGCS
NK6056	(gpt-lac)5 purC80::Tn10 lambda^{s}	Ibid
NK6069	proA82 (proA or proB) purD::Tn5 supE44	Ibid
NK6073	pro-82 purF91::Tn10 supE44	Ibid

(a) All strains are E coli K-12 derivatives

(b) Symbols are from Bachmann and Low

(c)

(1) Hirsch, 1971

(2) Sambrook et al, 1967

(3) Bachmann, 1973

(4) Herrington, Kohli, and Faraci, 1986.

(d) Coli Genetic Center, B. Bachmann, curator.

(e) Details for the strains constructed are given in Table 4.

(f) Tet^S strains were selected using the Bochner plate method, described in methods and materials, pp. 44.

Table 2. List of bacteriophage T4 strains

Strain	Affected gene	Affected gene product	References
T4	Wild type		
UAG mutant			
M103	e	lysozyme	Streisinger et al., 1960
JC1912	43	DNA polymerase	Edgar and Lielausis, 1964
JC1916	e	lysozyme	Person and Osborne,1968
UAA mutant			
JC1922	e	lysozyme	Yahata, Ocada and Tsugita 1970
UGA mutant			
eLP12	e	lysozyme	J.Owen, 1976
OPC100	23	head component	Wilson and Kells 1972
OPC105	34	tail fibre	Ibid

Table 3: **Plasmids**

Plasmid	Vector	Insert	Features	Reference
pAC5	pUC9	KpnI-PvuI	Contains the *folC* gene downstream from the lac promoter. ampicillin resistant.	Bognar,1987
pHC9800	pBR313	HindIII-Bgl II	Contains the complete *his* operon. tetracycline resistant, ampicillin resistant.	Bruni, 1983

of 15.0 g Bactoagar per 1 liter for plates and with 5.0 g/L for soft agar. The former was used for storage of the bacterial strain while the latter was used for fast phage assays. AB soft agar was stocked in 150.0 ml bottles and melted in a boiling water bath, and was then supplemented with thymidine at a final concentration of 20.0 μg/ml.

The LB medium, (Miller, 1972), used consists of 10.0 g tryptone, 5.0 g yeast extract and 5.0 g NaCl per liter of medium. When this media was used in plates, 10.0 g Sigma agar/liter was added.

The Minimal Media A used was the one described by Miller, 1972. It consists of 10.5 g K2HPO4, 4.5 g KH2PO4, 1.0 g (NH4)2SO4, 0.5 g sodium citrate.$2H_2O$ per liter of medium. After autoclaving 1.0 ml/L minimal medium of 20% MgSO4 and 10.0 ml/L of 20% glucose were added. Required supplements were usually added at 50.0 μg/ml for most of the purines and pyrimidines, and amino acids, with exception of adenine and adenosine which were added at 40.0 μg/ml. Vitamin supplements were added at a final concentration of 1.0 μg/ml. Minimal media A agar plates contained of 10.0 g/L of Sigma agar.

Superbroth was used in the preparation of the P1 lysates used for transductions. Superbroth consists of 32.0 g tryptone, 20.0 g yeast extract, 5.0 g Nacl and 5.0 ml of 1N NaOH per liter media (Howe, 1973).

JB medium is a defined media used for the preparation of the E.coli extracts for folate quantification studies (J. Basso, unpublished results). To make 100.0 ml of this media,

one must add 5.0 ml of 20X cocktail, 10.0ml of filtered sterilized concentrated (10X) M9 minimal media (Miller, 1971), and 85.0 ml of distilled water. To this is added the following salts at a final concentration of 0.001 mM $ZnCl_2$, 0.001 mM $FeSO_4$, and 0.02% $MgSO_4$. The 20X cocktail is prepared from sterile stock solutions and consists of the following ingredients present at a final concentration of 0.04 mg/ml nicotinate, 0.2 mg/ml histidine, 0.74 mg/ml valine, 0.45 mg/ml isoleucine, 0.6 mg/ml adenine, 1.0 mg/ml methionine, 0.6 mg/ml thymidine, 0.02 mg/ml thiamine, 1.0 mg/ml phenylalanine, 1.0 mg/ml tyrosine, and 1.0 mg/ml tryptophan.

Inoculum Broth For Folate Bioassay:

This inoculum broth is made by dispensing 2.5 ml of the dehydrated Difco media (0822) into screw cap tubes. To this is added 2.0 ng/ml folic acid solution in 0.05M phosphate ascorbate buffer (Reagent 1). This is loosely capped and autoclaved for 15 min at 121°C.

Agar media for stock culture:

Bacto-Lactobacilli Agar available from Difco was used. This media was hydrated by the addition of double distilled water and then was autoclaved for 15 minutes at 121°C.

Assay Media:

A commercially available dehydrated assay media was used for the folate bioassay. This media can be purchased from

Difco, bearing the code 0822. It is folate free, but contains all other essential nutrients to sustain the cultivation of L.casei ATCC 7409 and E.hirae ATCC 8043. Care must be taken while weighing out the media because it becomes readily contaminated with folates. The media should be checked for folate contamination, by incubating an inoculated sample containing no added folate. This media should be prepared only prior to its use.

PLATES:

Tetracycline Plates: (Maniatis, p.72)

These plates which were used for some strain constructions, consist of 10.0 g tryptone, 5.0 g yeast extract, 5.0 g sodium citrate, 1.0 g glucose and 10.0 g agar per liter of plates. Tetracycline was added at a final concentration of 25.0 μg/ml. Tetracycline stock consisted of 4.0 mg of tetracycline/ml in methanol and was stored at -20°C in a foil wrapped bottle.

LB Kanamycin Plates:

These plates consist of 10.0 g Tryptone, 5.0 g yeast extract and 5.0 g NaCl and 10.0 g agar per liter of plates. Kanamycin is added at a final concentration of 32.0 μg/ml.

Chloramphenicol Plates: (Miller,p.226)

These plates consist of 10.0 g tryptone, 5.0 g yeast extract, 0.5 g NaCl, 2.0 ml of 1.0 M NaOH adjusted to pH 7.0

with NaOH prior to adding 15.0 g of agar. After autoclaving, 10.0 ml of 20% glucose and 0.5 ml of 25.0 mg/ml chloramphenicol stock solution was added. The chloramphenicol stock solution is made by adding 25.0 mg chloramphenicol/ml absolute ethanol and was stored at -20°C.

Bochner Plates: (Bochner et al, 1980).

These plates consist of 10.0 g tryptone, 5.0 g yeast extract, 10.0 g NaCl, 2.0 g glucose, 0.05 g chlortetracycline hydrochloride, 10.0g $NaH_2PO4.H_2O$, and 15.0 g agar. After autoclaving for 20 min and cooled to room temperature, 6.0 ml of filtered sterilized fusaric acid (2.0 mg/ml), and 5.0 ml of ZnCl (20.0 mM) are added.

ASSAYS:

T4 MUTANT BACTERIOPHAGE ASSAYS:

In order to determine bacteriophage titers, and to obtain appropriate lysates, several dilutions of the various T4 phage mutants were plated on the appropriate permissive hosts: CAJ64,(UGA mutants), CA165, (UAA mutants), CR63 (UAG mutants), using the overlay method (Adams, 1959). The plates were then incubated at 37°C, overnight.

Lysates were prepared as follows: Single plaques were picked with a sterile Pasteur pipette and were suspended in 1.0 ml aliquots of AB medium. These phage suspensions were vortexed and diluted 10X. Then 0.1 ml of diluted phage was added to 0.1 ml of the bacterial permissive host and this was plated . The plates were incubated at 37°C overnight. Lysates

were prepared from plates where confluent lyses of bacteria had occurred. Approximately 3.0 ml of AB media were added to the top of the plates and swirled. The plates were then left to stand at room temperature for 2 to 3 hours with occasional swirling. The medium which contained the extracted phage was collected in screw cap tubes and extracted 3 times with an equal volume of chloroform to ensure that all the bacterial cells were lysed. The final lysate was stored with one-third volume of chloroform in tightly capped tubes at 4°C. Lysates were titred and the reversion frequency was determined by plating diluted phage on the permissive and non permissive, (MH128), hosts respectively.

SUPPRESSION ASSAYS:

Fast phage assays were developed by Cheung and Herrington (1982) for the quick screening of strains to see whether they had suppressor ability or not. In fast assays, 24 well cluster dishes are used. Each well contains 0.1 ml of an overnight culture of cells, with approximately 0.1 ml of diluted phage, so that approximately 100 plaques would be obtained on regular sized plates. To this different concentrations of test compound are added, along with 1.0 ml of soft AB (usually supplemented with 20.0 µg/ml thymidine). The well plates are then swirled so that the cells, phage and compound are mixed, and then they are incubated at 37°C. The fast assay is preferred because it gives a crude estimate of the plating efficiency, and allows for the quick and easy

testing of various amounts of phage, cells, and potential inhibitors or activators on suppression (Cheung and Herrington, 1982).

GENETIC PROCEDURES:

Transductions:

Preparation of P1CM lysates:

P1CM lysogens were prepared by streaking approximately 0.02-0.05 ml of a P1CM lysate on a chloramphenicol plate, followed by cross streaking with a heavy inoculum of the donor strain. These plates were incubated overnight at 31°C or until visible lysogens were present. Once a lysogen was obtained, 1.0 ml superbroth media was inoculated with a chloramphenicol resistant colony and incubated overnight at 31°C. This culture is diluted 50 fold with superbroth and transferred to a 250 ml Erlenmyer flask and incubated at 31°C in a shaking waterbath (1-2 hrs). The culture was transferred to 44°C for 30 minutes with aeration, and was then transferred back to 31°C until it cleared, (cells lysed). The phage lysate at this point was collected in screw cap tubes, chloroform treated, and then stored at 4°C.

Transductions with P1CM phage (Miller, 1972) were used to construct many of the strains used in this study, (Table 4). First an overnight culture of the recipient bacterial strain was grown in LB supplemented with 10.0 mM $CaCl_2$, 1.0mM $MgSO_4$. This was then subcultured (1/100) in the same media

Table 4: **Summary of P1CM Mediated Transductions used in Strain Constructions**

Cross #	Donor	Recipient	Selected Phenotype	Relevant Genotype	Constructed strain
1	NK6073	MH429	tet^R	purF91 thyA723	MH540
2	H642	MH429	tet^R	purH thyA723	MH543
3	NK6051	MH429	tet^R	purE79 thyA723	MH570
4	NK5526	MH429	tet^R	hisG thyA723	MH575
5	NK6056	MH429	tet^R	purC80 thyA723	MH578
6	MH128	MH429	thy+	metB lys	MH581
7	NK5526	MH540a	tet^R	purF91 hisG thyA723	MH616
8	NK6069	MH429	kan^R	purD81 thyA723	MH652
9	RS3032	MH429	tet^R	purB thyA723	MH653
10	CAG12099	UTH046	tet^R	hisI903	MH654a
11	MH654a	MH429	tet^R	hisI903 metB thyA723	MH654
12	CAG12099	HfrG6	tet^R	hisA323	MH655a
13	MH655a	MH429	tet^R	hisA323 metB thyA723	MH655
14	MH294	MH429	tet^R	hisB metB thyA723	MH656

Construction involved Tn10 (Tet^R) or Tn5 (kan^R) transposons located in or near the gene of interest.

and grown to an A_{600} of 0.3. This culture was then centrifuged for 10 minutes at 3400xg, and then concentrated 10X in MC buffer (0.1 M $MgSO_4$ and 0.005 M $CaCl_2$). This was shaken for 15 minutes at 37°C. This cell suspension (0.1 ml) and 0.1 ml of undiluted or diluted phage lysate were mixed and incubated with shaking at 37°C for 20 minutes. Approximately 0.2 ml of LB (20.0 μg/ml thymidine) was added and this was allowed to outgrow for one hour. This mixture is then plated on plates with the appropriate selection for the transposon insertions.

Transformations (Lederberg and Cohen, 1974):

Plasmids used in this study are described in Table 3.

Competent cells were made by growing an overnight in LB (20.0 μg/ml thymidine) and then subculturing (1/100) in the same media the following day and allowing it to grow to an $O.D_{600}$ of 0.2. The culture is then chilled on ice and centrifuged at 6750xg for 5 minutes at 4°C. The pellet is then resuspended in 1/10 the volume of cold 0.1 M MgCl2. This is centrifuged at 3444xg for 5 minutes at 4°C, and the pellet is resuspended in 0.2 ml of ice cold 0.1 M CaCl2. This is then left on ice for 1 hour. These competent cells are then mixed with about 50.0 ng of plasmid DNA and left on ice for 1/2 hour. The mixture is then heat shocked at 45°C for 30 seconds and left on ice for an additional 5 minutes. The mixture is then outgrown for about one hour and then plated on LB supplemented with the appropriate antibiotic.

Selection of Tet^s Strains:

Tetracycline sensitive strains were selected using the Bochner plate method. Overnights were cultured in AB medium, and the next morning they were washed three times in 1X minimal A medium. These cells were then diluted into a phosphate buffer salt solution to give a cell density of about 10^7/ml. A 0.1 ml amount of this suspension (ie.,10^6 cells) was spread on a Bochner plate, and incubated for 48h at 37°C. Colonies which had grown were purified and were tested for tetracycline sensitivity on LB plates with and without tetracycline.

Sulfisoxazole Sensitivity:

The sensitivity of different E.coli strains to sulfisoxazole was assayed on plates using the method of Bochner and Ames 1982. Minimal medium A plates were used to assay sensitivities. These plates were supplemented with 50.0 μg/ml of methionine and histidine, and 30.0 μg/ml of adenine. Thymidine was added either at 20.0 μg/ml or 500.0 μg/ml. Overnight cultures of the desired strains were grown in minimal medium A with the required supplements. Approximately 0.1ml of cells were washed 3 times in 1X minimal salts and were plated using F Top (8.0 g of NaCl and 5.0 g of agar). A 6mm disk impregnated with 10.0 μl of 1.0 mg/ml sulfisoxazole solution, was placed in the center of each agar plate. The plates were incubated overnight at 37°C and the diameter of the zone of inhibition was measured the following day. This

was done approximately ten times and an average diameter of the zone of inhibition was determined.

Folate Bioassay:

Solutions:

Phosphate Ascorbic Buffer, (Reagent 1).

This buffer consists of 0.05 M phosphate, 0.15% Ascorbate, pH 6.1, (Keagy, 1980). To make this buffer, dissolve 1.20 g Na_2HPO4, and 2.32 g $NaH_2PO4.H20$ in 500.0 ml double distilled water. Ascorbate (1.50 g/L) was added just before use since it is unstable at this pH.

0.1M Acetate Buffer, (Reagent 2).

This buffer is used to elute the conjugase from the Sephadex G-25 column. To make 100ml of this buffer (pH 4.8) dissolve 1.36 g of sodium acetate and 0.32 g cysteine.HCl in 100.0 ml double distilled H_20. This buffer was made fresh the day of the assay.

Folic Acid Solutions:

Folic acid (20.0 mg) was dissolved in 100.0 ml of 0.01 N NaOH in 20% ethanol. This solution was divided into aliquots, and stored in Eppendorf tubes at -20°C (Keagy, 1980). This solution is stable for at least one year at -20°C.
This 200.0 µg/ml solution of folic acid was diluted 1000 fold in a 0.01 N NaOH, 20% ethanol solution (200.0 ng/ml) which was stored in the dark at 4°C (Keagy, 1980).

The working solution of folic acid was diluted 200 fold in Reagent 1 to give a 1.0 ng/ml final concentration. This is the high standard. The high standard was diluted 1 to 10 with Reagent 1 to give the intermediate standard (0.1 ng/ml), and then 0.1 to 10 to give the low standard (0.01 ng/ml).

SEPHADEX G-25 COLUMN

A Sephadex G-25 column was utilized as recommended by Keagy 1981, for the removal of small molecules from hog kidney homogenates. To prepare the Sephadex beads 10.0 g of dry Sephadex G-25 were soaked in 100.0 ml of 0.1 M sodium acetate buffer overnight. The Sephadex was then poured into a glass column having the dimensions 1.5x30.0 cm (Keagy, 1980). To pour the beads, the Sephadex mixture is resuspended and is quickly poured in one motion to ensure that no air spaces are formed. The column must be kept moist at all times and can be reused after thoroughly washing the column with the 0.1 M acetate buffer. Continuous use of the column results in the accumulation of a hog kidney proteinaceous layer at the top that can be removed by using a long stem pasteur pipette.

PREPARATION OF EXTRACTS:

Hog Kidney Extract:

Fresh hog kidneys were used as the source of conjugase. The kidneys were homogenized in a blender with 3 volumes of

0.32% cysteine-HCL adjusted to pH 5.4. The homogenate was autolyzed 2hr at 37°C. It was then clarified by centrifuging 20 min at 1000xg at 4°C and then recentrifuged at 4000xg for 30min. The supernatant was adjusted to pH 4.5 with 1N HCl and treated with Dowex 1 (5.0 g/100.0ml) for 1 hr in an ice bath to remove endogenous folates. The mixture was then recentrifuged three times at 3400xg in the cold and the clear supernatant was stored at -20°C until used.

Preparation of Inoculum;

The cultures of the assay organisms were transferred aseptically from the stab vials to liquid inoculum medium, (see "media") and incubated 16-20hr. The morning of the assay the cultures were centrifuged in the clinical centrifuge at 5000 rpm for 15min. The pellets were then washed three times in sterile saline (0.9%), centrifuging at 5000 rpm for 15min. The cultures were diluted 30 fold in 0.9% saline and were used as the inocula.

E.coli extracts:

Cells were grown overnight in JB medium supplemented with either 20.0 μg/ml or 500.0 μg/ml thymidine. The next day they were subcultured in the same medium with low (20.0 μg/ml) or high (500.0 μg/ml) thymidine. Cells were allowed to grow to an $O.D_{600}$ of 1.0. Cultures were then removed from the shaking water bath and centrifuged at 3400xg for 10min at 4°C. Pellets were resuspended in 10.0 ml phosphate ascorbic buffer, (Reagent 1). The cells were then washed three times

(3400xg for 5min) in Reagent 1 to remove any traces of medium. Once washed the pellets were resuspended in 2.0 ml of Reagent 1 and the $O.D_{600}$ was taken after diluting 0.1 ml of cells in 0.9 ml water. The concentration of cells was adjusted so that all samples had an OD_{600} of 0.1. Several dilutions of bacteria from each culture were plated on AB (50.0 µg/ml thymidine) plates. The cultures were then sonicated at 6Hz for 10sec pulses, three consecutive times. Once the cultures had lysed they were centrifuged in the clinical centrifuge at 3400xg for 15min. The supernatants , were collected and stored at 0°C until the next day. On the day of the assay the extracts were diluted as follows: 25.0 µl of cell extract plus 100.0 µl of conjugase per 3.7 ml of Reagent 1. These mixtures were incubated at 37°C for 2hrs after which the mixture were autoclaved for 5min at 15psi in order to inactivate the conjugase. The mixture was cooled and added to the test tubes in varying amounts ranging from 10.0 µl to 200.0 µl. Reagent 1 was then added to bring the volume to 1.0 ml. Then 1.0 ml of assay media was added to each tube, and the tubes were autoclaved for 5 min at 121°C and after cooling were inoculated with either L.casei or E.hirae. The test tubes were then incubated at 37°C for 20-24hrs.

Preparation of Standard Curve:

Varying amounts (0-1.0 ml) of low, intermediate, and high standard folic acid solutions were added to assay tubes (16 x 100mm), and sufficient buffer (Reagent 1) was added to

bring the volume in each tube to 1.0 ml. To each tube was added 1.0 ml of assay medium, and the tubes were autoclaved for 15min at 121°C. After cooling the tubes were inoculated with appropriate inoculum.

Determination of Folic Acid Levels;

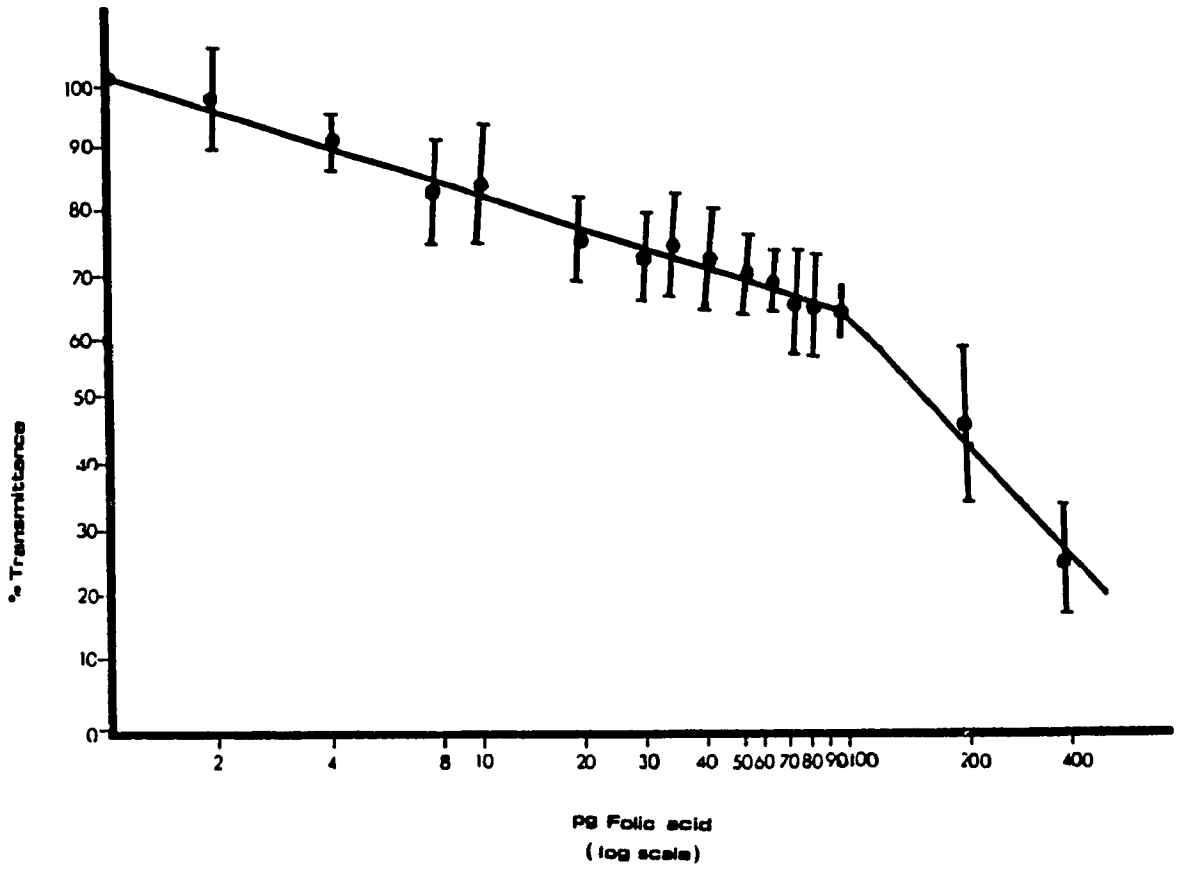
The percent transmitance of the samples was determined in a spectrophotometer set at 600nm. The values from the standard assay tubes were used to construct a standard curve by plotting percent transmitance against the log of picograms folic acid per tube in the standard series, (Fig. 8). The percent transmittance values of the experimental samples were used to estimate their folate content by comparison with the standard curve. Only values falling on the straight line portion (1-100.0 pg folic acid) of the curve were considered. The tube containing 0.0 pg folic acid was used as the blank.

To determine the concentration of folic acid per cell it was necessary to count cells microscopically and to estimate their volume. The cells were counted using a Reichert phase contrast microscope, and a Petroff-Hausser and Helber counting chamber. The bacterial cell volume was estimated by determining the wet weight of the bacterial pellet divided by the average bacterial density, (1.03 g/ml) (Gerhardt et al, 1981). The folate content of the samples extrapolated from the standard curve was then divided by the total number of

Figure 8

A Typical Lactobacillus casei Standard Curve

This figure represents a L.casei standard curve plotted on semilogarithmic graph paper, with percent transmission plotted on the linear ordinate and concentration of pteroylglutamate (folic acid) as the abscissa on the logarithmic side of the graph paper. This standard curve relates bacterial growth to concentration of reference standard, and is made up of two portions. The first portion, the so-called linear is the portion that is used for assay purposes. The second portion is relatively flat, since additional growth factor does not result in proportionally more growth.


% Transmittance
100
90
80
70
60
50
40
30
20
10
0
2
4
8
10
20
30
40
50
60
70
80
90
100
200
400
pg Folic acid
(log scale)

cells, and this number was then divided by the cell volume, to obtain the concentration in ng/μl.

Sources of chemicals:

The majority of the chemicals utilized were reagent grade and were purchased from either Sigma, Fisher, or Canlab. Ingredients for media were obtained from Difco.

RESULTS

PART I: Folate Involvement in Suppression.

Inhibition by Anti-folate Drugs:

Since our model suggests a link between intracellular folate pools and the inherent ability of thymidine auxotrophs to suppress, I attempted to determine if altering folate levels affected suppression. Suppression of the opal (UGA), lysozyme T4 mutant ELP12 by the Thy- strain MH429 was assayed in the presence of different sulphonamides (Table 5). These included sulfisoxazole, sulfathiazole, and sulfadiazine. All three of the sulphonamide derivatives assayed inhibited suppression of ELP12. The mutant phage was considered to be inhibited when there was 100 fold fewer plaques with the inhibitor, as compared to without. The slight variation in the minimum inhibitory concentration (M.I.C.), for the different sulphonamides can be attributed to their differential rates of uptake (Then and Angehrn, 1972). Inhibition of suppression by sulphonamides was specific to thymineless dependent suppression, since these drugs had no effect on plaque formation by wild type T4 phage nor on the suppression of ELP12 by its permissive host CAJ64 (Table 5). The anti-folate drugs, trimethoprim and aminopterin, also inhibited Thy- suppression (J.Basso, unpublished results).

Table 5. **Inhibition of thyA Dependent Suppression by Anti-Folate Drugs**

HOST	PHAGE	COMPOUND[a]	M.I.C.[b] (μM)
MH429	eLP12	Sulfisoxazole	25
MH429	T4	"	NI[c]
CAJ64	eLP12	"	NI
MH429	eLP12	Sulfathiazole	24
MH429	T4	"	NI
CAJ64	eLP12	"	NI
MH429	eLP12	Sulfadiazine	26
MH429	T4	"	NI
CAJ64	elp12	"	NI

Suppression was assayed semi-quantitatively using the fast assay (Cheung and Herrington, 1982). The mutant phage was considered to be suprressed when it gave at least 100 fold more plaques on Thy- strain than on Thy+.Suppression was inhibited when there was at least 100 fold fewer plaques in the presence of the inhibitor, than without.

[a] A stock solution of 1mg/ml was used and a range of concentrations from 12.0-250.0 μM were tested.

[b] M.I.C. refers to the minimum inhibitory concentration, the concentration above which there is no plaques but below which plaque formation is not inhibited.

[c] N.I. phage was not inhibited at concentrations equal to or greater than 25μM of sulphonamide.

Inhibition of Suppression of Other T4 Mutants:

Inhibition of Suppression of Other T4 Mutants:

To determine that the inhibition of Thy- suppression by sulphonamides was not confined to one suppressible mutation, a number of T4 nonsense mutants were assayed on strain MH429, and on the appropriate permissive host strains (Table 6). Results indicate that sulfisoxazole inhibited the suppression of all the mutants assayed, with an M.I.C. of 25.0 μM. This concentration did not inhibit cell growth. However suppression of JC1922 and OPC105 was more sensitive to inhibition, suggesting that suppression of these mutants was more sensitive to altered folate levels. This result is consistent with the observation that suppression of two of these mutants, JC1922 and OPC105 was more sensitive to other folate inhibitors (J.Basso, unpublished results). Since different suppressors are presumably altered in the modification of different tRNA's, it would not be surprising if sensitivity to folate levels were different in the mutants.

Recovery of Suppression from Sulphonamide Inhibition:

Sulphonamides are competitive inhibitors of the enzyme dihydropteroate synthase which compete for the same active site as p-aminobenzoic acid (p-ABA), (see figure 3.). This results in a decrease in dihydropteroate synthase activity (Brown and Williamson, 1987).

If inhibition of suppression by sulphonamides is due to lowered folate levels as a consequence of reduced folate

Table 6. Inhibition of *thyA* Dependent Suppression by Sulfisoxazole

STRAIN	PHAGE	TYPE OF MUTANT	M.I.C.[a] (μM)
MH429	T4	WILD TYPE[b]	NI
MH429	M103	UAG	25.0
MH429	JC1912	"	25.0
MH429	JC1916	"	25.0
MH429	eLP12	UGA	25.0
MH429	OPC100	"	25.0
MH429	OPC105	"	12.5
MH429	JC1922	UAA	12.5

Suppression was assayed as in Table 5. The compound sulfisoxazole was at a stock solution of 1mg/ml and a range of concentrations were assayed from 5.0-500 μM . The concentrations assayed did not affect cell growth.

[a] M.I.C. refers to the minimum inhibitory concentration.

[b] N.I. phage was not inhibited at the highest concentration of drug assayed.

biosynthesis, then supplying cells with exogenous p-ABA should prevent sulfisoxazole inhibition of suppression. Results indicate that suppression occurred in the presence of sulfisoxazole if exogenous p-ABA was added at a final concentration of 1.3 μM (Table 7). This result supports the idea that inhibition is a direct consequence of lowered folate biosynthesis.

Since the addition of p-ABA to the medium restored suppression, I wanted to test other metabolites to see if they could also restore suppression from sulfisoxazole inhibition (Table 7). These metabolites included the aromatic amino acids, histidine and methionine.

The aromatics were selected, since they are derived from chorismate, as is p-ABA. Methionine was selected because it is a folate endproduct, and has been documented to be a sulphonamide antagonist on E.coli (Martin, 1951). Histidine was selected for its possible regulatory role in folate biosynthesis.

The complete recovery of suppression can be obtained by the addition of tryptophan at a concentration of 3.2 mM (Table 7). The ability of tryptophan to recover suppression may be an indirect consequence of sparing chorismate levels, thus resulting in a greater flow to p-ABA. Consequently, the possible increase in internal p-ABA levels may rescue suppression by competing with sulfisoxazole. This hypothesis cannot be justified, since neither of the other aromatic

Table 7. **Effect of Different Compounds on Sulfisoxazole Inhibition of Suppression**

COMPOUND	CONCENTRATION	M.R.C.[a] (mM)
p-aminobenzoic acid	0.7μM-0.007mM	0.0013
tryptophan	80.0μM-4.0mM	3.2
phenylalanine	100.0μM-4.0mM	N.R.[b]
tyrosine	92.0μM-4.8mM	N.R.
tryptophan/histidine	80.0μM-3.2mM	1.6/1.6
histidine	80.0μM-3.2mM	P.R. (3.2)[c]
methionine	112.0μM-5.6mM	N.R.

Suppression of the T4 mutant eLP12 by the Thy- strain MH429 was assayed as in Table 5. Sulfisoxazole was present at the inhibitory concentration of 50.0μM.

[a]M.R.C. refers to the minimum recovery concentration- the concentration above which is required to recover from sulfisoxazole inhibition.

[b]N.R. Suppression was not rescued.

[c]P.R. refers to the minimum concentration above which is required to partially (approximately half the number of plaques as compared to complete recovery) rescue suppression from sulfisoxazole inhibition.

amino acids, tyrosine and phenylalanine, exhibit this rescuing effect at similar or greater concentrations (Table 7). Alternatively, the effect observed with tryptophan may result from a breakdown product of this amino acid. Indeed it has been observed that nicotinate, a precursor of tryptophan in Lactobacillus arabinosus (Martin ,1951), can rescue suppression from the action of sulfisoxazole (J.Basso, unpublished result).

The addition of histidine, at a similar concentration, (3.2 mM), gave approximately half the number of plaques as observed with p-ABA (Table 7). Higher concentrations of histidine had no greater effect. This recovery exhibited by histidine may be explained if histidine is degraded by E.coli. In S.typhimurium histidine is degraded and this results in the formation of N-formiminoglutamate, which in turn transfers its formimino group to tetrahydrofolate to form the N-5-derivative of formyl-THF. If this is also true for E.coli, the generation of additional N-5-formyl-THF may rescue suppression from sulfisoxazole inhibition, by resulting in a greater biosynthetic input of folate. Alternatively the presence of additional histidine in the medium might repress the histidine biosynthetic pathway and this may alter folates so that suppression can occur.

Recovery was also obtained when a combination of histidine (1.6 mM) and tryptophan (1.6 mM) was used. This suggested that these compounds complemented each other, and

that in combination their rescuing ability was greater; thus they were more effective at lower concentrations.

In contrast, methionine was not able to rescue suppression from sulfisoxazole inhibition. Although methionine has been known to antagonize sulphonamides (Martin, 1951), its antagonizing properties are confined to only certain types of sulphonamides. For example, methionine has been shown to antagonize sulphanilamides, but not sulfadiazine or sulfathiazole (Martin, 1951).

The Additive Effect of Thymidine on Sulfisoxazole Inhibition:

Suppression is also inhibited by high levels of thymidine (Cheung and Herrington, 1982; Herrington, Kohli, and Lapchack, 1984). Therefore I asked what effect combinations of sulfisoxazole and thymidine had on suppression. The inhibition of suppression by thymidine is additive to sulfisoxazole since suppression of JC1922 by MH429 could be inhibited by combinations at concentrations of sulfisoxazole (4.1 μM) and thymidine (0.4 mM) that represented less than half the minimum inhibitory concentrations of each (Table 8). These results suggest that thymidine potentiated the action of sulfisoxaole.

Table 8. Effect of Sulfisoxazole and Thymidine on Suppression

COMPOUND	M.I.C.[a]
Sulfisoxazole	12.5μM
Thymidine	0.4mM
Sulfisoxazole + Thymidine	4.1μM-0.15mM

Suppression of the T4 mutant JC1922 by the thymidine auxotroph MH429 was assayed as in Table 5. Stock solutions of sulfisoxazole (1mg/ml) and thymidine (5mg/ml) were used and a range of concentrations were assayed from 2.0-250.0μM and from 75.0-300.0mM respectively. This table was generated using a matrix of thymidine and sulfisoxazole concentrations.

[a]M.I.C refers to the minimum inhibitory concentration.

The Effect of 3-amino-1,2,4-triazole on Sulfisoxazole:

The herbicide amitrole, (3-amino-1,2,4-triazole), inhibits imidiazole glycerol phosphate dehydrogenase, an enzyme of histidine biosynthesis (Hilton et al, 1965), and also is implicated in the inhibition of purine synthesis (Hilton et al, 1965). It is believed that amitrole is converted to a derivative that inhibits purine biosynthesis.

Since amitrole inhibited suppression by Thy- cells (J. Basso, unpublished results) I asked whether it potentiated the action of sulphonamides on suppression. Amitrole was found to be additive to sulphonamides. This observation supported the postulate that amitrole like thymidine potentiated the action of sulfisoxazole possibly by contributing towards lowering folate levels (Table 9).

Sulfisoxazole Sensitivity:

Thymidylate synthase is the only reaction in E coli in which the folate cofactor N5-N10-methylenetetrahydrofolate is oxidized to dihydrofolate (O'Donovan and Neuhard, 1970). Strains of E.coli that are defective in their thyA gene, cannot carry out this reaction and consequently become auxotrophic for thymidine. The lack or decrease of thymidylate synthase activity may result in the accumulation of 5-10-methylene-THF, or any of its derivatives.

Table 9. **Additive Effect of Sulfisoxazole and 3-amino-1,2,4-triazole on Suppression**

COMPOUND	M.I.C.[a]
Sulfisoxazole	12.5 μM
3-amino-1,2,4-triazole	3.3 mM
Sulfisoxazole	5.8 μM
+	+
3-amino-1,2,4-triazole	1.6 mM

Suppression of the T4 mutant JC1922 by the thymidine auxotroph MH429 was assayed as in Table 5. Stock solutions of sulfisoxazole (1mg/ml) and 3-amino-1,2,4-triazole (1.0M) were used and a range of concentrations were assayed from 2.0-60.0 μM and from 0.85-27.0 mM respectively. This table was generated using a matrix of sulfisoxazole and 3-amino-1,2,4-triazole concentrations.

[a] M.I.C. refers to the minimum inhibitory concentration above which suppression is inhibited.

To determine if thymidine auxotrophs had significant differences in their folate levels or in the levels of folate biosynthetic enzymes, as compared to the wild type prototrophs, I assayed the sensitivity to sulfisoxazole of thymidine auxotrophs and prototrophs under suppressing (AB media) and nonsuppressing (MM) conditions. I predicted that Thy- mutants might accumulate folates and thus be more re nt than wild type strains. Furthermore, I expected t. thymidylate synthase mutant should be equally as sensi· ve as the wild type grown with high levels of exogenous thymidine, since thymidine might restore wild type folate levels, by decreasing folate biosynthesis.

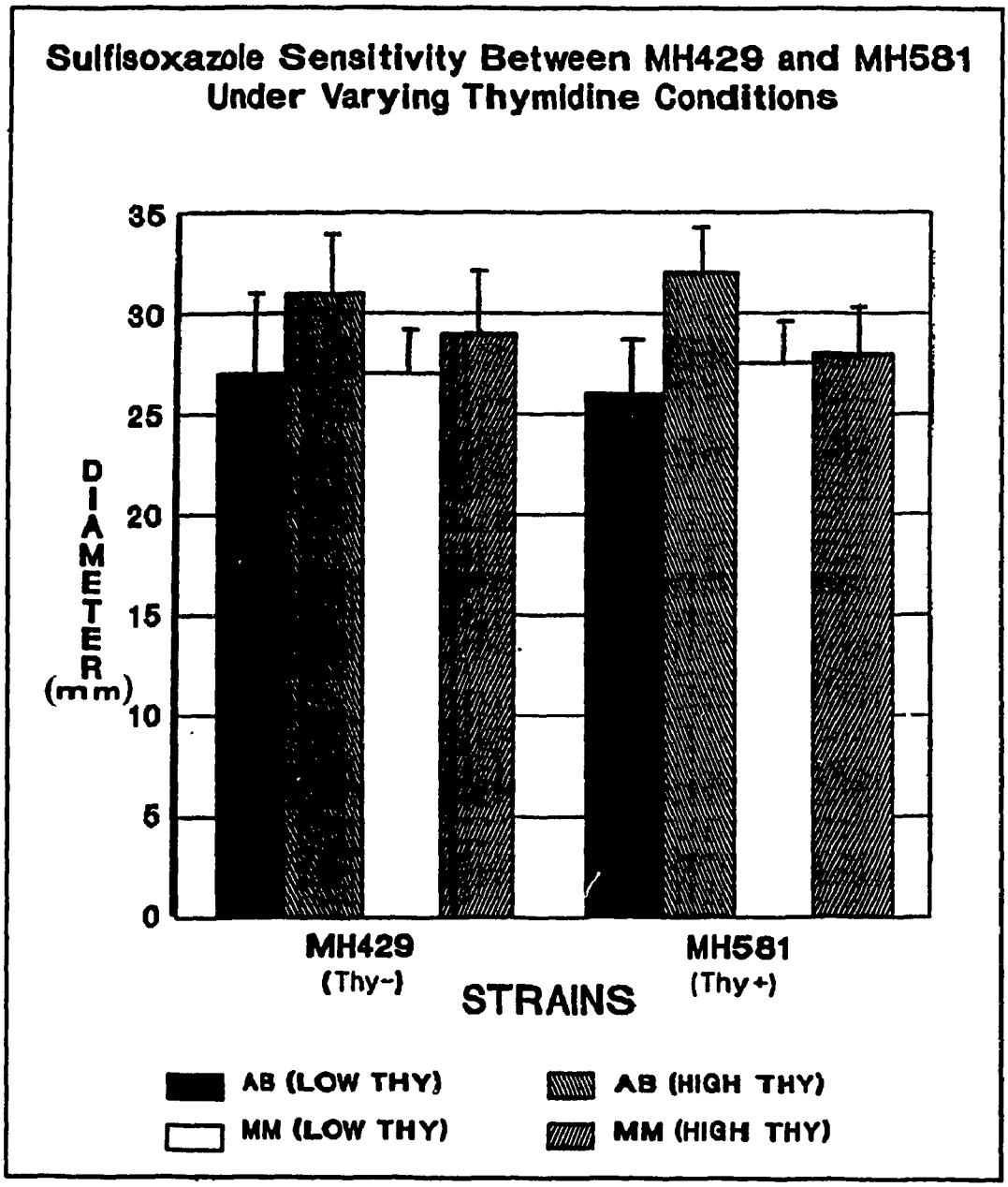
Results demonstrate that there was no significant difference in sulfisoxazole sensitivity between the ThyA mutant and the wild type strain, under suppressing (AB media) and non-suppressing (MM media) conditions (Fig. 9).

The sensitivity of the above assay was considered to be low, since the standard deviations were high, and since this assay did not allow me to look at folate levels directly. Therefore, I attempted to quantify folate levels using a folate bioassay, so that I can obtain a better indication of folate levels in the Thy- and Thy+ cells.

FIGURE 9

Sulfisoxazole Sensitivity Assay Between MH429 and MH581

The sensitivity of the Thy- strain MH429 and the Thy+ strain MH581 were assayed using the method of Bochner and Ames 1982. Overnight cultures of MH429 and MH581 were grown in AB and in MM media. Cells were washed three times in 1X minimal salts, and then 0.1ml of cells was plated on minimal medium plates. These plates were supplemented with 50.0μg/ml of methionine, 50.0μg/ml histidine, and 30.0μg/ml of adenine were used for the assay. Thymidine was added either at 20.0μg/ml or at 500.0μg/ml. A 6mm disk impregnated with 10μl of a 1mg/ml sulfisoxazole solution was then placed on to the center of each plate. Following incubation at 37°C, zones of inhibition were measured. The numbers on the diagram represent the average diameter in milimeters of the zones of inhibition around the disk. This experiment was replicated at least 10 times and the error bars represent the standard deviations.


Sulfisoxazole Sensitivity Between MH429 and MH581
Under Varying Thymidine Conditions
DIAMETER (mm)
35
30
25
20
15
10
5
0
MH429
(Thy-)
MH581
(Thy+)
STRAINS
AB (LOW THY)
AB (HIGH THY)
MM (LOW THY)
MM (HIGH THY)

Quantification of Folates:

The quantification of folates was done by a microbiological assay. The organisms used were auxotrophic for folates and hence their growth was limited by the amount of folic acid that is available. The three bacterial species which are most commonly used for folic acid assays are L.casei (ATCC 7469), E.hirae (ATCC 8043), and Pediococcus cerevisiae (ATCC 8081). These microorganisms differ in their ability to utilize folates. L.casei makes use of both methylated or nonmethylated derivatives of THF; whereas, E.hirae can only use non-methylated species of THF. In contrast P.cerevisiae is most fastidious in its folate requirements, being able to use only the tetrahydrofolate forms of folic acid. L.casei can only use mono, di, and tri-glutamate forms, while E.hirae uses the above mentioned forms as well as certain higher polyglutamates. Therefore, the levels of glutamation can be assessed by comparing folate samples treated with conjugase (γ-carboxylpeptidase-cleaves glutamic acid residues), to those that were not treated. In this assay I used L.casei and E.hirae as the test organisms.

Therefore by using both test organisms I was able to measure total folates as well as the fraction of total folates that are methylated present in isogenic Thy- and Thy+ strains.

When the amount of folic acid was expressed on a per cell basis, a large difference in the amount of total folates

is observed in the Thy- strain MH429, as compared to the isogenic Thy+ strain MH581, when cells were grown in low thymidine (Table 10, compare conjugase treated samples). In contrast, cells grown under high thymidine conditions (500.0 μg/ml), did not differ in their folate content, although they both contained less folate than cells grown in low thymidine (20.0 μg/ml), (Table 10).

The large difference observed might be explained by the fact that although the cultures were taken at the same OD_{600}, the concentration of cells as determined by microscopic examination varied. I found that there were about four times more Thy+ than Thy- cells/ml under low thymidine conditions (20.0 μg/ml), and that the Thy- cells were significantly larger. This difference in size and number between Thy- and Thy+ cells was not apparent under high thymidine conditions. Therefore intracellular folate concentrations were determined. In order to determine folate concentration I measured the volume of the cells. Volume was calculated by dividing the mass of the bacterial pellets by the average density of bacterial cells. The value for the density of the bacterial cells (1.03g/ml) is that given by (Gerhardt et al, 1981).

Table 10: **Folate content in E.coli cells.**

Assay Organism	Folate Source a'	Folate Content (10^{-6}pg/cell) in cells grown in Low Thymidine [U]b	Low Thymidine [C]c	High Thymidine [U]	High Thymidine [C]
L.casei	MH429	0.24±0.06	1.38±0.05	0.07±0.01	0.31±0.06
L.casei	MH581	0.06±0.02	0.35±0.02	0.05±0.01	0.21±0.02
E.hirae	MH429	0.61±0.03	0.91±0.09	0.15±0.05	0.23±0.07
E.hirae	MH581	0.14±0.02	0.22±0.04	0.12±0.02	0.20±0.03

[a] Results represent the average folate concentration determined on extracts from five independent cultures, expressed as ± standard deviations of the mean.

[a]'MH429 is Thy-, MH581 is Thy+

[b] [U] denotes untreated sample.

[c] [C] denotes conjugase treated.

The folate concentrations in the Thy- and Thy+ strains were significantly different as determined by a paired t-Test (Table 11, conjugase treated samples). The Thy- auxotroph had approximately 20% more folates than the Thy+ prototroph under low thymidine (20.0μg/ml) conditions. This difference in folate concentration was not observed under high thymidine (500.0μg/ml) conditions. There was also a significant decrease in folate concentrations in both the Thy- (38%) and Thy+ (53%) strains under high thymidine conditions (Table 11). This data demonstrates that the levels of the thyA mutant grown in high thymidine are quite compatible to the wild type under low thymidine conditions. This suggests that the 20-38% increases in pools in thyA mutants are sufficient to explain the effect of suppression, and this is corrected by the effect of thymidine in the medium. From this data, it was postulated that thymidine potentiated the action of sulphonamides by lowering folate levels.

I then attempted to look at folate distribution, by using E.hirae, in conjunction with L.casei. This should allow me to determine if a significant bias occurs in the distribution of total folates into their methylated and non-methylated derivatives.

When looking at folate distribution by comparing results obtained from both the L.casei and E.hirae assays, there is a significant difference in distribution between the Thy- auxotroph and Thy+ prototroph. Thy- cells had 24% more non-

Table 11: **Folate concentration in E.coli cells.**

Assay Organism	Folate Source [a']	Folate Concentration (µg/µl)[a] in cells grown in Low Thymidine [U][b]	Low Thymidine [C][c]	High Thymidine [U]	High Thymidine [C]
L.casei	MH429	0.08±0.02	0.45±0.02	0.07±0.01	0.28±0.02
L.casei	MH581	0.07±0.03	0.36±0.02	0.04±0.01	0.17±0.11
E.hirae	MH429	0.20±0.01	0.30±0.02	0.14±0.03	0.21±0.07
E.hirae	MH581	0.14±0.02	0.23±0.01	0.09±0.02	0.16±0.13

[a] Results of quintuple determinations, expressed as ± standard deviations of the mean.

[a'] MH429 is Thy-, MH581 is Thy+

[b] [U] denotes untreated sample.

[c] [C] denotes conjugase treated.

P values were calculated using a two-tailed t test, for the significance of the difference in total folate concentrations between Thy- and Thy+ cells. (P=0.002 Thy- vs Thy+ in low thymidine conditions. P=0.000 Thy- under low vs high thymidine. P=0.000 Thy+ under low vs high thymidine).

methyl folates than Thy+ cells did, under low thymidine conditions (Table 11). Furthermore, under low thymidine conditions there seems to be approximately 33% and 36% more methyl folates in both the Thy- and Thy+ cells respectively, as compared to under high thymidine conditions (Table 11).

Furthermore, results demonstrate that E.hirae was able to grow better than L.casei in untreated extracts (Table 10,11, compare conjugase treated with no conjugase treatment). This suggests that there are approximately 33-55% of the folates in E.hirae that have at least four or more glutamates. This result is not surprising, since as stated previously, E.hirae can grow on longer polyglutamates than can L.casei.

PART II: Genetic Modification of Suppression.

AICAR, the precursor of the proposed folate alarmone, ZTP, is made during histidine and purine biosynthesis. Two molecules of 10-formyl-THF are used for each purine molecule synthesized, while only one of these molecules is required for histidine biosynthesis. Thus, alterations in the flow of intermediates through the histidine and purine pathways might perturb folate levels and hence suppression. To determine if this was so, double mutants such as thyA purH and thyA hisI were constructed. Suppression and inhibition of suppression by sulfisoxazole and thymidine were assayed on these strains and the isogenic thy+ strains.

Overexpression of the folC gene or histidine enzymes might also perturb folate levels and thereby affect suppression. This was tested by introducing plasmids carrying either the folC gene or his operon (pHC9800) into Thy- and Thy+ cells.

Effect of Purine Pathway Mutations on the Inhibition of Thy- Suppression:

Purine auxotrophic derivatives of Thy- and Thy+ strains were constructed by transducing in mutations affecting six of the ten biosynthetic genes.

All the Thy- Pur- strains suppressed under low thymidine conditions (Table 12), whereas none of the Thy+ Pur- strains did. This suggested that suppression was not affected by the accumulation of any metabolite from the purine biosynthetic pathway, or by a lack of flow of metabolites through the pathway.

Suppression was inhibited by high thymidine in all Thy- Pur- strains, except in MH543 which has a purH mutation (Table 12). This effect might have been due to the presence of a linked marker arg::Tn10. However, suppression of the thyA pur+ argE::Tn10 sibling strain was inhibited by high thymidine, suggesting that the resistant phenotype was not due to the argE::Tn10.

Table 12. Suppression of JC1922 by Different Auxotrophic Derivatives of MH429 in the Presence of Thymidine and Sulfisoxazole

STRAIN	MUTANT ALLELE	THYMIDINE (20μg/ml)	THYMIDINE (500μg/ml)	SULFISOXAZOLE (50μg/ml)
MH429	thyA	+[a]	-[b]	-
MH540	thyA purF	+	-	+
MH543	thyA purH	+	+	-
MH570	thyA purE	+	-	-
MH578	thyA purC	+	-	-
MH652	thyA purD	+	-	-
MH653	thyA purB	+	-	-
MH657	thyA purF▲	+	-	-
MH658	"	+	-	-
MH659	"	+	-	+
MH660	"	+	-	-

Suppression was assayed as in Table 5. Stock solutions of sulfisoxazole (1mg/ml) and thymidine (5mg/ml) were used. Sulfisoxazole was present at a final concentration of 50.0μg/ml which was about 4X the minimum inhibitory concentration required to inhibit JC1922.

[a] +denotes cells suppressed in the presence of the inhibitors at the specified concentrations.

[b] - denotes inhibition of suppression.

▲ denotes deletion of Tn10.

Suppression by all the Thy- Pur- strains with exception of Thy- PurF- strains MH540, and MH659 was inhibited by sulfisoxazole (Table 12).

Strain MH540 contains a Tn10 inserted in the purF gene. Thus the resistance of suppression to sulfisoxazole in this strain, may be a consequence of the Tn10 insertion itself as opposed to the reduced flow through the purine biosynthetic pathway. For example many Tn10 insertions are polar, preventing the expression of distal genes in the same operon (Rolfes and Zalkin, 1988). To test this hypothesis, Tet^s derivatives of strain MH540 were selected on Bochner plates. Tet^s derivatives can arise by precise excision of Tn10 in which case the strain will not be auxotrophic for purines, or by imprecise excision resulting in the purine requirement. In this case these Tet^s derivatives were putative deletion mutants resulting from the imprecise excision of Tn10. Suppression by many of the Tet^s Pur- derivatives was not resistant to sulfisoxazole (Table 12.). Thus, the resistance of suppression by strains MH540 to sulfisoxazole inhibition probably results from the transposon insertion.

Potentiation of Sulphonamides:

Purines potentiate the toxicity of sulphanilamide to bacterial cells (Bruce et al, 1984). Consequently I tested whether I could inhibit suppression of JC1922 by the MH540 strain, with sulfisoxazole by adenine, guanine, or AICA which

can be converted to AICAR, the purine intermediate (Table 13). Furthermore other purine intermediates such as hypoxanthine and xanthine were also assayed.

The inhibition of suppression by MH540 (thyA purF) in the presence of sulfisoxazole, could be accomplished by the addition of equimolar concentrations of adenine or 5-amino-4-imidazole carboxamide riboside 5'-monophospate, AICA (2.3 mM) but not by adding guanine, xanthine, or hypoxanthine. This clearly suggests that adenine or AICA added exogenously potentiate the action of sulfisoxazole.

The Involvement of the Histidine Pathway in the Inhibition of Thy- Suppression:

AICAR (ZMP), can be made via the histidine or the purine biosynthetic pathway. AICAR synthesized via the purine pathway acts as an intermediate for purine biosynthesis. AICAR produced from the histidine pathway serves as a regenerative source of the ATP utilized to synthesize histidine. Consequently, since the AICAR derivative ZTP is postulated to be a folate alarmone (Bochner and Ames, 1982), the suppressing ability of various His- Thy- mutants was monitored (Table 14). I constructed histidine requiring derivatives of strain MH429 and MH581 by transduction of mutations affecting four of the nine histidine biosynthetic genes.

Table 13. Inhibition of Suppression of JC1922 by MH540 in the presence of Sulfisoxazole

COMPOUND	CONCENTRATION (mM)	SUPPRESSION[a]	M.I.C.[b] (mM)
-	-	+[c]	
AICA	0.4-4.0	-[d]	2.3
Adenine	0.4-4.9	-	2.3
Guanine	0.2-4.9	NI[e]	
Hypoxanthine	0.3-4.9	NI	
Xanthine	0.3-5.3	NI	

[a]Suppression was assayed as in Table 5. Sulfisoxazole was added at a final concentration of 50.0 μM, which is approximately four times the minimum inhibitory concentration for suppression by a Thy- strain.

[b] M.I.C. refers to the minimum inhibitory concentration.

[c] + denotes cells suppressed at 50.0μM sulfisoxazole.

[d] - denotes inhibition of suppression.

[e] NI denotes phage mutant was not inhibited at the above specified concentrations.

All the His- Thy- mutants assayed suppressed as did the His+ Thy- parent strain under 20.0μg/ml thymidine, while none of the His- Thy+ derivatives suppressed (Table 14).

The suppression by histidine mutants which were blocked prior to the bifurcation to AICAR, (hisG, I, A), was resistant to both sulfisoxazole and thymidine inhibition. Various hisG Tet^s derivatives were constructed by selection on Bochner plates. These hisG Tet^s derivatives were putative deletion mutants resulting from the imprecise excision of the Tn10, and were independently selected. Suppression by all of the Tet^s histidine mutants remained resistant to sulfisoxazole suggesting that the resistance was not due to an effect by the Tn10 insertion, as was the case of the Tet^s pur- mutants.

In addition a triple mutant was constructed by transducing the hisG mutation into strain MH658, a purF- Tet^s derivative which was inhibited by both inhibitors. The resulting strain (MH616) became resistant to both inhibitors, as resistant as strain MH575 (hisG)(Table 14). This suggests that the main determinative factor in sulfisoxazole resistance is the effect of metabolite flow through the histidine pathway rather than a decrease in sulfisoxazole penetration.

In contrast, suppression by a histidine mutant at a step past this bifurcation, (hisB), was as sensitive to sulfisoxazole and thymidine inhibition, as the suppression by

Table 14. **Suppression of JC1922 by Different Histidine Auxotrophic Derivatives of MH429 in the presence of Thymidine and Sulfisoxazole**

STRAIN	RELEVANT GENOTYPE	THYMIDINE (20μg/ml)	THYMIDINE (500μg/ml)	SULFISOXAZOLE (50μg/ml)
MH429	thyA	+[a]	-[b]	-
MH656	thyA hisB	+	-	-
MH654	thyA hisI	+	+	+
MH655	thyA hisA	+	+	+
MH575	thyA hisG	+	+	+
MH667	thyA hisG▲	+	+	+
MH668	"	+	+	+
MH669	"	+	+	+
MH616	thyA purF hisG	+	+	+

Suppression was assayed as in Table 5. Stock solutions of sulfisoxazole (1mg/ml) and thymidine (5 mg/ml) were used.

[a] + denotes cells suppressed in the presence of the inhibitors at the specified concentrations.

[b] - denotes inhibition of suppression.

▲ denotes deletion of Tn10.

His+ Thy- parent strain. These results suggests that the inhibition of suppression by sulfisoxazole or high thymidine may involve one of the histidine biosynthetic intermediates between the hisA and hisB step or the product of AICAR, (see Fig 7). This suggests that if AICAR is not made via the histidine pathway suppression can not be inhibited by sulfisoxazole or thymidine.

I then proceeded to see whether I could inhibit thyA suppression by increasing one of the histidine intermediates. Increasing the histidine enzymes can be obtained by either transforming a strain with the his operon (pHC9800), (Bruni et al, 1980) or by introducing the hisT mutation which results in the derepression of the operon.

The hisT gene encodes pseudouridine synthase I, which catalyzes the formation of pseudouridine residues in the anticodon stem and loop of several tRNAs. Mutations in the hisT gene alter the modification of several tRNAs, and this undermodification deacreases transcription termination at the his attenuator, resulting in derepression of the his operon (Winkler, 1987).

The parent stain MH429 was first transformed using plasmid pHC9800, which is derived from pBR313. A strain carrying this plasmid should express the histidine operon at high levels and hence may synthesize high levels of the histidine intermediates. Strain MH429/pHC9800 became two fold more sensitive to sulfisoxazole inhibition than the parent

strain (Table 15). This seemed to be in agreement with our hypothesis whereby an increase in one of the histidine intermediates results in increased sensitivity to sulfisoxazole inhibition.

On the other hand, strain MH429/pHC9800 remained as equally sensitive to thymidine inhibition as was the parent strain, MH429. This suggested that the mechanism of thymidine inhibition was different than the mechanism responsible for inhibition by sulfisoxazole.

To further test this hypothesis, I introduced the hisT mutation into strain MH429. Once again I expected that this mutation should result in an increase in the histidine enzymes and corresponding intermediates, and consequently should increase sensitivity to sulfisoxazole. Despite this presumption, MH429hisT remained as equally sensitive to sulfisoxazole as MH429 (Table 16). Hence, introducing the hisT mutation did not result in the same effect as did the plasmid. This may be explained by the fact that the hisT mutation only raises the histidine enzymes by 7X (Blasi et al, 1977), while the plasmid (pHC9800) raises them by at least 20X (Bruni et al, 1983).

Since the presence of the plasmid (pHC9800) resulted in increased sensitivity to sulfisoxazole, I tested to see whether the plasmid would make strain MH575(hisG), sensitive to the drug. The Thy-HisG- strain (MH575), transformed with this plasmid was as sensitive to sulfisoxazole, as the parent

Table 15. **Suppression of ϕLP12 by Various Thy-/pHC9800 Derivatives**

STRAIN	RELEVANT GENOTYPE	M.I.C.[b]	
		Sulfisoxazole	Thymidine
MH429	metB thyA	25.0μM	0.4mM
MH429/pHC9800		12.5μM	0.4mM
MH575	metB thyA hisG::Tn10	NI[c]	NI
MH575/pHC9800		25.0μM	0.4mM

Suppression was assayed as in Table 5. Stock solutions of sulfisoxazole (1mg/ml) and thymidine (5mg/ml).

[a] Plasmid pHC9800 is derived from plasmid pBR313, and contains the histidine operon.

[b] M.I.C. refers to the minimum inhibitory concentration.

[c] NI refers to phage mutant was not inhibited at the above specified concentrations.

Table 16. **Suppression of ϕLP12 by Various MH429/hisT Derivatives in the presence of Thymidine and Sulfisoxazole**

STRAIN	RELEVANT GENOTYPE	M.I.C.[a]	
		Sulfisoxazole	Thymidine
MH429	metB thyA	25.0μM	0.4mM
MH563	metB thyA hisT	25.0μM	0.4mM
MH575	metB thyA hisG	NI[b]	NI
MH571	metB thyA hisG hisT	NI	NI

Suppression was assayed as in Table 5. Stock solutions of sulfisoxazole (1mg/ml) and thymidine (5 mg/ml) were used, and a range of concentrations were assayed from 2.0-27.0μM and from 75.0-400.0μM respectively.

[a] M.I.C. refers to the minimum inhibitory concentration.

[b] NI refers to phage mutant was not inhibited at the above specified concentrations.

Thy- strain (MH429) (Table 15). However, under these circumstances it was necessary to determine whether the inhibition of suppression was due to the increase in one of the histidine intermediates or due to the increase in flow of the products through the pathway.

In order to determine this it was necessary to introduce the hisT mutation into strain MH575, which is blocked at the first step (hisG) of histidine biosynthesis. Since this strain is deficient in the first enzyme, the derepression of the histidine operon should only increase the histidine enzymes without affecting the flow through the pathway. Strain MH571 (hisGhisT) remained resistant to sulfisoxazole (Table 16), thus suggesting that it is the increased flow through the histidine pathway and not the increased levels of enzymes which results in the inhibition of suppression. Alternatively, this suggests that the inhibition observed by strain MH571 (hisGhisT) may be an effect of the hisG enzyme.

The Effect of folC Expression:

The enzyme folylpoly-γ-glutamate synthase (FPGS), is coded by the folC gene. This enzyme is bifunctional in nature, and is resposible for catalyzing the conversion of folates to polyglutamates, as well as the conversion of dihydropteroate to dihydrofolate (Bognar et al, 1989). Hence, this enzyme is essential for the de novo biosynthesis of folates.

Since the folC gene has the ability to alter the state in which intracellular folates are found, I tested whether overexpression of folC in cells transformed with plasmid pAC5 had any effect on suppression.

Plasmid pAC5 is a derivative of the multicopy plasmid pUC9, which contains only the folC gene, under the regulation of the lac promoter (Bognar et al, 1989). The suppressing ability of MH429 is completely abolished by the increased levels of folC expression (Table 17).

The inability of the Thy- strain to suppress in the presence of multiple copies of the folC gene may be a consequence of increased dihydrofolate synthase levels, instead of increased polyglutamylation. Overproduction of the folC gene is unlikely to increase the polyglutamate chain lengths of the folate cofactors since the folC gene product only synthesizes triglutamates (Brown and Williamson, 1987). Therefore, the only likely result of increased folC expression is an increase in cellular dihydrofolate levels. This increase in one type of folate cofactor may shift the distribution of the intracellular folates, so that the folate that might be required for suppression cannot be synthesized.

The sulfisoxazole resistant strains MH575(hisG) and MH540(purF) were also transformed with plasmid pAC5, in an attempt to see whether these strains were more tolerant to possible shifts in folate distribution. In both instances these strains remained capable of suppressing, and suppression was still resistant to the action of sulfisoxazole.

TABLE 17: Suppression of JC1922 by Various Thy-/pAC5 Derivatives in the Presence of Thymidine and Sulfisoxazole

STRAIN	RELEVANT GENOTYPE	THYMIDINE 20μg/ml	THYMIDINE 500μg/ml	SULFISOXAZOLE 3.3μg/ml
MH429	metB thyA	+[b]	-[c]	-
MH429/pAC5[a]		-	-	-
MH581	metB	-	-	-
MH581/pAC5		-	-	-
MH540	purF::Tn10	+	-	+
MH540/pAC5		+	-	+
MH575	hisG::Tn10	+	+	+
MH575/pAC5		+	+	+

Suppression was assayed as in Table 5. Stock solutions of sulfisoxazole (1 mg/ml) and thymidine (5mg/ml) were used.

[a]Plasmid pAC5 is derived from plasmid pUC9 which contains the folC gene under the regulation of the lac promoter.

[b] + denotes cells suppressed.

[c] - denotes the inhibition of suppression.

DISCUSSION

We have proposed that thyA dependent suppression may result from an alteration in the intracellular folate pools. To test this proposal I assayed the effect of altering folate metabolism on suppression. This was achieved by using the folate antagonists, sulphonamides (Then and Angerhn 1974).

Various sulphonamides were assayed in an attempt to determine whether reducing folate pools affected suppression. All the sulphonamides assayed were present at sublethal concentrations and were found to inhibit Thy- suppression. The only difference observed was that the minimum inhibitory concentration (M.I.C.) varied amongst the different sulphonamides. This difference can probably be attributed to differential uptake, whereby the ones with the lowest M.I.C. are probably internalized more readily (Then and Angerhn 1972). Sulfathiazole was found to inhibit suppression at the lowest concentration. This is probably due to the presence of the heterocyclic ring (thiazole), which has been proven to be most useful in retaining and enhancing the antibacterial effect of this sulphonamide (Jukes and Broquist, 1963). Since sulphonamides have been postulated to act towards the impairment of de novo folate synthesis (Martin, 1951), suppression was concluded to require elevated folate concentrations in Thy- auxotrophs as compared to Thy+ prototrophs. Further support for this conclusion is provided

by the observation that other antifolate drugs, such as aminopterin and trimethoprim, which inhibit dihydrofolate reductase, also inhibited suppression (J. Basso, unpublished results).

The inhibition of Thy- suppression by sulphonamides was not specific to one type of suppressible mutation. Suppression of all T4 nonsense mutants assayed on the Thy- strain MH429 was inhibited at approximately 25.0 μM of sulfisoxazole. However, two of the mutants, JC1922 (UAA) and OPC105 (UGA), could be inhibited by lower concentrations. No correlation between the increased sensitivity and type of suppressible mutation was found. This result is not surprising, since different suppressors are presumably altered in the modification of different tRNAs, and this might result in different sensitivity to folate levels.

Early studies have identified a compound, p-aminobenzoic acid (p-ABA), in yeast extract that was capable of counteracting sulphonamide toxicity (Then and Angerhn, 1974). p-ABA was postulated to be an essential metabolite for bacterial growth and sulphonamides were regarded as structural analogues of P-ABA, which interfere with bacterial growth by competing with P-ABA for the enzyme dihydropteroate synthase (Then and Angerhn 1974).

Consequently p-ABA was added to the medium in an attempt to rescue suppression from sulphonamide inhibition. The rescuing effect observed with p-ABA (1.3 uM) indicates that the action of sulfisoxazole on suppression may be due to its

interaction with dihydropteroate synthase, either by competing with the normal substrate (p-ABA) or by forming a product that cannot be utilized by the folate dependent enzymatic reactions. Hence this suggests that the primary mode of action of sulphonamides is on folate synthesis rather than on something other than folate biosynthesis.

Adding p-ABA exogenously probably results in an increase in folate biosynthesis since it outcompetes sulphonamides for the enzyme's active site. This is in agreement with our hypothesis that inhibition of Thy- suppression is a direct consequence of lowered folate biosynthesis. However, this finding contradicts studies conducted by Lampen and Jones (1946) which demonstrate that the inhibitory action of sulphonamides on E.coli is not reversed by p-ABA.

Kohn and Harris (1943) were the first to elaborate the theory of secondary inhibitors. These compounds were shown to be noncompetitive antagonists of sulphonamides and can be classified into four major groups: substances which increase growth rate and so mask sulphonamide action; essential metabolites which are displaced: compounds which combine with sulphonamides; and metabolites secondary to some prime reaction inhibited by the drug, (Martin, 1951). The last category include such things as amino acids, nucleic acids and their derivatives.

Several amino acids have been shown to be noncompetitive antagonists of sulphonamides. These include methionine, tryptophan, glycine, arginine, lysine, and serine (Martin,

1951). Consequently some of these amino acids as well as some others were assayed in an attempt to rescue suppression from sulfisoxazole inhibition.

Methionine added to the AB medium did not rescue suppression from sulfisoxazole inhibition. This was consistent with earlier studies that demonstrated that in E.coli methionine would antagonize only sulphanilamide but not sulfathiazole, sulfadiazine, or any of the other complex sulphonamides (Martin, 1951).

In contrast the addition of tryptophan into the assay medium, completely rescued suppression from sulfisoxazole inhibition. The rescuing effect observed with tryptophan was initially thought to be an indirect consequence of sparing chorismate levels, resulting in a greater flow to p-ABA. Hence, it would be the increase in p-ABA levels that was responsible for the recovery of suppression. If so, phenylalanine and tyrosine should also rescue suppression even though they may not spare as much chorismate as tryptophan (Pittard, 1987). They did not, suggesting that the tryptophan effect is not a result of sparing chorismate. Higher concentrations of phenylalanine and tyrosine did not rescue suppression either.

Alternatively, the effect observed with tryptophan may result from a breakdown product of this amino acid. It has been shown that tryptophan is a precursor of nicotinic acid, in Lactobacillus arabinosus (Martin, 1951), and nicotinate has been observed to rescue suppression from sulfisoxazole

inhibition (J Basso, unpublished results); hence, it may very well be that nicotinate derived from tryptophan is antagonizing the action of sulfisoxazole. This seems to be in agreement with studies conducted by Teply et al, (1943), who found that in L.arabinosus, sulphonamide bacteriostasis was completely overcomed by the addition of p-ABA or high levels of nicotinic acid.

Suppression was also partially rescued by the addition of histidine. The partial rescue of suppression by this amino acid may be explained if histidine is degraded in E.coli as it is in S.typhimurium. In S.typhimurium the breakdown of histidine by the hut genes results in an increase in the formation of N-formiminoglutamate, which in turn transfers its formimino group to THF to form the N-5 derivative of formyl-THF (Stryer 1981). This generation of N-5-formyl-THF may elevate folate levels sufficiently to rescue suppression from sulfisoxazole inhibition. However, there is no evidence for histidine degradation in E.coli.

Alternatively, the addition of high levels of l istidine might reduce flow through the histidine pathway by feedback inhibition of hisG enzyme and/or by repression of synthesis. This would be consistent with my observation with histidine mutants blocked in early steps of histidine biosynthesis. The only discrepancy is that the addition of histidine only partially rescues suppression from sulfisoxazole inhibition whereas a hisG mutant is completely resistant to sulfisoxazole. This may be explained by the fact that the

addition of histidine alone may not inactivate the synthesis of histidine to the same extent as does the mutation. In order to have complete inhibition of the histidine pathway, histidine must be present with ppGpp the alarmone for amino acid starvation. In this case histidine represses the hisG enzyme while ppGpp inhibits by attenuation. Since the cells presumably are not starved for amino acids in rich media, the histidine pathway would not be completely inhibited.

High thymidine (500.0 μg/ml) has been shown to inhibit thyA suppression, possibly by lowering intracellular folate levels (Cheung and Herrington, 1982). If thymidine inhibited suppression as a result of lowering folates, as did the sulphonamides, then I expected that both thymidine and sulfisoxazole should act additively. When both thymidine and sulfisoxazole were added in AB medium, suppression was inhibited. Thymidine was found to be additive to sulfisoxazole, suggesting that high levels of thymidine inhibit folate levels. The possible reduction in intracellular folates by thymidine made the suppression more sensitive to sulfisoxazole.

The addition of the herbicide amitrole was also shown to inhibit suppression (J. Basso, unpublished results). Since suppression was correlated to folates, it was postulated that amitrole might also affect suppression by lowering folate levels. In support of this postulate amitrole was shown to be additive to sulfisoxazole, thus suggesting that both these compounds acted towards lowering folate levels.

The above results indicate that suppression can be inhibited by factors which presumably result in a decrease of folates. Suppression may therefore result from the presence of higher folate pools in Thy- as compared to Thy+ cells. Alternatively, the folate pools may be the same in both Thy- and Thy+ but there may be differences in their distribution, so that one form is elevated in Thy- relative to Thy+. Consequently, the reduction in total folate pools after sulphonamide exposure may lead to non-suppressing levels of this type of folate. There are two observations that suggest that thyA suppression results not only from elevated folate pools, but shifts in folate distribution as well. First quantitative determination of folate levels demonstrated that there was a significant difference (P=0.002) in folate concentrations between Thy- and Thy+ strains. Thy- strains were shown to have approximately 20% more folates than Thy+ strains. This difference in folate concentration amongsts Thy- and Thy+ cells was not apparent under high thymidine (500.0μg/ml) conditions. Furthermore high concentrations of thymidine seemed to lower folate concentrations in both Thy-(38%) and Thy+(53%) cells. This suggests that the 20-38% increase in pools in thyA mutants are sufficient to explain the effect of suppression, and this effect seems to be corrected by the presence of high thymidine (500.0μg/ml) in the medium.

Furthermore when looking at folate distribution, Thy- strains were shown to have significantly (P=0.001) less

methyl folates than Thy+ strains under low thymidine conditions. This difference once again is not observed under high thymidine conditions. This suggets that Thy- strains may not require as many methyl folates as Thy+ strains, since they have a defective thymidylate synthase gene. Results also demonstrate that the methyl fraction of folates is considerably higher under suppressing conditions (20.0μg/ml) than under non-suppressing conditions (500.0μg/ml). Consequently, the additional reduction of methyl folates by subsequent addition of high thymidine may lead to non-suppressing levels of a characteristic methyl folate in Thy- cells, (for example, 5-methyl-THF).

Folate quantification was carried out on cells in the absence of T4 infection. It is still not clear whether phage infection is required for suppression. Consequently, it may be that following phage infection a difference in folate levels would have been observed. This seems to be consistent with earlier reports that demonstrate that following bacteriophage infection there is a shift in the distribution of intracellular folates (Nakamura and Kozloff, 1978).

From what has been presented thus far it appears that there is a distinct link between folates and the ability of Thy- cells to suppress. It appears that Thy- cells probably suppress not only because of elevated folate levels, but suppress as a result of altered folate distribution as well.

Genetic Modifications of the Purine Pathway:

Previous studies have demonstrated that in S. typhimurium a unique ribonucleotide, referred to as ZTP, accumulates whenever normal folate metabolism is altered (Bochner and Ames, 1982). This ribonucleotide is synthesized from a precursor ZMP which is a purine intermediate as well as a by-product of histidine biosynthesis. The accumulation of these nucleotides correlates specifically with folate deficiency; they do not accumulate under numerous other starvation conditions (Bochner and Ames, 1982). ZTP has therefore been proposed to be a folate alarmone which fluctuates in response to alterations in folate levels and specifically to 10-formyl-THF.

Studies have demonstrated that in E.coli there is a substantial accumulation of AICAR in certain rich media which contain casamino acids (Edward et al, 1951). These media ressemble AB medium used to assay suppression. AICAR can be formed via both the purine and histidine pathways.

Since ZTP which is derived from AICAR was postulated to be a folate alarmone, then manipulation of AICAR levels may alter ZTP levels which in turn might affect the suppression phenotypes. In an attempt to alter AICAR levels, strains were constructed with mutations blocking steps in the purine pathway, and in the histidine pathway.

Mutations affecting the first two steps of purine biosynthesis, alter usage of 10-formyl-THF and reduce total AICAR synthesis, since AICAR is now only synthesized through

the histidine pathway. These steps were blocked by purF and purD mutations. Both Thy-PurF- and Thy-PurD- suppressed, but responded differently to sulfisoxazole. Suppression by the Thy-PurF- mutant was insensitive to sulfisoxazole while suppression of Thy-PurD- was sensitive.

The resistant phenotype observed by the purF insertion mutant, was found to be a consequence of the insertion itself as opposed to a reduced flow through the purine pathway, since some purF deletion mutants became sensitive to sulfisoxazole inhibition. Both the purF insertion and the purF deletion mutants were expected to be null mutations of the purF gene. However, the Tn10 insertion mutant and presumably some of the deletion mutants should have been polar mutations whereas other deletion mutants may not be insertion mutants. The polar mutations might consequently cause the inactivation of genes which are located downstream from purF, and this might lead to the resistant phenotype observed. This seems to be consistent with what is known about polar effects at the purF locus.

The purF gene has been shown to be part of a polycistronic operon along with two genes of unknown function, dedE and dedF (Rolfes and Zalkin, 1988). The dedE gene is located immediately upstream of the purF and the dedF gene is located downstream. PurF polar mutations have been shown to inactivate the transcription of the downstream gene (approximately 100bp downstream)(Rolfes and Zalkin, 1988), which has been identified as the E.coli homolog for the S.

typhimurium ubiX gene. The ubiX gene has been shown to be responsible for altering the permeability characteristics of the plasma membrane, leading to reduced uptake of some antibiotics. Therefore the sulphanilamide resistant phenotype exemplified by the purF::Tn10 mutants, might be due to the polar inactivation of the downstream ubiX homolog, which possibly renders the cell impermeable to sulphonamides. This can be tested by assaying the sensitivity between purF deletion and purF::Tn10 insertion mutants to sulfisoxazole. The purF::Tn10 insertion mutants were extremely tolerant to high sulfisoxazole concentrations (1.0 mg/ml) suggesting that the sulphonamide may have been inefficiently taken into the cell (data not shown).

Suppression by the purF deletion mutants as well as purine mutants blocked after the first THF dependent step, (purF thyA, purE thyA, purH thyA, and purB thyA), suppressed, but were all sensitive to inhibition by sulfisoxazole. Therefore, changes in sulfisoxazole sensitivity in these purine mutants, as compared to purF mutants, could reflect changes in their amount of dihydropteroate synthase, or could reflect changes in the permeability of the cells towards sulfisoxazole.

Genetic alterations of the Histidine Pathway:

The other source of AICAR in cells is the histidine pathway. Furthermore, the herbicide 3-amino-1,2,4-triazole (amitrole), which has been shown to inhibit histidinol

phosphatase (Hilton et al, 1965), also inhibits suppression (J. Basso, unpublished results). For these reasons the effect of histidine pathway mutants on suppression was examined.

All Thy-His- strains with mutations blocking steps of pathway before the production of AICAR suppressed and their suppression was not inhibited by sulfisoxazole nor thymidine. This suggested that these mutants may have elevated folate biosynthetic enzymes (or at least high dihydropteroate synthase) with high folate levels. These high folate levels and folate biosynthetic enzymes may not be responsive to inhibitory levels of thymidine, or can not be lowered sufficiently by thymidine to non-suppressing levels. Furthermore, these results suggest that thymidylate stress is not required for suppression since the hisG thyA strain suppresses at thymidine concentrations which were 25 times (500.0μg/ml) that needed for growth (J. Basso, unpublished results).

In contrast mutants which were defective in enzymes after this bifurcation step, hisB, suppressed but were inhibited by sulfisoxazole and thymidine. This suggests that AICAR derived from the histidine pathway may have a regulatory role in folate metabolism.

The flow of intermediates through the histidine pathway might be increased by overproducing the intermediates or by introducing the hisT mutation, which leads to the derepression of the histidine operon.

Transforming strain MH429 with plasmid pHC9800 resulted in a two fold increase in sulfisoxazole sensitivity. That is, less sulfisoxazole was required to inhibit the suppression of ELP12 by MH429. This seemed to be in agreement with our hypothesis whereby an increase in one of the histidine intermediates results in increased sulfisoxazole sensitivity.

Transducing the hisT mutation into strain MH429 did not increase sulfisoxazole sensitivity as did the incorporation of the plasmid. This possibly can be explained by the fact that the plasmid pHC9800 raises the histidine enzymes to a greater level than does the hisT mutation. The plasmid has been reported to raise the enzymes about 20 fold (Bruni et al, 1983), while the mutation only raises them 7 fold (Blasi et al, 1977).

In addition, increasing the histidine intermediates by introducing the histidine plasmid (pHC9800) resulted in the inhibition of suppression by the previously resistant MH575(hisG) strain. However, it was not clear whether this sensitivity was brought about by the increase in the histidine intermediates per se or an increase in AICAR levels, resulting from the increase in flow through the pathway, or simply the presence of the hisG enzyme. The sensitivity in this case was possibly due to increased flow through the pathway since the hisT mutation did not produce the same phenotype as did the pHC9800 plasmid, in a thyA hisG background. This lends further support for AICAR's possible regulatory role in folate biosynthesis. However, the above

conclusion is not definitive because as stated above the plasmid pHC9800 increases the histidine enzymes to a greater extent than the hisT mutation. Therefore the lack of sensitivity to sulfisoxazole by hisG- hisT- thyA- may be a result of not having the elevation of histidine enzymes to levels comparable with those observed with the plasmid. Furthermore the amount of flow through a pathway is usually a function of the amount of product that can feed-back inhibit. Therefore in the case of the plasmid, the increase in histidine enzymes is not necessarily accompanied by an increase in flow through the pathway since the pathway may be repressed by end-product inhibition. Therefore sensitivity to sulfisoxazole may be due to the fact that the plasmid (pHC9800) simply establishes wild type hisG levels of expression.

The resulting scenario postulates that increased AICAR levels allow Thy- suppression to be inhibited by sulfanilamides and vice versa. This was further verified by inhibiting the suppression of strain MH575(hisG) and MH540(purF), by elevating AICAR levels.

AICAR levels were elevated by the exogenous addition of AICA, which is converted to AICAR. AICA is the only purine intermediate that can be exogenously supplied to the cell (Neuhard and Nygaard, 1987). Therefore adding increased amounts of AICA to the medium possibly results in the rapid internalization of this compound, which increases the already existing AICAR pools. Consequently this increase in AICAR

levels results in the observed inhibition pattern.

Alternatively the addition of AICA may simply be converted to adenine and hence AICA may indirectly potentiate sulfisoxazole toxicity by lowering folate biosynthesis. Therefore in the case of the purF::Tn10 insertion mutant, adding AICA in the presence of sulfisoxazole increases this sulphonamide's toxicity and hence less is required to inhibit.

The addition of adenine also permitted sulfisoxazole inhibition of suppression by thyA hisG and thyA purF strains. The mode of action of adenine may be explained in several ways. First, a derivative of adenine, ATP, has been demonstrated to inhibit the reaction involved in the oxidation of 5,10-methylenetetrahydro-pteroylglutamate to 5,10-methenyltetrahydroptroylglutamate, and this in turn results in the inhibition of formate of 10-formylTHF (Dalal and Gots, 1966). Consequently, if suppression is a function of AICAR levels, adenine may decrease 10-formyl-THF levels sufficiently, so that AICAR which utilizes this folate accumulates. This increase in AICAR may create non-permissive conditions for suppression. However this hypothesis cannot be justified since a purH mutant which should accumulates AICAR suppresses.

Alternatively, purines such as adenine have been shown to potentiate sulphonamide toxicity (Bruce et al, 1984). Therefore, the addition of adenine might lower folates and consequently the subsequent addition of sulfisoxazole may

result in an increased intracellular folate deficiency. This reduction in total folates may impair the synthesis of a folate coenzyme which is required for suppression.

Thymidine Inhibition:

Suppression by all the purine and histidine mutants previously described was also tested in the presence of inhibitory concentrations (500.0 µg/ml) of thymidine. Interesting enough, all the purine mutants constructed, with exception of the purH mutant (J.Basso unpublished results), were inhibited by high levels of thymidine. High thymidine concentrations had also no effect on some of the histidine mutants (hisG, hisA, hisI) as well.

High thymidine has been shown to repress glyA synthesis. A decrease in glyA consequently may shift the intracellular folate distribution, or decrease total folate pools since 5-10-methylene-THF can no longer be synthesized this way. However, E.coli cells also have the GCV (glucose cleavage system) pathway which may compensate for the lack of glyA expression and restore 5-10-methylene-THF levels. Therefore for thymidine to be effective as an inhibitor, both the glyA and GCV must be repressed. The resistance of suppression by purine and histidine mutants may be a consequence of a intermediate needed in conjunction with thymidine to repress glyA and GCV. For instance, the GCV pathway has been shown to be inhibited by inosine a purine intermediate following the AICAR step.

Inosine can be synthesized via the purine as well as the histidine pathways. Inosine synthesized via the histidine pathway results in a regenerative pathway for ATP used during histidine biosynthesis. Inosine synthesized via the purine pathway is for the synthesis of purines.

Under suppressing conditions, histidine and purines should be found in sufficient amounts, so this may result in the accumulation of AICAR and/or inosine. The accumulation of inosine with the subsequent addition of high thymidine (500.0μg/ml) may alter folate levels in Thy-, and render them compatible with levels found in Thy+. Therefore, all the purine mutants (purF → purC), and a histidine mutant (hisB) which may accumulate inosine, lose their ability to suppress in the presence of high thymidine. In contrast, mutants which may not accumulate inosine, (purH) (hisG,I,A), become non-responsive to inhibitory levels of thymidine, since they cannot repress GCV expression. The inability to inhibit GCV may maintain appropriate levels or distribution of folates required for suppression in the presence of thymidine.

The resulting scenario postulates that thymidine as in the case of sulfisoxazole, does eventually result in the lowering of folate levels, even though the mechanisms by which this occurs may be different. Thymidine seems to inhibit folate biosynthesis at the level of a gene as opposed to the inhibition of a specific de novo folate enzyme, as in the case of sulfisoxazole.

The Effect of *folC* Expression:

Another way in which I attempted to alter folate distribution was by overexpressing the *folC* gene. Overexpression of the *folC* gene was shown to inhibit *thyA* suppression.

The *folC* gene is a bifunctional enzyme that has dihydrofolate synthase (dHFS) activity. Therefore, the increase in dHFS may lead to the accumulation of dihydrofolate (not its polyglutamates because these would be produced as products of thymidylate synthase and folate biosynthesis). In contrast, one obvious effect of the *thyA* mutantion would be a decrease in cellular dihydrofolate. This decrease in dihydrofolate may not allow for the synthesis of tetrahydrofolate, and this may have regulatory consequences on a number of folate enzymes including methylene-THF reductase and thymidylate synthase. Therefore, an increase in the dihydrofolate synthase activity of *folC* might restore dihydrofolate levels in the cell and reverse some of the *thyA* phenotype.

Transforming strains MH575(*hisG*) and MH540(*purF*) with the *folC* plasmid, did not have any effect on their suppression patterns. These strains still remained resistant to sulfisoxazole and retained their ability to suppress. This suggested that these strains somehow nullified the effect exerted by increased dihydrofolate synthase activity.

The folC gene is closely linked to the hisT and purF polycistronic operons, at 50min in the E. coli linkage map (Makaroff and Zalkin, 1985). Genes located within this area, such as the hisT, dedA, dedB, and possibly folC have been postulated to be coordinately transcribed with one another. The folC gene has been shown by Bognar et al, (1989) to be regulated by two promoters, which are present 5' to the coding sequence of a upstream gene of unknown function. The downstream promoter has been identified as being essential for high folC expression, and these studies have also shown that the -35 region of this downstream promoter is located within the loop of a stable hairpin structure. Therefore, they postulate that the presence of this structure suggests that there may be a transcription activator that is required for expression of this promoter, and there may be a trans acting factor that binds to this region of DNA and controls the regulation of the folC gene.

The purF and hisG mutations result in a decrease in flow of intermediates through the purine and histidine pathways. The lack of flow through these pathways might in turn regulate folate biosynthesis and folC expression since folC is a de novo folate biosynthetic enzyme.

Suppression of T4 mutants by Thy- strains of E.coli has been shown to be a function of altered folate levels in Thy- as compared to Thy+ cells. The alteration of folates by various genetic and environmental factors has resulted in altered suppression patterns. Furthermore, conditions which

have been shown to reduce intracellular folate levels and inhibit suppression, were overcome by blocking a number of biosynthetic steps involving the histidine pathway.

Suppression was believed to require thymidine limitation and the ability of thymidine to inhibit suppression was possibly due to relieving thymidine stress. However this was not so since suppression was shown to occur at levels of thymidine which were 25 times (500.0μg/ml) greater than what is required for growth. Furthermore, thymidine was shown to be additive to sulfisoxazole which suggested that both these inhibitors lowered folates. This speculation was further justified by the fact that in the presence of high thymidine (500.0 μg/ml), folate concentrations were significantly reduced.

The mechanism of sulfisoxazole inhibition was shown to involve the purine intermediate, AICAR. Conditions which possibly led to the accumulation of AICAR in the presence of sulfisoxazole resulted in the inhibition of Thy- suppression. In contrast, conditions which elevated AICAR levels in the presence of thymidine, failed to inhibit suppression. This suggested that the mechanism of thymidine inhibition is different from that observed with sulphonamides, and should be dealt with independently.

Although this study did not unravel the mechanism of Thy- suppression, it brought some insight into some of the factors which are involved in suppression, under the above mentioned conditions. Further analysis of these factors is

required to determine the mechanism of suppression under different types of experimental conditions.

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