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The Role of Polyglutamylation in the Cytotoxicity of Methotrexate in *Neurospora crassa*

by

Rebecca Ellen Wrishko

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Biological Sciences

Edmonton, Alberta

Spring, 1996

ISBN 0-612-10767-1

University of Alberta

Library Release Form

Name of Author: Rebecca Ellen Wrishko

Title of Thesis: The Role of Polyglutamylation in the Cytotoxicity of Methotrexate in *Neurospora crassa*

Degree: Master of Science

Year this Degree Granted: 1996

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Date: Dec 5, 1996

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Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled The Role of Polyglutamylation in the Cytotoxicity of Methotrexate in *Neurospora crassa* by Rebecca Ellen Wrishko in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Methotrexate (MTX), an antineoplastic agent, may undergo metabolism to polyglutamate derivatives which have profound ramifications with regard to cytotoxicity. In the present studies, the effectiveness and selectivity of MTX towards wild type, and folylpolyglutamate deficient mutants, *met*-6 and *mac*, of *Neurospora crassa* were examined. Dose-response relationships as measured by mycelial dry weights revealed a greater sensitivity of *met*-6 and *mac* mutants than wild type as quantified by the IC_{50} values of 5.5 μM, 6.0 μM and 87.5 μM, respectively. When similar cultures were subjected to folinic acid rescue, following MTX exposure, a 50 μM concentration produced a *ca.* 20% recovery in the mutant strains, but only a *ca.* 8% recovery in wild type. Shorter exposures (6 hours) to MTX, followed by a 24 hour chase in the absence of this antifolate, provided evidence that growth during the recovery phase was more rapid in the mutants. The synthesis of MTX polyglutamate ($MTXGlu_n$) derivatives was evaluated using [^{3}H]-MTX and HPLC analyses. The extracted radioactivity from wild type corresponded to elution positions of MTX, $MTXGlu_2$ and $MTXGlu_3$ standards. In these pulse-chase experiments, MTX was the primary species; however there was evidence of $MTXGlu_2$ (2% of total) and $MTXGlu_3$ (1% of total). The results demonstrated the limited capacity of wild type to conjugate long chain polyglutamate derivatives. MTX polyglutamates were not detected in the *mac* mutant, and only very low levels of $MTXGlu_2$ were detected in *met*-6 after incubation in 1.0 μM [^{3}H]-MTX. Further, binding analyses did not suggest a MTX-binding protein other than DHFR in any of the three strains.

Additionally, DHFR was isolated from wild type, *met*-6 and *mac* strains and purified over 1400-fold, to homogeneity. Enzyme activity was fractionated using ammonium sulfate and chromatography on Sephadex G-75 and Matrex Green A. The levels of DHFR expression, based on the calculated activity, were not significantly different between the three strains. DHFR activity in each strain was associated with a M_r of 21.6 kD, based on the reduction of $H_2PteGlu$.

ACKNOWLEDGEMENT

I would like to thank my supervisor, Dr. Edwin A. Cossins, for his guidance and assistance throughout this project and for his financial support as provided by the Natural Sciences and Engineering Research Council of Canada.

I would also like to thank my parents, colleagues and friends for their support and encouragement.

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7-OH-MTX	7-hydroxy-methotrexate
7-OH-MTXGlu	7-hydroxy-methotrexate polyglutamate
5-HCO-H_4PteGlu	5-formyl-tetrahydrofolate (folinic acid; leucovorin)
10-HCO-H_4PteGlu	10-formyl-tetrahydrofolate
5-CH_3-H_4PteGlu$_n$	5-methyl-tetrahydrofolate polyglutamate
5,10-CH=H_4PteGlu$_n$	5,10-methenyl-tetrahydrofolate polyglutamate
5,10-CH_2-H_4PteGlu$_n$	5,10-methylene-tetrahydrofolate polyglutamate
[^{3}H]	tritium
ACN	acetonitrile
AMP	adenosine-5′-monophosphate
ADP	adenosine-5′-diphosphate
ATP	adenosine-5′-triphosphate
AdoMet	S-adenosylmethionine
AICAR	5-amino-4-imidazolecarboxamide ribonucleotide
ca.	circa
C_1	one-carbon
CHO	chinese hamster ovary
Da	Dalton
dTMP	deoxythymidine monophosphate
dUMP	deoxyuridylate monophosphate
DEAE	diethylaminoethyl
DHFR	dihydrofolate reductase
DPM	disintegration per minute
EDTA	ethylenediaminetetraacetic acid
FADH	flavin adenine dinucleotide
$FADH_2$	flavin adenine dinucleotide (reduced form)

FPGS	folylpolyglutamate synthetase
GGH	gamma-glutamyl hydrolase
GAR	glycinamide ribonucleotide
GTP	guanosine-5′-triphosphate
$H_2PteGlu$	dihydropteroylglutamic acid (dihydrofolate)
$H_2PteGlu_n$	dihydrofolate polyglutamate
$H_4PteGlu$	tetrahydropteroylglutamic acid (tetrahydrofolate)
$H_4PteGlu_2$	tetrahydrofolate diglutamate
$H_4PteGlu_3$	tetrahydrofolate triglutamate
$H_4PteGlu_4$	tetrahydrofolate tetraglutamate
$H_4PteGlu_6$	tetrahydrofolate hexaglutamate
$H_4PteGlu_n$	tetrahydrofolate polyglutamate
HPLC	high performance liquid chromatography
IC_{50}	concentration that inhibits activity by 50%
kD	kiloDalton
MTX	4-amino-10-methylpteroylglutamic acid (methotrexate)
$MTXGlu_2$	methotrexate diglutamate
$MTXGlu_3$	methotrexate triglutamate
$MTXGlu_4$	methotrexate tetraglutamate
$MTXGlu_5$	methotrexate pentaglutamate
$MTXGlu_n$	methotrexate polyglutamate
NAD^+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	nicotinamide adenine dinucleotide phosphate
$NADPH_2$	nicotinamide adenine dinucleotide phosphate (reduced form)
NMR	nuclear magnetic resonance
*p*ABA	*p*-aminobenzoic acid
PMSF	phenylmethylsulfonyl fluoride

PBS	phosphate-buffered saline
PteGlu	pteroylglutamic acid
$PteGlu_2$	pteroyldiglutamate
$PteGlu_3$	pteroyltriglutamate
$PteGlu_5$	pteroylpentaglutamate
$PteGlu_6$	pteroylhexaglutamate
$PteGlu_n$	pteroylpolyglutamate
PLP	pyridoxal-5′-phosphate
Pic A	tetrabutylammonium dihydrogen phosphate
SHMT	serine hydroxymethyltransferase
TS	thymidylate synthase
TCA	trichloroacetic acid
Ve	elution volume

INTRODUCTION

Folate coenzymes act as acceptors or donors of one-carbon units and are essential to cellular metabolism since these derivatives are involved in a variety of reactions leading to thymidylate, purine nucleotides and the amino acids serine, glycine and methionine (MacKenzie, 1984). Of all the coenzymes, tetrahydrofolate exhibits the most structural diversity. Originally tetrahydropteroylmonoglutamate was thought to be the principal coenzyme of one-carbon metabolism, but it has been known for several decades that the physiologically active forms of the coenzymes contain from four to seven glutamyl residues linked by amide bonds through the γ-carboxyl group (Schirch and Strong, 1989). Although these folate polyglutamates were originally thought to serve only a storage role, evidence now exists for their function as the preferred active coenzymes (Covey, 1980). Additionally, polyglutamylation appears to contribute significantly to the intracellular retention of folate cofactors.

Dihydrofolate reductase catalyzes dihydrofolate reduction to tetrahydrofolate which is required for subsequent one-carbon transfer reactions (Blakley, 1969). If this enzyme is blocked or deleted, the continued generation of dihydrofolate will ultimately deplete pools of reduced folates. Under these conditions, the tetrahydrofolate-dependent synthesis of DNA, as well as RNA and protein, would consequently cease (Matherly et al, 1987). Consequently, dihydrofolate reductase inhibitors like methotrexate have become a mainstay in chemotherapeutic treatments (Fleming and Schilsky, 1992).

In mammalian cells, methotrexate, like its physiological counterpart tetrahydrofolate, can serve as a substrate for folylpolyglutamate synthetase and be converted into methotrexate polyglutamates (Baugh et al, 1973). The therapeutic implications of methotrexate polyglutamates include their selective retention within cells and increased affinity for the target enzyme and other folate-dependent enzymes, both of which enhance the cytotoxicity of the drug (Kalman, 1990; Abraham et al, 1991). Hence, the ability of cells to synthesize methotrexate polyglutamates may be a prognostic indicator of therapeutic effectiveness, as demonstrated by the correlation between decreased polyglutamylation and resistance (Assaraf et al, 1992).

1.1. CHEMISTRY OF FOLIC ACID

The term, pterin, was originally used as a collective term for butterfly wing pigments or even for insect pigments in general and as such has no precise chemical meaning, since insect pigments include not only pteridines, but purine derivatives, phenoxazine derivatives and melanin-type pigments (Blakley, 1969). Pterin has also been used occasionally as an abbreviation for pteridine. During the same period that German chemists were isolating and characterizing the pigments from butterfly wings and elucidating the structure of these compounds, a number of apparently unrelated studies on nutritional factors were forming the bases for the discovery that another pteridine, folic acid, and its derivatives play a key role in metabolism.

In 1931, Lucy Wills reported that a component of the yeast extract "marmite" was effective in the treatment of tropical macrocytic anemia (Wills, 1931). Other studies reported that a factor in yeast, alfalfa and wheat bran stimulated the growth of chicks maintained on highly purified diets and that a factor in liver extracts prevented macrocytic anemia in chicks (Hogan and Parrott, 1940; Pfiffner et al, 1943). Further studies identified essential nutrients for lactic acid bacteria in liver, yeast and spinach extracts (Mitchell, 1941, 1944). Purification of these factors responsible for these nutritional effects in primates, chickens and bacteria revealed that several of the factors were identical and possessed a N-[4-{[(2-amino-4-hydroxy-6-pteridinyl) methyl] amino}benzoyl] glutamic acid structure (Blakley, 1969). The chemists who elucidated this structure, proposed the name 'pteroylglutamic acid' (Blakley, 1969). The alternative name, 'folic acid', was first proposed by Mitchell et al (1941) for the extract isolated from spinach which is a nutritional factor for *Streptococcus faecalis* R. The identification and determination of some or all of the derivatives of folic acid in serum, plasma, red cells or whole blood have been the subject of numerous investigations (Blakley, 1969). These folate derivatives occur in physiological fluids such as human milk and blood serum; in mammalian tissues including liver, kidney, spleen and intestine and, in microorganisms and plants (Blakley, 1969).

The growth promoting effect of these folate derivatives isolated from various bacteria formed the basis of microbiological assays in the course of the purification of these compounds from natural sources. Hence, bacteria have been extensively utilized for the assay of folate derivatives in natural materials. Since bacteria exhibit differential selectivity in their response to folate derivatives, identification of these derivatives proceeded through these means (Blakley, 1969). Refinement of these early assays was achieved by exploiting the specificity of the bacterial growth response. Consequently,

modern assays employ *Lactobacillus casei* [now known as *Lactobacillus rhaminosis* (ATCC 7469)] to detect all naturally occuring folates (Blakley, 1969). *Pediococcus cerevisiae* [now known as *Pediococcus acidilactici* (ATCC 8081)] only responds to highly reduced derivatives, such as 5-HCO-H_4PteGlu and 10-HCO-H_4PteGlu, that are not methylated (Blakley, 1969). *Streptococcus faecalis* [now known as *Enterococcus hirae* (ATCC 8043)] (Stover and Schirch, 1993) only responds to tetrahydrofolate (H_4PteGlu) and its formyl derivatives, not to methyl folates. These organisms do not respond quantitatively to polyglutamylated folate derivatives, thus they must be hydrolyzed before utilization in the microbiological assays (Krumdieck and Baugh, 1969; Cossins, 1980).

The classification and quantification of folates may be ascertained using other techniques, including paper and ion exchange chromatography, gel filtration, high performance liquid chromatography (HPLC) and competitive radioimmunoassays (Blakley, 1969; Cossins, 1984).

Chemically, folic acid is formed from three distinct moieties: a bicyclic, heterocyclic ring, 6-methylpterin; *p*-aminobenzoic acid (*p*ABA), which is itself required for the growth of many bacteria; and glutamic acid. In the structure of folic acid, 6-methylpterin is linked through the amino group of *p*ABA to form pteroic acid, which is linked in turn via an amide to glutamate, to form pteroylmonoglutamate. Polyglutamyl forms of the natural folates appear to be ubiquitous in nature, having been described in bacteria, plants, yeast and mammals; predominantly comprised of two to eight glutamate residues (McGuire and Bertino, 1981). These glutamate residues are linked to one another via a modified peptide bond between the α-amino group and the terminal γ-carboxyl group. Since the discovery of polyglutamates, a problem of nomenclature has arisen. Use of the term "folic acid" is generic and could be used to refer to: a molecule that initially contains one glutamic acid residue linked to pteroic acid (Covey, 1980), thus folylhexaglutamate contains seven glutamic acid residues; that is, six additional glutamate residues. Pteroylglutamic acid (PteGlu), the preferred nomenclature, refers to a compound that accounts for all glutamate residues. Hence, pteroylhexaglutamate ($PteGlu_6$) contains six glutamate residues (Covey, 1980).

Folate refers to a vitamin, based on its organic composition, which is found in minute quantities in most foods and is closely associated with the maintenance of normal physiological functions in mammals. Although all organisms require folate, not all are able to synthesize this compound. In mammals, folate is an essential nutrient and must be provided in the diet or by intestinal microorganisms. Most of the nutritional

folate is highly polyglutamylated and requires the enzymatic hydrolysis of the polyglutamate chain to permit its absorption (Kalman, 1990). A decrease in the activity of intestinal brush border conjugases may lead to certain folate deficiency syndromes. Gamma-glutamyl hydrolases (GGH) are primarily localized in the lysosomes of mammalian cells (McGuire and Coward, 1984) and function in the hydrolysis of glutamyl residues prior to folic acid absorption.

1.1.1. The Biosynthesis of Tetrahydrofolate

The three structural components of folate-namely pteridine, *p*-aminobenzoate and L-glutamate are incorporated into tetrahydrofolylpolyglutamate ($H_4PteGlu_n$) by a complex pathway in plants and bacteria, Figure 1. The pteridine component of plant folates, like bacteria and animal tissues, is derived from guanosine-5′-triphosphate (GTP) (Brown et al, 1975). The biosynthesis of 2-amino-4-hydroxy-6-hydroxymethyl dihydropteridine involves the conversion of GTP through 6-hydroxymethyl dihydroneopterin (Brown et al, 1975). This is followed by the conversion through a pyrophosphate intermediate to dihydropteroate which, in turn, is converted to dihydrofolate ($H_2PteGlu$) followed by $H_4PteGlu$ and $H_4PteGlu_n$.

1.1.1.1. The Biosynthesis of Pyrophosphorylmethyl dihydropteridine

The view that naturally occurring pterins are produced in animals from GTP with dihydroneopterin triphosphate as an intermediate has received support from reports that GTP cyclohydrolase I occurs in animal tissues (Brown and Fan, 1975; Brown et al, 1979). Understanding of the nature of the reactions involved in the transformation of a guanosine derivative into a pterin has been further advanced by the study of this transformation in cell-free extracts. Investigations by Brown et al (1975) have been directed toward an understanding of the enzymatic synthesis of the pterin precusor of folic acid in *Escherichia coli*. As with bacteria and animal tissues, the pteridine component of plant folates arises from GTP (Cossins, 1980).

The elucidation of the reactions in the biosynthetic pathway in *E. coli* between GTP and the pteridine moiety $H_2PteGlu$ indicates that the reaction scheme proceeds from GTP to dihydroneopterin triphosphate through triphosphate rather than monophosphate intermediates (Brown et al, 1975). The initial elimination reaction of formic acid from GTP, reaction [1], is catalyzed by GTP cyclohydrolase I (EC 3.5.4.16) (Iwai and Kobashi, 1975).

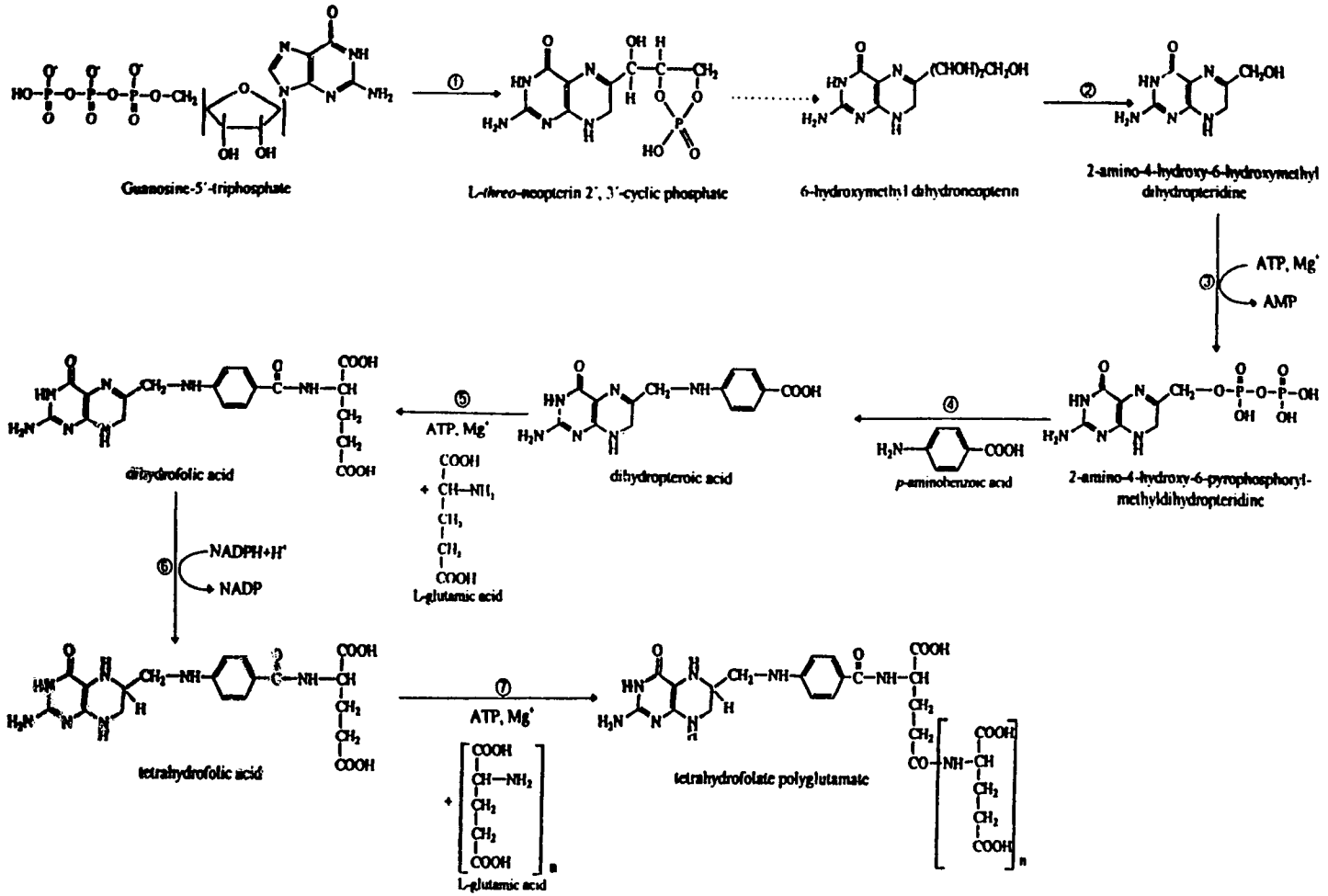


Figure 1. Biosynthesis of Tetrahydrofolate from Pterin Precursors. The successive reactions are catalyzed by the following enzymes: (1) guanosine triphosphate cyclohydrolase I; (2) dihydroneopterin aldolase; (3) hydroxymethyldihydropterin pyrophosphokinase; (4) dihydropteroate synthase; (5) dihydrofolate synthetase; (6) dihydrofolate reductase; and (7) folylpolyglutamate synthetase.

$$GTP + 2H_2O \rightarrow \text{formate} + \text{2-amino-4-hydroxy-6-dihydroneopterin triphosphate} \quad [1]$$

Direct evidence that dihydroneopterin triphosphate is the first pterin product of the pathway has resulted from investigations of the pterin products obtained by the action of *Pseudomonas* extracts on GTP (Blakley, 1969). A similar biosynthetic pathway, to that reported by Brown et al (1975), between GTP and dihydropteroate has been demonstrated in extracts of *Salmonella typhimurium*, *Lactobacillus plantarum* and *Brassica pekinensis* (Blakley, 1969).

Brown et al (1975) have suggested that in normal folate biosynthesis, phosphate residues are initially hydrolysed from the trihydroxypropyl side chain. Brown et al (1975) first presented evidence that a phosphatase, or a series of phosphatases, is (are) involved in the enzymatic conversion of dihydroneopterin triphosphate to 6-hydroxymethyl dihydropterin, the pterin compound used directly for the enzymatic synthesis of dihydropteroic acid. Dihydroneopterin triphosphate pyrophosphohydrolase from *E. coli* has a strict substrate specificity for the triphosphate ester (Brown et al, 1975). The resultant removal of inorganic pyrophosphate from the substrate produces the byproduct dihydroneopterin phosphate or the corresponding 2',3'-cyclic phosphoester. Since the phosphate esters of dihydroneopterin cannot be utilized for subsequent reactions, a phosphatase enzyme in *E. coli* catalyzes the hydrolytic removal of the remaining phosphate moiety from dihydroneopterin phosphate (Brown et al, 1975). This phosphate hydrolysis is followed by the removal of a two carbon compound having a 6-hydroxymethyl sidechain (Blakley, 1969).

Brown et al (1975) presented evidence that an enzyme in *E. coli* extracts converts dihydroneopterin to a more efficient precursor, dihydro-6-hydroxymethyl pterin. Similarly, extracts of *Brassica pekinensis* convert dihydroneopterin to dihydropteroate in the presence of *p*ABA, ATP and Mg^{2+} form dihydro-6-hydroxymethyl pterin (Blakley, 1969). The removal of the two carbon unit from dihydroneopterin is catalyzed by dihydroneopterin aldolase (EC 4.1.2.25) which produces glycolaldehyde and dihydro-6-hydroxymethylpterin, reaction [2] (Blakley, 1969; Brown et al, 1975; Albert, 1975; Wood, 1975).

$$\text{2-amino-4-hydroxy-6-dihydroneopterin} \rightarrow \text{glycolaldehyde} + \text{2-amino-4-hydroxy-6-hydroxymethyldihydropteridine} \quad [2]$$

Shiota (1984) and Brown et al (1975) demonstrated that extracts of *Lactobacillus plantarum* and *E. coli*, respectively require ATP and Mg^{2+} to synthesize a pterin diphosphate necessary to yield dihydropteroate. Although several pterins were found to be active in this reaction, 7,8-dihydroxymethylpterin was more active with *L. plantarum* extracts than other structurally modified pterins. Synthesis of H_2PteGlu and dihydropteroate from the pterin diphosphate also occurred in the presence of extracts of *Butyribacterium rettgeri*, *Fusobacterium fusiforma* and *Veillonella strain* V_2 (Blakley, 1969). The enzyme system which utilizes hydroxymethyl dihydropterin for H_2PteGlu synthesis in *E. coli* was reported to be resolved into two fractions. 7,8-dihydropterin pyrophosphokinase catalyzes the pyrophosphorylation of the hydroxymethyl dihydropterin derivative to the pyrophosphate derivative, reaction [3], while dihydropteroate synthase condenses diphosphate pterin with either *p*-aminobenzoate or *p*-aminobenzoyl glutamate (Shiota, 1984).

2-amino-4-hydroxy-6-hydroxymethyl dihydropteridine + ATP + Mg^{2+} →
2-amino-4-hydroxy-6-pyrophosphorylmethyl dihydropteridine + AMP [3]

The catalysis of dihydroxymethyl pterin to dihydropteroic acid in the presence of ATP and Mg^{2+} by pea seedlings appears to be due to a single protein with a molecular weight of 180,000 Da. In contrast to the *E. coli* pyrophosphokinase, having a molecular weight of 15,000 Da, the pea enzyme is a very large protein (Shiota, 1984). Furthermore, the pea seedling enzyme did not release the pyrophosphate derivative which remained enzyme-bound. Similar large molecular weight enzymes were reported in species of *Plasmodium* (Shiota, 1984).

1.1.1.2. Condensation of a Pyrophosphate Pterin with p-Aminobenzoate

Brown et al (1979) postulated that *in vivo* folic acid biosynthesis proceeds exclusively via dihydropteroate, which is formed by condensation of *p*-aminobenzoate with a diphosphate pterin precursor as depicted in reaction [4]. The enzyme that utilizes *p*-aminobenzoate is accordingly termed dihydropteroate synthase (EC 2.5.1.15).

2-amino-4-hydroxy-6-pyrophosphorylmethyl dihydropteridine +
p-aminobenzoate → dihydropteroic acid + $H_4P_2O_7$ [4]

Preliminary investigations utilized *E. coli* to form the basis of this hypothesis, however enzyme preparations from other bacteria, plants and *Plasmodium* demonstrate that either *p*ABA or the glutamate derivative can serve as a substrate with the pyrophosphate intermediate (Blakley, 1969).

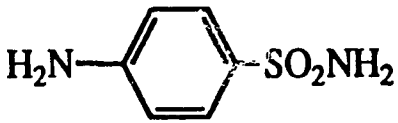
In 1940, British biochemist D. D. Woods proposed that the effect of sulfonamides on bacteria was due to competitive inhibition by these compounds of *p*ABA utilization (Blakley, 1969). Sulfonamides, such as sulfanilamide (Figure 2A), are antibiotics that are structural analogs of the *p*ABA component of H_4PteGlu. These antimetabolites competitively inhibit bacterial synthesis of H_4PteGlu at the *p*ABA incorporation step, thereby blocking H_4PteGlu-requiring reactions. The inability of mammals to synthesize folic acid leaves them unaffected by sulfonamides, which accounts for the medical utility of these antibacterial agents.

Supporting evidence of sulfonamide action was provided by the demonstration that microorganisms that fail to synthesize folate, are highly resistant to sulfonamides when supplemented with exogenous folate (Blakley, 1969). The inhibitory effects of sulfonamides on plant growth are also reportedly a result of interference with folic acid synthesis from *p*ABA and other precursors (Shiota, 1984). Therefore, sulfonamide inhibition of the growth of tomato roots was reversed by either *p*ABA or folic acid while sulfonamide inhibition of pea and wheat growth was surmounted by 10-HCO-H_4PteGlu$_n$ treatment (Blakley, 1969). Subsequently, it was demonstrated that the formation of reduced folate derivatives, which support *Pediococcus acidilactici* growth, was greatly decreased in sulfonamide-inhibited seedlings as compared to normal seedlings.

A relationship appears to exist between bacterial resistance to sulfonamides and the affinity of these antimetabolites for the strain's condensing enzyme. Additionally, alterations in the cellular permeability or *p*ABA overproduction may convey other forms of sulfonamide resistance. Although resistance to sulfonamides encoded by extrachromosomal DNA elements called R factors, or R-plasmids, in enteric microorganisms has been well documented, Wise and Abou-Donia conducted the initial investigations regarding the mechanism of plasmid-specified sulfonamide resistance (Shiota, 1984). They were able to demonstrate that clinical isolates of *E. coli*, *Citrobacter* and *Klebsiella pneumoniae* resistant to sulfonamides harbored plasmids that specified the synthesis of sulfonamide resistant dihydropteroate synthase.

The product of reaction [4], dihydropteroic acid, catalyzed by dihydropteroate synthase is modified to form dihydrofolic acid, a key intermediate in folate biosynthesis. Investigations of prokaryotic and eukaryotic species revealed that dihydrofolate

A



B

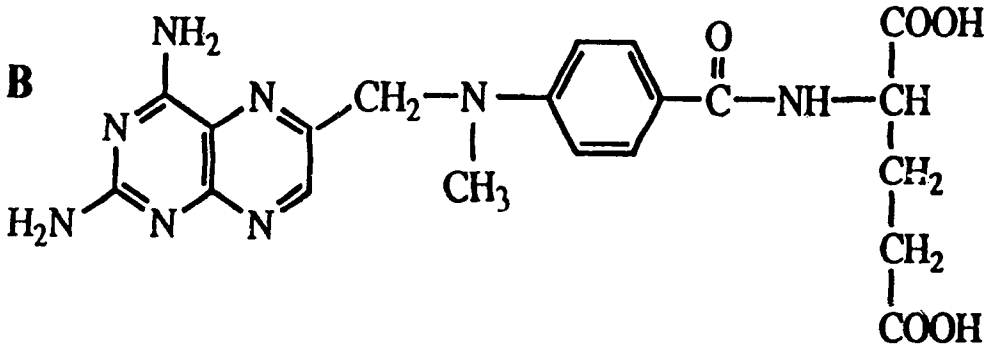


Figure 2. The Structures of Common Antifolates.

(A) Sulfanilamide, a structural analog of *p*ABA, competitively inhibits dihydropteroate synthase.

(B) Methotrexate, a structural analog of dihydrofolate/folic acid, a potent inhibitor of dihydrofolate reductase.

synthetase (EC 6.3.2.12) in the presence of a monovalent or a divalent cation, was responsible for the latter conversion, as presented in reaction [5].

$$ATP + Mg^{2+} + \text{dihydropteroic acid} + \text{L-glutamate} \rightarrow H_2PteGlu + ADP + P_i \quad [5]$$

The distribution of dihydrofolate synthetase activity was examined using cell-free extracts of *E. coli*, *Corynebacterium* species, *Saccharomyces cerevisiae*, *Bacillus megaterium*, and *Neurospora crassa* in addition to various other microorganisms (Griffin and Brown, 1964). Ikeda and Iwai (1970) demonstrated the existence of this enzyme in several plant species including pea, lettuce, cabbage, swiss chard and spinach. These studies indicated that these organisms were capable of *de novo* folate synthesis and therefore did not require additional folate supplementation for growth. Conversely, mammals, birds and bacteria including *Lactobacillus rhaminosis*, *Pediococcus acidilactici* and *Enterococcus hirae* require folate supplementation for growth and development, owing to a deficiency in dihydrofolate synthetase (Iwai et al, 1977).

1.1.1.3. The Reduction of Dihydrofolate to Tetrahydrofolate

The reduction of $H_2PteGlu$ to $H_4PteGlu$, reaction [6], is an obligatory step in the synthesis of the single carbon carrier forms of the molecule (Hamm-Alvarez et al, 1990). This conversion is catalyzed by dihydrofolate reductase (DHFR), EC 1.5.1.3. This enzyme also catalyzes the reduction of folate to $H_4PteGlu$ according to reaction [7], but at a much lower rate than $H_2PteGlu$ reduction (Blakley, 1969).

$$H_2PteGlu + NADPH + H^+ \rightarrow H_4PteGlu + NADP^+ \quad [6]$$

$$Folate + 2\ NADPH + 2\ H^+ \rightarrow H_4PteGlu + 2\ NADP^+ \quad [7]$$

Although levels of DHFR are very low in skeletal muscle, heart, lung and cerebral tissue, this enzyme is present in all dividing cells (Blakley, 1984). DHFR activity has been detected in human liver, kidney, gastric mucosa and bone marrow (Blakley, 1969). Embryonic tissues have a relatively high DHFR content, in both the rat and human embryo, the liver appears to have a higher enzyme level than other embryonic tissues (Blakley, 1969). DHFR activity has been detected in the liver, kidney, thymus, intestinal mucosa and spleen from rabbit, rat, guinea pig, hamster and mouse (Blakley,

1969). Examination of enzyme distribution in rat liver revealed the presence of DHFR in the cytoplasmic and microsomal fraction. The nuclei may contain significant levels of the enzyme for regeneration of H_2PteGlu formed during thymidylate synthesis in mitochondria (Blakley, 1969; Appling, 1991).

The DHFR of chicken liver, the first such enzyme to be partially purified, has a molecular weight in the 21,000-24,000 Da range (Blakley, 1969). Enzymes resembling the DHFR of vertebrate tissues have been identified in various bacteria including *Enterococcus hirae, Escherichia coli, Diplococcus pneumoniae, Lactobacillus leichmannii, Staphylococcus aureus* and *Proteus vulgaris*. The first bacterial species from which DHFR was purified was *E. hirae* (Blakley, 1969). A mutant strain of this bacterium contains two reductases, a wild type reductase similar in properties to that of the parent strain having a molecular weight of *ca.* 28,000 Da. The mutant-type reductase is more akin to the mammalian enzyme in that it reduces both folate and H_2PteGlu and has a molecular weight of *ca.* 20,000 (Blakley, 1969).

As previously stated DHFR isolated from vertebrates and bacteria is a monomeric protein having a molecular weight of *ca.* 20,000 Da (Blakley, 1984). Generally, mammalian DHFRs have molecular masses that are slightly higher (*ca.* 23 kD) than those of bacteria (ca. 18 kD) (Cella and Parisi, 1993). Interestingly, DHFRs from protozoa, including *Chritidia fasciculata* (Ferone and Roland, 1980) and *Leishmania tropica* (Meek et al, 1985), are homodimeric, with subunits ranging between 55 and 77 kD. Each monomer is bifunctional, having DHFR and thymidylate synthase domains. The sum of the molecular masses of monofunctional DHFR and thymidylate synthase from mammalian and bacterial sources correspond to the respective monomer molecular mass. Further, evidence has been presented that *Daucus carota* (Luo et al, 1993; Luo and Cella, 1995) and *Arabidopsis thaliana* (Lazar et al, 1993) possess bifunctional DHFR-thymidylate synthase proteins.

While the requirement for DHFR activity for plant cell growth has been demonstrated (Suzuki and Iwai, 1970), very little is known about the properties of this enzyme from plant sources, presumably due to the inherent low activity of the enzyme in plants (Ratnam et al, 1987). Identification and characterization of DHFR has been reported for *Pisum sativum* (Suzuki and Iwai, 1970; Crosti, 1981), *Glycine max* (Reddy and Rao, 1976; Ratnam et al, 1987), *Daucus carota* (Cella et al, 1983; Albani et al, 1985; Cella et al, 1987), *Oryza sativa* (Cella et al, 1983), *Petunia hybrida* (Barg et al, 1984, 1987) and *Zea mays* (Crosti, 1981) among plant species. The enzyme from various bacterial and yeast sources including *Lactobacillus rhaminosis* (Curtis et al,

1994), *Escherichia coli* (Hamm-Alvarez et al, 1990), *Neisseria* sp. (Averett et al, 1978), *Saccharomyces cerevisiae* (Nagelschmidt and Jaenicke, 1972; Wu et al, 1980; Fling et al, 1988) and *Candida albicans* (Baccanari et al, 1989) has been studied. While extensive physical and chemical investigations of the enzyme from various sources have been reported, to date DHFR has not been purified or characterized from a fungal source. Further investigations in this area are necessary to allow an adequate comparison between fungal DHFR and that of other species, including mammals, plants and bacteria.

The structure of DHFR has been the subject of intensive study over the last two decades. As a result, the structure of the protein has become one of the best understood. Many methods have been utilized in elucidating the structure of DHFR. Sequencing studies, X-ray crystallography and high-field nuclear magnetic resonance, NMR, (Blakley, 1984; Curtis et al, 1994) have resulted in the greatest advances in understanding DHFR.

There is much greater homology between DHFRs from vertebrate sources than between bacterial DHFRs, which may be attributed to the evolutionary process of mutations in bacterial strains, altering the structure of DHFR. (Blakley, 1984). The structure of bacterial DHFR is dominated by a twisted β sheet that contains eight strands, designated as βA to βH (Blakley, 1984). The βA strand is situated nearest the amino terminus while βH is located at the carboxy terminus, the order of strands in the sheet from left to right is G-H-F-A-E-B-C-D (Blakley, 1984). Four α helices are packed against the β sheet, and are designated αB, αC, αE, αF, the letter designating the β strand that follows the α helix in the primary sequence (Blakley, 1984). The remaining residues consist of loops that join the elements of secondary structure.

The vertebrate DHFRs contain sequences about 30 residues longer than those of the bacterial enzymes. Apart from six residues that lengthen the amino and carboxy termini, the additional residues are accommodated in eight insertions which, with one exception, occur in loops connecting elements of secondary structure (Blakley, 1984). The active site is a pronounced cavity located across one face of the enzyme and is lined by hydrophobic side chains, indicating that the majority of substrate and inhibitor binding must be due to hydrophobic and Van der Waals interactions (Blakley, 1984).

Based upon evidence from other oxido-reductions involving the nicotinamide adenine dinucleotides, a sequential mechanism for the action of dihydrofolate reductase was investigated. It has been suggested that NADPH binding occurs first, followed by H_2PteGlu binding, with release of H_4PteGlu preceding $NADP^+$ release

(Blakley, 1984). This theory assumes that H_2PteGlu complexes preferentially to enzyme-bound NADPH over the free enzyme, and NADPH binds to the free enzyme better than H_2PteGlu suggesting a kinetically important sequential mechanism (Blakley, 1969).

A means of detecting equilibria between conformational states of DHFR and its complexes is the use of NMR. Recent studies based on ^{1}H-, ^{15}N-, and ^{13}C-NMR experiments with labeled folates indicated that folate can bind in more than one conformation to the *Lactobacillus rhaminosis* DHFR (Curtis et al, 1994). Three different conformational states of the DHFR-folate complex have been detected, and their pH-dependence characterized. Two of the forms are similar and have identical pteridine ring conformations, while a third form has its pteridine ring orientation turned over by *ca.* 180° (Curtis et al, 1994).

The carbon transfer reactions leading to the purine nucleotides and amino acids, depicted in Figure 3, while mechanistically diverse, are similar in that carbon transfer to biosynthetic precursors regenerates unsubstituted tetrahydrofolate. This H_4PteGlu is free to reassociate with endogenous sources of one-carbon units. However, it is significant that the reaction catalyzed by thymidylate synthase consumes not only the carbon fragment but a reducing equivalent as well. Hence, the H_4PteGlu$_n$ is oxidized to form H_2PteGlu$_n$, a form with a redox potential between those of folic acid and H_4PteGlu$_n$. H_2PteGlu$_n$ is metabolically inert since it is incapable of directly participating in one-carbon transfer biosynthetic reactions without again being reduced to H_4PteGlu$_n$ (Matherly et al, 1987). H_2PteGlu$_n$ can only re-enter the active H_4PteGlu$_n$ pool following its reduction in the NADPH-dependent reaction catalyzed by DHFR. Thus it is apparent that the reaction catalyzed by DHFR is critical, if this enzyme is blocked or deleted, the continued generation of H_2PteGlu$_n$ during thymidylate biosynthesis would ultimately deplete reduced folate pools (Matherly et al, 1987). Under these conditions, the H_4PteGlu$_n$-dependent synthesis of DNA, as well as RNA and protein, would cease.

Specific inhibitors of DHFR have been derived empirically and, more recently, from structural and functional considerations. These inhibitors have found considerable utility as antibacterial, antiprotozoal and antineoplastic agents (Matherly et al, 1987). Two such compounds, aminopterin and methotrexate (MTX; Figure 2B), are folate analogs containing 4-amino substitutions on the pteridine ring, are amongst the most effective inhibitors of mammalian enzymes (Blakley, 1984).

Almost without exception, whenever folates are isolated from natural sources, they are found not as monoglutamate derivatives but as poly (γ-glutamyl) conjugates containing two to eight glutamate residues (McGuire et al, 1980: McGuire and Bertino, 1981; McGuire and Coward, 1984). The observation that all organisms have the ability to synthesize folylpolyglutamates, including organisms which are unable to synthesize folic acid *de novo*, indicated that folylpolyglutamates are vital to cellular metabolism. The realization that natural folates accumulated as polyglutamates in mammalian cells preceded the demonstration of an analogous metabolism for the structurally similar antifolates, MTX and aminopterin. This accumulation is considered to be important for maximum pharmacologic effects (Matherly et al, 1987; Synold et al, 1994).

1.1.1.4. The Production and Role of Folylpolyglutamates

Early literature contained frequent references to 'folic acid conjugates.' When the structures of these compounds were determined, one or more glutamic acid residues were bound by γ-carboxyl peptide bonds to folic acid forming folic acid polyglutamates (Covey, 1980).

Pteroylpolyglutamates ($PteGlu_n$) were first detected when folic acid extracted from yeast failed to serve as a growth factor for folate requiring bacteria (McGuire and Bertino, 1981). Elemental, chemical and microbiological analysis of this yeast extract revealed its structure to be folic acid with six additional molecules of glutamic acid in peptide linkage (Richardson et al, 1979; Covey, 1980; McGuire and Coward, 1984). All linkages were later unequivocally demonstrated to be γ-concatenates. Extracts which made this $PteGlu_n$ available as a bacterial growth factor contained an enzyme, γ-glutamyl hydrolase (GGH). Since only the hydrolyzed forms were active bacterial growth factors, and the monoglutamates (PteGlu) were active in folate-dependent enzyme reactions, it was assumed that the $PteGlu_n$ were merely storage forms of the vitamin (McGuire and Coward, 1984). It is now known that the requirement for hydrolysis of $PteGlu_n$ prior to bacterial utilization resulted from the ineffective intracellular transport of these forms rather than their inadequate utilization as coenzymes (McGuire and Bertino, 1981). The evidence that $PteGlu_n$ serve as coenzymes in folate-dependent reactions is obtained almost exclusively from investigations using isolated enzymes, and $H_4PteGlu_n$ synthesized chemically and enzymatically.

Generally, $H_4PteGlu_n$ are the major intracellular forms of folate. Since the chain length of these intracellular polyglutamates always exceeds the longest form that

can be transported efficiently, all cells contain folylpolyglutamate synthetase (FPGS), EC 6.3.2.17 (McGuire and Coward, 1984; Shane, 1989). FPGS catalyzes the ATP-dependent addition of glutamate moieties to $H_4PteGlu$ as depicted in reaction [8].

$$H_4PteGlu_n + \text{L-glutamate} + ATP + Mg^{2+} \leftrightarrow H_4PteGlu_{n+1} + ADP + P_i \qquad [8]$$

Progress in synthetase investigations has been slow due to the marked lability and low abundance of the protein. After preliminary characterization of crude enzyme preparations from sheep liver (Gawthorne and Smith, 1973) and partially purified enzyme from Chinese hamster ovary (CHO) cells (Taylor and Hanna, 1977), McGuire et al (1980) extensively characterized a 55-fold purified preparation from rat liver. Subsequently, the enzymes from mouse (Moran and Colman, 1984) and beef (Pristupa et al, 1984; Scrimgeour et al, 1985) were partially purified and characterized. Further, the pig liver synthetase has been purified to homogeneity (Cichowicz and Shane, 1987). Additionally, bacterial FPGS proteins have been purified to homogeneity from *Corynebacterium* (Shane, 1980), *Lactobacillus rhaminosis* (Bognar and Shane, 1983) and *E. coli* (Bognar et al, 1985) further, the *E. coli* gene has been cloned and sequenced (Bognar et al, 1985). Most synthetase activity is present in the cytoplasm of cells, although some activity is detected in the mitochondrial fraction (Gawthorne and Smith, 1973; McGuire and Coward, 1984).

The number of enzymes involved in the synthesis of $H_4PteGlu_n$ by a given organism has been investigated in several cases. The maximum number of enzymes would be required if either: a different enzyme was responsible for polyglutamylation of all lengths of each particular folate form; if all $PteGlu_2$ were synthesized by one enzyme, all $PteGlu_3$ by a second, etc.; or if each enzyme added only one glutamate to one folate form (McGuire and Bertino, 1981). A single enzyme could produce all lengths of each folate, or polyglutamylated one or more folates and rely on intracellular folate metabolism for the necessary intermediate(s) (McGuire and Bertino, 1981). The most convincing evidence suggesting that a single enzyme converts folates to polyglutamate forms has been reported in CHO cells. From wild type CHO cultures, which synthesize $PteGlu_5$, McBurney and Whitmore (1974) isolated a cell line following mutagenesis which was unable to synthesize $PteGlu_n$. The reversion frequency of the mutant was consistent with a single genetic lesion which caused the defective synthesis. Taylor and Hanna (1977) demonstrated that crude extracts and 25-fold purified FPGS from wild type CHO cells could synthesize both $PteGlu_2$ and higher lengths of $H_4PteGlu$. The purified FPGS could produce $PteGlu_n$ from a number of different fo-

late derivatives. However it lost the ability to generate the $PteGlu_2$ of any folate, and the ability to convert $H_4PteGlu_2$ or $H_4PteGlu_3$ to longer polyglutamates. This functional loss could not be attributed to the presence of a nondialyzable inhibitor, a very active conjugase, or the repression of FPGS under the permissive conditions required to grow the mutant. Extracts of spontaneous revertants of the mutant simultaneously regained the ability to synthesize all $PteGlu_2$, and greater lengths of $H_4PteGlu$. Further, the revertant cells contained a FPGS with altered properties suggesting that the original mutation was in the FPGS structural gene rather than a regulatory gene. The evidence accumulated is consistent with a single FPGS being responsible for synthesis of all $PteGlu_n$ in CHO cells (McGuire and Bertino, 1981). The demonstration that a partially purified rat liver FPGS (McGuire et al, 1980) could synthesize $PteGlu_5$ derivatives, the predominant length in rat liver, from PteGlu, and the observation that all $H_4PteGlu$ have similar optimal pH for activity, is consistent with rat liver also containing a single FPGS. In *Corynebacterium* (Shane, 1980) the relative activities of crude extracts and a 7000-fold purified FPGS with PteGlu and $PteGlu_n$ substrates are nearly identical suggesting that this organism also possesses a single enzyme. Although studies of $PteGlu_n$ deficiency in *Neurospora crassa* are less detailed, recent investigations suggest basic differences with the mammalian system. These studies raised the possibility that wild type polyglutamylation may proceed via a two-step process (Chan et al, 1984). The initial reaction appeared to involve the conjugation of glutamate to $H_4PteGlu$, yielding a $H_4PteGlu_2$ product; the second reaction appeared to add glutamate residues to $H_4PteGlu_2$, $H_4PteGlu_3$ and $H_4PteGlu_4$. For some time it was unclear whether one or more enzymes were involved. However, recent work with *N. crassa* suggests that the synthesis of $H_4PteGlu_n$ may be mediated by a single FPGS as reported for mammals (Chan et al, 1991; Atkinson et al, 1995).

Generally, the native molecular weight of FPGS is in the 60,000-70,000 Da range. Specifically, analysis of the rat liver FPGS revealed a molecular weight of 69,000 Da (McGuire et al, 1980) while *Neurospora crassa* FPGS had molecular weight of *ca.* 65,000 (Chan et al, 1991). Further, the pig liver enzyme and all purified bacterial synthetases are monomeric proteins (Shane, 1989).

Cells only transport folates which are smaller than those that predominate within that cell type. The highly anionic nature of $PteGlu_n$ limits its ability to diffuse through membranes, illustrating that polyglutamylation traps or retains folates intracellularly (Baugh et al, 1973). Experiments with *L. rhaminosis* demonstrated that both the affinity and maximum velocity of transport decreased with increasing polygluta-

mate chain length (Bognar and Shane, 1983). Assuming influx and efflux occurred by the same mechanism, the degree of retention would be directly related to chain length (McGuire and Bertino, 1981). At longer incubation times, as $PteGlu_n$ formed in *L. rhaminosis*, the efflux rate of [^{3}H]-folic acid decreased drastically, even though the intracellular folate concentration was the same in the short term (Bognar and Shane, 1983). This again suggests that longer polyglutamates were preferentially retained. Mammalian cells share this property of reduced permeability to $PteGlu_n$, whereas PteGlu is readily transported into and effluxes from mammalian cells (McGuire and Bertino, 1981).

In mammals, the major pathway for utilization of folate is by absorbing $H_4PteGlu$ across the intestinal mucosa into the blood stream. Nutritional sources of $H_4PteGlu_n$ are converted to the $H_4PteGlu$ moiety by GGH, EC 3.4.22.12. As depicted in reaction [9], this endopeptidase cleaves the innermost γ-peptide bond of folyl- or antifolylpolyglutamates to yield PteGlu products. GGH has been detected in saliva, pancreatic juice and the intestinal mucosa (Schirch and Strong, 1989).

$$PteGlu_n + H_2O \leftrightarrow PteGlu + Glu_{n-1} \qquad [9]$$

The metabolic importance of conjugated folates has been substantiated by studies of mutant cell lines that lack the ability to generate folylpolyglutamates *in vivo*. These investigations demonstrated that cells which lack the ability to synthesize $H_4PteGlu_n$ have nutritional requirements for the end products of one-carbon metabolism including thymine, methionine and adenine (Taylor and Hanna, 1977). McBurney and Whitmore (1974) as well as Taylor and Hanna (1977) investigated one wild type and two mutant CHO cell lines: AUXB1 is auxotrophic for glycine, adenosine and thymidine; AUXB3 requires only glycine and adenosine. The transport rate of folates and the levels of a number of folate-dependent enzymes were identical in the wild type and mutants, however the mutants were found to be unable to concentrate [^{3}H]-folate to the same extent as wild type cells. Chromatography of intracellular folates provided evidence that wild type cells contained predominantly $PteGlu_5$ while AUXB1 contained only PteGlu and AUXB3 contained PteGlu, $PteGlu_2$ and some $PteGlu_3$. The data suggested that a single defect in the biosynthesis of $PteGlu_n$ caused the multiple auxotrophy in these mutant cells. The decreased folate pools and extensive folate efflux found in the mutants is the result of their inability to synthesize $PteGlu_n$ and thus these $PteGlu_n$ derivatives aid in the retention of folates. Similarly, V-79 *ght*-1 mutant hamster lung cells contain negligible FPGS activity relative to its V-79 parent line

(Taylor and Hanna, 1977) and requires glycine, adenine and thymidine. Additionally, CHO cells expressing *E. coli* FPGS and containing $PteGlu_3$ were auxotrophic for glycine (Lin et al, 1993). Further, expression of FPGS is altered in a number of methionine auxotrophs of *Neurospora crassa* (Chan and Cossins, 1980, 1984; Chan et al, 1991). Although the evidence clearly supports the retention of $PteGlu_n$ by cells, the degree of retention is a function of both the $PteGlu_n$ length and the cell type.

The data on the predominant lengths of $PteGlu_n$ occurring in natural sources and the data on influx and efflux of $PteGlu_n$ suggests that cells have evolved $PteGlu_n$ biosynthetic machinery that synthesize lengths just longer than that which will pass the cellular membrane by active transport or diffusion (McGuire and Bertino, 1981). Cells can thus retain the levels of folates necessary for cellular growth while avoiding wasteful synthesis of excessively long $PteGlu_n$.

In addition to promoting enzyme stabilization, $PteGlu_n$ serve as allosteric effectors of non-folate reactions. $PteGlu_n$ may be important in regulating cellular levels of folate-dependent enzymes through substrate or inhibitor stabilization (McGuire and Bertino, 1981). Binding of a folate ligand could stabilize the enzyme against intracellular degradation and turnover, thus leading to an increased intracellular concentration. Since $PteGlu_n$ substrates and inhibitors generally have higher affinities for folate enzymes than PteGlu, $PteGlu_n$ could be more effective stabilizing agents. Cystathionine γ-synthase of *Neurospora crassa* catalyzes the formation of cystathionine, the immediate precursor of homocysteine. $5\text{-}CH_3\text{-}H_4PteGlu_n$ antagonize the feedback inhibition of cystathionine γ-synthase by S-adenosylmethionine, and serve as specific, essential activators of the enzyme (McGuire and Bertino, 1981). In several reactions the kinetic constants for other substrates are altered, that is the affinity is increased when $PteGlu_n$ is present as the substrate (McGuire and Bertino, 1981).

Originally considered to be storage forms of the folates, it is now clear that forms of $PteGlu_n$ are the preferred coenzymes for most dependent metabolic reactions. In almost every evaluation, the $PteGlu_n$ form of the required folate is the favored substrate and in no case has a clear specificity for the PteGlu or non-conjugated folate been demonstrated (Matherly et al, 1987). Increasing evidence also suggests that $PteGlu_n$ derivatives are channeled between the active site in multifunctional enzyme complexes, thereby further enhancing the catalytic efficiency for $PteGlu_n$ forms required in carbon transfer (Matherly et al, 1987). The properties of folate $PteGlu_n$ which render their formation within cells essential not only to biosynthetic efficiency but also cell survival are that these derivatives of the natural folates less readily per-

meate the membranes of mammalian cells allowing folates to be concentrated intracellularly relative to the extracellular medium. This is best exemplified by the fact that mammalian cells, are auxotrophic for the end products of one-carbon metabolism.

FPGS inhibitors are potentially cytotoxic agents with the unique ability to block all pathways of folate-dependent one-carbon metabolism interfering with *de novo* purine and DNA-thymine biosynthesis and amino acid metabolism, without affecting DHFR, the target of currently used antifolates. On the basis of these considerations, FPGS inhibitors should be effective cytotoxic agents and would find utility in cancer chemotherapy (Kalman, 1990).

The role of $PteGlu_n$ in biosynthetic processes has gained increasing attention in that they act as coenzymes within cells and are the preferred substrates for folate-dependent reactions in eukaryotic cells (Matherly et al, 1987). More recently, attention has focused on the potential importance of MTX polyglutamate derivatives that have been detected in normal and malignant cells both *in vivo* and *in vitro*. The biochemical transformation of this important chemotherapeutic agent is of particular significance since derivatives of MTX are not only potent inhibitors of the target enzyme, but have quite different cellular pharmacokinetics than the parent drug (Poser et al, 1981; Fry et al, 1982; Fry et al, 1983).

1.2. METABOLISM OF SINGLE-CARBON UNITS

Folate metabolism involves the reduction of the pyrazine ring of the pterin moiety to the coenzymatically active $H_4PteGlu$ form, and the elongation of the glutamate chain to form the physiologically active $H_4PteGlu_n$ form (MacKenzie, 1984; Shane, 1989). The coenzymatic function of $H_4PteGlu_n$ involves the mobilization and utilization of single-carbon functional groups. These intracellular folates are metabolically interconvertable via enzymic reactions as depicted in Figure 3. These reactions, referred to as one-carbon (C_1) metabolism, are critical for the synthesis of numerous cellular constituents required for cell growth.

1.2.1. Amino Acid Metabolism

$H_4PteGlu_n$ derivatives are found primarily in the cytosol and mitochondria (Schirch and Strong, 1987), serving as the C_1 donor in the biosynthesis of purines, pyrimidines and amino acids, Figure 3 (McGuire and Bertino, 1981). Degradation of serine, glycine, and histidine provide C_1 substituted folates. Additionally,

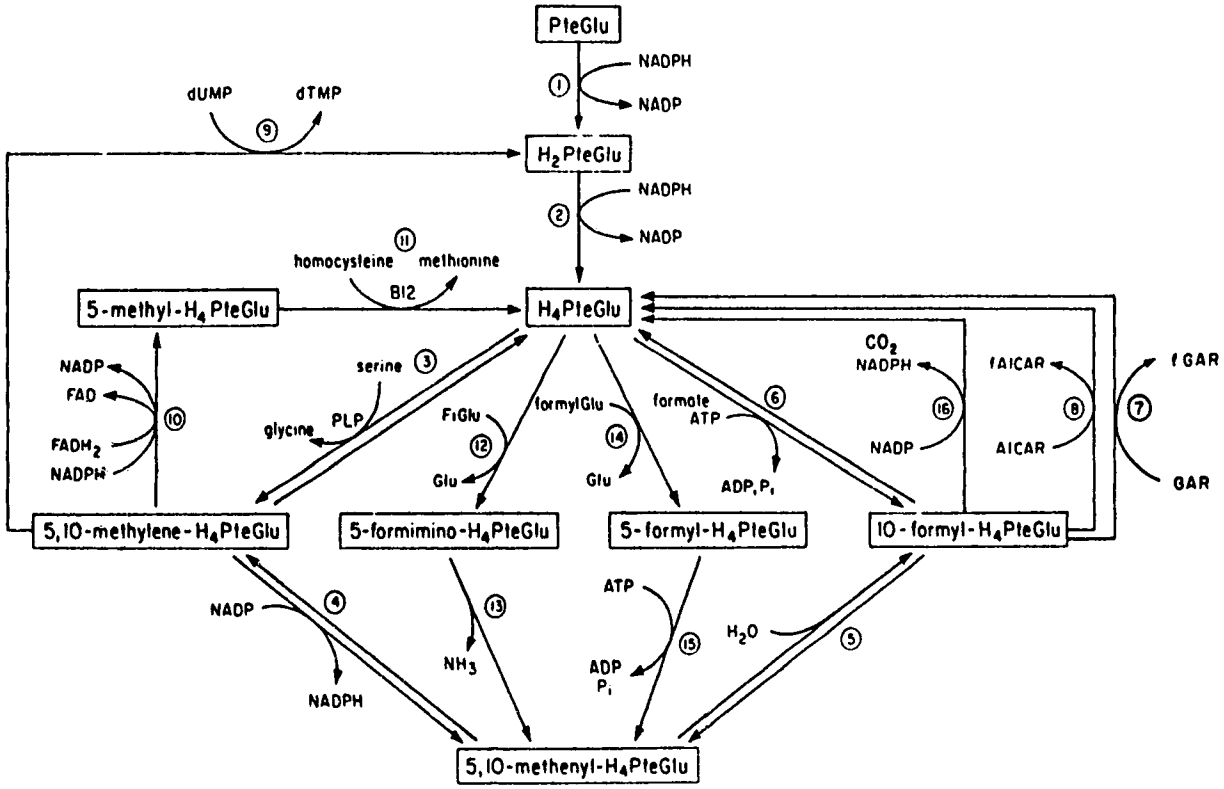


Figure 3. Generation and Utilization of One-Carbon Units. The enzymes involved in the following tetrahydrofolate-mediated reactions are: **(1)** and **(2)** dihydrofolate reductase; **(3)** serine hydroxymethyltransferase; **(4)** 5,10-methylenetetrahydrofolate dehydrogenase; **(5)** 5,10-methenyltetrahydrofolate cyclohydrolase; **(6)** 10-formyltetrahydrofolate synthetase; **(7)** glycinamide ribonucleotide (GAR) transformylase; **(8)** 5-amino-4-imidazolecarboxamide ribonucleotide (AICAR) transformylase; **(9)** thymidylate synthase; **(10)** 5,10-methylenetetrahydrofolate reductase; **(11)** methionine synthase; **(12)** formiminoglutamate formiminotransferase; **(13)** 5-formiminotetrahydrofolate cyclodeaminase; **(14)** *N*-formylglutamatetetrahydrofolate transformylase; **(15)** methenyltetrahydrofolate synthetase; and **(16)** 10-formyltetrahydrofolate dehydrogenase.

formaldehyde and formate may be utilized in folate metabolism (Pasternack et al, 1994). Quantitatively the major sources of C_1 units in most organisms are serine and glycine (Pasternack et al, 1994).

Serine hydroxymethyltransferase (SHMT), EC 2.1.2.1, catalyzes the reversible transfer of formaldehyde from serine to $H_4PteGlu_n$ to form 5,10-CH_2-$H_4PteGlu_n$ and glycine, reaction [10]. The reversal of SHMT has been demonstrated to be a source of serine, thus this enzyme is implicated in both the biosynthesis and catabolism of serine (Schirch, 1984).

$$\text{Serine} + H_4PteGlu_n \leftrightarrow 5,10\text{-}CH_2\text{-}H_4PteGlu_n + \text{Glycine} + H_2O \qquad [10]$$

The generation of C_1 units by this route occurs in both the cytosol and mitochondria as isoenzymes of SHMT exist in both compartments in mammals and higher plants (Cossins, 1980, 1987). This feature permits the transport of serine and glycine across the mitochondrial membrane, since evidence suggests that this membrane is impermeable to $H_4PteGlu_n$ derivatives (Schirch, 1984).

This enzyme, a 220 kD homotetramer, has been purified to homogeneity from several mammalian tissues. SHMT activity has also been recorded in plant tissues including cauliflower, mung bean and tobacco. More recently this enzyme has been purified from *Neurospora crassa* (Kruschwitz et al, 1993).

Evidence suggests that folates of glutamate chain length of three and longer are much more effective substrates of SHMT than the mono- and diglutamate forms (Shane, 1989). The rabbit liver SHMT displayed a Michaelis constant (K_m) for the triglutamate which was half that of the monoglutamate, indicating the enzyme has a higher affinity for substrates containing more glutamate residues (McGuire and Bertino, 1981).

An oxidative cleavage reaction first described by Sagers and Gunsalus (1961), catalyzed by glycine synthase (EC 2.1.2.10), produces 5,10-CH_2-$H_4PteGlu_n$ (reaction [11]) which supplies an additional C_1 unit to the folate pool. In eukaryotic cells, glycine synthase is exclusively mitochondrial and composed of four protein components designated as P-, H-, L- and T- proteins as reviewed by Schirch (1984). An enzyme in pea leaf mitochondria is composed of four different proteins with similar functions (Kikuchi, 1973; Walker and Oliver, 1986; Bourguignon et al, 1988).

$$\text{glycine} + H_4PteGlu_n + NAD^+ \leftrightarrow 5,10\text{-}CH_2\text{-}H_4PteGlu_n + CO_2 + NADH + NH_4^+ \qquad [11]$$

In most cells, serine and glycine are the major sources of C_1 units so entry to the active C_1 pool proceeds via 5,10-CH_2-H_4PteGlu$_n$. Histidine, considered to be a minor source, may also provide C_1 units to the H_4PteGlu$_n$ pool. The C-2 position of the imidazole ring of histidine provides C_1 units at the oxidation level of formate (Shane and Stokstad, 1984). Histidine, a gluconeogenic amino acid, is catabolized via the *N*-formimino-L-glutamate intermediate to glutamate in mammalian tissues. Formiminoglutamate forminotransferase (EC 1.1.2.15) catalyzes the transfer of a formimino group from formiminoglutamate to H_4PteGlu$_n$, reaction [12] (Shane, 1989).

formiminoglutamate + H_4PteGlu$_n$ →
5-formimino-H_4PteGlu$_n$ + glutamate [12]

The formimino moiety cannot be utilized in C_1 metabolism and must be converted to 5,10-CH=H_4PteGlu$_n$ in a 5-formiminotetrahydrofolate cyclodeaminase (EC 4.3.1.4) catalyzed reaction, as depicted in reaction [13] (Shane, 1989).

5-formimino-H_4PteGlu$_n$ + H^+ → 5,10-CH=H_4PteGlu$_n$ + NH_3 [13]

The folate product of serine and glycine cleavage, 5,10-CH_2-H_4PteGlu$_n$, is either utilized unaltered for thymidylate synthesis, is reduced to 5-CH_3-H_4PteGlu$_n$ for methionine biosynthesis, or is oxidized to 10-HCO-H_4PteGlu$_n$ for use in purine synthesis. The interconversion of 5,10-CH_2- and 10-HCO-H_4PteGlu$_n$ is central to the utilization of C_1 units.

The affinity of H_4PteGlu$_n$ for formiminotransferase increases with increased glutamate chain length, it reaches a maximum with the hexaglutamate and significantly decreases with the heptaglutamate derivative (Shane, 1989). The major changes in affinity occur as the glutamate chain is extended from the mono- to the tetraglutamate (McGuire and Bertino, 1981). Further, the K_m for the substrate for the cyclodeaminase, 5-formimino-H_4PteGlu$_n$, also decreases with the extension of the glutamate chain to tetraglutamate (Shane, 1989).

I.2.2. The Thymidylate Cycle

Folates are indirectly involved in the *de novo* synthesis of pyrimidines, more specifically, thymidylate. Thymidylate synthase (TS), EC 2.1.1.45, catalyzes the conversion of deoxyuridylate (dUMP) and 5,10-CH_2-H_4PteGlu$_n$ to deoxythymidine mono-

phosphate (dTMP) and dihydrofolate ($H_2PteGlu_n$), reaction [14] (Santi and Danenberg, 1984).

$$5,10\text{-}CH_2\text{-}H_4PteGlu_n + dUMP \rightarrow dTMP + H_2PteGlu_n \qquad [14]$$

In most eukaryotic cells, the level of TS is low, and hence decreased production of dTMP is believed to limit the rate of DNA replication (Santi and Danenberg, 1984). Expression of the synthase is highest during the S-phase of the cell cycle and the level of this enzyme is directly related to replication rates (Shane, 1989). TS is exclusively involved in *de novo* dTMP biosynthesis and is closely linked in function to dihydrofolate reductase (DHFR). Since inhibition of DHFR or TS result in the depletion of $H_4PteGlu_n$ and dTMP pools, which causes the subsequent cessation of DNA synthesis, both enzymes are obvious targets for chemotherapy in malignant diseases (Glynn and Albanes, 1994).

TS from a number of sources has been purified to homogeneity: *E. coli* (Haertle et al, 1979; Slavik and Slavikova, 1980), mammals (Staben and Rabinowitz, 1984) and carrot cell suspension cultures (Neilson and Cella, 1988). Studies on the mechanism of C_1 transfer by TS have been greatly facilitated by the availability of the crystalline enzyme from *L. rhaminosis* (Galivan et al, 1976). In these organisms TS exists as a dimer of 60-75 kD.

The K_m for $5,10\text{-}CH_2\text{-}H_4PteGlu_n$ decreases *ca.* 60% as the mono- proceeds to the diglutamate. K_m decreases a further 20% when another glutamate residue is added, forming the triglutamate (Shane, 1989). An additional extension of the glutamate chain length causes a modest increase in the K_m for deoxyuridylate. *L. rhaminosis* thymidylate synthase exhibits a specificity to the mammalian enzyme in that increased affinities for polyglutamate substrates occur primarily among the mono-, di- and triglutamate derivatives, but less dramatic changes occur beyond the triglutamate (Shane, 1989).

In most prokaryotic and eukaryotic organisms DHFR and TS are encoded by independent genes. However, the size of the protozoal bifunctional polypeptide approximates the sum of the monofunctional DHFR and TS enzymes suggesting that the gene encoding DHFR-TS may have resulted from the fusion of the monofunctional genes (Luo et al, 1993). Additionally, evidence of a bifunctional polypeptide of DHFR along with TS has been reported in carrot cells (Luo and Cella, 1995) and *Arabidopsis thaliana* (Lazar et al, 1993). The occurrence of a bifunctional DHFR-TS favors ex-

tensive communication between the two domains and allows channeling of the dihydrofolate ($H_2PteGlu_n$) from TS to DHFR (Ivanetich and Santi, 1990).

1.2.3. The Methionine Cycle

A major cycle of C_1 utilization involves the reduction of 5,10-CH_2-$H_4PteGlu_n$ to 5-CH_3-$H_4PteGlu_n$ followed by methyl group transfer to homocysteine to form methionine and regenerate $H_4PteGlu_n$ (Matthews, 1984). 5,10-CH_2-$H_4PteGlu_n$ reduction, reaction [15], is catalyzed by methylenetetrahydrofolate reductase (EC 1.1.1.68), which has been identified in a variety of bacterial, fungal, parasitic, plant and mammalian tissues (Matthews, 1984). This reaction is essentially irreversible under *in vivo* and *in vitro* conditions and is the committed step in methionine synthesis (Shane, 1989).

$$5,10\text{-}CH_2\text{-}H_4PteGlu_n + NADPH + H^+ \rightarrow 5\text{-}CH_3\text{-}H_4PteGlu_n + NADP^+ \qquad [15]$$

Substantial reduction in the K_m values was reported with increasing polyglutamate chain length for all methylenetetrahydrofolate reductases, indicating they were more efficient substrates (McGuire and Bertino, 1981; Shane, 1989). Effects of polyglutamate length on pig liver reductase displayed decreased K_m values with each additional glutamate up to the hexaglutamate, for a total reduction in K_m of 70-fold (McGuire and Bertino, 1981).

The terminal step in the *de novo* biosynthesis of methionine is the transfer of a methyl group from 5-CH_3-$H_4PteGlu_n$ to homocysteine, reaction [16], catalyzed by methionine synthase (EC 1.2.1.13).

$$5\text{-}CH_3\text{-}H_4PteGlu_n + \text{homocysteine} \rightarrow \text{methionine} + H_4PteGlu_n \qquad [16]$$

There are two different classes of methionine synthase enzymes: cobalamin (vitamin B_{12}) dependent and cobalamin independent (Matthews, 1984). Most fungi, higher plants and various microorganisms neither synthesize nor require cobalamin and contain only the cobalamin-independent enzyme (Cossins, 1980). In mammals, the essential amino acid methionine is adenylated to form the active methyl donor remethylating the homocysteine formed in the methylation of S-adenosylmethionine (AdoMet). Methionine synthase catalyzes the sole reaction whereby the methyl group of 5-CH_3-$H_4PteGlu_n$ can be utilized by mammalian cells.

The B_{12} dependent enzyme uses both mono- and triglutamate derivatives while the B_{12} independent synthase uses only the triglutamate (McGuire and Bertino, 1981). Although the exact polyglutamate moiety for the B_{12} independent enzyme is not known, methionine synthase is polyglutamate specific. The vitamin B_{12} independent methionine synthases of *Candida utilis*, *Caprinus lagopus*, *Aerobacter aerogenes*, *Salmonella typhimurium*, *Bacillus subtilis* and rat liver mitochondria all required a triglutamate for activity (McGuire and Bertino, 1981). *L. rhaminosis*, in common with green beans, spinach and barley sprouts, has a B_{12} independent enzyme which utilizes both mono- and pentaglutamate 5-CH_3-$H_4PteGlu_n$ derivatives, however the pentaglutamate was *ca.* 500 times more effective (McGuire and Bertino, 1981). The rat liver and bovine cerebral B_{12}-dependent methionine synthase displayed a greater affinity for polyglutamate substrates (Shane, 1989).

1.2.4. The Purine Cycle

Many organisms, including vertebrates, can also use formate as a C_1 source, and in this case the immediate product, 10-HCO-$H_4PteGlu_n$ must be reduced for incorporation into thymidylate, methionine and serine (MacKenzie, 1984). The interconversion of 5,10-CH_2-$H_4PteGlu_n$ and 10-HCO-$H_4PteGlu_n$ is central to the utilization of C_1 units. C_1 moieties at the formate oxidation level are utilized in *de novo* purine biosynthesis. They may arise by the oxidation of 5,10-CH_2-$H_4PteGlu_n$, which is catalyzed reversibly by methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5), reaction [17], and methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9), reaction [18].

$$5,10\text{-}CH_2\text{-}H_4PteGlu_n + NADP^+ \leftrightarrow 5,10\text{-}CH{=}H_4PteGlu_n + NADPH \qquad [17]$$

$$5,10\text{-}CH{=}H_4PteGlu_n + H_2O \leftrightarrow 10\text{-}HCO\text{-}H_4PteGlu_n + H^+ \qquad [18]$$

Alternatively, 10-HCO-$H_4PteGlu_n$ may be generated by the direct formylation of $H_4PteGlu_n$, reaction [19], catalyzed by formyltetrahydrofolate synthetase (EC 6.3.4.3).

$$\text{formate} + MgATP + H_4PteGlu_n \rightarrow 10\text{-}HCO\text{-}H_4PteGlu_n + MgADP + Pi \qquad [19]$$

In mammalian tissues, these dehydrogenase, cyclohydrolase, and synthetase activities are associated with a single trifunctional protein, termed C_1 tetrahydrofolate synthase (Paukert et al, 1976; Tan et al, 1977; MacKenzie, 1984). The cytoplasmic

and mitochondrial isoenzymes of *S. cerevisiae* are trifunctional (Appling and Rabinowitz, 1985), possessing an NADP-dependent methylenetetrahydrofolate dehydrogenase. However, a monofunctional, cytoplasmic NAD-dependent methylenetetrahydrofolate dehydrogenase has been identified in yeast species (Barlowe and Appling, 1990). Recent studies of spinach leaves (Nour and Rabinowitz, 1992) and pea cotyledons (Kirk et al, 1994; Kirk et al, 1995) have revealed that these tissues contain a monofunctional formyltetrahydrofolate synthetase protein that is readily separated from a protein with dehydrogenase-cyclohydrolase activities.

Methylenetetrahydrofolate dehydrogenase from *Clostridium cylindrosporum* exhibited a three-fold decrease in K_m as the glutamate chain length increased from mono- to triglutamate (McGuire and Bertino, 1981). The most complete study was undertaken using pig liver dehydrogenase, which is part of the trifunctional complex in eukaryotes. Studies with this enzyme suggested that the K_m decreased with increasing length up to the pentaglutamate derivative (Shane, 1989). Methenyltetrahydrofolate cyclohydrolase, the second activity of the bifunctional domain of the trifunctional protein, also exhibits a preference for folylpolyglutamate (Shane, 1989).

The clostridial formyltetrahydrofolate synthetase has been characterized with respect to polyglutamate specificity. The K_m for $H_4PteGlu_3$ in the forward reaction was ten-fold lower than that for $H_4PteGlu$ while the K_m for 10-HCO-$H_4PteGlu_3$ in the reverse direction was 100-fold lower than for the monoglutamate (McGuire and Bertino, 1981). The pigeon liver enzyme utilized saturating levels of mono-, tri-, and heptaglutamate derivatives at the same rate (McGuire and Bertino, 1981). Conversely, both the rat and bovine synthetases displayed dramatic declines in K_m as the chain length extended to the pentaglutamate, these large decreases were also observed with the pig liver enzyme but only to the level of triglutamate (Shane, 1989).

A number of complex reactions are involved in the biological production of purines of which two involve the donation of C_1 units by 10-HCO-$H_4PteGlu_n$ (Rowe, 1984). Glycinamide ribonucleotide (GAR) transformylase, EC 2.1.2.2, catalyzes the formyl group transfer from 10-HCO-$H_4PteGlu_n$ to GAR producing N-formylglycinamide ribonucleotide, reaction [20]. While 5-amino-4-imidazolecarboxamide (AICAR) transformylase, EC 2.1.2.3, catalyzes the formyl group transfer from 5,10-HCO-$H_4PteGlu_n$ to AICAR forming 5-foramido-4-imidazolecarboxamide, reaction [21].

Glycinamide ribonucleotide (GAR) + 10-HCO-$H_4PteGlu_n$ →
N-formyl-GAR + $H_4PteGlu_n$ [20]

5-amino-4-imidazolecarboxamide ribonucleotide (AICAR)
+ 10-HCO-H_4PteGlu$_n$ → formyl-AICAR + H_4PteGlu$_n$ [21]

Data available on purine biosynthetic enzymes isolated from uricotelic sources indicate GAR and AICAR transformylases copurify from chicken liver in association with C_1 synthase, suggesting a multi-enzyme complex for directing C_1 units into purine synthesis (Rowe, 1984).

The specificity of mammalian GAR transformylase for folylpolyglutamate substrates has received little attention. The transformylase from a human breast cancer cell line exhibits only a modest decrease in K_m for 10-HCO-H_4PteGlu$_5$ compared to the monoglutamate substrate (Shane, 1989). However, the activity of chicken liver AICAR transformylase was strikingly affected by folylpolyglutamate substrates. The K_m for its monoglutamate substrate was very high, and it was concluded that at physiological concentrations the monoglutamate would be inactive (McGuire and Bertino, 1981). Triglutamate and longer derivatives had K_m values which were three- to six-hundred-fold lower than the monoglutamate (Shane, 1989).

1.2.5. Initiation of Protein Synthesis

Prokaryotes require 10-HCO-H_4PteGlu$_n$ as the formyl donor in the formation of formylmethionyl-tRNA, which contributes to the complex that initiates the polypeptide chain (Staben and Rabinowitz, 1984). Although protein synthesis in the eukaryotic cytoplasm does not specifically require folate coenzymes or formylated tRNA derivatives, protein synthesis within the mitochondria and chloroplasts requires formylmethionyl-tRNA (Staben and Rabinowitz, 1984). Hence, methionyl-tRNA transformylase (EC 2.1.2.9) formylates methionyl-tRNA depicted in reaction [22], and its presence is presumed in all prokaryotes and in cellular organelles such as mitochondria and chloroplasts.

10-HCO-H_4PteGlu$_n$ + methionyl-tRNA → *N*-formylmethionyl-tRNA
+ H_4PteGlu$_n$ [22]

1.3. THE PHARMACOLOGY OF METHOTREXATE

Traditional methods for the discovery of chemotherapeutic agents can best be summarized as enlightened empiricism (Wood, 1975). Since the clinical introduction

of the diaminoantifolate, aminopterin, for treatment of childhood leukemia (Farber et al, 1948), considerable insight has been gained into the critical elements of pharmacologic activity for this class of drugs. It has become apparent that many folate antagonists, including aminopterin and MTX, are metabolized by man. The most important of these metabolic processes include the hydroxylation of the pteridine ring of diaminoantifolates (Bertino, 1981; Matherly et al, 1987), and the conversion to polyglutamates analogous to that of natural folates, first reported by Baugh et al (1973). For MTX, both of these structural alterations result in derivatives which exhibit pronounced differences in their biochemical and pharmacologic properties relative to the parent formulation (Fry et al, 1982). These changes affect the expression of antifolate activity both *in vivo* and *in vitro* (Fry et al, 1983). The extent of polyglutamylation of MTX and related antifolates in different tissues affects the expression of selective drug activity *in vivo* (Poser et al, 1981) and the biosynthetic utilization of exogenously supplied H_4PteGlu during 5-HCO-H_4PteGlu$_n$ rescue protocols (Dudman et al, 1982). Investigations into the formation and pharmacologic significance of these MTX metabolites have been a major focus of attention of many investigators.

1.3.1. The Clinical Applications of Methotrexate

Antifolates were discovered to be effective for the treatment of childhood acute leukemia in the 1940s. MTX, (4-amino-10-methyl pteroylglutamate; Figure 2B), remains an important folate antagonist used in cancer chemotherapy for many neoplasms including: acute lymphoblastic leukemia to maintain remission and to prevent central nervous system infiltration; non-Hodgkin's lymphoma; osteosarcoma; choriocarcinoma; Burkitt's lymphoma; as well as other solid tumors such as those of the head, neck, breast and lung (Winick et al, 1987; Fleming and Schilsky, 1992). MTX is also used in the treatment regimes of several non-neoplastic disorders, including: severe psoriasis unresponsive to other treatment; graft-versus-host disease; and in rheumatoid arthritis as immunosuppressive therapy (Fleming and Schilsky, 1992).

Organ distribution and MTX retention appears to reflect the presence or absence of specific transport mechanisms, as well as the levels of DHFR present in the cells, and perhaps the amount of conversion to polyglutamyl derivatives. The organs that contain the highest levels of MTX, with the longest retention rates are the liver and kidneys (Bertino, 1982). It has been clearly demonstrated that MTX present in liver and tumor tissues exists as MTX polyglutamates (MTXGlu$_n$). Since plasma

contains conjugase activity, $MTXGlu_n$ present in the blood would be hydrolyzed, and the monoglutamate moiety excreted in the urine (Bertino, 1982).

Toxicity to normal tissues occurs when cytocidal blood levels of MTX are maintained for sufficiently long periods of time. Both the plasma MTX concentrations and time of exposure are important in predicting toxicity (Bertino, 1982), which is a log function of the extracellular concentration multiplied by the duration of the concentration. MTX has the greatest toxic effect on the bone-marrow. Oral ulceration, stomatitis, pharnyngitis, glossitis and gingivitis are common, as are gastrointestinal disturbances, including anorexia, nausea, emesis and diarrhea (Bertino, 1982). During prolonged treatment hepatotoxicity is common resulting in hepatic fibrosis. The use of intrathecal MTX is often accompanied by headache and emesis and occasionally neurological disturbances may occur. MTX has been identified as a potent abortifacient, especially if administered during the first trimester of pregnancy (Bertino, 1982). Further, interstitial pneumonitis and osteoporosis have been reported with chronic low-dose MTX administration. Fever, seizures, phototoxicity and anaphylactoid reactions have been reported with high-dose administration (Bertino, 1982).

1.3.2. Binding of Methotrexate to Dihydrofolate Reductase

DHFR activity, studied as a function of the amount of enzyme added to a constant amount of MTX, demonstrated results characteristic of enzyme-inhibitor complexes with such low apparent dissociation constants they were small even compared with the enzyme concentration. This has been termed 'stoichiometric inhibition' (Blakley, 1969). At concentrations of a stoichiometric inhibitor inadequate to facilitate complete enzyme inhibition, most of the inhibitor is enzyme-bound, meaning the classical equations used by Lineweaver and Burk in their graphical representation of kinetic data are not appropriate. The classical kinetic treatment assumes that all inhibitor is free which is not the case in stoichiometric inhibition. However, the presence of a stoichiometric inhibitor increases both the slope and intercept of a double reciprocal plot (1/v versus 1/S) by the same factor, in the same manner as a classical noncompetitive inhibitor (Blakley, 1969) making the two inhibitors indistinguishable on a double reciprocal plot.

The degree of dissociation of the MTX-reductase complex appears to vary with the enzyme source (Cohen et al, 1978). DHFR forms a stable ternary complex with MTX and NADPH, increasing its antifolate affinity over that of a binary antifolate-DHFR complex (Kamen et al, 1983). The NADPH increases the affinity of

DHFR for MTX; however, when NADH was the cofactor, the inhibition of DHFR by MTX was not stoichiometric (Kamen et al, 1983). Detection of enzyme activity in the presence of NADH even in a 50-fold excess of MTX permits the hypothesis that natural MTX resistance may be a direct consequence of the decreased affinity of DHFR for NADPH or being in an intracellular milieu where NADH is the available cofactor. The process of inhibitor binding to DHFR is kinetically complex and occurs in distinct steps, an initial rapid formation of the MTX: enzyme: NADPH complex followed by a slow isomerization of the ternary complex (Matherly et al, 1987).

The X-ray data indicate that MTX binds to DHFR in an open conformation with the pteridine ring nearly perpendicular to the benzoyl ring (Blakley, 1984). The pyrimidine edge of the pteridine ring is deeply buried in the active site cavity, but the *p*ABA side chain extends out to the surface, and the glutamate moiety is draped across αB. The pteridine ring of MTX makes nonpolar contacts with the protein backbone and side chain atoms of βA, the loop βA to αB, and αB (Blakley, 1984). The additional contacts in the ternary complex between MTX and the nicotinamide moiety, together with the accompanying dissolution of the pteridine ring due to NADPH binding, likely explain the cooperative effects MTX and NADPH have on one another's binding (Blakley, 1984). Although MTX is a close structural analog of folic acid, X-ray and NMR stereochemical studies have reported that they bind differently to DHFR. The two molecules occupy essentially the same binding site in the enzyme (Bertino et al, 1964), but the pteridine ring of MTX is turned over by *ca.* 180° compared with its orientation in the enzymically productive form of the DHFR-folate complex (Curtis et al, 1994). Moreover, folate can bind in more than one conformation to DHFR, whereas MTX may bind only in a single conformation.

1.3.3. Methotrexate Rescue by 5-Formyl-Tetrahydrofolate

5-HCO-H_4PteGlu, known clinically as leucovorin or folinic acid, has been widely used for over 30 years in cancer chemotherapy, yet very little is known about its metabolic role (Stover and Schirch, 1993). Leucovorin can reverse the cytotoxic effects of antifolates, such as MTX, which primarily derive their effects from an inhibition of DHFR (Matherly et al, 1987). Leucovorin rescue involves the provision of a reduced folate cofactor in the form of 5-HCO-H_4PteGlu following MTX treatment and allows the administration of considerably higher doses of antifolate than would otherwise be tolerated. In theory, this allows the maximal penetration of antifolate into tumor cells, thereby maximizing long term cell kill (Matherly et al, 1987). The classical

view of leucovorin rescue was that exogenous 5-HCO-H_4PteGlu circumvents the block in *de novo* H_4PteGlu biosynthesis at DHFR in susceptible tissues by providing sufficient cofactor to sustain biosynthetic processes at a normal rate, as depicted in Figure 3 (Nixon and Bertino, 1971; Stover and Schirch, 1993). The biochemical basis for the putative selectivity of high-dose MTX-leucovorin rescue has long been elusive and only recently has a clear understanding emerged of the multiple cellular interactions between reduced folates and antifolates which contribute to the selectivity of the rescue phenomenon.

Expansion of intracellular folate pools by 5-HCO-H_4PteGlu exposure can decrease MCF-7 cell, a line of human breast cancer cells in continuous monolayer culture, sensitivity to MTX probably because high cellular folate contents decrease the capacity of the cells to metabolize MTX to polyglutamate derivatives (Jolivet et al, 1987). Possible sites of interaction between folates and antifolates leading to decreased MTX metabolism include membrane transport (Goldman et al, 1968) and FPGS (Baugh et al, 1973). In contrast to 5-HCO-H_4PteGlu, folic acid fails to afford protection against MTX effects (Mead et al, 1963; Jolivet et al, 1987). This phenomenon may be explained by evidence suggesting that reduced folates are better substrates for DHFR and FPGS than folic acid and MTX, respectively, and may decrease $MTXGlu_n$ synthesis *in vitro* when both substrates are coincubated (Jolivet et al, 1987). While high-dose MTX with leucovorin rescue has the theoretical ability to surmount many of the known mechanisms by which neoplastic cells become resistant to MTX, Allegra and Boarman (1990) provided a plausible explanation for the suboptimal clinical results using this strategy. The relatively greater polyglutamylation of MTX by malignant versus normal cells has been considered to be a critical factor in the selectivity of leucovorin rescue. While polyglutamylation provides some selectivity, even cells that extensively synthesize $MTXGlu_4$, like MCF-7 cells, may be rescued by leucovorin through the generation of H_2PteGlu (Allegra and Boarman, 1990). The ability of H_2PteGlu to diminish the intracellular concentration of MTX and $MTXGlu_n$ is of interest in that it suggests an additional rescue mechanism. Investigations utilizing Ehrlich ascites tumor cells demonstrated a marked antagonism by reduced folates to MTX binding to DHFR (Matherly et al, 1983). Of particular interest in these investigations is the finding that cellular energy metabolism plays a critical role in determining whether 5-HCO-H_4PteGlu induces the loss of MTX from DHFR. This may be related to the role of oxidative metabolism in determining the intracellular levels of reduced and oxidized pyridine nucleotides (Matherly et al, 1984). Hence, poorly oxy-

genated cells, such as solid tumors, with high glycolytic rates would probably have the highest ratios of reduced to oxidized pyridine nucleotides and would present the least favorable conditions for MTX loss from DHFR induced by reduced folates (Matherly et al, 1983). Conversely, well oxygenated cells, such as the highly perfused cells of the gastrointestinal tract, with lower levels of reduced pyridine nucleotides would present the most favorable conditions for the loss of MTX from its target enzyme. Accordingly, in the presence of NADPH, DHFR exhibits an enhanced affinity for MTX and a reduced binding of 5-HCO-H_4PteGlu relative to the binary $NADP^+$ complex. When the major portion of the enzyme is associated with MTX, H_2PteGlu accumulates within the cell to high levels and competes with MTX for DHFR, resulting in the net loss of MTX from the enzyme as extracellular and intracellular levels of free antifolate diminish (Matherly et al, 1983).

Conversely, leucovorin is ineffective in reversing the cytotoxic effects of MTX toward leukemic L1210 cells, following pretreatment with the antifolate so as to form polyglutamate derivatives, following which MTX is removed (Matherly et al, 1986). Since MTX is absent during the growth interval there can be no interaction between the cofactor and MTX at the membrane level. Hence, it appears that the intracellular antifolate level achieved is the critical element in establishing whether leucovorin can reverse the cytotoxic effects derived from DHFR inhibition. Matherly et al (1986) reported that the metabolism of MTX to $MTXGlu_n$ derivatives by L1210 cells limits the extent of rescue achieved by leucovorin *in vitro*. Similarly, T-lymphocytes and B-lymphocytes were less readily rescued following MTX treatment (Dudman et al, 1982). Only high leucovorin concentrations are able to rescue hepatoma cells containing $MTXGlu_n$ and only after a considerable lag period (Galivan and Nimec, 1983). Further, leucovorin was ineffective in reversing the MTX toxicity of fibroblasts if added to the medium following a preincubation in MTX (Rosenblatt et al, 1982). This suggests that H_2PteGlu can compete less well with $MTXGlu_n$ than with MTX for DHFR under cellular conditions. On the basis of these findings it is clear that the intracellular conversion of MTX to polyglutamyl derivatives limits the extent of rescue achieved with leucovorin.

1.3.4. The Metabolism of Methotrexate

1.3.4.1. The Polyglutamylation of Methotrexate

Recently attention has focused on the importance of the polyglutamyl derivatives of MTX that have been detected in normal (Rosenblatt et al, 1978; Gewirtz et al, 1980; Rosenblatt et al, 1981; Poser et al, 1981; Balinska et al, 1981; Balinska et al, 1982; Galivan et al, 1986) and malignant cells (Galivan, 1979, 1980; Jolivet and Schilsky, 1981; Fry et al, 1982; Krakower et al, 1982; Jolivet et al, 1982; Nimec and Galivan, 1983; Fry et al, 1983; McGuire et al, 1985) both *in vitro* and *in vivo* (Whitehead et al, 1975; Winick et al, 1987; Synold et al, 1994). The biochemical transformation of this important chemotherapeutic agent is of particular significance since polyglutamate derivatives of MTX are not only potent inhibitors of DHFR, but have different cellular pharmacokinetics than the parent monoglutamate. Consequently, FPGS appears to be important in cancer chemotherapy both because of its role in the metabolism of antifolates to intracellularly retained $PteGlu_n$ and because it is essential for cell survival (Moran et al, 1985).

A variety of analytical techniques have been employed to quantitate MTX polyglutamate derivatives, including diethylaminoethyl (DEAE) cellulose chromatography (Gewirtz et al, 1980; Poser et al, 1981; Balinska et al, 1982) and Sephadex gel filtration (Rosenblatt et al, 1978; Whitehead et al, 1975; Galivan, 1979, 1980; Rosenblatt et al, 1981; Balinska et al, 1981). The detailed description of the cellular kinetics of drug metabolism, the extent of glutamate conjugation, and the pharmacologic properties of the polyglutamyl drug forms, was virtually impossible prior to the routine application of high performance liquid chromatography (HPLC). HPLC separations have employed both reverse phase (Jolivet and Schilsky, 1981; Jolivet et al, 1982; Winick et al, 1987) and anion exchange methods (Fry et al, 1982; Krakower et al, 1982; Nimec and Galivan, 1983; Fry et al, 1983; McGuire et al, 1985; Galivan et al, 1986). This highly sensitive methodology has achieved a resolution of one to seven polyglutamyl forms of MTX which contrasts with the results obtained with previous gel filtration or ion exchange methods where the $MTXGlu_n$ derivatives were separated from MTX, however, the various polyglutamate forms were only poorly resolved.

Many investigators have demonstrated that the same enzyme, FPGS, catalyzes the γ-glutamylation of the folate coenzymes and folate analogs, such as MTX (Scrimgeour et al, 1985). Polyglutamylation of MTX, like $H_4PteGlu$, is a process by which successive glutamate residues are added in a peptide linkage to the existing

glutamate moiety through the γ-carboxyl group by FPGS. The initial metabolic transformation is thought to be the formation of a γ-acyl phosphate by reaction of the glutamate moiety of MTX with ATP. The acyl phosphate intermediate then reacts with the amino group of the incoming glutamate to form the γ-peptide bond with ejection of the phosphate. Kim et al (1993) utilized CHO cells expressing human and *E. coli* FPGS activities to assess the role of the FPGS level in MTX metabolism and cytotoxicity. The results indicated that MTX cytotoxicity is closely related to FPGS activity and cells that synthesize longer polyglutamate derivatives are more sensitive to the antifolate. Practically no $MTXGlu_n$ were detected in cells expressing low levels of human FPGS, despite their ability to accumulate and metabolize folate to $PteGlu_n$ derivatives. Defective polyglutamylation of MTX has been well recognized as an important determinant of MTX resistance (Assaraf et al, 1992). Resistance to MTX by this mechanism has recently been reported for a human leukemia cell line (McCloskey et al, 1991). Directly correlating the degree of MTX resistance to the reduced ability for polyglutamylation through decreased FPGS activity.

1.3.4.2. Properties of Methotrexate Polyglutamates

There are important pharmacological ramifications of the metabolism of methotrexate to its polyglutamyl derivatives. The polyglutamyl derivatives of MTX are at least equivalent inhibitors of DHFR as their parent drug (Galivan, 1979; Fry et al, 1982; Jaffe et al, 1983). Moreover, increased affinities of $MTXGlu_n$ for DHFR have been suggested (Galivan, 1980; Balinska et al, 1982; Kisliuk et al, 1983; Schilsky et al, 1983; Abraham et al, 1991; Assaraf et al, 1992). Like the polyglutamyl derivatives of natural folates, MTX polyglutamyl conjugates are retained intracellularly (Rosenblatt et al, 1978; Galivan, 1980; Fry et al, 1982; Jolivet et al, 1982; Galivan and Nimec, 1983; McGuire et al, 1985; Galivan et al, 1986). Generally, the loss of $MTXGlu_n$ from cells inversely correlates with the glutamyl chain length, no discernible loss of the longer derivatives can be detected. This in part, results from the continued synthesis of longer chain polyglutamates from the shorter chain derivatives in the absence of competing monoglutamate substrate. Moran et al (1985) reported long chain $MTXGlu_n$ derivatives (greater than $MTXGlu_3$) poorly traverse the cell membrane, while shorter chain derivatives ($MTXGlu_2$ and $MTXGlu_3$) seem to exit the cell more slowly than MTX. The efflux of MTX and $MTXGlu_n$ was examined with primary monolayer cultures of rat hepatocytes and also with monolayer cultures of a hepatocarcinoma cell line (H35 cells) (Balinska et al, 1981). When $MTXGlu_n$ were present in

hepatocytes in excess of MTX, efflux led to a further increase in the intracellular proportion of polyglutamates and a selective loss of MTX. Similar results were reported when MTX was the predominant intracellular species at the onset of efflux. These results demonstrate that both MTX and $MTXGlu_n$ may cross the cell membrane but that MTX does so much more rapidly, resulting in the selective retention of $MTXGlu_n$ derivatives. Similarly, in H35 cells, intact $MTXGlu_n$ can exit the cells, but at a slower rate than MTX efflux. Balinska et al (1982) suggested that $MTXGlu_n$ exit the cell by a slow loss of intact $PteGlu_n$ and also by intracellular cleavage to MTX followed by efflux. The preferential retention of $MTXGlu_n$ derivatives was also investigated in a human leukemia cell line, CCRF-CEM (McGuire et al, 1985) and Ehrlich ascites tumor cells (Fry et al, 1981). Not only were $MTXGlu_n$ derivatives retained within the cell, but as the ratio of $MTXGlu_n$ to MTX increases they rapidly displace the monoglutamate from DHFR with the subsequent rapid loss of MTX from the cell. Regardless of whether there is evidence of $MTXGlu_n$ diffusion from cells, it is clear that the primary effect arising from MTX metabolism is the conversion of a potent, albeit reversible, monoglutamate DHFR inhibitor to an essentially irreversible inhibitor of this intracellular target. This arises since polyglutamyl derivatives of MTX do not appreciably exit the cell.

Since folate $PteGlu_n$ have an enhanced affinity for certain folate-dependent enzymes compared to their PteGlu form, Allegra et al (1985) examined the capacity of MTX and $MTXGlu_n$ to directly inhibit $H_4PteGlu$-dependent enzymes in purine and thymidylate synthesis and various folate interconversions. A potent inhibition of AICAR transformylase by $MTXGlu_n$ derivatives has been reported. A 10-fold increase in binding affinity occurred with the addition of each new γ-glutamyl residue to MTX which is consistent with the free energy change required to form one additional hydrogen bond between the enzyme and the new glutamyl group (Allegra et al, 1985). Additionally, $H_2PteGlu_n$ that accumulate intracellularly, as the result of MTX induced DHFR inhibition, inhibit AICAR transformylase which contributes to the pharmacologic significance of this enzyme (Allegra et al, 1987). Further, $MTXGlu_n$ have greatly enhanced inhibitory effects on the catalytic activity of TS, as compared to MTX (Moran et al, 1979; Bunni et al, 1988), such that $MTXGlu_5$ has nearly 300-times the inhibitory activity of MTX. The parent drug displayed an uncompetitive inhibition of TS whereas the $MTXGlu_n$ derivatives display a competitive inhibition of TS such that the prior binding of either substrate or product is not required. This ability to bind to TS in the absence of other ligands may be the result of the presence of a polyglutamate

tail that markedly increases the affinity of binding to the unoccupied enzyme. Other enzymes including methylenetetrahydrofolate reductase, SHMT and GAR transformylase exhibited similar enhanced competitive inhibition by $MTXGlu_n$. The enhanced inhibition of certain folate-dependent enzymes by $MTXGlu_n$ as compared to weak inhibition by MTX indicates that these metabolites may have additional sites of action. The inhibitory potential of these compounds also depends on the state of polyglutamylation of the folate pool. In summary, enhanced inhibition of the folate-dependent enzymes may have the following consequences: direct suppression or inhibition of *de novo* purine synthesis or reduced availability of specific folate cofactors required for protein and/or nucleic production (Allegra et al, 1985).

Considerable evidence now substantiates the pharmacologic importance of cellular folylpolyglutamylation. Investigations have correlated the accumulation of $MTXGlu_n$ in various tumor cells with increased tumoricidal activity. Even in non-neoplastic tissues, a relationship between the extent of $MTXGlu_n$ synthesis and drug sensitivity exists. Implying that the level of MTX polyglutamylation influences the *in vivo* activity of the drug. High levels of $MTXGlu_n$ are formed in drug responsive tumors, where only low levels are formed in the drug proliferative compartments of bone marrow (Poser et al, 1981), or gastrointestinal epithelium (Poser et al, 1981; Fry et al, 1983). This differential mechanism would appear to represent at least one significant element in determining cell sensitivities to MTX. Only in cells which have accumulated appreciable levels of $MTXGlu_n$ would intracellular DHFR suppression be sustained as extracellular plasma drug levels decline (Fry et al, 1983; Matherly et al, 1987). This would occur in poorly perfused tumor cells; however, the sensitive bone marrow and gastrointestinal epithelial cells are highly perfused and thus form only low levels of $MTXGlu_n$. The cytotoxic effects of MTX toward these limiting host tissues would largely be independent of drug metabolism and occur primarily when the plasma MTX levels are high. Thereafter, as plasma and intracellular MTX levels decline, DHFR would be reactivated in these tissues, repleting $H_4PteGlu$ pools, and resuming folate-dependent biosynthesis.

1.3.4.3. Modulation of Methotrexate Polyglutamylation

Cellular $MTXGlu_n$ formation is dependent upon a number of critical elements. The intracellular level of antifolate, determined by the membrane transport process, determines the level of MTX substrate presented to the intracellular FPGS (Goldman et al, 1968; Galivan, 1979; Gewirtz et al, 1980; Holm et al, 1994). The kinetic charac

teristics of MTX influx involves a small, variable, and rapid uptake into cells which may partially represent surface adsorption of MTX (Holm et al, 1994), followed by a linear uptake phase. The rate of MTX flux into hepatocytes is similar to that observed in L1210 leukemia cells, such that transport represents the rate limiting component of MTX binding to DHFR (Goldman et al, 1968; Gewirtz et al, 1980). The initial linear uptake phase represents a unidirectional flux of transport across the cell membrane unperturbed by subsequent binding or cellular MTX metabolism. Gewirtz et al (1980) identified two remarkably similar influx routes for MTX. The properties of the second transport component indicate that it is a mediated process that is partly saturable and is inhibited by high MTX levels. Other investigations demonstrated that a part of the second process may represent passive diffusion which is uninfluenceable by the presence of any MTX cellular component (Gewirtz et al, 1980).

Since only unbound methotrexate is a substrate for polyglutamylation by FPGS, it is important to consider only the free intracellular drug concentration when assessing the availability of MTX for conversion to $MTXGlu_n$. Advocates of low-dose MTX argue that high MTX concentrations may impair MTX polyglutamylation through feedback inhibition, as has been demonstrated *in vitro* (McGuire et al, 1980; Balinska et al, 1982; Nimec and Galivan, 1983). Hence, high substrate concentrations result in predominantly shorter chain $MTXGlu_n$ moieties whereas relatively low substrate concentrations result in longer chain length derivatives. Conversely, MTX polyglutamylation in some mammalian tissues was reported to be time and dose-dependent (Fry et al, 1982; Abraham et al, 1991; Kim et al, 1993). In these investigations, μM MTX extracellular concentrations saturated DHFR within cells and achieved adequate intracellular levels of exchangeable MTX for polyglutamylation to proceed. The ability of MTX to sustain suppression of cellular DHFR activity is limited because: only a small fraction of DHFR is necessary to maintain $H_4PteGlu$ synthesis; and, the high $H_2PteGlu$ levels that accumulate in cellular pools as MTX associates with the major portion of the enzyme effectively compete with MTX for this small fraction of DHFR (Fry et al, 1982; White, 1974). Consequently, to inhibit $H_4PteGlu$ synthesis, free intracellular drug levels, must achieve orders of magnitude above the K_i (10^{-8} M).

1.3.4.4. Hydroxylation of Methotrexate by Hepatic Aldehyde Oxidase

The first indication that metabolic antifolate alterations could occur in mammals was demonstrated by the recovery of a metabolized form of 3′,5′-dichloromethotrexate in the urine from patients (Matherly et al, 1987). This metabolite was

identified as 7-hydroxydichloromethotrexate. Subsequent studies have confirmed that non-halogenated antifolates including MTX and aminopterin could be similarly metabolized (Bertino, 1982).

Early investigations indicated that the conversion of antifolates to their 7-hydroxylated forms occurred primarily in the liver and involved aldehyde oxidase (EC 1.2.3.1). This enzyme catalyzes the oxidation of a variety of aliphatic and aromatic aldehydes as well as several non-aldehyde heterocyclic compounds including antifolates. There is considerable interspecies variation regarding the capacity to oxidize the antifolates with the highest activity detected in rabbit liver (Matherly et al, 1987). Appreciable plasma levels of 7-hydroxymethotrexate (7-OH-MTX) have been reported in subjects given high doses of MTX, suggesting substantial hydroxylating activity in the human liver as well (Matherly et al, 1987; Belz et al, 1994). Additional studies have evaluated the substrate efficacy of mono-and polyglutamyl derivatives of MTX for hydroxylation by the aldehyde oxidase from rabbit liver (Fabre et al, 1985). In these investigations, the efficiency of the hydroxylation decreases as the glutamyl chain length increases. Hence, in hepatic tissue, 7-OH-MTXGlu$_n$ can be generated by either the hydroxylation of MTX with subsequent polyglutamate synthesis by FPGS, or MTX polyglutamylation with subsequent hydroxylation to form 7-OH-MTXGlu$_n$. Since polyglutamyl forms of MTX are poor substrates for hepatic aldehyde oxidase, it seems likely that the former pathway is of greater importance (Matherly et al, 1987).

Evidence which raises the possibility that 7-OH-MTX may modulate MTX pharmacological activity *in vivo*, arises through interactions: (i) with the carrier which it shares with MTX and other reduced folates, (ii) with the FPGS enzyme, or (iii) as a direct inhibitor of intracellular enzymes (Matherly et al, 1987). MTX influx is depressed by 7-OH-MTX, and this reduces the net intracellular accumulation of the parent compound. Even though 7-OH-MTX cannot itself compete effectively with MTX for DHFR binding sites it nonetheless can diminish the pharmacologic effects of MTX by reducing the free intracellular drug level, thereby decreasing the suppression of DHFR (Matherly et al, 1987). Further, diminishing the free MTX level under these conditions also results in reduced MTXGlu$_n$ accumulation (Fabre et al, 1985).

The 7-OH-MTX metabolite is cytotoxic to tumor cells *in vitro*, but far less so than the parent compound, MTX (Matherly et al, 1987). Since the hydroxy-metabolite only weakly inhibits DHFR, other potential cellular targets have been examined in an attempt to further elucidate the biochemical basis for *in vitro* cytotoxicity. Matherly et al (1987) demonstrated 7-OH-MTX and its tetrapolyglutamyl derivative

inhibit two folate-dependent enzymes, TS and AICAR transformylase. While both enzymes were only weakly inhibited by 7-OH-MTX, the inhibitory potencies increased with the polyglutamylated cofactors in the order of 50- to 100-fold against TS and 550- to 1300-fold against AICAR transformylase.

1.3.4.5. Methotrexate Resistance

Cytotoxicity of MTX depends on the function of at least three processes including: intracellular drug accumulation against a concentration gradient, via a carrier-mediated system; the presence of an intact high binding affinity intracellular target enzyme, DHFR; and prolonged intracellular retention due to the action of FPGS, that concatenates γ-glutamyl residues to MTX. Two major impediments toward successful chemotherapy are inherent and acquired drug resistance (Assaraf, 1992). Quantitative or qualitative alterations in each of the above cellular processes have been widely recognized as important determinants of MTX resistance. These alterations include: increased DHFR activity as a result of gene amplification (Assaraf et al, 1992); diminished or absent MTX uptake (Wu et al, 1993); increased MTX-binding proteins different from DHFR accompanied by a reduction in the affinity for MTX binding (Barg et al, 1984; Barg et al, 1987); enhanced γ-glutamyl hydrolase activity (Rhee et al, 1993); methionine inhibition of polyglutamylation (Rhee et al, 1989); and reduced formation of $MTXGlu_n$, resulting from decreased FPGS activity (McCloskey et al, 1991). The existence of each of the various modes of MTX resistance has been described in mammalian and plant tissues.

1.4. THE PRESENT STUDY

The importance of $MTXGlu_n$ cytotoxicity in mammalian cells has been well documented, however its implication has yet to be determined in plants and fungal species. As decreased polyglutamylation of MTX reduces intracellular retention of the drug, it therefore increases MTX resistance. The ability of viable wild type and methionine auxotrophs of *Neurospora crassa* to convert [^{3}H]-MTX to its various polyglutamyl derivatives was examined via HPLC analysis. For the purpose of this study, *met*-6 and *mac* mutants of *Neurospora crassa* were utilized. Folylpolyglutamate deficiencies, detectable by reduced FPGS activity, result in failure to generate $PteGlu_n$ *in vivo*. The mutation at the *mac* locus prevents the generation of $PteGlu_2$ derivatives (reaction [23]), whereas the *met*-6 lesion prevents the conversion of $PteGlu_2$ to

$PteGlu_6$ derivatives (reaction [24]). These mutations result in auxotrophies for methionine, normally generated by the folate-dependent pathways of C_1 metabolism.

$$H_4PteGlu + ATP + L\text{-glutamate} \rightarrow H_4PteGlu_2 + ADP + P_i \quad [23]$$

$$H_4PteGlu_2 + 4\ ATP + 4\ L\text{-glutamate} \rightarrow H_4PteGlu_6 + 4ADP + 4P_i \quad [24]$$

Unlike comparable mammalian enzymes, *N. crassa* FPGS has a much narrower specificity for folate substrates (Chan et al, 1991). The present study will demonstrate MTX inhibition of *N. crassa in vivo* and measure the extent of $MTXGlu_n$ synthesis in the three *N. crassa* forms to ascertain the role of FPGS activity in fungal MTX polyglutamylation. Additionally, 5-HCO-H_4PteGlu was incorporated into these investigations to determine whether the reduced folates produced, from the incorporation of this exogenous folate cofactor into the H_4PteGlu utilization pathway, could rescue the cells from $MTXGlu_n$.

Recalling that increased activity of DHFR, is another mechanism of resistance, this enzyme was isolated and purified in *N. crassa*, wild-type, *met*-6 and *mac* forms, to determine whether MTX associated cytotoxicity or insensitivity was a direct result of altered FPGS activity or due to changes in DHFR affinity or increased gene expression.

MATERIALS AND METHODS

2.1. CHEMICALS

Reagent-grade chemicals were supplied by Sigma Chemical Company (St. Louis, Mo., USA), Fisher Scientific and BDH Chemicals (Edmonton, Alberta). [3',5',7-^{3}H]-MTX with a specific activity of 24.1 Ci mmol^{-1} was purchased from Moravek Biochemicals Incorporated (Brea, CA., USA). MTX was supplied by Sigma Chemical Company, however MTXGlu$_n$ (n=2-4) were obtained from Dr. B. Schircks Laboratories (Jona, Switzerland). Matrex Green A was purchased from Amicon, Oakville, Ontario. Sephadex G-75 was obtained from Pharmacia, Uppsala, Sweden. Molecular weight marker proteins were purchased from Sigma, and ACS scintillation fluor was supplied by Amersham (Oakville, Ontario).

2.2. FUNGAL MATERIAL

Neurospora crassa Lindegren A wild type (FGSC 853), and two methionine auxotrophic strains; *met*-6 (FGSC 1330) and *mac* (FGSC 3609) were maintained and cultured in liquid or solid Vogel's medium N (Davis and de Serres, 1970). The L-methionine supplement for mutant cultures was 10 mM. Large scale conidiospore production was achieved by inoculating 2500 mL Fernbach flasks containing 500 mL of Vogel's medium with conidia. The inoculum from an agar slant, was resuspended in sterile double-distilled water. The resulting cultures were incubated at 30°C for 72 hours. Each Fernbach flask was fitted with a two-hole stopper equipped with a long inlet tube and a short outlet tube. A sterile 500 mL Erlenmeyer flask containing water connected to a drying tube packed with sterile glass wool was attached to the inlet tube to filter-sterilize the entering air. Compressed air, humidified by passage through the water, was forced into the Fernbach flask by connecting the tubing leading from the outlet tube of the Fernbach flask to a vacuum line. Growth with aeration continued for three days under room lighting at ambient temperature (25°C) (Davis and de Serres, 1970). *Neurospora* conidia were harvested by suspension in cold, sterile, double-distilled water followed by filtration through sterile cheesecloth. The filtrate was then decanted into 50 mL polycarbonate Nalgene (Rochester, N.Y.) Oak Ridge centrifuge tubes and centrifuged at 3000 x g for 10 minutes. The supernatant was removed and the pellet was resuspended in cold, sterile double-distilled water and

centrifuged at 3000 x g for a further 10 minutes; this washing procedure was repeated twice. The supernatant was again removed and the freshly harvested conidiospores (20-25 mg dry wt) were stored at 2°C.

2.3. *NEUROSPORA CRASSA* GROWTH INHIBITION

Freshly harvested conidia were resuspended in a volume of Vogel's medium to give an absorbance of *ca.* 0.080 at 350 nm. Subsequently, 1.0 mL aliquots of resuspended conidia were added to 29 mL of Vogel's medium contained in 125 mL Erlenmeyer flasks, under sterile conditions. Aliquots of methotrexate or other supplements were then added to a final volume of 30 mL and the flasks were incubated at 30°C for 24 hours in a shaking water bath.

After 24 hours of growth, mycelia were harvested by vacuum filtration through Whatman No. 1 filter paper. Cultures that did not yield clear filtrates (which suggests microbial contamination) were discarded. Mycelia were extensively washed with double-distilled water and placed in preweighed aluminum dishes and dried at 50°C for 48 hours before determination of dry weights.

2.3.1. Methotrexate Treatment

Methotrexate solutions, of known concentration were stored as 100 µL aliquots, in the dark at -20°C. Several drops of 1N NaOH were required to facilitate complete dissolution of MTX, hence the pH of these MTX solutions was routinely measured as 13.35. Only small aliquots (10-500 µL) were added to the cultures and the pH of the media was only altered by 0.03. Prior to use, MTX aliquots were thawed and filter-sterilized, by passage through sterile Millex-GS (Millipore) 0.22 µm filter units. Appropriate aliquots were added to each culture (see **2.3.**) to attain the desired concentration in 30 mL. The incubation and harvest proceeded as described above (**2.3.**).

2.3.2. Effects of Amino Acid Supplementation on Methotrexate Treatment

The standard culture conditions, described in **2.3.**, were followed. The defined media contained either no supplementation, or the addition of 10 mM L-methionine or 10 mM glycine, added prior to media sterilization. The effect of these amino acids was investigated in *N. crassa* wild type to determine what effect, if any, the L-meth-

ionine supplementation employed in growth of *met*-6 and *mac* might have on MTX cytotoxicity. The incubation and harvest conditions were identical to those described in section **2.3.**.

2.3.3. Sulfanilamide Treatment

The conidiospores, as in **2.3.**, were inoculated into 30 mL of defined media containing varying amounts of sulfanilamide. The stability of sulfanilamide permitted its addition to the defined media prior to sterilization. The cultures were then incubated under the conditions used in section **2.3.**.

In other experiments, 100 μM sulfanilamide, which resulted in a *ca.* 50% inhibition of growth (100 μM), was included in the defined media. Appropriate aliquots of MTX were then added to each culture as in the MTX treatment studies **(2.3.1.)**. The incubation and harvest conditions were identical to those utilized in previous trials **(2.3.)**.

2.4. FOLINIC ACID RESCUE OF *NEUROSPORA CRASSA* GROWTH

Standard folinic acid (5-HCO-H_4PteGlu) solutions were prepared with several drops of 1N KOH to facilitate complete dissolution. These were stored at 4°C in the dark.

Freshly harvested conidiospores were resuspended in a volume of defined media which resulted in an absorbance of *ca.* 0.080 at 350 nm. Aliquots (1 mL) of this suspension were added under sterile conditions to 29 mL of Vogel's medium contained in 125 mL Erlenmeyer flasks. An aliquot of MTX was then thawed and filter-sterilized, through a sterile Millex-GS 0.22 μM filter unit, prior to addition to the cultures. The resulting final concentration of MTX was 20 μM in 30 mL. The flasks were then incubated at 30°C for 24 hours in a shaking water bath. After this 24-hour MTX treatment, the media were removed by aspiration and each culture was washed with MTX-free Vogel's medium and transferred into another 29 mL volume of Vogel's medium, under aseptic conditions. An aliquot of folinic acid was filter-sterilized, through a sterile Millex-GS 0.22 μM filter unit, and appropriate volumes were added to the defined media to achieve the desired concentrations in total volumes of 30 mL. The cultures were incubated for a further 24 hours at 30°C in a shaking water bath.

After the folinic acid treatment, mycelia were harvested by vacuum filtration through Whatman No. 1 filter paper. The mycelia were extensively washed with double-distilled water and dry weights were determined (see **2.3.**).

2.5. RECOVERY OF MYCELIAL GROWTH AFTER 24 HOUR MTX TREATMENTS

Aliquots (1 mL) of freshly harvested conidia (see Section **2.3.**) were added to 29 mL of Vogel's medium contained in 125 mL Erlenmeyer flasks, under aseptic conditions. An aliquot of MTX was thawed, filter-sterilized, and added to the cultures to give a final concentration of 20 μM in 30 mL. The flasks were then incubated at 30°C for 24 hours in a shaking water bath. To initiate efflux of the antifolate, the MTX-containing media was removed by aspiration and each culture was washed and transferred to 29 mL samples of MTX-free Vogel's medium, under sterile conditions. Subsequently, the cultures were incubated at 30°C in a shaking water bath for 3, 6, 9, 12 and 24 hours.

After MTX efflux for the indicated times, mycelia were harvested by vacuum filtration through Whatman No. 1 filter paper. Mycelial dry weights were then determined (see **2.3.**).

2.6. MTX TREATMENT OF 36-HOUR PREINCUBATED CULTURES

Aliquots (500 μL) of a conidial suspension (see Section **2.3.**) were added to 29.5 mL of Vogel's medium contained in 125 mL Erlenmeyer flasks, under aseptic conditions. The cultures were then incubated for 36 hours in a 30°C shaking water bath. After this 36-hour preincubation, the media were removed by aspiration and each culture was transferred into another 29 mL of Vogel's medium, under sterile conditions. Appropriate volumes of filter-sterilized MTX required to attain the desired concentrations in 30 mL were added to these cultures, which were then incubated for 6 hours at 30°C in a shaking water bath. After the MTX treatment, the cultures were each washed with 29 mL of MTX-free Vogel's medium to remove exogenous MTX. Efflux of the antifolate was initiated by transfer of the cultures to 30 mL of Vogel's medium in 125 mL Erlenmeyer flasks, under sterile conditions, following aspiration of the wash media. The cultures were then incubated for a further 24 hours in a 30°C shaking water bath.

After this 24 hour efflux period, mycelia were harvested by vacuum filtration through Whatman No. 1 filter paper. Dry weights were determined as described in Section **2.3.**.

2.7. MTX TREATMENT OF 14-HOUR PREINCUBATED CULTURES

Aliquots (500 μL) of a conidial suspension (see Section **2.3.**) were added to 29.5 mL of Vogel's medium contained in 125 mL Erlenmeyer flasks, under aseptic conditions. These cultures were preincubated for 14 hours in a 30°C shaking water bath. After this 14 hour preincubation, the media were removed by aspiration and each culture received 29 mL of fresh Vogel's medium, under sterile conditions. Appropriate volumes of filter-sterilized MTX, necessary to achieve the desired concentrations in 30 mL, were added to these cultures, which were then incubated for either 6 or 24 hours at 30°C in a shaking water bath. After the MTX treatment, the cultures were each washed with 30 mL of MTX-free Vogel's medium to remove exogenous MTX. Efflux was initiated by transfer of the cultures to 30 mL of Vogel's medium in 125 mL Erlenmeyer flasks, under sterile conditions, following aspiration of the wash media. The cultures were incubated for a further 24 hours in a 30°C shaking water bath.

Following efflux, mycelia were harvested and dry weights were determined (see Section **2.3.**).

2.8. EXTRACTION OF MTX AND MTX POLYGLUTAMATES

Freshly harvested conidiospores (20-25 mg dry weight) were preincubated in 10 mL of sterile Vogel's medium for 14 hours with shaking in a 30°C water bath. Subsequently, 500 μL inocula were removed and transferred to sterile liquid shake cultures containing final concentrations of 0.03, or 1.00 μM [^{3}H]-MTX and defined media to achieve a total volume of 1.0 mL. These cultures were incubated for a further 24 hours at 30°C. Following the aspiration of the media, the cultures were each resuspended in 1.0 mL of sterile defined media for 15 minutes to remove exogenous [^{3}H]-MTX. MTX efflux was initiated by transfer of the cultures to 30 mL of Vogel's medium in 125 mL Erlenmeyer flasks under sterile conditions, following removal of the wash media. Subsequently, the cultures were incubated for a further 24 hours in a shaking 30°C water bath. After this 24-hour efflux, *Neurospora* mycelia were har-

vested and the protocol of Jolivet and Schilsky (1981) was modified to permit the extraction of unconjugated and conjugated MTX from sample extracts.

The medium was aspirated from each of the flasks and the mycelia were washed twice with 30 mL aliquots of ice-cold phosphate-buffered normal saline (PBS) pH 7.4. The mycelia (*ca.* 1 g fresh weight) were then weighed and homogenized in a mortar with an equal weight of acid-washed sand, and 1.0 mL of ice-cold PBS and 4.0 mL of ice-cold 10% w/v trichloroacetic acid (TCA). Aliquots (1 mL) of the homogenate were transferred into 1.5 mL Eppendorf tubes and subsequently centrifuged in an Eppendorf bench-top Microcentrifuge at 14,000 rpm for 10 minutes. The clarified homogenates were filtered through Millipore (Bedford, MA., USA) Millex-GS syringe adaptable filter units and then injected onto Waters (Milford, Ma., USA) Sep-Pak C_{18} cartridges. To perform reverse phase (C_{18}) chromatography with the Sep-Pak cartridges, a series of solvents of decreasing polarity was utilized. The cartridges were conditioned with 6.0 mL of 100% acetonitrile (ACN) followed by 6.0 mL of filtered milli-Q water (Millipore). The *Neurospora* extracts, injected onto these pretreated cartridges, were removed by flushing the cartridges with 3.0 mL of milli-Q water. MTX and its metabolites were retained by the cartridge packing material, and 2.0 mL of 100% ACN were required to elute these components. The labeled samples were evaporated to dryness under argon, to remove all traces of ACN, and then redissolved in 350 µL of mobile phase buffer. The resuspended samples were placed in Ultrafree-MC microcentrifuge filters (Millipore) and subsequently centrifuged for 2 minutes at 14,000 rpm in an Eppendorf Microcentrifuge to remove any particulate matter exceeding 0.22 µm. Each filtrate was then analyzed by HPLC for MTX and $MTXGlu_n$.

2.9. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC analyses of cell extracts were performed using reverse phase chromatography (Jolivet and Schilsky, 1981). A Varian (Walnut Creek, CA, USA) chromatographic system equipped with a 3.9 mm x 30 cm steel µBondapak C_{18} column (Waters Associates) and a Rheodyne injector was used for MTX and $MTXGlu_n$ separations. The initial buffer was reduced from 30% v/v ACN in 5 mM tetrabutylammonium dihydrogen phosphate (Pic A) to 15% v/v ACN in 5 mM Pic A to permit resolution of the monoglutamate and diglutamate MTX standards. A third buffer, consisting of 40% v/v ACN in 5 mM Pic A, allowed separation of higher polyglutamate chain lengths. The HPLC program included an initial 6 minute isocratic wash with the mobile phase buffer (15% ACN/5 mM Pic A) followed by the first linear gradient from

15% to 30% ACN in 5 mM Pic A for 30 minutes then a second linear gradient from 30% to 40% ACN in 5 mM Pic A for another 30 minutes. Subsequently, a 20 minute isocratic wash of 40% ACN in 5 mM Pic A was employed and finally, the column was re-equilibrated with 15% ACN in 5 mM Pic A for 15 minutes. The chromatographic program was run at a flow rate of 1 mL min^{-1} at ambient temperature (25°C) following a 100 µL sample injection. One minute fractions were collected directly into scintillation vials using a Redifrac fraction collector (Pharmacia) and assayed for radioactivity by liquid scintillation counting.

Prior to sample chromatography, aliquots of chemically synthesized $MTXGlu_n$ were prepared to permit assignment of their elution positions by monitoring ultraviolet (UV) absorbance at 254 nm on a Varian UV-50 detector. 1 mM solutions of $MTXGlu_{1-4}$ were prepared and each was injected onto a conditioned Sep-Pak C_{18} cartridge. Each cartridge was then washed with 3.0 mL of milli-Q water followed by 2.0 mL of 100% ACN to elute the $MTXGlu_{1-4}$ standards. The samples were then evaporated to dryness under argon and redissolved in 350 µL of mobile phase buffer (15% ACN/5 mM Pic A). Particulate matter was removed by centrifugation using Ultrafree-MC microcentrifuge filters prior to injection onto the column. A mixture of the four standards, 14.29 nmol of each, was prepared and injected to obtain the elution profile.

2.10. DETERMINATION OF PROTEIN-BOUND VERSUS FREE [^{3}H]-MTX

Freshly harvested conidiospores (20-25 mg dry weight) were preincubated in 10 mL of sterile Vogel's medium for 14 hours in a 30°C shaking water bath. 500 µL inocula were removed and transferred to sterile shake cultures containing final concentrations of 0.03 or 1.00 µM [^{3}H]-MTX and defined media to achieve a total volume of 1.0 mL. These cultures were incubated for a further 24 hours at 30°C. Following the aspiration of the media, the cultures were each resuspended in 1.0 mL of sterile Vogel's medium for 15 minutes to remove any [^{3}H]-MTX adsorbed to the hyphal surface. MTX efflux was initiated with the transfer of these cultures to 30 mL of Vogel's medium in 125 mL Erlenmeyer flasks under sterile conditions, following removal of the wash media. The cultures were then incubated for a further 24 hours in a shaking 30°C water bath. After the 24 hour efflux, mycelia were harvested and the procedure described by McGuire et al (1989) was modified to permit the isolation of the enzyme-ligand complex.

The medium was aspirated from each of the flasks and the mycelia were washed twice with 30 mL aliquots of ice-cold double-distilled water. The mycelia were weighed and homogenized in a mortar with Buffer A at a 1:2 ratio (grams of tissue : mL of Buffer A) and an equal weight of acid-washed sand. The homogenates were collected and 1.0 mL aliquots were transferred to 1.5 mL Eppendorf tubes and centrifuged at 14,000 rpm for 10 minutes in an Eppendorf Microcentrifuge. Total intracellular label was estimated by mixing 500 µL of the supernatant extract with 4.0 mL of ACS scintillation fluor and quantitating in a liquid scintillation counter. Protein-bound radiolabel was determined using disposable PD-10 columns (Pharmacia) containing Sephadex G-25 to separate bound from free drug. Prior to sample application, the gel was equilibrated with 25 mL of Buffer A. A 500 µL aliquot of each supernatant fraction (see above) was layered evenly onto the gel bed and allowed to penetrate, this was followed by a 2.0 mL wash with Buffer A. The eluent was collected directly into scintillation vials. Elution of protein-bound [^{3}H]-MTX was achieved by a further 3.5 mL wash of Buffer A, collected directly into scintillation vials. Fractions collected from each PD-10 column were quantified, as described above.

Initial experiments indicated that this procedure permitted the isolation of the DHFR-[^{3}H]-MTX complex, which passes through the minicolumn in a small volume, from the free [^{3}H]-MTX which is retained completely by the column. Accordingly, a 500 µL aliquot of purified chicken liver DHFR (0.35 mg/mL, 3.7 units/mg) was applied onto a conditioned PD-10 column. The volume required to elute the protein (Ve) was calculated by measuring the absorbance at 280 nm. Consequently, the Ve for DHFR was determined to be 5.5 mL, that is 3.5 mL after an initial 2.0 mL wash following a 500 µL sample application.

2.11. MEASUREMENT OF RADIOACTIVITY

Tritiated samples were quantified by liquid scintillation counting following the addition of 4.0 mL of ACS scintillation fluor to each sample. A Beckman LS 6000TA scintillation spectrometer was routinely utilized to measure the disintegrations per minute (DPMs) of each sample.

2.12. PURIFICATION OF DIHYDROFOLATE REDUCTASE

2.12.1. Preparation of Dihydrofolate Substrate

Dihydrofolic acid was prepared according to the method of Futterman (1957). Folic acid (16 mg) was dissolved in 2 mL of 0.1N NaOH and then added to 5 mL of 10% w/v sodium ascorbate (pH 6.0). Next, 222 mg of sodium dithionite were added slowly with stirring which continued for an additional 30 minutes. The pH of the solution was then reduced to pH 3.0 by the dropwise addition of HCl, with stirring, in an ice bath. The solution was stirred for an additional 5 minutes after which the precipitate (H_2PteGlu) was removed by bench-top centrifugation at 3000 rpm for 4 minutes. The final H_2PteGlu product was recovered by repeated (10 x) resuspension in HCl containing 200 mM 2-mercaptoethanol followed by centrifugation, as before. H_2PteGlu was resuspended in 2 mL of 1 mM 2-mercaptoethanol and 2 drops of 1N KOH and stored as 250 µL aliquots at -20°C.

The concentration of H_2PteGlu was measured in the presence of excess DHFR using an NADPH extinction coefficient of 6.22 mM at 340 nm. The assay involved the stepwise addition of 100 µL of 0.8 M KH_2PO_4 buffer (pH 7.5), 100 µL of 100 mM 2-mercaptoethanol, 50 µL of 25-fold diluted DHFR (0.35 mg/mL, 3.7 units/mg) purified from chicken liver (Sigma), 10 µL of freshly prepared H_2PteGlu and 590 µL of double-distilled water. The reaction was initiated by addition of 100 µL of 1 mM NADPH + H^+. An equal volume of double-distilled water was added to the reference cuvette. The yield of H_2PteGlu was *ca.* 4.58 µmoles or 1.5274 mM as calculated from the total decrease in absorbancy due to the utilization of H_2PteGlu and NADPH + H^+ to produce H_4PteGlu and $NADP^+$ respectively.

2.12.2. Measurement of DHFR Activity

The DHFR assay protocol developed by Mathews, Scrimgeour and Huennekens (1963) was modified whereby, enzyme activity could be measured spectrophotometrically at 340 nm. The standard assay was performed in 100 µL of 0.8 M KH_2PO_4 buffer (pH 7.5), with 100 µL of 0.1 M 2-mercaptoethanol, 25 µL of 1.5 mM dihydrofolate and 100 µL of 1 mM NADPH + H^+, in a final volume of 1.0 mL at 30°C. The reference cuvette contained all of the components except NADPH + H^+, which was replaced by an equal volume of double-distilled water. The reactions were initiated with the addition of varying aliquots of enzyme extracts. Changes in absor-

bance (A_{340}) were recorded for a total period of five minutes. DHFR activity causes a decrease in absorbance at 340 nm due to the oxidization of NADPH + H^+ to $NADP^+$ as $H_2PteGlu$ is reduced to $H_4PteGlu$. One unit of activity is therefore defined as the amount of enzyme required to reduce 1 μmole of dihydrofolate per mL per minute based on an extinction coefficient of 6.55 at pH 6.4.

The efficacy of the assay reagents was investigated by consecutively excluding them while continuing to monitor the change in absorbance as depicted in Figure 4. No decrease in absorbancy was detected when either DHFR, $H_2PteGlu$ or NADPH + H^+ were omitted from the assay mixture. This finding demonstrated that all of the reagents, at their specified concentrations were necessary for the reaction to proceed.

2.12.3. Preparation of Cell Free Extracts

Freshly harvested conidiospores of *N. crassa*, having an optical density of 0.350 to 0.400 at 350 nm, were inoculated into 3.0 L of sterile defined medium. These cultures were aerated with an aseptic central inlet tube under sterile conditions. After 72 hours of growth at 30°C in a water bath, *Neurospora* mycelia were harvested by vacuum filtration through Whatman No. 1 filter paper. Only cultures yielding clear filtrates (which suggests no microbial contamination) were processed further. Harvested mycelial pads were extensively washed with double-distilled water. Homogenate preparation and all protein purification steps were performed at 4°C.

The filtered mycelial pads were weighed and mixed with an equal weight of acid-washed sand in a mortar. The mixture was homogenized in 50 mM K_2HPO_4 buffer (pH 7.0) containing 20% v/v glycerol, 10 mM 2-mercaptoethanol, 1 mM EDTA and 1mM PMSF (Buffer A) using a 1:2 ratio (grams of tissue : mL of Buffer A) with a pestle until a smooth paste was obtained (Sebald et al, 1979). The homogenate was then filtered through four layers of cheesecloth prior to centrifugation at 5000 x g for 10 minutes.

2.12.4. Fractionation of DHFR Activity

The clarified homogenate (Step 1) was treated with streptomycin sulfate, to give a final concentration of 1% v/v. This reagent was added slowly over one hour and the homogenate was stirred for one additional hour. Nucleic acids were removed by centrifugation at 7800 x g for 10 minutes. The resultant streptomycin sulfate supernatant (Step 2) was fractionated using ammonium sulfate. Initial studies revealed

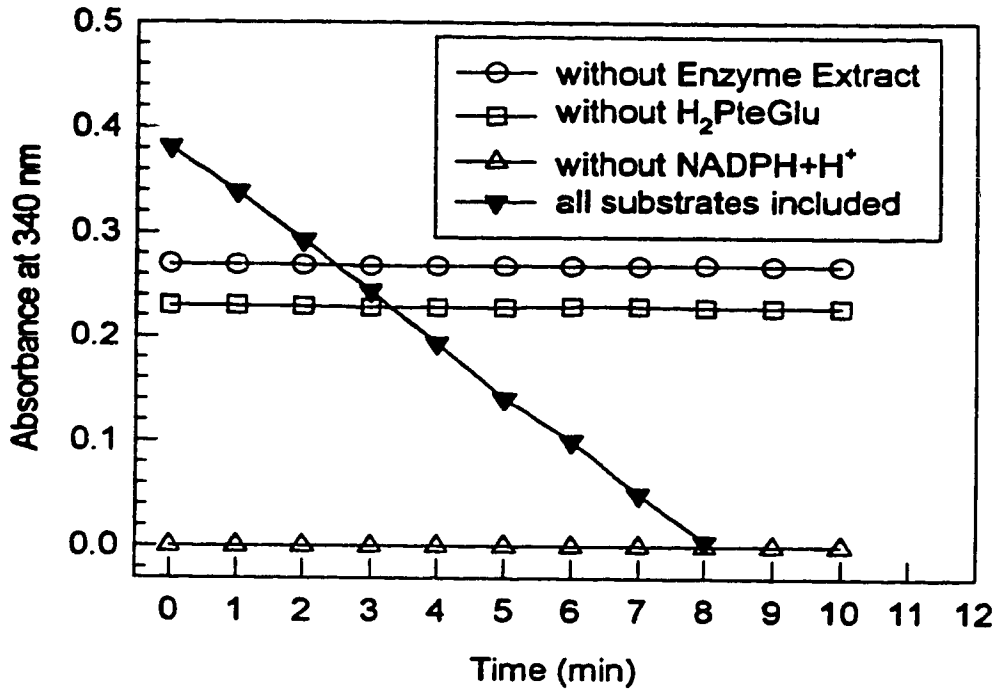


Figure 4. Assay of DHFR Activity. Aliquots of Step 3 protein were examined. Complete reaction systems (▼) and those lacking the indicated component, were assayed under standard conditions as described in Section **2.12.2.**

that DHFR activity was recovered at 50-80% of saturation with ammonium sulfate. Accordingly, the first precipitate (0-50% range of saturation), recovered after one and one-half hours of stirring, was removed by centrifugation at 7800 x g for 10 minutes and discarded. Further additions of ammonium sulfate were made in order to recover protein (50-80% of saturation) from the supernatant. The suspension was stirred for another one and one-half hours and protein containing DHFR activity was recovered after centrifugation at 7800 x g for 10 minutes.

The DHFR-containing pellets (Step 3) were redissolved in a minimal volume (*ca.* 4 mL) of Buffer A and pumped onto a 2.5 x 70 cm column of Sephadex G-75 previously equilibrated with Buffer A. Fractions (6 mL) were collected at a flow rate of 20 mL hr^{-1}. The DHFR-active fractions, containing at least 40% of peak activity, were pooled and concentrated using an Amicon 200 mL stirred ultrafiltration cell. Protein concentration was achieved under pressure (10 psi) from an argon cylinder. The membrane inserted into the stirred cell excluded samples with molecular weights greater than 10 kD hence the enzyme of interest was retained and concentrated. Determinations of apparent native molecular weight were based on measurements of the volumes required for elution (Ve) of molecular weight marker proteins from a 2.5 x 70 cm Sephadex G-75 column. Fractions of 2 mL were collected at a flow rate of 20 mL hr^{-1} and elution positions ascertained by measuring absorbance at 280 nm. The marker proteins included bovine serum albumin (66,000), bovine erythrocyte carbonic anhydrase (29,000), horse heart cytochrome *c* (12,400) and bovine lung aprotinin (6,500). The elution positions of DHFR was determined by the standard enzyme assay rather than protein absorbance at 280 nm.

Further purification of DHFR was achieved by applying the Sephadex G-75 concentrate (Step 4) onto a 1.5 x 10 cm column of Matrex Green A equilibrated with *ca.* 60 mL of Buffer A. DHFR activity was eluted with a 0.20-0.40 M KCl linear gradient in Buffer A following removal of unbound protein by washing the column with 50 mL of Buffer A. Fractions of 3 mL were collected at a flow rate of 42 mL hr^{-1}. Those DHFR-active fractions containing at least 40% of peak activity were combined and placed in an Amicon 200 mL stirred ultrafiltration cell, as before, to yield a concentrated protein (Step 5).

2.12.5. IC_{50} Determination

The concentration of MTX and $MTXGlu_3$ necessary to inhibit dihydrofolate reductase activity by 50% (IC_{50}) was determined by a modification of the standard as-

say. Inhibitors of known concentration in assay buffer (0.8 M KH_2PO_4 buffer, pH 7.5) were prepared, and varying aliquots were added *in lieu* of an equal volume of double-distilled water. The DHFR sample, 4.64 units, from Step 4 was incubated with individual MTX congeners, 0.8 M KH_2PO_4 buffer (pH 7.5), 0.1 M 2-mercaptoethanol, 1.5 mM dihydrofolate, in a final volume of 1.0 mL for 5 minutes before the reaction was initiated with 1.0 mM NADPH + H^+. Interpretation of the percentage of enzyme activity versus inhibitor concentration in the reaction mixture were used to calculate the IC_{50}.

2.12.6. Determination of Protein Contents

Protein concentrations were determined using the Bio-Rad (Mississauga, Ontario) Protein Assay, based on the method of Bradford (1976). Protein was quantified colormetrically at 595 nm by the addition of an acidic dye. Known concentrations of bovine serum albumin were used to construct standard curves for macro- and microassays in this procedure.

2.12.7. SDS-Polyacrylamide Gel Electrophoresis

The discontinuous buffer systems proposed by Laemmli (1970) were employed. The polyacrylamide gels were run on a Bio-Rad mini-PROTEAN II slab cell, consisting of a 12% separating (lower) gel and a 4% stacking (upper) gel. Once the gels were cast, enzyme extracts which had been diluted with sample buffer containing sodium dodecyl sulfate (SDS) and heated to 95°C in a water bath for 10 minutes were loaded. Finally, electrode running buffer was added to the assembly and electrophoresis proceeded at 200 volts for *ca.* 45 minutes.

The molecular weight of DHFR was estimated using a low molecular weight protein kit (Bio-Rad) for electrophoresis, under denaturing conditions. The protein kit contained six proteins, namely rabbit muscle phosphorylase b (97,000 Da), bovine serum albumin (66,200 Da), hen egg white ovalbumin (45,000 Da), bovine carbonic anhydrase (31,000 Da), soybean trypsin inhibitor (21,500 Da) and hen egg white lysozyme (14,400 Da).

The protein bands were revealed by either Coomassie blue or silver stain following the Bio-Rad protocol. The apparent molecular weight of DHFR was estimated graphically from its electrophoretic migration as compared to those demonstrated by the protein standards.

RESULTS

3.1. *NEUROSPORA CRASSA* GROWTH INHIBITION

In the present study, the effectiveness and selectivity of MTX towards wild type, *met*-6 and *mac* strains of *N. crassa* was examined to confirm the inhibitory activity of this antifolate on the growth of these cultures. Dose-response relationships calculated as percentages of untreated controls revealed greater MTX sensitivity in *met*-6 and *mac* mutants than wild type as depicted in Figure 5. The IC_{50} values for wild type, *met*-6 and *mac* were 87.5 μM, 5.5 μM and 6.0 μM, respectively. A significant difference, derived from a population parameter of 'n-1' (standard deviation), between the mutant strains and wild type was established. The statistical variance between the IC_{50} values of the two mutant strains was insignificant. The lack of complete growth inhibition observed in these experiments, may be due to dihydrofolate pools which are sufficient to compete with MTX for DHFR. Additionally, high levels of DHFR activity may provide a plausible explanation for the inability of MTX to completely inhibit fungal growth (Fry et al, 1982; White, 1974). Furthermore, the greater resistance of wild type may be attributed to either a higher conidiospore folate concentration (Chan and Cossins, 1984) or increased MTX-binding proteins other than DHFR accompanied by a reduction in the affinity of DHFR for MTX binding (Barg et al, 1984; Barg et al, 1987).

The ability of exogenous methionine to enhance the accumulation of folates thereby impairing the MTX glutamylation has been investigated in mammalian cells (Matthews, 1984; Rhee et al, 1989). To examine this possibility, amino acid supplements, L-methionine and glycine, were included in the MTX treatments. The results suggest, as depicted in Figure 6, that the inclusion of L-methionine significantly reduces the MTX IC_{50} value from 87.5 μM to 33.0 μM in the wild type strain. Whereas glycine significantly increases the concentration of MTX required to achieve growth inhibition. In this regard, 100 μM MTX only inhibits wild type growth by *ca.* 12%. The pronounced effect of L-methionine could conceivably result from decreased rates of *de novo* folate synthesis (Chan and Cossins, 1980; Rhee et al, 1989) or by increasing folate turnover and thereby affecting the distribution of the polyglutamate pool (Chan and Cossins, 1980; Rhee et al, 1989). Glycine increases carbon flow and therefore stimulates folate synthesis, hence growth in glycine-supplemented media is per

Figure 5. Methotrexate Inhibition of *N. crassa* Growth. Resuspended conidiospores (1.0 mL) were inoculated into 29 mL of Vogel's medium, under sterile conditions. Appropriate aliquots of MTX were added to each culture to attain the desired concentration in 30 mL. Mycelia were harvested after 24 hours of shake culture at 30°C (see Materials and Methods Sections **2.3.** and **2.3.1.**). The IC_{50} values for each strain are means derived from six separate cultures. Error bars were computed from the standard error. Where not shown, the error bars fall within the symbol size.

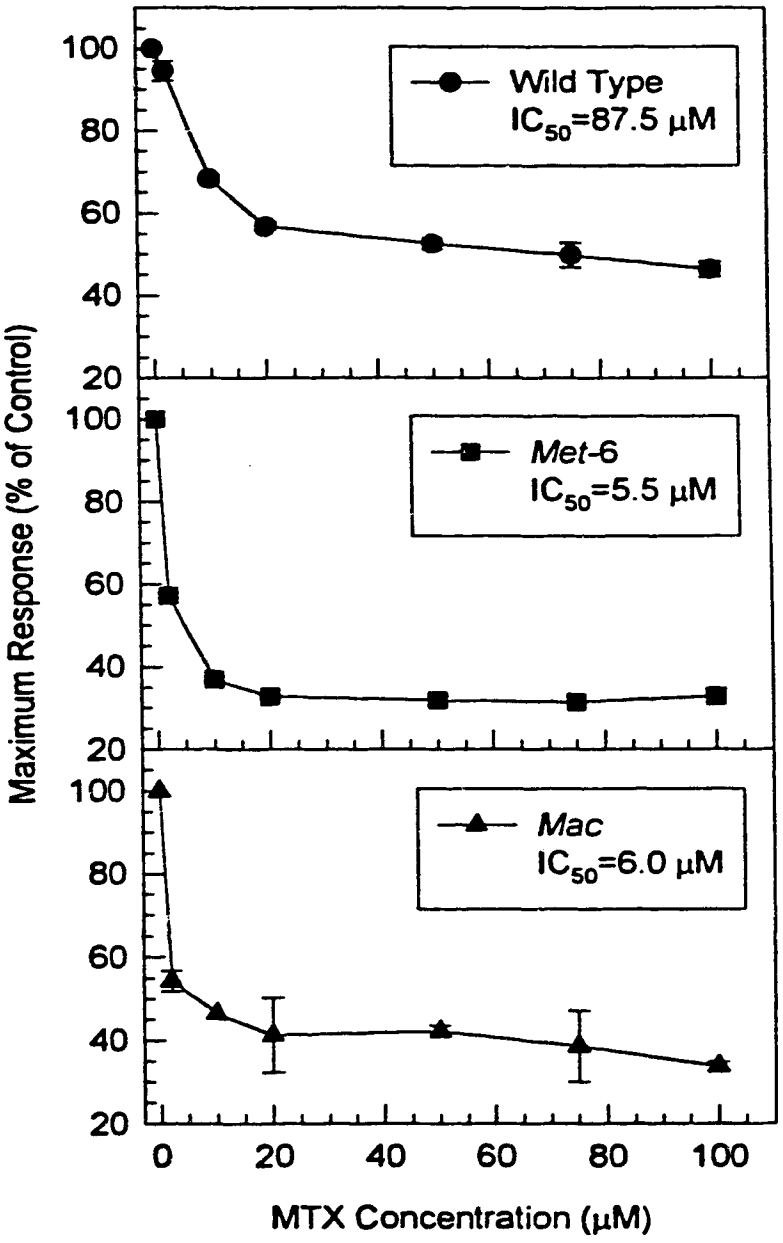
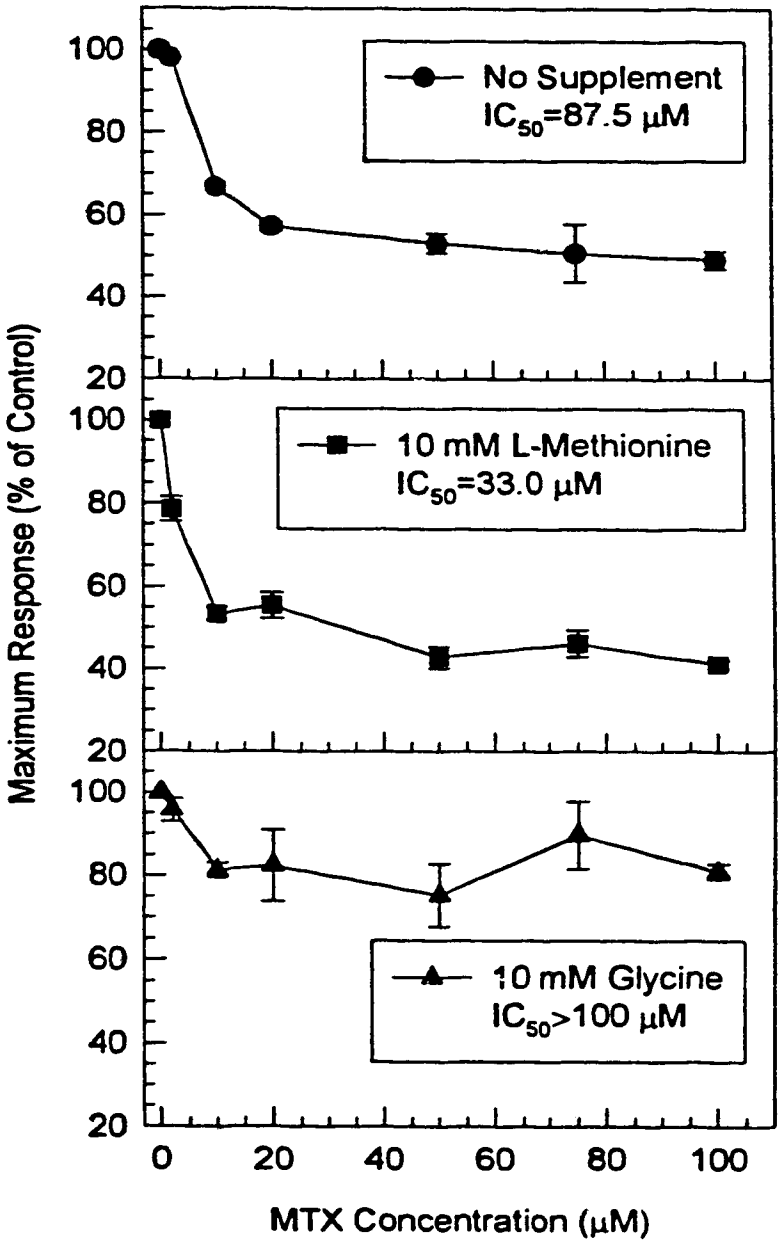

Wild Type
IC50=87.5 μM
Met-6
IC50=5.5 μM
Mac
IC50=6.0 μM
Maximum Response (% of Control)
MTX Concentration (μM)
100
80
60
40
20
0
20
40
60
80
100

Figure 6. Methotrexate Inhibition of Wild Type *N. crassa* Growth: The Effect of Methionine and Glycine Supplementations. The standard culture conditions, described in Materials and Methods Section **2.3.**, were followed but the defined medium contained no supplementation, 10 mM L-methionine or 10 mM glycine respectively. Appropriate aliquots of MTX were added to each culture to attain the desired concentration in 30 mL. Mycelia were harvested after 24 hours of shake culture at 30°C (see Materials and Methods Sections **2.3.** and **2.3.1.**). The IC_{50} values are the means derived from four cultures. Error bars were calculated from the standard error. Where not visible, the error bars fall within the symbol size.


No Supplement
IC50=87.5 μM
10 mM L-Methionine
IC50=33.0 μM
10 mM Glycine
IC50>100 μM
Maximum Response (% of Control)
MTX Concentration (μM)
100
80
60
40
20
0
20
40
60
80
100

haps related to the general stimulatory effect of this amino acid on folate biosynthesis presumably involving at least one reaction in C_1 metabolism (Cha[illegible] and Cossins, 1980).

Sulfonamides, such as sulfanilamide, competitively inhibit bacterial synthesis of H_4PteGlu at the *p*ABA incorporation step, thereby blocking the $H_4PteGlu_n$-requiring reactions. To elucidate sulfanilamide action in a fungal species, this antimetabolite was included in the culture conditions. *Neurospora*, like microorganisms which synthesize folates *de novo*, is sensitive to sulfanilamide, as illustrated in Figure 7. The inhibitory effects of sulfanilamide on *Neurospora* growth are a result of interference with folic acid synthesis from *p*ABA and other precursors (Shiota, 1984). The formation of reduced folate derivatives, 5-HCO-H_4PteGlu and 10-HCO-H_4PteGlu, was impaired in sulfanilamide-inhibited organisms (Shiota, 1984). In this regard, MTX inhibition of wild type growth during concurrent sulfanilamide treatment (see Figure 8) demonstrated that the MTX IC_{50} value significantly decreased from 87.5 μM to 1.25 μM, when 0.10 mM sulfanilamide was incorporated into the culture conditions, which is consistent with impaired formation of reduced folate derivatives. Sulfanilamide enhances MTX cytotoxicity through sustained suppression of DHFR activity because high levels of H_2PteGlu cannot accumulate; and the ability of MTX to attain a higher degree of polyglutamylation as a direct consequence of the reduction in the levels of preferred reduced folate cofactors.

3.2. RESCUE OF GROWTH BY FOLINIC ACID

Folinic acid reverses the cytotoxic effects of antifolates, such as MTX, which inhibit DHFR (Matherly et al, 1987). Clinically, rescue following MTX treatment involves the provision of a source of reduced folate cofactors in the form of 5-HCO-H_4PteGlu. The classical view of folinic acid rescue was that exogenous 5-HCO-H_4PteGlu, by providing sufficient cofactor to sustain biosynthetic processes at a normal rate, circumvents the block in *de novo* H_4PteGlu biosynthesis at DHFR in susceptible tissues (Nixon and Bertino, 1971; Stover and Schirch, 1993). In contrast to 5-HCO-H_4PteGlu, PteGlu fails to afford protection against MTX effects (Mead et al, 1963; Jolivet et al, 1987). This may be explain why reduced folates are better substrates for DHFR and FPGS than folic acid and MTX. As a result, $MTXGlu_n$ synthesis *in vitro* is decreased when PteGlu and MTX are coincubated (Jolivet et al, 1987).

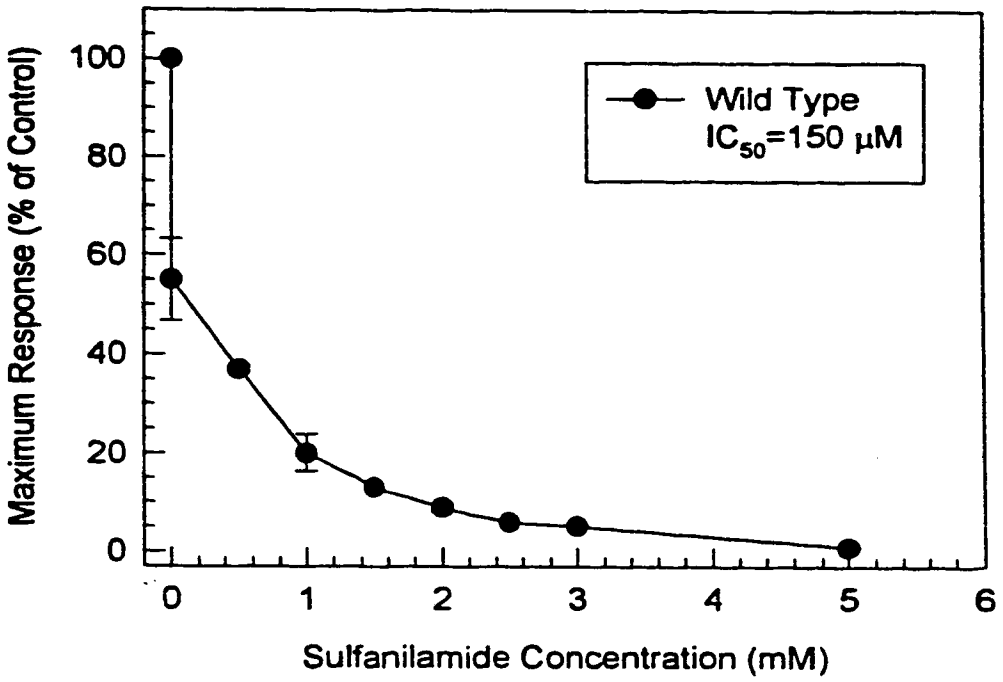
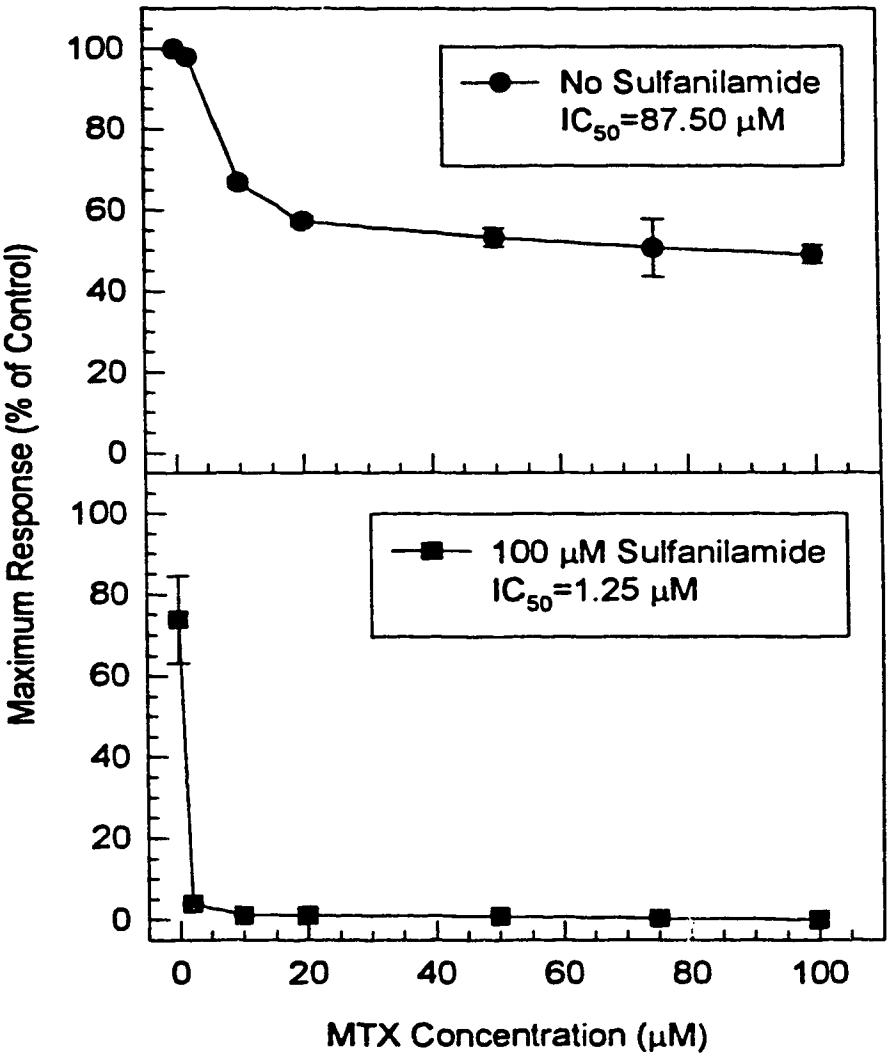


Figure 7. Sulfanilamide Inhibition of Wild Type *N. crassa* Growth. Resuspended conidia, as in Materials and Methods Section 2.3., were inoculated into 30 mL of Vogel's medium containing varying amounts of sulfanilamide. The cultures were incubated under the standard conditions described in Materials and Methods Section 2.3. The IC_{50} value is the mean of four cultures. Error bars were calculated from the standard error. Where no shown, the error bars fall within the symbol size.

Figure 8. Methotrexate Inhibition of Wild Type *N. crassa* Growth During Concurrent Sulfanilamide Treatment. The concentration of sulfanilamide, which resulted in a 50% inhibition of growth (100 μM), was included in the Vogel's medium. Resuspended conidia (1.0 mL) were added to this medium in addition to aliquots of methotrexate necessary to attain the desired concentrations in 30 mL. The incubation and harvest conditions were identical to those used in previous experiments (see Materials and Methods Section 2.3. and 2.3.1.). The IC_{50} is the mean value calculated from four separate cultures. The error bars were derived from the standard error. Where not shown, the error bars fall within the symbol size.


No Sulfanilamide
IC50=87.50 μM
100 μM Sulfanilamide
IC50=1.25 μM
Maximum Response (% of Control)
MTX Concentration (μM)
0
20
40
60
80
100

The important ramifications of folinic acid rescue in mammalian systems have been well documented (Mead et al, 1963; Dudman et al, 1982; Matherly et al, 1983; Galivan and Nimec, 1983; Matherly et al, 1984; Matherly et al, 1986; Allegra and Boarman, 1990), but there is little information on this topic for fungi. The effect of 5-HCO-H_4PteGlu following MTX treatment was therefore examined in this study. Varying concentrations of folinic acid were added to mycelia pre-treated with 20 μM MTX, which produced a *ca.* 30% inhibition of growth in all three strains, resulting in growth recovery (see Figure 9). A more pronounced growth recovery; 19% and 22% response occurred in *met*-6 and *mac* mutants, respectively, with 50 μM folinic acid. Whereas growth recovery was only measured as an 8% response at the same concentration in wild type.

In wild type, unlike *met*-6 and *mac*, an active polyglutamylation of MTX may affect the subsequent growth rate during folinic acid rescue. While polyglutamylation may provide some selectivity, even animal cells that extensively synthesize $MTXGlu_n$ can be rescued, most probably through the generation of H_2PteGlu (Allegra and Boarman, 1990). The data suggests that H_2PteGlu can compete less effectively with $MTXGlu_n$ than with MTX for DHFR under cellular conditions. Additionally, cellular energy metabolism may be implicated in 5-HCO-H_4PteGlu inducing the loss of MTX from DHFR (Matherly et al, 1984). Well oxygenated cells, like those in the present study, have lower levels of reduced pyridine nucleotides presenting the most favorable conditions for the loss of MTX from its target enzyme (Matherly et al, 1984).

3.3. RECOVERY OF MYCELIAL GROWTH FOLLOWING MTX TREATMENTS

Several approaches were used in this study to determine whether $MTXGlu_n$ play a significant role in MTX toxicity. Due to the limited capacity of each strain to conjugate long chain polyglutamate derivatives (data presented later in Table 1), a 24-hour exposure to 20 μM MTX followed by removal of MTX from the medium was implemented to provide an optimal environment for MTX polyglutamylation (see Figure 10). If $MTXGlu_n$ derivatives were present in sufficient quantities in wild type, this would result in less recovery of growth than observed in the mutants. However, the data did not support this hypothesis. Rather, MTX-treated wild type recovered to 80% of untreated controls whereas *met*-6 and *mac* only recovered to 20% and 42%, respectively, of untreated controls. This observation may be attributed to the higher

Figure 9. Folinic Acid Rescue of *N. crassa* Growth Following MTX Exposure. Aliquots (1.0 mL) of resuspended conidia were inoculated, under sterile conditions, into Vogel's medium followed by an aliquot of MTX to give a final concentration of 20 μM in 30 mL. The cultures were then incubated at 30°C for 24 hours, after which time the media were removed and each culture was washed and transferred to MTX-free Vogel's medium. Aliquots of folinic acid were then added to attain the desired concentrations in a final volume of 30 mL. The cultures were incubated for a further 24 hours at 30°C (see Materials and Methods Section **2.4.**). Each data point, calculated as percentages of untreated controls, is based on four cultures of each strain. Error bars were computed from the standard error. Where not visible, the error bars fall within the symbol size.

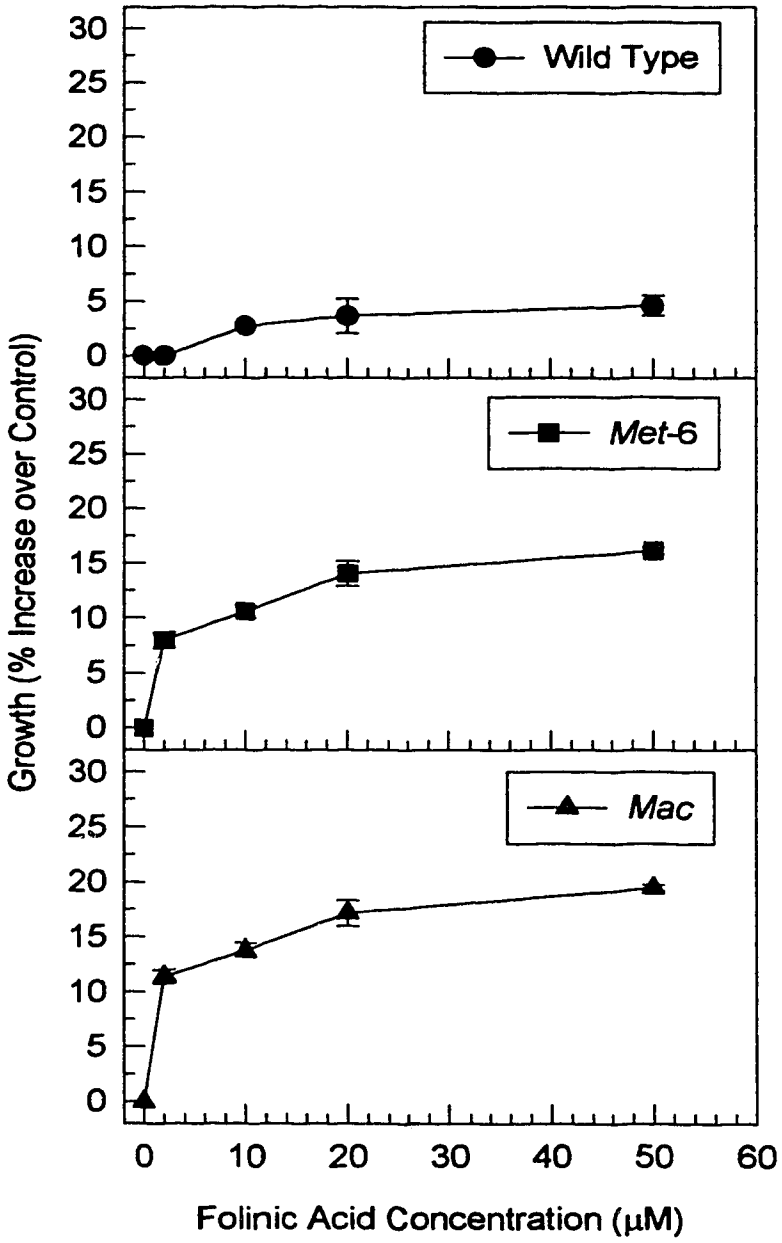
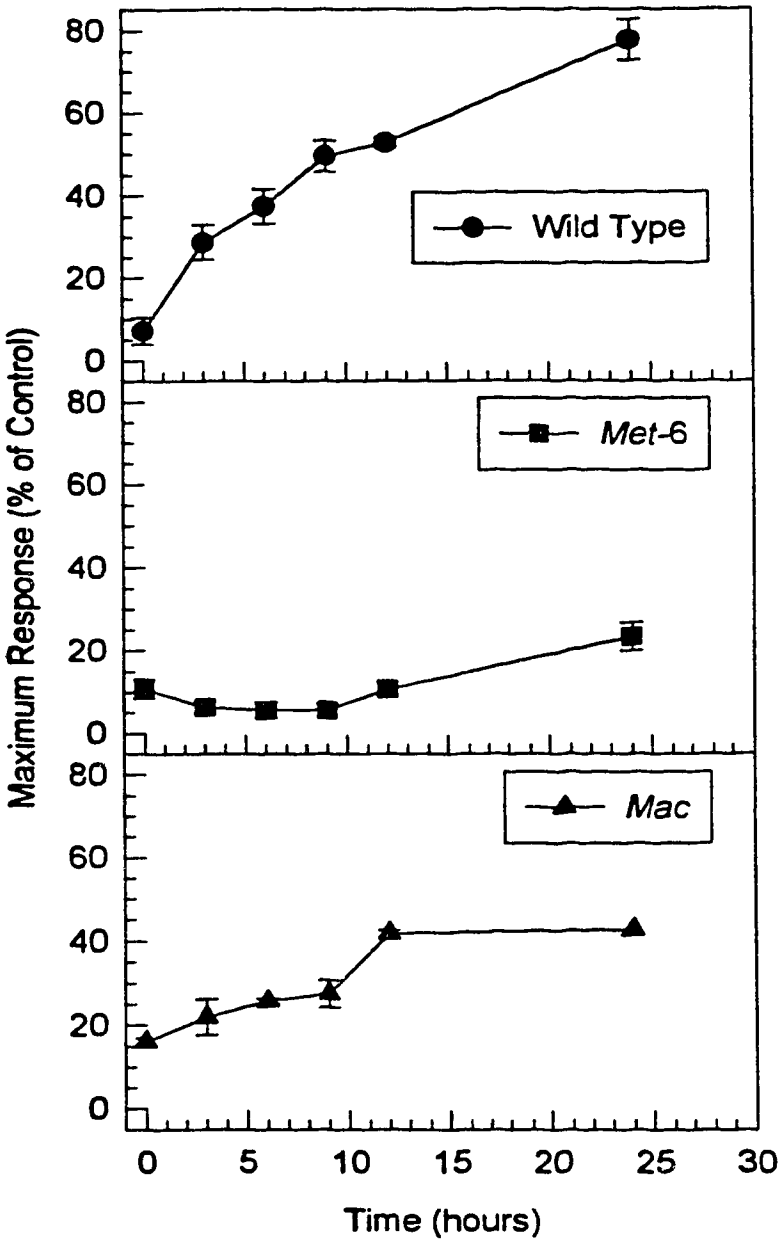

Wild Type
Met-6
Mac
Growth (% Increase over Control)
Folinic Acid Concentration (μM)
0
5
10
15
20
25
30
40
50
60

Figure 10. Recovery of Mycelial Growth Following 24 Hour MTX Treatments. Aliquots (1.0 mL) of freshly harvested conidia were added to Vogel's medium, under sterile conditions. An aliquot of MTX was then added to the cultures to give a final concentration of 20 μM in 30 mL. The cultures were then incubated at 30°C for 24 hours. To initiate efflux of the antifolate, the media were removed and each culture was washed and transferred to 29 mL samples of MTX-free Vogel's medium. The cultures were then incubated at 30°C for 3, 6, 9, 12 and 24 hours respectively. After MTX efflux for the indicated times, mycelia were harvested (see Materials and Methods Section **2.5.**). Data are expressed as percentages of untreated controls and are based on four cultures of each strain. Error bars were derived from the standard error. Where not shown, the error bars fall within the symbol size.


Maximum Response (% of Control)
80
60
40
20
0
Wild Type
Met-6
Mac
0
5
10
15
20
25
30
Time (hours)

initial levels of intracellular folate in wild type conidiospores. (Chan and Cossins, 1984) which may provide an effective means of overcoming MTX inhibition. These endogenous substrates may also compete for DHFR and FPGS and thereby reduce the deleterious effects of MTX and its polyglutamate derivatives. The extracellular concentration of MTX may also have been inadequate to achieve an intracellular level which was in excess of that tightly bound to DHFR and thus would not become polyglutamylated by FPGS.

MTX is known to be more cytotoxic on confluent cells, thus conidiospores were incubated for 36 hours prior to a pulse MTX exposure. Wild type growth recovery after 24 hours in MTX-free media was measured as a 15% response at 20 μM MTX, and was much lower than recovery observed in the *met*-6 and *mac* mutants, 80% and 78%, respectively (see Figure 11). The data suggests that MTX inhibition may depend on the generation of polyglutamyl derivatives in actively dividing cells.

Time-dependent inhibition of cell growth was examined in biphasic incubations. In the first phase, pre-incubated conidiospores were incubated with MTX for either 6 or 24 hours, then mycelia were placed in drug-free medium that supported cell growth. Following the 24-hour incubation (see Figure 12) the growth recovery at 50 μM MTX, calculated as percentages of untreated controls, measured 72%, 50% and 62% for wild type, *met*-6 and *mac*, respectively. Following the 6-hour incubation (see Figure 13) the growth recovery at 50 μM MTX measured 80%, 70% and 62%, respectively. The data suggests enhanced formation of polyglutamate derivatives may be dependent upon the increased length of exposure to MTX. This is consistent with the proposal that cellular MTX accumulation and metabolism to $MTXGlu_n$ appear to be time-dependent in mammalian cells (Fry et al, 1982). The recovery in *mac* does not appear to change between the 6-hour and 24-hour incubation, the inability of this mutant to generate polyglutamate derivatives may provide a plausible explanation for this phenomenon.

3.4. THE FORMATION OF $MTXGLU_N$ DERIVATIVES

Several analytical techniques quantified polyglutamate derivatives of MTX, including DEAE cellulose chromatography (Gewirtz et al, 1980; Poser et al, 1981; Balinska et al, 1982) and Sephadex gel filtration (Rosenblatt et al, 1978; Whitehead et al, 1975; Galivan, 1979, 1980; Rosenblatt et al, 1981; Balinska et al, 1981) and

Figure 11. Recovery of Mycelial Growth After 6 Hour MTX Treatments. Aliquots (500 µL) of a conidial suspension were inoculated into Vogel's medium and incubated for 36 hours at 30°C. After this 36-hour preincubation, the media were removed and each culture was transferred to fresh Vogel's medium. Appropriate volumes of MTX, required to attain the desired concentrations in 30 mL, were added to these cultures, which were then incubated for 6 hours at 30°C. After the MTX exposure, the cultures were each washed with MTX-free Vogel's medium and efflux was initiated by transfer of the cultures to 30 mL of Vogel's medium. The cultures were then incubated for 24 hours at 30°C, subsequently the mycelia were harvested (see Materials and Methods Section 2.6.). Data for each strain are the mean values calculated from four incubations. Error bars were derived from the standard error. Where not visible, the error bars fall within the symbol size.

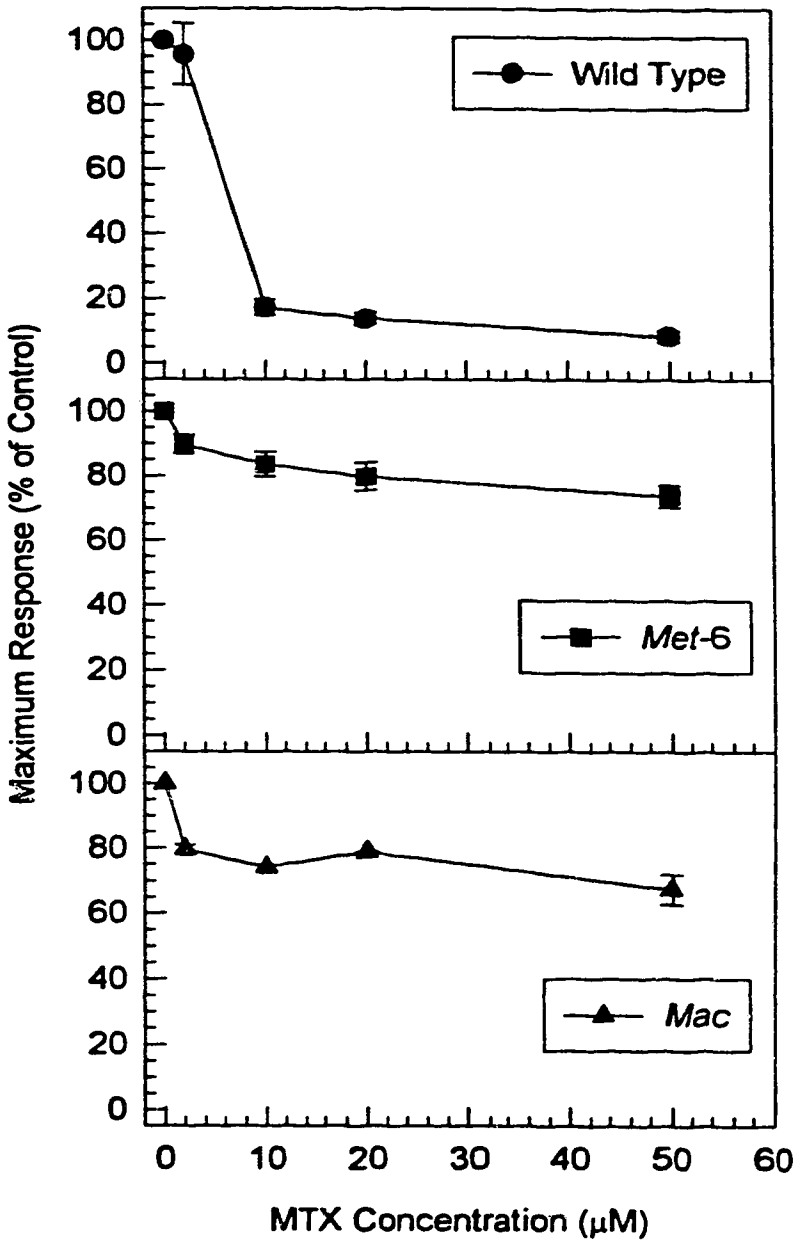

Maximum Response (% of Control)
100
80
60
40
20
0
Wild Type
Met-6
Mac
0
10
20
30
40
50
60
MTX Concentration (μM)

Figure 12. Mycelial Preincubation Followed by 24 Hour MTX Treatment and Growth Recovery. Resuspended conidia (500 μL) were inoculated into Vogel's medium and incubated for 14 hours at 30°C. Cultures were then transferred to Vogel's media containing various concentrations of MTX in a total volume of 30 mL. These cultures, were then incubated 24 hours at 30°C. After the MTX treatment, the cultures were each washed with MTX-free Vogel's media and efflux was initiated by transfer of the cultures to 30 mL of Vogel's media. Each culture was incubated for a further 24 hours at 30°C, after which the mycelia were harvested (see Materials and Methods Section 2.7.). Data are mean values obtained from nine separate cultures of each strain. Error bars were derived from the standard error. Where not shown, the error bars fall within the symbol size.

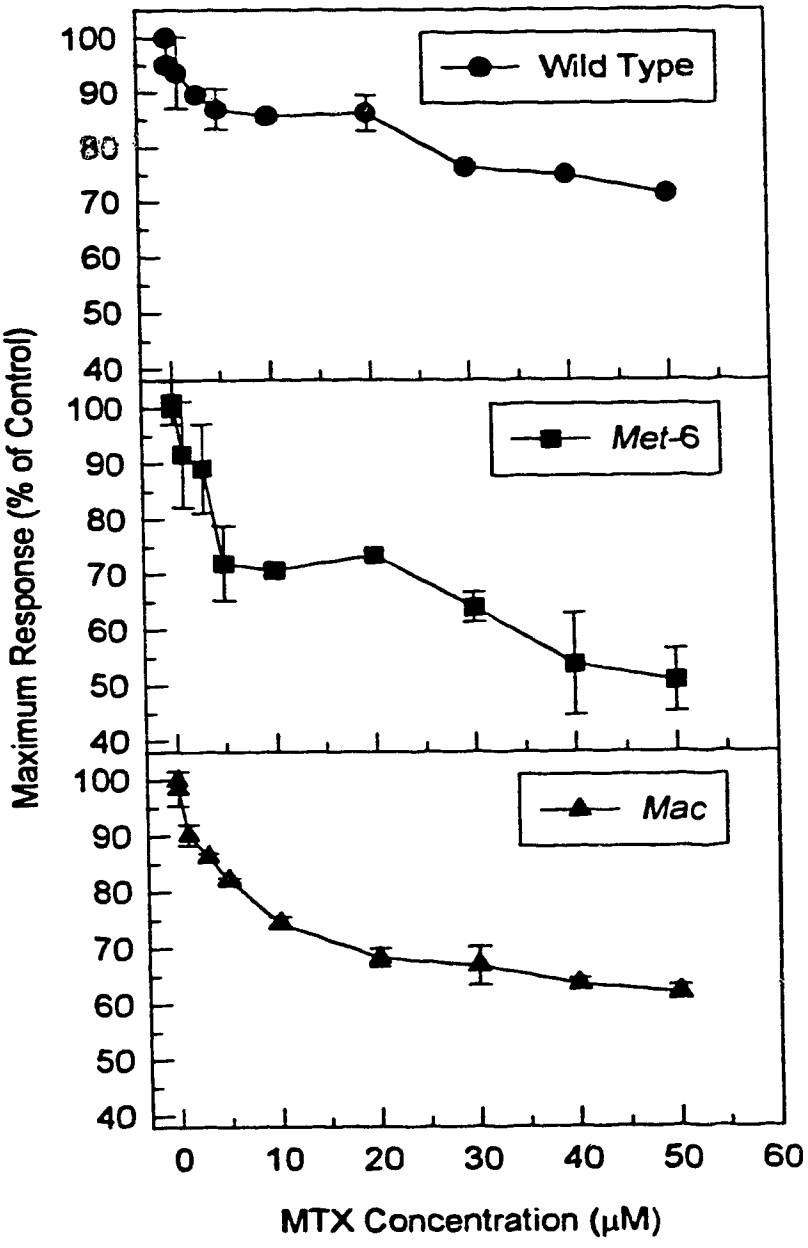
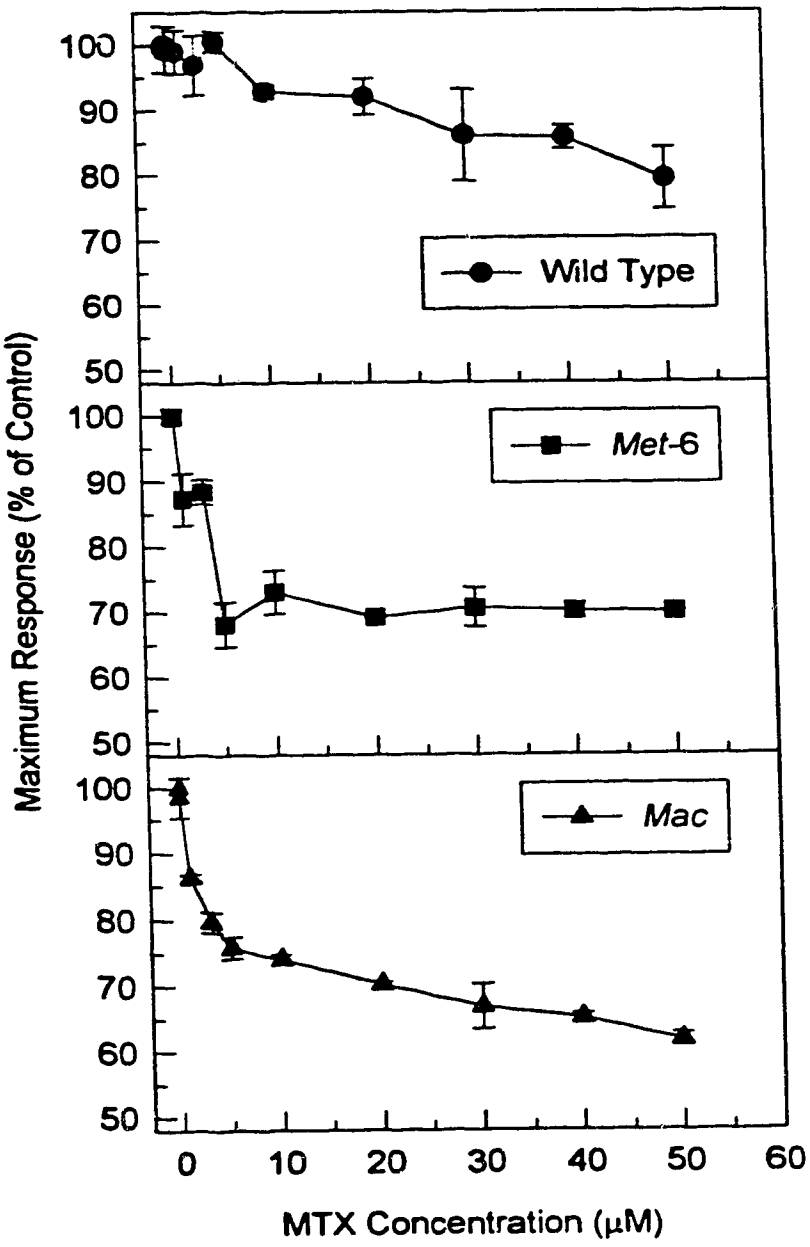

Wild Type
Met-6
Mac
Maximum Response (% of Control)
MTX Concentration (μM)
100
90
80
70
60
50
40
0
10
20
30
40
50
60

Figure 13. Mycelial Preincubation Followed by 6 Hour MTX Treatment and Growth Recovery. Resuspended conidia (500 μL) of were inoculated into Vogel's medium and incubated for 14 hours at 30°C. After which, cultures were transferred into Vogel's medium containing various concentrations of MTX in a total volume of 30 mL. These cultures, were then incubated for 6 hours at 30°C. After the MTX treatment, each culture was washed with MTX-free Vogel's medium and efflux was initiated by transfer of the cultures to 30 mL of Vogel's medium. Each culture was incubated for a further 24 hours at 30°C, after which the mycelia were harvested (see Materials and Methods Section 2.7.). Data for each strain are means of six separate experiments. Error bars were derived from the standard error. Where not visible, the error bars fall within the symbol size.


Maximum Response (% of Control)
100
90
80
70
60
50
Wild Type
Met-6
Mac
0
10
20
30
40
50
60
MTX Concentration (μM)

HPLC separations (Jolivet and Schilsky, 1981; Fry et al, 1982; Krakower et al, 1982; Jolivet et al, 1982; Nimec and Galivan, 1983; Fry et al, 1983; McGuire et al, 1985; Galivan et al, 1986; Winick et al, 1987). This highly sensitive methodology has achieved a resolution of one to seven polyglutamyl forms of MTX which contrasts with the results obtained with previous gel filtration or ion exchange methods where the $MTXGlu_n$ derivatives were only poorly resolved.

In an attempt to ascertain which HPLC method would provide superior detection of $MTXGlu_n$ in *N. crassa* extracts, several techniques were examined. Nimec and Galivan (1983) utilized an initial isocratic wash with 0.1 M acetate buffer (pH 5.5) containing 4% ACN for 10 minutes followed by a linear gradient from 4 to 13% ACN in 0.1 M acetate (pH 5.5) for 30 minutes. The elution positions of MTX and $MTXGlu_2$ standards were determined by monitoring the UV absorbance at 254 nm. The data (not shown) indicated that $MTXGlu_2$ was not retained by the column. In an attempt to achieve a better resolution, the method of Fry et al (1982) was employed which consisted of a 40 minute linear gradient from 0 to 13% ACN in 0.1 M acetate buffer (pH 5.5) was employed. The elution positions of authentic standards, MTX and $MTXGlu_2$, were determined. The data (not shown) suggested that $MTXGlu_2$ was not retained by the column. The method of Jolivet and Schilsky (1981) was also examined, as previous investigations indicated that this method allowed the separation of MTX and its polyglutamated derivatives in increasing order of glutamate chain length. This method also resolves the pteridine cleavage products of [^{3}H]-MTX prior to MTX, thereby eliminating interference from contaminants. The initial buffer was reduced from 30% v/v ACN in 5mM Pic A to 15% v/v ACN in 5mM Pic A to permit proper resolution of the monoglutamate and diglutamate MTX standards (see Figure 14). A third buffer, consisting of 40% v/v ACN in 5mM Pic A, allowed separation of higher polyglutamate chain lengths (see Figure 14).

The formation of $MTXGlu_n$ was examined in wild type, *met*-6 and *mac* cultures that had been maintained in Vogel's medium for 14 hours prior to incubation with either 0.03 μM or 1.0 μM [^{3}H]-MTX. Since MTX is known to be more cytotoxic during cell division, these analyses were undertaken with cultures which were actually forming hyphae. The capacity to accumulate MTX may correlate with the length of time the cells have been in culture, thus cultures incubated in [^{3}H]-MTX were followed by a 24-hour efflux to conceivably provide optimal conditions for $MTXGlu_n$ formation in the absence of the competing substrate. The formation of $MTXGlu_n$ was initially examined in extracts continuously incubated with 0.03 μM

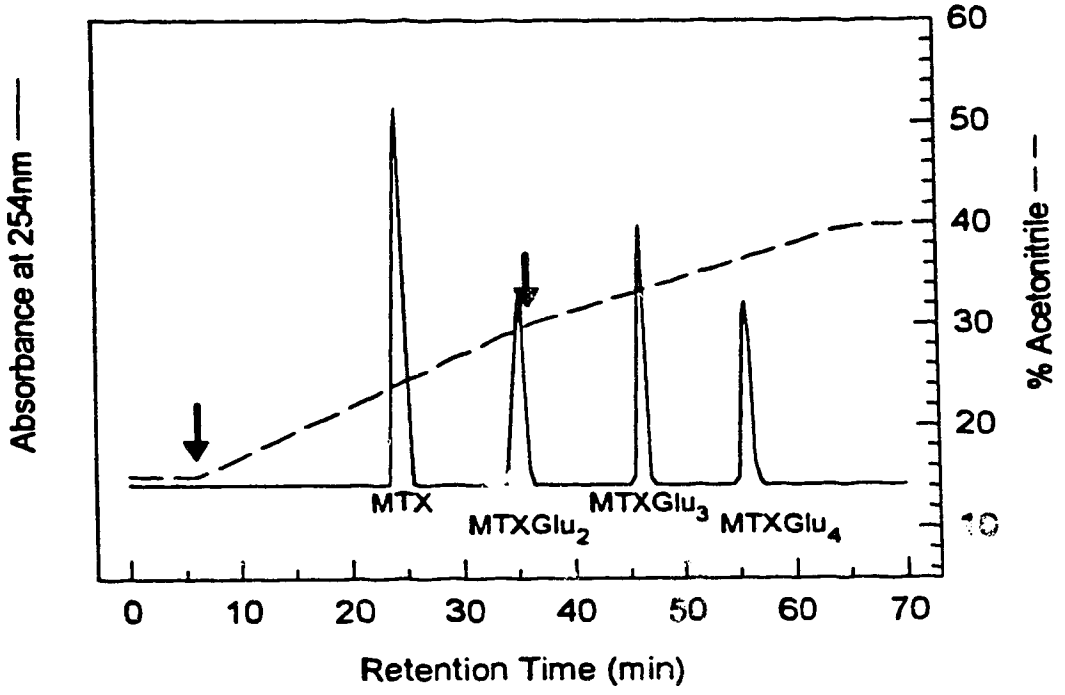


Figure 14. High Performance Liquid Chromatographic Separation of MTX Polyglutamates. A 100 µL mixture of four $MTXGlu_n$ standards (14.3 nmol of each) was injected into a Varian chromatographic system equipped with a µBondapak C_{18} column (see Materials and Methods Section 2.9.). The elution positions were determined by monitoring the UV absorbance at 254 nm. Resolution of the standards was achieved using an ACN gradient. The initial gradient was from 15-30% ACN; the second gradient was from 30-40% ACN (see arrows). The average retention times for $MTXGlu_n$ standards (glutamyl residues numbered 1 to 4) were 24, 35, 46, and 56 minutes, respectively, as calculated from three separate sample injections.

[^{3}H]-MTX (data not shown). Following a 6-hour incubation, 99.7% of the intracellular radioactivity in wild type consisted of a peak that cochromatographed with authentic MTX, while a very small peak constituting 0.1% of the total disintegrations per minute cochromatographed with authentic $MTXGlu_2$. After a 24-hour incubation followed by a 24-hour efflux, $MTXGlu_2$ had increased to 1.9% of the total disintegrations per minute. A third peak appeared that reflected 1.3% of the total intracellular radioactivity and had a retention time consistent with $MTXGlu_3$ (Table 1). In total, $MTXGlu_n$ accounted for 3.2% of the total intracellular drug after 24-hour incubation and efflux, which represents a 30-fold increase over $MTXGlu_n$ accumulation after a 6-hour exposure. Increasing the extracellular MTX concentration from 0.03 μM to 1.0 μM resulted in an increase in the conversion to $MTXGlu_n$ derivatives, most notably in wild type, whether measured as the amount of MTX converted into the polyglutamate pool or as the amount of glutamylation (Table 1). After the incubation in 1.0 μM [^{3}H]-MTX and efflux, $MTXGlu_2$ and $MTXGlu_3$ accounted for 2.9% and 1.3% of the total radioactivity, respectively. In summation, 4.6% of the total intracellular drug was in the form of $MTXGlu_n$ after incubation in 1.0 μM [^{3}H]-MTX compared to a 3.2% value obtained after exposure to 0.03 μM [^{3}H]-MTX. Under these conditions, *met*-6 produced only very low levels of $MTXGlu_2$ (0.7% of total) and only after incubation in 1.0 μM [^{3}H]-MTX, whereas the *mac* mutant failed to catalyze the addition of a single glutamate residue to MTX (Table 1). These results are consistent with the loss of a diglutamate forming synthetase at the *mac* locus (Chan and Cossins, 1984), thus the *mac* mutant contain only pteroylmonoglutamates. Mutation at the *met*-6 locus affects the expression of a second, synthetase activity that produces hexaglutamate from $H_4PteGlu_2$ or $MTXGlu_2$ (Chan and Cossins, 1984) whereby the mutant forms only pteroyldiglutamates. These results, like those derived from work on mammalian cells (Kim et al, 1993), demonstrate that the level of FPGS may directly related to $MTXGlu_n$ formation. Accordingly, the cellular accumulation and metabolism of MTX to $MTXGlu_n$ appears dependent upon the level of FPGS activity.

Generally, loss of $MTXGlu_n$ from cells inversely correlates with the glutamyl chain length so that no discernible loss of the longer derivatives can be detected (Rosenblatt et al, 1978; Galivan, 1980; Fry et al, 1982; Jolivet et al, 1982; Galivan and Nimec, 1983; McGuire et al, 1985; Galivan et al, 1986). In this study, HPLC analyses of the efflux medium followed by quantification of the radioactivity suggested that a single peak with a retention time identical to that of MTX was present in all three strains (data not shown). Failure to detect $MTXGlu_2$ in the efflux medium of *met*-6

Table 1. MTX Uptake and Distribution of Intracellular $MTXGlu_n$ in *Neurospora* Extracts.

		[^{3}H] Recovered (DPM)				Distribution of [^{3}H]-$MTXGlu_n$ (DPM)				
[^{3}H]-MTX (μM)	Strain	In Media Following Incubation	In Wash Media	In Media Following Efflux	In Extracts	MTX	$MTXGlu_2$	$MTXGlu_3$	$MTXGlu_4$	Total $MTXGlu_n$
0.03	Wild Type	14.2×10^5	3.6×10^5	0.8×10^5	0.2×10^5	14.9×10^3	0.3×10^3	0.2×10^3	n.d.	0.5×10^3
	Met-6	14.2×10^5	2.4×10^5	2.0×10^5	0.03×10^5	2.3×10^3	n.d.	n.d.	n.d.	n.d.
	Mac	14.3×10^5	2.5×10^5	2.0×10^5	0.03×10^5	2.3×10^3	n.d.	n.d.	n.d.	n.d.
1.0	Wild Type	17.1×10^6	5.1×10^6	1.0×10^6	0.1×10^6	13.2×10^4	0.4×10^4	0.2×10^4	n.d.	0.6×10^4
	Met-6	17.3×10^6	3.9×10^6	2.1×10^6	0.02×10^6	1.4×10^4	0.01×10^4	n.d.	n.d.	0.01×10^4
	Mac	17.3×10^6	4.1×10^6	2.2×10^6	0.02×10^6	1.2×10^4	n.d.	n.d.	n.d.	n.d.

Conidiospores were initially incubated in Vogel's medium for 14 hours at 30°C. Aliquots (500 μL) were removed and transferred into defined media containing 0.03 or 1.0 μM [^{3}H]-MTX and incubated for 24 hours at 30°C. Following the aspiration of the media, each culture was resuspended in 1 mL of MTX-free Vogel's medium for 15 minutes. MTX efflux was initiated by transfer of the cultures to 30 mL of Vogel's medium. Each culture was incubated for a further 24 hours at 30°C, after which the mycelia were harvested. The mycelia were then extensively washed with PBS and homogenized with an equal weight of acid-washed sand, 1 mL of PBS and 4 mL of 10% TCA. The homogenates were collected by centrifugation at 14,000 rpm for 10 minutes. The clarified homogenates were filtered and then injected onto Sep-Pak C_{18} cartridges (see Materials and Methods Section 2.8.). Cellular extracts were then analyzed by HPLC for $MTXGlu_n$ (see Materials and Methods Section 2.9.). One-minute fractions were collected and the radioactivity was quantified by liquid scintillation counting (see Materials and Methods Section 2.11.). Tabulated results are means (± 1%) of two separate experiments for each strain. Where n.d. denotes radioactivity not detected or quantified as less than twice background levels.

and wild type may be due largely to its preferential retention. Efflux was greater in the two mutants than in the wild type (Table 1), which demonstrates the ability of nonglutamyl conjugated MTX to rapidly cross the cell membrane. The preferential retention of $MTXGlu_n$ derivatives has been demonstrated in leukemia cells (McGuire et al, 1985), Ehrlich ascites tumor cells (Fry et al, 1981), rat hepatocytes and hepatocarcinoma cells (Balinska et al, 1981). It is possible that the primary effect arising from MTX metabolism in *Neurospora*, as in other species, relates to the conversion of a potent monoglutamate inhibitor of DHFR to an essentially irreversible inhibitor of this intracellular target.

Studies of L1210 leukemia cells suggest that the intracellular antifolate level is of critical importance to the level of $MTXGlu_n$ synthesis since this determines the level of MTX substrate presented to intracellular FPGS (Goldman et al, 1968; Galivan, 1979; Gewirtz et al, 1980; Holm et al, 1994). Since only MTX in excess of that tightly bound to DHFR is a substrate for polyglutamylation by FPGS, it was important to consider this level when assessing MTX available for conversion to $MTXGlu_n$. Hence, protein-bound and free [^{3}H]-MTX were examined in all three strains following a 24-hour incubation in 0.03 μM and 1.0 μM [^{3}H]-MTX and 24-hour efflux (Table 2). Accumulation of exchangeable, free, and non-exchangeable, bound, [^{3}H]-MTX was concentration-dependent. At 1.0 μM [^{3}H]-MTX the protein-bound drug represented a very small proportion (1.3%) of the total intracellular level in all three strains. The data demonstrates that bound (non-exchangeable) and free (exchangeable) [^{3}H]-MTX account for 1.3% and 98.6% of total intracellular radioactivity, respectively and are comparable in all three strains incubated in 1.0 μM [^{3}H]-MTX. Similar results were obtained from the cultures incubated in 0.03 μM [^{3}H]-MTX, suggesting that any antifolate resistance exhibited by the *met*-6 and *mac* mutants may not have been due to a MTX-binding protein other than DHFR or to alterations in the level of [^{3}H]-MTX available for polyglutamylation.

3.5. DHFR ASSAY

Extensive physical and chemical investigations of DHFR from various sources have been reported however to date the enzyme has not been purified or characterized from *Neurospora*. The optimal conditions were determined for this enzyme assay using a standard protocol developed by Mathews, Scrimgeour and Huennekens (1963) for the mammalian protein. The requirements for the reaction included 0.8 M KH_2PO_4

Table 2. DHFR-Bound Versus Unbound [^{3}H]-MTX in *Neurospora* Extracts.

[^{3}H]-MTX (μM)	Strain	[^{3}H]-MTX Recovered Per Culture (DPM)				
		In Media Following Incubation	In Media Following Efflux	In Extracts	Protein-Bound [^{3}H]-MTX	Free [^{3}H]-MTX
0.03	Wild Type	14.9 x 10^5	0.7 x 10^5	0.4 x 10^5	1083.0	3.9 x 10^4
	Met-6	13.7 x 10^5	2.7 x 10^5	0.08 x 10^5	209.0	0.8 x 10^4
	Mac	13.9 x 10^5	2.9 x 10^5	0.07 x 10^5	191.3	0.7 x 10^4
1.0	Wild Type	15.8 x 10^6	0.9 x 10^6	0.5 x 10^6	6712.0	4.9 x 10^5
	Met-6	16.3 x 10^6	1.3 x 10^6	0.04 x 10^6	411.0	0.4 x 10^5
	Mac	16.2 x 10^6	1.6 x 10^6	0.04 x 10^6	404.0	0.4 x 10^5

Conidiospores were initially incubated in Vogel's medium for 14 hours at 30°C. Aliquots (500 μL) were removed and transferred into defined media containing 0.03 or 1.0 μM [^{3}H]-MTX and incubated for 24 hours at 30°C. Following the aspiration of the media, each culture was resuspended in 1 mL of MTX-free Vogel's medium for 15 minutes. MTX efflux was initiated by transfer of the cultures to 30 mL of Vogel's medium. Each culture was incubated for a further 24 hours at 30°C, after which the mycelia were harvested. The mycelia were then extensively washed with double-distilled water and homogenized in a mortar with Buffer A at a *1 : 2 ratio (grams of* tissue:mL of Buffer A) and an equal weight of acid-washed sand. The homogenates were collected by centrifugation at 14,000 rpm for 10 minutes. DHFR-bound radiolabel was determined using PD-10 columns containing Sephadex G-25. A 500 μL aliquot of the clarified homogenate was layered onto the equilibrated gel bed followed by a 2 mL wash with Buffer A (see Materials and Methods Section **2.10.**). The radioactivity of the column effluent was quantified by liquid scintillation counting (see Materials and Methods Section **2.11.**). Tabulated results are means (± 1%) of two separate experiments for each strain.

buffer (pH 7.5), 0.1 M 2-mercaptoethanol, 1.5 mM dihydrofolate and 1mM NADPH + H^+ (outlined in Materials and Methods Section 2.12.2.). The efficacy of the assay reagents were examined, as illustrated in Figure 4. The results suggested that all of the reagents, at their specified concentrations, were necessary for the assay to proceed optimally.

3.6. PURIFICATION OF DHFR PROTEIN

The protocol for DHFR purification (see Materials and Methods Section 2.12.4.) used to isolate this protein from wild type, *met*-6 and *mac* strains has been summarized in Tables 3, 4 and 5, respectively.

The initial step; homogenization of filtered mycelial pads followed by centrifugation, removed any cellular debris. The clarified homogenate was adjusted to 1% streptomycin sulfate to remove nucleic acids and other polyanionic proteins. This step increased the specific activity by *ca.* 1.0-fold. The resulting supernatant was fractionated with ammonium sulfate, and protein containing DHFR activity (Step 3) was recovered at 50-80% of saturation. The DHFR-containing precipitate was redissolved and applied to a column of Sephadex G-75 (Figure 15). DHFR activity emerged as a distinct peak, at approximately fraction number 34, subsequently the DHFR-active fractions were pooled and concentrated (Step 4) resulting in a 50 to 60-fold purification. This degree of purification was achieved due to the majority of the other proteins in the extracts having much higher molecular weights than DHFR-active protein. Further purification (*ca.* 1400-fold) resulted from the application of the combined Sephadex G-75 fractions to a column of Matrex Green A (Figure 16). DHFR was eluted with an increasing linear KCl gradient. The active fractions were pooled and concentrated (Step 5) resulting in an increase in the specific activity to 5580 units/mg, 1058 units/mg and 893 units/mg for wild type, *met*-6 and *mac*, respectively. Purification of *N. crassa* DHFR, from all three strains, apart from being of intrinsic interest, permitted an adequate comparison between each strain (see Table 6). DHFR activity expressed as units per gram of mycelia, from wild type, *met*-6 and *mac* crude extracts, were recorded as 13.2, 13.3, and 13.2, accordingly. A comparison of DHFR activities of Step 4 protein from wild type, *met*-6 and *mac* strains (Table 6) suggests that MTX associated cytotoxicity or insensitivity did not result from differences in DHFR gene expression.

Table 3. Purification of DHFR Activity from Wild Type *Neurospora crassa*.

Fractionation Step	Volume (mL)	DHFR (units/mL)	Protein (mg/mL)	Specific Activity (units/mg)	Total (units)	Purification (x-fold)	Recovery (%)
1. Crude extract	106.0	8.4	2.2	3.9	890.4	1.0	100
2. Streptomycin SO_4	105.0	7.7	1.8	4.2	808.5	1.1	90.8
3. 50-80% $(NH_4)_2SO_4$	4.0	80.5	14.9	5.4	322.0	1.4	36.2
4. Sephadex G-75	8.5	69.3	0.2	346.5	589.1	88.8	66.2
5. Matrex Green A	3.7	85.9	0.004	21475.0	[illegible]	5506.4	35.7

Mycelia were harvested after 72 hours of growth at 30°C (see Materials and methods Section **2.12.3.**). Subsequently, DHFR activity was fractionated and purified as discussed in Section **2.12.4.** The values for enzyme activity, protein content and volume reflect the mean of triplicate measurements from a single large purification (67.5 g wet weight). 1 unit of activity is expressed as 1 nmole of dihydrofolate reduced per minute.

Table 4. Purification of DHFR Activity from *Neurospora crassa Met*-6 Mutant.

Fractionation Step	Volume (mL)	DHFR (units/mL)	Protein (mg/mL)	Specific Activity (units/mg)	Total (units)	Purification (x-fold)	Recovery (%)
1. Crude extract	65.0	7.4	10.2	0.7	481.0	1.0	100
2. Streptomycin SO_4	63.0	6.7	6.7	1.0	422.1	1.4	87.8
3. 50-80% $(NH_4)_2SO_4$	4.0	43.3	21.1	2.1	173.2	2.9	36.0
4. Sephadex G-75	8.0	39.1	0.9	43.4	312.8	62.1	65.0
5. Matrex Green A	7.6	22.8	0.003	7600.0	173.3	10857.1	36.0

Mycelia were harvested after 72 hours of growth at 30°C (see Materials and Materials Section **2.12.3.**) and DHFR activity was fractionated and purified as discussed in Section **2.12.4.** The values for enzyme activity, protein content and volume reflect the mean of triplicate computations from a single large purification (36.1 g wet weight). 1 unit of activity is expressed s 1 nmole of dihydrofolate reduced per minute.

Table 5. Purification of DHFR Activity from *Neurospora crassa Mac* Mutant.

Fractionation Step	Volume (mL)	DHFR (units/mL)	Protein (mg/mL)	Specific Activity (units/mg)	Total (units)	Purification (x-fold)	Recovery (%)
1. Crude extract	37.0	8.4	7.4	1.1	310.8	1.0	100
2. Streptomycin SO_4	36.0	7.4	4.6	1.6	266.4	1.4	85.7
3. 50-80% $(NH_4)_2SO_4$	4.0	28.3	24.2	1.2	113.2	1.1	36.4
4. Sephadex G-75	8.0	25.8	0.4	64.5	206.4	58.6	66.4
5. Matrex Green A	3.5	32.0	0.01	3200.0	112.0	2909.1	36.0

Mycelia were harvested after 72 hours ofgrowth at 30°C (see Materials and Methods Section **2.12.3.**). Subsequently, DHFR activity was fractionated and purified as discussed in Section **2.12.4.** The values for enzyme activity, protein content and volume reflect the mean of triplicate measurements from a single large purification (23.5 g wet weight). 1 unit of activity is expressed as 1 nmole of dihydrofolate reduced per minute.

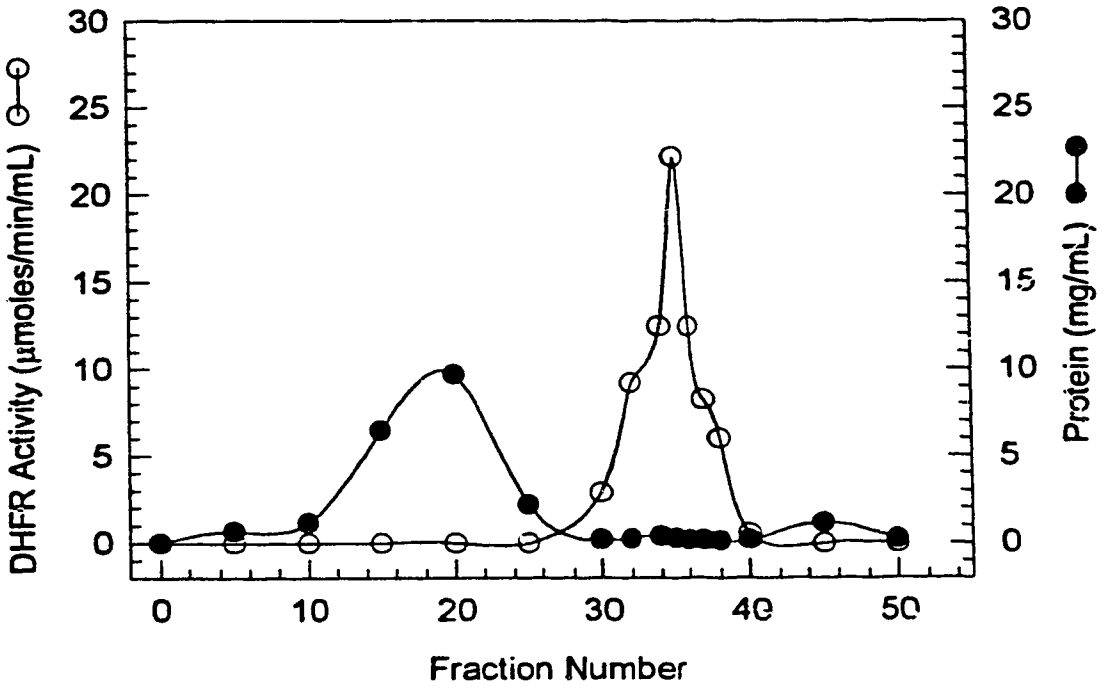


Figure 15. Chromatography of DHFR protein on Sephadex G-75. Step 3 protein was redissolved in a minimal volume of Buffer A and applied to a Sephadex G-75 column (2.5 x 70 cm) previously equilibrated with Buffer A (see Materials and Methods Section 2.12.4.). DHFR activity was eluted with Buffer A. Fractions of 6 mL were collected at a flow rate of 20 mL hr^{-1} and assayed for DHFR activity and protein concentration.

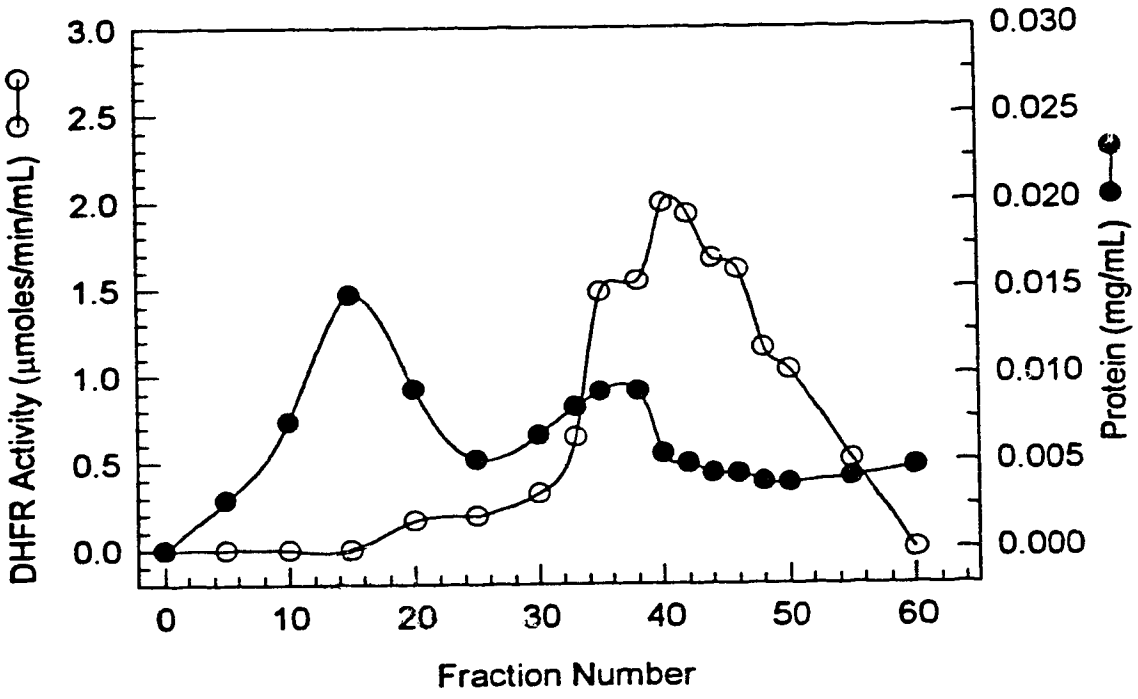


Figure 16. Matrex Green A Chromatography. Step 4 protein was applied to a Matrex Green A column (1.5 x 10 cm) equilibrated with Buffer A (see Materials and Methods Section 2.12.4.) The column was washed with 50 mL of Buffer A followed by a 0.2-0.4 M KCl linear gradient (100 mL) in Buffer A commencing at fraction 16. Fractions of 3 mL were collected at a flow rate of 42 mL hr^{-1} and assayed for DHFR activity and protein concentration.

Table 6. Comparison of DHFR Activities of Wild Type, *Met*-6 and *Mac* Strains of *Neurospora crassa*.

Strain	Volume (mL)	Mycelium (g)	DHFR (units/mL)	Protein (µg/mL)	Specific Activity (units/mg)	DHFR/g mycelia (units/g)
Wild type	8.5	67.5	69.3	197.1	346.5	8.7
Met-6 mutant	8.0	36.1	39.1	858.1	45.6	8.7
Mac mutant	8.0	23.5	25.8	437.0	59.1	8.8

After 72 hours of growth at 30°C, mycelia were harvested (see Materials and Methods Section **2.12.3.**). The values reflect DHFR activity, based on the means of triplicate determinations of Step 4 protein (see Materials and Methods Section **2.12.4.**). 1 unit of activity is expressed as 1 nmole of dihydrofolate reduced per minute.

3.7. MOLECULAR WEIGHT DETERMINATION

Gel filtration of DHFR activity, from Step 4 *mac* extracts, on a calibrated column of Sephadex G-75 resulted in an apparent M_r of 21,600 Da (Figure 17). Similar results were obtained from of wild type and *met*-6 extracts. The molecular weight of DHFR was examined by SDS-PAGE by subjecting the DHFR-active fractions, collected after each purification step, to SDS-PAGE and staining with Coomassie blue (Figure 18). A single protein band with an apparent M_r of 24,000 Da, was revealed after purification on a Matrex Green A column. The staining intensity of this band corresponded to the DHFR activity of this peak from the Matrex Green A column (data not shown). Similar electrophoretic patterns were observed when wild type and *met*-6 were examined. SDS-PAGE of concentrated Step 5 protein from each of the three strains, followed by silver staining, revealed a single protein band with an apparent M_r of 22,000 Da. The results are consistent with the M_r values reported for DHFR from various vertebrate, bacterial and plant tissues. The DHFR protein isolated from vertebrates, including chicken, bovine and human tissues (Blakley, 1984), has an apparent M_r of 21 kD, 21 kD and 22 kD, respectively. The reported M_r of this protein from bacteria, including *Lactobacillus rhaminosis* (Curtis et al, 1994), *Escherichia coli* (Hamm-Alvarez et al, 1990), *Saccharomyces cerevisiae* (Wu et al, 1980; Fling et al, 1988) and *Candida albicans* (Baccanari et al, 1989), are 18 kD, 18 kD, 26 kD and 25 kD, respectively. The apparent M_r of soybean seedling DHFR protein is 22 kD (Ratnam et al, 1987). Thus, a high degree of homology may exist between vertebrate, fungal, some plant and bacterial DHFRs. Interestingly, *N. crassa* does not possess a large bifunctional DHFR-thymidylate synthase protein like that from *Daucus carota* (Luo and Cella, 1995), *Arabidopsis thaliana* (Lazar et al, 1993) and some protozoa.

3.8. INHIBITION OF *NEUROSPORA* DHFR BY MTX AND MTXGLU$_3$

The inhibition of *Neurospora* DHFR by MTX and MTXGlu$_3$ was examined using Step 4 protein. The enzymatic assay was performed using an equal number of enzyme units from all three fungal strains in the presence of different concentrations of these inhibitors.

The data, depicted in Figure 20, show that MTX is a very effective inhibitor of *Neurospora* DHFR. The inhibitor concentrations required for 50% inhibition of enzymatic activity (IC_{50}) were similar for all three strains. The IC_{50} values of 9.0 nM,

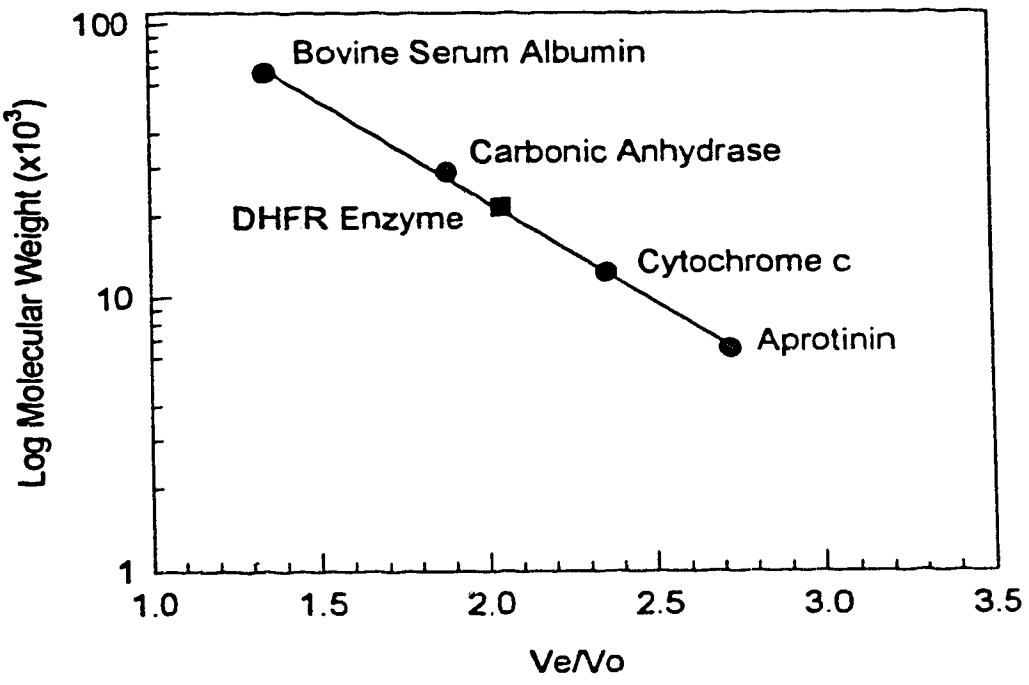


Figure 17. Molecular Weight Determination of *Neurospora* DHFR. Molecular weight marker proteins were applied to a 2.5 x 70 cm column of Sephadex G-75 (see Materials and Methods Section **2.12.4.**). The volume required to elute each protein (Ve) was determined by measuring the absorbance at 280 nm. The Ve of DHFR, from *mac* extracts, was calculated using the standard enzyme assay (see Materials and Methods Section **2.12.2.**). The void volume (Vo) was determined by measuring the absorbance at 280 nM of Blue Dextran. The apparent native molecular weight of DHFR, based upon the mean of three determinations, was calculated as 21,600 Da (± 2200 Da). Similar results were obtained upon examination of wild type and *met*-6 extracts.

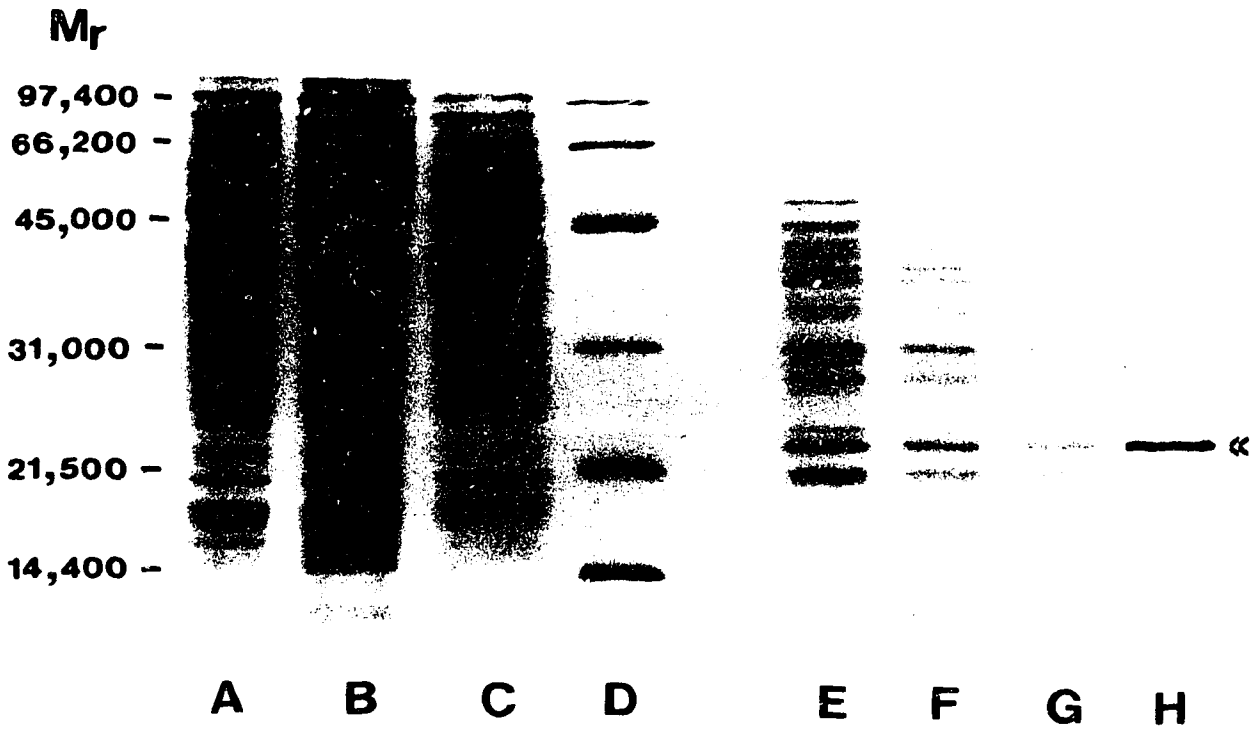


Figure 18. SDS-Page of *Mac* Mutant Extracts. DHFR was purified over 1400-fold from all three strains of *Neurospora* crassa (See Materials and Methods Section 2.12.4.). Mycelia were homogenized in Buffer A and centrifuged. The clarified homogenate (lane A; 74.1 μg) was treated with streptomycin sulfate to remove nucleic acids (lane B; 46.4 μg). DHFR protein in the 50-80% range of $(NH_4)_2$ SO_4 saturation was redissolved in Buffer A (lane C; 121.1 μg) and applied to a Sephadex G-75 column. DHFR activity emerged as a sharp peak in the 20-30 kD range. Active fractions were pooled and concentrated (lanes E, F and G; 14.9, 8.7 and 6.6 μg, respectively) and applied to a Matrex Green A column. Unbound protein was washed from the column using Buffer A prior to DHFR elution with a linear 0.2-0.4 M KCl gradient in Buffer A. Active fractions were pooled and concentrated (lane H; 0.2 μg). Coomassie blue stain revealed a single protein band with an apparent M_r of 24 kD as estimated from a low molecular weight protein kit for electrophoresis (lane D) as described in Materials and Methods Section 2.12.7. Similar electrophoretic patterns were obtained when wild type and *met*-6 extracts were examined.

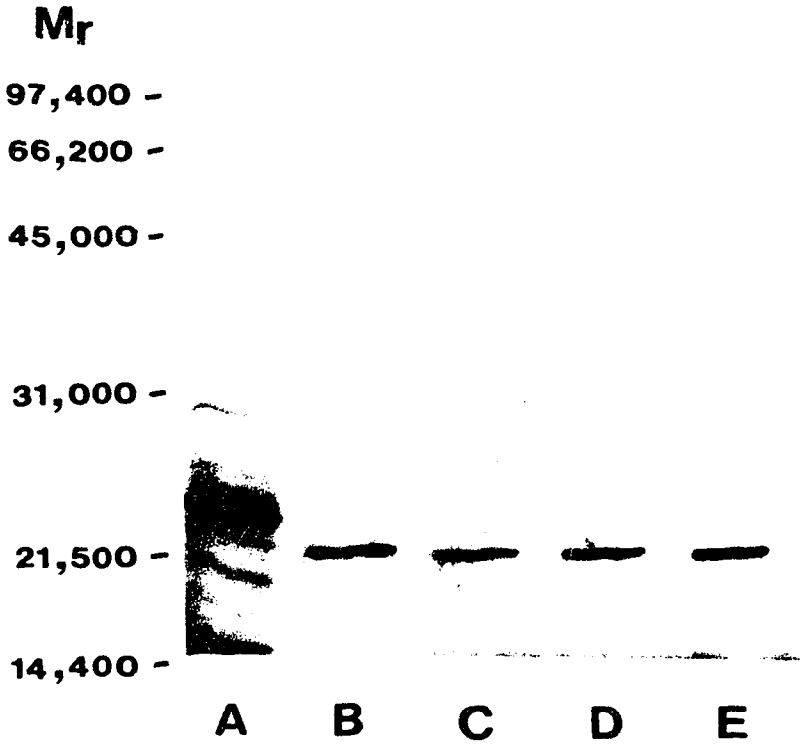


Figure 19. SDS-PAGE of Wild Type, *Met*-6 and *Mac* Extracts. DHFR protein from all three strains was chromatographed on Matrex Green A. Unbound protein was removed from the column using Buffer A (lane A; 0.2 μg wild type protein) whereas elution of DHFR required a linear 0.2-0.4 M KCl gradient in Buffer A. Active fractions were pooled and concentrated from each of the three strains. Silver staining revealed a single protein band with an apparent M_r of 22 kD from each of wild type (lane B; 0.2 μg), *met*-6 (lane C; 0.2 μg) and *mac* (lane D and E; 0.2 μg) strains as estimated from a low molecular weight protein kit for electrophoresis (see Materials and Methods Section 2.12.7.).

11.0 nM and 9.8 nM for wild type, *met*-6 and *mac*, respectively, are not significantly different when calculated from the standard deviation. Crosti (1981) determined the IC_{50} values of MTX for several DHFRs. The IC_{50} values of the antimetabolite were 2.0 nM, 1.0 nM, 1.7 nM and 4.0 nM for *Z. mays, P. sativum, N. crassa* and *Euglena gracilis*, respectively.

The data illustrated in Figure 21 shows that $MTXGlu_3$ is a more potent inhibtor of *Neurospora* DHFR than the monglutamate progener. Again, the IC_{50} values for each strain were similar; accordingly, these values of 2.8 nM, 2.5 nM and 2.8 nM for wild type, *met*-6 and *mac* were not significantly different as derived from the standard deviation. At pH 6.4 the addition of two glutamate residues to MTX increased its potency by 30% in *Neurospora* extracts. Similarly, Schilsky et al (1983) and Kisliuk et al (1983) reported that for *Lactobacillus rhaminosis*, inhibition by MTX was influenced by polyglutamylation whereby addition of two glutamate residues increased the potency by at least 30%.

Figure 20. Effect of MTX Concentration on DHFR Activity. The IC_{50} of MTX on DHFR was determined by a modification of the standard assay. Step 4 protein, 4.64 units, was incubated with individual MTX congeners, 0.8 M KH_2PO_4 buffer (pH 7.5), 0.1 M 2-mercaptoethanol, and 1.5 mM dihydrofolate, in a final volume of 1.0 mL for 5 minutes before the reaction was initiated with 1.0 mM NADPH + H^+ (see Materials and Methods Section **2.12.5.**). The IC_{50} values are means derived from duplicate determinations. Error bars were calculated from the standard error of the enzyme activity measurements. Where not shown, the error bars fall within the symbol size.

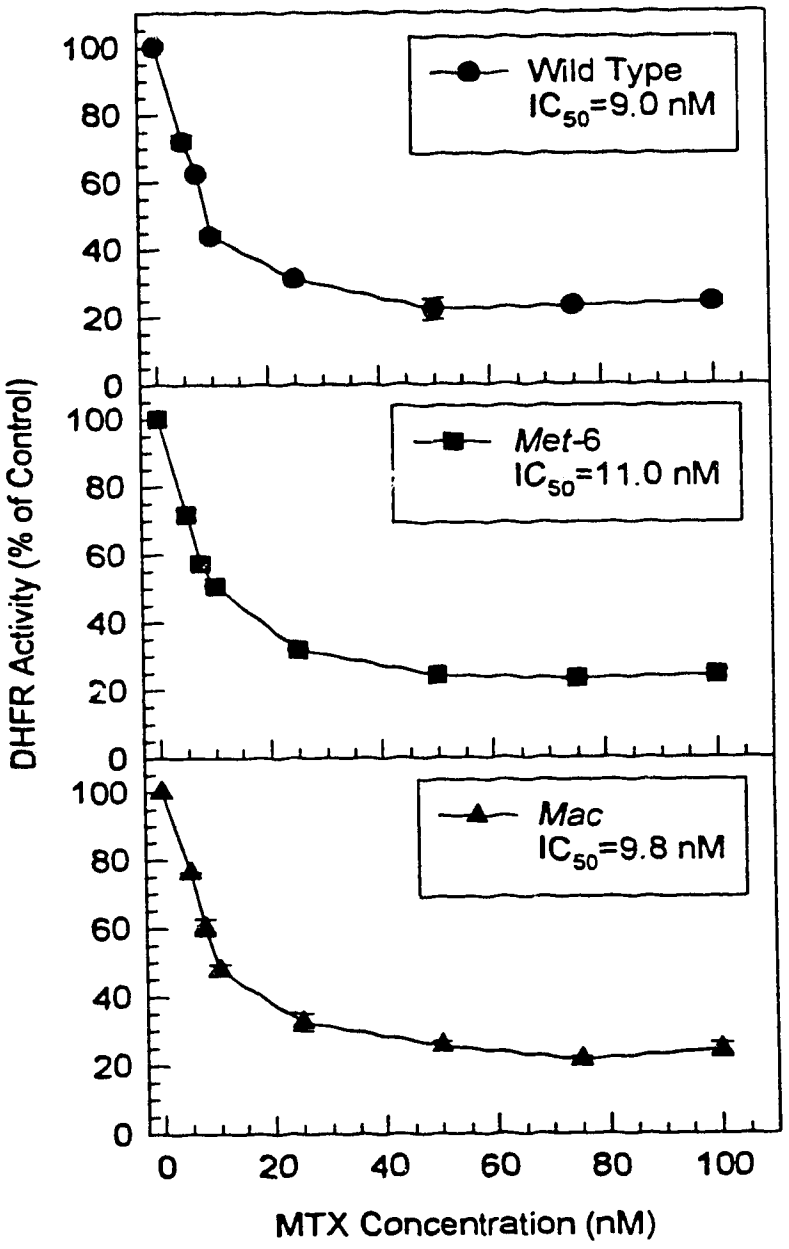
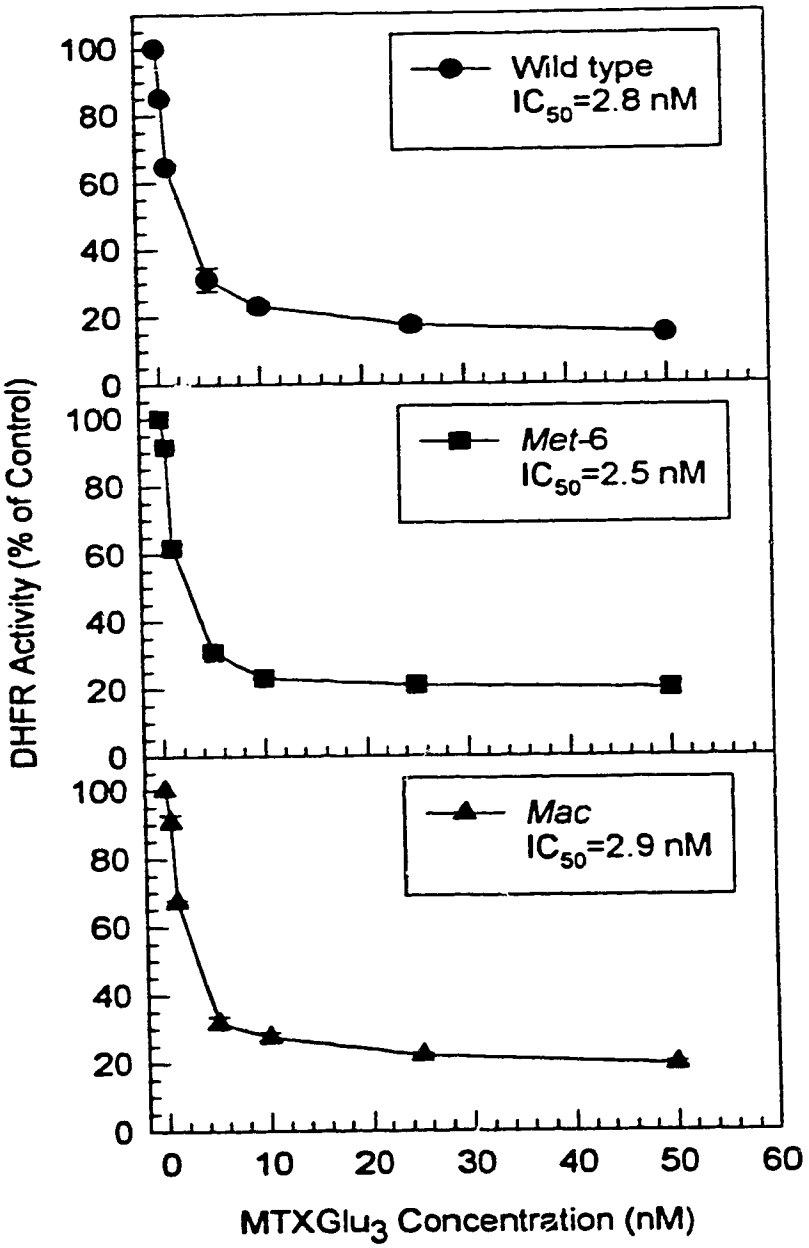

Wild Type
IC50=9.0 nM
Met-6
IC50=11.0 nM
Mac
IC50=9.8 nM
DHFR Activity (% of Control)
MTX Concentration (nM)
0
20
40
60
80
100

Figure 2 .. Effect of $MTXGlu_3$ Concentration on DHFR Activity. The IC_{50} of $MTXGlu_3$ on DHFR was determined by a modification of the standard assay. Step 4 protein, 4.64 units, was incubated with individual $MTXGlu_3$ congeners, 0.8 M KH_2PO_4 buffer (pH 7.5), 0.1 M 2-mercaptoethanol, and 1.5 mM dihydrofolate, in a final volume of 1.0 mL for 5 minutes before the reaction was initiated with 1.0 mM NADPH + H^+ (see Materials and Methods Section **2.12.5.**). The IC_{50} values are means of duplicate determinations. Error bars were calculated from the standard error of the enzyme activity measurements. Where not visible, the error bars fall within the symbol size.


Wild type
IC50=2.8 nM
Met-6
IC50=2.5 nM
Mac
IC50=2.9 nM
DHFR Activity (% of Control)
MTXGlu3 Concentration (nM)
0
20
40
60
80
100
0
10
20
30
40
50
60

DISCUSSION

4.1. SENSITIVITY TO MTX INHIBITION

Methotrexate, one of the most widely used and effective antineoplastic agents, is believed to exert its antitumor effects by virtue of its high affinity for DHFR resulting in metabolic disturbances at the level of the intracellular reduced folate pools.

Although DHFR inhibition in growing cells should deplete cellular reduced folate pools, MTX treatment of *N. crassa* cultures resulted in only partial growth inhibition (see Figure 5). One possible explanation for this phenomenon is the preservation of a major portion of reduced folates as observed in Ehrlich ascites tumor cells (Fry et al, 1982). As a consequence of DHFR inhibition, the intracellular pools of reduced folate are converted to H_2PteGlu by the TS catalyzed reaction and cannot be reduced to the H_4PteGlu state required for cofactor activity thus providing a mechanism whereby reduced folates would ultimately become trapped as H_2PteGlu (Allegra et al, 1987). However, the H_2PteGlu that accumulates due to MTX presence may also inhibit TS activity (Sur et al, 1985), resulting in some preservation of the reduced folate pool. It is likely that the reduced folate pool is only partially depleted after MTX treatment. In myeloid precursor cells, Baram et al (1986) demonstrated that MTX caused a 50% depletion of 5-CH_3-H_4PteGlu and H_4PteGlu whereas pools of 10-HCO-H_4PteGlu were depleted by 30-40%, thus the biologically important 10-HCO-H_4PteGlu, required for *de novo* purine synthesis, appeared to be partially conserved at the expense of pools 5-CH_3-H_4PteGlu and H_4PteGlu.

In order to maintain complete inhibition of DHFR activity, it is usually necessary to establish levels of MTX that are in excess of that bound to DHFR (Jackson et al, 1977; Matherly et al, 1987). In the absence of high levels of free intracellular MTX, the effects of the enzyme-bound drug on H_4PteGlu pools and folate-dependent reactions are readily reversed as the intracellular drug declines when extracellular MTX is removed. Matherly et al (1984) demonstrated that in Ehrlich ascites tumor cells, reduced folates can induce the net dissociation of DHFR-bound MTX in the absence of free intracellular MTX. The reversible nature of antifolate suppression of mammalian DHFR activity is based on the premise that only about 5% of the total DHFR is necessary to sustain adequate levels of H_4PteGlu for cell replication (Matherly et al, 1987). On the other hand, the rapid reversibility of MTX inhibition of DHFR is probably due to the high levels of H_2PteGlu substrate that accumulate and

become competitive with MTX for this percentage of total DHFR activity (Fry et al, 1982). MTX cytotoxicity in the present study was enhanced during concurrent sulfanilamide treatment (Figure 8), likely due to the inability to accumulate H_2PteGlu. The competition between H_2PteGlu and MTX continues until *ca.* 95% of DHFR is bound with MTX because of the conversion of folate cofactors to H_2PteGlu through TS-mediated reactions (Matherly et al, 1987). This explains continued growth even when exogenous MTX concentrations are high. DHFR is present in excess of its preferred substrate H_2PteGlu (Matherly et al, 1987). This fact, in conjunction with competition between H_2PteGlu and MTX for DHFR, may explain why relatively high (10 μM) MTX concentrations were required to inhibit growth in *Neurospora* (see Figure 5). Higher concentrations of MTX may overcome H_2PteGlu competition and deplete reduced folate pools necessary for continued thymidylate and purine synthesis. If MTX cannot bind to DHFR, due to the presence of the preferred substrate, this reverses its antifolate pharmacologic effect. It is because of this reversibility of drug binding to DHFR that the conversion of MTX to polyglutamate derivatives which cannot readily penetrate cell membranes takes on a particular importance.

The presence of reduced folates in *E. coli* cells that lack DHFR implies the presence of another enzyme or metabolic pathway responsible for the generation of H_4PteGlu (Hamm-Alvarez et al, 1990). This could be another mechanism by which an organism like *Neurospora crassa* could circumvent MTX induced growth inhibition. A recently identified dihydropteridine reductase may regenerate a quinoid-H_2PteGlu species to H_4PteGlu (Hamm-Alvarez et al, 1990). In the presence of this enzyme H_4PteGlu pools might be protected under conditions where DHFR activity was minimal.

The increased growth recovery in the *N. crassa* mutant strains following a 36-hour preincubation (see Figure 11) is best explained by the presence of a large mycelial folate pool. MTX cytotoxicity may be dependent upon the extent of cellular folate pools, as observed in rat hepatoma cells (Galivan et al, 1983). The mycelial folate content of wild type is 75% of that detected in the *met*-6 mutant (Chan and Cossins, 1984). This may have affected growth recovery of wild type because of an inherently lower mycelial folate pool, which may be unable to compete with MTX for DHFR and other H_4PteGlu-requiring enzymes. Likewise, in folate depleted media MTX reduces H_2PteGlu accumulation and inhibits growth and thymidylate synthesis in murine leukemia L1210 cells (Bunni et al, 1988). However, results obtained in this study indicate a greater resistance and recovery exhibited by wild type as compared to the *met*-6

and *mac* mutants after conidiospore exposure to MTX (see Figures 5, 10, 12, 13). Chan and Cossins (1984) reported that the conidiospores of wild type had an *ca.* two-fold greater folate content than spores of the *met*-6 and *mac* mutants. Cultures of wild type that have been recently generated from a spore inoculum may have an increased folate reserve which could provide an effective means of surmounting MTX inhibition under these conditions. In this regard, folate analyses combined with the antifolate treatments should be undertaken to ascertain the degree of folate deficiency that may be occurring under the experimental conditions.

The addition of L-methionine to wild type cultures of *N. crassa* resulted in increased MTX cytotoxicity in this study (see Figure 6). L-methionine, acting as a methyl donor may reduce intracellular folate production. The effect of methionine on MTX metabolism has been investigated in rat hepatocytes (Rhee et al, 1989), and the results suggested that a significant increase in H_4PteGlu, 5,10-CH_2-H_4PteGlu and 10-HCO-H_4PteGlu was accompanied by a decrease in 5-CH_3-H_4PteGlu. These findings indicated that the lack of growth inhibition and decreased MTX glutamylation could be due to sustainable levels of reduced folates that effectively compete with MTX as a substrate for FPGS (Rhee et al, 1989). In the present study, the effect observed may be best explained by the work of Chan and Cossins (1980), who demonstrated that L-methionine supplemented media resulted in accumulations of AdoMet. Accordingly, AdoMet controls methionine synthesis by inhibiting 5,10-CH_2-H_4PteGlu reductase and cystathionine γ-synthase. These culture conditions thereby reduce 5-CH_3-H_4PteGlu generation which may account for a significant decrease in conidiospore folate pools thereby enhancing MTX cytotoxicity. Results from the aforementioned authors suggest that effects of exogenous methionine on folate metabolism support interspecies variability.

4.2. FOLINIC ACID RESCUE

In this study, MTX inhibited *met*-6 and *mac* cultures demonstrated an enhanced growth recovery following folinic acid rescue (see Figure 9). Whereas, wild type cultures only exhibited a partial growth recovery under the same conditions. Folinic acid rescue circumvents the MTX block in *de novo* H_4PteGlu biosynthesis at DHFR by serving as an intermediate in folate biosynthesis (Nixon and Bertino, 1971; Stover and Schirch, 1993). The data reported in this study, like that of Rosenblatt et al (1982) indicates that folinic acid reversed the effects of MTX on cell growth when

present in conjunction with MTX in all cultures. This was most probably related to impaired MTX uptake, by competitive binding to the membrane transport carrier thereby decreasing the effective dose of MTX presented to the cell, and reduced polyglutamate formation. Direct studies utilizing [^{3}H]-folinic acid may provide further information on the effects of MTX on uptake of this folate. Moreover, the competitive relationship between MTX and folinic acid observed under these conditions may have been due to H_2PteGlu accumulation which acts as a competitive substrate with the inhibitor MTX for DHFR. Matherly et al (1986) reported that folinic acid rescue in murine L1210 leukemia cells involves a non-anabolic role for the cofactor. The competition between MTX and H_2PteGlu for DHFR led to the suggestion that exogenous reduced folate, such as folinic acid, might elevate the level of H_2PteGlu which could competitively displace the bound antifolate. This would restore H_4PteGlu biosynthesis, thereby reversing the pharmacologic effects of the drug. Consistent with this possibility are studies indicating that exogenous reduced folates, if present in sufficient concentrations, can promote a dissociation of MTX bound to DHFR in tumor cells or from purified DHFR (Matherly et al, 1983). Clearly, the level of free intracellular MTX is a critical determinant of the extent of antifolate displacement from DHFR since if this free drug component is elevated no significant competition at DHFR is observed.

Since folinic acid is a recognized cofactor in C_1 metabolism it affects MTX binding by producing a competitive substrate, H_2PteGlu, for DHFR. Well oxygenated cells, like those in the present study, with high levels of oxidized pyridine nucleotides favor MTX dissociation from DHFR. This is due to significantly reduced cooperative binding of the antifolate relative to natural cofactors (Matherly et al, 1983; Matherly et al, 1984). This phenomenon could contribute to the 8% rescue of wild type following MTX and folinic acid treatment in this study. The present data (see Figure 9) suggests that folinic acid preferentially reverses the cytotoxic effects of MTX in the two mutant strains, following pretreatment with the antifolate. One proposed reason for the less than 100% rescue of wild type is that of measurable accumulation of intracellular $MTXGlu_n$. If the sustained concentrations of $MTXGlu_n$ are inhibiting folate-dependent enzymes other than DHFR, sufficient concentrations of appropriate substrates must accumulate within the cell from folinic acid to surmount $MTXGlu_n$ inhibition and to serve as C_1 donors. These investigations also suggest that H_2PteGlu can compete less well with $MTXGlu_n$ than MTX for DHFR under cellular conditions. On the basis of these findings, it is clear that the intracellular conversion of MTX to poly-

glutamyl derivatives limits the extent of rescue achieved with folinic acid. Similarly, it has been demonstrated that folinic acid was ineffective in reversing the MTX toxicity of fibroblasts if added to the medium following a preincubation in MTX which resulted in the derivatization of MTX to its polyglutamyl forms (Rosenblatt et al, 1982).

There are two known enzymes that may be involved in 5-HCO-H_4PteGlu metabolism (Kruschwitz et al, 1994). Methenyltetrahydrofolate synthetase catalyzes the irreversible ATP-dependent conversion of 5-HCO-H_4PteGlu to 5,10-CH=H_4PteGlu in what has been termed a salvage reaction (Stover and Schirch, 1993). This reaction is responsible for the *in vivo* conversion of folinic acid to $H_4PteGlu_n$ derivatives that may serve as C_1 donors and thus relieve MTX toxicity. In the presence of glycine, SHMT catalyzes the hydrolysis of 5,10-CH=H_4PteGlu to 5-HCO-H_4PteGlu (Kruschwitz et al, 1994). Methenyltetrahydrofolate synthetase may represent a rate-limiting step in folinic acid rescue, as the monoglutamate substrate is supplied rather than the preferred polyglutamate derivative. Consequently, 5-HCO-H_4PteGlu utilization by the present *N. crassa* cultures may not be optimal especially in the presence of competing $MTXGlu_n$. Further, Kruschwitz et al (1994) reported that the physiological polyglutamate forms of 5-HCO-H_4PteGlu are slow binding inhibitors of *N. crassa* SHMT which may account for the differential rescue observed in the present study. The folylpolyglutamate deficient mutants are unable to generate $PteGlu_n$, whereas wild type may conceivably produce 5-HCO-$H_4PteGlu_n$ which may inhibit cytosolic SHMT activity thereby reducing the rescue effect by blocking 5,10-CH_2-H_4PteGlu synthesis. In this regard, direct studies with radiolabeled folinic acid, in *Neurospora*, could elucidate its intracellular metabolism into the various C_1 substituted folate and polyglutamate pools (Boarman and Allegra, 1992).

4.3. POLYGLUTAMYLATION OF MTX

$PteGlu_n$ derivatives of natural folates permeate cellular membranes less readily than their non-conjugated forms. This allows an increased intracellular concentration relative to the extracellular concentration. The folylpolyglutamate deficient *N. crassa* mutants are unable to synthesize long chain MTX polyglutamates, therefore the monoglutamates can readily exit the cell reducing MTX cytotoxicity (see Table 1).

Polyglutamyl derivatives were detected in wild type cultures in the present study (see Table 1). These derivatives of MTX have increased affinities for DHFR (Schilsky et al, 1983; Abraham et al, 1991; Assaraf et al, 1992), and are retained intra-

cellularly (Rosenblatt et al, 1978; Galivan, 1980; Fry et al, 1982; Jolivet et al, 1982; Galivan and Nimec, 1983; McGuire et al, 1985; Galivan et al, 1986).

In the present study, MTX uptake (see Table 1) was not apparently impaired or inhibited in any of the three *Neurospora* strains. It therefore follows that the affinity of the carriers for the antifolate is probably not changed by the *met*-6 and *mac* mutations. Conceivably, similar levels of MTX would be presented to FPGS, the enzyme responsible for γ-glutamylation of MTX and natural folates.

The results in Table 2 indicate that the accumulation of free and bound [^{3}H]-MTX was concentration-dependent. Protein-bound MTX represented a small proportion of the total cellular level and the levels of ^{3}H in this fraction were similar in all three strains. Based on previous investigations (McGuire et al, 1989), it is likely that this ^{3}H is mainly DHFR-bound. If this assumption is correct it appears unlikely that the mycelial extracts contained differing levels of other MTX-binding proteins.

Neurospora crassa, in common with human breast cancer cells (Schilsky et al, 1983), synthesizes $MTXGlu_n$ in a time- and dose-dependent manner. Increasing the extracellular MTX concentration results in increased conversion to $MTXGlu_n$ derivatives, most notably in wild type, whether measured as the amount of MTX converted into the polyglutamate pool or as the amount of total glutamylation (see Table 1). Conversely, McGuire et al (1980), Balinska et al (1982), and Nimec and Galivan (1983) proposed that high substrate concentrations result predominantly in shorter chain length derivatives whereas relatively low substrate concentrations result in primarily longer chain $MTXGlu_n$ moieties. In the present investigations an extracellular concentration of 1.0 μM [^{3}H]-MTX was necessary for measurable polyglutamylation to proceed because at 0.03 μM [^{3}H]-MTX quantification of polyglutamylation was not significantly higher than background levels. This may be attributed to a species specific concentration which is required to saturate DHFR within cells and to achieve adequate levels of exchangeable intracellular MTX (Fry et al, 1982; Abraham et al, 1991; Kim et al, 1993). The duration of exposure is dictated by the pharmacokinetics for MTX in each tissue, which in turn appears to be a function of FPGS in each cell type. The data from these investigations (see Table 1) suggest that unlike human FPGS, the *Neurospora* enzyme does not support extensive polyglutamylation of MTX, a result, consistent with previous work on human leukemia cells (Fabre et al, 1985), *Neurospora crassa* cultures (Chan et al, 1991) and auxotrophic CHO cells (Kim et al, 1993).

The presence of $MTXGlu_n$ may contribute to overall therapeutic responsiveness, by inhibiting other enzymes of folate metabolism. This study indicates that $MTXGlu_3$ is a more potent inhibitor than MTX of DHFR in all three strains as characterized by the mean IC_{50} values of 2.7 nM and 10.0 nM, respectively (see Figures 20, 21). Schilsky et al (1983) and Kisliuk (1983) reported that in *Lactobacillus rhaminosis*, MTX inhibition increased by 30% when the diglutamate was provided. Likewise, the MTX IC_{50} values appeared to vary for different DHFR proteins (Crosti, 1981). In the present study, enzyme extracts collected and concentrated after Sephadex G-75 chromatography were utilized in the determination of MTX IC_{50} values, which enabled the removal of any inhibitory proteins that may have interfered with MTX-induced inhibition. Allegra et al (1985) examined the capacity of MTX and $MTXGlu_n$ with 1-4 additional glutamyl moieties in γ-peptide linkage to directly inhibit folate-dependent enzymes. These MTX polyglutamate derivatives were found to be pure competitive inhibitors of purified human AICAR transformylase. There was a 10-fold increase in the binding affinity with the addition of each glutamate residue (Allegra et al, 1985), which is consistent with the free energy change required to form one additional hydrogen bond between the enzyme and the new glutamyl group. $MTXGlu_5$ has been established as having 300 times the inhibitory effect on the catalytic activity of TS, as compared to the parent compound (Moran et al, 1979; Bunni et al, 1988). Moreover, MTX displayed an uncompetitive inhibition of TS while $MTXGlu_n$ derivatives non-competitively inhibited the enzyme such that the inhibitor binding does not require the prior binding of either substrate or product. Methylenetetrahydrofolate reductase and GAR transformylase exhibited a similar enhanced inhibition when presented with $MTXGlu_n$ derivatives rather than the parent, MTX. These previous investigations indicate that $MTXGlu_n$ may have additional sites of action, hence their capacity to inhibit $H_4PteGlu$-dependent enzymes in *N. crassa* should be ascertained.

Based on the present studies, it appears that MTX polyglutamylation in *Neurospora* is only slight and may not be significant to the overall sensitivity of growth in this antifolate. The intracellular levels of competing substrates must be considered in assessing the inability to conjugate long chain $MTXGlu_n$ derivatives. Reduced folate pools effectively compete with MTX for DHFR, as previously described, similarly these endogenous cofactors may compete with MTX for FPGS. The increased folate concentration found in wild type conidiospores (Chan and Cossins, 1984) provides a means whereby MTX polyglutamylation may be restricted. Nimec

and Galivan (1983) demonstrated that any reduction in cellular folate pools could effectively decrease the available FPGS for MTX polyglutamylation. Moreover, the subcellular distribution of the folate-dependent enzymes in *Saccharomyces cerevisiae* suggests the existence of a mitochondrial and cytoplasmic localization of DHFR. Likewise, examination of DHFR distribution in rat liver revealed the presence of the enzyme in the cytoplasmic and microsomal fraction (Blakley, 1969). The nuclei may contain significant levels of the enzyme for H_2PteGlu regeneration formed during thymidylate synthesis in mitochondria (Appling, 1991). This may provide an additional means of surmounting MTX inhibition through the continuous generation of reduced folate cofactors (Zelikson and Luzzati, 1977) as MTX may not effectively cross the mitochondrial membrane (Barrueco et al, 1992). Furthermore, the mitochondrial membrane would be impermeable to any $MTXGlu_n$, this would circumvent any potential inhibitory effects on other H_4PteGlu-requiring enzymes. In this regard, the cytosolic folate pool may represent the only site of MTX action. These investigations confirm that the ability of cells to synthesize $MTXGlu_n$ derivatives may, indeed, be a prognostic indicator of therapeutic effectiveness.

4.4. SUGGESTIONS FOR FURTHER RESEARCH

Folylpolyglutamate synthetase, an enzyme essential for cell survival, also has an important role in the metabolism of antifolates. Consequently, it represents a novel target enzyme for the design of cytotoxic antifolates. In this regard, it would be of considerable interest to determine the effectiveness of aminopterin or 3′,5′-dichloro-MTX on *Neurospora* cultures, as these strong inhibitors of DHFR are better substrates for FPGS and the MTX-H_4PteGlu transport carrier in human leukemia cells (Fabre et al, 1985). The cytotoxicity of non-polyglutamyl antifolates such as trimetrexate or piritrexim could be ascertained to formulate a comparison between cytocidal activity as a function of polyglutamylation versus inhibition of DHFR, alone.

ELISA experiments using polyclonal antisera to DHFR could be employed to determine DHFR content when this enzyme is complexed with MTX. An immunological assay would enable the measurement of DHFR content which is otherwise inactive in the spectrophotometric assay. Consequently, immunoblots performed with extracts from cells grown in the presence of MTX could reveal any significant change in DHFR content following exposure to MTX. Additionally, the subcellular distribution of DHFR activities in *Neurospora* should be investigated. This would facilitate an understanding of the primary cellular site(s) of action of MTX and other antifolates.

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