METHOTREXATE RESISTANCE IN L5178Y MOUSE LEUKEMIA CELLS

By

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Date **July 21st, 1982**
Methotrexate, a folic acid antagonist, has been used in the clinical treatment of a wide variety of malignant neoplasms for over 20 years, either as a single agent or in combination with other antineoplastic agents. It is a cell cycle specific inhibitor and kills cells only in the S phase of growth. MTX is a potent inhibitor of the enzyme dihydrofolate reductase (5,6,7,8-tetrahydrofolate: NADP⁺ oxidoreductase, EC 1.5.1.3.), which catalyses the NADPH dependent reduction of dihydrofolate acid and folic acid to tetrahydrofolic acid: the metabolically active coenzyme form of folic acid essential in the biosynthesis of dTMP from dUMP by thymidylate synthetase. Inhibition of DHFR therefore leads to the inhibition of DNA synthesis and cell death.

Methotrexate has many favourable properties; for instance, it interacts directly with intracellular sites without the need for prior metabolic transformation. It can be administered in large doses because toxicity to normal cells can be minimized by the administration of folinic acid (N⁵ formyl tetrahydrofolic acid) shortly after the administration of MTX. However, the effectiveness of MTX is inevitably compromised by the emergence of drug resistance, which can be either intrinsic, i.e. the tumour cells are resistant to MTX at the outset, or the tumour cells acquire resistance after exposure to MTX. An understanding of the mechanisms of resistance to MTX is therefore very important if treatment with this potent antineoplastic agent is to be improved.

Three mechanisms of resistance to MTX have been determined from studies with experimental tumour systems: impaired uptake of MTX; increased levels of dihydrofolate reductase; and appearance of altered
dihydrofolate reductase with a lower affinity for MTX. Impaired uptake of MTX and increased levels of DHFR can both theoretically be overcome by sustaining increased concentrations of free intracellular MTX. This can be achieved by exposing the cells to higher concentrations of MTX, and many chemotherapeutic regimens now use 'high-dose' MTX which can achieve plasma concentrations of MTX as high as $10^{-3}$ M. However, resistance to MTX is still a major clinical problem and the use of 'high-dose' MTX has not significantly increased the therapeutic index of MTX treatment.

Appearance of DHFR with a lower affinity for MTX suggests as an alternative the synthesis of an agent which would be a potent inhibitor of the altered enzyme, and requires the detailed characterization of the properties of this enzyme. If the altered enzyme retains some affinity for MTX, the administration of MTX and the more potent agent would result in better growth inhibition of the resistant tumor.

In this thesis, a mouse leukemia cell line (L5178Y) grown in suspension culture was used to isolate two MTX-resistant cell lines and these were used to study the mechanisms leading to MTX resistance.

Both resistant cell lines exhibited impaired MTX uptake when exposed to $10^{-6}$ M MTX but not when exposed to $10^{-4}$ M MTX. Both lines also had elevated DHFR levels (7 to 9 fold). A variant form of DHFR present in small amounts in both cell lines was isolated by MTX-sepharose affinity chromatography. The altered DHFR differed from the major form of reductase present in these cells in its markedly lower affinity (100,000 fold) for MTX. The two forms of the enzyme were
iv

purified from the most resistant cell line and their properties compared. They were found to differ moderately in their Km for substrates, however, the Ki of MTX differed by a factor of 100,000 for the two forms. In addition there were marked differences in their heat stability, isoelectric points and sensitivity to p-chloromercuriphenylsulphonate, and a minor difference in their molecular weights. It is concluded that the presence of a highly resistant form of DHFR in these cell lines represents an important mechanism in conferring a high degree of resistance to these cells. The importance of this form of DHFR in MTX resistance is discussed in relation to impaired transport and elevated DHFR levels. Experiments to determine the amino acid sequence of the altered enzyme are underway and once determined should facilitate the synthesis of specific inhibitors of its activity.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xi</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xiii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1. Cytotoxic mechanism of action of methotrexate</td>
<td>1</td>
</tr>
<tr>
<td>2. Properties of dihydrofolate reductase</td>
<td>3</td>
</tr>
<tr>
<td>3. Mechanisms of resistance to methotrexate</td>
<td>10</td>
</tr>
<tr>
<td>(1) Increased levels of dihydrofolate reductase.</td>
<td>12</td>
</tr>
<tr>
<td>(2) Impaired transport of methotrexate.</td>
<td>14</td>
</tr>
<tr>
<td>(3) Altered dihydrofolate reductases with lowered affinity for methotrexate.</td>
<td>18</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>23</td>
</tr>
<tr>
<td>1. Chemicals</td>
<td>23</td>
</tr>
<tr>
<td>2. Cell culture</td>
<td>23</td>
</tr>
<tr>
<td>3. Procedures for establishing methotrexate-resistant cells</td>
<td>24</td>
</tr>
<tr>
<td>4. Measurement of the initial rate of entry and the intracellular steady-state concentrations of methotrexate</td>
<td>24</td>
</tr>
<tr>
<td>5. Preparation of extracts</td>
<td>25</td>
</tr>
<tr>
<td>6. Assay for folate reductase activity</td>
<td>26</td>
</tr>
<tr>
<td>7. High pressure liquid chromatography of reduced and oxidized folates</td>
<td>27</td>
</tr>
<tr>
<td>8. Procedures for the isolation and purification of folate reductase</td>
<td>27</td>
</tr>
<tr>
<td>(a) MTX-sepharose affinity chromatography</td>
<td>28</td>
</tr>
</tbody>
</table>
(b) Sephadex G-100 chromatography
(c) Chromatography of Form 2 folate reductase on DEAE Affigel Blue

9. Gel Electrophoresis
10. Isoelectric focusing

RESULTS
1. Growth properties of the MTX-sensitive and MTX-resistant L5178Y mouse leukemic cells.
2. Properties of MTX uptake into L5178Y(S) and (R₃) cells.
3. Evidence of the presence of two forms of folate reductase in MTX-resistant L5178Y cells.
4. Purification of Forms 1 and 2 of DHFR from L5178Y(R₄) cells.
   (a) Affinity chromatography
   (b) Sephadex G-100 chromatography
5. Molecular weight determination

Properties of the MTX-sensitive (Form 1) and MTX-insensitive (Form 2) folate reductases.
   (a) pH optima
   (b) Heat inactivation
   (c) Effect of pCMS on enzyme activities
   (d) Kinetic properties
   (e) Electrophoretic properties

DISCUSSION AND CONCLUSIONS

REFERENCES
LIST OF TABLES

Table 1. Physical and kinetic properties of known dihydrofolate reductases. 7

Table 2. Properties of L5178Y (S), (R₃) and (R₄) cells. 37

Table 3. Properties of ³H-MTX influx into L5178Y(S) cells when exposed to 10⁻⁶ M MTX. 42

Table 4. Properties of ³H-MTX influx into L5178Y(S) and (R₃) cells when exposed to 10⁻⁴ M MTX. 43

Table 5. Purification of Form I and Form 2 folate reductases from L5178Y (R₄) cells. 59

Table 6. Summary of the kinetic properties of Form 1 and Form 2 folate reductases. 75
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.</td>
<td>Structural formulae of methotrexate and folic acid.</td>
<td>2</td>
</tr>
<tr>
<td>Figure 2.</td>
<td>Summary of pathways of 'one-carbon' transfer reactions.</td>
<td>5</td>
</tr>
<tr>
<td>Figure 3.</td>
<td>Diagrammatic illustration of a carrier transport model to account for the unidirectional influx of MTX which occurs prior to saturation of the dihydrofolate reductase binding sites.</td>
<td>16</td>
</tr>
<tr>
<td>Figure 4.</td>
<td>Growth curve of L5178Y(S) cells.</td>
<td>34</td>
</tr>
<tr>
<td>Figure 5.</td>
<td>Effect of MTX on proliferation of L5178Y(S), (R₃) and (R₄) cells.</td>
<td>36</td>
</tr>
<tr>
<td>Figure 6.</td>
<td>Time course of uptake of ^3H-MTX by L5178Y(S) and (R₃) cells.</td>
<td>40</td>
</tr>
</tbody>
</table>
| Figure 7. | Methotrexate-sepharose affinity chromatography of cellular lysates from A. L5178Y(S) cells  
B. L5178Y (R₃) cells  
C. L5178Y (R₄) cells.                                                                                                                         | 46   |
| Figure 8. | High pressure liquid chromatography of various folate compounds and the reaction products of folate reductase assays. A. HPLC of a standard mixture of folates. B. HPLC of the reaction products of folate reductase activity in L5178Y(S) lysate. C. HPLC of the reaction products of Form 2 folate reductase activity. | 50   |
| Figure 9. | Inhibition of folate reductase activity from MTX-sensitive and MTX-resistant cells by MTX.                                                                                                                   | 55   |
| Figure 10. | Gel filtration on Sephadex G-100 of folate reductase Forms 1 and 2 from L5178Y (R₄) cells. A. Form 1  
B. Form 2.                                                                                                                                 | 58   |
| Figure 11. | Photograph of SDS-polyacrylamide disc gel electrophoresis stained with Coomassie blue. A. Form 2 folate reductase from R₄ cells  
B. Form 1 folate reductase from R₄ cells  
C. Molecular weight standards.                                                                                                                   | 62   |
Figure 12. Photograph of SDS-polyacrylamide slab gel electrophoresis stained by the silver staining method. Electrophoresis of Form 2 folate reductase at various stages of purification. 64

Figure 13. Chromatography of Form 2 folate reductase on DEAE Affigel Blue. 66

Figure 14. Effect of temperature on the enzyme activities of Form 1 and Form 2 folate reductases
A. Effect of heat on the enzyme activities of L5178Y(S) and (R₄) lysates.
B. Effect of heat on the enzyme activities of partially purified Form 1 and Form 2 folate reductases. 69

Figure 15. Effect of pCMS on the enzyme activities of Form 1 and Form 2 folate reductases. 71

Figure 16. Double reciprocal plots of initial velocity patterns.
A. Km for folic acid of Form 2 folate reductase
B. Km for folic acid of Form 1 folate reductase
C. Km for NADPH of Form 1 folate reductase
D. Km for NADPH of Form 2 folate reductase. 74

Figure 17. Dixon plots for the determination of MTX dissociation constants.
A. L5178Y(S) folate reductase 77
B. L5178Y(R₃) Form 2 folate reductase 78
C. L5178Y(R₄) Form 2 folate reductase. 78

Figure 18. Electrophoresis of Form 1 and Form 2 folate reductases on 7.5% non-denaturing polyacrylamide gels.
A. Stained for enzyme activity in the absence of MTX.
B. Stained for enzyme activity in the presence of MTX. 81

Figure 19. Photograph of Giemsa-trypsin-stained metaphase chromosomes from:
A. L5178Y (S) cells. 83
B. L5178Y (R₄) cells. 84
Figure 20. Schematic representation of the effect of MTX on folate metabolism in MTX-sensitive and MTX-resistant L5178Y cells.
AH Sepharose 4B: Aminohexyl sepharose 4B.

DEAE: Diethylaminoethyl.

DHFR: Dihydrofolate reductase or folate reductase depending on whether dihydrofolic acid or folic acid is used as substrate.

DNA: Deoxyribonucleic acid.

dTMP: Deoxythymidine monophosphate.

dUMP: Deoxyuridine monophosphate.

E. Coli: Escherischia Coli.

EDC: 1-Ethyl-3(3-Dimethyl-aminopropyl)-carbodiimide hydrochloride.

HnRNA: Heterogeneous nuclear RNA.

HPLC: High pressure liquid chromatography.

HSR: Homogeneous staining region.

KHz: Kilohertz.

L. Casei: Lactobacillus Casei.

mRNA: Messenger RNA.

MTT: 3(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide.

MTX: Methotrexate.

NADP: Nicotinamide adenine dinucleotide phosphate.

NADPH: Nicotinamide adenine dinucleotide phosphate, reduced form.

pCMB: Para-chloromercrubenzoate.

pCMS: Para-chloromercubiphenylsulphonate.

Poly(A): Polyadenylate.

Q₁₀: Change in reaction velocity for every 10° difference in the temperature of reaction.
| **RNA:**   | Ribonucleic acid.                |
| **SDS:**   | Sodium dodecyl sulphate.         |
| **TEMED:** | N,N,N',N'-Tetramethylethylenediamine. |
| **U.V.:**  | Ultraviolet.                     |
| **Vmax:**  | Maximum velocity of reaction.    |
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INTRODUCTION

1. CYTOTOXIC MECHANISM OF ACTION OF METHOTREXATE.

In the early 1940's, nutritional studies designed to discover the cause of certain forms of anaemia indicated the lack of an important factor in the diets of subjects exhibiting such a disorder, (Pfiffner et al., 1943). This factor was isolated and its chemical and physical properties characterized. It was shown to be a pteridine derivative and was called "Folic acid," (Latin, folium, leaf). The structure of folic acid was elucidated in 1946, (Angier et al., 1946) and was soon followed by the synthesis and subsequent investigation of the biological activity of a number of potent antagonists. The three most widely studied and clinically useful folate antagonists were found to be aminopterin, methotrexate (MTX), and 3', 5' dichloro-MTX. These compounds are all 4-amino analogs of folic acid and are powerful inhibitors of the enzyme dihydrofolate reductase (DHFR). Of these compounds MTX is the most potent inhibitor of DHFR and has been in clinical use as an antineoplastic agent since the early 1950's. The structural formulae of folic acid and MTX are shown in Fig. 1. Dihydrofolate reductase carries out the NADPH dependent reduction of folic acid and dihydrofolic acid to tetrahydrofolic acid, (Futterman, 1957; Osborn et al., 1958; Peters et al., 1959). Tetrahydrofolic acid is the metabolically active coenzyme form of folic acid, and functions as a carrier of "one carbon" units in the biosynthesis of the purine ring, thymidylate, methionine, serine and histidine (Huennekens, 1963; Huennekens et al.,
Pteric Acid

P - Amino Benzoic Acid

Glutamic Acid

FOLIC ACID (Pteroylglutamic Acid)

METHOTREXATE

(2, 4 diamino N\textsubscript{10} Methyl Pteroylglutamic Acid)
A summary of these various pathways is shown in Fig. 2. A lack of this coenzyme, therefore, caused either by dietary deficiency or the administration of a folate antagonist results in decreased synthesis of purines, thymidylate and proteins. Although there have been reports indicating that in certain tissues a defect in purine synthesis is responsible for the effects of antifolates (e.g. MTX), (Goldthwait et al., 1952; Sartorelli et al., 1958), a number of studies showed that an inhibition of thymidylate synthesis is the primary event leading to cell death after exposure to MTX. (Totter et al., 1955; Borsa et al., 1969; Capizzi et al., 1971). This type of cell death is called "Thymineless" death due to the decrease in the intracellular pools of thymidylate. MTX has been shown to be most potent during the S phase of the cell cycle, (Hryniuk et al., 1969; Bruce, 1970). In addition to being a very strong inhibitor of DHFR, MTX has also been found to be a weak, competitive inhibitor of thymidylate synthetase, (Borsa and Whitmore, 1969). Since the early studies on the mechanism of action of MTX, it has become increasingly apparent that the primary intracellular target of MTX is dihydrofolate reductase, and the subsequent decrease in the levels of tetrahydrofolic acid coenzymes, (especially methylene tetrahydrofolic acid) leads to a decrease in intracellular thymidylate pools and to decreased and unbalanced DNA synthesis.

2. PROPERTIES OF DIHYDROFOLATE REDUCTASE

All known dihydrofolate reductases readily reduce 7, 8-dihydrofolate [EQ. (1)] and, more slowly, folic acid [EQ. (2)], to tetrahydrofolate (Blakeley, 1969; Huennekens et al., 1971).
A summary of pathways illustrating the central role of dihydrofolate reductase in 'one-carbon' transfer reactions leading to the biosynthesis of purines, pyrimidines and amino acids.
FA
2NADPH
2NADP
FH4 + Serine
ATP ADP+Pi
FH4 + HCO3H
N0-Formyl
FH4
N8, N10-Methylene FH4
N8-Methyl FH4
FH4
Homocysteine
NAD NADH
NADP
NADPH
dUMP
DNA
Glycine
Pyridoxal Phosphate
N8, N10-Methylene FH4
FH4
Purines

1. Dihydrofolate Reductase
2. Serine Hydroxymethyl Transferase
3. Thymidylate Synthetase
4. Cyclohydrolase
5. FH4 Formylase
[EQ. (1)] \[ 7,8\text{-dihydrofolate} + \text{NADPH} + \text{H}^+ \rightarrow 5,6,7,8\text{-tetrahydrofolate} + \text{NADP}^+ \]

[EQ. (2)] \[ \text{Folate} + 2\text{NADPH} + 2\text{H}^+ \rightarrow 5,6,7,8\text{-tetrahydrofolate} + 2\text{NADP}^+ \]

Most of the known dihydrofolate reductases are strongly inhibited by MTX; (Werkheiser, 1961; Bertino et al, 1964) the inhibition is pH dependent and stoichiometric at acid pH, and competitive with dihydrofolic acid, at physiological pH; the reported Ki values being approximately \(10^{-9}\) M or lower, (Huennekens, 1968a; Blakeley, 1969). Exceptions to this observation have been reported however, and will be discussed more fully in the section on mechanisms of resistance to MTX. Dihydrofolate reductases have been purified and characterized from a variety of sources, including several normal and malignant cell lines. While all share one common property, i.e., catalysis of the reactions in EQ. (1) and (2), these enzymes exhibit considerable diversity with respect to both structural and kinetic parameters. These properties are summarized in Table 1. The molecular weights can be grouped into four general categories, with the DHFRs from mammalian sources (with the exception of calf thymus enzyme-MWt. 33,500) having molecular weights ranging from 20,000 to 23,000. Most of the mammalian reductases also exhibit double pH optima, but there is considerable variation in the turnover numbers and the Km values for the substrates, dihydrofolate and NADPH.
<table>
<thead>
<tr>
<th>SOURCE</th>
<th>MWt.</th>
<th>SAC*</th>
<th>pH of Assay</th>
<th>NADPH</th>
<th>Km (M) Dihydrofolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef Liver</td>
<td>20,000</td>
<td>1.09</td>
<td>6.25</td>
<td></td>
<td>1.5x10^-5 6x10^-6</td>
</tr>
<tr>
<td></td>
<td>21,100</td>
<td>103</td>
<td>6.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pig Liver</td>
<td>20,000</td>
<td></td>
<td>7.2</td>
<td></td>
<td>3.1x10^-7 5x10^-8</td>
</tr>
<tr>
<td>Chicken Liver</td>
<td>22,000</td>
<td>15</td>
<td>7.4</td>
<td></td>
<td>1.8x10^-6 1.2x10^-7</td>
</tr>
<tr>
<td>Calf Thymus</td>
<td>33,500</td>
<td>16.3</td>
<td>6.0</td>
<td></td>
<td>3.3x10^-5 2.3x10^-6</td>
</tr>
<tr>
<td>Ehrlich Ascites Cells</td>
<td>20,200</td>
<td>1.0</td>
<td>7.5</td>
<td></td>
<td>5.6x10^-6 4x10^-7</td>
</tr>
<tr>
<td>L1210 Cells (R)a</td>
<td>20,000</td>
<td>44.5</td>
<td>7.0</td>
<td></td>
<td>5.0x10^-6 4x10^-7</td>
</tr>
<tr>
<td>in mice L1210 Cells (R)</td>
<td>20,000</td>
<td>23</td>
<td>7.0</td>
<td></td>
<td>3.8x10^-6 3x10^-7</td>
</tr>
<tr>
<td>in culture Sarcoma 180 cells (R)</td>
<td>21,000</td>
<td>7.2</td>
<td>7.0</td>
<td>1.7x10^-6</td>
<td>3x10^-7</td>
</tr>
<tr>
<td>Baby Hamster kidney cells (R)</td>
<td>23,000</td>
<td>4.7</td>
<td>7.0</td>
<td>1.6x10^-4</td>
<td></td>
</tr>
</tbody>
</table>

* Specific activity is expressed in μmoles/min/Mg protein.

a. Resistant to folate antagonist.
From Huennekens et al, (1976).
The amino acid sequences of dihydrofolate reductases from
Streptococcus faecium (Gleisner et al., 1974), Escherichia coli (Bennet et
al., 1977; Stone et al., 1977), Lactobacillus casei (Bitar, K.G. et al.,
1977), chicken liver (Kumar et al., 1980), L1210 mouse leukemia cells
(Stone et al., 1979) and porcine liver (Smith et al., 1979), have been
determined so far. Considerable homology, especially in the first
N-terminal 30-35 amino acid residues, exists between the above
mentioned mammalian reductases (Freisheim et al., 1979). X-ray
crystallographic studies of the bacterial enzymes have identified some of
the amino acids involved in the binding of the substrates (dihydrofolic
acid or folic acid and NADPH) and MTX (Mathews et al., 1978), and
most of these are conserved in all the reductases sequenced so far.

Several of the mammalian dihydrofolate reductases have two
cysteine residues per molecule, one buried and one accessible.
Modification of the accessible residue by organic mercurial compounds
such as pCMS, pCMB, or methylmercuric hydroxide (MeHgOH) results,
in some instances, to an increase in catalytic activity of the enzyme
(Perkins and Bertino, 1966; Kaufman 1964, 1966; Freisheim et al.,
1979; Goldie et al., 1981). However, bacterial reductases are inhibited
by these compounds (Warwick and Freisheim, 1975).

The various dihydrofolate reductases also differ with respect to
their pH profiles, e.g., the reductase from chicken liver cells,
described by Mathews and Huenekeens, (1963) has a double pH optimum
(with higher activity at acidic pH values); that from Lactobacillus
Leuhranii, a single pH optimum (Kessel and Roberts, 1965); and that
from human leukocytes, a single but very broad optimum over a wide pH range (Bertino et al., 1970.)

Kinetic data for numerous dihydrofolate reductases are available and the Km values for the substrates, dihydrofolic acid and NADPH are listed in Table 1. Analyses of kinetic data have revealed that both the L1210 mouse leukemia (McCollough et al., 1971), and E. Coli (Burchall, 1970) enzymes utilize a random type mechanism, i.e. either dihydrofolate (or folic acid), or NADPH may bind first. Ki values for MTX of most DHFRs are of the order of $10^{-9}$ M or lower.

A very interesting feature of dihydrofolate reductases is their ability to exist in multiple forms that can be separated electrophoretically or chromatographically. Electrophoresis of partially purified preparations from chicken liver and L1210 mouse leukemia cells on cellulose acetate membranes, followed by staining for enzyme activity, revealed the presence of at least 2 forms of reductases (Mell et al., 1968). Further work with the enzyme from MTX-resistant L. Casei showed that the 2 forms of reductases were related by the presence or absence of one equivalent of NADPH, which appeared to be tightly bound in L. Casei but only weakly bound in the chicken liver enzyme, (Dunlap et al., 1971).

Some of the multiplicity observed with dihydrofolate reductase is however due to the expression of genetically different forms of the enzyme by both procaryotic and eucaryotic cells. S. Faecuim var. durans A contains two reductases; one is specific for dihydrofolate whereas the other can utilize both dihydrofolate and folate as substrate
(Nixon et al, 1968; Albrecht et al, 1969). It has been shown that there are several distinct types of *E. Coli* which contain extrachromosomal R-factors and produce both the normal trimethoprim (a DHFR inhibitor) sensitive chromosomal dihydrofolate reductase and novel trimethoprim resistant R-factor reductase (Skold et al, 1974; Pattschall et al, 1977). In addition, *E. Coli* RT500 has two genetically independent isoenzymes, as revealed by their differences in primary structure (Baccanari et al, 1979).

Genetically different forms of dihydrofolate reductases have also been shown to be present in mammalian cells, however, the appearance of these forms is usually associated with resistance to MTX. In addition, the different forms tend to have different binding capacities for MTX with the resistant cells producing reductases with reduced affinities for MTX (Albrecht et al, 1972; Flintoff et al, 1976, 1980; Goldie et al, 1980, 1981; Melera et al, 1980; Haber et al, 1981).

3. MECHANISMS OF RESISTANCE TO MTX

Methotrexate is one of the most useful antineoplastic agents presently available, having a broad spectrum of antitumour activity. It has been found clinically useful against a variety of cancers, either as a single agent or in combination with other antineoplastic drugs. Being a cell cycle specific inhibitor, killing cells only in the S phase of growth, it is most potent against tumours with large growth fractions and short generation times and least potent against tumours with a small growth fraction and long generation times.
MTX interacts directly at intracellular sites without the need for prior metabolic transformation. It can be administered in large doses because toxicity to normal cells can be reversed by the administration of folinic acid (N\textsubscript{5} formyl tetrahydrofolic acid), the so-called "Folinic acid rescue." In spite of these favourable properties, the clinical usefulness of MTX is limited inevitably by drug resistance: either intrinsic resistance, or acquired resistance after exposure of the tumour to MTX. From numerous studies on experimental tumour systems, three mechanisms of acquired resistance have been determined:

(1) Increased levels of dihydrofolate reductase (Fischer, 1961; Chang and Littlefield (1976); Schimke et al., 1978; Niethammer and Jackson, 1975; Dolnick et al., 1979; and Goldie et al., 1980).

(2) Impaired transport of MTX (Fischer, 1962; Harrap et al., 1971; Jackson et al., 1975).

(3) Generation of altered dihydrofolate reductase with a lower affinity (increased Ki) for MTX (Jackson et al., 1976; Flintoff et al., 1976, 1980; Gupta et al., 1977; Goldie et al., 1980, 1981; Haber et al., 1981).

Other possible mechanisms of resistance have been reported in the literature and include increased levels of thymidylate synthetase (Maley and Maley, 1971) and the induction of the thymidine kinase salvage pathway (Wilmanns, 1971); however, the latter mechanism depends upon the presence of relatively high levels of exogenous thymidine not usually encountered under physiological conditions.
Each of the mechanisms of resistance will now be considered in more detail:

(1) **Increased levels of dihydrofolate reductase.**

This phenomenon was first described by Hakala *et al.*, (1961) who found that cultured Sarcoma 180 cells gradually became resistant to MTX upon exposure to the drug, and at the same time dihydrofolate reductase activity increased proportionally with the degree of resistance. The enzyme from the resistant cells (elevated up to 154 fold) did not differ from the enzyme from sensitive cells in terms of $K_m$ for the substrates or turnover number per MTX binding site, and it was deduced that resistance in this case resulted from the production of a large excess of normal DHFR which immobilized all of the intracellular MTX, leaving enough free enzyme to carry on its normal functions. Similar observations were made by Fischer, (1961) in MTX-resistant L5178Y cells. Since that time many other cell lines resistant to MTX exhibited this phenomenon. However, the mechanism for the increased levels of DHFR were not clear until 1972 when Nakamura and Littlefield demonstrated by immunologic and inhibitor titration studies that this elevation in DHFR activity was due to an increase in the reductase protein resulting from an increase in the relative rate of DHFR synthesis. This was substantiated later and was found to be due to an alteration in the regulatory mechanism controlling the rate of synthesis of the enzyme (Hailingi and Littlefield, 1976; Alt *et al*, 1976). The increased rate of synthesis of DHFR was shown to be related to elevated quantities of DHFR mRNA which was capable of synthesizing DHFR in a cell-free system derived from wheat germ (Chang and Littlefield, 1976; Kellems *et al*, 1976). Some of the MTX-resistant cell
lines have up to 300 fold elevations in the amount of DHFR (representing up to 6% of the total soluble protein in these cells), as compared with the parent sensitive cells. The simultaneous increase in the mRNA for the enzyme facilitated the \textit{in vitro} synthesis of the corresponding complimentary DNA (cDNA), and utilizing this probe Alt et al, (1978) and Schimke et al, (1978) were able to demonstrate selective gene multiplication leading to an increase in the number of dihydrofolate reductase genes.

This amplification of the dihydrofolate reductase genes in stably MTX-resistant cell populations has been found to be associated with a long homogeneously staining chromosomal region (usually on chromosome 2) upon Trypsin-Giemsa banding analysis of metaphase chromosomes. Non banding (homogeneous) chromosomal regions in cells producing high dihydrofolate reductase levels was first demonstrated by Biedler et al, in 1974. Similar findings have been reported since in MTX-resistant sarcoma S-180 cells (Numberg et al, 1978), in MTX-resistant L5178Y cells (Dolnick et al, 1979; Berenson et al, 1981) and in MTX-resistant mouse lymphoma EL4 cells and mouse melanoma P619 cells (Bostock and Tyler-Smith, 1981). In all of these cases resistance was shown to be stable i.e. MTX resistance and DHFR gene copy number were stable when cells were grown in the absence of selection pressure (i.e. MTX). MTX resistant cell lines exhibiting unstable amplification of DHFR gene sequences (loss of resistance upon removal of selection pressure and simultaneous loss of amplified DHFR DNA sequences) have been found to be associated with small, paired chromosomal elements denoted "double minute chromosomes." (Kaufman et al, 1979; Bostock and Tyler-Smith, 1981).
The mechanisms by which the amplified DHFR sequences become incorporated into normal chromosomes or appear as "double minute chromosomes" involve extensive chromosomal rearrangement with many intermediate chromosomal forms leading, either to stable, or unstable amplification (Tyler-Smith and Alderson, 1981; Bostock and Tyler-Smith, 1981; Tyler-Smith and Bostock, 1981), the details of which are beyond the scope of this thesis.

The appearance of amplified DHFR gene sequences in MTX-resistant mouse S-180 cells has led to the elucidation of the structure and organization of mouse DHFR gene (Nunberg et al., 1980). The gene contains a minimum of five intervening sequences and spans a minimum of 42 kilobase pairs on the genome. Four of the intervening sequences occur within the protein-coding region of the gene, and one intervening sequence occurs within the 5' untranslated region. Such a large amount of intervening (untranslated) sequences has not been found in any other gene analyzed to date, and its significance is not clear. The active mRNA was found to be only 1600 nucleotides long and contained a 3' poly(A) tail.

2) Impaired Transport of Methotrexate.

A high-affinity transport system for MTX has been established in a number of mammalian cells which conform to a carrier model that MTX shares with \(N_5\)-methyl tetrahydrofolate and \(N_5\)-formyl tetrahydrofolate (Goldman, 1971; Bender, 1975). The mechanism for active influx of MTX into cells is illustrated in Figure 3. The relationship between the extracellular MTX concentration and the unidirectional influx of MTX adheres to Michaelis-Menten kinetics, and 5-methyl tetrahydrofolate and 5-formyl tetrahydrofolate are competitive inhibitors of this process.
A carrier transport model to account for the undirectional influx of MTX which occurs prior to the saturation of dihydrofolate reductase binding sites. Membrane carrier is denoted by 'C'. The dissociation constant for the MTX-carrier reactions at the outer and inner cell membrane are denoted as $k_{-1}/k_1$ and $k_{-3}/k_3$ respectively.

Adapted from Goldman (1971).
EXTRACELLULAR

MTX
MTX
MTX
MTX
MTX

MTX MEMBRANE

MTX
MTX
MTX
MTX
MTX

|MTX-C|MTX-C|

k-2

k-4

k-1

k1

k2

k3

k4

C

C

INTRACELLULAR

+MTX

Binds to Dihydrofolate Reductase

MTX

Binds to Dihydrofolate Reductase

MTX
Influx is highly temperature dependent and pH dependent and the carrier-MTX interaction involves sulphydryl groups, since influx is strongly inhibited by p-hydroxy mercuribenzoate (Goldman et al., 1968; Sinotnak et al., 1968) and p-chloromercuriphenyl sulphonate (pCMS) (Rader et al., 1974). Plasma membrane-associated protein components appearing to play a role in MTX transport in L1210 lymphoma cells have been identified and partially characterized (McCormick et al., 1979).

The steady-state intracellular level of MTX not only depends on the rate of influx of MTX, but also on the concentration of DHFR and on the rate of efflux of MTX. The latter has been shown to be inhibited by metabolic inhibitors (sodium azide and dinitrophenol), and thus exposure of cells to MTX in the presence of metabolic inhibitors results in a net increase in the intracellular steady state level (Hakala, 1965; Goldman, 1969). Dembo and Sirotnak (1976) have provided data for the hypothesis that influx and efflux of MTX take place via different carriers thus accounting for the fact that influx and efflux of MTX are differentially affected by metabolic inhibitors.

Impaired membrane transport of MTX as a mechanism of cellular resistance to this agent was first observed in a strain of L5178Y leukemia cells in vitro by Fischer (1962). Later, the relationship between influx of MTX in vitro and cytotoxicity in vivo was correlated for a variety of murine leukemias (Kessel et al., 1965). It has been shown that free intracellular MTX in excess of the DHFR-binding capacity is needed to suppress cellular dihydrofolate reduction (White and Goldman, 1976). When free intracellular MTX is low but dihydrofolate is high, displacement of bound MTX by dihydrofolate plays an important role in reversing the toxic effects of MTX (White,
1979). The amount of free intracellular MTX is related to its influx kinetics. Impaired transport in MTX-resistant human lymphoblastoid cell line was attributed to a marked decrease in the influx Vmax for the MTX carrier system (Niethammer and Jackson, 1975), and impaired transport in resistant lines of L1210 mouse leukemia cells has been related to an increase in the influx Km (Jackson et al, 1975; Sirontrak et al, 1968). Hill et al, (1979; 1982), have shown that while the difference in MTX transport in sensitive and resistant L5178Y cells is profound at low concentrations, this difference is diminished as the extracellular concentrations are increased. Hence, when the extracellular MTX level is sufficiently high, resistance to this agent should be overcome.

3) Altered dihydrofolate reductases with lowered affinity for MTX.

As mentioned earlier, genetically different forms of dihydrofolate reductases have been shown to be present in mammalian cells. These variant forms of DHFR are most often associated with resistance to MTX and the enzymes usually have a lower affinity (higher Ki) for MTX than the DHFRs from the parent MTX-sensitive cells.

Intrinsic resistance to MTX of cultured mammalian cells has been correlated with the Ki's of dihydrofolate reductases for MTX (Harrap et al, 1971; Jackson et al, 1976). In the study by Harrap et al, the cell lines: Yoshida ascites sarcoma, mouse leukemia L1210 and mouse leukemia L5178Y, were evaluated with respect to sensitivity to MTX (dose of MTX required to reduce the surviving fraction of cells to less than 10%). Yoshida cells were 10 fold more resistant than L5178Y cells which were 10 fold more resistant than L1210 cells. The Ki's of DHFRs for MTX
from these three cell lines followed this same order, i.e. the Yoshida DHFR had a higher Ki (lower affinity) for MTX than L5178Y DHFR which had a higher Ki than L1210 DHFR. All three cell lines transported the drug at comparable rates and the specific activities of dihydrofolate reductases were also comparable. It appeared therefore that in these three cell lines differences in MTX sensitivity could be directly correlated with the KIs of dihydrofolate reductases for MTX. These results were confirmed by Jackson et al. (1976) who carried out similar studies with more stringent analyses of the Ki values. Human melanoma cells have been reported to be intrinsically resistant to MTX (Kufe et al., 1980), and although the resistance in this case was attributed to elevated intracellular levels of DHFR, the presence of DHFR with a lower affinity of MTX could not be eliminated because of the method used for the assay for DHFR activity (³H-MTX ligand binding assay). Proliferating normal rabbit epidermal cells have also been found to be intrinsically resistant to MTX (Harper and Flaxman, 1981), although the mechanism of resistance in this instance was not clear.

Mammalian cells, initially highly sensitive to MTX, acquire resistance to the drug upon exposure to increasing concentrations of MTX. Albrecht et al., (1972) reported the synthesis of altered DHFR in two MTX-resistant lines of Chinese hamster cells. Ki values for MTX of the DHFR from these cells were not determined; however, whereas the enzyme from the parent MTX-sensitive cells interacted stoichiometrically with MTX, the inhibition of DHFR from the resistant cells was reversible, reflecting weak interaction. In addition, some other properties of the enzyme were different from those of DHFR from sensitive cells in terms of temperature sensitivity, pH optima, and the effect of NADPH on the inhibition of DHFR activity.
Jackson and Niethammer (1977) described the properties of an altered dihydrofolate reductase from MTX-resistant human lymphoblastoid cells. These cells were resistant to \(10^{-6}\) M MTX; the Km for dihydrofolate was 18-fold higher than that of the DHFR from the parent line, and the affinity for MTX was 50-fold lower. The "low-affinity" DHFR differed in its temperature sensitivity (more heat labile) from the parent DHFR but both forms of the enzyme had similar molecular weights (22,500). In addition to the structural alteration of the DHFR from MTX-resistant cells, the total activity of the enzyme had increased greatly over that in the parent cells (230 fold higher), probably by the mechanism of gene amplification.

Flintoff et al, (1976) demonstrated the presence of normal amounts of an altered DHFR with decreased affinity for MTX from MTX-resistant Chinese hamster ovary cells. Selection for increased resistance produced cells which appeared to possess increased activity of altered enzyme (Gupta et al, 1977). The mutant enzymes (6 to 8 fold more resistant to inhibition by MTX than wild type enzyme) and the wild type enzyme also demonstrated small differences in pH optima, Km for folate and heat stability. Further characterization of the wild type and altered reductases by two-dimensional gel electrophoresis failed to reveal any charge differences between the two forms.

Most recently, Haber et al, (1981) demonstrated the presence of elevated levels of altered DHFR with a lower affinity for MTX in highly MTX-resistant 3T6 mouse embryo fibroblasts. This enzyme exhibited a 270-fold reduction in binding affinity for MTX. In addition the Km for dihydrofolic acid was increased 3-fold, the pH optimum was different and, although the molecular weight was similar to the enzyme from parent cells, the altered enzyme demonstrated a significant basic shift in electrophoretic migration.
Ample evidence therefore exists for the presence of altered forms of DHFRs in MTX-resistant cells. Although the alterations are associated with decreased affinity for MTX in all of these cases, the degree of loss of affinity varies considerably, as do the alterations in other properties. It therefore appears that in as much as amino acid substitution at various points in the dihydrofolate reductase molecule can effect MTX-binding (Matthews et al., 1977), a variety of altered enzymes which differ in physical and kinetic properties can be expected. Selection with stepwise increases in concentrations of MTX initially appear to yield MTX-resistant cells expressing wild type DHFR expressed at high levels, however, growth at high MTX concentrations result in the prevalence of cells expressing elevated levels of DHFR with reduced affinity for MTX (Haber et al., 1981).

It has been noted by Biedler et al. (1972), that the correlation between DHFR activity and the level of drug resistance varied for different sublines of Chinese hamster cells grown in increasing inhibitor concentrations. The cells containing lower levels of DHFR than expected for their degree of resistance were found to contain an enzyme with reduced affinity for MTX (Albrecht et al., 1972).

MTX-resistant cells exhibiting two of the three mechanisms of resistance have been isolated. For example cells may have impaired uptake of MTX as well as elevated levels of DHFR, or elevation of DHFR is also accompanied by an alteration in the structure of the enzyme leading to decreased affinity for MTX.

The present investigation was undertaken with the aim of studying these three mechanisms further and to assess the importance of each in imparting a high degree of resistance to the cell. In addition, if an
altered form of DHFR with a lower affinity for MTX was present in MTX-resistant cells, isolation, purification and characterization of its physical and chemical properties could be carried out and compared with the enzyme from MTX-sensitive cells i.e. one with a high affinity for MTX. These experiments would lead to an insight into the nature of the alterations leading to reduced affinity, and would facilitate the synthesis of more potent inhibitors of its activity.
METHODS AND MATERIALS

1. Chemicals

\[ {^2}{^{14}}\text{C}] \text{Folic acid (55mCi/m mole)}, \left[3,5,9^3\text{H}\right] \text{MTX}, \left[3,5,7,9^3\text{H}\right] \text{Folic acid (29Ci/m mole)} \text{ and PCS liquid scintillation fluid were purchased from Amersham Corp., Oakville, Ontario.}

Fisher's medium for leukemic cells of mice, horse serum (dialyzed and undialyzed), glutamine and penicillin/streptomycin were all obtained from Grand Island Biological Co., Grand Island N.Y.

Dihydrofolic acid, NADPH, MTT, AH-Sepharose-4B, EDC, pCMS, 5-methyltetrahydrofolic acid, p-aminobenzoic acid, p-aminobenzoyl glutamate, tetrahydrofolic acid were purchased from Sigma Chemicals, St. Lo., Miss.

\( \text{N}_{5}\)-Formylfolic acid was purchased from ICN Pharmaceuticals Inc., Plainview, N.Y.

Methotrexate was purchased from Lederle, Montreal.

Acrylamide, Methylenebisacrylamide, ammonium persulphate, TEMED, SDS, DEAE Affigel blue were purchased from Bio-Rad Laboratories, Richmond, California.

Sephadex G100 was obtained from Pharmacia (Canada) Inc., Dorval, Quebec.

2. Cell culture:

L5178Y mouse leukemia cells were initially passaged in the peritonium of BDF\(_1\) mice. One week after the inoculation the cells were removed and were grown at 37°C as suspension cultures in plastic flasks or tubes (Corning) under an atmosphere of 5% \(\text{CO}_2\) in air. The
growth medium was Fisher's medium supplemented with 10% dialyzed horse serum, 1% glutamine and 100U/ml penicillin - 100μl/ml streptomycin (Grand Island Biological Co., Grand Island, New York). Cell stocks were diluted every one or two days with fresh medium to maintain the cultures in exponential growth. Under these conditions the doubling time for all three cell lines was approximately 12 hours. Total cell numbers were determined by counting on a Model ZB1 Coulter Counter (Coulter Electronics, Hialeah, Fla.).

3. Procedures for establishing MTX-resistant cells.

Two stable MTX-resistant cell lines were developed from the parent L5178Y cells, designated L5178Y(S). The properties of these cells are summarised in Table 2. The two resistant sublines were designated L5178Y(R₃) and L5178Y(R₄). The resistant sublines were obtained by subculturing the cells in progressively increasing sublethal concentrations of MTX until the desired level of resistance had been achieved. Both resistant lines were maintained in MTX containing medium; the R₃ cell line grew in 10⁻⁵M MTX and R₄ cells in 10⁻³M MTX. R₄ cells were derived from R₃ cells.

4. Measurement of the initial rate of entry and the intracellular steady-state concentrations of MTX.

L5178Y lymphoblasts in exponential growth at a density of 5-7 x 10⁵ cells/ml were dispensed in 2 ml aliquots into stoppered tubes. MTX-resistant cells were washed twice with MTX-free Fisher's medium without serum and allowed to grow for 2-3 days in drug-free media, with serum, prior to experimentation.
Radiolabelled MTX was added to the shaking cell suspensions at 37°C to a final concentration of $10^{-6}$ M or $10^{-4}$ M. At various times, tubes were removed from the water bath, plunged into ice and 8 ml ice-cold 0.9% sodium chloride solution was added to each tube and the cells immediately removed from suspension by centrifuging at 350 g. for 6 min. at 4°C. The cells were washed twice further with ice-cold 0.9% NaCl solution. The resulting cell pellets were solubilized in 0.5 ml IN NaOH at 37°C for 12 hours. 0.25 ml of the resulting solutions were added to 10 ml PCS liquid scintillation fluid. 50µl 6N HCl was added to each scintillation cocktail to neutralize the alkali and 0.7ml distilled water was added to obtain a homogeneous, clear scintillation cocktail. Radioactivity was determined using a Searle Mark III liquid scintillation spectrometer. Efficiency of counting was 40% for $^3$H. Initial rate of influx (entry) studies were carried out by stopping the reaction at 2 min. Intracellular steady state concentrations were determined by stopping the reaction at 30 min.

5. Preparation of Extracts.

Approximately $10^9$ cells in logarithmic growth were removed from suspension by centrifuging at 250 g. at 4°C for 6 min. (Prior to precipitation of cell extracts, $R_3$ and $R_4$ cells were grown in MTX-free medium at 37°C for 24 hours to remove all enzyme-bound MTX). The cells were washed twice in ice-cold PBS (0.15 M NaH$_2$PO$_4$·H$_2$O; 0.15 M Na$_2$HPO$_4$ pH 7.2 in 0.9% NaCl) and then resuspended in 4-6 ml ice-cold 0.05 M Tris-HCl pH 7.5. The cells were disrupted by sonicating at 20
KHZ for 30 seconds at 4°C (Branson Sonifier, cell disrupter 350, Branson Sonic Power Co., Connecticut). The lysate was then centrifuged at 100,000 g. for one hour at 4°C in a Beckman L5 Ultracentrifuge. The supernatant was termed 'crude lysate'.

6. Assay for Folate Reductase Activity:

Folate reductase activity was measured using the radiolabelled folic acid method of Littlefield (1969). The 100 μl incubation mixture consisted of 20 μl 0.01 M potassium acetate buffer, pH 5.0; 30 μl 3.33 mM NADPH; 30 μl 330μM [2-14C] Folic acid (S.Ac.:55mCi/mmol), or [3',5',7,9-3H] Folic acid (S.Ac.:29Ci/mmol) and 20μl enzyme preparation. Inhibitors (10 μl) were incubated with the enzyme preparation for 5 min. at room temperature prior to starting the reaction with labelled folic acid. After incubation at 37°C for 20 minutes, the reaction mixture was chilled on ice and 20μl 0.027 M unlabelled folic acid was added. Unreduced folic acid was precipitated by the addition of 20 μl 0.17 M zinc sulphate and 5 μl glacial acetic acid. After 10 minutes in ice the mixture was centrifuged at 1,000 g. for 15 minutes at 4°C. 50 μl of the supernatant fraction was added to 10 ml PCS scintillation cocktail and counted in a Searle Mark III liquid scintillation spectrometer. All assays were corrected for a blank reaction mixture lacking enzyme which showed less than 2% of the added radioactivity. 1 unit of activity is defined as that amount of enzyme reducing 1 nmole of folic acid in 20 minutes per ml. Protein was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Chemical Division, Technical Bulletin 1051, April 1977), which is based on the observation that the absorbance maximum of Coomassie Brilliant Blue G-250 dye shifts from 465nm to 595nm when binding to protein occurs.
7. High pressure liquid chromatography of reduced and oxidized folates.

The products of the radioactive folate reductase assay were determined by HPLC. The method used was a modification of Stout et al., (1976), for the separation of substituted pteroyl monoglutamates and pteroyl oligo- -glutamates.

Tetrahydrofolates, dihydrofolate and unreduced folic acid were separated by anion exchange chromatography on a Model 7000 B liquid chromatograph (Micromeretics, Nocross, Ga., U.S.A.) using a Reeve-Angel partisol 10 SAX column 25 x 4.6mm i.d. (Whatman, Clifton, N.J., U.S.A.). The eluent (0.16M NaCl solution containing 4mM NaNH₄HPO₄ pH6.5) was formed by mixing distilled water (primary reservoir) and 2M NaCl containing 0.05M NaNH₄HPO₄ pH6.5 (secondary reservoir.). The run was carried out isocratically with the secondary reservoir set at 8%. The column temperature and flow rate were maintained at 29°C and 2.0 ml/min respectivley. Before the chromatographic run, the column was equilibrated with 0.6M NaCl obtained by the use of a 30% setting for the secondary reservoir. Standard solutions of reduced and oxidized folates were detected in the column effluent by their U.V. absorbance at 254 nm., using a chromonitor 785 flow spectrophotometric detector (Micromeretics). Fractions of the column effluent (1ml.) were also collected automatically into scintillation vials on a fraction collector. PCS scintillation fluid (10ml.) was added to each vial and the radioactivity counted on a Mark III liquid scintillation spectrometer.

Radiopurities of ³H Folic acid and ¹⁴C Folic acid were determined by HPLC on an anion exchange column as described above. Both were found to be 98% pure with the main impurities being para-aminobenzoic acid.
8. Procedures for the isolation and purification of folate reductase.

(a). **MTX-Sepharose Affinity Chromatography:** AH-sepharose - 4B (5 g.) was swollen and washed with 1 liter 0.5 M NaCl on a sintered glass filter (G3). The gel was then washed with 500 ml of distilled water (made pH 4.5 with 1 N HCl). The gel was resuspended in 25 ml distilled water (pH 4.5); MTX (10 mg for S cells; and 50 mg for R₃ and R₄ cells) and EDC (88 mg and 440 mg respectively) were added to the gel and incubated for 16 hours at room temperature on a multi purpose rotator. The MTX sepharose was then washed with 0.05 M Tris-HCl, pH 8.0; 2 M KCl and poured into a 1 cm x 8 cm glass column. The packed column was then equilibrated with 200 ml 0.05 M Tris-HCl pH 7.5 containing 10⁻⁵ M NADPH. 'Crude lysate' (4 ml) was then applied to the column and chromatography was carried out using 20 ml 0.05 M Tris-HCl pH 7.5; 10⁻⁵ M NADPH (Buffer 1), followed by 30 ml 0.2 M Tris-glycine buffer pH 9.5; 2 M KCl, 10⁻⁵ M NADPH (Buffer 2) and the folate reductase was then eluted with 0.2 M Tris-glycine pH 9.5, 2 M KCl and 0.5 mM dihydrofolic acid (Buffer 3). Fractions (3 ml) were collected automatically on a Gilson micro fractionator and scanned for protein (U.V. absorbance at 280 nM) and folate reductase activity.

(b). **Sephadex G-100 Chromatography:** The fractions containing folate reductase activity in the Buffer 1 wash of the MTX-sepharose affinity column (Form 2) were pooled, dialyzed against 4mM Tris-HCl pH 7.5, lyophilized and dissolved in 3 ml of distilled water. Aliquots (1.5-2.0 ml) were applied to Sephadex G-100 columns (1.6 x 81 cm) and elution was carried out with 50 mM Tris-HCl (pH 7.5) at a flow rate of 24 ml/hr. Fractions (6 ml each) were collected and assayed for protein (O.D. 280 nm) and folate reductase activity. The active fractions were
pooled (total volume - 18 ml), dialyzed extensively against 2.8 mM Tris-HCl (pH 7.5), lyophilized and dissolved in 1 ml distilled water.

The fractions containing folate reductase activity in the Buffer 3 wash of the MTX-sepharose affinity column (Form 1) were dialyzed extensively against 50 mM Tris-HCl (pH 7.5) to remove all of the dihydrofolic acid. The active fractions were then pooled, dialyzed against 5 mM Tris-HCl (pH 7.5), lyophilized and dissolved in 2 ml of distilled water. Aliquots (2 ml) were applied to Sephadex G-100 columns and chromatographed as described above for Form 2 activity.

(c). Chromatography of Form 2 folate reductase on DEAE Affigel blue:

DEAE Affigel blue is a crosslinked agarose which has both Cibacron blue and diethylaminoethyl groups covalently linked to it. Form 2 folate reductase from R₄ cells, after fractionation through MTX-Sepharose affinity column and gel filtration through G100 Sephadex was applied to a DEAE Affigel blue column (1.5x5.4 cm.). Prior to equilibration of the column the gel was washed with 50 ml 0.1M acetic acid, pH 3.0 containing 1.4M NaCl and 40% isopropanol. The column was then equilibrated with at least 5 bed volumes of starting buffer which was 50mM Tris-HCL pH 7.5. Approximately 6.0ml (2.0mg) of the enzyme preparation from G100 sephadex in 50mM Tris-HCL, pH 7.5 was applied, and eluted with 5 bed volumes of the starting buffer. The column was then eluted with a step gradient from 1.0M NaCl to 2.5M NaCl in starting buffer.
Gel Electrophoresis: Samples (50-75 µl) containing 25 µg protein were electrophoresed on 7.5% polyacrylamide gels (acrylamide: methylenebisacrylamide, 29.2 : 0.8) according to the procedure of Huennekens et al, (1971). The running buffer was 25 mM Tris-glycine (pH 8.3) and electrophoresis was carried out for 1.5 hr. at 4°C using a current of 2.2 mA per tube. The gels were stained for dihydrofolate reductase activity in the presence and absence of MTX (10^-5 M), as described by Huennekens et al, (1971).

Electrophoresis in the presence of sodium dodecyl sulphate (SDS) was carried out using the procedure of Laemmli, (1970). Samples (75µl) containing 25-50 µg protein for detection with Coomassie blue, or 2 to 4µg for detection by the silver staining technique, were electrophoresed using a running buffer consisting of 0.025 M Tris, 0.192 M glycine, 0.1% SDS (pH 8.3). After electrophoresis for 4hr. at 4°C and 2.2 mA per tube, the gels were either stained in 0.2% Coomassie blue 'R' in 50% trichloroacetic acid for 2h at room temperature and destained by diffusion in 7% acetic acid, 10% isopropanol; or were stained by a modification of the silver staining technique (Switzer et al, 1979), described below. Silver staining of 13% SDS-PAGE slab gels was carried out as follows: The gel was removed from the electrophoresis apparatus and fixed for 2 periods of 30 min.in 25% methanol/10% acetic acid. The gel was then rinsed in distilled water (3x20min.), followed by fixation in 10% gluteraldehyde solution for 30 min.; it was then rinsed free of fixative with distilled water (3x10 min.) and left overnight in the same. The gel was next placed in diamine solution (0.36% NaOH (46ml); concentrated NH₄OH (3.1ml); 20%(w/v) AgNO₃ (9ml) and 20% ethanol (165ml)) for 15min, rinsed with distilled water (3x10 min.)
and then placed in the developing solution containing 100 ml. absolute ethanol, 1% citric acid (5 ml.), 3.7% formaldehyde (5 ml.) and 890 ml. distilled water. The bands developed almost immediately and the reaction was stopped by rinsing the gel with several washes of distilled water, once the desired intensity of the bands had been achieved. The dark background and the surface deposits of silver were removed as per Switzer et al, (1979).

Isoelectric focusing: Isoelectric focusing was carried out in 130 x 2.5 mm glass tubes as described by O'Farrell, (1975). Gels contained a final concentration of 2% Ampholines (LKB), 1.5% pH range 5 to 7 and 0.5% pH range 3.5 to 10. Following electrophoresis for 12 hr. at 400 volts the gels were extruded into 50% methanol, 10% acetic acid containing 0.03% Coomassie Brilliant Blue ("R" grade, Sigma) for 2 hr. Gels were then destained overnight against 7% acetic acid, 5% methanol.

To determine the pH gradient, gels were sliced into 1 cm sections and incubated in sealed vials with 5 ml of degassed distilled water. After 1 hr. of shaking at 23°C the pH was measured using an Orion Research pH meter.
RESULTS

1. Growth properties of the MTX-sensitive and MTX-resistant L5178Y mouse leukemic cells.

A typical growth curve for L5178Y(S) cells is shown in Fig. 4. The growth of these cells could be divided into 3 phases: a lag phase, followed by an exponential phase and finally a stationary phase. An initial lag phase was always present at low cell concentrations (i.e. less than 50,000 cells/ml). This was followed by an exponential phase characterized by a doubling time of 11 to 14 hours. Cell growth began to slow down at a cell concentration of greater than 700,000 cells/ml with zero growth at cell concentrations of 10^6/ml. Growth could be stimulated with the addition of fresh medium containing serum. Routine cultures were maintained at a cell concentration of 2 to 6 x 10^5 cells/ml, i.e. in the exponential phase.

Upon exposure of the S cells to MTX during the selection of MTX-resistant cells, the doubling time slowed down considerably; however, the cells were kept exposed to the drug in culture medium until the cell number doubled every 11 to 14 hours. At this point the selection process of resistant cells to that particular concentration of MTX was regarded to be essentially complete. This was substantiated by determining the I.D.50 (i.e. the dose of MTX required to inhibit cell proliferation by 50% of control) for MTX of these cells.

The inhibition of L5178Y(S), (R3), and (R4) cells with increasing concentrations of MTX is shown in Fig. 5., and the I.D.50 values are listed in Table 2. Thus, L5178Y(R3) cells were approximately 1000 fold, and R4 cells 180,000 fold more resistant to MTX than the parent L5178Y(S) cells.
FIGURE 4

A typical growth curve of L5178Y(S) cells under the conditions described in the text. The figure shows these cells growing with a doubling time of 12 hours. Once established, $R_3$ and $R_4$ cells exhibited similar growth profiles and doubling times. Cell numbers were determined by counting on a Model Zb1 Coulter Counter.
FIGURE 5

Determination of I.D.\textsubscript{50} values (i.e. concentration of MTX required to inhibit proliferation of cells by 50% of control) for MTX-sensitive and MTX-resistant cells. Cells, at a concentration of approximately 5\times10^{-4} cells/ml were allowed to grow under standard conditions in increasing concentrations of MTX for 24 hours. Cell numbers were counted at this time and the results plotted as % increase in cell number of control against MTX concentration. Each point is the mean of three separate experiments.

L5178Y(S) ○ ; L5178Y(R\textsubscript{3}) □ ; L5178Y(R\textsubscript{4}) ● .
### TABLE II

Properties of L5178Y MTX-Sensitive and MTX Resistant Cell Lines.

<table>
<thead>
<tr>
<th>Cell-Line</th>
<th>I.D. (_{50}) (MTX)</th>
<th>Folate Reductase S.Ac. (units/Mg Protein)</th>
<th>% Folate Reductase Activity Represented By Low-Affinity Variant.</th>
<th>Intracellular Steady State Level (nmoles/10(^9) cells) of MTX at 'Low' and 'High' Extracellular MTX.</th>
</tr>
</thead>
<tbody>
<tr>
<td>L5178Y(S)</td>
<td>2.8x10(^{-8})M</td>
<td>1.0</td>
<td>0%</td>
<td>1.3 32.3</td>
</tr>
<tr>
<td>L5178Y(R(_3))</td>
<td>2.6x10(^{-5})M</td>
<td>7.0</td>
<td>2%</td>
<td>0.3 30.0</td>
</tr>
<tr>
<td>L5178Y(R(_4))</td>
<td>5.0x10(^{-3})M</td>
<td>8.6</td>
<td>8%</td>
<td></td>
</tr>
</tbody>
</table>
2. Properties of MTX uptake into L5178Y(S) and (R₃) cells

MTX transport into L1210 murine leukemia and Ehrlich ascites tumour cells is compatible with a carrier transport model (Lichtenstein et al., 1968; Goldman et al., 1971; Mckormick et al., 1979), and evidence exists that the mode of transport in L5178Y cells is similarly carrier mediated (Harrap et al., 1971). The sequence of events when carrier interacts with MTX during the influx process can be accounted for by a series of four reversible reactions as illustrated in Fig.3. The time course of uptake of MTX can therefore be divided into three phases. During the initial phase, the carrier from which MTX has dissociated at the inner cell membrane reorients towards the cell exterior in the unloaded condition. During this phase, the net uptake of MTX is equal to its unidirectional influx velocity, and the uptake is a linear function of time. Following saturation of all the intracellular binding sites (mainly dihydrofolate reductase), free intracellular MTX appears and can interact with the carrier at the inner cell membrane to produce a unidirectional efflux which leads to a declining net uptake until a steady state is reached, when these bidirectional fluxes are equal.

These three phases are illustrated for the uptake of MTX into L5178Y(S) cells in Fig.6 (a).: a linear phase during the first 5 min. of exposure to 10⁻⁶ M MTX, followed by a declining uptake phase and then a plateau phase (steady state phase) after 20 min. exposure. Under these conditions the Km for influx was 9.1μM and Vmax was 0.4nmol/min/10⁹ cells. In contrast, MTX (10⁻⁶M) uptake into R₃ cells was greatly impaired, achieving 23% of the intracellular steady state MTX concentration of S cells. Uptake of MTX into L5178Y(S) cells was found to be temperature dependent (Q₁₀ = 3.0) and the influx was strongly
FIGURE 6

The time course of uptake of $^3$H-MTX into L5178Y(S) and (R$_3$) cells.

A. Cells were exposed to 10$^{-6}$M MTX for various time periods (from 0 to 40 min.) and the intracellular levels of $^3$H-MTX determined as described in the text. L5178Y (S) •; L5178Y(R$_3$) o.

B. Cells were exposed to 10$^{-4}$M MTX and treated as described above. L5178Y(S) •; L5178Y(R$_3$) o.

In both cases the experiment was carried three times with identical results.
inhibited by p-chloromercuriphenylsulphonate (pCMS) (Table 3.), suggesting the involvement of sulphydryl groups in the carrier-MTX interaction. The effect of 5-methyl tetrahydrofolate and folinic acid (5-formyl tetrahydrofolate) on MTX influx and on the intracellular steady-state concentration of the drug was also determined. (Table 3.) It was noted that both of these folate compounds reduced the level of the steady-state concentration of MTX, though this was much less than the inhibition seen on the initial rate of influx (Table 3). The above studies could not be carried out on the R₃ cells since the influx of MTX was so slow and the extent of drug uptake so limited that accurate determinations were not possible.

Next, the extent of MTX uptake with time in the two cell lines was determined when the extracellular concentration of drug was increased to 10⁻⁴M. (Fig 6 b.). It is now seen that significant amounts of drug entered both cell lines. At all points the amount of MTX associated with both cell types was nearly identical, with no significant difference observed in either the initial rate of entry of MTX or in the intracellular concentrations achieved at steady-state levels after a 30 min. incubation. At this 10⁻⁴ MTX concentration not only are higher intracellular levels achieved, but there is also a considerable quantity of drug associated with the cells at zero time, which is consistent with earlier reports and is considered to represent the component bound to the cell surface (Goldman, 1975; Hill et al., 1979). When either S or R₃ cells were exposed to 10⁻⁴M MTX for either 2 or 30 mins. at 37°C, drug uptake was not significantly influenced by the presence of 70 μM pCMS, 10⁻⁴M methyl tetrahydrofolate or 10⁻⁴M folinic acid (Table 4).
### TABLE III
Properties of $^3$H-MTX Influx into L5178Y(S) Cells at $10^{-6}$M Extracellular MTX.

<table>
<thead>
<tr>
<th></th>
<th>Initial rate of influx* (nmoles/10^9 cells)</th>
<th>% Inhibition of Steady-State initial rate</th>
<th>Steady-State Concentration** (nmoles/10^9 cells)</th>
<th>Inhibition of Steady-State</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX only (Control)</td>
<td>0.256 ± 0.16</td>
<td>89%</td>
<td>1.58 ± 0.36</td>
<td>-</td>
</tr>
<tr>
<td>MTX + pCMS (70μM)</td>
<td>0.029 ± 0.001</td>
<td>75%</td>
<td>0.41 ± 0.084</td>
<td>74%</td>
</tr>
<tr>
<td>MTX + 5-CH₃FH₄ (10⁻⁶M)</td>
<td>0.063 ± 0.005</td>
<td>60%</td>
<td>1.06 ± 0.10</td>
<td>33%</td>
</tr>
<tr>
<td>MTX + Folinic acid (10⁻⁶M)</td>
<td>0.105 ± 0.012</td>
<td>75%</td>
<td>1.21 ± 0.07</td>
<td>23%</td>
</tr>
</tbody>
</table>

*S cells were exposed to $10^{-6}$M $^3$H-MTX for 2 min. in the presence or absence of the above compounds.

**S cells were exposed to $10^{-6}$M $^3$H-MTX for 30 min. in the presence or absence of the above compounds.

Each point represents the mean of 3 values ± standard error.
TABLE IV
Properties of $^3$H-MTX Influx into L5178Y(S) and (R$_3$) Cells at $10^{-4}$M Extracellular MTX.

<table>
<thead>
<tr>
<th></th>
<th>MTX-sensitive cells</th>
<th>MTX-resistant cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Inhibition of</td>
<td>% Inhibition of</td>
</tr>
<tr>
<td></td>
<td>Initial rate of</td>
<td>Steady-State</td>
</tr>
<tr>
<td></td>
<td>influx*</td>
<td>Concentration**</td>
</tr>
<tr>
<td>MTX only</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(Control)***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTX + pCMS</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>(70µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTX + $5$-CH$_3$FH$_4$</td>
<td>11%</td>
<td>10%</td>
</tr>
<tr>
<td>($10^{-4}$M)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTX$_4$ + Folinic acid</td>
<td>0%</td>
<td>10%</td>
</tr>
<tr>
<td>($10^{-4}$M)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Cells were exposed to $10^{-4}$M $^3$H-MTX for 2 min. in the presence of the above compounds.

**Cells were exposed to $10^{-4}$M $^3$H-MTX for 30 min. in the presence of the above compounds.

***Initial rate of influx in nmoles/min/10$^9$ cells in control cells was $3.9 \pm 0.4$ and $3.6 \pm 0.4$ for S and R cells respectively. Steady-State concentrations in nmoles/10$^9$ cells in control cells were $32.3 \pm 1.2$ and $29.5 \pm 1.3$ for S and R cells respectively (see Figure 3).

Each point represents the mean of 3 estimations.
These data therefore suggest that at high extracellular drug concentration the impairment in drug uptake in MTX-resistant cells can be overcome, but that under these conditions the mechanism by which MTX enters the cells (both the sensitive and the resistant variants) may differ from the apparent active transport process demonstrated at the lower drug concentration of $10^{-6}$M in the L5178Y(S) cells.

3. Evidence for the presence of two forms of Folate reductase in MTX resistant L5178Y cells. Folate reductase activity in the lysates of $S$, $R_3$ and $R_4$ cells was assayed as described in the Methods and Materials section. The specific activities (in Units/Mg protein) of DHFR in these cell lines are shown in Table 2. Both resistant cell lines had elevated levels of DHFR, being 7-fold higher in the $R_3$ cells and 8.6-fold higher in the $R_4$ cells, as compared to the level in the parent $S$ cells.

Affinity chromatography of cellular lysate from L5178Y(S) cells on MTX-sepharose affinity column resulted in the binding of the total DHFR activity applied. This is apparent from Fig. 7a. where DHFR activity is absent in the buffer 1 and buffer 2 elutions, which resulted in the elution of large amounts of protein. (solid line Fig. 7a.). DHFR activity was only eluted with buffer 3 which contained the substrate dihydrofolinic acid.

In contrast, chromatography of $R_3$ lysate resulted in the elution of a DHFR peak in buffer 1 in addition to the bound activity eluted with buffer 3 (Fig. 7b.). The activity eluted with buffer 1 therefore represented a portion of the total DHFR activity which did not bind to the affinity column.
Methotrexate-sepharose affinity chromatography of lysates from L5178Y(S), (R₃) and (R₄) cells. Cellular lysates (μl.) were applied to MTX-sepharose affinity columns and eluted stepwise with the following buffers:

Buffer 1: 0.05M Tris-Cl, pH7.5; 10⁻⁵ M NADPH.

Buffer 2: 0.2M Tris-Glycine, pH9.5 in 2M KCl; 10⁻⁵ NADPH.

Buffer 3: 0.2M Tris-Glycine, pH9.5 in 2M KCl; 5mM dihydrofolic acid.

3.5-ml fractions were collected and protein was estimated by absorbance at 280nM. Fractions eluted with Buffer 3 were dialyzed at 4°C for 18 h. against two changes of 2 liters of 0.05M Tris-Cl, pH7.5, before assaying all fractions for enzyme activity ○, A₂₈₀ profile;

●, enzyme activity (units). A, cellular lysate from L5178Y(S) cells; B, cellular lysate from L5178Y(R₃) cells; and C, cellular lysate from L5178Y(R₄) cells.
A

[Graph showing folate reductase activity and protein A280 levels across fraction number.

- Buffer 1
- Buffer 2
- Buffer 3]
Chromatography of L5178Y(R₄) lysate showed a similar profile of protein and DHFR activity elution (Fig.7c.) as R₃ lysate; however, the proportion of unbound activity in buffer 1 was greater than that seen in R₃ cells.

The question now arose as to whether the unbound activities eluting with buffer 1 upon chromatography of R₃ and R₄ lysates represented a different form of the enzyme with a lower affinity for MTX, or 'spill-over' of the bound enzyme due to the saturation of binding sites on the column. Another possibility could be that the activity observed in buffer 1 elution was artifactual resulting from insufficient precipitation of the unreduced radiolabelled folic acid.

To answer these questions, fractions with DHFR activity in buffer 1 elution were pooled, concentrated and assayed in the absence of NADPH. No enzyme activity was detected. In order to show that the products of this NADPH dependent enzymatic reaction was indeed tetrahydrofolic acid, the supernatant of the radioassay was subjected to HPLC on an anion exchange column (see Methods and Materials).

Fig.8a. shows the separation of a standard mixture of N⁵ formyl tetrahydrofolic acid, N⁵ methyl tetrahydrofolic acid, tetrahydrofolic acid, p-aminobenzoylglutamate, p-aminobenzoic acid, dihydrofolic acid and folic acid

Fig.8b. represents the radioactive profile of the reaction products of the MTX sensitive DHFR present in the cell lysate from L5178Y(S) cells. Three radioactive peaks are evident with two of them corresponding to the retention times of the tetrahydrofolate cofactors. These two peaks were therefore tentatively identified as one or more forms of tetrahydrofolate.
FIGURE 8

Separation of folate compounds by high pressure liquid chromatography on an anion exchange column. Experimental details are given in the text.
A, Chromatography of a standard mixture of folate compounds.
B, Chromatography of the reaction products of folate reductase activity in L5178Y(S) lysate.
C, Chromatography of the reaction products of Form 2 folate reductase activity.
• $^{3}$H-CPM; ——— O.D.254 profile of separation of a standard mixture of folate compounds.
P-AMINO BENZOYL GLUTAMATE

5-FORMYL-TETRAHYDROFOLIC ACID/TETRAHYDROFOLIC ACID

5-METHYL-TETRAHYDROFOLIC ACID

DIHYDROFOLIC ACID

FOLIC ACID

O.D. 254 nm

TIME (MIN)
Fig. 8c. shows the radioactive profile of the products of the DHFR enzyme activity eluted in buffer 1 upon chromatography of R₃ lysate. Four radioactive peaks were present this time with two of these peaks corresponding to the retention times of tetrahydrofolate cofactors. It was concluded therefore that this latter activity represented DHFR capable of generating tetrahydrofolic acid from folic acid. However the problem of 'spill-over' still remained. The unbound activities from both R₃ and R₄ cells were therefore assayed in the presence of increasing concentrations of MTX to determine the sensitivity of these enzymes to MTX. This is shown in Fig. 9, where the percentage inhibition of the enzyme of control is plotted against increasing MTX concentration. Included for comparison is the sensitivity of DHFR's derived from S cells, and the affinity column bound reductase from R₄ cells. It can be seen that both sensitive enzymes were 100% inhibited by 10⁻⁶ M MTX. In contrast the unbound DHFR from R₃ cells was only inhibited 50% by 10⁻³ M MTX and that from the R₄ cells was inhibited less than 40% by 10⁻³ M MTX.

It was therefore concluded that R₃ and R₄ cells have in addition to MTX sensitive DHFR (affinity column bound activity) a variant form of DHFR characterized by marked insensitivity to MTX and inability to bind to MTX-sepharose affinity column. Hereafter, the DHFR activity from R₃ and R₄ cells which bound to the affinity columns was called Form 1, and the activity which did not bind to the columns was called Form 2.

4. Purification of Forms 1 and 2 of DHFR from L5178Y (R₄) cells.

(a). Affinity chromatography: As described earlier, affinity chromatography of the R₄ cell lysate on MTX-sepharose columns resulted in the complete separation of the two forms of reductase. This
Inhibition of folate reductase activity from MTX-sensitive and MTX-resistant cells by MTX. Enzyme preparations were incubated with NADPH (1mM) and MTX in reaction buffer (0.01M potassium acetate, pH5.0) at room temperature for 5 min. The enzyme reaction was then started by the addition of 2-\(^{14}\)C folic acid (100\(\mu\)M). ● L5178Y(S) lysate; □ L5178Y(R\(_4\)) Form 1 DHFR; ■ L5178Y(R\(_3\)) Form 2 DHFR; ○ L5178Y(R\(_4\)) Form 2 DHFR.
DO METHOTREXATE CONCENTRATION (M)

% FOLATE REDUCTASE ACTIVITY OF CONTROL

METHOTREXATE CONCENTRATION (M)

10^-9 10^-8 10^-7 10^-6 10^-5 10^-4 10^-3
procedure also achieved substantial purification of both forms of the enzyme (see later and Table 5).

(b). Sephadex G-100 chromatography: Further purification of the two dihydrofolate reductases from the R₄ cells was carried out by means of gel filtration through Sephadex G-100. Both forms eluted in a position corresponding to an apparent molecular weight of approximately 20,000 (Fig. 10a) and b)). Similar results were obtained in low and high salt concentrations.

The extent of purification obtained at each stage is shown in Table 5. The total enzyme activity applied to the affinity column was 155.1 nmoles/20 min. As indicated in Table 5, 8.3% of this activity was found to be due to Form 2 dihydrofolate reductase, determined by integrating the areas under Peaks I and II following MTX-sepharose affinity chromatography (Fig. 7C). On the basis of this assumption the purification of Form 1 dihydrofolate reductase after affinity chromatography was 211 and that of Form 2 reductase was 31.5. Chromatography of Form I through sephadex G-100 resulted in a total purification of 1767 fold. Some high molecular weight proteins apparently bind to methotrexate-sepharose affinity columns and are eluted with Form 1 dihydrofolate reductase (Kaufman and Pierce, 1971)). These proteins are eluted in the void volume when applied to G-100 sephadex (Fig. 10a). A 741 fold overall purification was achieved for the Form 2 reductase after Sephadex G-100 chromatography.

Molecular weight determination: Form 1 (from affinity column) and Form 2 (from Sephadex G-100) enzymes were electrophoresed in the presence of sodium dodecyl sulphate as described in the Methods Materials section. These gels were stained with Coomassie blue and
FIGURE 10

Gel filtration on Sephadex G-100 of folate reductase Forms 1 and 2 from L5178Y(R₄) cells. Samples (1.5 to 2.0ml) were applied to a sephadex G-100 column (1.6x81cm) and elution was carried out with 50mM Tris-HCl, pH7.5, at a flow rate of 24ml./hr. 6ml. fractions were collected and assayed for protein (absorbance at 280nM) and folate reductase activity ———, the A₂₈₀ profile; ●, enzyme activity (units).

A. Fractions eluted with Buffer 3 from methotrexate-sepharose affinity column and containing reductase activity (Form 1) were pooled, dialyzed at 4°C for 14hr. against 5mM Tris-HCl, pH7.5, lyophilized, dissolved in 2ml. of water and applied to the column.

B. Fractions eluted with Buffer 1 from methotrexate-sepharose affinity column and containing reductase activity (Form 2) were pooled, dialyzed at 4°C for 14hr. against 4mM Tris-HCl, pH 7.5, lyophilized, dissolved in 3ml. of water and applied to the column.
<table>
<thead>
<tr>
<th></th>
<th>Total Activity</th>
<th>Protein</th>
<th>Specific Activity</th>
<th>Purification</th>
<th>Yield&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/20 min</td>
<td>µg</td>
<td>nmol/20 min/µg</td>
<td>-fold</td>
<td>%</td>
</tr>
<tr>
<td>Form I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell lysate</td>
<td>142&lt;sup&gt;b&lt;/sup&gt;</td>
<td>48,000</td>
<td>0.0030</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Affinity column</td>
<td>114</td>
<td>180</td>
<td>0.63</td>
<td>210</td>
<td>88</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>106</td>
<td>≤20</td>
<td>5.30</td>
<td>1770</td>
<td>81.5</td>
</tr>
<tr>
<td>Form II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell lysate</td>
<td>12.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>48,000</td>
<td>0.00027</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Affinity column</td>
<td>12.7</td>
<td>1,500</td>
<td>0.0085</td>
<td>31.5</td>
<td>98</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>4.0</td>
<td>≤20</td>
<td>0.2</td>
<td>740</td>
<td>32</td>
</tr>
</tbody>
</table>

<sup>a</sup>These values were based on the assumption that Forms I and II contribute 91.7 and 8.3%, respectively, of the total activity in the crude lysate.

<sup>b</sup>These values were calculated on the assumptions that Form II contributes 8.3% of the total activity in the crude lysate. This percentage was determined by integrating the areas under Peaks I and II following methotrexate-Sepharose affinity chromatography (Fig. 1B). This value was further substantiated by the heat stability studies performed on the L5178Y (R<sub>4</sub>) crude lysate in which 70% of the total enzyme activity corresponds to the heat-stable Form II dihydrofolate reductase (see Fig. 5). Since the Vmax of Form II enzyme is approximately 9.0-fold lower than Form I enzyme (Table II), the actual concentration of Form II enzyme in L5178Y R<sub>4</sub> cells is approximately 38% of the total dihydrofolate reductase concentration.
single protein bands were observed for both reductases corresponding to apparent molecular weights of 18,500 (Form 1) and 20,500 (Form 2). Molecular weight standards were electrophoresed simultaneously (Fig. 11). Because of the unusual nature of Form 2 DHFR in terms of its very low affinity for MTX, it was desirable to be able to determine the amino acid sequence of this enzyme. In order to do this the enzyme had to be purified to homogeneity. Recently a highly sensitive silver staining technique for the visualization of proteins in polyacrylamide gels has been reported in the literature (Switzer et al, 1979). This technique has been used to reveal proteins not visible with Coomassie blue; a modification of the method was therefore used to determine the homogeneity of Form 2 reductase after filtration through G-100 sephadex. As shown in Fig.12.(lane 2), the main band corresponds to DHFR (M.Wt 20000), however, three or four minor bands, not previously observed upon staining with Coomassie blue, were present. Further purification was therefore essential and was carried out by chromatography on a DEAE affigel blue column as described in the Methods and Materials section. The enzyme bound tightly to the column and was eluted with 1.5M NaCl in 50mM Tris-Cl pH 7.5 (Fig. 13.) The fractions under this peak were pooled and concentrated by ultrafiltration through a 10,000 M.Wt. filter (Amicon). Polyacrylamide gel electrophoresis in the presence of SDS followed by silver staining now revealed an essentially homogeneous enzyme corresponding to a M.Wt. of 20,000 with one minor contaminating band of lower M.Wt. (Fig.12.-lane 4).

Isoelectric focusing—Isoelectric focusing on pH gradients of 3.5 to 9.5 resulted in single peaks corresponding to pl values of 8.4 for Form 1 and 6.0 for Form 2.
FIGURE 11

Molecular weights of folate reductase Forms 1 and 2 from L5178Y(R₄) cells in polyacrylamide-sodium dodecyl sulphate gels. Samples (75μl) containing 25-50μg of protein were applied to 10% gels, prepared as described by Leammli (1970), and electrophoresed at 4°C for 4 hr. at 2.2mA/tube. The gels were stained in 0.2% Coomassie blue R. in 50% trichloroacetic acid and destained in 7% acetic acid, 10% isopropyl alcohol. In Gel A, enzymatically active fractions from sephadex G-100 chromatography of Form 2 reductase were pooled, dialyzed at 4°C for 14hr. against 2 liters of 3mM Tris-HCl, pH7.5, lyophilized, and dissolved in 1ml. of water. Gel B, Form 1 reductase, obtained from sephadex G-100 chromatography as described above. Gel C, molecular weight standards (Bio-Rad Laboratories, Mississauga, Ontario). From top to bottom: phosphorylase b, 94,000; bovine serum albumin, 68,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soyabean trypsin inhibitor, 21,000; lysozyme, 14,300.
Polyacrylamide-sodium dodecyl sulphate gel electrophoresis of Form 2 folate reductase from L5178Y(R$_4$) cells. Samples (50µl) containing not more than 6µg of protein were applied to a 13% slab gel described by Laemmli (1970), and electrophoresed at 10°C for 4 to 5 hr. at 30mA total. The gel was stained by the silver staining technique described in detail in the text. The gel was photographed immediately after development. Lane 1: Molecular weight standards (described in Fig. 11); Lane 2: Form 2 folate reductase preparation from sephadex G-100; Lane 4: Form 2 folate reductase after subsequent chromatography on DEAE affigel blue. Lane 3: No protein applied. The two bands appearing in all lanes are artifacts of electrophoresis and staining since they appear in the blank lane (lane 3) as well.
FIGURE 13

Chromatography of Form 2 folate reductase on DEAE affigel blue. Form 2 folate reductase activity from three sephadex G-100 columns was pooled and applied to the column. The column (1.5x8cm) was eluted step-wise with the buffers indicated and folate reductase activity was determined in each fraction by measuring the rate of decrease in absorbance at 340nm (i.e. oxidation of NADPH to NADP) in the presence of dihydrofolinic acid.
Properties of the MTX-sensitive (Form 1) and MTX-insensitive (Form 2) folate reductases

a) **pH Optima:** The pH optimum for the Form 1 reductase was found to be 4.0, whereas that for Form 2 was between 4.5 and 5.0 (data not shown). These findings are consistent with previous reports (Smith et al., 1979) in that when folic acid is used as a substrate, activity is limited to the acidic pH.

b) **Heat Inactivation:** The heat stability of the two forms of dihydrofolate reductase present in the R₄ cells was compared to that of the reductase present in the lysate of the parent sensitive S cells. Incubation of the L5178Y(S) lysate at 60°C, resulted in total inactivation of folate reductase activity in 10 min (Fig. 14). Incubation of the L5178Y(R₄) lysate at 60°C resulted in inactivation of 92% of the activity; however, the remaining 8% was stable for up to 34 min of incubation at this temperature (Fig. 14). Partially purified Form 1 and Form 2 reductases were incubated at 60°C for 30 min. Whereas Form 1 lost 98% of its activity within 10 min (and 100% by 30 min), Form 2 was completely stable for the entire incubation period (Figs. 14).

c) **Effect of para-chloromercuri phenylsulphonate on folate reductase activities:** The two forms of reductase responded very differently to the organic mercurial, pCMS. Partially purified enzyme preparations were incubated with various concentrations of pCMS, ranging from $10^{-9}$ to $10^{-4}$ M, for 5 min at room temperature, prior to assaying for enzyme activity. Fig. 15. shows the effect of increasing concentrations of pCMS on the activity of the two forms of dihydrofolate reductase. Form
FIGURE 14

Effect of temperature on the enzyme activities of the two folate reductases from L5178Y(R₄) cells. Enzyme preparations (50μl) were incubated at 60°C for various time periods and cooled in ice prior to assaying for enzyme activity using the assay described in the text.

A. Effect of heat on the enzyme activities of L5178Y(S) (●) and L5178Y(R₄) (○) cell lysates.

B. Effect of heat on the enzyme activities of partially purified Form 1 (○) and Form 2 (●) folate reductases.
FIGURE 15

Effect of pCMS on the enzyme activities of the two folate reductases from L5178Y(R₄) cells. Samples (20μl) containing 50μg of protein were incubated with various concentrations of pCMS at 25°C for 5 min. prior to assaying for folate reductase activity. ○, effect of pCMS on the enzyme activity of partially purified Form 1 folate reductase; ●, effect of pCMS on the enzyme activity of partially purified Form 2 folate reductase. V₀, enzyme activity in the absence of pCMS; V, enzyme activity after exposure to pCMS.
2 was found to be very sensitive to low concentrations of pCMS, the activity almost doubling in the presence of $10^{-7} - 10^{-5}$ M pCMS. However, the activity dropped to below that obtained in the absence of pCMS, at higher concentrations ($10^{-4}$ M). Form 1 appeared to be activated very slightly by pCMS, the activity increasing with increasing concentrations of pCMS such that at $10^{-4}$ pCMS it was approximately 1.1 x the level obtained in the absence of pCMS.

d) **Kinetic properties**: Dissociation constants for folic acid and NADPH with both forms of the enzyme were determined from Lineweaver-Burk double reciprocal plots (Lineweaver and Burk, 1934), (Fig. 16 a-d). In each case one substrate was varied from 10 - 100 μM while the other was held constant. A summary of the constants from a fit of data to the equation is shown in Table 6. The Km for NADPH is approximately $4 \times 10^{-5}$ M for both forms. However, the Km for folic acid of Form 2 ($1.66 \times 10^{-4}$ M) is approximately 2.5 fold higher than that of Form 1 ($6.8 \times 10^{-5}$ M). Moreover, the Vmax for Form 2 with either substrate is substantially less than the Vmax for Form 1.

The Ki for MTX of DHFR from S cells as well as the two forms of reductase present in R4 cells were determined in partially purified preparations with the use of Dixon plots (Dixon, 1955), (Fig 17.a, b, and c.) (Appendix I). The inhibitor dissociation constants (Ki) are also summarized in Table 6. The Ki for MTX of the L5178Y(S) reductase was approximately $2.6 \times 10^{-9}$ M. Form 1 enzyme from R4 cells had Ki for MTX of $2.0 \times 10^{-8}$ M, a 10-fold apparent increase. These values are only approximate because of the tight binding nature of MTX to sensitive dihydrofolate reductases and also the higher molar concentration of the
FIGURE 16

Double reciprocal plots of initial velocity patterns with folic acid or NADPH as the varied substrates.

A, determination of $K_m$ for folic acid of Form 2 folate reductase. The NADPH concentration was kept constant at 1mM.

B, determination of $K_m$ for folic acid of Form 1 folate reductase.

C, determination of $K_m$ for NADPH of Form 1 folate reductase. The folic acid concentration was kept constant at 100µM.

D, determination of $K_m$ for NADPH of Form 2 folate reductase. In all cases, the enzyme concentration was 0.7mg/ml. Points were fitted with a straight line estimated by least squares linear regression analysis.
TABLE VI
A Summary of the Kinetic Properties of DHFRs From L5178Y(S) and (R₄) Cells.

<table>
<thead>
<tr>
<th>DIHYDROFOLATE REDUCTASE</th>
<th>Vmax (units)</th>
<th>Km (Folic Acid) Molar</th>
<th>Km (NADPH) Molar</th>
<th>Ki (MTX) Molar</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>12.3</td>
<td>$1.72 \times 10^{-5}$</td>
<td>-</td>
<td>$2.6 \times 10^{-9}$</td>
</tr>
<tr>
<td>R₄ 'High Aff'</td>
<td>83.3</td>
<td>$6.8 \times 10^{-5}$</td>
<td>$5.0 \times 10^{-5}$</td>
<td>$2.0 \times 10^{-8}$</td>
</tr>
<tr>
<td>R₄ 'Low Aff'</td>
<td>10.0</td>
<td>$1.7 \times 10^{-4}$</td>
<td>$2.7 \times 10^{-5}$</td>
<td>$7.5 \times 10^{-4}$</td>
</tr>
</tbody>
</table>
FIGURE 17

Dixon plots of the reciprocal of initial velocity of reaction against MTX concentration at two substrate (folic acid) concentrations. Two lines with different slopes are obtained and intersect at a point to the left of the ordinate axis. This point lies at a value of $-K_i$ (see appendix I). Enzyme assays were carried out as described in the text. The NADPH concentration was kept constant at 1mM in all cases. A, L5178Y(S) folate reductase. B, Form 2 folate reductase from L5178Y(R₃) cells. C, Form 2 folate reductase from L5178Y(R₄) cells. Points were fitted with a straight line estimated by least squares linear regression analysis.
enzyme in R₄ cells. The Ki for MTX of Form 2 enzyme (7.5x10⁻⁴ M) was approximately 2x10⁵ fold higher than Form 1 reductase and indicates a very low (but detectable) affinity for MTX.

e) Electrophoretic properties: Electrophoresis of the two forms of folate reductase on non-denaturing polyacrylamide gels demonstrated a marked difference in electrophoretic mobility (Fig. 18A). The gels were stained for activity as described by Huennekens et al., (1971). Both forms of the enzyme were also incubated with 10⁻⁷ M MTX in the presence of NADPH at 4°C for 30 min., then electrophoresed as above and stained for activity in the presence of 10⁻⁶ M MTX for 15 min. Fig 18(B) shows the formation of a complex between Form 1 and MTX, as indicated by an increase in the R_f value. In contrast, there is no change in the R_f of Form 2 dihydrofolate reductase, indicating the lack of complex formation between MTX and the enzyme. Staining the gels for enzyme activity in the presence of 10⁻⁶ M MTX resulted in a band of very low intensity for Form 1. However, the intensity of the Form 2 enzyme remained the same in the presence and absence of MTX (Fig 18B). An increase in R_f with ternary complex formation between DHFRs, NADPH and MTX has been demonstrated before by several workers (Neef and Huennekens, 1975; Gundersen et al., 1972).
Electrophoresis of dihydrofolate reductases Form 1 and 2 on 7.5% non-denaturing polyacrylamide gels. Samples (50-75μl) containing 25μg protein were electrophoresed on 7.5% polyacrylamide gels in 25mM Tris-glycine, pH8.3, at 2.2mA/tube for 1.5h. at 4°C. The gels were stained for dihydrofolate reductase activity by placing the gels in a freshly prepared solution containing NADPH, dihydrofolic acid and 3(4,5-dimethylthiazoly(-2)-2, 5-diphenyltetrazoluim bromide in 50mM Tris-Cl, pH 8.0. After staining the gels were washed with distilled water, followed by 5% acetic acid and then photographed.

A. Gel A: Form 1 reductase; Gel B: Form 2 reductase.

B. Samples were incubated with $10^{-7}$M methotrexate for 30 min. at 4°C before electrophoresis. The gels were stained for activity in the presence of $10^{-6}$M MTX. Gel A: Form 2 reductase; Gel B: Form 1 reductase.
FIGURE 19

Giemsa-trypsin-stained metaphase chromosomes. Cells (3x10^4 cells/5ml) were incubated for 72hr. at 37°C in Fisher's medium containing 10% horse serum. Colcemid was added to a final concentration of 16ng/ml. and after 90 min. at 37°C cells were centrifuged at 300g. for 3 min. and resuspended in 5ml. of 75mM KCl (37°C) for 8 min. Cells were centrifuged as above and resuspended gently in 3ml. methanol:acetic acid (3:1 V/V), centrifuged as before and resuspended in 5ml. of the same solution. The cell suspension was then dropped on wet slides and allowed to air dry. Slides were stored at room temperature for 1 week, heated for 16hr. at 56°C, incubated in 25mM potassium phosphate (pH 6.8: 56°C) for 8 min., and then stained with Giemsa-Trypsin. Slides were rinsed in distilled water, air dried, and examined by light microscopy. A, L5178Y(S); B, L5178Y(R_4). The homogeneous staining region on one of the chromosomes of R_4 cells is indicated by the arrow.
DISCUSSION

The effectiveness of MTX as a cytotoxic agent depends on its potency as an inhibitor of dihydrofolate reductase. In cells resistant to MTX the inhibition of the DHFR activity may not be complete. This may be due to insufficient intracellular levels of MTX because of reduced uptake; increased levels of DHFR resulting in the immobilization of all of the intracellular MTX leaving free DHFR; or, the appearance of altered DHFR with reduced affinity for MTX resulting in incomplete inhibition of DHFR activity. In the MTX-resistant cells examined in this thesis all three mechanisms were found to be present to some extent.

L5178Y(S) cells, growing rapidly with a population doubling time of 11 to 14 hours are very sensitive to MTX (Fig. 5). The I.D.\textsubscript{50} of these cells was $2.8 \times 10^{-8}$ M MTX, with total inhibition of cell proliferation occurring at $10^{-6}$ M MTX. L5178Y (R\textsubscript{3}) cells derived from S cells by growing them in media containing progressively increasing sublethal concentrations of MTX (starting from $10^{-9}$ M), had an I.D.\textsubscript{50} of $2.5 \times 10^{-5}$ M. This constitutes a 1000-fold increase in resistance over the S cells. R\textsubscript{3} cells also had a population doubling time of 11 to 14 hours. The properties of S and R\textsubscript{3} cells were studied in terms of MTX influx and the amount and type(s) of dihydrofolate reductase. Tables 2 and 3 show that whilst uptake of MTX into S cells at "low" ($10^{-6}$ M) extracellular concentration was significantly inhibited by pCMS, 5-methyl tetrahydrofolate and 5-formyl tetrahydrofolate, uptake into S and R\textsubscript{3} cells at "high" ($10^{-4}$ M) extracellular concentration was not influenced by these compounds. At "low" extracellular MTX concentrations drug uptake in S cells appears to occur via the carrier
mediated transport process demonstrated in other cell types (Harrap et al., 1971; Hill et al., 1979), and is defective in the R₃ cells, resulting in considerable lower uptake of MTX (Fig. 6a). In contrast, at "high" MTX concentrations, both drug-sensitive and resistant lines took up equivalent amounts of MTX (Fig. 6b). In addition, the lack of competition for uptake between MTX and 5-methyl tetrahydrofolate or 5-formyl tetrahydrofolate, strongly suggest that most of the MTX was not taken up via the physiological carrier that accounts for transport at "low" extracellular concentrations. The nature of this different process is not known; however, the marked reduction in the sensitivity of uptake to pCMS indicates that sulphydryl groups are probably not involved in contrast to the uptake at "low" MTX concentrations which is inhibited by pCMS. The resistant cells were markedly less susceptible to MTX cytotoxicity despite the fact that potentially cytocidal intracellular concentrations of drug were achieved. Therefore other factors must be contributing to the resistance of these cells.

L5178Y(R₄) cells were derived from R₃ cells by exposing them to increasing concentrations of MTX, up to 10⁻³ M. R₄ cells had an I.D₅₀ of between 5x10⁻³ M and 10⁻² M MTX, representing a 200,000-fold increase in resistance. Despite being extremely resistant to MTX, these cells demonstrated identical drug uptake properties to R₃ cells (described above). It would therefore appear that the transport barrier is not a significant mechanism of resistance in these two highly MTX-resistant cell lines.

Folate reductase activity, assayed by the radiolabelled folic acid method, was next determined in the cellular lysates of L5178Y(S), (R₃)
and (R₄) cells. The specific activities are listed in Table II. Folate reductase activity in R₃ cells was 7-fold higher than in S cells and the activity in R₄ cells was approximately 9-fold higher. As explained in the 'Introduction' of this thesis, many MTX-resistant cell lines have elevated levels of folate reductase, some as much as 300-fold higher than the level in the corresponding wild-type cells. This elevation in DHFR level has been associated with the specific amplification of the DHFR gene (Alt et al, 1978; Schimke et al, 1978). In addition, the amplification has been correlated with the appearance of a homogeneously staining chromosomal region (HSR) upon Trypsin-Giemsa banding analysis of metaphase chromosomes (Numberg et al, 1978; Dolnick et al, 1979; Berenson et al, 1981). It was of interest therefore to see if the moderate elevation in DHFR activity in R₄ cells was associated with an HSR region. Fig. 19(b) shows just such a region in one of the chromosomes of R₄ cells, and is not present in any of the chromosomes of S cells (Fig. 19(a)). R₄ cells grown in the absence of MTX for over 100 generations retained the 9-fold elevation in the DHFR level and their resistance to MTX. This observation substantiates the finding that stably resistant cells with DHFR gene amplification are associated with an HSR region on the chromosomes whereas unstably resistant cells with gene amplification are associated with 'double minute' chromosomes which are lost, together with the elevated levels of DHFR, upon removal of the selection pressure (Kaufman et al, 1979).

R₃ and R₄ cells, in addition to the elevation of DHFR activity also contained two forms of the enzyme. The principal form (Form 1) in R₃ and R₄ cells exhibited high affinity for MTX and bound tightly to the MTX-sepharose affinity column. The second form (Form 2) present in
smaller amounts in both cell lines, failed to bind to the MTX-affinity column and exhibited markedly lower affinity for MTX (see Table VI). In the parent sensitive cells there were no detectable amounts of the Form 2 type of enzyme (Fig. 7). The Form 2 DHFR in the resistant cells retained its ability to reduce folic acid, suggesting capability of physiological function in the intact cell. R₄ cell-line, the most resistant subline, had higher levels of Form 2 DHFR and this enzyme had also a higher Ki (lower affinity) for MTX than the Form 2 reductase from R₃ cells. Therefore a distinct correlation can be made between the amount of 'low-affinity' (Form 2) enzyme present, its Ki for MTX and the degree of resistance of the cells (Table VI).

Purification of the two forms of DHFR was carried out from the R₄ cell line since this subline contained the highest levels of both forms of the enzyme. It was carried out by affinity chromatography on MTX-sepharose columns, followed by gel filtration on sephadex G-100. These two steps afforded 1760-fold purification of Form 1 (high affinity) DHFR and 740-fold purification for Form 2 (low affinity) DHFR. Further purification of the Form 2 enzyme had to be carried out on DEAE-Affigel blue columns to achieve homogeneity as determined by SDS polyacrylamide gel electrophoresis and staining for protein with the very sensitive silver staining method (see Results Section).

The apparent molecular weights of the two forms of the enzyme differ by 2000, both being in the range of previously reported molecular weights for mammalian dihydrofolate reductases (~20,000) (Bertino et al, 1965; Perkins et al, 1967; Neef and Huennekens, 1975; Smith et al, 1979). The apparent molecular weights determined by gel-filtration through sephadex G-100 (Fig. 10) were consistent with
those determined by SDS polyacrylamide gel electrophoresis (Fig. 11 and 12). The two forms of the enzyme had different isoelectric points (pI). Form 1 had a pI of 8.4 and agrees well with the pIs of DHFRs exhibiting high affinities for MTX (Flintoff et al., 1976; Kaufman and Kemerer, 1977; and Smith et al., 1979). The isoelectric point of Form 2 enzyme was 6.0 and represents a considerable acidic shift. These results suggest that the alteration in the structure of DHFR (Form 2) resulting in a huge reduction in affinity for MTX is probably associated not only with changes in the amino acids normally responsible for the tight binding of MTX but also in the amino acids elsewhere in the molecule.

This difference in the overall charge and the affinity for MTX (and therefore the ability to form a stable ternary complex with MTX and NADPH) was demonstrated by carrying out electrophoresis on 7.5% polyarylamide gels under non-denaturing conditions (i.e. in the absence of SDS). Gels were stained for enzyme activity by placing them in a freshly prepared solution containing NADPH, dihydrofolic acid and 3(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide in 50mM Tris-Cl, pH 8.0. DHFR activity was indicated by a purple zone of reduced tetrazolium. Fig. 18 (a and b) shows the differential migration of the two forms of DHFR under these conditions. Staining for enzyme activity in the presence of MTX (10^{-6} M) diminished the intensity of the colour due to the activity of Form 1 but not Form 2 reductase (Fig. 18). Polyacrylamide gel electrophoresis has been used by Gundersen et al., 1972 and Neef and Huennekens, 1975 to visualize complex formation between DHFRs and their substrates and inhibitors. These investigations have shown an increase in the R_f value of enzyme-NADPH-MTX complex. Upon pre-incubation of the two forms of
DHFRs with NADPH and MTX ($10^{-7}\text{M}$) for 30 min. at 4°C, an increase in the $R_f$ value of Form 1 was observed indicating the formation of a ternary complex (Fig. 18). However, the $R_f$ value of Form 2 enzyme (low-affinity enzyme) remained the same and indicated clearly the inability of this enzyme to bind MTX (Fig. 18). This was a 'visual' confirmation of the differences in the MTX binding capacities of the two forms of DHFR.

Dihydrofolate reductase activity can be stimulated by organic mercurial compounds such as pCMB and pCMS (Kaufman, 1964; Perkins and Bertino, 1964). The latter authors reported that whereas pCMB stimulated the activity of DHFR from Ehrlich ascites cells 3-4-fold when dihydrofolic acid was used as the substrate, no stimulation of activity occurred when folic acid was used as the substrate. In this present study, only a slight increase in activity of Form 1 enzyme occurred upon incubation with pCMS (upto $10^{-4}\text{M}$) (Fig. 15), whereas Form 2 activity was stimulated almost 2-fold by pCMS ($10^{-7}$ to $10^{-5}\text{M}$) using folic acid as substrate (Fig. 15). Further increasing the pCMS concentration to $10^{-4}\text{M}$ was inhibitory to the enzyme activity. An observation similar to this one was reported by Blumenthal and Greenberg (1970), who showed that the DHFR activity from a MTX-resistant mouse lymphoma cell line was stimulated by low concentrations of pCMB, but was inactivated by higher concentrations ($3\times10^{-4}\text{M}$). In contrast, the activity of DHFR from their MTX-sensitive cell line was only stimulated slightly. The differential response of the two forms of DHFR, reported in this thesis, to pCMS would suggest the presence of cysteine residues at different positions in the two enzymes, Form 2 having cysteine groups which are
more readily accessible to pCMS; the interaction of pCMS with these cysteine groups then causing the enzyme to undergo a conformational change resulting in a more rapid reduction of folic acid.

The marked difference observed in the stability of the two enzymes to heat (Fig. 14) suggests different tertiary structures and together with the differences in the overall charge and the differential effect of pCMS on enzyme activity suggests substantial alteration in the amino acid sequence of Form 2 reductase from that of Form 1 reductase.

Enzyme kinetic studies of the two enzymes showed that while the Ki for MTX was very different for the two forms, the Km for folic acid and NADPH (Table 6) were relatively similar. In order to explain this phenomenon one has to speculate that although MTX and folic acid (or dihydrofolic acid) compete for the active site at physiological pH, they do not bind to the active site in the same way; since, it appears that the binding site for folic acid is relatively unaltered, whereas that for MTX is very different, perhaps due to the substitution of one or several amino acids crucial to the tight binding of MTX. In this regard, Hood and Roberts (1978) and Charlton et al., (1979) have shown by x-ray diffraction and stereochemical studies, respectively, that folic acid and dihydrofolic acid bind to DHFR in the same orientation, whereas MTX binds to the enzyme-NADPH complex in the reverse orientation.

The Vmax of Form 2 DHFR was lower than that of Form 1 by a factor of 9.0, so that alterations leading to decreased affinity for MTX also led to diminished enzyme activity. Such a reduction in the Vmax has recently been reported by Haber et al., (1981) for an altered DHFR with a 250-fold decrease in affinity for MTX in MTX-resistant 3T6 mouse fibroblasts.
In light of the data discussed so far, three points need to be raised. Firstly, whether exposure of sensitive cells to MTX induces mechanisms which lead to resistance or whether variant cell types expressing the mechanisms of resistance appear during normal growth and are selected for upon MTX exposure; secondly, what are the molecular mechanisms for the phenotypic expression of altered dihydrofolate reductases; and thirdly, does the presence of altered DHFRs with substantially lower affinities for MTX constitute an important mechanism of resistance, and if so what types of compounds may be more potent than MTX in inhibiting their activity.

The question of whether MTX induces mutations leading to resistance (i.e. by epigenetic processes), or whether variant cell types expressing the mechanisms of resistance appear during normal growth as a result of spontaneous mutations (i.e. genetic processes) is still under considerable debate. These phenomena apply to drug resistance in general in both procaryotic and eucaryotic systems. Substantial evidence exists for the latter process, i.e. drug resistant phenotypes arise spontaneously and with a definite frequency (Luria and Delbrück, 1943; Law, 1952; Brockman, 1974; Poche et al, 1975; Ling et al, 1975). Utilizing the 'fluctuation test' Luria and Delbrück (1943) were able to show the occurrence of this process for bacterial populations resistant to viruses. Law (1952) utilized the same test to show that spontaneous mutation and selection constitute the mechanism by which MTX-resistant L1210 mouse leukemic cells develop in vivo. Siminovich (1976) has suggested that a similar process exists for the appearance of resistant cells to many of the currently used chemotherapeutic agents, although an epigenetic process may be a factor in the case of some of these
agents. Goldie and Coldman (1979) have recently developed a mathematical model which relates drug sensitivity of tumours to their spontaneous mutation rate towards phenotypic drug resistance. Analysis of this model indicated that the probability of the appearance of drug resistant phenotypes increases with the mutation rate.

The answer to the second question can at best be very speculative at the present time because the study of gene amplification and altered dihydrofolate reductases is very recent and any significant understanding of molecular mechanisms must await further work.

Whether alteration in the structure of dihydrofolate reductase occurs as a result of errors during translation or transcription; or whether separate genes coding for different forms of DHFRs exist, remains an open question. It is unlikely that errors during translation would lead to such drastically altered species of enzymes, and in addition any mutations leading to errors in the translation of proteins would probably be lethal to the cells. Errors during the transcription of DHFR gene, or during the processing of mRNA from HnRNA, may be possible. The dihydrofolate reductase gene from a MTX-resistant mouse cell line has been shown to be 42 kilobase pairs long and to contain a minimum of five intervening sequences (four of which are in the protein coding region) (Nunberg et al, 1980). If such a large amount of intervening sequence is a common property of all mouse DHFR genes then clearly, errors in the transcriptional process, due to either, point mutations in the genetic sequences of 'start' and 'stop' codons (resulting in the expression of 'noncoding' sequences), or due to errors in the processing of mRNA after transcription, could lead to the expression of mRNA coding for altered DHFRs. The existence of separate genes coding for altered DHFRs is also a distinct possibility.
Perhaps such genes are repressed in MTX-sensitive cells, and, those few cells that express these genes due to some mutational event, are selected for upon exposure to increasing concentrations of MTX. In the case of L5178Y R₄ cells which express two distinct species of enzymes, it is difficult to visualize how the expression of two such different proteins can take place from a single gene. Rather, it is very likely that two genes, one coding for Form 1 and one for Form 2 enzyme exist in these cells and both are expressed.

In this regard multiple functional mRNA species have been shown to be present in MTX-resistant cells (Dolnick and Bertino, 1981; Setzer et al, 1980). The expression of multiple mRNAs for a single enzyme is rather unique, and if it is a common property of DHFR expression, one can speculate that at least some of these mRNAs code for more than one species of enzyme.

Therefore, in resistant cells which express a single species of altered enzyme with a moderate decrease in affinity for MTX (Haber et al, 1981), errors in the transcription of 'normal' DHFR (i.e. one usually coding for high affinity DHFR) or errors in subsequent processing of mRNA, probably constitute the mechanism; whereas the expression in the same cell, of low affinity DHFR of the Form 2 type together with high affinity DHFR probably results from the expression of two separate genes. It is entirely possible that the MTX-resistant cell populations such as R₃ and R₄ are not homogeneous, but composed of several cell types expressing different mechanisms of resistance. Therefore, the 'low-affinity' and 'high-affinity' DHFRs in these cell lines may be expressed by two different cells. The only certain way for establishing
this fact is to localize DHFR activity in these cells by histochemical means, in the presence and absence of MTX. Although a histochemical technique for the detection of DHFR has been reported (Tzortzatou and Hayhoe, 1974), it is fraught with problems relating to non-specific background staining, and also difficulty in quantitation, and therefore is not very accurate.

The frequency of spontaneous mutation to an altered dihydrofolate reductase has been calculated as $10^{-9}$ with the number of mutants present in an uncloned population estimated at $2\times10^7$ (Flintoff et al., 1976; Flintoff et al., 1976). Most cell lines (including the ones described in this thesis) which have been treated with stepwise increasing concentrations of MTX have yielded resistant cells containing wild type dihydrofolate reductase expressed at high levels (Raunio and Hakala, 1967; Nakamura and Littlefield, 1972; Hänggi and Littlefield, 1976; Alt et al., 1976). Whereas the highly MTX-resistant L5178Y cells described in this thesis expressed elevated levels of DHFR, a proportion of which constituted an enzyme species with drastically reduced affinity for MTX, the study of other cells resistant to progressively increasing MTX concentrations (Albrecht et al., 1972; Biedler et al., 1972; Haber et al., 1981), has shown that growth at high drug concentrations can result in the prevalence of cells expressing elevated levels of a DHFR with reduced affinity for MTX. The question remains as to whether the mutational event leading to the expression of altered reductase occurred in the parental diploid L5178Y(S) population, and cells having a genotype for this alteration were selected for upon exposure to MTX, or, whether it occurred in cells already containing amplified DHFR genes i.e. the expression of altered DHFR is secondary
to gene amplification. Some cells are found to be intrinsically resistant to MTX (Vanden Berg et al, 1981; Kufe et al, 1980) and if such cells are found to express normal amounts of DHFR with a low affinity for MTX then this would mean that mutations leading to altered DHFR with reduced affinity can occur in the absence of an increase in gene dosage. Flintoff et al, (1976 and 1980) treated Chinese hamster ovary cells with a single high dose of MTX. Resistant cells were found to express normal levels of altered DHFR with sevenfold reduction in affinity for MTX. Therefore exposure of cells to high concentrations of MTX may select for cells with altered reductases in the absence of gene amplification. However, it is also conceivable that extended periods of growth in the presence of MTX selection, of cells with an increased number of target genes would increase the probability of the expression of an altered DHFR gene.

Regardless of the mechanism for the expression of altered reductases with lower affinity for MTX, considerable evidence exists for the presence of these forms in MTX-resistant cells. In the highly resistant L5178Y (R3) and (R4) cells described in this thesis, altered DHFR with a very low affinity for MTX exists together with a moderate elevation in the total DHFR activity, and transport defect for 'low' extracellular concentration of MTX. The significance of this altered form of DHFR in relation to elevation in DHFR activity and impaired drug transport, needs to be established.

From the experiments carried out on the uptake of MTX by R3 and R4 cells at 'low' and 'high' extracellular MTX concentrations, it would appear that growth of these cells in high concentrations of drug allows the achievement of potentially cytocidal intracellular drug levels. Despite this fact the cells were still not susceptible to MTX cytotoxicity. The appearance of cells expressing impaired uptake of
'low' \((10^{-6} \text{M})\) extracellular concentrations of MTX maybe an early event in the stepwise selection of resistant mutants. These cells, resistant to low concentrations of the drug, were probably so by virtue of impaired uptake alone and did not have elevated levels of DHFR or 'low-affinity' DHFR. With the exposure of these cells to increasing concentrations of MTX, cytocidal levels of intracellular MTX would be achieved and this new selection pressure would select for cells also expressing elevated levels of DHFR and/or 'low affinity' DHFR.

Although the most resistant cell line (L5178Y \(R_4\)) had a 9-fold elevation in DHFR level over the wild type S cells, 8\% of this was represented by the 'low-affinity' (Form 2) enzyme. Since the Vmax of the Form 2 enzyme was approximately 9-fold lower than that of Form 1 ('high-affinity') enzyme, the actual concentration of Form 2 DHFR in \(R_4\) was approximately 38\% of the total DHFR concentration (Table 5). Therefore the net elevation in DHFR activity with high affinity for MTX was only 5.6 fold over that in S cells. Clearly, an elevation of approximately 6-fold in the 'high affinity' DHFR activity of \(R_4\) cells could not alone account for the 200,000 fold increase in resistance of these cells to MTX.

The amount of DHFR represented by the 'low-affinity' enzyme in \(R_4\) cells is therefore 3.4 fold higher than the total DHFR in S cells. It has been estimated (Jackson and Harrap, 1973; White and Goldman, 1976) that in murine tumour cells no more than 5\% of the DHFR activity is required to generate sufficient tetrahydrofolate cofactors to maintain cell viability. Low affinity DHFR activity in excess of this amount is present in \(R_4\) cells and, as shown in Fig. 9, at a concentration of MTX
whereby inhibition of the high affinity DHFR is complete, there is no significant effect on the activities of low affinity DHFR (Form 2) form R₃ or R₄ cells.

Fig. 20 schematically illustrates how a moderate elevation in high affinity DHFR, along with the presence of significant levels of low affinity DHFR function together in imparting a high degree of resistance to MTX in cell types such as L5178Y (R₄).

In conclusion, the present investigation has shown that extreme resistance to MTX in L5178Y mouse leukemia cells is associated with the expression of an altered target enzyme (DHFR) with a very low affinity for MTX. Although these cells exhibited impaired uptake of 'low' concentrations (10⁻⁶M) of MTX; when exposed to 'high' concentrations (10⁻⁴M) of the drug, equivalent intracellular steady state levels were achieved in both sensitive and resistant cells. The moderate (9-fold) elevation in the DHFR concentration cannot account for the approximately 200,000 fold increase in resistance of these cells. These results agree with those reported by Biedler et al., (1972) and Albercht et al., (1972) who noted that the correlation between DHFR activity and the level of drug resistance varied for different sublines of Chinese hamster cells growing in increasing inhibitor concentrations, and that the cells containing lower levels of DHFR than expected for their degree of resistance were found to contain an enzyme with reduced affinity for MTX.

The appearance of altered DHFRs in extreme resistance to MTX is quite common (Haber et al., 1981), although the finding of this investigation, i.e. the presence of significant amounts of a DHFR with very low affinity for MTX together with a DHFR with high affinity for MTX, is unique.
The mechanisms of resistance to MTX in the clinical situation are far from clear. If dihydrofolate reductases with very low affinities for MTX are expressed in human MTX-resistant tumour cells in vivo, then compounds with significant inhibitory effects towards these types of enzymes should prove very useful in overcoming resistance to MTX. If, in addition, these variants are expressed together with normal DHFRs with high affinity for MTX, then the administration of high-doses of MTX together with compounds capable of inhibiting the low affinity DHFRs (either simultaneously or in some kind of sequence) should result in better growth inhibition of the resistant tumours.

The synthesis of compounds capable of inhibiting altered DHFRs must await the elucidation of the amino acid sequence as well as the secondary and tertiary structures of these enzymes. Amino acid sequencing and x-ray crystallographic data on the 3-dimensional structure of DHFRs from E. Coli and chicken liver (Matthews et al, 1977; Volz et al, 1982) have shown that MTX is bound in a cavity, 15Å deep. The presence of an amino group on position 4 (see Fig. 1) of MTX increases the basicity of the pteridine ring by about three pK units resulting in the protonation of the ring at the nitrogen on position 1. This protonated nitrogen interacts strongly with the side chain of an aspartate residue (in E. Coli). This interaction apparently enhances the binding of MTX relative to folic acid and dihydrofolic acid, which do not have the amino group on position 4 (Fig. 1). It can therefore be visualized that the substitution of a less polar amino acid for the aspartate residue, or the substitution of a highly basic amino acid (such as arginine) close to the aspartate residue would result in a substantial decrease in the binding affinity of MTX to the altered DHFR. The latter of the two processes has recently been described by
Baccanari et al., (1981) for an altered form of E. Coli. DHFR with a lower affinity for MTX.

Although altered DHFRs reported so far have different kinetic and physical properties, it is very likely that only a limited number of changes in the amino acids responsible for the binding of MTX at the active site, lead to a decreased affinity for it. Once these changes have been established, compounds capable of binding to this altered active site can be synthesized.

The present investigation has therefore demonstrated the existence of a biochemical target which can be exploited by chemotherapy in overcoming the heretofore unresolved problem of resistance to MTX.
Schematic representation of the effect of MTX on folate metabolism in MTX-sensitive and MTX-resistant L5178Y cells.

A. MTX-sensitive cells: MTX is actively taken up by the cells and binds strongly to the single species of 'high-affinity' folate reductase present. Inhibition of the enzyme results in the depletion of tetrahydrofolate pools with the consequent decrease in dTMP levels, leading to unbalanced and decreased DNA synthesis. The tendency of the increase in dihydrofolic acid levels (due to the inhibition of folate reductase) to displace MTX from the enzyme can be overcome by sustaining free intracellular MTX.

B. MTX-resistant cells: When exposed to 'high' concentrations ($10^{-4}$M) MTX, the drug enters the cell and binds strongly to the elevated levels of 'high-affinity' folate reductase (HA), inactivating it and leaving very little free intracellular MTX. The 'low-affinity' folate reductase (LA) (with a considerable higher $K_i$ for MTX) present in these cells will continue to be functional at the free intracellular concentrations of MTX now present. The uninhibited activity of the 'low-affinity' enzyme ensure the synthesis of sufficient amounts of tetrahydrofolate cofactors and thus of dTMP.
REFERENCES


APPENDIX I

A graphical method of obtaining values for $K_i$ in the case of competitive and non-competitive inhibitors was described by Dixon in 1953. With a competitive inhibitor the effect of varying independently both $[S]$ and $[I]$ has to be determined in order to obtain $K_i$. However, if $1/v_i$ (where $v_i$ is the initial reaction velocity) is plotted against $[I]$, keeping $[S]$ constant, a straight line is obtained, and if this is done at two different substrate concentrations, $[S_1]$ and $[S_2]$, the resultant lines will intersect at a point to the left of the ordinate axis (as in Fig. 17). This point lies at a value of $-K_i$ which can thus be read directly from the graph. This may be proved as follows:

$$E + S \xrightarrow{k+1} ES \xrightarrow{k+2} E + P; \quad E + I \rightleftharpoons EI.$$  

where $S$ is the substrate, $E$ the enzyme, $I$ the inhibitor, and $P$ the product of the reaction. It is assumed that $EI$ does not undergo further reaction. Inhibitor concentration is denoted by $[I]$ and the dissociation constant of enzyme-inhibitor complex by $K_i$. For equilibrium conditions:

$$(i.e \quad k_{+1} \ll k_{-1})$$

The rate equation is:

$$v = k + \frac{2}{2} [ES]$$

and the conservation equation

$$[E_0] = [E] + [ES] + [EI]$$
also \[ K_s = \frac{[E][S]}{[ES]} \]

\[ K_i = \frac{[E][I]}{[EI]} \]

Hence \[ [E_0] = \frac{K_s}[ES] + [ES] + \frac{K_s}[ES] \frac{[I]}{[S]} \]

Thus \[ \nu = \frac{k+2[E_0]}{1+\frac{K_s}{[S]}(1+\frac{1}{K_i})} \]

\[ \ldots \ldots . .1 \]

For steady state conditions the relationship is:

\[ \nu = \frac{k+2}{K_m \left(1 + \frac{1}{K_i}\right)} \]

\[ \ldots \ldots . .2 \]

where \[ K_m = \frac{k+2 + k-1}{k+1} \]

Rearranging Eq. 2 in the form

\[ \frac{1}{V} = \frac{K_m \left(1 + \frac{1}{K_i}\right)}{k+2} \frac{1}{[E_0]} - \frac{1}{k+2} - \frac{[E_0]}{[S]} \]

\[ \ldots \ldots . .3 \]

and replacing \( k + 2 \) \([E_0]\) by \( V \) as follows:

\[ \frac{1}{V_i} = \frac{K_m}{V} \left(1 + \frac{[I]}{K_i}\right) \frac{1}{[S]} + \frac{1}{V} \]

\[ \ldots \ldots . .4 \]

The equation represents each line and at the point of intersection \( \frac{1}{V_i} \) and \([I]\) will be the same for both lines as also will be \( V \). Consequently

\[ (1 + \frac{[I]}{K_i}) \frac{K_m}{[S_1]} = (1 + \frac{[I]}{K_i}) \frac{K_m}{[S_2]} \]

This can be true only if either \([S_1] = [S_2] \) or if \([I] = -K_i \), and since the former is not true, \([I] \neq -K_i \).
From Dawes (1972).

This method of determining values for $K_i$ is not very accurate for 'tight-binding' inhibitors i.e. inhibitors which interact stoichiometrically with the enzyme. Therefore $K_i$ values of MTX obtained for the dihydrofolate reductase from MTX-sensitive cells are only estimates and not strictly accurate. However, the DHFRs from the MTX-resistant cells exhibit higher I.D. $^{50}$ values for MTX and therefore MTX does not interact stoichiometrically with these enzymes, and the $K_i$ values obtained for these DHFRs are probably quite accurate as determined by Dixon plot.
PUBLICATIONS:


ABSTRACTS:


ARTICLES:


5. Inhibition by folic acid antagonists of a methotrexate-insensitive dihydrofolate reductase from methotrexate-resistant L5178Y cells (In Preparation).