THE UNIVERSITY OF CALGARY

AMPLIFICATION AND MUTATION OF IMP DEHYDROGENASE IN MYCOPHENOLIC ACID RESISTANT NEUROBLASTOMA CELLS

by

STEPHEN D. HODGES

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THE UNDERSIGNED CERTIFY THAT THEY HAVE READ, AND RECOMMEND TO THE FACULTY OF GRADUATE STUDIES FOR ACCEPTANCE, A THESIS ENTITLED, "AMPLIFICATION AND MUTATION OF IMP DEHYDROGENASE IN MYCOPHENOLIC ACID RESISTANT NEUROBLASTOMA CELLS" SUBMITTED BY STEPHEN HODGES IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE.

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Abstract

Mouse neuroblastoma cells (NB) have been adapted for growth in the presence of mycophenolic acid, an inhibitor of inosinate dehydrogenase (IMP·NAD Oxidoreductase, EC 1.1.1.205), by successive incremental increases in drug concentration of approximately 10% per passage. Mycophenolic acid caused 50% growth inhibition at 0.1 μM for NB and 1mM for the resistant (NB-Myco) cells representing a 10,000-fold increase in resistance. The specific activity of inosinate dehydrogenase was increased 25-fold, from 3.1 to 75 nmole/min·mg protein, and a 56.7 kDa peptide was increased in abundance 200 - 500-fold in the NB-Myco as compared to NB cells. Culture of the NB-Myco cells in the absence of mycophenolic acid resulted in a parallel decline in inosinate dehydrogenase activity, 3-fold over 80 days, and the abundance of the 56.7 kDa protein, 4-fold over 90 days. The stepwise selection, unstable phenotype, increased activity and protein abundance are indirect evidence that gene amplification has taken place; however, neither the increase in activity by itself nor the amount of the 56.7 kDa protein corresponds to the degree of increased drug resistance.

Kinetic findings are indicative of inosinate dehydrogenase having undergone a mutation. The Michaelis constants were unchanged for IMP, 14 and 13 μM, and increased for NAD^+ from 25 to 94 μM, for NB and NB-Myco respectively. The enzyme from the resistant cell is markedly less sensitive to mycophenolic acid: the K_i was increased 2400-fold from 1.4 nM to 3.4 μM for NB versus NB-Myco cells and the K_i for XMP was increased 4-fold from 78μM to 336 μM. The kinetic studies are consistent with an ordered bi bi reaction mechanism where IMP binds first and XMP is released last. Mycophenolic acid and high

concentrations of NAD^+ exhibited uncompetitive inhibition with IMP, consistent with the formation of dead end E•XMP•inhibitor complexes.

In addition a comparison of the pH optimum of the NB and NB-Myco cells showed the pH optimum to be the same for both, pH 8.0. Of three other enzymes examined in addition to inosinate dehydrogenase in the NB and NB-Myco cells only purine nucleoside phosphorylase showed a change with a 1.5-fold decrease in the NB-Myco cells. Guanylate synthase and hypoxanthine phosphoribosyltransferase showed no changes.

Nucleotide levels were perturbed in the resistant cells both in the presence and absence of mycophenolic acid. With mycophenolic acid present CTP, UTP and GTP concentrations were increased 1.3, 1.7 and 2-fold respectively. Upon removal of the mycophenolic acid, ATP continued to remain essentially unchanged but within hours GTP increased to 4.5-fold that of NB cells. UTP and CTP returned to normal levels.

The 56.7 kDa protein copurified with inosinate dehydrogenase activity from the mycophenolic acid resistant cells. In these cells it was greatly overproduced. Using a simple protocol of ammonium sulphate fractionation and gel exclusion chromatography, inosinate dehydrogenase was purified to apparent homogeneity. The subunit molecular weight was estimated to be 56.7 kDa, and the protein was shown to be N-terminally blocked. Amino acid composition was obtained for the purified protein and five peptides from endoproteinase lys-C digestion yielded limited amino acid sequence. A peptide sequence of 11 residues was used to predict a nucleotide sequence utilizing predictions of the most frequent codon usage. A unique 32 mer oligonucleotide sequence was predicted to have 82% homology with the actual mRNA sequence.

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List of Abbreviations

ADP, dADP	Adenosine and deoxyadenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
ATP, dATP	Adenosine and deoxyadenosine 5'-triphosphate
BPB	Bromophenol blue
BSA	Bovine serum albumen
cDNA	Complementary DNA
cm	centimeter
CTP	Cytidine 5'-triphosphate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EBQ	Enzyme•B•Q complex
EDTA	Ethylenediaminetetra acetic acid
EQI	Enzyme•Q•Inhibitor complex
FPLC	Fast performance liquid chromatography
GDP, dGDP	Guanosine and deoxyguanosine 5'-diphosphate
GMP	Guanosine 5'-monophosphate
GTP, dGTP	Guanosine and deoxyguanosine 5'-triphosphate
HPLC	High performance liquid chromatography
HPRT	Hypoxanthine phosphoribosyltransferase
HSR	Homogeneous staining regions
IMP	Inosine 5'-monophosphate
K_{iMyco}	Inhibitor constant for mycophenolic acid
K_{iNAD^+}	Inhibitor constant for NAD^+
K_{iXMP}	Inhibitor constant for XMP

Kb	Kilobase
kDa	Kilodalton
LD_{50}	Lethal dose 50%
mA	Milliamps
mCi	MilliCurie
mg	Milligram
K_{mIMP}	Michaelis Menten constant for IMP
K_{mNAD^+}	Michaelis Menten constant for NAD^+
mL	Millilitre
mM	Millimolar
mm	Millimeter
mmole	Millimole
MNNG	N-methyl-N-nitroso-N'-nitroguanidine
mPa	Milipascal
Myco	Mycophenolic acid
µC	MicroCurie
µg	Microgram
µL	Microliter
µM	Micromolar
NAD^+	β-Nicotinamide adenine dinucleotide
NADH	β-Nicotinamide adenine dinucleotide, reduced form
NADP	β-Nicotinamide adenine dinucleotide phosphate
NB	Mouse neuroblastoma cells
NB-Myco	1 mM mycophenolic acid resistant NB cells
NB-Myco-200	200 µM mycophenolic acid resistant NB cells
nm	Nanometer
nM	Nanomolar

nmole	Nanomole
OD_X	Optical density (absorbance) at X nm
PBS	Phosphate buffered saline
PEI	Polyethyleneimine
PNP	Purine nucleoside phosphorylase
PRPP	5'-Phosphoribosyl-1-pyrophosphate
RMS	Root mean squared
RNA	Ribonucleic acid
mRNA	Messenger RNA
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
TFA	Trifluoroacetic acid
TTP	Thymidine 5'-triphosphate
UV	Ultraviolet
UTP	Uridine 5'-triphosphate
XMP	Xanthosine 5'-monophosphate

I. Introduction

I.A. Inosinate dehydrogenase.

Inosinate dehydrogenase (IMP:NAD^+ oxidoreductase, EC 1.1.1.205) catalyses the conversion of inosinate (IMP) to xanthylate (XMP) in a reaction requiring NAD^+ (Abrams and Bentley 1959), and in the mammalian system, potassium (Anderson and Sartorelli, 1968).

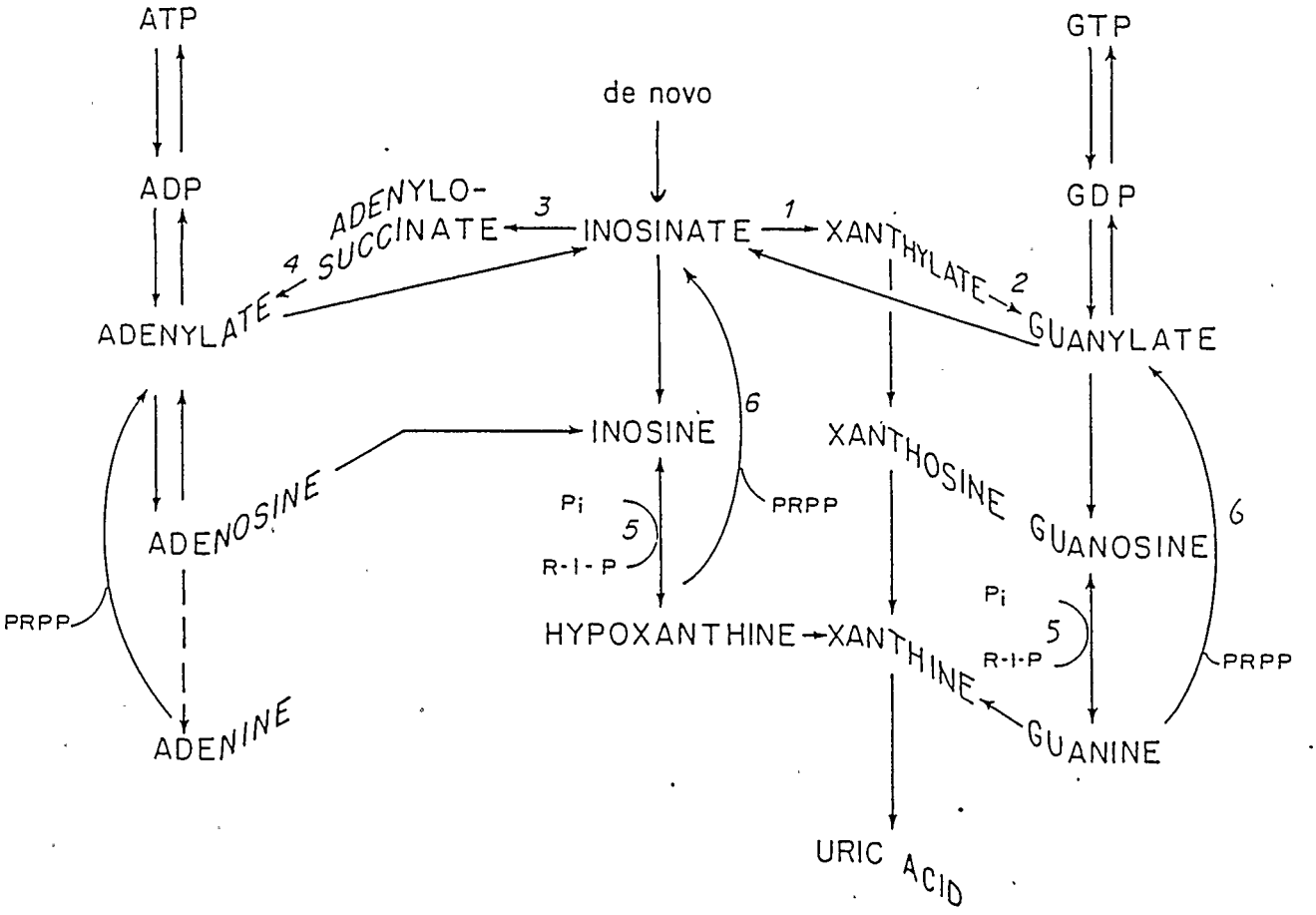
$$\text{Inosinate} + NAD^+ \xrightarrow{K^+} NADH + \text{Xanthylate}$$

Xanthylate is then converted to guanylate (GMP) in an ATP and glutamine requiring reaction. Both of these reactions are essentially irreversible (Hartman 1970). Inosinate can only be formed from guanylate in an NADPH requiring deamination of guanylate which releases NH_3 and $NADP^+$ and is catalysed by the enzyme GMP reductase.

The metabolic pathway (Figure one) shows the cumulative and central position of inosinate in the purine de novo and interconversion pathways. Inosinate may be derived from three primary sources, the first being from de novo purine synthesis, the second the purine salvage and reutilization pathway involving hypoxanthine phosphoribosyltransferase (HPRT), and the third purine nucleotide interconversion. Inosinate is the end-product of the de novo pathway, a series of 10 reactions which starts with the conversion of 5-phosphoribose 1-pyrophosphate (PRPP), in a reaction requiring glutamine, to 5-phosphoribosylamine. The enzyme that catalyzes the formation of PRPP is ribose phosphate pyrophosphokinase (or PRPP

Figure One: Pathways of purine metabolism and interconversion.

Enzymes indicated by the numbers are 1) Inosinate dehydrogenase; 2) Guanylate synthase; 3) Adenylosuccinate synthase; 4) Adenylosuccinate lyase; 5) Purine nucleoside phosphorylase; 6) Hypoxanthine phosphoribosyltransferase.



synthase). PRPP participates in a number of important reactions of pyrimidine metabolism and is an important metabolite in the first step of purine nucleotide biosynthesis. PRPP synthase is subject to inhibition by a number of purine and pyrimidine nucleotides and these include ATP, ADP, CTP, GTP and GDP and other triphosphate products, which act in a competitive manner with respect to Mg-ATP. Mg-GTP acts as an inhibitor but not in a competitive manner. Inosinate is also derived from the salvage pathway which uses the free base, hypoxanthine, a product of endogenous nucleic acid catabolism, dietary sources, or inosinate catabolism. HPRT catalyzes the conversion of hypoxanthine to inosinate in a PRPP requiring reaction. The third source of inosinate is the interconversion of nucleotides, primarily AMP. Adenylate and guanylate formation from inosinate are essentially irreversible reactions and, therefore, to form inosinate from these two sources specific enzymes are required. Adenylate conversion to inosinate is catalysed by the enzyme adenylate deaminase, whereas the conversion of guanylate to inosinate, found only in low activities in mammalian cells, is catalysed by the enzyme guanylate reductase (Hartman, 1970).

Inosinate is at the central branch point for three principal routes of inosinate metabolism. The first route is the catabolic path in which inosinate is dephosphorylated to inosine and then onto hypoxanthine by the enzyme purine nucleoside phosphorylase (PNP). Hypoxanthine can then be converted to xanthine and on to uric acid. Alternatively hypoxanthine once formed may be converted back to inosinate in a cyclic reaction. The first committed steps in the formation of the guanine and adenine nucleotides respectively comprise

the remaining two routes. Inosinate is converted to adenylosuccinate by adenylosuccinate synthase, which in turn is converted to adenylate by adenylosuccinate lyase. The first committed step of guanine nucleotide synthesis is the conversion of inosinate to xanthylate by inosinate dehydrogenase; guanylate synthase converts xanthylate to guanylate. The relative utilization of inosinate in purine metabolism and interconversion is determined by the relative activities of the three enzymes at the inosinate branch point, adenylosuccinate synthase, inosinate dehydrogenase, and 5'-nucleotidase, with each one of these enzymes controlling the amount of inosinate flowing into each specific pathway. The activity of each of these three enzymes is independently regulated (Hartman, 1970).

Attempts have been made to use inosinate dehydrogenase activity levels as a marker of malignant transformation (Jackson and Weber, 1975; Weber et al., 1980). In one study all hepatomas examined, from those dividing very rapidly to the slowest growing tumors, showed an increased level of inosinate dehydrogenase activity and this activity correlated with the growth rate (Jackson and Weber, 1975). In other reports the activities of 4 key enzymes studied in hepatomas, CTP synthase, thymidine kinase, inosinate dehydrogenase, and ribonucleotide reductase were all shown to markedly increase in parallel with tumor growth rates (Weber et al, 1980). More recently, alterations in the inosinate branch-point enzymes, particularly inosinate dehydrogenase and adenylosuccinate synthase, were examined in cultured human lymphoblasts. Inosinate dehydrogenase activity and malignancy of a cell showed a positive correlation. However, so did inosinate dehydrogenase activity and growth rate for non-malignant

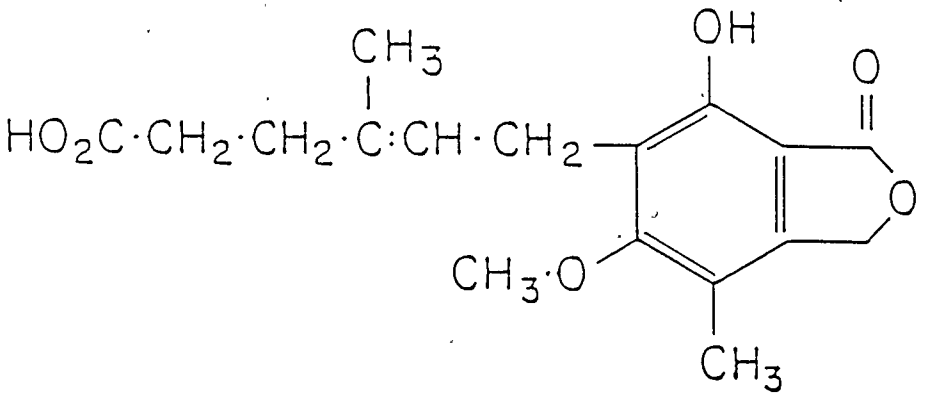
cells (Gruber et al, 1985). Therefore, correlation of cell growth rate and inosinate dehydrogenase activity for malignant (Jackson and Weber, 1975, Weber et al., 1980) and non-malignant (Gruber et al., 1985) cell lines, suggests inosinate dehydrogenase activity is inter-dependent with conditions that affect growth rate, rather than being a marker of malignant transformation (Gruber et al., 1985). In tumor cells, inosinate dehydrogenase inhibitors caused a dose dependent reduction in growth rate (Lui et al., 1984; Sweeney et al.,1972b), and in the human promyelocytic HL-60 leukemia cell line inhibition of inosinate dehydrogenase caused a dose-dependent induction of cell maturation (Sokoloski et al., 1986; Lucas et al.,1983). These observations have led to the suggestion that inosinate dehydrogenase activity and the production of guanine nucleotides are involved in regulation of growth and differentiation of mammalian cells.

In summary, the metabolic branch point at inosinate is of key importance in the cell. Studies of alterations in the activity and regulation of inosinate dehydrogenase, an enzyme at this branch point, may provide insight into aspects of purine regulation.

I.B. Mycophenolic acid.

Mycophenolic acid is a metabolically stable compound with the structure shown in figure two. It was first described by Gozio et al (1896) as a phenol isolated from the broth of a culture of Penicillium glaucum, a mold found on corn (Sweeney et al, 1972a). Alsberg and Black (1913) found several phenols in cultures of Penicillium stoloniferum (Sweeney et al, 1972a) and named one "mycophenolic acid"; it was considered to be the same compound described by Gozio.

Figure Two: Structure of mycophenolic acid.



Birkinshaw et al (1952) published a tentative formula and reported the complete structure for mycophenolic acid (Sweeney et al, 1972a). It was initially recognized as having antibacterial and antifungal activity (Abraham, 1945; Florey et al, 1946; Korzybski et al, 1967; Sweeney et al, 1972a), and by the mid 1960's as having antiviral activity (Williams et al, 1968; Sweeney et al, 1972a). Over the last 20 years interest has centered primarily on its use as an experimental oncolytic agent (Williams et al, 1968; Franklin and Cook, 1969; Sweeney et al, 1972a), and it indeed has been demonstrated to have a marked activity against a range of solid tumors in mouse and rat (Franklin and Cook, 1969; Sweeney et al, 1972a).

The biological activity of mycophenolic acid results from its inhibition of the interconversion of inosinate to xanthylate and xanthylate to guanylate by the inhibition of the enzymes inosinate dehydrogenase and guanylate synthase. RNA and protein synthesis are partially inhibited by 1μM concentrations of mycophenolic acid and DNA synthesis is substantially (90%) inhibited (Lowe et al, 1977). Concentration dependent inhibition of glycoprotein synthesis has also been recorded in sarcoma 180 cells (Sokoloski and Sartorelli, 1985).

As a result of the specific action on inosinate dehydrogenase and guanylate synthase, mycophenolic acid has been used in many studies to further understanding of the controlling factors of nucleotide metabolism and interconversion. Examples include the work of Sweeney et al (1972b) who examined β-glucuronidase, and HPRT levels in a number of different tumors. Mycophenolic acid has also been used to manipulate nucleotide pools (Cass et al, 1977) and study purine de novo synthesis

when guanine synthesis is blocked (Willis and Seegmiller, 1980; Gruber et al, 1985).

In conclusion, mycophenolic acid is a very specific inhibitor of inosinate dehydrogenase and guanylate synthase, the first committed reactions of guanine nucleotide metabolism. It is a compound of some antiquity which is being studied most recently as an oncolytic agent. Numerous studies have used this drug to look at various aspects of purine metabolism and interconversion.

I.C. Drug resistance and the selection of an amplified phenotype.

There are many reports of cultured cells that have been subject to growth in the presence of a selection agent, such as a drug. The presence of these selection agents forces the cell to adapt and overcome the growth restraints they impose. With each selection agent a different response may be elicited by the cell to overcome the growth restraint. In some the response is to promote mutagenesis and in others it is to promote gene amplification. Examples of drug induced amplification include the amplification of the dihydrofolate reductase gene in methotrexate resistant cell lines (Alt et al, 1978), or adenosine deaminase amplification as a result of coformycin resistance (Debatisse, et al, 1984).

Gene amplification is currently believed to occur as a result of DNA over-replication; that is over-replication within a single cell cycle (Stark and Wahl, 1984). Over-replication is proposed to result from the accumulation of an increased capacity for initiation of DNA synthesis in cells where DNA synthesis is partially inhibited, and where RNA and especially protein synthesis can continue (Schimke et

al, 1986). Therefore, the amplification events will theoretically be more likely to occur if DNA synthesis is partially inhibited by a specific drug or agent, with protein and RNA synthesis being essentially unaffected. In the case of mycophenolic acid it has been demonstrated to inhibit DNA synthesis in a dose dependent manner, and to have minimal effect on RNA and protein synthesis (Lowe et al, 1977), thereby making it a good agent for the selection of a gene amplified phenotype (Schimke et al, 1987). Conversely, agents that inhibit protein synthesis without blocking DNA synthesis are unlikely to generate resistance via a mechanism of gene amplification. There are multiple mechanisms by which a cell may acquire resistance to a drug, and switching from one mechanism to another may occur as cells displaying a low cellular resistance to a drug are subjected to greater drug concentrations. For example a cell may undergo amplification, then as the cells are subject to higher concentration of a drug a mutation may then occur (Haber and Schimke, 1981; Schimke et al, 1987). Alternatively a mutation may occur at low drug concentrations and at higher drug concentrations amplification may provide resistance (Flintoff et al, 1976; Schimke, 1984).

A population of cells which over-produce a protein as a result of gene amplification can best be selected by growing the cells in a drug concentration which is increased incrementally with each passage, in conjunction with the cloning of these cells and the selection of those that display resistance. Most cell lines maintained in continuous culture are suitable for the selection of a resistant line by a suitable drug or physical agent (as defined earlier, such as mycophenolic acid or methotrexate) by the use of a progressive

stepwise selection procedure, though this is not universal (Schimke et al, 1987). If gene amplification requires DNA synthesis, it logically follows that cells subjected to selection must be in an active state of growth (rapidly proliferating) in order to undergo gene amplification. The highest frequency of amplification occurs when cells are placed under selective conditions in early to mid log growth phase (Brown et al, 1983), as opposed to late growth phase or beyond. In the case of adherent cells (such as neuroblastoma cells) they often can require a period after subculture to progress into the rapid logarithmic growth phase, though longer recovery times are more likely to be required for cells that require trypsinization for subculture. Selection regimes are very important and can greatly affect the phenotype selected. For example, selection of methotrexate resistance at a concentration 20 times the LD_{50} yields methotrexate resistant colonies at a rate of 1×10^{-6} of which none were amplified phenotypes, whereas cells subjected to 5 -10 times LD_{50} give a higher frequency of 1×10^{-5} resistant clones with 50% displaying amplified phenotypes (Schimke et al, 1987). Overall, a selection regime that uses small incremental increases in drug is more likely to result in the emergence of an amplified resistant phenotype than a single large step.

Having established a resistant cell line there are a number of criteria which demonstrate that a selected phenotype is a consequence of gene amplification. Gene amplification can result in either stable or unstable resistant phenotypes. Stable phenotypes are amplified gene sequences which have been integrated into the chromosomes and are cytogenetically referred to as homogeneous staining

regions (HSR) (Biedler and Spengler, 1986). The unstable phenotype generally reflects amplified gene sequences residing in extrachromosomal minute chromosomes which often occur as doublets (Kaufman et al, 1979; Schimke et al, 1987). A stable amplified chromosome may be lost, but less rapidly than the unstable amplified chromosome which by virtue of the lack of a centromeric region can be unequally distributed in daughter cells resulting in its very rapid loss. In mouse cell lines, double minutes occur more often than HSR's (Schimke et al , 1987). Typically the hallmark of amplification is the unstable nature of the drug resistant phenotype.

Karyotypic analysis of metaphase chromosome spreads which demonstrate minute chromosomes or expanded regions of the chromosome also adds further evidence for gene amplification, though this needs to be approached with caution as the number and size of the minute chromosomes can vary greatly. The only conclusive proof with karyotypic techniques is in situ hybridization of the chromosomal spreads to localize and identify multiple copies of the particular gene.

A third method of documentation of a gene amplification is evidence of overproduction of a protein, due to gene amplification, the result of which is drug resistance. If purification is to be attempted, overproduction of the protein of interest can be of great assistance.

Finally, the ultimate and necessary proof of an amplification event is the demonstration that the copy number of a DNA sequence has increased and that this corresponds to overproduction of its mRNA and resultant protein.

A number of mammalian cells have been described which show increased resistance to mycophenolic acid and increased inosinate dehydrogenase activity (Table one). Examples in which mutagenesis has been used to obtain mycophenolic acid resistance are mouse T-lymphoma S49 cells (Ullman 1983) and a Chinese hamster V79 cell line (Huberman et al., 1981; Collart and Huberman, 1987). Resistant lines that have been selected without mutagenesis are murine leukemia L1210 cells (Cohen, 1987) and mouse neuroblastoma cells (Hodges et al., 1986).

In conclusion, a number of factors are involved in the selection of an amplified phenotype in a cell line that is forced to adapt and overcome growth restraints imposed by a specific inhibitor, in particular the drug and the selection protocol used are of key importance. Gene amplification may be inferred from specific characteristics, such as the stability of the drug resistance, the presence of double minutes and protein overproduction, but can only be conclusively identified from studies of the copy number of a specific DNA and mRNA sequence that code for an overproduced protein.

I.D. Kinetics of inosinate dehydrogenase.

The kinetics of inosinate dehydrogenase have been examined in many different tissues and cell lines including mammalian cells such as mouse sarcoma 180 (Anderson and Sartorelli, 1968; Miller and Adamczyk, 1976), human placenta (Holmes et al., 1974), human blood (Becher and Lohr, 1979), Yoshida sarcoma ascites (Shimura et al., 1983; Okada et al., 1983), rat hepatoma 3924a (Jackson et al., 1977) and mouse neuroblastoma cells (Hodges et al., 1986). Similar studies

Table One: Mycophenolic acid resistance and inosinate dehydrogenase activities in mammalian cell lines.

Cell Line	Mutagen used	Mycophenolic Acid Growth Resistance	Inosinate Dehydrogenase Activity	Reference
		Fold Increase		
Mouse Neuroblastoma	None	5000	11	Hodges et al, 1986
Murine Leukemia L1210	None	125	56	Cohen, 1987
Murine (S49) T-Lymphoma	MNNG	50	10	Ullman et al, 1983
Chinese (V79) Hamster	MNNG	500	10	Collart et al, 1987

Summary table of four cell lines resistant to mycophenolic acid. Two were obtained by mutagenesis and single step selection and two by incremental increases in the drug concentration. Shown for each is the fold increase in the mycophenolic acid resistance to growth with respect to matching control cells, and the fold increase in the inosinate dehydrogenase activity.

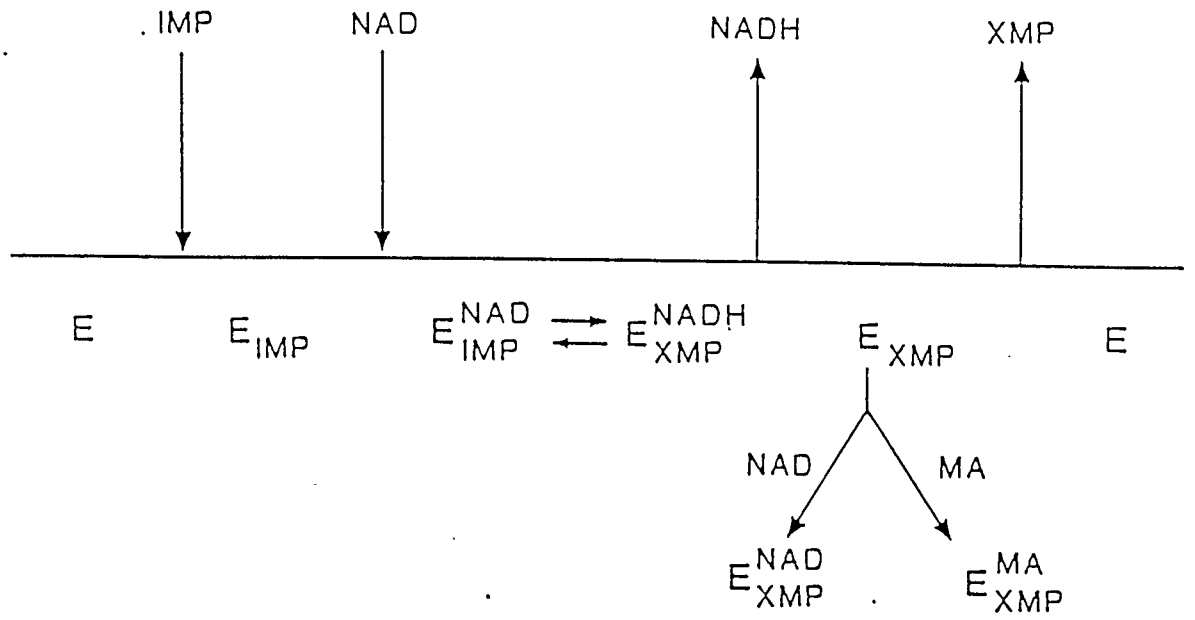
have also been conducted in non-mammalian systems, including parasitic protozoa (Verham et al., 1987; Hupe et al., 1986), nitrogen fixing nodules of cowpea (Atkins et al., 1985), Aerobacter aerogenes (Brox and Hampton, 1968; Hampton et al., 1969), and E.coli (Powell et al., 1969; Gilbert et al., 1979; Lowe et al., 1980).

Mammalian inosinate dehydrogenase exhibits Michaelis -Menten (hyperbolic) kinetics (Anderson and Sartorelli, 1968; Holmes et al, 1974; Jackson et al, 1977) unlike some bacterial studies which have suggested allosteric properties for inosinate dehydrogenase (Buzzee and Levin, 1968). Initial velocity and product inhibition studies are consistent with an ordered sequential reaction mechanism as shown in Figure three and evidence supporting this has been found in mammalian (Holmes et al., 1974), protozoan, (Verham et al., 1987; Hupe et al.,1986) and bacterial enzymes (Brox and Hampton, 1968; Hampton et al., 1969). In this scheme inosinate binds to the free enzyme first followed by NAD^+ with NADH being released first and followed lastly by XMP.

Though much work has been done on studying the mode and degree of inhibition by purine ribonucleotides and their numerous analogues on inosinate dehydrogenase from both mammalian and bacterial sources (Holmes et al, 1974; Powell et al, 1969; Miller and Adamczyk, 1976), the mechanism of inhibition by mycophenolic acid has remained unknown. Recent detailed kinetic studies have been conducted with mycophenolic acid to provide a rationale for a mechanism of inhibition (Hupe et al, 1986; Verham et al, 1987). The conclusions from these studies are illustrated in Figure three as the dead-end Enzyme•XMP•Inhibitor complex.

Figure Three: Reaction mechanism for inosinate dehydrogenase.

An ordered bi bi reaction mechanism is shown where NAD^+ and mycophenolic acid exhibit dead-end Enzyme▪XMP▪inhibitor complex formation.



I.E. Cellular nucleotides

Primary gout is characterized by hyperuricemia and an increased rate of purine synthesis is postulated as one cause (Wyngaarden and Kelley, 1976). In some inborn errors of purine metabolism, for example the Lesch-Nyhan syndrome resulting from a complete deficiency of hypoxanthine phosphoribosyltransferase (Seegmiller et al, 1967), apparent purine overproduction and hyperuricemia are key characteristics. In some instances of hyperuricemia, however, the metabolic defect is as yet unknown. An increased rate of purine synthesis has been shown to result from the inhibition of guanylate synthesis, as seen for cells exposed to mycophenolic acid (Ullman, 1983; Willis and Seegmiller, 1980). This has led to the suggestion that some patients having hyperuricemia may well have a partial deficiency of inosinate dehydrogenase (Willis and Seegmiller, 1980; Snyder et al, 1980; Ullman, 1983; Gruber et al., 1985).

Alterations in the nucleotide levels resulting from mycophenolic acid inhibition of inosinate dehydrogenase have been studied in a number of cell lines. These studies have shown that mycophenolic acid produces a marked reduction in cellular GTP (Ullman, 1983; Willis and Seegmiller, 1980; Lowe et al, 1977; Lee et al, 1985; Cohen et al, 1981) and a lesser decrease in dGTP (Lowe et al, 1977; Cohen et al, 1981). Marginal increases in CTP, UTP (Lowe et al, 1977; Lee et al, 1985) and TTP (Lowe et al, 1977) were observed with no change or a marginal decrease in ATP (Willis and Seegmiller, 1980; Lowe et al, 1977). These changes were noted for cells cultured in concentrations of mycophenolic acid which caused a loss of viability (Lowe et al, 1977; Lee et al, 1985; Cohen et al, 1981) or partial growth inhibition

(Ullman, 1983; Willis and Seegmiller, 1980). Nucleotide measurements in a mycophenolic acid resistant line showed increased GMP and GTP concentrations (Ullman, 1983).

I.F. Purification of inosinate dehydrogenase.

Many attempts have been made to purify inosinate dehydrogenase from a number of different sources and using different methods (Table two). The extent of purification varied from 48-fold to approximately 1000-fold (Jackson et al., 1977; Gilbert et al., 1979; Okada et al., 1983; Atkins et al., 1985; Verham et al., 1987). Yields were on average 25% and ranged from 11% to 50%. Only one purification attempt has been made on the protein from cells having an amplified phenotype but no details are available regarding the procedure (Collart and Huberman, 1987).

Two purification attempts of particular interest are those from Yoshida sarcoma ascites tumor cells (Okada et al., 1983), and the Chinese hamster V79 cells (Collart and Huberman, 1987). The Yoshida sarcoma ascites tumor enzyme provided the first, and currently only, amino acid composition data available for the mammalian enzyme. Comparison of this composition with that of E.coli inosinate dehydrogenase shows that the two are quite similar except for the higher glycine and lower alanine levels found in the Yoshida sarcoma ascites tumor cells (Okada et al., 1983) (Table three), though the molecular weights differed considerably with 68 kDa and 54 kDa respectively for the Yoshida sarcoma ascites and E.coli. Inosinate dehydrogenase was purified from the mycophenolic acid resistant Chinese hamster cell line by unspecified procedures. An anti-serum

Table Two: Purification of inosinate dehydrogenase; Summary of reported protocols.

Source	Methodology	Subunit Size (k daltons)	Subunit number	Reference
Rat Hepatoma	105000g supernatant Ammonium Sulphate Sephadex A-50 Sephadex G-150 DE-52	-	-	Jackson et al.,1977
E.coli	Ammonium Sulphate Agarose Column Affinity Column	54,000		Krishnaiah, 1975
Yoshida Sarcoma Ascites Cells	96,000g supernatant Ca-phosphate gel Ultrogel AcA 34 Blue Sepharose	68,000	4 (2)	Okada et al., 1983
Cowpea	Centrifugation Ammonium Sulphate Blue affi-gel Sephacryl S-200	50,000	4	Atkins et al., 1985
Protozoan	Ammonium Sulphate Bio-gel A-0.5m DEAE-Sepharose Cibacron blue PAGE	58,000	6 (4)	Verham et al., 1987
Chinese Hamster V79	No Details	56,000	-	Collart et al.,1987

Six protocols which yielded purified or partially purified inosinate dehydrogenase. The subunit size and number estimated for the purified protein are also presented.

Table Three: Amino acid composition of inosinate dehydrogenase from Yoshida sarcoma ascites cells and E. Coli.

Amino Acid	Residues per subunit	
	Sarcoma ascites	E.Coli
Lysine	43	20
Histidine	13	8
Arginine	29	24
Aspartic acid	58	32
Threonine	29	25
Serine	40	21
Glutamic acid	64	48
Proline	19	17
Glycine	128	40
Alanine	40	44
Valine	45	32
Methionine	12	8
Isoleucine	36	20
Leucine	56	33
Tyrosine	19	8
Phenylalanine	23	12
Tryptophan	0	8

Amino acid composition of inosinate dehydrogenase from purified Yoshida sarcoma ascites tumor cells (Okada et al, 1983), and E.coli (Krishnaiah, 1975), reported as residues per subunit size (nearest integer). The subunit size is 68K daltons and 54K daltons for the Yoshida sarcoma ascites cells and E.coli protein respectively.

raised against this protein was used to screen a \gt 11 expression library derived from mouse bone marrow. From this a cDNA insert was obtained and used to study mRNA levels in poly A^+ RNA of the normal and mycophenolic resistant cell lines. This work showed a 27-fold increase in the levels of inosinate dehydrogenase mRNA (a similar fold increase to that noted for the protein) in the mycophenolic resistant cell lines (Collart and Huberman, 1987).

I.G. Aims of this study.

The pathways of purine metabolism and interconversion as they pertain to inosinate and guanine nucleotide production have been discussed (I.A.) particularly with respect to the inhibitory effects mycophenolic acid can have on inosinate dehydrogenase and its role in studying this important branchpoint point (I.B.)

The aim was to select and examine a neuroblastoma cell line resistant to mycophenolic acid. The stepwise protocol favored the selection of a phenotype displaying an amplification of the cellular target of mycophenolic acid, inosinate dehydrogenase (I.C.).

With such a resistant phenotype available a number of studies were of interest. These included characterizing the degree of growth resistance; a kinetic study of the enzyme inosinate dehydrogenase to define the reaction mechanism of the mammalian enzyme and the mechanism of mycophenolic acid inhibition (I.D.); an examination of the cellular nucleotides; and purification of the amplified inosinate dehydrogenase.

II. Materials and Methods

II.A. Materials.

When not specified, all materials were the highest grade available from standard sources.

Minimum essential medium (Eagle) with Earle's salts, L-glutamine (200 mM), fetal bovine serum (mycoplasma and virus tested) and culture flasks were obtained from Gibco Laboratories. Sodium pyruvate (100 mM) was obtained from Flow Laboratories, and mycophenolic acid (100 mg vials) from Calbiochem. [8-^{14}C]IMP (specific activity 56 mCi/mmole) and [8-^{14}C]XMP (56 mCi/mmole) were purchased from Moravek Biochemicals, Inc and [8-^{14}C]inosine (57 mCi/mmole) and [8-^{14}C]hypoxanthine (55 mCi/mmole) from Amersham Canada. All sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) materials were electrophoresis grade and purchased from Bio-Rad. Polyester polyethyleneimine cellulose plates were purchased from Sigma, and cellulose plates (13254) with fluorescent indicator were from Eastman Kodak. DEAE cellulose chromatography paper (DE-81) was purchased from Whatman. Superose 12 (prep grade) media and two gel filtration calibration standards kits, a low molecular weight range (containing ribonuclease A, chymotrypsinogen A, ovalbumin, bovine serum albumen) and a high molecular weight range (containing aldolase, catalase, ferritin, thyroglobulin), were purchased from Pharmacia Fine Chemicals. Spectrapor membrane tubing was purchased from Spectrum Medical Industries. Endoproteinase lys-C (3 U) was purchased from Boehringer Mannheim.

The SDS-PAGE apparatus used was a Protean I system with a gel size of 18 x 16 cm using 0.75 mm spacers and 10 or 15 well combs, and a Protean II mini system with 7 x 8 cm gel size with 0.75 mm spacers and 5 and 10 well combs. The fast protein liquid chromatography (FPLC) system was from Pharmacia Fine chemicals and was composed of a GP-250 gradient programmer with two P-500 high precision pumps, a two channel REC-481 recorder, a FRAC-100 fraction collector, a UV-2 dual path UV monitor equipped with 254 and 280 nm detectors, and a V-7 manual valve attached to a P-1 peristaltic pump. In addition, an HR 16/50 column and HR 16 packing column were also purchased from Pharmacia Fine Chemicals. Nucleotide high performance liquid chromatography (HPLC) was achieved on a Spectra Physics 8000B equipped with a Whatman Partisil 1025 SAX column. Protein composition, peptide separation and peptide sequencing was conducted by the Protein Sequencing Laboratory, Health Sciences Centre, University of Calgary.

II.B. Methods.

II.B.1. Cell maintenance and harvesting.

II.B.1.a) Cell maintenance.

Mouse neuroblastoma cells adapted for growth from mouse C-1300 neuroblastoma cells (Seeds et al., 1970; Snyder et al., 1978) were used for the selection of resistance to mycophenolic acid.

Cells were maintained in minimum essential medium (Eagle) supplemented with 2 mM L-glutamine, 10% fetal calf serum (heat inactivated for 1 hour at 60°C), and 1 mM sodium pyruvate. Five to seven

mL cultures were maintained in 25 cm^2 polystyrene flasks, 15 mL cultures were maintained in 80 cm^2 flasks, and 30 mL cultures were maintained in 175 cm^2 flasks. Cells were grown to confluency on the large flat surface of the flasks. All cultures were maintained at 37°C under 10% CO_2 in the gas phase. At each subculture, when the cells were confluent, the media was carefully removed and replaced with 5-10 mLs of fresh media. The cells were dislodged from the flasks by vigorous tapping and then diluted 1 in 4 into new flasks containing fresh media. Cells grew to confluency within 4 days in the absence of mycophenolic acid and within 5 to 6 days in the presence of mycophenolic acid.

Mycophenolic acid was dissolved in either ethanol or at high concentrations (0.4 M) dimethyl sulphoxide (DMSO). Each was prepared in a sterile cryotube, and stored at 4°C. Mycophenolic acid was added to culture flasks containing fresh media prior to the cells being added. Using a sterile pipette tip the mycophenolic acid in solvent was dropped onto the growing surface of the flask and mixed thoroughly with the media until all was dissolved. The cells were then added and gently mixed by rocking the flask.

Cells were demonstrated free from mycoplasma by the enzyme assay described by Hatanaka et al (1975). In this assay adenine formation from adenosine was measured. Mammalian cells are unable to convert adenosine to the free base, adenine, whereas the activity is characteristic of mycoplasma.

II.B.1.b) Selection of mycophenolic acid resistant cells.

Mycophenolic acid resistant cells were obtained by incremental

increases in the concentration of mycophenolic acid of 5-10% with each passage. No mutagenizing agent was used. At passage 109, when the cells were growing in 200 μM mycophenolic acid the cells, NB-Myco-200, were cloned by dilution into 96 well microtitre plates, and several colonies were obtained which were grown from a theoretical less than one cell concentration. One of these clones was subsequently subjeced to further incremental increases in mycophenolic acid concentrations to a new level of resistance of 1 mM mycophenolic acid, NB-Myco.

II.B.1.c) Mycophenolic acid resistance analysis.

Growth inhibition of the cells by mycophenolic acid was determined by growing the cells in increasing drug concentrations and counting them using a model ZB1 Coulter counter. Cells were counted at intervals and the doubling time of mycophenolic acid treated cultures was measured over a 3 to 6 day growth period and compared with the doubling time of cells cultured in the absence of drug. The reciprocal fraction of the mean doubling time in the absence of drug was plotted against the drug concentration.

II.B.1.d) Cell harvesting.

Cells were grown for 1 to 4 days in the absence of mycophenolic acid prior to harvesting. When the flasks were ready for harvest the media was removed and replaced with 5-10 mL of fresh media. The flasks were then vigorously tapped to release the cells into the media, transferred to a 50 mL centrifuge tube and centrifuged at 300 x g for 5 minutes. The pellet was transferred to a 1.5 mL snap-cap,

washed 3 times with cold phosphate buffered saline (PBS) (pH 7.2) and finally resuspended in 20 mM phosphate buffer (pH 7.0) at a ratio of 1:1 (volume/volume) buffer to pellet. Cells were disrupted by 3 freeze-thaw cycles in liquid nitrogen and centrifuged at 12,000 x g for 15 minutes at 4°C. The supernatant was removed and stored at -20°C until required and the pellet was discarded. This crude enzyme preparation was used for all enzyme assays, purification attempts, and crude lysate SDS-PAGE runs.

II.B.2. Enzyme assays.

II.B.2.a) Inosinate dehydrogenase.

(1) Spectrophotometric.

The inosinate dehydrogenase assay mix contained 100 mM Tris-HCl (pH 7.5), 100 mM KCl, 0.3 mM NAD^+, and 1.0 mM IMP in an adaptation of the method described by Magasanik et al (1957). Approximately 40 µg of crude lysate from the mycophenolic acid resistant cells and 80 µg of crude lysate from the control cells was used in each assay with a typical reaction volume of 400 µL. The reaction was run at 37°C in a temperature controlled spectrophotometer cuvette and monitored at 340 nm to measure the conversion of NAD^+ to NADH. A value for the OD change over time (OD_{340}/Minute) was obtained and the specific activity was determined.

$$\text{Specific Activity (nmoles/min·mg protein)} = \frac{\text{OD/Minute}}{\text{Factor}} \times \frac{\text{Reaction Vol (mL)}}{100} \times \frac{1 \times 10^{-9}}{\text{mg prot.}}$$

(2) Radiochemical.

The inosinate dehydrogenase assay mix contained 100 mM Tris-HCl (pH 8.0), 100 mM KCl, 3 mM EDTA, 0.5 mM NAD^+, and 40 µM [^{14}C] IMP in an adaptation of the method described by Holmes et al (1974). Protein was added at a concentration of approximately 30 µg per assay for the control cells and approximately 2 µg per assay for the mycophenolic acid resistant cells. BSA was added to a final concentration of 0.05 mg/mL in the mycophenolic acid resistant cell lysate reaction mix to maintain a minimum protein concentration. Typically a 60 µL reaction volume was used and the concentrations of each component fixed unless otherwise specified. The reaction was run at 37°C in a shaking water bath, typically with two time points at 20 and 40 minutes. The reaction was stopped by boiling the tubes and contents (100°C) for two minutes. The tubes were stored on ice before use, then the liquid was spun into the bottom of the tube before spotting. The assay mix (25 µL) was spotted on polyester polyethyleneimine cellulose plates, up to 10 samples per plate, and the spots dried with a hair drier. Each sample spot was overlaid with 5 µL of standard containing approximately 50 mM XMP and 50 mM IMP. The plates were developed in 1 M sodium formate pH 3.4 until the solvent front had reached the top of the plate, approximately 3-4

hours. XMP and IMP marker spots were visualized with a short wavelength UV light at Rf values of approximately 0.14 and 0.40, respectively. The spots marking XMP and IMP were cut out and counted in toluene/omnifluor, and the enzyme activity was calculated as described later (Section II.B.2.e).

II.B.2.b) Purine nucleoside phosphorylase.

The reaction mix contained 100 mM phosphate buffer (pH 7.0) and 0.5 mM [^{14}C] inosine. Approximately 10 µg protein was added to the assay mix to give a final reaction volume of 100 µL. The reaction was run for 20 minutes at 37°C, quenched with the addition of 40 µL of 8 M formic acid and cooled on ice. Inosine and hypoxanthine were separated by thin layer chromatography. The assay mix (25 µL) was spotted on cellulose plates with fluorescent indicator, up to 10 samples per plate. The spots were dried with a hair drier and each sample overlaid with 5 µL of a saturated inosine/hypoxanthine solution. The samples were separated using a 1-Butanol, propionic acid, and boric acid (4%) [3:1:1] solvent system, developed until the solvent front had reached the top of the plate, approximately 3 hours (Snyder et al, 1976). Inosine and hypoxanthine were visualized with short wavelength UV light, the inosine being the lower spot and hypoxanthine the upper spot. They were cut out, counted in toluene/omnifluor and enzyme activity calculated as described later (Section II.B.2.e).

II.B.2.c) Hypoxanthine phosphoribosyltransferase.

The reaction mix contained 80 mM Tris-HCl (pH 7.2), 10 mM $MgCl_2$, 1 mM PRPP, 25 µg BSA, and 0.1 mM [^{14}C]-hypoxanthine. Approximately 5 µg protein was added to the reaction mix to give a final reaction volume of 50 µL. The reaction was run at 37°C for 60 minutes, and quenched by spotting a 25 µL sample on a DE-81 paper square (2 X 2 cm). The squares were washed 3-4 times in 2L of 1 mM ammonium formate to remove the hypoxanthine, given a final rinse in ethanol, dried and counted in toluene/omnifluor (Snyder et al, 1976). The enzyme activity was calculated as described later (Section II.B.2.e).

II.B.2.d) Guanylate synthase.

The reaction mix contained 40 mM Tris-HCl (pH 7.6), 2 mM glutamine, 10 mM $MgSO_4$, 0.3 mM ATP, and 1.8 mM [^{14}C] XMP, an adaptation of the method described by Spector et al (1976). Typically 20 µg per assay of crude protein lysate was used in a reaction volume of 100 µL, and the assay run for 60 minutes at 37°C. The reaction was stopped by the addition of 10 µL 4.4 M HCl, with the tubes then being sealed and boiled for 1 hour. Twenty five µL aliquots were spotted on cellulose plates and overlaid with 5 µL of guanine and xanthine saturated in an aqueous solution. These were developed with a methanol/formic acid/water (100:30:10) solvent system until the solvent had reached the top, approximately 3 hours. The spots of guanine and xanthine were visualized using shortwave UV light, cut, counted in toluene/omnifluor and the enzyme activity calculated as described later (Section II.B.2.e).

II.B.2.e) Isotope factor and specific activity determination.

For each radioisotope assay, a factor is determined which relates the concentration of the radioactive substrate and the counts it generates. The calculation for this is shown below, equation (1).

$$\text{Factor} = \frac{\text{nmoles of } {}^{14}C \text{ spotted}}{\text{Counts}} \qquad (1)$$

In order to determine the concentration of the specific substrate a dilution was usually required, and then the optical density at Y_{max} was taken and used to calculate the concentration as shown in equation (2).

$$\text{Concentration(M)} = \frac{OD_{Ymax} \text{ X dilution}}{\text{Extinction coefficient}} \qquad (2)$$

For inosine, inosinate, hypoxanthine, and xanthylate the Y_{max} and the extinction coefficients (E) at pH 6.0 (for a 1 cm path length) are shown in the following table:

Substrate	Y_{max}	Extinction coefficient
Inosine	248.5	12.3×10^3
Inosinate	248.5	12.3×10^3
Hypoxanthine	248	10.8×10^3
Xanthylate	263	8.95×10^3

These factors are determined by counting a known amount of each purine on the matrix (DE-81 or PEI cellulose) that will be used in the assay, thereby accounting for the different matrices applicable to the system and the counting efficiency of the liquid scintillation counter.

II.B.3. Kinetic studies.

II.B.3.a) Initial velocity studies with varied substrate.

Initial velocity studies were conducted by varying the two substrates of the bi-reactant system, IMP and NAD^+, and measuring inosinate dehydrogenase activity. The IMP concentrations used were 10, 13.3, 20, 26.7 and 40 μM for the NB and 4, 5, 6.66, 10, and 20 μM for the NB-Myco assay at changing fixed concentrations of NAD^+ of 0.1, 0.167 and 0.4 mM for the NB and 0.1, 0.167 and 0.5 mM for the NB-Myco assay.

The initial velocity studies provide information as to whether the reaction mechanism is sequential or ordered, the determination of the Michaelis constants for IMP and NAD^+ and the calculation of the maximal velocity (V_{max}). Results are presented as reciprocal plots

of velocity versus [IMP], with each line representing a different concentration of NAD^+.

II.B.3.b) Product inhibition studies.

Inhibition studies for the product XMP were conducted in a similar manner to the initial velocity studies by varying the substrate (IMP) at changing fixed concentrations of the product (XMP). The IMP concentrations used were 10, 13.3, 20, 26.7 and 40 μM for both the NB and NB-Myco at changing fixed concentrations of XMP of 0, 40, 80, 120 and 160 μM for the NB and 0, 0.5, 1.0 and 1.5 mM for the NB-Myco enzyme. The NAD^+ concentration was 0.5 mM and all other components were held constant.

Product inhibition studies allow for an ordering of the reactants and products, determination of the inhibition constant (K_{iXMP}), the Michaelis constant for IMP (K_{mIMP}) and the maximal velocity (V_{max}). Results are presented as reciprocal plots of velocity versus [IMP] with each line representing a different XMP concentration.

II.B.3.c) Substrate inhibition studies.

Previous studies had suggested that NAD^+ could inhibit inosinate dehydrogenase. Substrate inhibition studies were conducted by varying the substrate IMP in the same range used in the initial velocity studies, at changing fixed concentrations of NAD^+ greater than 0.4 mM. NAD^+ concentrations were 0.4, 0.8, 1, 2.5, and 5mM for the NB and 0.5, 1, 2.5, 5, 7.5 and 10 mM for the NB-Myco enzyme.

Substrate inhibition studies provide information on the mechanism of substrate inhibition, and the determination of the inhibition

constants for NAD^+ ($K_{iNAD}+$), the Michaelis constant for IMP (K_{mIMP}) and the maximal velocity (V_{max}). Results are presented as a reciprocal plot of velocity versus [IMP] for increasing NAD^+ concentrations.

II.B.3.d) Inhibition by mycophenolic acid.

Inhibition studies for the inhibitor, mycophenolic acid, were conducted by varying the IMP concentration at different concentrations of the drug. The IMP concentration used were 10, 13.3, 20, 26.7 and 40 μM for both the NB and NB-Myco cells at changing fixed concentrations of mycophenolic acid of 0, 2.5, 5, 10 and 20 nM for the NB and 0, 1.5, 3, 6 and 12 μM for the NB-Myco cells. The NAD^+ concentration was 0.5 mM and all other components were constant.

Inhibition studies provide information on the kinetic mechanism of the inhibitor, and the determination of an inhibition constant for mycophenolic acid (K_{iMyco}), the Michaelis constant for IMP (K_{mIMP}), and the maximal velocity (V_{max}). Results are presented as a reciprocal plot of velocity versus [IMP] for increasing mycophenolic acid concentrations.

II.B.3.e) Data analysis

The kinetic parameters were calculated in two ways. Firstly with the use of reciprocal plots and replots, the equations that define the intercepts and slopes provided definitions of the kinetic parameters. Secondly with the use of a computer program, "Enzyme", compiled in Basic for the IBM-PC by Lutz and Rodbard (Lutz et al, 1986).

In the first approach, the equation of each line of a reciprocal plot of velocity versus [IMP] is calculated using least squares linear regression. Equations for the line of a replot of the slopes or 1/velocity versus the changing fixed component, for example [NAD^+], were determined by linear regression. The kinetic parameters were derived from the intercepts and slopes of the replots as taken from the equation of the line. The definition of these intercepts and slopes, and the full rate equation are detailed in the appendix. This approach was used in the determination of an inhibitory constant for NAD^+, and a comparison of the Michaelis constants obtained for IMP and NAD^+ from the initial velocity studies.

Enzyme-substrate-inhibitor analysis was assisted by the use of a computer program "Enzyme". This program uses the classification of models, terminology and nomenclature of Wong (1975), and allows for weighted nonlinear least squares fitting with iterative re-weighting to estimate parameters that obey Michaelis-Menten kinetics. The program first provides initial estimates of V_{max} and K_m based on Eadie plot linearization. Then using non-linear least squares analysis the estimates are refined and the procedure repeated for data with different inhibitor concentrations. The effects of inhibitors on the maximal velocity (V_{max}) and the Michaelis-Menten constant (K_m) are used to automatically select the most plausible model of inhibition using a slightly modified two sided unpaired student t-test. The program selects the simplest model that fits the data. For example, if the inhibitor causes only the apparent K_m to increase with increasing inhibitor concentration, then the inhibition is either pure or partial competitive, and the program will choose pure competitive

inhibition. The selected model can then be compared with various alternatives and a different model selected if desired. The program then iterates the data and calculates the V_{max}, K_m, and the inhibitor K_i. The standard errors and the weighted RMS error value for these are also calculated showing the deviation of the points from the predicted curve.

The equations that detail the calculations for the program "Enzyme", and descriptions of the various models are discussed in the appendix.

II.B.4. SDS-polyacrylamide gel electrophoresis.

II.B.4.a) Gel Preparation.

SDS polyacrylamide gels were prepared and run according to the method of Laemmli (1970). Typically a 12% gel was composed of 0.375 M Tris-HCl (pH 8.8), 0.1% SDS, 2.5% glycerol, 12% acrylamide, and 0.3% bis-acrylamide. A 4.5% stacking gel composed of 0.125 M Tris-HCl (pH 6.8), 4.5% acrylamide and 1.2% bis was poured on top of the polymerized running gel. Two sizes of gel systems were used, the larger being the Protean I system (Bio-Rad laboratories) with a gel size of 18 x 16 cm, the smaller system being the mini Protean II (Bio-Rad laboratories) with a gel size of 8 x 7 cm. The spacer used for both was 0.75 mm, with 10 and 15 well combs for the Protean I and 5 and 10 well combs for the Protean II mini system.

II.B.4.b) Sample preparation.

Samples were prepared by mixing the protein with sample buffer (2x sample buffer containing 0.2 M Tris-HCl, 20% sucrose, 4% SDS, and 0.004% bromophenol blue (BPB)). Samples were then heated to 95°C for 5 minutes, cooled on ice and briefly spun before loading. An average load of a sample with a broad spectrum of proteins was 20 µg per lane for the protean I system and 5 µg per lane for the mini protean II. For a single species of protein the minimum load that could be easily visualized was 1 µg using the protean II mini gel system.

II.B.4.c) Running conditions.

The gel running buffer was 0.025 M Tris-Base, 0.15 M glycine, and 0.15% SDS. Protean I gels were run at a constant current setting of 30 mA limiting with the voltage at 1000 V for approximately 3 hours, and the mini protean II gels run at a constant current of 50 mA limiting with a voltage setting of 200 V for 45-60 minutes. The power was the same for each at 50 W. Protean I gels were run with water cooling and the protean II run at room temperature.

II.B.4.d) Staining conditions.

Once the BPB dye front had just run off the bottom of the gel, the gel was removed and stained in Coomassie blue stain for 20 minutes: methanol, glacial acetic acid and water (9:2:9) with 0.126% Coomassie brilliant blue. It was then destained overnight in destain solution: methanol, glacial acetic acid and water (10:15:175). The gels were photographed on a light box using a Y_2 yellow filter and

Polaroid type 52 film (F_{16}, 1/60), and stored moist in a sealed plastic bag at 4°C.

II.B.5. Cellular nucleotides.

II.B.5.a) Acid extraction of cells.

Nucleotide analysis was conducted on acid extracts of exponentially growing cells (Snyder et al, 1973). Subcultures for harvest were established so that confluency would be obtained prior to the time of harvest. For the determination of cellular nucleotide pools of cells grown in the absence of mycophenolic acid the following protocol was used. At time zero the drug containing media was gently removed, discarded, and the cells still adhering to the flask were rinsed with 5 mLs of complete media. Then 30 mLs of fresh media (without mycophenolic acid) was added to the cells and the flask was returned to 37°C. Cells grown in the absence of mycophenolic acid longer than 24 hours were subcultured as usual into fresh mycophenolic acid free media.

To harvest and extract the cells for nucleotide determinations the cells were detached and transferred in media into a 50 mL conical centrifuge tube on ice. The culture flask was rinsed with 5 mL of fresh, 4°C, media and pooled. Cells were pelleted (300 x g, 2.5 minutes), the supernatant was carefully removed and the pellet resuspended in approximately 150 µL of fresh media , 4°C, (gassed with CO_2) and a measured volume, 110 µL, transferred to a 12 x 75 mm glass test tube on ice. Ten µL was added to saline, and the cell number determined. The remaining 100 µL of cell suspension was lysed and

deproteinized by the addition of 15 µL of 21% perchloric acid, followed by vortexing and placement on ice for 5 minutes. Extracts were centrifuged at 10,000 x g for 2 minutes and 100 µL of the supernatant was removed and neutralized with 10 µL of 4 M KOH. The $KClO_4$ pellet was removed and discarded by centrifugation and the supernatant was stored at -70°C for analysis.

II.B.5.b) HPLC separation of the nucleotides.

Nucleotide analysis of cellular extracts was conducted on a Spectra Physics 8000B equipped with a Whatman Partisil 1025 SAX column. Separation was achieved using a two-component gradient: buffer A, 5mM KH_2PO_4 (pH 4.0), and buffer B, 1 M KH_2PO_4 (pH 4.0). The gradient was 100 to 90% A from 0 to 12 minutes, 90 to 50% A from 12 to 47 minutes, constant at 50% A from 47 to 70 minutes, with a flow rate of 1.5 mL/min at 30°C (Lukey and Snyder, 1983). Nucleotides were identified by retention time, and the absorbance at 254 and 280 nm was used for the quantitation by comparison with standards. HPLC separation analysis was conducted by E. Fung, Biochemical Genetics Laboratory, Alberta Childrens Hospital, Calgary.

II.B.6. Ammonium sulphate fractionation.

The 30 - 40% ammonium sulphate fraction of a cell lysate was obtained by the following procedure. A 75% ammonium sulphate solution (516 g/L) and a 75% glycerol solution were added to the cell lysate in minimal quantities to provide a final concentration of 30% ammonium sulphate and 10% glycerol. This was stored on ice for 15 minutes and then centrifuged for 15 minutes at 6,000 x g. The pellet

was discarded and 75% ammonium sulphate and 75% glycerol were added to the supernatant to give final concentrations of 40% ammonium sulphate and 10% glycerol in a minimal volume. This was left on ice for a further 15 minutes and centrifuged as before. The supernatant was discarded and the pellet washed once with a fresh solution of 40% ammonium sulphate and 10% glycerol, and finally dissolved in the buffer used for the Superose column run.

The optimal range for ammonium sulphate fractionation of inosinate dehydrogenase was determined by fractionating crude cell lysates at 20, 25, 30, 35 and 40% ammonium sulphate each with 10% glycerol using the procedure described above. The pellet and supernatant from each were examined by SDS-PAGE to identify the fraction containing the 56.7 kDa protein.

II.B.7. Fast Performance Liquid Chromatography, gel filtration.

II.B.7.a) Column preparation.

Pharmacia Superose 12 (prep grade) gel is a high performance gel filtration media designed for preparative purification of biomolecules. Superose 12 prep grade is a cross linked agarose based medium with an exclusion limit (MW) of approximately 2×10^6, a separation range (MW) of 1×10^3 - 3×10^5 and a particle size limit (μm) of 20 - 40.

Column media came stored in 20% ethanol, and for column preparation 105 mL was washed with distilled water on a 4 - 5.5 μm scintered glass filter and finally made up to a 180 mL slurry in buffer A (20 mM Tris-HCl, 0.1 M KCl, pH 8.0). The slurry was poured (in a single

pour) into a Pharmacia HR 16/50 column using a Pharmacia HR 16 packing column. The gel was packed at a rate of 2 mL/min until settled. The packing column was then removed and the gel further packed at 6 mL/min for approximately 60 minutes. The maximum allowable back-pressure that the gel can withstand is 0.7 MPa; typically the back pressure was less than 0.1 MPa. When not in use the gel was stored in a Tris azide solution (Buffer A with 1% sodium azide).

All buffers used for FPLC were prepared at room temperature, and filtered through a 0.45 µm filter, and degassed for 15 minutes prior to use. Before any sample was loaded onto the sample loop, the pump was flushed through with the new buffer and the column washed with 2-4 void volumes of fresh buffer.

II.B.7.b) Column standards

From the Pharmacia high and low molecular weight standards the following were chosen: chymotrypsinogen A, 25 kDa; bovine serum albumen, 67 kDa; catalase, 232 kDa; and thyroglobulin, 669 kDa. Two hundred µL of a 10 mg/mL solution of each was mixed with 200 µL of blue dextran, 2,000 kDa (which was used to determine the void volume), to give a sample volume of 1 mL. This was run through the column with buffer B (20 mM Tris-HCl, 0.5 M KCl, and 5 % ammonium sulphate, adjusted to pH 7.0).

II.B.7.c) Column run.

Typically 25 - 30 mg of crude cell lysate were used per purification run of which approximately 10-15 mg were obtained following the ammonium sulphate fractionation, and loaded into the gel filtration

column. The pellet from the ammonium sulphate fractionation was resuspended in buffer B and loaded into the sample loop using the peristaltic pump (sample loop size varied from 10 μL up to 2 mLs), and run into the column with buffer B. Typically the program used a one buffer system with a flow rate of 1-2 mL/min and a chart speed of 0.3 cm/mL. Collection was started at approximately 28 mLs which was less than the void volume, and was stopped after the collection of 100 mL. The column effluent was monitored at OD_{280} with the sensitivity being varied according to the protein load. Fractions (1 mL) were collected into 1.6 mL snap-caps and stored at -20°C. The fractions were subject to protein determination, inosinate dehydrogenase activity measurements, and SDS-PAGE. Suitable fractions were pooled, dialysed against 20 mM Tris HCl, pH 7.0, concentrated on the Speedi Vac and stored at -20°C. These samples were used for composition and sequence determination.

II.B.8. Protein composition and sequence determination.

II.B.8.a) Composition determination.

Composition determination was conducted by B.S. Renaux and D.J. McKay of the Protein Sequencing Laboratory, Health Sciences Centre, University of Calgary. In this procedure purified protein was subject to 24 hour acid hydrolysis (6N HCl, 0.1% phenol, and 0.1% thioglycolic acid) at 110°C, followed by HPLC separation using a reverse-phase C_{18} column to allow quantitation and identification of the amino acids.

II.B.8.b) Carboxymethylation and enzyme digestion.

The protein sample for sequencing was mixed with 7.5 M guanidine-HCl in 0.5 M Tris (pH 8.5) buffer to give a final guanidine-HCl concentration of 5M. A typical reaction volume of 500 µL contained 800 µg (14 nmoles) of protein which was mixed thoroughly to ensure that all the precipitate was dissolved. All components used were freshly prepared and stored in the dark and the carboxymethylation reaction was conducted in the dark at room temperature. To the protein, 10 µL of 50 mM dithiothreitol (DTT) was added, mixed and left for 30 minutes, then 10 µL of 0.25 M iodoacetic acid was added and stirred for 60 minutes. Excess iodoacetic acid was neutralized by the addition of one drop of mercaptoethanol. The sample was then dialysed overnight at 4°C against 0.2 M ammonium bicarbonate, pH 8.5 containing 0.25 M guanidine-HCl. It was then dialysed for 4 hours at 4°C against 0.2 M ammonium bicarbonate, pH 8.5 and monitored carefully to ensure that no precipitate formed during this period.

The final volume following dialysis was 1.5 mL, and this sample was subjected to digestion with Endoproteinase lys-C. One hundred µL of enzyme (2 units in 50 mM Tris-HCl buffer, pH 8.5) was added and incubated at 37°C for 24 hours in a shaking water bath. Once complete, this sample was concentrated to 300 µL and 105 µL (7 nmoles) was used for sequence determination.

II.B.8.c) HPLC separation and peptide sequencing.

Peptides modified by the endoproteinase lys-C digestion of the purified protein were passed on to D.J. McKay and B.S. Renaux of the

Protein Sequencing Laboratory, Health Sciences Centre, University of Calgary. The peptides were separated on a reverse-phase Vydac C_{18} column with 500 µL fractions being collected. The buffers used were buffer A, acetonitrile, with 0.095% trifluoroacetic acid (TFA); and buffer B, water, with 0.1% TFA. The gradient used was 0 - 50 % buffer A between 0-50 minutes, and 50 - 85% buffer A between 50-65 minutes then back to 0% buffer A by 73 minutes. Fractions representing peaks were pooled, concentrated and subjected to sequencing cycles on an Applied Biosystems 470A gas phase protein sequencer (Hewick et al, 1981). The amino acids were identified by reverse-phase HPLC using an Econosphere C_{18} column in a Varian 5000 connected to a Waters model 44 absorbance detector (Zimmerman et al, 1977; McKay et al, 1985).

III. Experimental

III.A. Selection of cells resistant to mycophenolic acid

III.A.1. Introduction

A number of protocols can be used to obtain a cell line that displays resistance to a specific inhibitor and these affect the way in which the cell adapts to overcome the inhibitory effects. When a chemical mutagen/carcinogen, such as N-methyl-N' -nitro -N-nitrosoguanidine (MNNG), is used to induce a mutation, cells capable of growing in higher concentrations of a specific inhibitor are selected (Ullman, 1983). Alternatively a high concentration of a specific inhibitor can been used in a single one step procedure without the use of a mutagen to select a resistant cell line (Brown et al, 1983). In both these cases the most likely product is a cell that has achieved its acquired resistance by a mutational change, with very few, if any, colonies displaying a mechanism of resistance involving amplification. In another approach the specific inhibitor is added to the exponentially growing cells at low concentrations and is progressively increased with each passage or as the cells adapt to each new concentration of inhibitor (Alt et al, 1978). When the inhibitor concentration is increased in small incremental stages the drug resistance will often be due, in whole or in part, to increased expression of the inhibitor target. Cellular resistance to an inhibitor due to an amplification of a specific gene will often result in overproduction of a specific protein, though amplification and mutational events are not mutually exclusive. As well as the method

of selection of resistance there are many other factors that dictate whether a cell will develop resistance to a specific inhibitor by a mutational event, an amplification or another method. These include the cell type, the selection regime and the mode of inhibition of the inhibitor as reviewed earlier (I.C).

Inosinate dehydrogenase is one of the rate controlling enzymatic steps of nucleic acid biosynthesis and a correlation has been reported between its activity and cellular proliferation suggesting that inosinate dehydrogenase may be involved in growth regulation (Jackson et al, 1975; Weber et al, 1983). Mammalian cells in culture are dependent for growth on the conversion of inosinate to xanthylate. This in turn is converted to guanylate providing the first committed reaction towards guanine nucleotide synthesis. Mycophenolic acid is a potent inhibitor both of inosinate dehydrogenase (Franklin and Cook, 1969; Sweeney et al, 1972b; Smith et al, 1974; Cass et al, 1977; Cohen et al, 1981) and guanylate synthase (Sweeney et al, 1972b; Cohen et al, 1981). Potent and specific inhibitors of enzymes essential for the growth of a cell in culture have been used in many cases for selecting cell lines that overproduce a target enzyme. Examples of this include the dihydrofolate reductase gene multiplication in methotrexate variants (Alt et al, 1978) and the amplification of the first three enzymes of UMP synthesis in N-(Phosphonacetyl)-L - aspartate-resistant cells (Whal et al, 1979).

Mechanisms of cellular resistance to a specific inhibitor include reduced intracellular conversion to an active form of the inhibitor, increased production of a cellular target, structural alterations in

the cellular target, or decreased transport into the cell of the inhibitor.

The mode of resistance for mycophenolic acid acting on inosinate dehydrogenase and guanylate synthase does not involve the cellular conversion of mycophenolic acid to an active form; the native form is the active form. The only metabolic alteration of the mycophenolic acid reported in whole animal metabolism studies was its conjugation with glucuronic acid, a normal mechanism of detoxification of phenolic compounds (Sweeney et al, 1972b). This conjugation reaction affects its transport into the cell as the mycophenolic acid glucuronide is unable to cross the cell membrane, whereas the unconjugated form is very lipid soluble. β-Glucuronidase activity is reported to correlate with the sensitivity of tumors to mycophenolic acid, its mode of action being to hydrolyse the glucuronide conjugates. With increased β-glucuronidase activity a greater concentration of unconjugated mycophenolic acid would be available for entry into the cell decreasing its resistance (Sweeney et al, 1972b). Mycophenolic acid conjugation generally occurs in the liver and therefore, as a mode of resistance, is not of concern with cultured cells.

The most likely causes of altered resistance for cells in culture are an increased cellular target and/or a structural alteration of the cellular target. Examples of cells resistant to mycophenolic acid as a result of mutagenisis are Chinese hamster V79 cells (Huberman et al, 1981; Collart and Huberman, 1987) and murine lymphoma cells S49 (Ullman, 1983) which were both subject to MNNG treatment. Following mutagenesis, they were placed in a high concentration of mycophenolic acid. Those cells able to grow in this inhibitor concentration were

subjected to another round of mutagenesis and selection. Chinese hamster V79 cells were able to grow in 50 μg/mL mycophenolic acid (6.4 μM), a 500-fold increase in resistance over the control cells (Collart and Huberman, 1987) and lymphoma S49 cells displaying a 50-fold increase in resistance were able to grow in 20 μM mycophenolic acid.

Without the use of a mutagen, murine L1210 cells were obtained that were able to grow in 20 μM mycophenolic acid and displayed 125-fold increase in resistance (Cohen, 1987) and NB-Myco-200 cells were obtained that were able to grow in 200 μM mycophenolic acid, representing a 5000-fold increase in resistance (Hodges et al, 1986). To obtain mycophenolic acid resistant leukemia L1210 cells, wild type cells were grown in medium containing mycophenolic acid. Every three weeks the concentration of mycophenolic acid was doubled. Periodically cells were plated in media similar to that in which they were growing, except agarose was also included, and individual colonies selected and expanded into cell lines. Eventually a number of clones were obtained that were 2 to 125-fold more resistant to mycophenolic acid than the parent wild type cells (Cohen et al, 1987). The report on neuroblastoma cells forms a part of this work and is reported in more detail here (Hodges et al, 1986; Section III.A.2.).

III.A.2. Results

Mouse neuroblastoma (NB) cells were adapted for growth in mycophenolic acid by successive incremental increases in the concentration of mycophenolic acid, approximately 10% per passage. Neuroblastoma cells were cloned at passage 109, when the cells were

growing in 200 μM mycophenolic acid(NB-Myco-200), and were eventually selected for growth in 1 mM mycophenolic acid (NB-Myco).

The growth inhibitory response of the control, NB, and the mycophenolic acid resistant neuroblastoma cells at passage 25 (p25), passage 99 (p99) and the NB-Myco-200 and NB-Myco are compared (Figure four and five), and the 50% growth inhibition and fold resistance are compared to the parental cell line, NB (Table four). 50% growth inhibition occurred at 0.0001, 0.5 and 1 mM for the NB, NB-Myco-200 and NB-Myco respectively, representing a 10,000-fold increase in resistance to the mycophenolic acid for NB-Myco as compared to the NB cells.

III.A.3. Discussion

Described here is a derivative of the 200 μM resistance mouse neuroblastoma cell line, NB-Myco-200 (Hodges et al, 1986), which has been adapted for growth in 1 mM mycophenolic acid, NB-Myco, by successive incremental increases in mycophenolic acid. Several mammalian cell lines have been selected for their resistance to mycophenolic acid, and these include mouse T-lymphoma S49 cells, 20 μM (Ullman, 1983); Chinese hamster V79 cells, 3.1 μM (Huberman et al, 1981) and 156 μM (Collart and Huberman, 1987); and mouse leukemia L1210 cells, 34 μM (Cohen, 1987).

Growth inhibition studies showed a steady increase in the concentration of mycophenolic acid required to elicit 50% growth inhibition as the cells were selected for growth in higher concentrations of mycophenolic acid, as shown in p25, p99 and p109 (Figure four and five, Table four). The NB-Myco-200 displays a 5000-fold increase in

Figure Four: Growth inhibition by mycophenolic acid for control and mycophenolic acid resistant neuroblastoma cells.

The growth inhibitory effect of increasing concentrations of mycophenolic acid on NB (0) and mycophenolic acid resistant (Δ , □) cells is given. The mycophenolic acid resistant cells are upper panel p25 (Δ) and p99 (□) and the lower panel NB-Myco-200 a (Δ) and b (□). Each point represents the reciprocal fraction of the mean doubling time of mycophenolic acid-treated cultures measured over a 3 to 6 day growth period compared to the doubling time in the absence of drug as described in the methods (II.B.1).

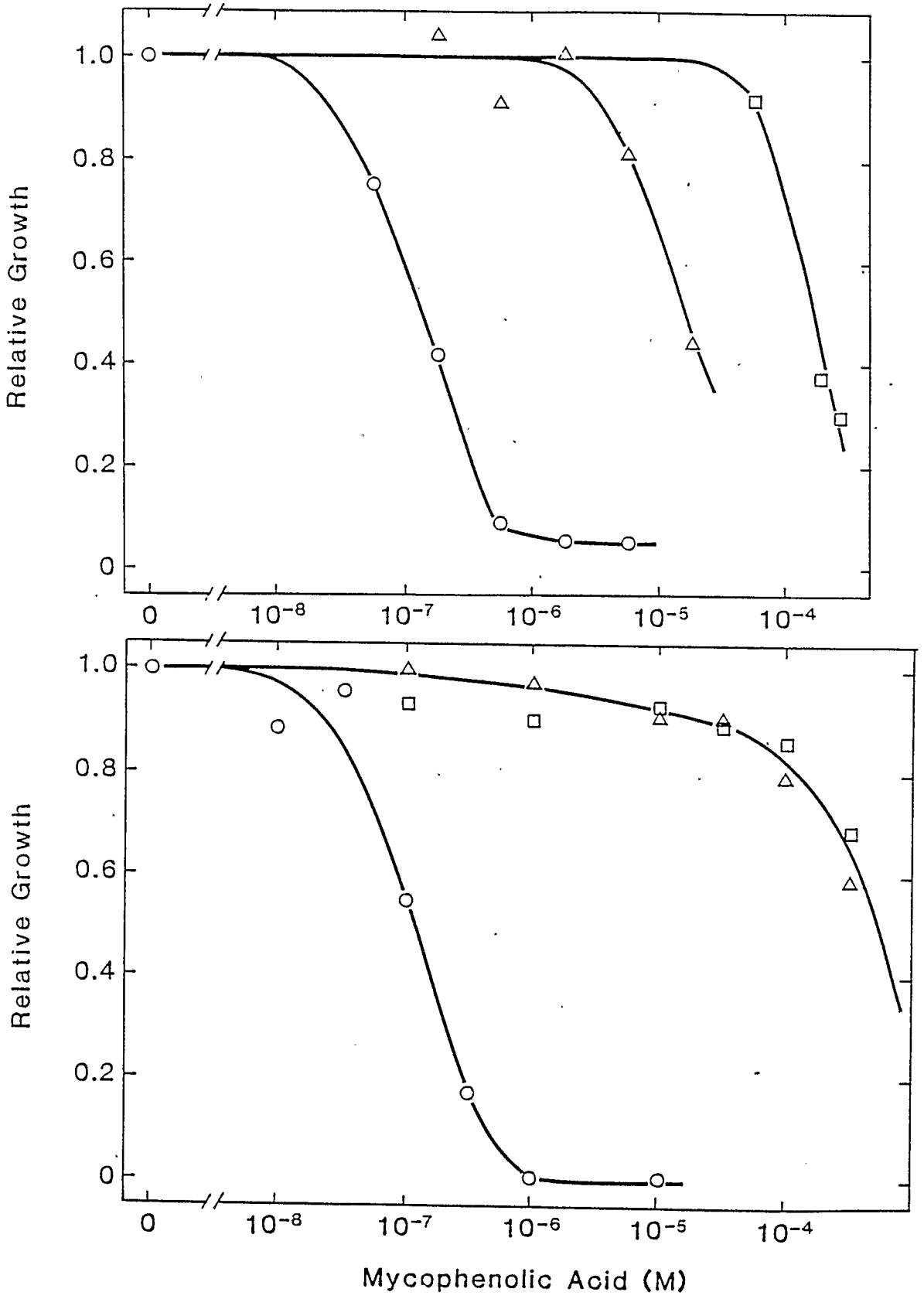

Relative Growth
1.0
0.8
0.6
0.4
0.2
0
0
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
Relative Growth
1.0
0.8
0.6
0.4
0.2
0
0
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
Mycophenolic Acid (M)

Figure Five: Growth inhibition by mycophenolic acid for control (NB) and 1 mM mycophenolic acid resistant (NB-Myco) neuroblastoma cells.

The growth inhibitory effect of increasing concentrations of mycophenolic acid on NB (O) and NB-Myco (△) cells is given. Each point represents the reciprocal fraction of the mean doubling time of mycophenolic acid-treated cultures over a 3 to 6 day growth period compared to the doubling time in the absence of drug as described in the methods (II.B.1).

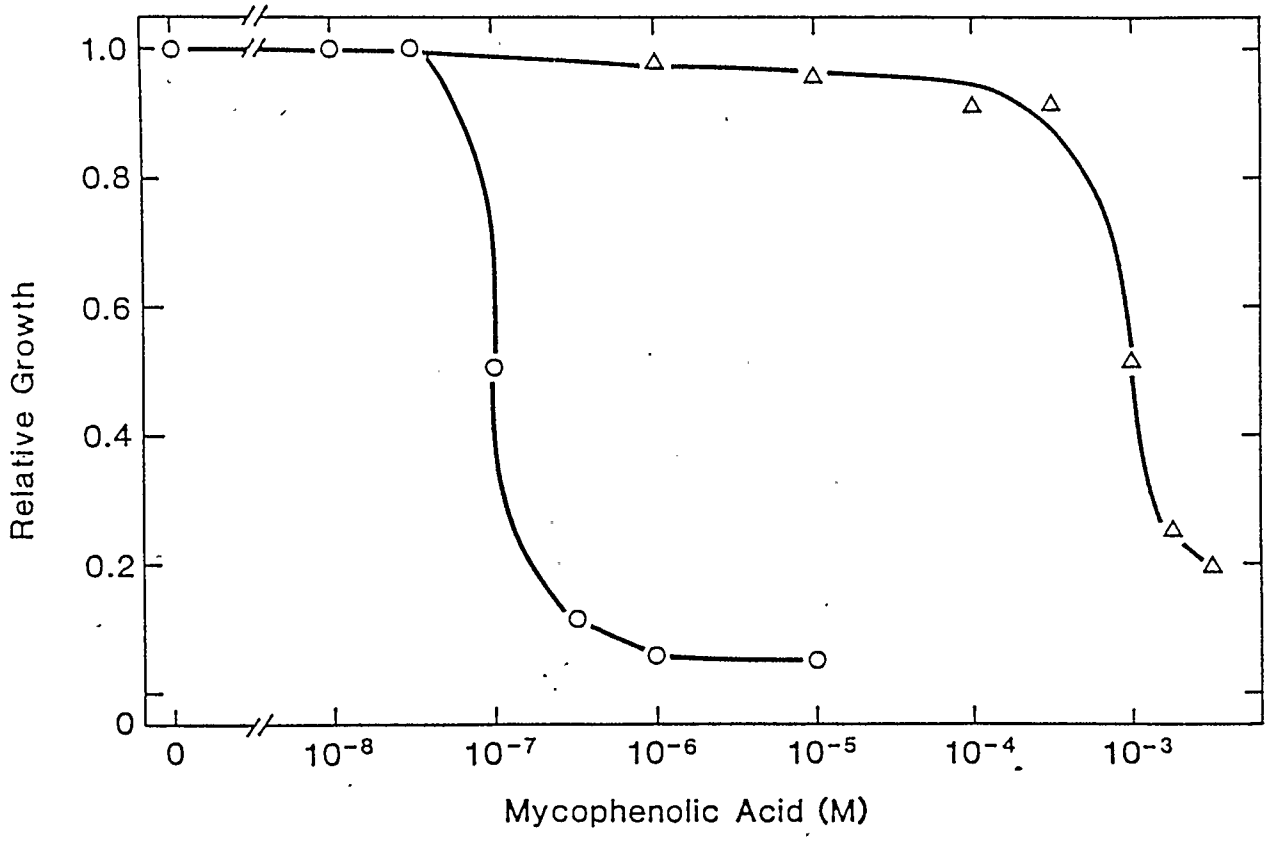


Table Four: Mycophenolic acid sensitivity in control and mycophenolic acid resistant neuroblastoma cells.

Cell line	Mycophenolic acid Concentration Causing 50% Growth Inhibition (μM)	Fold resistance To Mycophenolic Acid
Control (NB)	0.1	0
Passage 25 (p25)	10	100
Passage 99 (p99)	100	1,000
NB-Myco-200a/b (p109)	500	5,000
NB-Myco	1,000	10,000

Mycophenolic acid concentration causing 50% growth inhibition and the fold resistance with respect to the control (NB) is reported. Growth curves from which these data are obtained were conducted as described in the methods.

resistance and the NB-Myco shows a 10,000-fold increase in resistance to mycophenolic acid, as compared to NB cells.

Previously reported levels of mycophenolic acid resistance were in the range 50 - 500-fold either with or without mutagenesis (Ullman, 1983; Cohen, 1987; Collart and Huberman, 1987). Thus the NB-Myco cells represent a considerably greater fold increase in the resistance obtained than any of these.

The mode of resistance cannot be defined from the growth inhibition data alone, though the fact that an incremental increase in a specific inhibitor was used, combined with the known effect of mycophenolic acid on DNA, RNA and protein synthesis would suggest that an amplified cellular target is probable. However a mutation cannot be ruled out (Alt et al, 1978; Whal et al, 1979; Cohen, 1987).

In conclusion, we have obtained, by progressive selection, a neuroblastoma cell line which displays 10,000-fold increased resistance to mycophenolic acid as compared to the wild type.

III.B. Characterization of the mycophenolic acid resistant neuroblastoma cells.

III.B.1. Introduction

A number of mammalian cell lines have been described which show altered resistance to mycophenolic acid including those selected as a result of mutagen treatment (Huberman et al, 1981; Collart and Huberman, 1987; Ullman, 1983), and those selected for resistance to mycophenolic acid without mutagenesis (Cohen, 1987; Hodges et al, 1986). In each case, increased inosinate dehydrogenase activity was

a primary characteristic of the resistant phenotype. The specific activity was increased in most of the cases 10 - 16-fold compared with the normal range (Huberman et al, 1981; Ullman, 1983; Collart and Huberman, 1987), and in one case as high as 56-fold (Cohen, 1987). The degree of mycophenolic acid resistance however, did not correlate with the increased inosinate dehydrogenase activity.

Specific proteins were increased in two mycophenolic acid resistant cell lines. A 57 kDa protein was increased in a resistant leukemia L1210 cell line as observed by SDS-PAGE (Cohen, 1987), and a 56 kDa protein increased 27-fold in mycophenolic acid resistant Chinese hamster V79 cell lines as observed using an immunoblotting technique (Collart and Huberman, 1987). In each case the increased abundance of the specific protein band correlated with the level of resistance achieved.

Other characteristics described in mycophenolic resistant cell lines concerned the stability of the phenotype. Cohen (1987) described two different clones of mycophenolic acid resistant leukemia L1210 cells, each achieved without the use of mutagenizing agents. In the first case, a cell line, named MA5, displayed 50% growth inhibition at 5 μM mycophenolic acid. When this line was grown for 3 months in the absence of mycophenolic acid it lost its resistance to mycophenolic acid, exhibiting 50% growth inhibition at 1.8 μM as compared with 0.27 μM for the control. In the second case, the cell line, MA20, displayed 50% growth inhibition at 34 μM mycophenolic acid. When MA20 was grown in the absence of mycophenolic acid for 3 months it showed no alteration in the concentration of mycophenolic acid required to attain 50% growth inhibition (Cohen, 1987).

Measurement of the inosinate dehydrogenase levels in the mycophenolic resistant cells (MA5 and MA20) and those grown in the absence of mycophenolic acid for 3 months (MA5R and MA20R) showed that a 3-fold reduction in the activity of inosinate dehydrogenase had occurred for both the MA5 and the MA20. Examination of the protein profiles of these cells grown in the absence of mycophenolic acid showed that the protein which was markedly increased in the MA5 compared with the wild type was no longer visible in the MA5R cell line (Cohen, 1987).

Inosinate dehydrogenase amplification has been shown in only one case (Collart and Huberman, 1987). In this report the inosinate dehydrogenase protein was purified and used to develop an antibody. With the use of this antibody a λgt11 expression library, derived from mouse bone marrow, was screened and a cDNA for inosinate dehydrogenase obtained. Hybridization of northern blots using a radiolabelled cDNA probe showed that poly A^+ RNA had increased in the mycophenolic acid resistant cell line, and southern blots showed an increased level of DNA coding for inosinate dehydrogenase. These increases correlated with an increased level of inosinate dehydrogenase protein as observed by immunoblotting.

There are no reports of guanylate synthase activity, nor any other enzymes of purine synthesis or catabolism being studied in any of the mycophenolic acid resistant cell lines.

III.B.2. Results

A comparison of the activity of inosinate dehydrogenase in the NB, NB-Myco-200, NB-Myco cells and at a number of intermediate

Table Five: Inosinate dehydrogenase activities of the control (NB) and mycophenolic acid resistant (NB-Myco) neuroblastoma cells.

Cell Line	Method of Assay	Activity nmoles/min•mg protein
Control (NB)	Spectrophotometric	5.85 ± 0.94
	Radiochemical	3.06 ± 0.23
Passage 93 (p93)	Spectrophotometric	22.9
Passage 97 (p97)	Spectrophotometric	26.6
Passage 102 (p102)	Spectrophotometric	56.3
NB-Myco-200 (p109)	Spectrophotometric	a) 64 ± 7.9
		b) 64 ± 9.6
NB-Myco	Radiochemical	75 ± 2.6

For the NB, NB-Myco-200 and NB-Myco the mean and standard deviations of 3-5 determinations are reported, for the remainder a single assay result is reported. The assays were conducted as described in the methods.

passages is presented in Table five. In all mycophenolic resistant cell lines the activity of inosinate dehydrogenase is increased. A progressive increase in the inosinate dehydrogenase activity levels is observed, as the mycophenolic acid resistance increases, from a 3.5-fold increase at Passage 93, to an 8.7-fold increase at passage 102, an 11-fold increase in the NB-Myco-200 cells, and finally a 24-fold increase in the NB-Myco cells. These comparisons are made on results obtained using the same enzyme assay methods, as different methods gave slightly differing results.

Changes in the soluble protein profiles in the NB, NB-Myco-200 and the NB-Myco cell lines were examined by SDS-PAGE, Figures six and seven. A single band with a molecular mass of approximately 56.7 kDa which is only faintly, if at all, visible in the NB soluble protein profile is considerably increased in the NB-Myco-200 soluble protein profile, and is the most prominent band in the NB-Myco soluble protein profile. Densitometry scans of the NB-Myco profile show this band to be in excess of 20% of the total protein.

Changes in the inosinate dehydrogenase activities measured for cells grown in the absence of mycophenolic acid are given in Table six. The NB-Myco-200 were grown in the absence of mycophenolic acid for 17 days and in this time the inosinate dehydrogenase decreased 3-fold. The NB-Myco cells were grown in the absence of mycophenolic acid for 80 days, 5-fold longer, and the inosinate dehydrogenase activity also decreased approximately 3-fold.

The soluble protein profiles for the NB-Myco-200 and NB-Myco cells grown in the absence of mycophenolic acid are shown in Figures six and seven. Densitometer scans of the 56.7 kDa protein, show that

Figure Six: Soluble protein profiles for NB, NB-Myco, NB-Myco-200 and NB-Myco-200 cells cultured in the absence of mycophenolic acid for the time indicated.

Cells were cultured in the absence of mycophenolic acid and the 10,000 x g supernatant of cell lysates was prepared. SDS polyacrylamide, 12%, gel electrophoresis was run with 20 µg of protein per lane as described in the methods (II.B.4). The molecular mass of the standards is given in kDa.

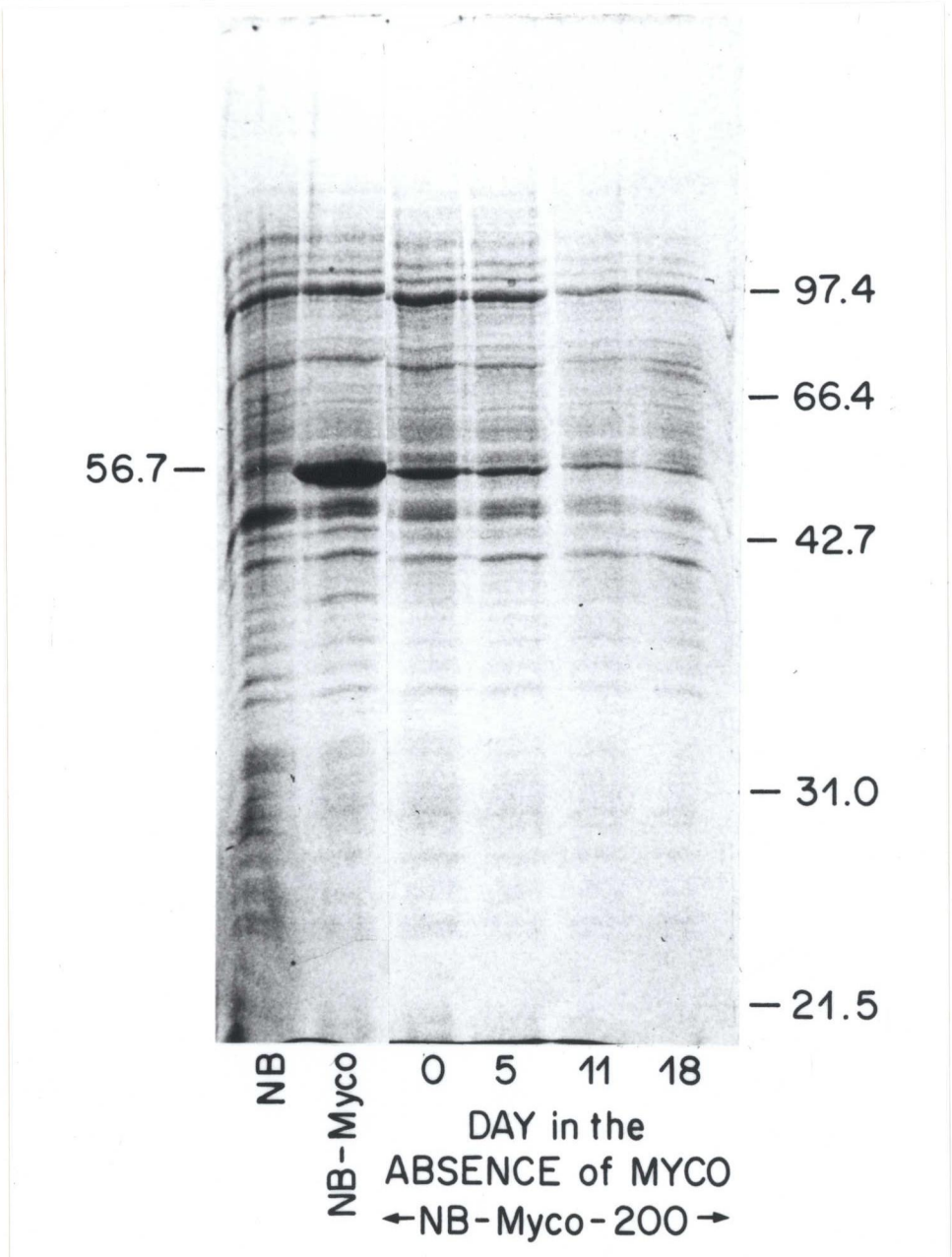


Figure Seven: Soluble protein profiles for NB and NB-Myco cells cultured in the absence of mycophenolic acid for the time indicated.

Cells were cultured in the absence of mycophenolic acid and the 10,000 x g supernatant of the cell lystates was prepared. SDS polyacryamide, 12%, gel electrophoresis was run with 20 µg protein per lane as described in the methods (II.B.4). The molecular mass of standards is given in kDa.

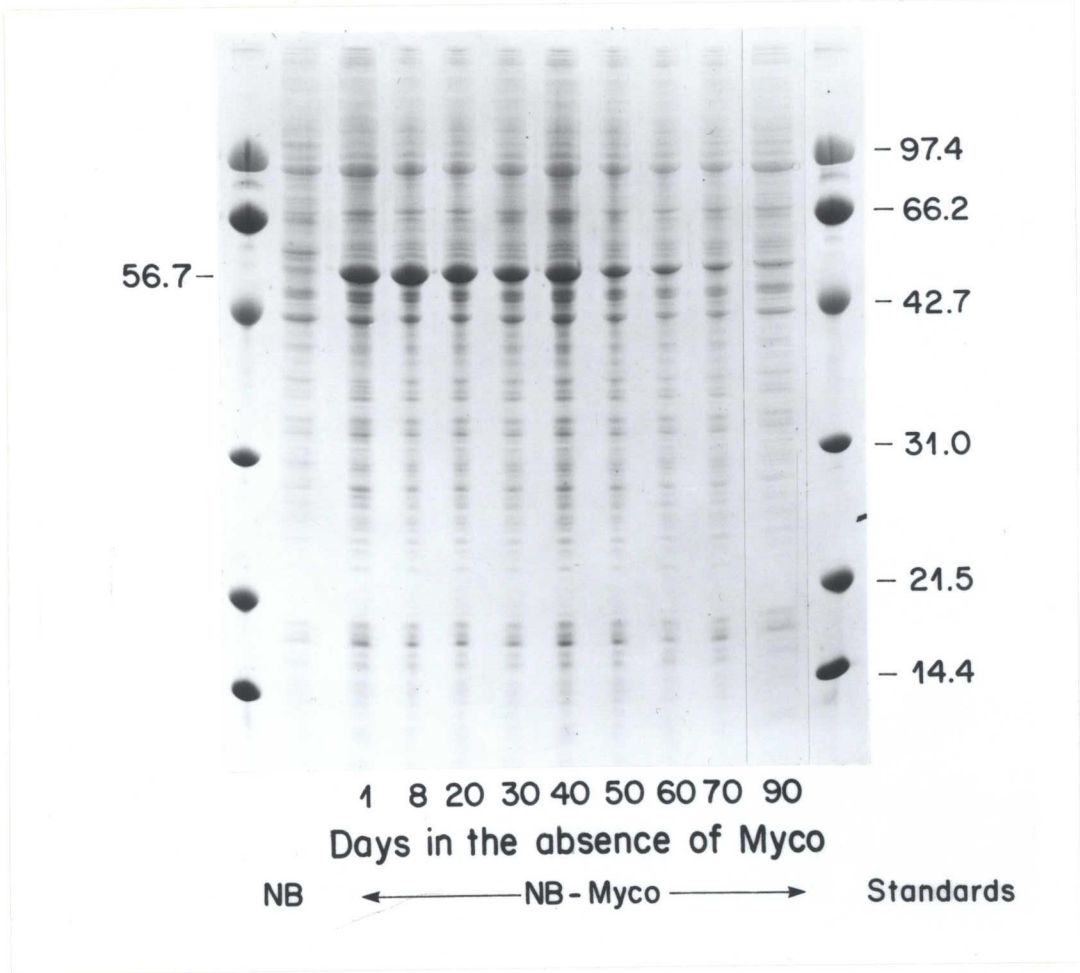


Table Six: Inosinate dehydrogenase activity in the NB-Myco-200 and NB-Myco cells grown in the absence of mycophenolic acid.

	Days in Absence of mycophenolic Acid		Activity nmole/min•mg protein		Relative activity	
NB-Myco-200	A	B	A	B	A	B
	0	0	73.2	68.5	1.00	1.00
	4	5	52.5	38.1	0.71	0.56
	10	11	29.2	27.8	0.40	0.41
	17	18	24.6	17.8	0.34	0.26
NB-Myco						
	1		85		1.00	
	8		81		0.95	
	14		64		0.75	
	25		57		0.67	
	30		59		0.69	
	40		59		0.69	
	45		53		0.62	
	60		42		0.49	
	70		28		0.32	
	80		26		0.31	

Cells were harvested on the days indicated and the lysates prepared and assayed as described in the methods. Relative activity is given with respect the inosinate dehydrogenase activity in cells growing in the presence of mycophenolic acid (NB-Myco-200) or cells grown in the absence for 1 day (NB-Myco). For the NB-Myco-200 cells two cell lines were examined A and B, for the NB-Myco one cell line.

it decreased by 4-fold in the NB-Myco cell line over 90 days. For the NB-Myco-200 cells the starting level of the 56.7 kDa staining band is considerably fainter, but its rate of decline is estimated to be approximately the same though the densitometer scans are not available to support this.

The activities of 4 enzymes of the purine metabolism and salvage pathways examined in the NB and NB-Myco cell lysates, are shown in Table seven. GMP synthase and hypoxanthine phosphoribosyltransferase (HPRT) show no change between the NB and NB-Myco cells. The purine nucleoside phosphorylase (PNP) activity showed a marginal, 1.5-fold, decrease in the NB-Myco compared with the NB cells.

III.B.3. Discussion

Inosinate dehydrogenase activity showed an 11-fold increase in the NB-Myco-200 and a 24-fold increase in the NB-Myco when compared with the NB cells (Table five). In addition the inosinate dehydrogenase activity measured in p25, p97 and p102 cells showed a steady increase in inosinate dehydrogenase activity as the level of mycophenolic acid resistance increased. In other reports of cell resistance to mycophenolic acid very little correlation exists between the inosinate dehydrogenase activity and the fold increase in resistance to mycophenolic acid. The increases in inosinate dehydrogenase activity were for mouse lymphoma S49 cells, 15-fold (Ullman, 1983), mouse L1210, 56-fold (Cohen, 1987), and Chinese hamster V79 cells 10-fold (Collart and Huberman, 1987) and 16-fold (Huberman et al, 1981), whereas the resistance varied in these cells between 50 and 500-fold. The only conclusion we can draw therefore is that as the

Table Seven: Activities of four enzymes of purine metabolism in the control (NB) and mycophenolic acid (NB-Myco) resistant neuroblastoma cells.

Enzyme	NB	NB-Myco
	nmole/min•mg protein	
Inosinate Dehydrogenase	3.1 ± 0.2	75 ± 2.6
Guanylate Synthase	8.0 ± 2.5	7.6 ± 0.8
Purine Nucleoside Phosphorylase	54 ± 4	31 ± 2.4
Hypoxanthine phosphoribosyltransferase	1.57 ± 0.21	1.48 ± 0.20

Means and standard deviations of 3 to 5 determinations are reported. Assays were conducted as described in the methods.

mycophenolic acid resistance increases, inosinate dehydrogenase activity increases and contributes in whole or in part to the increased growth resistance to the drug.

A difference was seen between the results obtained from the spectrophotometric method and the radiochemical method (Table five) for inosinate dehydrogenase activity. For the NB cells the difference between the results obtained from these two methods is 2-fold, and this disparity most likely reflects the inadequate sensitivity and the large baseline fluctuations observed with the spectrophotometric technique . This method has a greater standard error, 16%, than that observed for the radiochemical method, 7.5%, when examining the NB cells. In cells with greater inosinate dehydrogenase activity, inaccuracies due to poor sensitivity have a lesser effect for both the spectrophotometric method; standard error 12%; and the radiochemical technique; standard error 3.4%.

A 56.7 kDa protein has increased over 200-fold in the NB-Myco cells to represent approximately 20% of the soluble protein (Figure seven). The only other reported increase in a specific protein in a mycophenolic acid resistant cell is a 27-fold increase in a 56 kDa protein corresponding to inosinate dehydrogenase described for the 156 μM mycophenolic acid resistant V79 cells (Collart and Huberman, 1987). Similarly a 57 kDa protein increased in the mycophenolic acid resistant L1210 cells (Cohen, 1987), though it was not identified nor was the degree of increase determined. The subunit molecular weight reported for mammalian inosinate dehydrogenase is in the range 56-68 kDa (Okada et al, 1983; Cohen, 1987; Page et al, 1987; Collart and Huberman, 1987). Subsequent work has shown inosinate dehydrogenase

activity and the 56.7 kDa species from the NB-Myco cells co-purify (Section III.E), clearly showing that the amount of inosinate dehydrogenase protein has increased. These results support the idea that an amplification of the inosinate dehydrogenase gene has occurred. The one contradiction is that the extent of the increased activity and the increased abundance of the 56.7 kDa band, however do not correspond.

The specific activity of inosinate dehydrogenase decreased progressively with time when NB-Myco-200 and NB-Myco cells were grown for an extended period of time in the absence of mycophenolic acid. Inosinate dehydrogenase activity of the NB-Myco-200 decreased 3-fold in 17 days and for NB-Myco cells, 80 days to decrease the same amount (Table six). Examination of the soluble protein profiles of cells grown in the absence of mycophenolic acid shows that there is a progressive decline in the abundance of the 56.7 kDa species for both the NB-Myco-200 (Figure six) and the NB-Myco cells (Figure seven). Densitometer scans of the NB-Myco showed an approximately 4-fold decrease over 90 days. These changes are similar to the changes observed in the MA5 L1210 clone, though they are quite different from the changes observed in the MA20 clone (Cohen, 1987). No other soluble protein profiles from mycophenolic acid resistant cells grown in the absence of mycophenolic acid have been examined. The lack of stability of the 56.7 kDa protein is very suggestive of an amplified gene coding for inosinate dehydrogenase. An unstable phenotype is typical of amplification occurring on acentromeric extrachromosomal minute chromosomes (Schimke et al, 1987).

Examinations of PNP, HPRT and GMP synthase showed no change in the latter two, a 1.5-fold decrease in the former in the NB-Myco as compared to the NB cells (Table seven). GMP synthase is specifically inhibited by mycophenolic acid and might be expected to have been altered with the increased resistance to mycophenolic acid but it was not. The reason for the decrease in the PNP activity in the NB-Myco cells is unknown, and certainly has not been looked at nor discussed in any other reported work.

These studies support the notion that the inosinate dehydrogenase gene has undergone amplification in the NB-Myco cells. Though no direct evidence for amplification has been obtained the following are properties characteristic of cells that have undergone amplification: 1, the stepwise selection protocol without mutagenesis; 2, the increased enzyme activity and increased inosinate dehydrogenase protein and 3, the unstable phenotype. The instability of the phenotype may also indicate that the amplification is extrachromosomal, a characteristic of amplified genes not uncommon in murine cells (Schimke et al, 1987).

III.C. Kinetics of inosinate dehydrogenase.

III.C.1. Introduction

Inosinate dehydrogenase catalyzes the NAD^+ dependent conversion of inosinate to xanthylate. This reaction is the first in a branched pathway leading to the synthesis of GMP and consequently it is a potential site of considerable regulatory importance (Holmes et al, 1974). It is one of the rate controlling enzymes in nucleic acid

biosynthesis and thereby proposed to be involved in regulating the growth rate of rapidly proliferating cells (Jackson et al, 1977; Weber et al, 1983)

Detailed characterization of the kinetics of inosinate dehydrogenase in the mammalian system has provided strong evidence for an ordered sequential bi bi reaction in which IMP binds to the free enzyme first, followed by NAD^+, with NADH being released first followed lastly by XMP (Anderson and Sartorelli, 1968; Jackson et al, 1977; Holmes et al, 1974). Added to this mechanism is the proposal that substrate inhibition occurs at high NAD concentrations with the formation of a ternary complex between the enzyme, XMP and NAD (Anderson and Sartorelli, 1968; Jackson et al, 1977). A similar ordered bi bi system has been proposed for Aerobacter aerogenes (Brox and Hampton, 1968; Hampton et al, 1969) and Escherichia coli (Powell et al, 1969) though no NAD substrate inhibition has been reported in these cases. In parasitic protozoa the same mechanism has been reported and it would seem that the protozoan enzyme kinetics closely resemble those of the mammalian system (Hupe et al, 1986; Verham et al, 1987). The full reaction mechanism is detailed in Figure two.

Hupe et al (1986) provided evidence for a kinetic model describing the inhibition of inosinate dehydrogenase by mycophenolic acid. The possibility was advanced that mycophenolic acid may be acting on the same enzyme form as inhibitory concentrations of NAD^+. In the protozoan, inhibition by mycophenolic acid with respect to NAD^+ and inosinate is uncompetitive, suggesting that mycophenolic acid and NAD^+ bind to the enzyme•XMP complex. This has been supported by other

protozoan work (Verham et al, 1987) but has not been investigated to date for the mammalian enzyme.

The K_m values reported for IMP and NAD^+ in mammalian cells (Table eight) show that K_{mIMP} varies within the range of 12 - 30 μM and that the $K_{mNAD}+$ varies within the range of 24 - 46 μM, these being determined from work on sarcoma 180 (Anderson and Sartorelli, 1968), human placenta (Holmes et al, 1974), rat liver (Jackson et al, 1977), Chinese hamster V79 (Huberman et al, 1981), lymphoma S49 (Ullman, 1983) and Yoshida sarcoma ascites cells (Okada et al, 1983). In the protozoan a similar K_{mIMP} range is observed, 18 - 30 μM (Table eight), whereas the $K_{mNAD}+$ is considerably higher than the mammalian enzyme at 150 μM in E. tenella (Hupe et al, 1986) and 340 μM in T. foetus (Verham et al, 1987). The $K_{mNAD}+$ for the mammalian cells is well below the values for cytosolic NAD^+ concentrations. For example, in rat liver the NAD^+ concentrations are approximately 700-900 μM compared to the $K_{mNAD}+$ for inosinate dehydrogenase of 24 μM (Williamson, 1974). The cytosolic concentration of IMP can be inferred from the reported cytosolic concentration of ATP of 3 mM (Bridger and Henderson, 1983), and the reported ratio of ATP to IMP of 1:40-50 (Nuki et al, 1977), suggesting a cytosolic IMP concentration in the order of 60 to 75 μM. This concentration is approximately 2 - 5-fold the reported K_{mIMP} values.

For cells resistant to mycophenolic acid the kinetics of inosinate dehydrogenase have been studied in a few cases. For Chinese hamster V79 cells there were no significant differences in the $K_{mNAD}+$ and K_{mIMP} between wild type cells and cells mutagenized to yield mycophenolic acid resistant cells (Huberman et al, 1981).

Table Eight: Michaelis-Menton and inhibitor constants for inosinate dehydrogenase from mammalian and protozoan sources.

Source	$K_{m\ IMP}$ (μM)	$K_{m\ NAD^+}$ (μM)	$K_{i\ Myco}$ (nM)	Reference
Mammalian				
Mouse Sarcoma 180	25	46	-	Anderson et al., 1968
Human Placenta	14	46	-	Holmes et al., 1974
Rat liver	12	24	-	Jackson et al., 1977
Mouse Yoshida sarcoma ascites	12	25	-	Okada et al., 1983
Mouse Lymphoma, S49 (wild type)	30	45	-	Ullman, 1983
Chinese Hamster V79	22	29	16	Huberman et al., 1981
Cowpea nodules	9	35	-	Atkins et al., 1985
Mouse Neuroblastoma	10	-	6	Hodges et al., 1986
Mouse Leukemia L1210	32	-	46	Cohen, 1987
Protozoan				
Eimeria tenella	30	150	-	Hupe et al., 1986
Tritichomonas foetus	18	340	-	Verham et al., 1987

Summary of Michaelis constants for NAD^+ and IMP and the inhibitor constants for mycophenolic acid reported for mammalian and protozoan enzymes.

Cohen et al (1987) also found no change between the K_{mIMP} for murine leukemia L1210 control cells and non mutagenized mycophenolic acid resistant cells. For lymphoma S49 cells there was no increase in the K_{mNAD}^+, 45 μM for the control versus 50 μM for the mutagenized mycophenolic acid resistant cells, but the K_{mIMP} was increased over 10-fold from 30 μM in the control cells to 300-400 μM in the mycophenolic acid resistant cells (Ullman, 1983).

The K_{iMyco} studied in the V79 cells gave a value of 16 nM for the control and 12 nM for mycophenolic acid resistant cells (Huberman et al, 1981), and for L1210 cells, 46 nM for the control and 58 nM for the mycophenolic acid resistant clones (Cohen et al, 1987). In protozoan a higher K_{iMyco} has been reported with 0.178 μM for E. tenella (Hupe et al, 1986), and 9.4 μM for T. foetus (Verham et al, 1987), both in wild type cells. Only two reports have been made for a $K_{i\ app\ NAD}^+$, both for the protozoan enzyme. The reported value for wild type cells is 2 mM for E. tenella (Hupe et al, 1986), and 370 μM for T. foetus (Verham et al, 1987). No reports have been made regarding an altered K_{iMyco} or $K_{i\ app\ NAD}^+$ in any mycophenolic acid resistant cells.

The only reports of K_{iXMP} have been in wild type cells and these are 109 μM for Yoshida sarcoma cells (Okada et al, 1983), 30 μM for human placenta (Holmes et al, 1974), 21 μM for the protozoan E. tenella (Hupe et al, 1986), and 610 μM for the protozoan T. foetus (Verham et al, 1987). No studies have been done with mycophenolic acid resistant cells.

In this study the kinetic parameters of inosinate dehydrogenase in the NB and NB-Myco cell lines are compared. The aim of this study

was not to conduct a rigorous kinetic analysis of inosinate dehydrogenase but rather to: 1, provide enough evidence to confirm the reaction mechanism as outlined by Holmes et al (1974) and Hupe et al (1986); 2, determine the nature of the NAD^+ and mycophenolic acid inhibition for the mammalian enzyme; and 3, compare the kinetic parameters of the NB and the NB-Myco enzyme in order to determine if a mutational event has taken place that might account in whole or in part for the altered sensitivity of the NB-Myco cell to mycophenolic acid.

III.C.2. Results

Inosinate dehydrogenase exhibited a sharp pH optimum at pH 8.0 in both the NB and the NB-Myco cells (Figure eight), similar to that previously reported (Jackson et al, 1977; Okada et al, 1983).

Initial velocity studies at different substrate concentrations of inosinate and changing fixed concentrations of NAD^+ produced an intersecting pattern at low NAD^+ concentrations (Upper panel, Figures nine and ten). The apparent Michaelis constants, K_{mIMP} and K_{mNAD^+} for the NB and NB-Myco cells are given in Table nine. The K_{mIMP} was unchanged at 14 μM for the NB and 13 μM for the NB-Myco cells, and the K_{mNAD^+} increased 4-fold from 25 μM to 94 μM for the NB and NB-Myco cells respectively. At NAD^+ concentrations greater than 0.5 mM, when compared against varying substrate (IMP) concentrations, an uncompetitive pattern of inhibition is observed for increasing [NAD^+] (lower panels, Figures nine and ten). The $K_{i\ app\ NAD^+}$ for NB and NB-Myco cells are 1.3 and 1.5 mM respectively. A replot of the reciprocals

Figure Eight: pH activity profile for inosinate dehydrogenase from control (NB) and mycophenolic acid (NB-Myco) resistant cells.

Inosinate dehydrogenase activity from NB (□) and NB-Myco (△) resistant cells was determined as described (II.B.2.b.2).

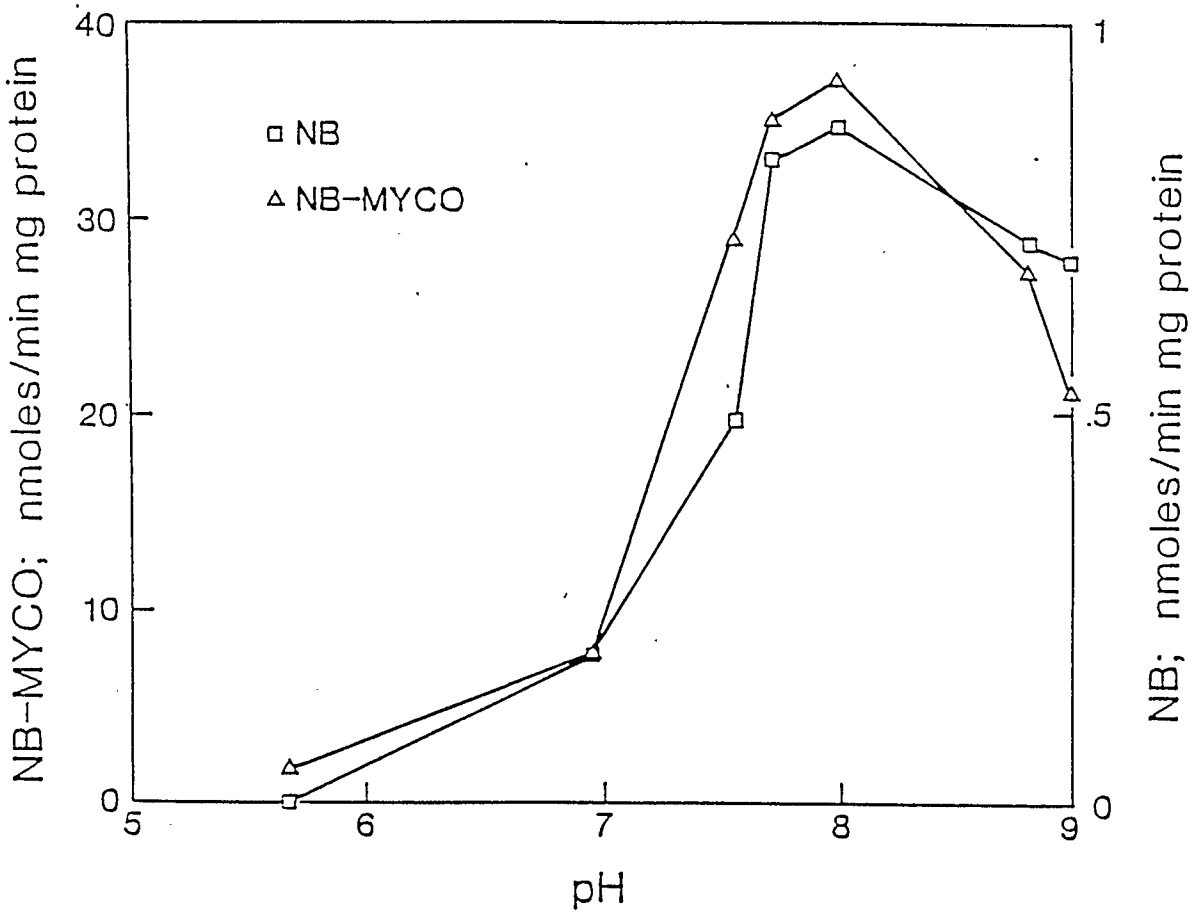


Figure Nine: Initial velocity of inosinate dehydrogenase from control (NB) neuroblastoma cells with IMP as the variable substrate and changing fixed concentrations of NAD^+.

IMP was varied as follows, 10, 13.3, 20, 26.7, and 40 μM, at the following changing fixed concentrations of NAD^+, 0.1, 0.167, 0.4, 0.8, 1.0, 2.5 and 5.0 mM. All other components were constant and the assay conducted as described in the methods (II.B.2.b.2). The reciprocal of velocity (nmoles/min•mg protein) versus IMP (μM) is plotted. The lower panel illustrates the change in pattern observed at high NAD^+ concentrations where the convergent pattern changed to parallel lines.

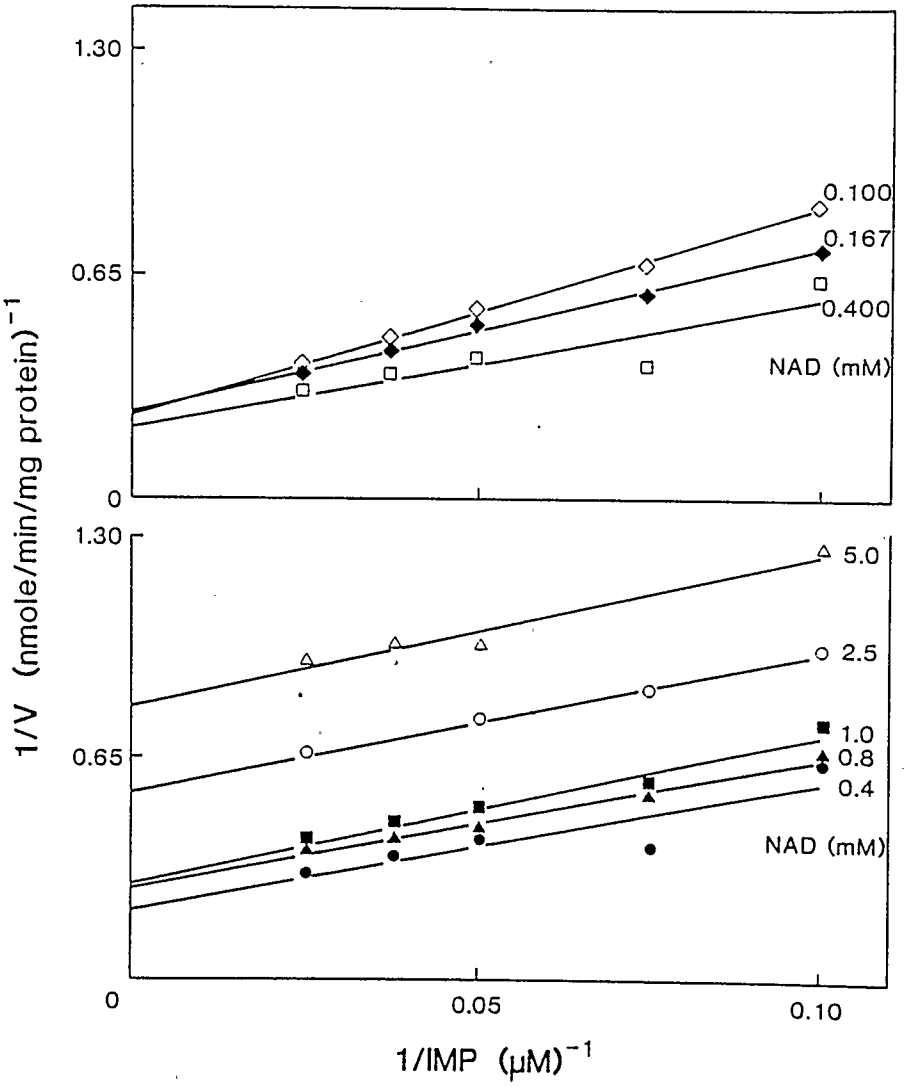
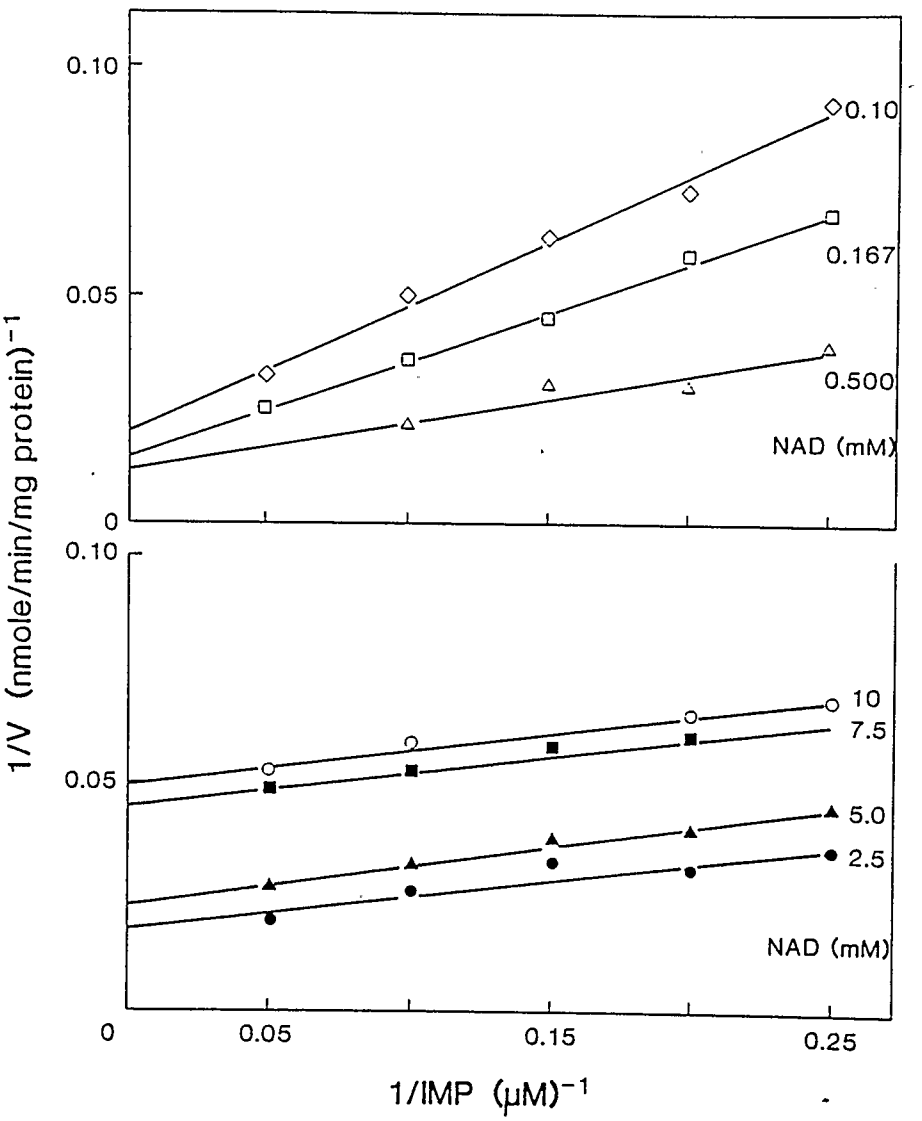

1.30
0.65
0
0.100
0.167
0.400
NAD (mM)
1.30
0.65
0
5.0
2.5
1.0
0.8
0.4
NAD (mM)
0.05
0.10
1/V (nmole/min/mg protein)$^{-1}$
1/IMP (μM)$^{-1}$

Figure Ten: Initial velocity of inosinate dehydrogenase from mycophenolic acid resistant (NB-Myco) neuroblastoma cells with IMP as the variable substrate and changing fixed concentrations of NAD^+.

IMP was varied as follows, 4, 5, 6.7, 10, and 20 μM, at the following changing fixed concentrations of NAD^+, 0.1, 0.167, 0.4, 0.8, 1.0, 2.5 and 5.0 mM. All other components were constant and the assay conducted as described in the methods (II.B.2.b.2). The reciprocal of velocity (nmoles/min•mg protein) versus IMP (μM) is plotted. The lower panel illustrates the change in pattern observed at high NAD^+ concentrations where the convergent pattern changed to parallel lines.


0.10
0.05
0
0.10
0.167
0.500
NAD (mM)
0.10
0.05
0
10
7.5
5.0
2.5
NAD (mM)
0.05
0.15
0.25
1/V (nmole/min/mg protein)$^{-1}$
1/IMP (μM)$^{-1}$

of V_{max} versus NAD^+ shows the dependence of the maximal velocity on the NAD^+ concentration (Figure eleven).

XMP displays a competitive pattern of product inhibition with respect to IMP, producing a converging family of lines which intersected on the 1/velocity axis (Figure twelve). The K_{iXMP} has increased 4-fold from 78 μM to 336 μM for the NB and NB-Myco cells respectively (Table nine).

Mycophenolic acid inhibition is uncompetitive with respect to IMP, with a parallel family of lines being attained from the reciprocal plot of velocity versus [IMP] (Figure thirteen). The K_{iMyco} is increased 2600-fold from 1.3 nM for NB to 3.4 μM for NB-Myco cells (Table nine).

III.C.3. Discussion

A comparison of NB and the NB-Myco cells showed the pH optima to be the same at pH 8.0 (Figure eight). For Yoshida sarcoma ascites Okada et al (1983) noted a very sharp maximum between pH 7.8 and 8.0, and in rat liver and rat hepatoma 3924A Jackson et al (1977) noted a maximum at pH 8.1 for both malignant and non malignant tissue. The protozoan inosinate dehydrogenase activity examined as a function of pH showed no distinct maxima, but the activity increased with increasing pH up to pH 8.5 (Hupe et al., 1986).

Initial velocity, substrate inhibition, product inhibition, and inhibitor studies using mycophenolic acid were conducted for NB and NB-Myco cells. The aim of these four studies was to examine the reaction mechanism, the nature of the enzyme inhibitor complex for inhibition by the substrate, NAD^+, and the inhibitor, mycophenolic

Figure Eleven: Dependence of velocity on the concentration of NAD^+ for the control (NB) and mycophenolic acid resistant (NB-Myco) cells.

A reciprocal plot of velocity versus NAD^+ showing that maximal velocity occurs between 0.4 - 0.5 mM NAD^+. The data were obtained from the initial velocity studies given in figures 9 and 10.

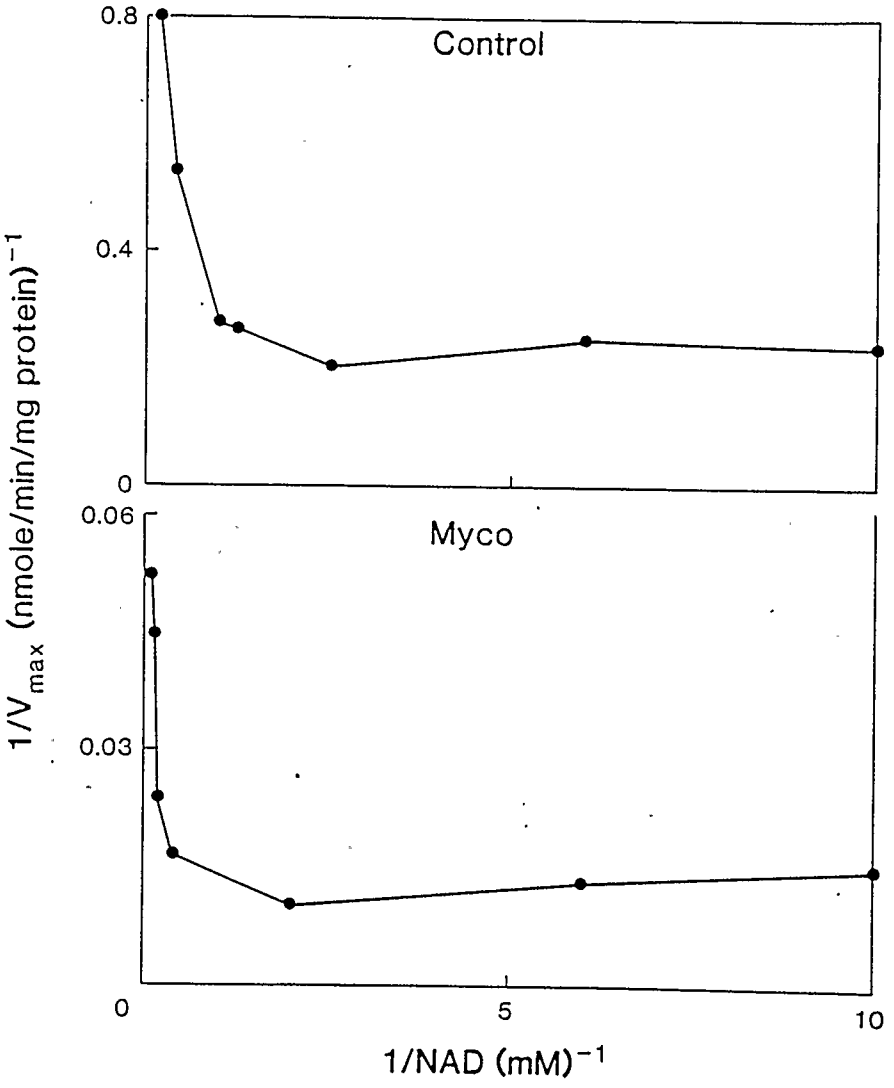

Control
Myco
0.8
0.4
0
0.06
0.03
0
5
10
1/NAD (mM)$^{-1}$
1/V_{max} (nmole/min/mg protein)$^{-1}$

Figure Twelve: Product inhibition of inosinate dehydrogenase by XMP versus IMP at fixed concentration of NAD^+.

IMP concentration was varied as follows, 10, 13.3, 20, 26.7, and 40 μM at a fixed concentration of NAD^+, 0.5 mM. The XMP concentration was 0, 40, 80, 120, 160, μM for the control (NB) and 0, 0.5, 1.0 and 1.5 mM for the mycophenolic acid resistant (NB-Myco) enzyme assay. All other components were constant and the reaction conducted as described in the methods (II.B.2.b.2). A reciprocol plot of velocity (nmoles/min•mg protein) versus IMP (μM) at changing fixed concentration of XMP is shown.

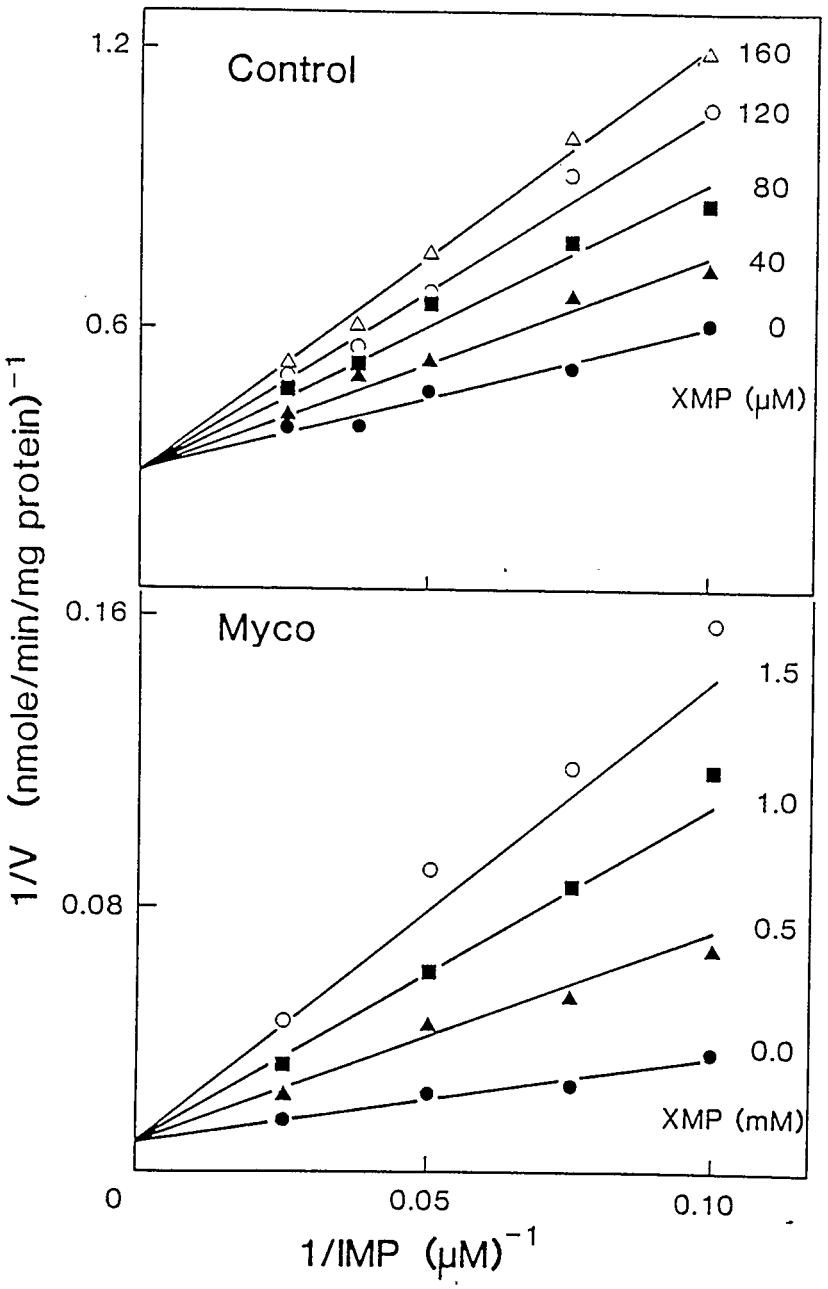

Control
Myco
160
120
80
40
0
XMP (μM)
1.5
1.0
0.5
0.0
XMP (mM)
1.2
0.6
0.16
0.08
0
0.05
0.10
1/V (nmole/min/mg protein)$^{-1}$
1/IMP (μM)$^{-1}$

Figure Thirteen: Inhibition of inosinate dehydrogenase by mycophenolic acid versus IMP at a fixed concentration of NAD^+

IMP concentration was varied as follows, 10, 13.3, 20, 26.6, 40 μM at a fixed NAD^+ concentration of 0.5 mM. Mycophenolic acid was 0, 2.5, 5, 10, and 20 nM for the control (NB) and 0, 1.5, 3, 6, 12 μM for the mycophenolic acid (NB-Myco) resistant enzyme study. All other components were constant and the reaction was conducted as described in the methods (II.B.2.b.2). The reciprocal plot of velocity (nmoles/min•mg protein) versus IMP for changing fixed concentrations of mycophenolic acid shows the results.

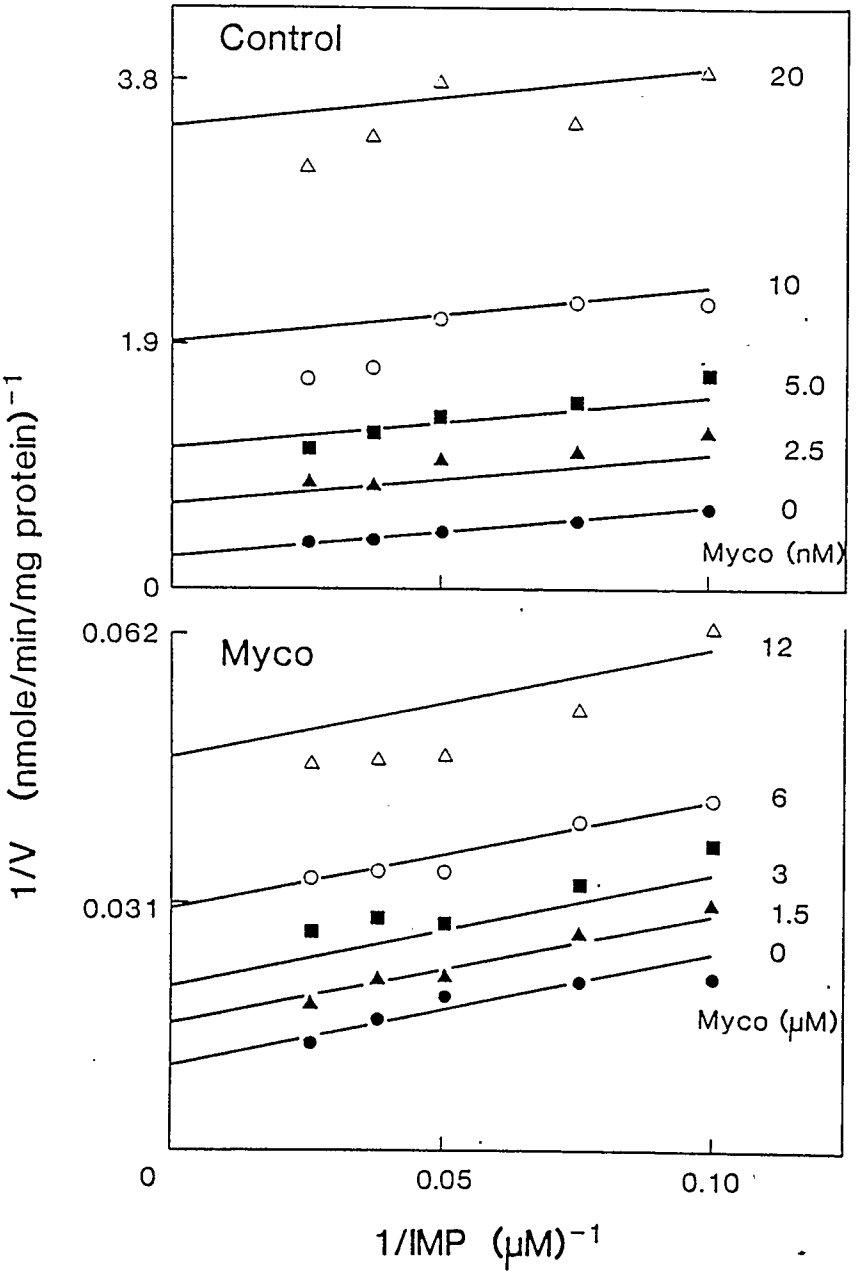

Control
3.8
1.9
0
20
10
5.0
2.5
0
Myco (nM)
Myco
0.062
0.031
0
12
6
3
1.5
0
Myco (μM)
0.05
0.10
1/V (nmole/min/mg protein)$^{-1}$
1/IMP (μM)$^{-1}$

Table Nine: Summary of kinetic parameters for control (NB) and mycophenolic acid (NB-Myco) resistant cells.

Parameter	NB	NB-Myco
$K_{m\ IMP}$ (μM)	13.7 ± 0.3 [9]	12.7 ± 2.1 [10]
	11.1 ± 1.1 [12]	7.5 ± 2.3 [12]
	17.8 ± 3.4 [13]	13.9 ± 2.6 [13]
$K_{m\ NAD^+}$ (μM)	25 ± 8 [9]	94 ± 39 [10]
$K_{i\ XMP}$ (μM)	78 ± 7 [12]	336 ± 34 [12]
$K_{i\ NAD^+}$ (mM)	1.27 ± 0.24 [9]	1.52 ± 1.0 [10]
$K_{i\ Myco}$ (μM)	$1.4 \times 10^{-3} \pm 0.34 \times 10^{-3}$ [13]	3.44 ± 0.32 [13]
V_{max} (nmole/min·mg protein)	4.78 ± 0.004 [9]	104 ± 35 [10]
	3.67 ± 0.13 [12]	114 ± 12 [12]
	4.61 ± 0.42 [13]	96 ± 7 [13]

Superscript numbers indicate the figures containing the data from which these kinetic constants were obtained. The mean and standard error are displayed.

acid, and compare a number of kinetic parameters for the NB and NB-Myco enzyme inosinate dehydrogenase.

The initial velocity studies in which IMP was varied at fixed variable concentrations of NAD^+ less than or equal to 0.5 mM are given in Figures nine and ten (upper panel). The same pattern, intersecting to the left of the Y (1/velocity) -axis and above the X -axis, is seen for each. A plot of 1/velocity versus $1/[NAD^+]$ (not shown) gave the same pattern of intersecting lines. This pattern is indicative of an ordered reaction, though no information is gained about the order of the reactants. An ordered reaction mechanism has been reported for inosinate dehydrogenase from a number of mammalian sources (Anderson and Sartorelli, 1968; Holmes et al, 1974; Jackson et al, 1977) and in protozoa (Hupe et al, 1986; Verham et al, 1987).

Product inhibition studies in which IMP was varied at fixed variable concentrations of the product (XMP) provides information about the reaction order. A reciprocal plot of velocity versus [IMP] for increasing XMP concentrations gave a pattern intersecting on the Y-axis (Figure twelve). The pattern obtained for both the NB (Figure twelve, upper panel) and the NB-Myco (Figure twelve, lower panel) was the same, the only difference being the concentration of XMP required to elicit comparable inhibition. The intercepts are constant and the slopes increases as the inhibitor concentration increases, indicating that XMP is a competitive inhibitor with respect to IMP. This can only occur if XMP and IMP bind to the same enzyme form, and in the case of an ordered reaction system this can only be the free enzyme. If this is the case then IMP must bind to the free enzyme first, and XMP be released last. The competitive product inhibition by XMP with

respect to IMP is in agreement with a number of reports for mammalian sources (Anderson and Sartorelli, 1968; Holmes et al, 1974) and for the protozoan enzyme (Hupe et al, 1986; Verham et al, 1987).

At NAD^+ concentrations greater than or equal to 0.5 mM a reciprocal plot of velocity versus [IMP] for changing fixed concentrations of NAD^+ gave a pattern in which the slope is constant and the intercept increases. This is in contrast to the pattern demonstrated at NAD^+ concentrations less than 0.5 mM in which both the intercepts and the slope change with changing $[NAD^+]$. A reciprocal plot of V_{max} versus $[NAD^+]$ (Figure eleven) shows the dependence of $1/V_{max}$ on the $[NAD^+]$, and that the optimum NAD^+ concentration was between 0.4 mM and 0.5 mM for both the NB and NB-Myco cell. This pattern is as expected for an ordered bi-bi reaction sequence due to the formation of a dead-end EBQ complex in which NAD^+ is B and XMP is Q (Segel, 1975), and is the same as that previously described in mammalian systems (Anderson and Sartorelli, 1968; Jackson et al, 1977) and in protozoa (Hupe et al, 1986).

The inhibition pattern of mycophenolic acid on inosinate dehydrogenase is very similar to that of NAD^+. A reciprocal plot of velocity versus [IMP] for various fixed and variable concentrations of mycophenolic acid was the same for NB (Figure thirteen, lower panel) and NB-Myco cell (Figure thirteen, lower panel) where the slope was constant, and the intercept increased with increasing concentration of inhibitor; the only difference was the concentration of mycophenolic acid required to elicit the same response. This uncompetitive pattern of inhibition suggests that the enzyme form to which the mycophenolic acid binds is not present when there is no IMP present,

suggesting that an enzyme•XMP•Mycophenolic acid complex is being formed. In order for this to be conclusive an additional study in which NAD^+ and mycophenolic acid were varied would need to be completed, and if it gave an uncompetitive inhibition profile the model would have conclusive support. Such studies were conducted for the protozoan enzyme and the inhibition studies supported the model of a dead-end EQI complex being formed (Hupe et al, 1986). These patterns of inhibition indicate that mycophenolic acid and NAD^+ (at high concentrations) are binding to the same enzyme form, the E•XMP complex. This model has been advanced for the protozoan (Hupe et al, 1986) but not for the mammalian enzyme.

The kinetic parameters calculated from the kinetic studies are summarized in Table nine. The K_{mIMP}, K_{mNAD^+} and V_{max} were calculated from all the studies, except the NAD^+ inhibition study, and the K_i calculated from the appropriate inhibition study. The V_{max} increased 25-fold from 4.35 nmoles/min•mg protein in the NB to 104 nmoles/min•mg protein in the NB-Myco cells. Because the enzyme was not purified from either source the increased V_{max} does not indicate greater catalytic efficiency but rather the presence of a more abundant enzyme. In fact the total amount of inosinate dehydrogenase protein has increased 200-fold and the activity only 25-fold suggesting, therefore, that the catalytic activity may be reduced for the inosinate dehydrogenase from the NB-Myco cells as a consequence of a mutational event. The K_{mIMP} was unchanged in the NB and the NB-Myco cells and the K_{mNAD^+} increased 4-fold from 25 μM to 94 μM in the NB and NB-Myco cells. The K_m's obtained for the NB cells fall within the range of 12 - 30 μM and 24 - 46 μM for the K_{mIMP} and K_{mNAD^+}

respectively reported for other mammalian tissues (Anderson and Sartorelli, 1968; Holmes et al, 1974; Jackson et al, 1977; Huberman et al, 1981; Okada et al, 1983; Ullman, 1983). No other reports have been made of an altered K_{mNAD^+} in a mycophenolic acid resistant line.

For product inhibition, the K_{iXMP} has increased 4-fold from 78 μM in the NB to 336 μM in the NB-Myco cells; for substrate inhibition, the $K_{i\ app\ NAD^+}$ has remained essentially unchanged in the NB and the NB-Myco cells at 1.27 mM and 1.52 mM respectively. The K_{iMyco} increased from 1.4 nM for NB to 3.44 μM for NB-Myco cells, the largest change for any of the parameters, approximately 2600-fold. In the only other kinetic studies of inosinate dehydrogenase for mycophenolic acid resistant clones the K_i was not reported for NAD^+ and XMP and remained the same for mycophenolic acid in the control and mycophenolic acid resistant cells (Huberman et al, 1981; Cohen, 1987). The K_{iXMP} for NB cells falls within the range of reported values for other mammalian tissues of 26 - 109 μM (Holmes et al, 1974; Jackson et al, 1977; Okada et al, 1983).

The results presented here suggest an ordered bi bi mechanism for inosinate dehydrogenase with IMP binding to the free enzyme first followed by NAD^+, and XMP being released last. In addition, dead-end substrate inhibition formation is supported with the substrate NAD^+, at high concentrations, combining with the enzyme•XMP. Similarly an enzyme•XMP•inhibitor complex is proposed for mycophenolic acid inhibition. The ordered mechanism for inosinate dehydrogenase is well documented for both mammalian (Anderson and Sartorelli, 1968; Holmes et al, 1974; Jackson et al, 1977) and protozoan (Hupe et al, 1986; Verham et al, 1987) systems with NAD^+ acting as a dead-end substrate

inhibitor (Anderson and Sartorelli, 1968; Jackson et al, 1977; Hupe et al, 1986).

The increased activity, as displayed by the 25-fold increase in the V_{max} in the NB-Myco cells over the NB, is alone insufficient to account for the degree of resistance to growth inhibition. The increased K_i values for XMP and mycophenolic acid combined with the increased K_{mNAD^+} observed in the NB-Myco compared with the NB cells provide some direct evidence that a mutational event has occurred. Therefore, this work is consistent with the idea that an alteration in the primary structure of the inosinate dehydrogenase protein has occurred, and that a mutation has occurred at some stage in the selection of resistance to mycophenolic acid.

In conclusion, kinetic analysis of the inosinate dehydrogenase in the NB and NB-Myco cells is in agreement with an ordered sequential reaction where IMP binds first, followed by NAD^+ and XMP is the last product released. NAD^+ and mycophenolic acid inhibit the inosinate dehydrogenase showing uncompetitive patterns with respect to IMP which are consistent with the formation of Enzyme•XMP•Inhibitor complexes. The NB and NB-Myco cells display the same mechanism. Also it could be suggested that in addition to an amplification of the protein in the NB-Myco cells these results are also indicative of a mutational event having taken place as demonstrated by the altered kinetic parameters displayed in the NB-Myco cells when compared with the NB cells.

III.D. Cellular nucleotides.

III.D.1. Introduction

Specific enzyme abnormalities have been assigned to several inherited clinical syndromes, some of which characteristically have increased rates of purine de novo synthesis and hyperuricemia, though the metabolic defect is unknown in a portion of cases involving hyperuricemia. Examples of defects of enzymes of the purine biosynthetic pathway that show purine overproduction are the complete (Seegmiller, et al, 1967) and partial hypoxanthine phosphoribosyltransferase (HPRT) deficiencies (Kelley et al, 1967); an increase in PP-ribose -P synthase activity (Becker et al, 1973); and a deficiency in purine nucleoside phosphorylase (PNP) activity (Cohen et al, 1976). Thus excessive purine synthesis can result from increases in enzymes of the purine biosynthetic pathway or deficiencies in specific enzymes involved in purine interconversion, and it is quite conceivable that a defect in any of the interconversion enzymes, particularly those at branch points which have regulatory importance, could result in an increased rate of purine synthesis. Purine overproduction, as a result of drug induced deficiencies of certain purine inter-conversion enzymes, has been studied. These include inhibition of the inosinate branchpoint enzymes, inosinate dehydrogenase by mycophenolic acid or adenylosuccinate synthase by alanosine (Seegmiller et al, 1959; Snyder et al, 1980; Willis and Seegmiller, 1980; Gruber et al, 1985). The possibility has been advanced that mutations resulting in decreased but not deficient inosinate dehydrogenase activity may phenotypically resemble other

purine overproduction disorders with gout and hyperuricemia (Willis and Seegmiller, 1980; Ullman, 1983; Gruber et al, 1985). This has led to the suggestion that screening of patients who overproduce uric acid and potentially have undescribed purine metabolic defects could identify abnormalities in the inosinate branchpoint enzymes (Ullman, 1983; Gruber et al, 1985).

In order to understand the utilization of inosinate it is of importance to consider the inosinate branchpoint enzymes, particularly inosinate dehydrogenase and adenylosuccinate synthase, and the factors which regulate them. The activities of these enzymes within the cell can be altered in a variety of ways, such as by inhibition with drugs or increasing activity following preincubation with specific substrates. These manipulations allow the changes in the synthesis of GTP and ATP and also the balance of the relative intracellular concentrations of each to be studied. ATP production is dependent on the presence of GTP, a required substrate for adenylosuccinate synthase. GTP production is dependent on ATP, a requirement for the enzyme guanylate synthase (Smiley et al, 1967). Several nucleotides have an inhibitory and potential regulatory effect on these enzymes as summarized in the following table:

Enzyme	Inhibitor	(Reference)
Inosinate dehydrogenase	XMP > GMP > AMP	(Holmes et al, 1974)
Guanylate synthase	AMP ≥ dAMP > GMP ≥ XMP	(Spector et al, 1976)
Adénylosuccinate synthase	GDP > AMP ≥ GMP ≥ dGMP	(Henderson and Paterson, 1973)

The inosinate dehydrogenase inhibitors are competitive with respect to inosinate, and in addition ATP and GTP are weak inhibitors (Hampton and Nomura, 1967; Henderson and Paterson, 1973). Because of the importance of inosinate dehydrogenase in guanylate biosynthesis, studies of alterations of GTP (and ATP) that result from modifications in the inosinate dehydrogenase activity assist in furthering the understanding of the regulation of guanylate nucleotide production. For example, studies in intact Ehrlich ascites tumor cells showed that GTP concentrations could be increased by preincubation with guanine. The conversion of IMP to XMP by inosinate dehydrogenase was strongly inhibited when GTP concentrations increased and this was most likely due to increased GMP or GDP (Snyder and Henderson, 1973).

Incubation of mouse T-lymphoma (S-49) cells with toxic concentrations of mycophenolic acid produces a depletion of both dGTP and GTP.

In addition, growth inhibition, partial reduction in RNA synthesis and drastic inhibition of DNA synthesis occurred (Cohen et al, 1981). Mycophenolic acid inhibition of growth of normal S-49 cells and HPRT deficient S-49 cells was equally well prevented by guanosine or deoxyguanosine. Both are converted to guanine, by PNP, and then to GMP, by HPRT, and on to GDP and dGDP, thus bypassing the block caused by mycophenolic acid. Deoxyguanosine is more effective than guanosine in elevating the dGTP levels, because as well as the salvage pathway just described, deoxyguanosine can be converted directly to dGMP by deoxycytidine kinase. In an HPRT deficient S-49 clone deoxyguanosine was unable to prevent mycophenolic acid toxicity, though normal dGTP levels were achieved, via the deoxycytidine kinase pathway, suggesting that depleted GTP levels are responsible for toxicity (Cohen et al, 1981; Nguyen et al, 1983). This again shows the important role GTP has in cell growth and regulation.

In other studies, cells have been exposed to sub lethal concentrations of mycophenolic acid or adapted by mutation or incremental increases in drug concentration to grow in high and ordinarily lethal concentrations of mycophenolic acid. At sub-lethal concentrations of mycophenolic acid, cellular nucleotide levels studied in HPRT-deficient and normal lines derived from spleen and peripheral blood gave the following results. Hypoxanthine excretion is greatly increased, and the total rate of purine de-novo synthesis is markedly elevated. Guanylate and adenylate synthesis is considerably reduced. Over a 4 hour period of study following mycophenolic acid inhibition, GTP rapidly diminishes, ATP increases transiently and then decreases, and UTP and CTP also increase with time. IMP increased rapidly over the

first 120 minutes and continued to increase thereafter more slowly. There were no differences in nucleotides between normal and HPRT deficient cell lines (Willis and Seegmiller, 1980). Long term (14 day) cultures showed IMP increased 10-fold, and ATP and GTP decreased to 80% and 17% of the controls respectively (Willis and Seegmiller, 1980).

Murine lymphoma L5178Y cells treated with 1 μM mycophenolic acid produced the following changes within 2 hours (Lowe et al, 1977). Firstly, GTP decreased rapidly to less than 10% of the controls, UTP and CTP markedly increased and ATP remained unchanged. In addition, dGTP decreased and TTP increased. DNA synthesis was markedly inhibited, at a rate similar to GTP reduction, and RNA and protein synthesis were reduced slightly (Lowe et al, 1977).

In other work mouse neuroblastoma cells were incubated with 0.07 μM mycophenolic acid and Cass et al (1977) noted that in a 24 hour period proliferation was inhibited 50% and cell viability reduced by 83%. Within 3 hours intracellular GTP was reduced by 70%, CTP and UTP significantly increased and ATP was marginally increased. In mouse lymphoma S-49 cells 1 μM mycophenolic acid reduced GTP levels, marginally increased CTP and UTP, but had no effect on ATP (Ullman, 1983).

In conclusion, cells in culture, subject to inosinate dehydrogenase inhibition exhibit a marked depletion in cellular GTP (Lowe et al, 1977; Cass et al, 1977; Willis and Seegmiller, 1980; Cohen et al, 1981; Ullman, 1983; Lee et al, 1985), a lesser decrease in dGTP (Lowe et al, 1977; Cohen et al, 1981), and marginal increases in CTP, UTP (Lowe et al, 1977; Cass et al, 1977; Lee et al, 1985) and

TTP (Lowe et al, 1977), with no change or a marginal decrease in ATP (Lowe et al, 1977; Cass et al, 1977; Willis and Seegmiller, 1980). The cells also exhibited loss of viability, growth inhibition, and a reduction in RNA and DNA synthesis (Lowe et al, 1977; Cass et, al, 1977; Cohen et al, 1981; Lee et al, 1985). This provides insight into the changes that occur in a cell following a block in guanylate synthesis and the ways the cell adapts to these changes.

The foregoing may be compared with the changes in cells capable of growing in high concentration of mycophenolic acid, and the changes caused by the removal of the mycophenolic acid from such a system. Ullman (1983) working with murine T-lymphoma S-49 cells has provided information regarding nucleotide levels in mutagenesis induced mycophenolic acid resistant clones. Upon examination of the nucleotide levels in the control cells when subject to 1μM mycophenolic acid and 1 μM mycophenolic acid resistant (MYCO-1a) cells when subject to 5 μM mycophenolic acid, similar effects were seen. These were a 90% depletion of GTP and GMP, an increase in IMP and no change in ATP. A 2-fold increase in the rate of de novo purine synthesis with massive inosine excretion into the medium was also seen. Cells resistant to 20 μM mycophenolic acid (MYCO-1a-20) incubated with 25 μM mycophenolic acid showed no change in purine production, nor any nucleotide pool perturbations. In both the MYCO-1a and the MYCO-1a-20 clones, the intracellular GMP and GTP were elevated 10-fold. When the cells were grown in the absence of mycophenolic acid over a 4 hour time period GTP increased a further 2-fold (Ullman, 1983).

In an attempt to understand the basis for the 25-fold increase in inosinate dehydrogenase activity found in the mycophenolic acid resistant NB-Myco cells, and to study the effect of removal of the mycophenolic acid from cells capable of growing in high concentration of the drug, the nucleotide pools were examined in these cells and are reported here.

III.D.2. Results

Nucleotide concentrations were measured in extracts harvested from mycophenolic acid resistant cells (NB-Myco), the controls (NB) and the NB-Myco cells in the absence of mycophenolic acid as shown in Table ten. Acid extracts were prepared on a known number of cells, the nucleotides determined by HPLC, and expressed on a per cell basis.

NB-Myco cells in the presence of 1 mM mycophenolic acid have normal levels of ATP and ADP, increased GTP, 2-fold, and GDP, 2-fold, and marginally increased CTP, 1.7-fold, and UTP, 1.3-fold, (Table ten).

Removal of the drug resulted in a further increase in the GTP pool to 4.5-fold that of NB cells within 12 hours with a similar trend observed for GDP. Upon removal of the mycophenolic acid, CTP and UTP levels declined back to normal wild type levels over the 11 day period. ATP and ADP remained essentially unchanged when the fluctuations observed are considered with respect to the standard deviation involved. Changes in GTP, ATP, CTP, and UTP are shown between 0 and

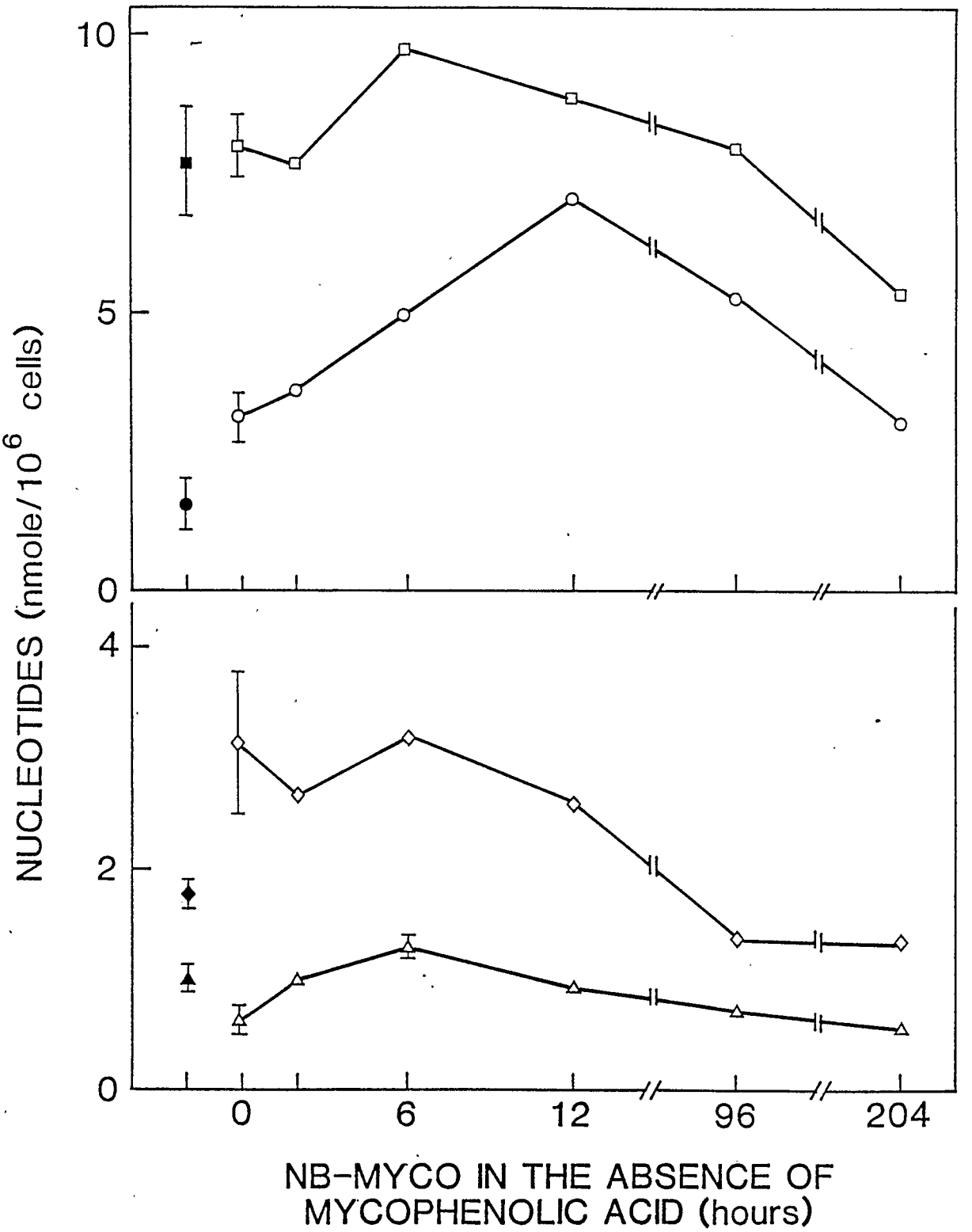
Table Ten: Cellular nucleotide concentrations for control (NB) and mycophenolic acid resistant (NB-Myco) cells in the presence and the absence of mycophenolic acid.

	GTP	GDP	ATP	ADP	CTP	UTP
			(nmole per 10^6 cells)			
NB	1.6 ± 0.5	0.1 ± 0.1	7.7 ± 1.0	0.7 ± 0.2	1.0 ± 0.2	1.8 ± 0.2
NB-Myco	3.1 ± 0.4	0.4 ± 0.2	8.2 ± 0.6	0.9 ± 0.1	0.6 ± 0.1	3.2 ± 1.3
NB-Myco in the absence of mycophenolic acid						
2 hr	3.6 ± 0.1	0.7 ± 0.1	7.7 ± 0.2	1.4 ± 0.3	1.0 ± 0.3	2.7 ± 1.0
4 hr	3.9 ± 0.2	0.7 ± 0.1	8.4 ± 0.8	1.3 ± 0.1	1.2 ± 0.2	2.8 ± 0.4
6 hr	5.0 ± 0.5	0.5 ± 0.2	9.8 ± 1.7	1.0 ± 0.2	1.3 ± 0.4	3.2 ± 0.7
12 hr	7.09	0.26	8.85	0.66	0.94	2.56
14 hr	5.01	0.55	9.38	1.15	1.13	2.32
20 hr	6.62	0.46	11.71	1.02	1.34	2.10
26 hr	5.06	0.65	8.59	1.37	0.99	1.92
48 hr	6.49	0.51	12.94	0.96	1.59	3.06
4 day	5.30	0.11	8.02	0.45	0.75	1.40
11 day	3.07	0.10	5.36	0.37	0.58	1.45

Nucleotides were determined by HPLC analysis of acid soluble extracts as described in the methods. Results are the mean and standard deviation for three independent preparations for the NB, NB-Myco and 1 - 6 hour time course data points. The remaining time course data points are a single determination.

Figure Fourteen: Cellular ribonucleoside triphosphate concentrations for control (NB) and mycophenolic acid resistant (NB-Myco) cells in the presence and absence of mycophenolic acid.

Cellular nucleotides were determined by HPLC analysis from acid soluble extracts as described in the methods (II.B.5). Closed symbols represent nucleotide determinations from control (NB) cells and open symbols those from the mycophenolic acid resistant (NB-Myco) cells in the absence of mycophenolic acid for the time (in hours) indicated. The nucleotides represented are ATP (□), GTP (0), UTP (◇), and CTP (△) in nmoles per 10^9 cells.


10
5
0
4
2
0
NUCLEOTIDES (nmole/10^6 cells)
0
6
12
96
204
NB-MYCO IN THE ABSENCE OF
MYCOPHENOLIC ACID (hours)

264 hours in Figure fourteen. The ATP/ADP and GTP/GDP ratios for each harvest remained constant at approximately 10/1.

III.D.3. Discussion

NB-Myco cells cultured in the presence of mycophenolic acid have normal levels of ATP and ADP and increased GTP, CTP and UTP (Table ten). Thus NB-Myco cells appear to have excess capability for GTP synthesis and as a result minor corrections occurred in the CTP and UTP concentrations. In all other studies where nucleotides were examined following mycophenolic acid exposure, GTP was greatly reduced as a result of inosinate dehydrogenase inhibition (Cass et al, 1977; Lowe et al, 1977; Lee et al, 1985).

In contrast, cells which have been adapted to grow in the presence of mycophenolic acid have raised GTP concentration (Ullman, 1983). Ullman (1983) noted in two murine T-lymphoma S-49 cell lines, mutated so that one would grow in 1 μM and the other in 20 μM mycophenolic acid, that GTP and GDP had increased at least 10 fold, when compared with the controls.

In the NB-Myco-200 cells the ratio of guanine to adenine ribonucleotides remained the same compared with the NB cells, and the XMP concentration in the NB-Myco-200 was marginally less than that from the NB cells (Hodges et al, 1986).

The raised levels of GTP, GDP or, by inference, GMP could inhibit the inosinate dehydrogenase and thus control the level of the guanine nucleotide products. In addition, one must consider that GTP is increased 2-fold and the inosinate dehydrogenase protein over 200-fold. A mutant form of inosinate dehydrogenase may be controlled

differently by GTP and/or GDP; this could be tested by measuring the K_i for GTP, GDP and GMP in the NB and the NB-Myco cells. We have shown that the K_{iXMP} is increased in the NB-Myco, and similar changes may have occurred in the K_i's for GTP, GDP or GMP.

Removal of the mycophenolic acid from the NB-Myco cells increased the level of GDP and GTP 4.5-fold compared with the level of the control cells with a corrective increase in the CTP and a normalization of the UTP. Ullman (1983) reported, in mycophenolic acid murine T-lymphoma S49 cells grown in the absence of drug, an increase of GTP and GDP at least 2-fold compared with the mycophenolic acid resistant cells, which already displayed a 10-fold increase compared with the controls. As GDP and GTP increase they may inhibit inosinate dehydrogenase, thereby inhibiting the further production of GTP. Thus the rapid rise in the GTP and GDP following mycophenolic acid removal and the slow decline is expected. Despite the fact that in the NB-Myco cells the inosinate dehydrogenase activity decreases with time in the absence of mycophenolic acid which could assist in reducing GTP, these changes occur slowly as compared to the decrease in GTP. ATP was unaffected in the NB-Myco cells in the presence or absence of the drug most likely because adenylosuccinate synthase activity requires GTP and this was never depleted so ATP production would be unperturbed.

These cells have been of value in examining the changes caused by the inosinate dehydrogenase inhibition and the controlling aspects at the inosinate branch point under severely perturbed conditions such as is observed following the mycophenolic acid removal from the NB-Myco cells. Deductions and conclusions are very difficut to obtain from a

study of this sort due to the many variables, often unknown, which are involved but it is of value to examine the trends established. In conclusion, two clear trends can be observed. One is the excess capability for GTP synthesis seen in the NB-Myco cells, particularly when the cells are grown in the absence of mycophenolic acid. The second is the minimal changes observed in the UTP, CTP, and ATP levels even under the severely perturbed conditions indicating how well regulated are the level of these nucleotides.

III.E. Purification and characterization of inosinate dehydrogenase.

III.E.1. Introduction

The enzyme inosinate dehydrogenase has been purified from a wide range of sources with varying degrees of success. Partial purification of the mammalian enzyme includes work on mouse sarcoma 180 (Anderson and Sartorelli, 1968), human placenta (Holmes et al, 1974), and rat liver and hepatoma 3924A cells (Jackson et al, 1977). Purifications from other sources include Escherichia coli (Powell et al, 1969), and the nitrogen fixing nodules of Cowpea (Atkins et al, 1985). Only one mammalian inosinate dehydrogenase has been purified to apparent homogeneity, that being the enzyme from rat Yoshida sarcoma ascites tumor cells (Okada et al, 1983). The enzyme from protozoan Tritrichomonas foetus (Verham et al, 1987), and E.coli (Gilbert et al , 1979; Lowe et al, 1980) have also been purified to homogeneity. The only report of a purification attempt from mycophenolic acid resistant cells is that of Chinese Hamster V79 cells

(Collart and Huberman, 1987). In this report a purification is stated but insufficient details are provided to study it.

There are methodological similarities between the two purification procedures that have yielded a homogeneous protein. Okada et al (1984) used a four step process. In the first step, the property of forming high molecular weight aggregates was exploited as previously demonstrated for the E.coli enzyme (Krishnaiah, 1975). High molecular weight complex formation was shown to occur more readily in mammalian tumor cells than mammalian liver cells (Shimura et al, 1983). Following pelleting of the enzyme at 96,000 x g all the subsequent steps were done in the presence of 10% glycerol and 10% ammonium sulphate $\{(NH_4)_2SO_4\}$ to reduce the ionic and hydrophobic interactions which were postulated to be the cause of the aggregation. Further separation was achieved with a calcium phosphate gel column which provided ion exchange chromatography; followed by gel exclusion chromatography on an ultrogel AcA 34 column; and finally, affinity chromatography on a blue Sepharose column, in which the dye and proteins which have an NAD^+ binding siteform a complex. In a similar approach Verham et al (1987), using a 5 step purification scheme, isolated inosinate dehydrogenase from the protozoan T. foetus. The first step was a wide range ammonium sulphate fractionation 45 - 75%. This was followed by gel filtration using Bio-gel A; ion exchange chromatography using DEAE Sepharose; and affinity chromatography using Cibacron blue, a dye that also binds proteins with an NAD^+ binding site. Following this, the final step used was SDS-polyacrylamide electrophoresis and gel isolation which provided approximately 50% of the purification.

The subunit molecular weights reported for inosinate dehydrogenase are for the mammalian enzyme: 57 kDa (Cohen, 1987), 56 kDa (Collart and Huberman, 1987), 68 kDa (Okada et al, 1983), and 65 kDa (Page et al, 1987). Other reported molecular weights are for cowpea, 50 kDa (Atkins et al, 1985), E.coli, 54.5 and 58 kDa (Tiedeman and Smith, 1985; Gilbert et al, 1979), and for the protozoan, 58 kDa (Verham et al, 1987). The native protein may exist as a dimer and/or a tetramer giving a native mass in the order of 100 - 200 kDa (Okada et al, 1983; Atkins et al, 1985; Verham et al, 1987), and suggestions of strong hydrophobic or ionic interactions holding the subunits together have been made (Okada et al, 1983).

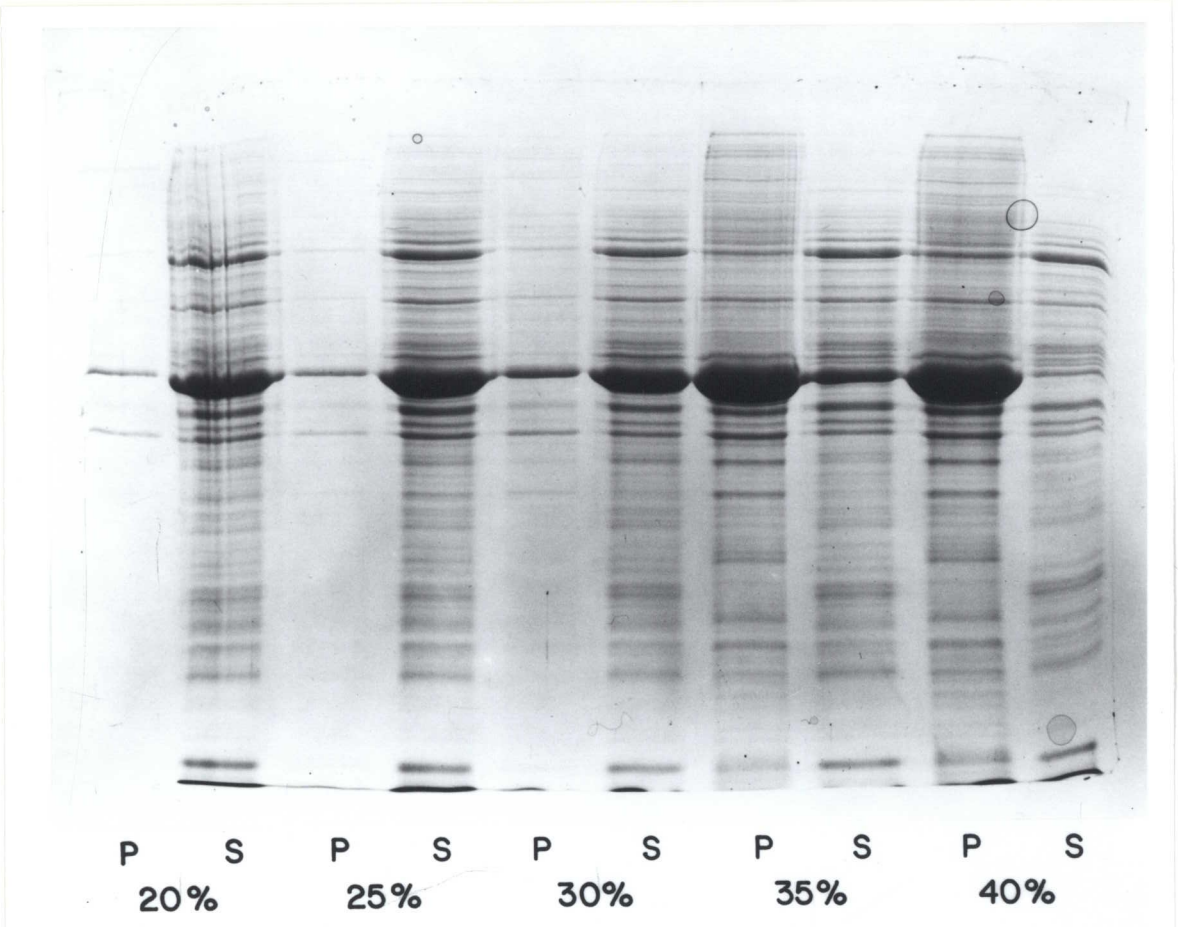
One amino acid composition has been reported for mammalian inosinate dehydrogenase (Okada et al, 1983) but no primary structure of the protein or nucleotide sequence information has been reported. For the E.coli enzyme an amino acid composition has also been reported, the composition closely resembling that reported for the mammalian system (Krishnaiah, 1975; Okada et al, 1983). Also reported for E.coli is the complete nucleotide sequence of the guaB locus that codes for inosinate dehydrogenase, the coding region being 1.533 Kb long with a predicted subunit molecular mass of 54,512 daltons (Tiedeman and Smith, 1985).

III.E.2. Results

The determination of the optimum ammonium sulphate range for the fraction of inosinate dehydrogenase was conducted first. NB-Myco crude lysate was fractionated between 20 and 40% ammonium sulphate in the presence of 10% glycerol and the pellet and supernatant examined

Figure Fifteen: Soluble protein profiles of various ammonium sulphate fractions of a mycophenolic acid resistant cell lysate.

Mycophenolic acid resistant cell lysates were subjcted to fractionation at 20, 25, 30, 35 and 40% ammonium sulphate containing 10% glycerol as described in the methods (II.B.6). Approximately 0.4 mg of lysate protein was used in a total volume of 200 µL. A 15 µL portion of the pellet (P), solubilized in 200 µL Tris-HCl buffer, and 15 µL of the supernatant (S) were run on a 12% SDS-PAGE gel.



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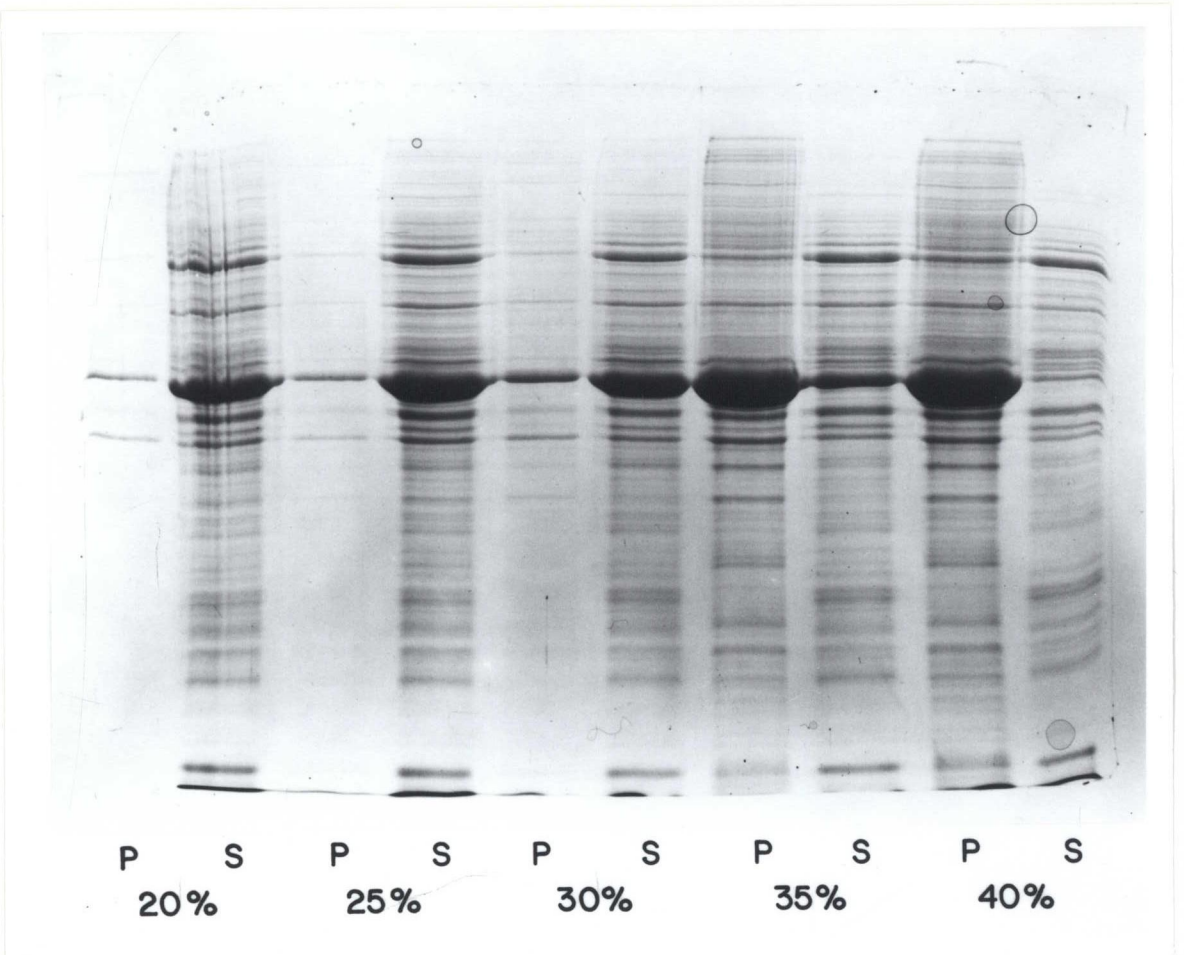


Table Eleven: Purification of inosinate dehydrogenase from mouse neuroblastoma cells.

	Vol	Total Protein	Total Activity	Specific Activity	Fold Purification	Yield
	ml	mg	nmole/min	nmole/min•mg protein		%
Crude Extract	1.4	23.4	2170	92	1	100
Ammonium Sulphate	1.0	3.5	1089	311	3	50
Superose 12	8:0	0.49	408	830	9	19

The ammonium sulphate fractionation and Superose 12 gel filtration protocols are described in the methods. These results are typical for a preparation from 20-30 mg of crude lysate. Fold purification is calculated from the specific activity (nmole/min•mg protein), the yield (%) is calculated from the total activity (nmole/min).

by SDS-PAGE (Figure fifteen). The 56.7 kDa protein started precipitating in 30% ammonium sulphate and was all precipitated by 40% ammonium sulphate.

The purification of inosinate dehydrogenase from neuroblastoma cells followed the two step purification scheme summarized in Table eleven. The neuroblastoma cell line NB-Myco was used for the purification attempt as it displays a 10,000-fold increase in mycophenolic acid resistance and a 56.7 kDa band, suspected to be inosinate dehydrogenase, which was increased 200-fold over control cells. The first step was 30-40% ammonium sulphate fractionation which yielded 50% of the total inosinate dehydrogenase available from the crude lysate and a 3-fold purification. The second step was gel filtration on a Superose 12 column having a fractionation range of 25 to 600 kDa. The elution profile was monitored on a flow through UV cell at 280 nm (Figure sixteen); inosinate dehydrogenase activity was determined in a number of the fractions (Figure sixteen); and a number of the fractions were examined by mini SDS-PAGE gels (Figure seventeen). Two pooled fractions were obtained: one representing fractions 11 - 14 that appeared to contain the 56.7 kDa protein partially purified (Fraction 1) and the other representing fractions 15 - 21 which appeared to contain only the 56.7 kDa protein (Fraction 2). Fraction 2 represented 20% of the total starting inosinate dehydrogenase and a 9-fold purification was achieved compared to the NB-Myco cell lysate.

Following dialysis the two pooled fractions were examined on a 12% SDS-PAGE mini gel over the range of protein concentrations between 3 and 12 µg (Figure eighteen). The first fraction showed a few faint secondary bands, and the second pool appeared to be a single species

Figure Sixteen: Superose 12 gel filtration chromatography

The inosinate dehydrogenase preparation from the ammonium sulphate fractionation (3.5 mg) was applied to a Superose 12 gel exclusion column as described in the methods (II.B.6), and 1 mL fractions collected. Inosinate dehydrogenase activity, (0) in nmole/min and the 280 nm absorbance were determined.

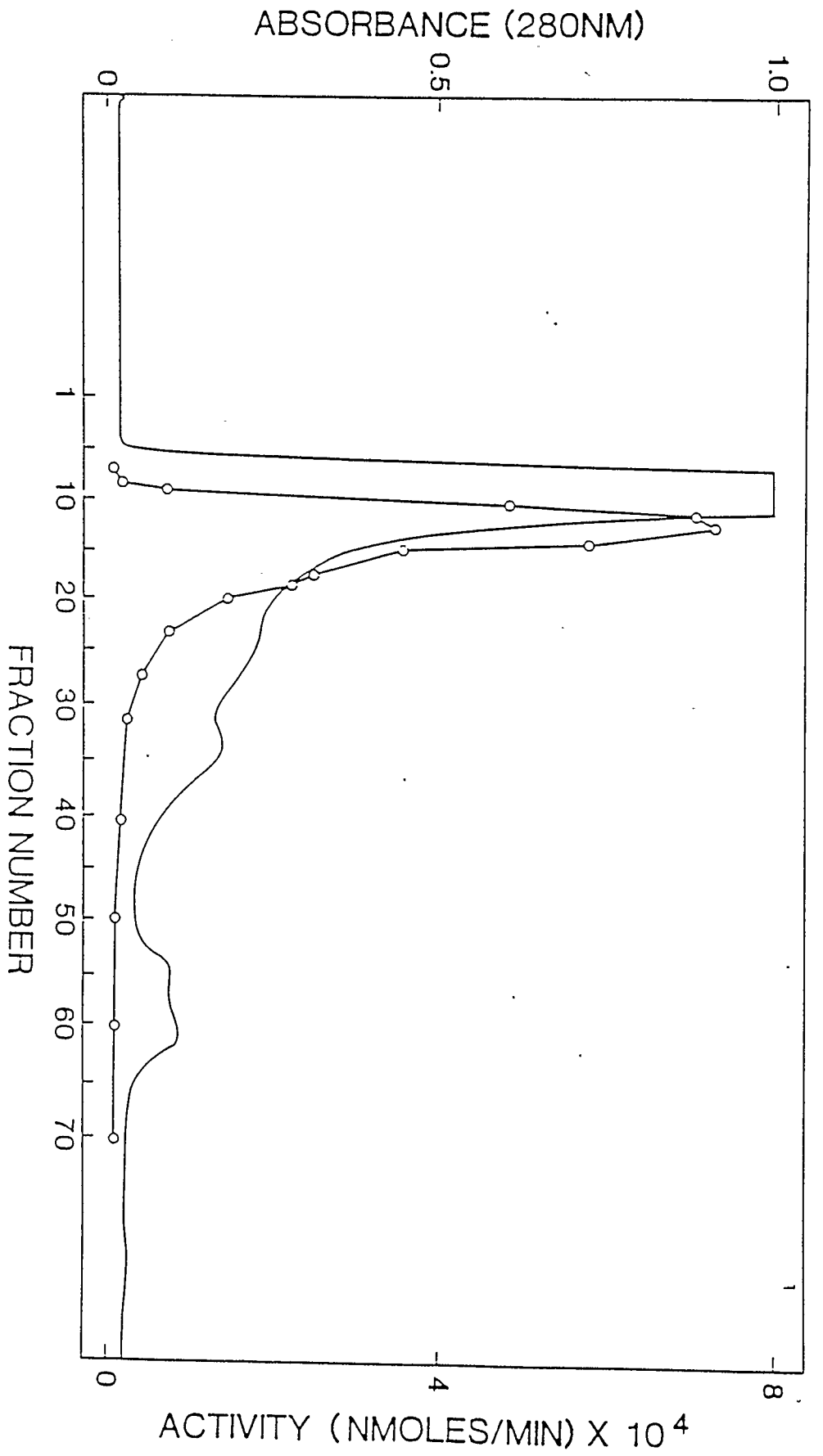

ABSORBANCE (280NM)
0
0.5
1.0
FRACTION NUMBER
1
10
20
30
40
50
60
70
0
4
8
ACTIVITY (NMOLES/MIN) X 10 4

Figure Seventeen: Soluble protein profiles of eluted fractions from Superose 12 exclusion column

Fraction number correspond to those given in figure 16. 15 µL of the 1 mL fractions were loaded per lane and examined on a 12% SDS polyacrylamide gel as described in the methods (II.B.4). Fractions 11 - 14 were pooled to form Fraction 1 and fractions 15 - 21 to form Fraction 2.

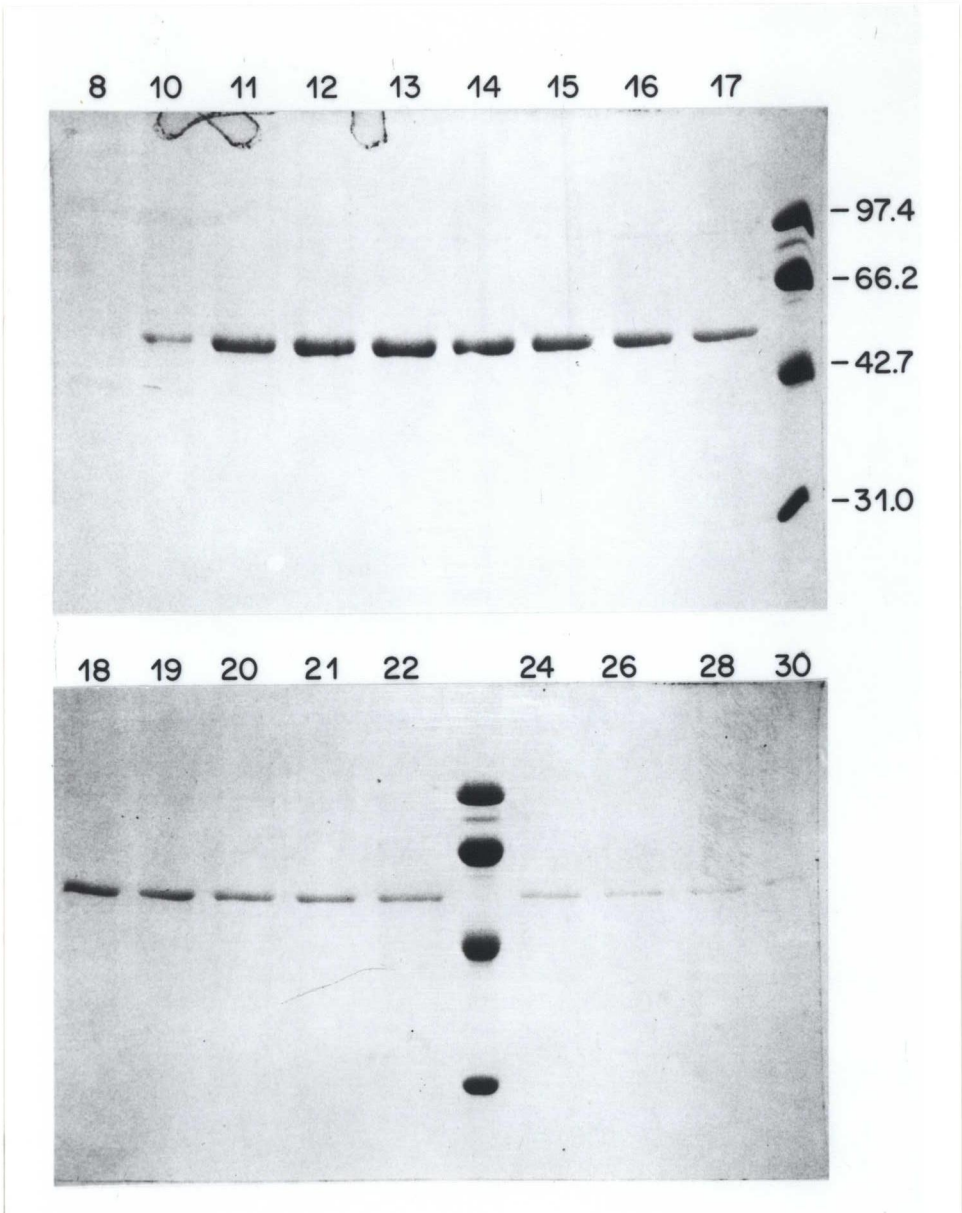
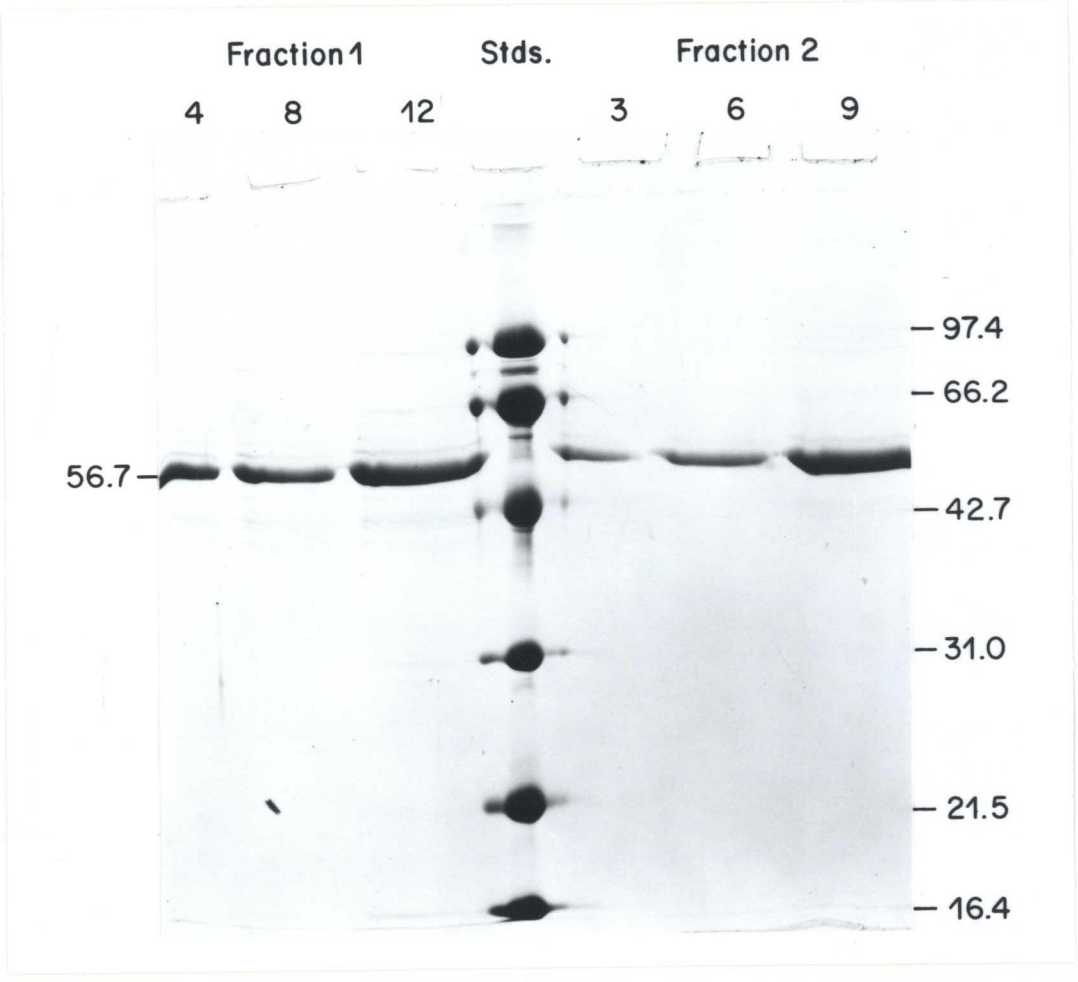


Figure Eighteen: Denaturing gel electrophoresis of purified inosinate dehydrogenase.

Fraction 1 and Fraction 2 were dialysed and concentrated and examined on a 12% SDS polyacrylamide gel as described in the methods (II.B.6). 3 - 12 µg of protein were loaded per lane as indicated.



even when 12 μg was loaded. The subunit molecular weight of the purified protein was estimated to be 56.7 kDa based on the SDS-PAGE standards.

Fraction two was subjected to 24 hour acid hydrolysis and the amino acid composition was determined. Based on a subunit molecular weight of 56.7 kDa the composition shown in Table twelve was obtained. The intact protein was subjected to sequencing and was found to be N-terminally blocked. Therefore, for sequence determination, the purified protein was subjected to carboxymethylation, using dithiothreitol and iodoacetic acid, and digested using endoproteinase lys-C which cleaves at lysine residues (Titani et al, 1984). The resultant peptides were separated on a reverse phase column and 0.5 mL fractions collected (Figure nineteen). Five of the eluted peaks were sequenced using a gas phase sequencer and the amino acid sequences obtained are shown in Table thirteen.

III.E.3. Discussion

We have purified the enzyme inosinate dehydrogenase to apparent homogeneity. The first major step in the purification scheme was the selection of the mycophenolic acid resistant cell line, NB-Myco, which displayed a 200-fold overproduction of a 56.7 kDa protein which was shown ultimately to be inosinate dehydrogenase, a cellular target of mycophenolic acid. Purification of this protein from NB-Myco cells yielded 0.49 mg of inosinate dehydrogenase from 23 mg of crude lysate. The overall yield was 19% and a 9-fold purification was achieved.

In each of the two cases already described where inosinate dehydrogenase has been purified to apparent homogeneity, the mammalian

Table Twelve: Amino acid composition data for the mycophenolic acid resistant (NB-Myco) neuroblastoma cells, Yoshida sarcoma ascites tumor cells, and E.coli.

Amino Acid	Residues per 56.7 kDa protein		
	NB-Myco	Sarcoma Ascites	E.coli
Lysine	38	36	21
Histidine	11	10	8
Arginine	27	24	25
Aspartic Acid	49	48	34
Threonine	29	24	26
Serine	36	33	22
Glutamic acid	58	53	50
Proline	23	16	18
Glycine	49	107	42
Alanine	47	33	46
Valine	24	37	34
Methionine	13	10	8
Isoleucine	32	29	21
Leucine	49	46	35
Tyrosine	16	16	8
Phenylalanine	17	20	13
Tryptophan	N/A	N/A	8

The amino acid composition of inosinate dehydrogenase purified from mycophenolic acid resistant (NB-Myco) cells was determined as described in the methods and reported per subunit size (56.7K dalton). The amino acid composition for inosinate dehydrogenase purified from Yoshida sarcoma ascites tumor cells (Okada et al, 1983), and E.coli. (Krishnaiah, 1975) is also presented for comparison. The amino acid compositions for each of these two have been adjusted to reflect a subunit size of 56.7 kDa corresponding to that determined for the NB-Myco cells rather than that reported for each at 68 kDa and 54 kDa respectively.

Figure Nineteen: Separation of peptides from an endoproteinase lys-C digestion of inosinate dehydrogenase.

Fraction 2 was digested with endoproteinase lys-C, and separated on a reverse-phase Vydac C_{18} column with buffer A (acetonitrile containing 0.095% TFA) and buffer B (water containing 0.1% TFA) as described in the methods (II.B.8). 500 µL fractions were collected and the peaks marked A, B, C, D, E concentrated and subjected to sequencing.

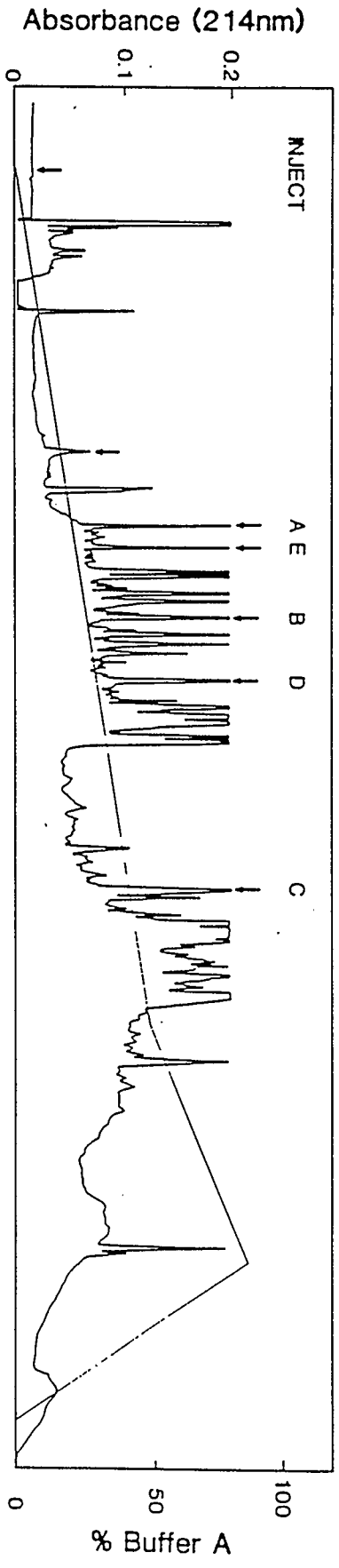

Absorbance (214nm)
0
0.1
0.2
INJECT
A
E
B
D
C
0
50
100
% Buffer A

Table Thirteen: Limited amino acid sequences of mycophenolic acid resistant (NB-Myco) neuroblastoma inosinate dehydrogenase.

Fragment	Sequence	Residues
A	Tyr Ile	2
B	Asp Arg Val Arg Asp Val Phe Glu Ala Lys	10
	Ser Leu Thr Glu	4
C	Phe Val Leu Tyr Leu Ile Ala Gly Ile (Ser) (His)	11
	Thr Pro Pro Val	4
D	Arg Glu Asp Leu Val Val Ala Pro Ala Gly Val (Glu)(Leu)	13
E	Val Ala Glu Gly Val Ser Gly Ala Val Gln Asp	11

Peptides from endo-lys cleavage of purified inosinate dehydrogenase were sequenced as described in the methods (II.B.8) and the amino acid sequence is shown. Where ambiguity over the residue exists the most likely one is shown in parenthese. If more than one residue is detected for a specific cycle then both are reported giving the major and minor peptides as shown for fragments B and C.

enzyme from Yoshida sarcoma ascites cells (Okada et al, 1983) and the protozoan Tritrichomonas foetus (Verham et al, 1987), the cells had not been previously exposed to mycophenolic acid. In these examples the starting material was 2,700 or 3,700 mg of protein and 0.6 or 1 mg of protein was finally obtained having achieved approximately 1,000-fold purification with a 22-30% yield. The 9-fold purification observed in our procedure versus the 1000-fold reported by Okada et al (1983) and Verham et al (1987) undoubtedly reflects the difference in the starting material. For the sarcoma cells and the protozoan sources, the abundance of the inosinate dehydrogenase could be estimated to be at about 0.1% and a 1000-fold purification would be expected to yield a homogeneous protein. Inosinate dehydrogenase in the NB-Myco cells was increased so that it was estimated to represent as high as 20% of the total soluble protein. Therefore, a 9-fold purification is consistent with having purified this protein to homogeneity.

Examination of the purified protein by SDS-PAGE demonstrated a single staining band with a molecular weight of 56.7 kDa. Molecular weight estimates of inosinate dehydrogenase have been reported in the range 50-68 kDa (III. G. 1), the mammalian range being 56-68 kDa (III. G. 1). Thus this estimated value for the molecular weight falls within this range.

In the purification scheme of Verham et al (1987) only 50% purification was achieved following the 3 different column separations, with the remaining 50% separation being achieved on an SDS-PAGE gel. Other proteins co-purified with inosinate dehydrogenase which was most likely due to hydrophobic and ionic interactions. In

contrast Okada et al (1984) achieved purification to homogeneity with the use of the 3 steps. The buffers at each step contained 10% glycerol and 10% ammonium sulphate, in attempts to minimize ionic and hydrophobic interactions.

In the gel filtration procedure described here the buffer comprised, amongst others, 5% ammonium sulphate and 0.5M potassium chloride in order to minimise ionic interactions. Without these components being added to the buffers the proteins eluted in quite a different fashion from the Superose column. In particular the fractions displaying inosinate dehydrogenase activity eluted earlier than if no ammonium sulphate was present in the buffer suggesting that inosinate dehydrogenase was co-purifying with a number of other proteins or that several subunits combined having an increased apparent mass. Mini SDS-PAGE of fractions eluted without ammonium sulphate present showed a large number of proteins co-purified with the inosinate dehydrogenase, these being both of higher and lower molecular weights. Although not conclusive these results are consistent with the ammonium sulphate and potassium chloride having minimized the ionic and hydrophobic interactions, thereby improving the usefulness of this step in purifying inosinate dehydrogenase.

The amino acid composition for the mammalian and E.coli enzymes are shown in Table three. In Table twelve the compositions are again shown but they have been adjusted to reflect a molecular weight of 56.7 kDa. Also shown in Table twelve is the amino acid composition for the NB-Myco inosinate dehydrogenase. Clearly the compositions are very similar with the majority of amino acids differing by only a few residues. The only major difference is seen for glycine.

Okada et al (1984) reported 107 residues of glycine for Yoshida sarcoma ascites; Krishnaiah (1975) for E.coli reported 40 residues; for the NB-Myco cells 49 residues of glycine were found. A closer look at the protocol of Okada et al (1984) shows that the purified protein was dialysed prior to composition determination against a buffer containing, amongst other things, 0.12 mM IMP. IMP would be converted to glycine when the protein was subjected to acid hydrolysis, thus greatly increasing the apparent glycine composition of the protein.

In addition to composition analysis the protein was used to obtain some limited amino acid sequence. The protein was carboxymethylated, cut at the lysine residues using the enzyme endoproteinase lys C, and the peptides were separated on a reverse-phase column (Figure nineteen). Of the 30 plus peaks obtained, 5 fragments were sequenced and the results given in Table thirteen. From fragment A only 2 residues were obtained. One possible reason for this is that fragment A was a di-peptide of Thr-Ile with no lys at its C-terminus; however, this is in disagreement with its apparent abundance. Fragment B was subjected to 20 cycles with a major and a minor sequence being identified. The minor sequence could not be followed after 4 cycles and the major peak yielded 10 residues. Fragment C also contained two peptides; one of these was 4 residues long, the other 11 residues, though after 9 cycles the yields declined making it difficult to be certain of the last two residues. Fragments D and E gave the least ambiguous sequence and therefore of the greatest use. Fragment D was subjected to 15 cycles and gave a sequence 11 amino acids long. Fragment E was subjected to 20 cycles

and gave a sequence 13 amino acids in length, the peptide appearing to be only 15 residues long in total. The yields from fragment E declined noticeably after residue 3 most likely because residues 4-13 are hydrophobic and consequently are more soluble in the organic solvents used to wash or extract the peptide during each cycle.

The amino acid sequence can be used to generate an oligonucleotide which is specific for the inosinate dehydrogenase gene. Such an oligonucleotide probe may be used: 1, as a primer for mRNA sequencing; 2, for screening a cDNA library; and 3, for measuring mRNA and DNA levels. Fragments D and E provided the longest unambiguous amino acid sequence which could be used to generate a corresponding nucleotide sequence.

An approach to predicting a unique oligonucleotide sequence from an amino acid sequence based on theoretical and practical considerations was applied to fragments D and E (Lathe, 1985). This approach is based on most probable codon usage (and its probability), and provides guidelines for the minimum probe length requirement, and the most suitable annealing temperature for hybridization based on the probe length and probability and sequence complemetality. Using the approach laid out by Lathe (1985) the best nucleotide sequence and its probability are detailed in Table fourteen. With this information an oligonucleotide could be synthesized from peptide sequences D and / or E.

Table Fourteen: Most probable codon usage for two amino acid sequences (fragments D and E) of inosinate dehydrogenase from a mycophenolic acid resistant (NB-Myco) cell.

Fragment	Amino acid/Nucleotide Sequence and codon probability %
D	Arg Glu Asp Leu Val Val Ala Pro Ala Gly Val
	CGG.GAG.GAC.CTG.GTG.GTG.GCC.CCT.GCT.GGC.GTG.
	65, 87, 90, 69, 83, 83, 84, 81, 84, 81, 83.
E	Val Ala Glu Gly Val Ser Gly Ala Val Gln Asp
	GTG.GCC.CAG.GGC.GTC.TCT.GGC.GCT.GTG.CAG.GAC.
	83, 84, 91, 81, 83, 60, 81, 84, 83, 91, 90.

For fragment D and E each amino acid is shown, its optimum codon and a certainty factor for this codon. The certainty factor is the overall % homology predicted between the optimum codon and the target sequence for each amino acid based on codon utilization in human protein-coding sequences (Lathe, 1985).

IV. Summary

IV.A. Amplification and / or mutation; what is the evidence?

The mycophenolic acid resistance attained in cultured mouse neuroblastoma, NB-Myco, cells could result from at least four mechanisms: altered transport of the mycophenolic acid into the cell; reduced conversion of the mycophenolic acid to an active form; overproduction of the cellular target of mycophenolic acid, inosinate dehydrogenase; and finally reduced affinity of inosinate dehydrogenase for mycophenolic acid. A number of experiments have been conducted on the control (NB) and the mycophenolic acid resistant (NB-Myco) neuroblastoma cell lines to establish the cause of the increased resistance to mycophenolic acid in the neuroblastoma, NB-Myco, cells.

As mycophenolic acid is able to readily cross the cell membrane, resistance due to altered transport into the cell is unlikely. However, a correlation between sensitivity of a tumor to mycophenolic acid and β-glucuronidase activity has been identified (Sweeney et al, 1972b). A mycophenolic acid glucuronide conjugate is formed as one method of detoxification and excretion of mycophenolic acid. Once conjugated the mycophenolic acid is water soluble, therefore more easily excreted, and is unable to cross the cell membrane whereas the unconjugated form is very lipid soluble. Tumor cells with a lower activity of β-glucuronidase were identified as being more resistant to mycophenolic acid. Despite this correlation no reports have been made regarding a change in this enzyme as a result of an induced resistance of a cultured cell line. As detoxification by conjugation in mammals occurs in the liver it is unlikely to be a method of

resistance for cultured neuroblastoma cells. Thus amplification and / or mutation are the most likely events to have occurred and these are now addressed.

There are three basic mechanisms that could account for gene amplification in somatic cells: uptake of DNA from killed cells and subsequent replication and integration; some form of unequal cross over event, such as sister chromatid exchange; or disproportionate replication wherein a portion of the genome is replicated more than once in a single cell cycle (Schimke, 1984). Though clearly gene amplification can occur by more than one mechanism, the current concept of the mechanism for mammalian cells, though not uniformly accepted nor entirely documented, is the latter mechanism which is based on the idea that there can occur more than a single initiation of replication within a chromosome region during a single cell cycle (Schimke et al, 1987). The disproportionate replication model fits some of the data available regarding amplification as does the model of unequal sister chromatid exchange, though neither fits all the data (see Stark and Whal, 1984), leaving them in need of further clarification

If a mouse neuroblastoma cell line was resistant to mycophenolic acid due to gene amplification of inosinate dehydrogenase the properties expected to result would be: a cell which was the product of a stepwise selection process; overproduction of the target protein inosinate dehydrogenase; and an unstable phenotype, though stable phenotypes do also occur in an amplified cell. The NB-Myco cells meet all three criteria in that they were selected by incremental increases in mycophenolic acid of 5 - 10% per passage; they display an

overproduction of inosinate dehydrogenase (200-500 fold); and have an unstable phenotype as observed by SDS-PAGE of the inosinate dehydrogenase protein, and inosinate dehydrogenase activity measurements. In addition, double minute chromosomes have been observed in the NB-Myco-200 cells, not reported here, but have not been examined in the NB-Myco cells. This is consistent with the report that highly aneuploid lines, such as mouse, are more likely to form double minutes rather than homogeneous staining regions integrated into the chromosome in order to maintain the amplified state (Schimke, 1984). Double minutes are more unstable than HSR which is consistent with the relatively unstable phenotype observed.

It has been observed in methotrexate resistant lines that initially unstable amplified dihydrofolate reductase lines can occur and as the selection is maintained for some time the emerging population often contains a more stable amplified gene (Schimke, 1984). This change could be due to double minutes being present initially and integration occurring later forming homogeneous staining regions with continued selection pressure. As the double minutes contain no centromere they can be proportioned unequally into daughter cells at mitosis and hence can be lost very rapidly under non-selective conditions. Homogeneous staining regions are lost under non-selective conditions, though this occurs at a much slower rate than that for double minutes, and therefore they are more stable than double minutes. In the mycophenolic acid resistant mouse neuroblastoma cells the stability of the phenotype, as observed by the overproduction of the inosinate dehydrogenase protein and the enzyme activity, is reduced in the NB-Myco-200 cells compared with the NB-Myco cells which

had been subject to the selection for considerably longer. Double minutes existed in the NB-Myco-200 cells, but in the NB-Myco cells, due to the increased stability of the phenotype, as seen by the slower decline of the inosinate dehydrogenase activity and protein in the absence of mycophenolic acid, the amplified inosinate dehydrogenase gene may be present as a homogeneous staining region though this has not been examined. Double minutes and homogeneous staining regions of the same sequence have not been seen to coexist in the same cell (Stark and Whal, 1984), though changes in stability are most likely due to a transition from extrachromosomal (unstable) to chromosomal (stable) amplified genes upon prolonged selection (Schimke, 1984). Like a number of other reports, the properties of gene amplification have been demonstrated for the NB-Myco cells but have not yet been confirmed by gene quantitation data (see Schimke et al, 1987).

Amplification does not account for all the features of the NB-Myco cells regarding the increased resistance of the NB-Myco cells to mycophenolic acid. Of particular interest is the fact that the fold increase in inosinate dehydrogenase protein and activity do not correlate with each other nor do they correlate with the fold increase in the resistance to mycophenolic acid. This suggests that, in addition to the amplification event, a mutational event has occurred. Reports of methotrexate resistance provide examples where a mutation in the dihydrofolate reductase gene has occurred secondary to initial gene amplification (Haber and Schimke, 1981) and, conversely, where low resistance to methotrexate was due to a mutation in the dihydrofolate gene and subsequently higher levels of methotrexate resistance were due to overproduction of the altered enzyme (Flintoff

et al, 1976). Thus one mechanism of resistance can be superimposed on another. In the case of the selection of mycophenolic acid resistant neuroblastoma cells it is unclear if a mutational event preceded or followed amplification, but evidence is presented indicating that both have occurred. The evidence for a mutation are the increased Michaelis constant (K_m) for NAD^+, and increased inhibitor constants (K_i) for XMP and mycophenolic acid in the NB-Myco compared with the NB cells, the largest change being the 2400-fold increase in K_i for the mycophenolic acid in the NB-Myco cells.

We can, therefore, conclude that the gene coding for the inosinate dehydrogenase has undergone both mutation and amplification during the process of selection of a mycophenolic acid resistant NB.Myco cell line.

IV.B. Kinetics of mammalian inosinate dehydrogenase and the nature of the NAD^+ and mycophenolic acid inhibition complex.

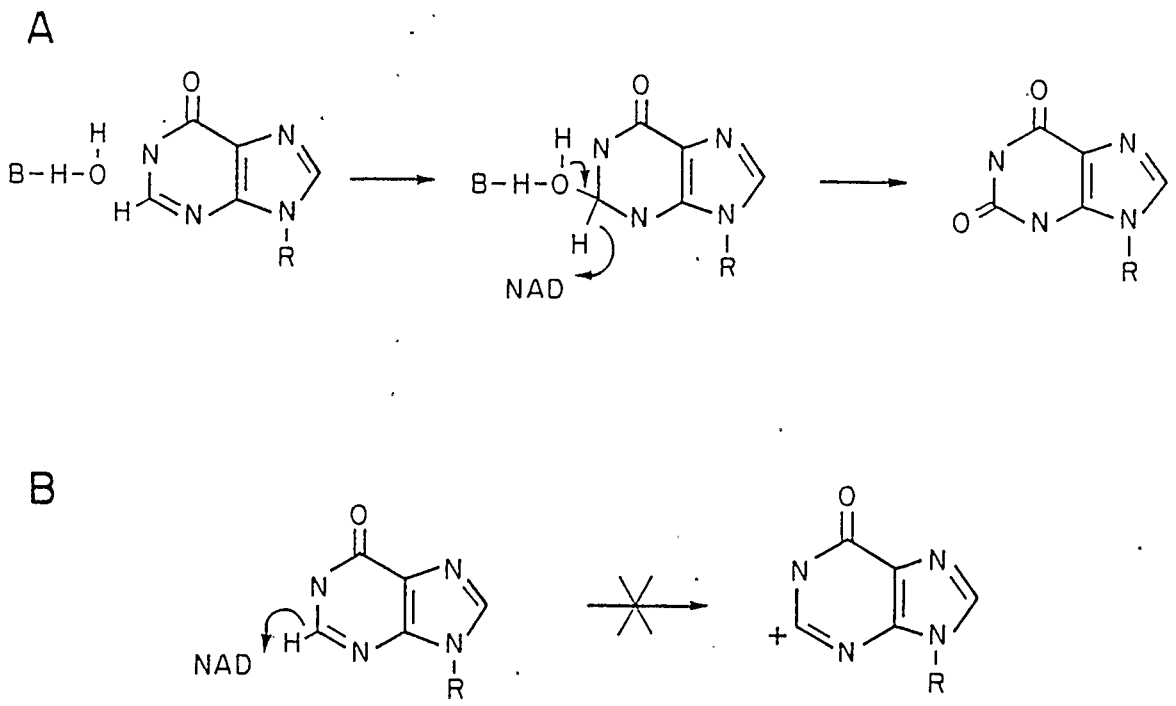
As described earlier the results presented here provide support for an ordered bi bi reaction in which IMP binds first and XMP is released last, with inhibition by both mycophenolic acid and high concentrations of NAD^+ resulting from E▪XMP▪I complex formation (III.C.3). A question raised is why does the mycophenolic acid binds to E▪XMP so tightly and does not bind to E▪IMP, and can this provide information regarding the decreased sensitivity of the NB-Myco cells to mycophenolic acid?

The bacterial enzyme does not exhibit inhibition at high $[NAD^+]$ and is not inhibited by mycophenolic acid. This adds support to the notion that mycophenolic acid inhibition and NAD^+ inhibition may

involve the same site. A modification to this site could affect the K_{iMyco}, and also the K_{mNAD+} of the inosinate dehydrogenase. The protozoan enzyme has a kinetic mechanism similar to the mammalian system (Hupe et al , 1986; Verham et al, 1987). The reported K_{mIMP} and K_{iNAD+} for the protozoan and mammalian enzymes are very similar, whereas a difference is reported between the K_{iMyco} and K_{mNAD+}. The protozoan enzyme has a K_{mNAD+} in the range 150 - 340 μM compared to that reported for mammalian cells of 24 - 46 μM. In addition, the protozoan enzyme has a K_{iMyco} of 0.2 - 9 μM versus a K_{iMyco} of 12 - 46 nM for mammalian cells (III.C.1). A correlation between an increased Michaelis constant for NAD^+ and an increased inhibitor constant for mycophenolic acid (K_{iMyco}) would appear to exist. In the case of the NB and NB-Myco cells the K_{mNAD+} has significantly increased in the NB-Myco cells and this correlates with an increasing K_{iMyco} which is in the nM range for the NB cells and the μM range for the NB-Myco cells. Interestingly the K_{iNAD+} is unchanged in protozoan and mammalian cells including cells that are resistant to mycophenolic acid.

Hupe et al (1986) proposed a reaction scheme in which mycophenolic acid and NAD^+ bind at the same site. The catalytic reaction shown in Figure Twenty a requires the donation of an oxygen from water to IMP to derive the product, XMP, coupled with a H: transfer to NAD^+. In contrast, the reaction shown in Figure 20b is unlikely due to the instability of the carbonium ion intermediate. With such a mechanism inosinate dehydrogenase would have to be configured so as to stabilize the complex formed by the addition of

Figure Twenty: Reaction schemes for inosinate dehydrogenase.

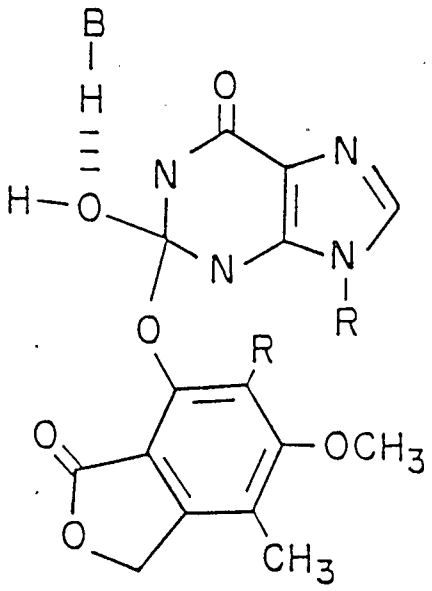


water. In this event a speculative proposal on the mode of mycophenolic acid inhibition can be made which explains why mycophenolic acid binds only to the E▪XMP complex and not to E▪IMP. The intermediate E▪XMP▪NAD^+ complex can be represented as shown in Figure Twenty one b and in the same manner mycophenolic acid could neutrophilically add to E▪XMP forming a similar complex, Figure Twenty one a. Such an inhibitor complex would be stable since it resembles the transition state for the H: transfer and could not be formed with E▪IMP due to the requirement of a hydroxyl group at C_2 of the purine ring for stabilization. This model allows predictions to be made regarding the effect of structural modifications to mycophenolic acid which adds further support to the model. In the case of the NB-Myco cells a reduction in the stability of this complex due to a mutation of the inosinate dehydrogenase enzyme, could cause an increase in the K_{iMyco}. The stability of the transition complex would likely also be expected to be affected causing an increase in the K_{mNAD+}. In the case of the NB and NB-Myco cells the K_{iMyco} did increase parallel with an increase in the K_{mNAD+} in the NB over the NB-Myco cells. A change in the $K_{iNAD}+$ could also be expected but this was not seen for the protozoan nor the NB-Myco cells.

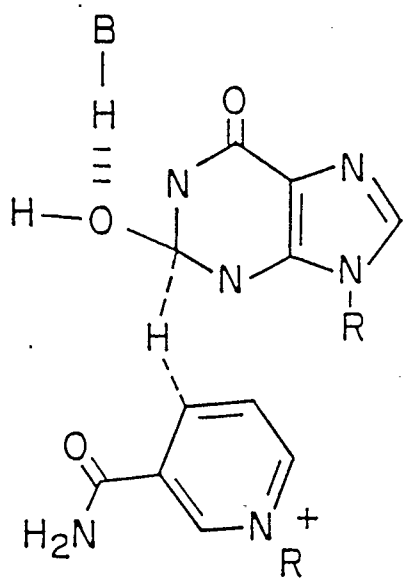
IV.C. Composition and sequence information. Further work.

With two peptide sequences available, fragments D and E (III.E.3.), the sequence of an oligonucleotide can be predicted and synthesized according to the best codon usage protocol presented by Lathe (1985). In this protocol a unique oligonucleotide sequence is

Figure Twenty One: Proposed XMP•NAD$^+$ and XMP•Myco complexes.



A



B

selected based on the most frequent codon usage, as calculated by Lathe (1985) based on known human codon usage. Further, an estimate of the required oligonucleotide length to ensure sufficient specificity over random nucleotide sequence matches can be made, and estimates of an annealing temperature providing hybridization of the probe to the target with sufficient specificity have been made.

Methods have been developed and optimized for the direct sequencing of relatively rare abundance mammalian messenger RNA (Joshi and Snyder, 1988). Those approaches will be applied in attempts to generate some unique sequence for inosinate dehydrogenase from mRNA prepared from NB-Myco cells.

V. Conclusions

In conclusion, we have described a mouse neuroblastoma cell line resistant to mycophenolic acid, obtained by incremental increases in the drug at each passage. This cell line, NB-Myco, displays a 10,000-fold increase in resistance to mycophenolic acid, the inosinate dehydrogenase specific activity is increased 25-fold and the protein level, with a subunit molecular weight of 56.7 kDa, increased 200-500-fold compared with the control, NB, cells. The NB-Myco cells grown in the absence of mycophenolic acid showed a parallel decline in inosinate dehydrogenase specific activity and protein level. These factors are consistent with the suggestion that the gene coding for inosinate dehydrogenase has been amplified.

Kinetic studies conducted with the enzyme from NB and NB-Myco cells show that each have the same ordered bi bi reaction mechanism in which IMP binds first and XMP is released last. Mycophenolic acid and high concentrations of NAD^+ exhibit uncompetitive inhibition with IMP consistent with the formation of a dead-end E•XMP•inhibitor complex.

The Michaelis constants for IMP and the apparent inhibition constant for NAD^+ were unchanged in the NB and NB-Myco cells, whereas the Michaelis constant for NAD^+ and the inhibition constants for XMP and mycophenolic acid were all increased to varying degrees in the NB-Myco cells. These findings provide evidence that the inosinate dehydrogenase has undergone a mutation.

Purification of inosinate dehydrogenase from the NB-Myco cells allowed for the determination of the amino acid composition and a limited amino acid sequence which should be sufficient for the

isolation of the gene coding for inosinate dehydrogenase using oligonucleotide probes.

VI. References

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APPENDIX

I. Introduction

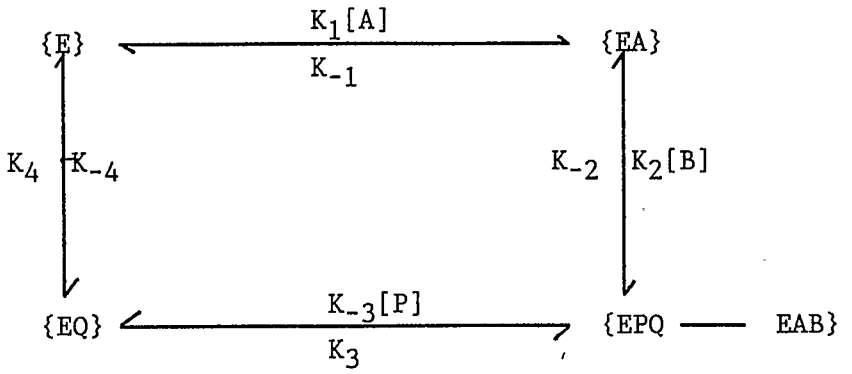
This appendix provides details of the kinetics as they apply to the data obtained in the investigation of inosinate dehydrogenase. It is not intended to be a complete exposition of the subject, but rather detailed information of specific areas is given.

II. Derivation of the steady state velocity equation for an ordered bi bi reaction using the King-Altman and Cleland coefficient method.

Firstly the ordered bi reactant reaction in both directions can be defined as follows:

$$E + A \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} EA + B \underset{K_{-2}}{\overset{K_2}{\rightleftharpoons}} (EAB \underset{K_{-p}}{\overset{K_p}{\rightleftharpoons}} EPQ) \underset{K_{-3}}{\overset{K_3}{\rightleftharpoons}} EQ + P \underset{K_{-4}}{\overset{K_4}{\rightleftharpoons}} E + Q$$

This can also be written in King-Altman notation which has four enzyme forms and consequently four corners to the diagram as follows:



The distribution of the enzyme forms {E}, {EA}, {EQ} and {EPQ-EAB} can therefore be obtained from the four, three lined, conversion patterns and given as a proportion of the total enzyme, $[E]_t$:

$$\frac{[E]}{[E]_t} = \frac{K_4K_{-1}K_{-2} + K_{-3}[P]K_{-2}K_{-1} + K_2[B]k_3K_4 + K_{-1}K_3K_4}{\text{denominator}}$$

$$\frac{[EA]}{[E]_t} = \frac{K_4K_1[A]K_{-2} + K_1[A]K_{-3}[P]K_{-2} + K_{-4}[Q]K_{-3}[P]K_{-2} + K_3K_4K_1[A]}{\text{denominator}}$$

$$\frac{[EAB\text{-}EPQ]}{[E]_t} = \frac{K_4K_1[A]K_2[B] + K_1[A]K_2[B]K_{-3}[P] + K_{-4}[Q]K_{-3}[P]K_2[B] + K_{-1}K_{-4}[Q]K_{-3}[P]}{\text{denominator}}$$

$$\frac{[EQ]}{[E]_t} = \frac{K_{-2}K_{-1}K_{-4}[Q] + K_1[A]K_2[B]K_3 + K_{-4}[Q]K_2[B]k_3 + K_{-1}K_{-4}[Q]K_3}{\text{denomnator}}$$

The denominator, $[E]_t$, is the sum of the numerators of all of the enzyme forms which when combined and the terms grouped is:

$$\begin{aligned}\text{Denominator} = {} & K_4K_{-1}(K_{-2}+K_3) + [A]K_1K_4(K_3+K_{-2}) + [B]K_2K_3K_4 + \\ & [P]K_{-1}K_{-2}K_{-3} + [Q]K_{-1}K_{-4}(K_{-2}+K_3) + [Q][P]k_{-3}k_{-4}(K_{-2}+K_{-1}) \\ & + [Q][B]K_2K_3K_{-4} + [A][P]K_1K_{-2}K_{-3} + [A][B]K_1K_2(K_3+k_4) \\ & + [A][B][P]K_1K_2K_{-3} + [B][P][Q]K_2K_{-3}K_{-4}\end{aligned}$$

The steady state net velocity is defined as the difference between the forward and the reverse velocities at any step. Therefore we can use the following to define the net velocity (v):

$$v = K_1[A]E - K_{-1}EA$$

Redefining this by replacing the expressions for the enzyme forms in terms of the rate constants and the ligand constants we obtain the following:

$$\frac{v}{[E]_t} = \frac{K_1[A](K_2[B]K_3K_4+K_{-2}K_{-3}K_{-1}[P]+K_4K_{-1}K_{-2}+K_4K_{-1}K_3) + K_{-1}(K_1[A]K_4K_3 + K_4K_1[A]K_{-2}+K_1[A]K_{-2}K_{-3}[P]+K_{-4}[Q]K_{-3}[P]K_{-2})}{\text{denominator}}$$

Simplfying the numerator the following is the result:-

$$\frac{v}{[E]_t} = \frac{K_1K_2K_3K_4[A][B] + K_{-1}K_{-2}K_{-3}K_{-4}[P][Q]}{\text{denominator}}$$

The above equation can now be written in the coefficient form of Cleland as follows:

$$\frac{v}{[E]_t} = \frac{\text{Num 1 [A][B] + Num 2 [P][Q]}}{\begin{array}{l}\text{Const + Coef A [A] + Coef B [B] + Coef P [P] + Coef Q [Q]} \\ \text{+ Coef Q [Q] + Coef PQ [P][Q] + Coef QB [Q][B] +} \\ \text{Coef AP [A][P] + Coef AB [A][B] + Coef ABP [A][B][P] +} \\ \text{Coef BPQ [B][P][Q]}\end{array}}$$

Where:-

$$K_{ma} = \frac{\text{Coef B}}{\text{Coef AB}}, \quad K_{ma} = \frac{\text{Coef A}}{\text{Coef AB}}, \quad K_{ia} = \frac{\text{Coef P}}{\text{Coef AB}} = \frac{\text{Const}}{\text{Coef A}} = \frac{\text{K-1}}{\text{K1}}$$

$$K_{ia} = \frac{\text{Coef PQ}}{\text{Coef BPQ}} = \frac{\text{K-1 + K-2}}{\text{K-3}}, \quad K_{mp} = \frac{\text{Coef Q}}{\text{Coef PQ}}, \quad K_{mq} = \frac{\text{Coef P}}{\text{Coef PQ}}$$

$$K_{ia} = \frac{\text{Coef AB}}{\text{Coef ABP}} = \frac{K3 + K4}{K\text{-}3}, \qquad K_{iq} = \frac{\text{Coef B}}{\text{Coef BQ}} = \frac{\text{Const}}{\text{Coef Q}} = \frac{K4}{K\text{-}4}$$

$$V_{maxf} = \frac{\text{Num 1}}{\text{Coef AB}}, \qquad V_{maxr} = \frac{\text{Num 2}}{\text{Coef PQ}}, \qquad K_{eq} = \frac{\text{Num 1}}{\text{Num 2}}$$

Now by multplying the top and the bottom of the equation with with $\frac{\text{Num2}}{\text{CoefAB CoefPQ}}$ and by replacing the coefficients with the relavent Michealis constants one is able to simplify the equation to the following:

Equation 1).

$$v = \frac{V_{maxf} V_{maxr} ([A][B] + \frac{[P][Q]}{Keq}}{V_{maxr}K_{ia}K_{mb} + K_{mb}V_{maxr}[A] + K_{ma}V_{maxr}[B] + \frac{K_{mq}V_{maxf}[P]}{K_{eq}} + \frac{K_{mp}V_{maxf}[Q]}{K_{eq}} + \frac{V_{maxf}[P][Q]}{K_{eq}} + \frac{V_{maxr}[Q][B]K_{ma}}{K_{iq}} + \frac{K_{mq}[A][P]V_{maxfY}}{K_{ia}K_{eq}} + [A][B]V_{maxr} + \frac{V_{maxr}[A][B][P]}{K_{ip}} + \frac{[B][P][Q]V_{maxf}}{K_{ib}K_{eq}}}$$

This equation can be further simplified by dividing the top and the bottom by V_{maxr} giving :

Equation 2).

$$v = \frac{- V_{maxf}\left([A][B] + \frac{[P][Q]}{Keq}\right)}{K_{ia}K_{mb} + K_{mb}[A] + K_{ma}[B] + \frac{K_{mq}V_{maxf}[P]}{V_{maxrKeq}} + \frac{K_{mp}V_{maxf}[Q]}{V_{maxKeq}} + \frac{V_{maxf}[P][Q]}{V_{maxr}K_{eq}} + \frac{[Q][B]K_{ma}}{K_{iq}} + \frac{K_{mq}[A][P]V_{maxf}}{V_{max}K_{ia}K_{eq}} + [A][B] + \frac{[A][B][P]}{K_{ip}} + \frac{[B][P][Q]Vmaxf}{V_{maxr}K_{ib}K_{eq}}}$$

This equation (2) represents the full rate equation.

III. Initial velocity studies

The use of initial velocity studies allows one to learn a great deal about the reaction mechanism of a particular enzyme. When the concentration of the products are set equal to zero, then the rate equation for the reaction becomes an equation describing the initial velocity of the enzymatic reaction. These rate equations are much simpler than the full rate equation (2) which defines the forward and reverse reactions as they represent only the forward reaction and thus are easier to work with. For initial rate studies there are no products and therefore no reverse reaction therefore all components

containing [P], [Q] and V_{maxr} reduce to zero. The velocity equation can be written as shown.

Equation 3).

$$\frac{v}{V_{maxf}} = \frac{[A][B]}{K_{ia}K_{mb} + K_{mb}[A] + K_{ma}[B] + [A][B]}$$

In order to conduct an initial velocity study, one substrate is varied at fixed variable concentrations of the second substrate. The velocity measurements obtained are plotted as a reciprocal plot of velocity (v) versus variable substrate (A) for each varied, yet fixed, concentration of B with all other variables remaining constant. The result is a family of lines for which either the slope or the intercept, or both, vary when [B] is varied. The intercept of the reciprocal plot represents the reciprocal velocity at infinite substrate concentration, the slope is a measure of how fast the reaction slows down as the substrate concentration decreases from infinity to zero. In order to identify the slope and the intercept equations it is of value to rewrite the equation to the form shown.

Equation 4).

$$\frac{1}{v} = \text{Slope}\left(\frac{1}{[A]}\right) + \text{Intercept}$$

Rewriting the initial velocity rate equation into the format defined in equation 4 for the forward reaction, when [A] is varied, yields the equation shown.

Equation 5).

$$\frac{1}{v} = \frac{K_{ma}}{V_{max}}\left(1 + \frac{K_{ia}K_{mb}}{K_{ma}[B]}\right)\frac{1}{[A]} + \frac{1}{V_{max}}\left(1 + \frac{K_{mb}}{[B]}\right)$$

In this case the slope and intercept are defined as follows:

$$\text{Intercept} = \frac{1}{V_{max}}\left(1 + \frac{K_{mb}}{[B]}\right)$$

$$\text{Slope} = \frac{K_{ma}}{V_{max}}\left(1 + \frac{K_{ia}K_{mb}}{[B]K_{ma}}\right)$$

The calculation of the kinetic parameters can be best achieved by replots of the slope or 1/intercept versus the variable fixed substrate concentration, B, taken from the reciprocal plot of v versus the varied substrate, A. Definitions of the slopes and intercepts from the replots are as follows. Firstly for the $1/v_{max\ app}$ versus 1/[B] replot, and secondly the Slope versus 1/[B] replot.

1) The Slope $= \dfrac{K_{mb}}{V_{max}}$

The Intercept $= \dfrac{1}{V_{max}}$

2) The Slope $= \dfrac{K_{ia}K_{mb}}{V_{max}}$

$$\text{The Intercept} = \frac{K_m}{V_{max}}$$

If equation (3) is re-written to consider the example when [B] is varied then the following is obtained.

Equation 6).

$$\frac{1}{v} = \frac{K_{mb}}{V_{max}} \left(1 + \frac{K_{ia}}{[A]} \right) \frac{1}{[B]} + \frac{1}{V_{max}} \left(1 + \frac{K_{ma}}{[A]} \right)$$

The intercept and slope are therefore defined as:-

$$\text{The slope} = \frac{K_{mb}}{V_{max}} \left(1 + \frac{K_{ia}}{[A]} \right)$$

$$\text{The intercept} = \frac{1}{V_{max}} \left(1 + \frac{K_{ma}}{[A]} \right)$$

Similarly replots of 1/intercept versus [A] or 1/slope versus [A] provide a way of calculating the kinetic parameters.

IV. Determination of Reaction Mechanism from Initial Velocity studies.

Different types of reaction mechanisms give different initial velocity equations. By comparing the data obtained from initial velocity studies and using the equations that describe different reaction mechanisms the mechanism may be deduced. Cleland described a method whereby rate equations could be determined for each reaction mechanism and experimental data assigned to each equation to identify

the reaction mechanism to which it best fitted (Cleland, 1963a). In a later discussion by Cleland this task was further addressed, and the problems associated with deriving the rate equation for each reaction mechanism acknowledged (Cleland, 1963c). The difficulty was that in-order to be sure one has obtained the correct mechanism for the data in hand one had to examine every possible mechanism, and reject those that didn't fit, both time consuming and difficult. Therefore Cleland proposed a number of rules to assist in the selection of the correct model (Cleland, 1963c). Using kinetic data plotted as a reciprocal plot of v versus [A], as previously described, Cleland outlined the following:-

1) A reactant affects the <u>intercept</u> of a reciprocal plot when it combines reversibly with an enzyme form other than the one the variable substrate combines with and thereby changes the reaction velocity in a manner which cannot be eliminated by saturation with the variable substrate.

2) A reactant affects the <u>slope</u> of a reciprocal plot when it and the variable substrate either combine with the same form of the enzyme or are separated in the reaction sequence by a series of irreversible steps (Cleland, 1963c).

If the effects being analyzed are only for one enzyme form then the effect is always linear; that is a replot of the slopes or the intercepts versus the concentration of inhibitor or versus the reciprocal concentration for substrates, are a straight line. If the line is not linear, such as a hyperbola or a curve, then effects other than those which are within the scope of this appendix have occurred (Cleland, 1963c).

Applying Cleland's rules to a sequential mechanism (ordered, Theorell-Chance, and also rapid equilibrium random) would give a family of lines that would intercept to the left of the vertical axis. The intersection point could be above, below or on the horizontal axis, depending on the ratio of K_{ia} to K_a though the vertical coordinate will be the same regardless of which substrate is varied. By comparison, if the reaction was a ping pong mechanism then the rate equation would be such that the reciprocal plot would produce a family of parallel lines in which the slope remained constant and the intercepts changed for each changing fixed substrate concentration. To look at it another way, the apparent constant in equation 3) can either increase, decrease, or remain constant with increasing concentrations of B, dependent on the ratio of K_{ia} to K_a. If the apparent K decreases or remains constant the reaction must be sequential. The apparent K only increases with increasing concentrations of B for a ping-pong mechanism.

Therefore by a simple comparison of the reciprocal plots and replots of the slopes and intercepts versus the fixed changing substrate concentration, an insight into the reaction mechanism can be obtained by initial velocity studies. Though these studies assist in defining a type of mechanism, they provide no information on the order of a sequential reaction. This information can be obtained from product inhibition studies.

V. Inhibition Studies

Patterns of inhibition by substrates, products, or dead-end inhibitors all provide useful information in determining the kinetic

mechanism. In conducting studies of this nature a substrate is varied at various fixed levels of inhibitor including zero, all other substrate concentrations being held constant at non-saturating levels. Using saturating levels of substrate may also be useful as it can provide additional kinetic information for certain types of inhibition, for example the inhibition may be overcome by the saturation, but in the cases discussed here only non-saturating levels are considered.

As with initial velocity studies, reciprocal plots of velocity versus the varied substrate concentration plotted for each fixed and varied concentration of inhibitor allows us to examine the patterns obtained and to draw deductions about the mode of inhibition. The raw data could be examined for each individual rate equation that could be deduced for each type of inhibition and the one to which the data fit best would likely point to the possible type of inhibition, but the complexity of this approach versus the ease of examining the patterns makes it less appealing so this discussion primarily centers on examination of the various patterns.

Three basic types of inhibition are discussed, the distinction between each being shown in terms of the reciprocal plots of v versus a varied substrate concentration for a range of fixed and changing inhibitor concentrations, from zero up. Such a plot is composed of a family of lines with each line representing a different inhibitor concentration.

V.A. Competitive inhibition

If the slopes of the lines are affected by the inhibitor, but the intercepts are not, then the pattern intercepts on the vertical axis

and the inhibition is called competitive. This pattern shows a mutually exclusive binding of the inhibitor and substrate (being varied) and suggests that the two bind to the same enzyme form (Cleland, 1963b). Exceptions to this exist but are not addressed here. The rate equation for simple competitive inhibition in the reciprocal form is shown in (Segal, 1975).

Equation 7).

$$\frac{1}{v} = \frac{K_m}{V_{max}} \left(1 + \frac{[I]}{K_i} \right) \frac{1}{[S]} + \frac{1}{V_{max}}$$

V.B. Uncompetitive inhibition.

The second inhibition pattern consists of a series of parallel lines where the inhibitor affects the intercepts and not the slopes and is called uncompetitive. This pattern of inhibition disappears as the substrate concentration is extrapolated to zero thereby showing that the enzyme form to which the inhibitor binds is not present when the variable substrate concentration is zero (Cleland, 1963b). For simple uncompetitive inhibition the reciprocal form of the velocity equation is shown (Segal, 1975).

Equation 8).

$$\frac{1}{v} = \frac{K_m}{V_{max}} \left(1 + \frac{[I]}{K_i} \right) \frac{1}{[S]} + \frac{1}{V_{max}} \left(1 + \frac{[I]}{K_i} \right)$$

The third pattern of inhibition shows variation in both the intercepts and the slopes with varied inhibitor concentration and is

called noncompetitive. The effect of the inhibitor on the slopes and intercepts are usually not equal, there being no reason to assume they should be. —The crossover point of the pattern therefore may be above or below but not on the horizontal axis. The noncompetitive pattern of inhibition shows that the substrate and inhibitor combine with different enzyme forms and that the inhibitor causes inhibition at high or low substrate concentrations (Cleland, 1963b). The reciprocal rate equation for noncompetitive inhibition is shown (Segal, 1975).

Equation 9).

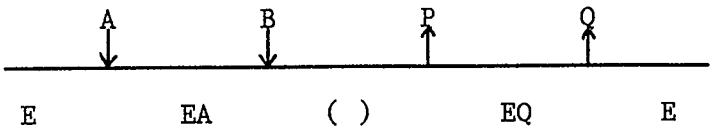
$$\frac{1}{V} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \left(1 + \frac{[I]}{Ki} \right)$$

In each of the inhibition patterns detailed above replots of the slopes or intercepts should be replotted versus the changing fixed inhibitor concentration to identify if the inhibition is linear. In the majority of cases they are, but a parabolic or hyperbolic replot can also provide useful information in deducing the mechanism.

V.C. Product inhibition.

In theory the products of a reaction should always inhibit it, since they are substrates for the reverse reaction and will thus form complexes with the enzyme. In practice the concentration of the product required may exceed its solubility for the reaction, but most

product-inhibition studies can be practical and useful to fill in some of the gaps left by initial velocity studies. Consider then an ordered reaction such as the case outlined below.



Q and A both bind to the same enzyme form E, thus Q is competitive versus A (and vice versa), and all other combinations (Q versus B and P versus A or B) are all noncompetitive as in each case the product binds to a different enzyme form than the varied substrate. Differences exist between saturating and non saturating conditions for example in the case of P versus A the pattern of inhibition changes from noncompetitive to uncompetitive as B becomes saturating whereas inhibition of P versus B remains noncompetitive at saturating A. Obviously if the reaction order was different then different inhibition patterns would be observed providing further identification of the reaction mechanism and the ordering of the substrates and products.

Rate equations for any product inhibition are obtained from the full velocity equation (3) by setting the concentrations of all other products to zero. The reaction will always be linear if no alternative reaction exists (Cleland, 1963b). The rate equation, when Q is the inhibitor and A is the variable substrate, is shown (Segal, 1975).

Equation 10).

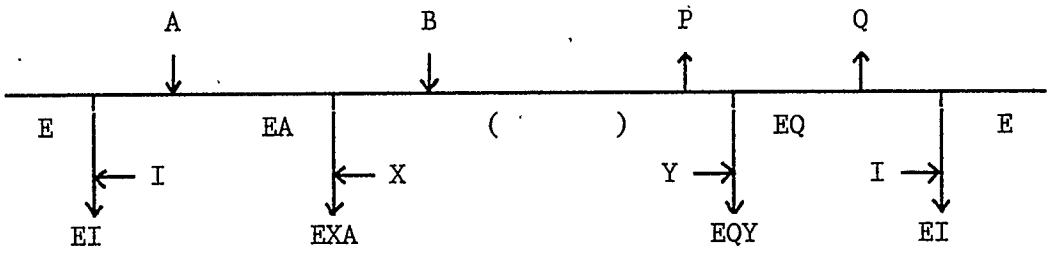
$$\frac{v}{V_{max}} = \frac{[A]\,[B]}{K_{ma}\left(1 + \frac{[Q]}{K_{iq}}\right)\left(1 + \frac{K_{ia}K_{mb}}{K_{ma}[B]}\right) + [A]\left(1 + \frac{K_{mb}}{[B]}\right)}$$

The resultant reciprocal plot and replots of the slope and intercepts versus the changing fixed inhibitor concentration can be used to calculate the kinetic parameters (Segal, 1975).

V.D. Inhibition with and inhibitor other than a substrate or product.

In a similar manner an inhibitor, which is neither a substrate nor a product, can be studied and the mode of inhibition understood by the examination of inhibition patterns. Inhibitors usually form complexes with one or more of the various enzyme forms and thus lower the amount of enzyme available for the normal reaction sequence. Unlike inhibition involving the majority of products and substrates in which the normal reaction complexes are formed, which can break down either in the reverse or the forward direction to release the products or substrates, inhibitors may form "dead-end" complexes. In such a case the the enzyme {with or without associated product(s) or substrate(s) attached} forms a complex with the inhibitor which cannot undergo a reaction other than inhibitor dissociation. Because dead-end inhibitors cannot reverse a reaction, as can product inhibition, they influence the slopes of the reciprocal plots only when the combination of the variable substrate follows the addition of the inhibitor in the reaction sequence, and is reversibly connected to it (Cleland, 1963c). Inhibitors may add to more than one enzyme form

but the inhibition should remain linear (observed on the replots) as the inhibitor concentration of the other normal enzyme forms are unaffected. — The greatest use of dead-end inhibitors is that they allow one to establish the order in a mechanism, for example consider the ordered bi bi sequence shown below with three possible points of inhibition.

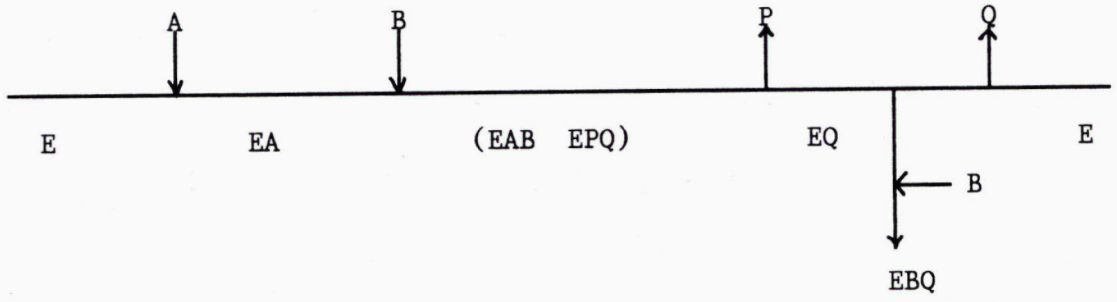


The dead-end inhibitor I is competitive with A and a noncompetitive inhibitor with respect to B. Inhibitor X is competitive with B and uncompetitive with A, and as X adds after A there is no slope effect. Inhibitor Y is uncompetitive with respect to both A and B. In the reverse direction, Y is competitive with P and uncompetitive with Q. I is competitive with Q and a mixed-type inhibitor with respect to P. In general all reciprocal plots in the presence of dead-end inhibitors are linear and all slope and intercepts versus inhibitor concentrations replots are linear (Cleland, 1963c). In calculating the rate equation for the reaction where a dead-end inhibitor binds with one enzyme form one needs to multiply certain terms or parts of terms in the denominator of the rate equation by the factor $(1 + I/K_i)$ where K_i is the dissociation constant for the complex containing the inhibitor. The terms multiplied by this factor are those appearing in the numerator of the distribution equation which describe the proportion of the

enzyme form with which the inhibitor reacts with (Cleland, 1963b; Cleland, 1963c).

V.E. Substrate inhibition

In general most cases of substrate inhibition result from the combination of a substrate with the wrong enzyme form, and are only apparent at high substrate concentrations. Substrate inhibition can be a very valuable diagnostic tool (Cleland, 1963c). To determine the nature of the substrate inhibition, the non-inhibitory substrate is varied at fixed levels of the inhibitory substrate, both less than and at the substrate-inhibition concentration. Changes in the slope, intercepts or both reveal the nature of the inhibition. Most substrate inhibition is linear, that is 1/v versus high concentrations of the substrate inhibitor is a linear function, corresponding to dead-end inhibition by the inhibitory substrate. In an example of dead-end inhibition consider an ordered bi-bi reaction were B reacts with EQ as well as EA, producing uncompetitive inhibition with respect to A. If B prevents the release of Q from EBQ then the inhibition is linear, whereas if it cannot totally prevent the release of Q then the inhibition is hyperbolic.



In this case therefore where B reacts with EQ in a dead end fashion as shown above. There is no rapid equilibrium counterpart as the level of EQ is negligible. Generally uncompetitive substrate inhibition by B can be recognized from a reciprocal plot of v versus [A]. In such a plot, at low concentrations of B, v progressively decreases (slope of 1/v increases). At the optimum concentration of B a minimum for 1/v is observed. As the concentration of B increases from the optimum there is a marked decrease in V_{max} seen as a rapid increase in the slope as 1/[B] approaches the $1/V_{max}$ axis. Calculations of the constants are obtained by extrapolation of the asymptote, and are thus only apparent constants.