GENETIC AND BIOCHEMICAL STUDIES WITH CHINESE HAMSTER OVARY CELL MUTANTS RESISTANT TO THE PURINE NUCLEOSIDE ANALOGS: TOYOCAMYCIN, FORMYCIN A AND FORMYCIN B

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TO MY PARENTS

ABSTRACT

The aim of this study was to investigate the mechanism of cellular resistance and toxicity to the purine nucleoside analogs toyocamycin, formycin A and formycin B by using genetic, biochemical and immunological approaches.

To investigate the similarity or differences in the mechanism of action of various pyrrolopyrimidine nucleosides, second-step toyocamycin resistant mutants (Toy^{rII} mutants) of Chinese hamster ovary cells were isolated from a cell line which exhibited similar degree of resistance to toyocamycin and tubercidin. These second-step mutants exhibited a further 8- to 9-fold increase in resistance to toyocamycin but no concurrent increase in their resistance towards tubercidin. The Toy^{rII} mutants were found to be very similar to the first-step mutants in their levels of adenosine kinase activity (< 1%), as well as cellular uptake and phosphorylation of adenosine and its analogs. The increased resistance of the Toy^{rII} mutants to toyocamycin but not to tubercidin provides strong evidence that the mechanism of cellular toxicity of these two analogs is different and suggests that these mutants may be affected in a cellular component which is specifically involved in the toxicity of toyocamycin. The Toy^{rII} mutants also exhibit increased resistance to sangivamycin and the tricyclic pentaazaacenaphthylene ribonucleoside, indicating that the mechanism of cellular toxicity of these two analogs may be similar to that of

toyocamycin.

The genetic and biochemical approach was also used to investigate the mechanism of resistance and metabolism to another group of nucleoside analogs in which the base is linked to ribose moiety by a C-C linkage. Studies presented showed that stable mutants which are approximately 3- and 8-fold resistant to the C-nucleoside, formycin A (Fom^R mutants) could be obtained in a single step in CHO cells. In cell extracts, the Fom^R mutants contained no measurable activity of the enzyme adenosine kinase. In cell hybrids formed between formycin A resistant and sensitive cells (Fom^S) as well as formycin A resistant and toyocamycin resistant cells (Toy^r), the drug resistant phenotype of Fom^R mutants behaved codominantly as indicated by the degree of resistance of the hybrid cells to formycin B. However, extracts from these hybrid cells contained either $\approx 50\%$ (Fom^R x Fom^S) or $< 1\%$ (Fom^R x Toy^r) AK activity, indicating that the lesion in these mutants neither suppresses the wild-type AK activity nor complements the AK deficiency of the Toy^r mutants. Cross-resistance studies with various adenosine analogs show that these mutants are distinct from the Toy^r mutants, which also contained no measurable AK activity in cell extracts. The Fom^R mutants exhibited a high degree of cross-resistance to different C-nucleosides but did not show appreciable cross-resistance to different N-nucleosides examined. In contrast, mutants selected in presence of toyocamycin exhibited a high degree of cross-resistance to both N- as well as C-nucleosides which are phosphorylated via adenosine kinase. Studies on the cellular uptake and phosphorylation of

radiolabelled N- and C-nucleosides by various mutant lines showed that, unlike the Toy^r mutants which show greatly reduced phosphorylation of all adenosine analogs (both N- and C-nucleosides), the Fom^R mutants showed reduced cellular phosphorylation of only C-nucleosides but not of N-nucleosides. The normal level of phosphorylation of N-nucleosides in the Fom^R mutants suggested that the Fom^R mutants contain normal levels of AK activity *in vivo*. The above observations together with the specific cross-resistance of these mutants to C-nucleosides as well as the reduced phosphorylation of such nucleosides, provides strong suggestive evidence that the Fom^R mutants contain a novel genetic lesion affecting adenosine kinase which specifically affects the phosphorylation of only C-purine nucleosides.

Since formycin A under the normal cell culture condition is rapidly deaminated to the inosine analog, formycin B, cellular toxicity and resistance of formycin B was also investigated. Mutants of CHO cells selected for resistance to formycin B (Fom^r mutants) were found to be 5- to 8-fold resistant to this drug. Cross-resistance studies with these mutants revealed that they exhibit increased resistance to all adenosine analogs (N- and C-nucleosides); as well as reduced cellular uptake and phosphorylation. However, unlike the Fom^R and Toy^r mutants, which contained no AK activity in their cell extracts, the Fom^r mutants were found to contain between 60 - 110% of WT activity in their cell extracts. The AK activity present in both Fom^r mutant cell extracts differed from the WT AK activity in terms of its specific activity as well as in its ability to phosphorylate adenosine analogs.

The AK activity from the Fom^r mutants was found to have less affinity for phosphorylation of the formycin A derivative, Bbb-85. Like Toy^r mutants, Fom^r mutants were found to show recessive behaviour in cell hybrids.

Biochemical studies on the metabolism of formycin B indicated that upon incubation with CHO cells, [^{3}H]formycin B is metabolized into formycin B-5'-monophosphate, formycin A-5'-monophosphate and higher phosphorylated derivatives of formycin A which are incorporated into RNA. All three different classes of mutants affected in AK exhibit appreciable cross-resistance as well as reduced cellular uptake and phosphorylation of formycin B. These observations strongly indicate that in CHO cells, formycin B is phosphorylated via AK and like other nucleoside analogs, its phosphorylation is essential for the cellular toxicity. Formycin B-5'-monophosphate and formycin A-5'-monophosphate have been found to inhibit the purine nucleotide biosynthetic enzyme adenylosuccinate synthetase.

To gain further insight into the nature of genetic and biochemical alterations in different types of mutants affected in adenosine kinase, this enzyme from CHO cells was purified to homogeneity. Antibodies which specifically cross-react with adenosine kinase have been raised. Immunoblot analyses using these antibodies showed that all three classes of mutants i.e., Toy^r, Fom^R and Fom^r contained nearly similar amounts of cross-reacting material that had a similar electrophoretic mobility to the enzyme in the WT cells. These results indicate that the lesion in these mutants does not involve a

deletion or regulatory type of genetic alteration in the AK gene nor a nonsense type of mutation which may cause premature chain termination. Instead, these mutants may contain a missense type of alteration in the structural gene of AK. Using these antibodies, regions (or spots) on two-dimensional gels that correspond to the AK protein have been identified. Comparison of the 2-D gel electrophoretic patterns of total cellular proteins from different mutant lines indicates that some of the mutants show a specific alteration in this region. This supports the inference that these mutants may contain a missense type of mutation in the structural gene of AK.

The results presented in this thesis have been presented or submitted in the following publications.

1. Mehta, K.D. and Gupta, R.S. (1983) Formycin B-Resistant Mutants of Chinese Hamster Ovary Cells: Novel Genetic and Biochemical Phenotype Affecting Adenosine Kinase. Mol. Cell. Biol. 3, 1468.
2. Gupta, R.S. and Mehta, K.D. (1984) Genetic and Biochemical Studies on Mutants of CHO Cells Resistant to 7-Deazapurine Nucleosides: Differences in the Mechanisms of Action of Toyocamycin and Tubercidin. Biochem. Biophys. Res. Commun. 120, 88.
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LIST OF ABBREVIATIONS

ACS	aqueous counting scintillant
ADA	adenosine deaminase
ADP	adenosine-5'-diphosphate
AK	adenosine kinase
AMP	adenosine-5'-monophosphate
APRT	adenine phosphoribosyltransferase
ASS	adenylosuccinate synthetase
ATP	adenosine-5'-triphosphate
Bbb-73	N^7-benzyl formycin A
Bbb-85	N^7-(Δ^2-isopentenyl) formycin A
BSA	bovine serum albumin
CHO	Chinese hamster ovary
CM-cellulose	carboxymethyl cellulose
CRM	cross-reacting material
2-D gels	two-dimensional gel electrophoresis
DEAE-cellulose	diethylaminoethyl cellulose
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EHNA	erythro-9-(2-hydroxyl-3-nonyl) adenine
EMS	ethyl methanesulfonate
FoA-MP	formycin A-5'-monophosphate
FoB-MP	formycin B-5'-monophosphate

GTP	guanosine-5'-triphosphate
HAT	hypoxanthine-aminopterin-thymidine
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
IEF	isoelectrofocussing
IMP	inosine-5'-monophosphate
KCl	potassium chloride
KOH	potassium hydroxide
6-MeAPR	6-methylaminopurine ribonucleoside
MEM	minimal essential medium
6-MeMPR	6-methylmercaptopurine ribonucleoside
$MgCl_2$	magnesium chloride
M_r	relative molecular mass
2-MSH	2-mercaptoethanol
NAD^+	nicotinamide-adenine dinucleotide
NH_4OH	ammonium hydroxide
P-100	100 mm diameter dish
PBS	phosphate buffer saline
PCA	perchloric acid
PEG	polyethylene glycol
PNP	purine nucleoside phosphorylase
POPOP	1,4-bis-2-(5-phenyloxazolyl)benzene
PPO	2,5-diphenyloxazole
n-PrOH	normal propanol
R_F	ratio of distance travelled by solute to that of solvent

RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
rRNA	ribosomal ribonucleic acid
tRNA	transfer ribonucleic acid
SDS	sodium dodecyl sulphate
TCN	3-amino-1,5-dihydro-5-methyl-1-β-D-ribo-furanosyl-1,4,5,6,8-pentaazaacenaphthylene
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris (hydroxymethyl) aminomethane
V	volts
WT cells	wild type cells

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1. INTRODUCTION

> "The scientific dreams and expectations of one generation become the techniques that later generations take for granted. It has always been so, and it should be no surprise that startling new techniques continue to appear, making possible work that was previously unimaginable.... Already these techniques have shed light on the mechanisms of gene control in prokaryotic and eukaryotic systems, and they promise to be used at increasing rates for the study of gene expression, for potential treatment of human disease, for agriculture, and all other fields of genetics."
>
> T.A. Friedman (1979)

In 1907 Harrison first demonstrated that cells of an animal can grow outside the body as single cells (Harrison, 1907). Since then, interests in using cell culture techniques to study various aspects of biology and medicine has been steadily increasing. The ability to culture mammalian cells for extended periods of time outside the body has led to the development of the field of somatic cell genetics. It has now become possible to isolate variant cell lines affected in specific cellular functions which are proving extremely useful to investigate fundamental questions in genetics and biochemistry. Though it is a young branch of science, somatic cell genetics provides a means for analyzing the complex genetic organization and regulation of higher eukaryotes (Puck and Kao, 1982; Ringertz and Savage, 1976; Ruddle, 1981a,b; Wright et al.,1980).

Somatic cell genetics has proven very useful in understanding the mechanism of action of many different drugs and inhibitors of cellular functions (Gupta, 1983c; Lewin, 1980; Wright et al., 1980;

Schimke, 1984a). This has become possible by isolating from established cell lines, mutants which are resistant to these drugs. Investigation of drug resistance has given new insights into the mode of action of various compounds, some of which are medically important from a chemotherapeutic point of view. In addition, this approach has created new possibilities for studying the genetic origin of resistance to clinically important drugs for the development of more rational chemotherapeutic combinations (Curt et al., 1984; Goldie and Coldman, 1984; Schimke, 1984a). Furthermore, mutants affected in specific cellular functions have provided a unique opportunity to study the structure, role and regulation of the affected function in mammalian cells. With the application of the somatic cell genetics approach to teratocarcinoma cells, it has now become possible to study the role of a mutated function on the growth and development of an intact animal. This has been accomplished by transplanting teratocarcinoma cells affected in a function into early mouse embryo, which leads to the development of a mouse deficient in that particular function (Dewey et al., 1977; Goldstein et al., 1979; Illmensee and Stevens, 1979; Watanabe et al., 1978). The selection systems for drug resistant mutants have also shown much promise in providing important applications in quantitative mutagenesis studies and for assessing human genetic risk due to environmental mutagenic agents (Gupta, 1984; Howard-Flander, 1981; Hsie et al., 1981; 1982). Many of the mutants obtained in cultured cells, such as those resulting from deficiencies of enzymes in purine and pyrimidine metabolism, have provided excellent

models with which to study human genetic diseases in cell culture (Martin and Gelfand, 1981; Osborne, 1981). Furthermore, resistant cell lines overproducing specific mRNAs and proteins serve as models for investigating the mechanisms of gene amplification as they operate in evolution (Schimke, 1980; 1982; 1984b; Stark and Wahl, 1984).

Advances in somatic cell genetics have also made possible the mapping of different genes to specific human (or other species) chromosomes or their parts. The chromosomal assignment of genes is usually done by correlating a particular donor gene or its product with a specific donor chromosome or subchromosomal fragment. The transfer of genetic information between two cells can be accomplished by a number of different methods including, somatic cell hybridization, micro cell-mediated transfer, or DNA-mediated gene transfer. By selecting a particular gene transfer system, the amount and size of donor material transferred to the recipient cell can be controlled. By using these techniques, hundreds of human genes have been mapped, including those which produce various genetic diseases (Klobutcher and Ruddle, 1981; McKusick and Ruddle, 1977; Medrano and Dutrillaux, 1984; Ruddle, 1981a). Increased knowledge of the human gene map is providing an understanding about how genes function individually and as coordinated sets in man. Such information is essential for defining all aspects of normal and abnormal human biology and development (Shows et al., 1982).

The combination of gene transfer and recombinant DNA technology has provided a quantum leap in the powers of somatic cell genetics.

Mammalian gene mapping techniques are now sufficiently advanced to contribute significantly to prenatal diagnosis and to human molecular genetics (Ruddle, 1981b). For example, restriction fragment mapping has been used to find the polymorphic genetic markers at random sites within the genome, and these sites can be used to assign genes responsible for a disease condition to a specific chromosomal region (Gusella et al., 1980; Kan and Dozy, 1978; Woods et al., 1980). The isolation and chromosomal assignment of polymorphic fragments of DNA promises to be a powerful tool in analyzing the Mendelian inheritance of linked genetic loci involved in inherited diseases (Bostein, 1980). Furthermore, combination of DNA-mediated gene transfer and recombinant DNA technology has already lead to the isolation and characterization of cellular "oncogenes" which has led to a better understanding of the genetic nature of neoplasia and to a unified concept of chemical and viral carcinogenesis (Cooper, 1982; Weinberg, 1982).

Finally, somatic cell genetics has great potential in several areas of biology including possible therapy of genetic diseases, in producing therapeutic agents, in food production, and in energy production and industrial processes (Shows and Sakaguchi, 1980).

1.1 Somatic Cell Lines

The advantages of the somatics cell genetics approach lie in the use of established cell lines. There are a number of benefits stemming from the use of established cell lines (Puck and Kao, 1982; Siminovitch, 1976; Thompson, 1979; Wright et al., 1980). First, since individual cells divide by asexual reproduction and form discrete

colonies, a genetically uniform population can be obtained and eventually grown in large batch cultures. Secondly, the composition of the medium can often be manipulated by the investigator so that the cellular phenotype can be observed under a variety of environmental conditions. The ability to grow various cell lines in completely defined growth medium (Hamilton and Ham, 1981) is expected to further enhance the potential of somatic cell genetics. Thirdly, the generation time of cultured cells is usually between 12 to 24 hours which is much shorter ($\approx 10^4$-fold) than the generation time of a whole organism, such as man. As a result many more experiments can be performed with cells in culture than with the animals from which they were obtained. Furthermore, the genetic properties of a particular cell line are not masked by the presence of other types of somatic cells, as can be the case in a multicellular organism like man which contains approximately 10^{13} cells (Wright et al., 1980).

During the past two decades, a large number of cell lines from many different sources have been establised. Table I presents a partial list of these cell lines. In addition, there are differentiated cell lines representing a number of tissue types as well as established lines from persons with different types of genetic defects (Augusti-Tocco et al., 1969; Weiss, 1982; Yaffe, 1968).

Among the established cell lines, Chinese hamster ovary (CHO) cells have been extensively used for genetic studies (Siminovitch, 1976; Thompson, 1979; Tijo and Puck, 1958). This cell line exhibits a number of favourable properties which have led to its wide spread use

Table I. List of Some of the Established Somatic Cell Lines

Name	Organism and tissue of origin	Chromosome complement of species (2n)	Current Mode and range of chromosome number in cell line
HeLa	human cervical carcinoma (fibroblast)	46	heteroploid ~71 (38-106)
L	mouse connective tissue (fibroblast)	40	heteroploid ~70 (40-115)
3T3	mouse embryo (fibroblast)	40	heteroploid ~76 (70-80)
S49	mouse lymphoma	40	pseudo diploid (39-41)
Friend	mouse erythroleukemia	40	pseudo diploid ~39 (35-43)
CHO	Chinese hamster ovary (fibroblast)	22	pseudo diploid ~21 (20-22)
V79	Chinese hamster lung (fibroblast)	22	pseudo tetraploid
BHK 21	Syrian hamster kidney (fibroblast)	44	pseudo diploid ~44 (42-45)
BSC 1	African green monkey kidney	60	pseudo tetraploid ~115
RAG	mouse renal adenocarcinoma (epithelioid)	40	heteroploid ~74
S2	Drosophila melanogaster embryo (epithelial)	4	diploid

Table I was taken from Lewin (1980).

for genetic studies: (i) the karyotype of CHO cells appears to be stable from clone to clone in different cells within a clone, and upon repeated subcloning, which ensures that any mutants that are isolated can be compared directly with the parental cell line; (ii) CHO cells can be grown under a variety of culture conditions such as in suspension culture, in soft agar, on top of soft agar, as well as in monolayer; (iii) the CHO cells have very high plating efficiency (nearly 100%) which is an important property for various genetic and biochemical studies; (iv) the short generation time of CHO cells (approximately 12 hours) make it possible to obtain large cell populations in a short time; (v) a large number of autosomal recessive mutants have been isolated in this cell line indicating that these cells may be functionally hemizygous at a number of genetic loci of interest.

1.2 Types of Somatic Cell Mutants

During the past 10-15 years, a large variety of mutants exhibiting altered stable phenotype have been isolated in CHO cells. These mutants can be divided into two main classes, auxotrophic or conditional-lethal mutants (Kao and Puck, 1972; Patterson, 1976; Puck and Kao, 1967; Thompson et al., 1970; 1971), and mutants resistant to various cytotoxic agents including drugs and inhibitors (Gottesman, 1980; Siminovitch, 1976; Stanley, 1980; Thompson, 1979; Thompson and Baker, 1973; Wright, 1979; Wright et al., 1980).

As a general class, the drug resistant mutants have been studied more often than any of the others because of the wide variety

of selective agents available and the ease with which specific mutants can be obtained. Since the usefulness of the drug resistant mutants is the main focus of the thesis, it will therefore receive the most emphasis.

1.3 Selection of Drug Resistant Mutants

Drug resistant mutants can in principle be obtained to any compound that is cytotoxic to cells and for isolation of drug resistant mutants, two types of procedures have been employed. These are a single-step protocol or a multi-step protocol. In single-step protocols, acquisition of resistance is determined following a single exposure to a given toxic concentration of the drug (Chu and Powell, 1977; Lewin, 1980; Thompson, 1979; Thompson and Baker, 1973). Depending upon the nature of genetic alterations, a series of resistant lines may be obtained having varying levels of resistance. Sometimes it is difficult to acquire a high level of resistance in a single step, in such circumstances, discrete multiple single steps can be used (Gupta, 1981). The Luria-Delbruk fluctuation test (Luria and Delbruk, 1943) suggests that the single-step protocol selects mutants already present in the population and excludes the possibility that the selective agents has induced the variants. In the multi-step protocol, the parental line is exposed to gradually increasing concentrations of the selective agent for as long as may be necessary to obtain the desired level of resistance. Most of the time, variants obtained by this protocol revert back to parental phenotype in the absence of selection pressure and are thus not mutants in the true sense. Such

variants can contain multiple single mutations and a large number of variants selected by this protocol have been found to involve gene amplification leading to increased synthesis of the target protein which is inhibited by the selective agents (Schimke, 1982).

1.4 Mutation and Epigenetic Variation

Since the first isolation of drug-resistant mutants, a great deal of effort has gone into elucidating the actual genetic mechanisms underlying altered phenotypes (Caskey and Kruh, 1979; De Mars, 1974; Lewin, 1980; Siminovitch, 1976). Before variant cells isolated in culture can be used as a model system for studying basic genetic problems, it is necessary to establish that the altered phenotype results from a genetic event and is not due to epigenetic changes. Therefore, much attention has been focussed on determining whether the altered phenotypes observed in cell culture were due to changes in DNA (mutations) or were the result of stable directed shifts in genetic expression (epigenetic events).

In the past, a number of criteria have been used to assess the mutational origin of the phenotypic variation in cultured mammalian cells (Chasin, 1979; Davidson, 1979). These criteria are: (a) low spontaneous frequencies of forward and reverse mutation; (b) the frequency of mutant appearance is induced by mutagen treatment; (c) the mutant phenotype should breed true and it should be stably transmitted under non-selective conditions; (d) alteration or deficiency of specific protein is demonstrable; (e) mutation can be assigned to certain chromosome or to a specific chromosome region; (f) an altered

gene can be demonstrable at the DNA level. During the past decade, abundant evidence has accumulated suggesting that a large number of mutants isolated in culture satisfy the majority of the above criteria, thus confirming their genetic origin. However, it is important to mention that the non-mutational mechanism of "gene inactivation" or "gene silence" is still a viable concept and some of the variants may be originating by this mechanism (Bradley, 1979; 1983; Bradley and Letovanec, 1982; Milman et al., 1976; Morrow, 1977; Turker et al., 1984).

1.5 Mechanisms of Drug Resistance in Mammalian Cells

Mutant cell lines obtained for resistance to various drugs exhibited resistance due to one of the following mechanisms:

(a) decreased cellular uptake of the drug

(b) lack of conversion of drug into a cytotoxic form (lethal synthesis)

(c) increased metabolism of the drug or its conversion to an inactive form

(d) altered intracellular nucleotide pools

(e) an increase in the level of the cellular target for the drug

(f) alteration in the target site.

A few selected examples of each of these mechanisms are summarized in Table II. As can be seen, there are various mechanisms available for cells to develop resistance through mutation in DNA.

1.6 Antimetabolites

The term antimetabolites refers to a group of natural and synthetic substances, with very heterogeneous chemical structures and

Table II. Mechanisms of Resistance to Cytotoxic Drugs

Mechanism	Drug	Specific alteration
Defective transport	Adenosine	Decreased cellular uptake (Cohen et al., 1979)
	Cytarabine	Decreased membrane nucleoside binding sites (Wiley et al., 1982)
	VM-26	Increased efflux (Gupta, 1983b; Lee and Roberts, 1984)
Defect in the synthesis of lethal metabolite(s)	Adenosine	Alteration in adenosine kinase (McBurney and Whitmore, 1974a)
	8-Azaadenine 2,6-Diaminopurine	Decreased adenine phosphoribosyltransferase (Jones and Sargent, 1974; Taylor et al., 1977)
	5-Azacytidine	Decreased uridine-cytidine kinase activity (Vesely et al., 1967)
	Cytarabine	Decreased deoxycytidine kinase activity (Tattersall et.al., 1974)
	5-Fluorouracil	Decreased uridine kinase activity (Reyes and Hall, 1969)
	6-Mercaptopurine 6-Thioguanine	Decreased hypoxanthine-guanine phosphoribosyltransferase activity (Brockman, 1963; Caskey and Kruh, 1979)
	Toyocamycin Tubercidin Pyrazofurin	Lack of adenosine kinase activity (Chan and Juranka, 1981; Dix et al., 1979;

Mechanism	Drug	Specific alteration
	9-β-D-arabino-furanosyl adenine 6-MeMPR	Gupta and Siminovitch, 1978b; Gupta and Singh, 1983; Rabin and Gottesman, 1979; Thacker, 1980)
Increased drug inactivation	Bleomycin	Increased bleomycin-hydrolase activity (Akiyama et al., 1981)
	Cytarabine	Increased cytidine deaminase activity (Stewart and Burke, 1971)
	6-Mercaptopurine 6-Thioguanine	Increased membrane alkaline phosphatase (Lee et al., 1978; Scholar and Calabresi, 1979)
	Cisplatin	Increased intracellular metallothionein (Bakka et al., 1981)
Altered nucleotide pools	Cytarabine	Increased intracellular CTP and dCTP pools (Desaintvincent and Butten, 1979)
Gene amplification	Azauridine or Pyrazofurin	Increase in UMP synthetase (Suttle, 1983)
	5-Fluorodeoxyuridine	Thymidylate synthetase amplified (Rossana et al., 1982)
	Doxorubicin Vinca alkaloids Colchicine	Increase in membrane glycoproteins (Biedler, 1981; Ling 1982; Robertson et al., 1984)
	Methotrexate	Increase in dihydrofolate reductase activity (Flintoff, 1976; Littlefield, 1969; Schimke, 1982)

Mechanism	Drug	Specific alteration
	Mycophenolic acid	Inosine-5'-monophosphate dehydrogenase amplified (Huberman et al., 1981)
	PALA	Increase in carbamyl-P-synthetase, aspartate transcarbamylase and dihydroorotase (Kempe et al., 1976)
Altered targets	Allyl alcohol	Altered alcohol dehydrogenase (Thirion and Talbot, 1978)
	α-Amanitin	RNA polymerase II becomes resistant (Chan et al., 1972; Ingles et al., 1976)
	Colcemid	Tubulin (Ling et al., 1979; Keates, 1981)
	Diphtheria toxin	Failure of elongation factor -2 to become ADP-ribosylated (Moehring and Moehring, 1977; Gupta and Siminovitch, 1978a)
	Emetine	Altered 40S ribosomal subunit (Gupta and Siminovitch, 1976; 1977)
	Methotrexate	Altered dihydrofolate reductase (Flintoff et al., 1976; Gupta et al., 1977; Melera et al., 1980)
	Ouabain	Altered Na^+/K^+ ATPase (Baker et al., 1974; Lever and Seegmiller, 1976)
	Podophyllotoxin	Alteration in microtubule associated protein (Gupta et al., 1982; Gupta and Gupta, 1984)

mechanisms of action, but having in common the fact that their inhibitory effects can be antagonized by one or more metabolites. Generally, but not always, their chemical structures are analogous to those of the antagonistic metabolites.

The antimetabolites can be divided into two large groups on the basis of their mechanisms of action:

I. those that are incorporated into "informational" polymers (DNA, RNA and proteins) in place of natural monomers and change the information content; and

II. those that inhibit the formation of essential metabolites.

1.6.1 Nucleoside Analogs

Among the various antimetabolites of group I, nucleoside analogs represent a diverse group of biological compounds structurally related to the purine and pyrimidine nucleosides and/or their nucleotides found in the cell. Nucleoside analogs have proven very useful as important biochemical tools in studying a variety of problems such as, mechanisms of protein, RNA and DNA synthesis, regulation of purine and pyrimidine nucleotide synthesis, mechanisms of enzymatic reactions, subcellular organizations, etc. (Daves and Cheng, 1976; Suhadolnik, 1970; 1979).

Although purine nucleoside and nucleotide analogs have been known for a long time, it is only relatively recently that they have been considered to be useful as antiparasitic, antitumor and antiviral agents (Daly, 1982; Daves and Cheng, 1976; De Clercq, 1984; Fox et al., 1983; Sidwell, 1979; Suhadolnik, 1979).

1.6.2 Classification of Nucleoside Analogs

To date, about seventy naturally occurring nucleoside analogs have been isolated and they can be classified on the basis of their structures into five groups: (i) purine nucleosides or nucleotides analogs; (ii) pyrimidine nucleosides analogs; (iii) pyridine nucleosides analogs; (iv) diazepine nucleosides analogs; and (v) maleimide nucleosides analogs (Suhadolnik, 1981). There are thirty nine purine-like, twenty six pyrimidine-like, two with the pyridine aglycon, two with the diazepine ring and only one with the maleimide aglycon.

Naturally occurring purine nucleoside analogs can be broadly divided based on ribosidic linkage into two groups, N- and C-nucleosides. In N-nucleosides, like adenosine the base is joined to a sugar moiety by a nitrogen-carbon bond, but on the otherhand, in C-nucleosides there is a carbon-carbon bond. The pyrrolopyrimidine nucleosides and pyrazolopyrimidine nucleosides are examples of N-nucleosides and C-nucleosides, respectively. Because of the longer carbon-carbon glycosidic bond (1.55 angstrom) in comparison to carbon-nitrogen bond (1.47 angstrom), the energy barrier for rotation is low for C-nucleosides and as a result C-nucleosides can exist in syn-anti equilibrium rather than anti as is the case for adenosine (Prusiner et al., 1973; Ward and Reich, 1968).

Since the work described in this thesis is concerned with the mechanism of action and cellular resistance of pyrrolopyrimidine and pyrazolopyrimidine nucleosides, the earlier work on these purine

nucleoside analogs is reviewed below in somewhat greater detail.

1.6.3 Pyrrolopyrimidine Nucleosides

The structurally related pyrrolopyrimidine nucleosides, tubercidin, toyocamycin and sangivamycin represents an important class of nucleoside antibiotics which possess useful antibacterial, antifungal, antiviral as well as antiparasitic activities (Jaffe, 1975; Kouni et al., 1983; Suhadolnik, 1970; 1979). Compounds of this group are highly cytotoxic to mammalian cells in culture and have been shown to be effective against a variety of experimental tumors such as sarcoma 180, Ehrlich ascites tumor, Jensen carcinoma and also against nodular basal cell carcinomas (Klein et al., 1975; Owen and Smith, 1964).

These three pyrrolopyrimidine nucleosides have been isolated from thirteen Streptomyces cultures in independent laboratories (Anzai and Marumo, 1957; Hingashide et al., 1966; Nishimura et al., 1956; Rao et al., 1969). Structures of these three nucleosides have been elucidated and their total chemical syntheses have been reported (Suzuki and Marumo, 1961; Taylor and Hendess, 1965; Tolman et al., 1969). The chemical structures of tubercidin, toyocamycin and sangivamycin (Fig. 1) are 4-amino-7-(β-D-ribofuranosyl) pyrrolo [2,3-d]pyrimidine, 4-amino-5-cyano-7-(β-D-ribofuranosyl) pyrrolo [2,3-d]pyrimidine, and 4-amino-5-carboxamido-7 β-D-ribofuranosyl)pyrrolo [2,3-d]pyrimidine, respectively.

Pyrrolopyrimidine nucleosides are 7-deaza derivatives of adenosine and are commonly referred as adenosine analogs. Because of

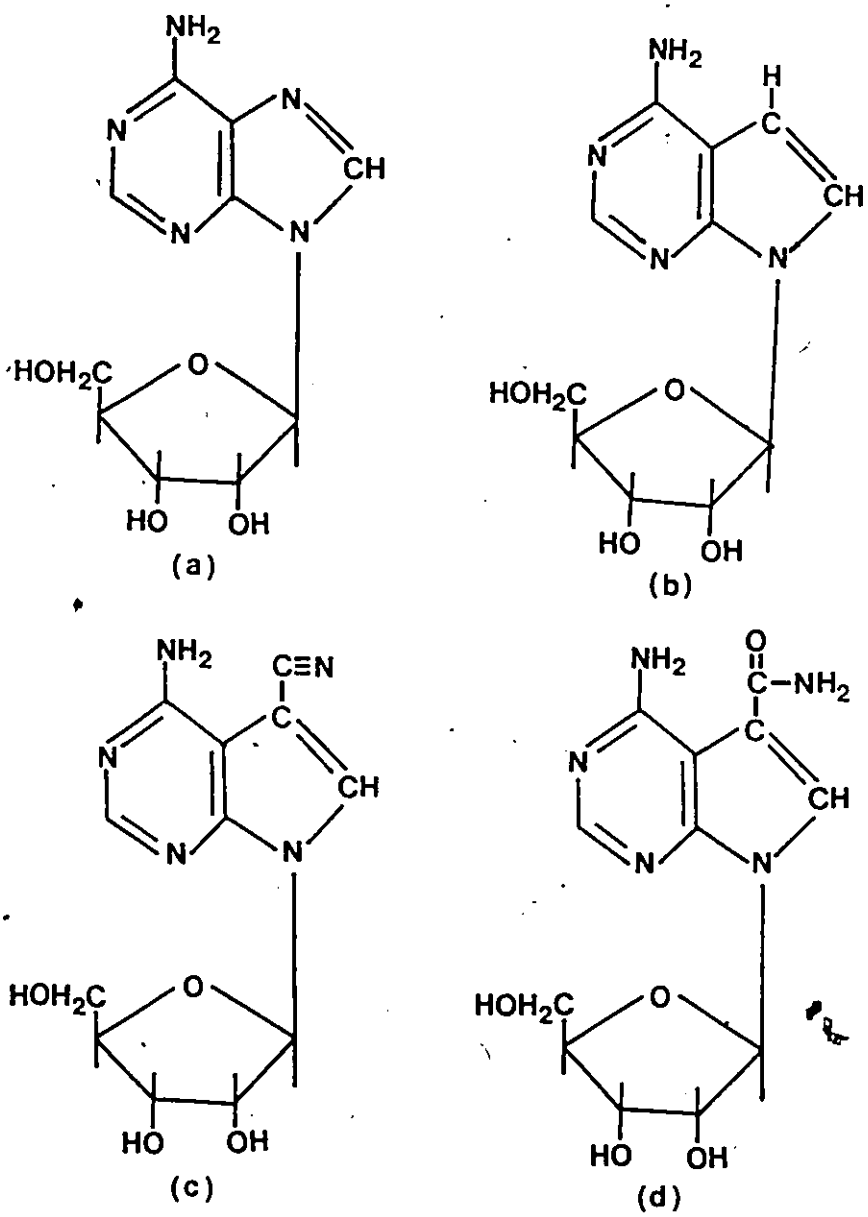


Figure 1: Structural formulae of the adenosine and the pyrrolopyrimidine nucleosides. (a) Adenosine, (b) Tubercidin, (c) Toyocamycin, (d) Sangivamycin.

their close structural relationship to adenosine, they are excellent substrates for adenosine kinase (Schnebli et al., 1967; Miller et al., 1979b), but are not subject to phosphorolysis or deamination. After phosphorylation, their phosphorylated derivatives can take the place of, or interfere with the metabolism of adenosine-5'-monophosphate (AMP), adenosine-5'-diphosphate (ADP) and adenosine-5'-triphosphate (ATP), in a wide variety of cellular reactions.

Tubercidin undergoes intracellular phosphorylation by adenosine kinase and is readily converted to the nucleotide form. The nucleotide thus formed from tubercidin has been shown to be incorporated into RNA and DNA of both bacterial as well as mammalian cells (Acs et al., 1964; Bloch et al., 1967). Tubercidin inhibits proteins and nucleic acid synthesis (Acs and Reich, 1967; Acs et al., 1964), ribosomal RNA (rRNA) processing (Suhadolnik, 1970) as well as causes visible nuclear damage (Bassleer et al., 1976). The other pleiotropic effects of tubercidin have appeared in studies in which tubercidin replaces the adenosine moiety of nicotinamide adenine dinucleotide (NAD^+); Baxter and Bovoet, 1974; Bloch et al., 1967), S-adenosylmethionine (Wainfan and Landsberg, 1973), S-adenosylhomocysteine (Chang and Coward, 1975) and cyclic AMP (Blecher et al., 1971; Walter, 1976). Bloch et al. (1967) found that glucose utilization in bacteria was blocked by tubercidin and inhibition of growth in bacterial cells can be prevented by addition of purine and pyrimidine nucleosides, ribose-5'-phosphate, pyruvate and certain amino acids. They have correlated these effects with the synthesis of a tubercidin derivative of NAD^+. Tubercidin is also known

to inhibit mitochondrial respiration in Ehrlich-Lettré tumor cells (Miko and Drobnica, 1975). The substitution of tubercidin for adenosine in S-adenosylhomocysteine has been reported to yield a potent inhibitor of transfer RNA (tRNA) methylase (Borek and Kerr, 1972; Coward et al., 1974; 1977; Wainfan and Landsberg, 1973), catechol-o-methyltransferase, indolemethylamine N-methyltransferase (Coward et al., 1974), methylation of tRNA in lymphocytes (Chang and Coward, 1975), methylation of nuclear RNA in L1210 cells (Stern and Glazer, 1980) and polyamine biosynthesis (Coward et al., 1977).

Toyocamycin is known to be converted to its 5'-triphosphate and incorporated into the RNA of Ehrlich ascites tumor cells (Suhadolnik et al., 1967). A more complete study showed that its incorporation into the RNA of mouse L cells selectively inhibits rRNA synthesis (Tavitian et al., 1968; 1969). Studies on the effect of toyocamycin on rRNA maturation in cultured Novikoff hepatoma cells has shown that the processing of 45S to the 38S RNA is not inhibited, however, the formation of mature 28S and 18S RNA is inhibited (Hadjiolova et al., 1981; Weiss and Ritot, 1974). The inhibition of rRNA maturation by tubercidin and toyocamycin suggests a possible mode of their antineoplastic activity. Toyocamycin has been reported to interfere with the metabolism of RNA by preventing polyadenylation and/or methylation of adenosine residues (Swart and Hodge, 1978).

Sangivamycin is one of the few nucleoside analogs that have been selected for clinical studies because it has strong antileukemic activity (Cairns et al., 1967). This adenosine analog undergoes

intracellular phosphorylation by adenosine kinase (Hardesty et al., 1969; 1974; Miller et al., 1979b) and is incorporated into RNA and DNA (Hardesty et al., 1974). Sangivamycin has also been shown to inhibit de novo purine synthesis (Bennett et al., 1978), inhibits RNA synthesis (Suhadolnik et al., 1968), and interferes with amino acid activation by reduction of tRNA acceptor activity (Uretsky, 1968). In addition, trancription of several species of messenger RNA (mRNA) in L1210 cells is inhibited by sangivamycin (Glazer and Peale, 1980). Recently, sangivamycin was found to be selectively incorporated into poly-adenylated RNA (Ritch et al., 1981; 1982a; 1982b) and the modified mRNA showed a diminished translational capacity (Glazer and Hartman, 1983).

1.6.4 Pyrazolopyrimidine Nucleosides

The pyrazolopyrimidine nucleosides include formycin A, formycin B and oxoformycin B which have been isolated from culture filtrates of Nocardia interforma species (Hori et al., 1964), Streptomyces levendulae (Aizawa et al., 1965), and Streptomyces gummaenis (Nakayama and Kunishima, 1968). Formycin A and formycin B possess very useful biochemical and medicinal properties including antiviral, anticancer antileishmanial and immunosuppressive activity (Carson and Chang, 1981; Ishida et al., 1967; Ishizuka, 1968; Kunimoto et al., 1968; Spermulli et al., 1983; Willemot, 1979). Their structures were established through degradation studies, by UV and NMR spectral interpretations, and by X-ray crystallography (Koyama et al., 1966; Robins et al., 1966). The chemical structures of formycin A and formycin B (see Fig. 2) are 7-amino-3-(β-D-ribofuranosyl) pyrazolo [4,3-d]pyrimidine (or

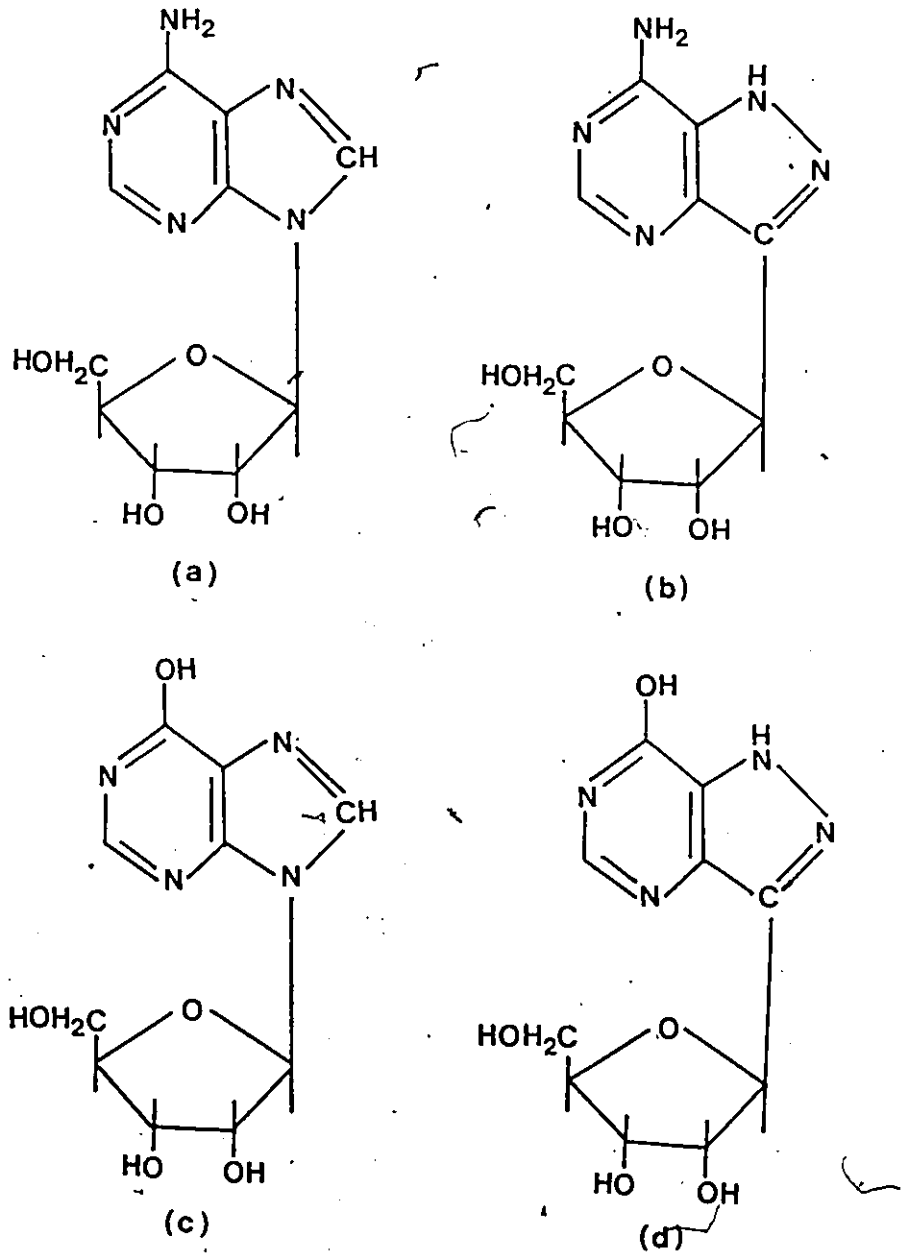


Figure 2: Structural formulae of the purine nucleosides and the pyrazolopyrimidine nucleosides. (a) Adenosine, (b) Formycin A, (c) Inosine, (d) Formycin B.

8-aza-9-deazaadenosine), and 3-(β-D-ribofuranosyl) pyrazolo [4,3-d]6(H)-7-pyrimidine (or 8-aza-9-deazainosine), respectively. Oxoformycin is an oxidized product of formycin B (Sheen et al., 1970). Formycin A and formycin B closely resemble adenosine and inosine respectively in structure and are commonly referred as adenosine and inosine analogs. In them, the N-atom at position 9 and C-atom at position 8 of adenosine and inosine, respectively, are interchanged resulting in a C-C glycosyl bond, unlike adenosine which has a N-C glycosyl bond. As a result of this structural difference, they are hydrolytically stable and therefore a potentially important pathway for the catabolism of nucleosides or nucleotides, i.e. hydrolysis of the base from the sugar, does not affect these compounds (Snyder and Henderson, 1973). In addition, this structural change permits them to exist in an equilibrium of syn-anti forms, which differs from adenosine that exists in anti conformation only (Ward and Reich, 1968).

Formycin A has been shown to be phosphorylated enzymatically to the 5'-mono-, di- and triphosphates and deaminated to formycin B in mammalian cells (Umezawa et al., 1967). Formycin A-5'-triphosphate has been shown to be incorporated into RNA and is a good substrate for RNA polymerases from different sources (Asano et al., 1971). It is also a substrate for aminoacyl-tRNA synthetases and tRNA-CCA pyrophosphorylase (Majima et al., 1977) and interact with pyruvate carboxylase (Attwood et al., 1984). Haar and Cramer (1976) have shown that the tRNA in which the 3'-terminal adenosine is replaced with formycin A, accepts the amino acid from the amino acid-tRNA complex to the nonaccepting

adenosyl hydroxyl (i.e., the 3'-hydroxyl) and amino acid occupies a position that is not accessible to the groups on the synthetase to make corrections for misactivation. Detailed studies on the effect of formycin A on RNA synthesis in posterior silk glands of Bombyx mori have suggested that formycin A blocks tRNA synthesis and this has been attributed to the failure of processing of formycin A-containing 4.5S precursor RNAs into normal 4S tRNA (Majima et al., 1977). However, recently the incorporation of formycin A into DNA has been shown to correlate closely with its lethal effects on mammalian cell viability (Glazer and Lloyd, 1982). Formycin A has also been shown to replace the adenosine moiety in NAD^+ (Suhadolnik et al., 1977) as well as convert into cyclic AMP derivatives in mammalian cells (Rossomando et al., 1981).

Earlier biochemical studies with formycin B have suggested that unlike formycin A and other adenosine analogs, formycin B, which is an inosine analog, is not phosphorylated in mammalian cells (Müller et al., 1974; Umezawa et al., 1967). However, formycin B has been reported to be a potent competitive inhibitor of purified human purine nucleoside phosphorylase (PNP) (Cowan et al., 1981) and aldehyde oxidase (Sheen et al., 1970). Cowan et al. (1981) have shown a close relationship between formycin B inhibition of PNP and decrease in DNA synthesis in phytohemagglutinin stimulated human peripheral blood lymphocytes. On the contrary, Osborne et al., (1980) have reported earlier that inhibition of PNP is a secondary effect of formycin B in human lymphocytes and lymphoblastoid cell lines but the primary site of

action was not identified in these studies. Formycin B has also been reported to be a competitive inhibitor of NAD^+ in reactions catalyzed by purified chromatin bound and soluble polyadenosine diphosphoribose polymerase. The nicotinamide derivative of formycin B has been shown to be equally effective in replacing NAD^+ as an inhibitor of DNA synthesis. The ability of adenosine or NAD^+ to reverse the inhibition of exponentially growing mouse L cells by formycin B has been interpreted to indicate that the inhibition of cellular poly (ADP-ribosylation) is more sensitive to formycin B than are DNA, RNA and protein synthesis (Müller et al., 1974). Recently several groups of investigators have shown that formycin B is phosphorylated to its 5'-monophosphate derivative which is subsequently converted into formycin A nucleotides in various Leishmania species (Carson and Chang, 1981; Nelson et al., 1982; Rainey and Santi, 1983), in human macrophages (Berman et al., 1983) and in mouse L cells (Lafon et al., 1983; Spector et al., 1984). The incorporation of formycin A nucleotide into RNA has been suggested as a possible mechanism for the toxicity of the drug in Leishmania (Nelson et al., 1982; Rainey and Santi, 1983).

1.7 Application of Somatic Cell Genetics to Investigate the Mechanism of Action of Purine Nucleoside Analogs

As mentioned earlier, purine nucleoside analogs being structurally similar to the normal purine nucleosides uses the same transport system as normal nucleosides to cross the cell membrane (Cohen and Martin, 1977; Sirotnak et al., 1979; Müller, 1979).

Furthermore, because of structural similarity, purine nucleoside analogs or their base analogs are readily converted to toxic metabolite(s) by the cellular salvage enzymes. The first step in their toxic conversion involves either phosphorylation by adenosine kinase (AK) or phosphoribosylation by hypoxanthine-guanine phosphoribosyltransferase (HGPRT) (Fig. 3). Their phosphorylated derivatives follow the same metabolic routes as the normal purine nucleosides, and as a result, they can take the place of normal nucleotides or interfere with their metabolism. For most of the nucleoside analogs it is not clear whether the cytotoxicity of the above drugs is due to inhibition of, or interference with one particular reaction, or whether it is a cumulative effect of interference with a large number of cellular reactions (Smith et al., 1980). In the past, the major problem encountered in determining their mechanism of action was to distinguish the toxic reaction from other cellular reactions in which the toxic metabolite participates but does not lead to cellular toxicity.

The identification of the crucial step required for the drug to cause cellular toxicity can be made by using a combination of genetic and biochemical techniques. The basic scheme used in this approach is summarized in Fig. 4. If mutants are selected for resistance to purine nucleoside analogs in culture, resistance in mutant lines can develop due to one of the following mechanisms.

1. Defect in the nucleoside transport system

One of the mechansims of cellular resistance is the reduced

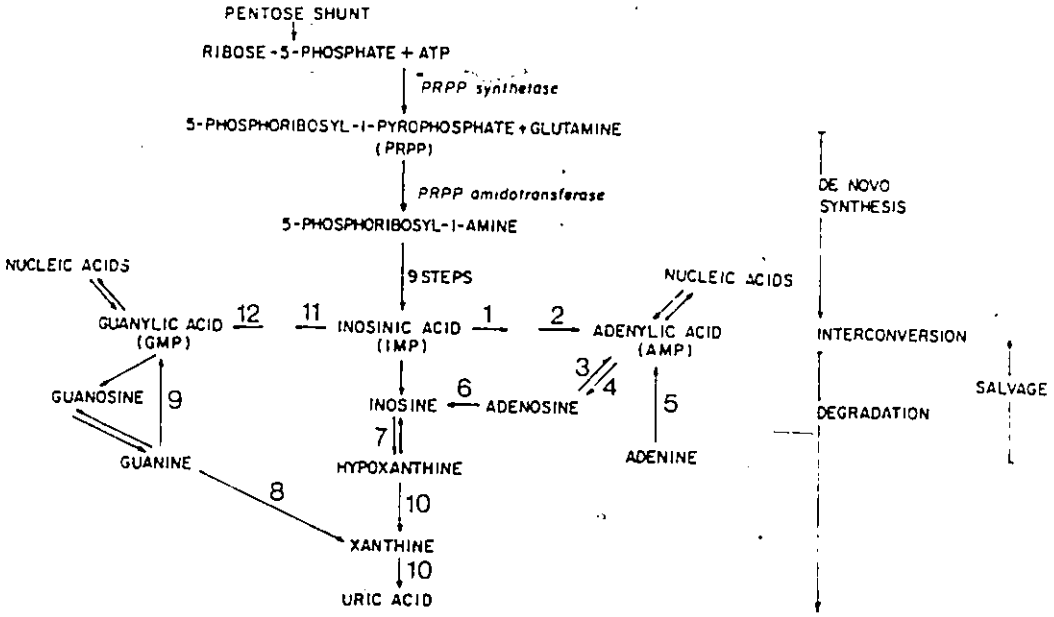


Figure 3: Pathways of purine metabolism in mammalian cells: the enzymes responsible for catalysing different reactions are: 1) adenylosuccinate synthetase, 2) adenylosuccinate lyase, 3) adenosine kinase, 4) 5'-nucleotidase, 5) adenine phosphoribosyl-transferase, 6) adenosine deaminase, 7) purine nucleoside phosphorylase, 8) guanine deaminase, 9) hypoxanthine-guanine phosphoribosyltransferase, 10) xanthine oxidase, 11) inosine-5'-monophosphate dehydrogenase, 12) guanosine-5'-monophosphate synthetase. This figure was modified from Sperling et al., 1977.

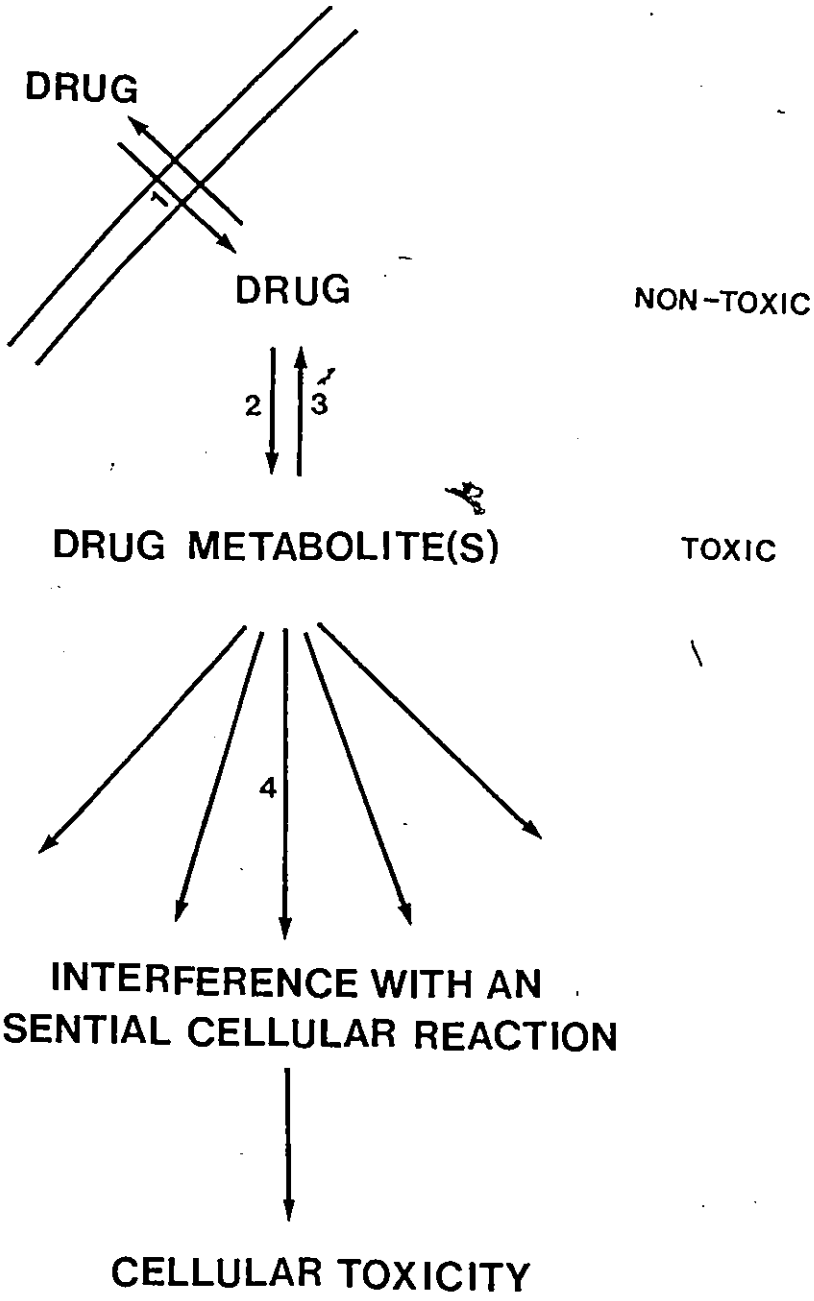


Figure 4: Mechanisms of resistance to purine nucleoside analogs in mammalian cells. Cellular resistance can develop due to one of the following mechanisms: 1) defect in the nucleoside analog transport, 2) alteration in the metabolic activation step, 3) increased inactivation of the toxic metabolite into nontoxic form, or 4) alteration in the target site.

permeability of the drug. Recently, Cohen et al. (1979) have described adenosine resistant mutants affected in the nucleoside transport system and have demonstrated that their resistance is due to a decrease in the permeability of these mutants to adenosine. Such mutants exhibit increased resistance to a variety of nucleosides and their analogs. However, this is not a very common mechanism leading to resistance towards nucleoside analogs.

2. Alteration in the metabolic activation step

As mentioned earlier, nucleoside analogs exert their toxicity only after their conversion to the phosphorylated derivatives by the appropriate phosphorylating enzyme. These enzymes are mainly salvage pathway enzymes, which are not essential for growth of cells under normal growth conditions. Due to this, the most likely mechanism for development of resistance to purine analogs is due to alteration in the biochemical step leading to their toxic conversion. The alteration may involve either partial or complete loss of activity of the activation enzyme or alteration in its substrate affinity.

One of the major purine phosphorylating activities in mammalian cells is the enzyme adenosine kinase which catalyzes the transfer of phosphate group from ATP to adenosine in cells and is also known to phosphorylate various adenosine analogs such as toyocamycin or tubercidin. Chinese hamster cells selected for resistance to toyocamycin or tubercidin usually contain no measurable activity of the enzyme AK, indicating that cellular resistance is due to the lack of conversion of these analogs to phosphorylated forms (Gupta and

Siminovitch, 1978b; Rabin and Gottesman, 1979).

There are two purine phosphoribosyltransferase activities in mammalian cells. One enzyme HGPRT, phosphoribosylate hypoxanthine, guanine or their analogs while the other enzyme, adenine phosphoribosyltransferase (APRT) is specific for adenine or its analogs. Both activities are not required for proliferation of cells because they are involved in salvage pathways (see Fig. 3). It has been shown in the past that mammalian cells resistant to 6-thioguanine or 8-azaguanine exhibit resistance due to partial or total loss of HGPRT activity and in some cases resistance is due to the alteration in substrate affinity of HGPRT (Brockman, 1963; Caskey and Kruh, 1979; Epstein et al., 1979; Meyers et al., 1980). Similarly in 8-azaadenine or 2,6-diaminopurine resistant cell lines, resistance is caused by the partial or total loss of APRT activity (Chasin, 1974; Jones and Sargent, 1974; Meuth and Arrand, 1982; Simon and Taylor, 1983; Taylor et al., 1977).

Resistance can also develop due to an alteration in a step leading to the formation of toxic metabolite from the activated nucleoside, i.e. due to mutation in a step other than the phosphorylation or ribophosphorylation step. For example, antitumor effects of tiazofurin results from conversion of its phosphate to a derivative of nicotinamide adenine dinucleotide, i.e. TAD^+, which inhibits the activity of the enzyme Inosine-5'-monophosphate (IMP) dehydrogenase. (Earle and Glazer, 1983; Jayaram, et al., 1982b). Resistant clones obtained after multi-step exposure to tiazofurin were

found to be defective in the formation of the toxic metabolite TAD^+ (Jayaram et al., 1982a).

3. Increased inactivation of the nucleoside or its analog

Increased inactivation of the nucleoside analogs decreases the net effective toxic concentration and as a result leads to resistance. This mode of resistance has been observed in cells plated in medium containing toxic concentrations of adenosine as well as inhibitors of de novo purine synthesis and adenosine deaminase (ADA). Mutants capable of growing in such media have been shown to have elevated levels of the enzyme adenosine deaminase, which detoxifies adenosine by converting it into inosine (Yeung et al., 1983; Meija-Fernandez et al., 1984). Some of the mutants resistant to purine base analogs such as 6-thioguanine and 6-mercaptopurine, contain increased levels of membrane alkaline phosphatase activity, which may be involved in the detoxification of the nucleotides of these purine bases (Lee et al., 1978; Scholar and Calabresi, 1979).

4a. Overproduction of the target enzyme

This mode of resistance is not commonly observed in mutant lines selected for resistance to purine nucleoside analogs. This has only been observed in the case of purine nucleoside analog pyrazofurin. Pyrazofurin, a C-nucleoside when metabolized to the monophosphate, acts as a potent inhibitor of the de novo pyrimidine pathway enzyme orotidine-5'-phosphate decarboxylase (Dix et al., 1979). Some of the mutants resistant to this drug contain large excess of the target enzyme orotidine-5'-phosphate decarboxylase (Suttle, 1983), indicating

that gene amplification may be the basis of resistance in some mutants.

4b. Alteration in the target site

This mode of resistance has not been observed in single step selection to any nucleoside analogs which require cellular activation for toxicity. Recently, it has been shown that mutation at the target site is responsible for resistance to an adenosine analog, 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole, which does not require cellular activation for toxicity (Weinmann et al., 1984).

Thus by obtaining mutant cells resistant to purine nucleoside analogs, one can obtain a better understanding of the cellular enzymes involved in their conversion to toxic metabolite(s) and in some cases conclusive information about the target site of the drugs. By studying the genetic alteration in mutant cells, one can easily distinguish the reaction which leads to cellular toxicity from those reactions in which activated nucleoside analogs participate but which are not primarily responsible for the observed cellular toxicity.

1.8 Scope of This Work

As mentioned earlier, nucleoside analogs constitute an important group of antimetabolites which have found widespread applications in antiviral and anticancer chemotherapy. In recent years, extensive efforts have been made by using biochemical approaches to understand their mechanisms of action. However, for many of these compounds, the mechanisms of action and cellular toxicity is currently not understood. Pyrrolopyrimidine nucleosides and pyrazolopyrimidine nucleosides are examples of such nucleoside analogs. The major problem

encountered in these and other related compounds was the close structural similarity between normal cellular nucleosides and the above nucleosides. As a result, their phosphorylated derivatives can take the place of or interfere with the metabolism of normal nucleotides in a wide variety of cellular reactions. Therefore, the major problem encountered in these studies was to decide whether their cytotoxicity is due to the inhibition of or interference with one particular reaction, or it is a cumulative effect of interference with a large number of cellular reactions. Thus the issue of defining exactly which mechanism is responsible for their action and which may result in cellular toxicity is often clouded. Since these nucleoside analogs possess useful medicinal properties, it is important to understand their mechanisms of action before they are utilized for clinical purposes.

In an attempt to understand their mechanism of action, a combined genetic and biochemical approach was undertaken to study the mechanisms by which the resistance to them develops in mammalian cells. The basic approach is described on page 24 and summarized in Fig. 4. Therefore, stable mutants were isolated using the pyrrolopyrimidine nucleoside, toyocamycin as well as pyrazolopyrimidine nucleosides, formycin A and formycin B in CHO cells. Various genetic and biochemical studies with these mutants have given useful insights into the metabolisms of these nucleoside analogs as well as the mechanism by which mammalian cells can develop resistance to them.

2. MATERIALS AND METHODS

2.1 Materials

(a) Chemicals and Reagents

Adenosine-5'-triphosphate (ATP), bovine serum albumin (BSA), formycin A, formycin B, guanosine-5'-triphosphate (GTP), inosine-5'-monophosphate (IMP), α-ketoglutarate, magnesium chloride ($MgCl_2$), 6-methylaminopurine ribonucleoside (6-MeAPR), 6-methylmercaptopurine ribonucleoside (6-MeMPR), sodium deoxycholate, tris(hydroxymethyl)aminomethane(Tris)-HCl and Tris base and zinc sulphate were purchased from Sigma Chemical Co. 8-Azaadenosine was obtained from Vega Biochemicals and N^7-benzylformycin A (Bbb-73) and N^7-(Δ^2-isopentenyl) formycin A (Bbb-85) were kindly provided by Dr. R.H. Hall, Department of Biochemistry, McMaster University. Pyrazofurin was a gift from Dr. R.L. Hamil of Lilly Research Laboratories, Indianapolis, IN and 9-Deazaadenosine was obtained from Dr. R.S. Klein of Sloan-Kettering Institute for Cancer Research, Rye, NY. Tiazofurin (NSC-286193), toyocamycin (NSC-63701), sangivamycin (NSC-65346) and the tricyclic nucleoside pentaazaacenaphthylene (TCN; NSC-154020) were obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute, National Institute of Health, Silver Spring, MD. Formycin A-5'-monophosphate (FoA-MP) and formycin B-5'-monophosphate (FoB-MP) were purchased from Calbiochem. Alanosine monosodium salt was kindly provided by Dr. L. Coronelli of Gruppo Lepetit, Milan and erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) was

purchased from Burroughs Wellcome Co. All of the nucleoside analogs and drugs were freshly dissolved in water and filter sterilized before each experiment. Polyethylene glycol (PEG) 6000, sodium dodecyl sulphate (SDS) and ethylenediaminetetraacetic acid were obtained from BDH Chemicals Ltd. Acrylamide, bis acrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), biolyte 3/10 and biolyte 4/6 were purchased from Bio-Rad Laboratories. MEM alpha medium, fetal bovine serum and dialyzed fetal bovine serum were purchased from Grand Island Biological Company, NY. 5'-AMP-Sepharose 4B, carboxymethyl (CM)-cellulose, diethylaminoethyl (DEAE)-cellulose and protein A-Sepharose were obtained from Pharmacia and nitrocellulose membrane was obtained from Mandell Scientific Co. Chromatography paper, GF/C filters, as well as DE81 filter papers were obtained from Whatman and polyethyleneimine-impregnated MN-300 cellulose sheets were purchased from Brinkman Instruments Inc, NY. All the other chemicals were of the highest analytical reagent grade available.

(b) Enzymes

Alkaline phosphatase (EC 3.1.3.1) and L-glutamate-oxaloacetate transaminase (EC 2.6.1.1) were purchased from Sigma Chemical Company and Boehringer-Mannheim, respectively.

(c) Radiochemicals

[^{3}H]Formycin A (5 Ci/mmol) and [^{3}H]formycin B (3 Ci/mmol) were purchased from Moravek Biochemicals. [^{3}H]Adenosine (36 Ci/mmol), [^{14}C]hypoxanthine (40 mCi/mmol), [^{3}H]leucine (47 Ci/mmol), [^{3}H]thymidine (20 Ci/mmol) and [^{3}H]uridine (38.8 Ci/mmol) were

purchased from New England Nuclear Corporation. [^{35}S]-Methionine (600 Ci/mmol), [^{125}I]goat anti-rabbit IgG $(Fab)_2$, L-[4-^{14}C]aspartic acid (40 mCi/mmol), [8-^{3}H]guanosine (9.4 Ci/mmol) and [8-^{14}C]inosine (58 mCi/mmol) were obtained from Amersham Corporation. [^{3}H]Tubercidin was obtained by custom labelling of tubercidin by a catalytic exchange procedure (New England Nuclear) and then was purified by paper chromatography (100 mCi/mmol) using ethylacetate:isopropanol:H_2O (7:2:3, v/v/v) as solvent system.

(d) Cell Culture and Cell Lines

The different CHO lines employed in this study are listed in Table III. All cells were routinely grown in monolayer culture in alpha minimal essential medium (α-MEM; Stanners et al., 1971) supplemented with 5% fetal calf serum and maintained at 37°C in a 5% CO_2 atmosphere. However, all experiments involving various nucleoside analogs and their effects on macromolecular synthesis were carried out in α-MEM supplemented with 5% dialyzed fetal bovine serum. The doubling time of CHO cells in both these media under the conditions described above was about 14 h. Labelling of cells with [^{35}S]methionine was carried out in growth medium lacking methionine and supplemented with 10% fetal bovine serum. Phosphate buffer saline (PBS) used for washing of cells contained 0.8% sodium chloride, 0.02% potassium chloride (KCl), 0.115% disodium hydrogen orthophosphate and 0.008% potassium dihydrogen phosphate. The cell count measurements were made with a Coulter Electronic Counter (Model Z_F).

2.2 Methods

Table III. Characteristics of CHO Cell Lines Used In the Present Study

Cell Line	Phenotype and Origin
WT (Pro^-)	Proline requiring CHO line used for various mutant selections (Siminovitch, 1976; Tijo and Puck, 1958). A detailed karyotype of the CHO line in use has been reported by Worton et al. (1977)
Fom^R2 and Fom^R4	Formycin A-resistant mutants of WT cells, selected in the presence of 10 µg/ml of formycin A (Section 3.2)
Fom^R10 and Fom^R12	Mutants obtained for resistance to formycin B by plating WT cells in the presence of 10 µg/ml of formycin B (Section 3.3)
Toy^r4, Toy^r10 and Toy^r11	Toyocamycin resistant mutants selected from WT cells in the presence of 10 ng toyocamycin per ml (Gupta and Siminovitch, 1978b; Section 3.2)
Tub^r12 and Tub^r22	Mutants obtained for resistance to tubercidin by plating WT cells in presence of 0.1 µg/ml of the drug (Section 3.4)
Mpr^r16 and Mpr^r20	6-MeMPR resistant mutants selected from WT cells in the presence of 20 µg/ml of 6-MeMPR (Section 3.4)
Pyr^r13 and Pyr^r19	Mutants obtained for resistance to pyrazofurin by plating WT cells in the presence of 10 µg pyrazofurin/ml (Section 3.4)
EOT	Multiply marked CHO line resistant to emetine, ouabain, and 6-thioguanine, selected from WT cells in earlier studies (Gupta et al., 1978a)

Cell Line	Characteristics
EOT Toy^{r}_{2}	Toyocamycin resistant mutant selected from EOT line in the presence of 10 ng of toyocamycin per ml (Gupta and Siminovitch, 1978b)
Gat^{-} WT (CHO)	A glycine, adenosine, and thymidine auxotroph of CHO cells (McBurney and Whitmore, 1974b)
Gat^{-} Toy^{rI}_{16}	First-step toyocamycin resistant mutants selected from Gat^{-} WT (CHO) cells in the presence of 10 ng of toyocamycin per ml (Gupta and Siminovitch, 1978b)
Gat^{-} Toy^{rII}_{1} Gat^{-} Toy^{rII}_{2}	Selection of second-step mutants was carried out by plating a mutagen-treated culture of the Gat^{-} Toy^{rI}_{16} cell in the presence of 0.4 µg/ml of toyocamycin (Section 3.1)
HeLa	Human cervical carcinoma cells isolated by Gey et al. (1952)

2.2.1 Measurement of Degree of Resistance

The degree of resistance of mutants towards various drugs was determined by seeding 100 and 200 cells in duplicate (in a 0.5-ml volume of growth medium) into the wells of 24-well tissue culture dishes. To each well, 0.5 ml of the various dilutions of the drugs made at two times the final concentrations desired in the growth medium was added. The dishes were incubated for 6 to 7 days at 37°C, after which they were stained with 0.5% (w/v) methylene blue in 50% (w/v) methanol; then the number of colonies was counted. From the averages of the numbers of colonies observed at different concentrations of the drugs, relative plating efficiencies were determined as the ratios of the number of colonies at a given drug concentration to that obtained in the absence of the drug. The D_{10} value of a drug towards a cell line refers to the dose of the drug which reduces relative plating efficiency of the cell line to 10%.

2.2.2 Effect of Formycin B on the Growth of CHO and HeLa Cells

Effect of formcyin B on the growth of CHO and HeLa cells was determined by plating 2 x 10^5 cells each in a large number of 60 mm dishes. Formycin B at the indicated concentration was added to the dishes at zero time and at different time intervals cells were trypsinized, suspended in equal volume of medium and counted.

2.2.3 Selection of Mutants

An exponentially growing culture of WT cells was treated with a freshly prepared solution of the mutagen ethylmethanesulfonate (EMS) at a concentration of 300 µg/ml for 20 h. This treatment results in about

50% cell killing. The mutagen-treated cells were grown for 3 days in non-selective medium to allow time for mutation fixation. The selection of mutants was carried out by plating 10^6 cells per 100 mm diameter dish (P-100 dish) on several dishes in medium containing toxic concentrations of adenosine analogs (see Table III). The plating efficiencies of the cells at the time of plating were determined by plating a known number (usually 250) of cells in non-selective medium in 60 mm diameter dishes, and the mutation frequencies observed were corrected for this.

2.2.4 Cell Hybridization Protocol.

Somatic cell hybrids were constructed between the mutants isolated in the present studies and EOT cell line. The latter cell line, which is derived from the WT cells, has been selected for resistance to ouabain (Oua^R) and is also unable to grow in hypoxanthine-aminopterin-thymidine (HAT) supplemented medium due to a deficiency of the enzyme HGPRT (HAT^S). The hybrids between the EOT cell line and the appropriate mutants can then be selected in HAT medium supplemented with 2×10^{-3} M ouabain in which none of the parental cell lines can grow. For hybrid formation, one day prior to cell fusion 1×10^6 cells each of EOT and other mutant cell lines were seeded into a 60 mm diameter tissue culture dish. The next day, the confluent monolayer of cells were treated for 1 min with a 44% solution of PEG 6000 in 10% dimethyl sulfoxide (DMSO) in α-MEM without serum (Davidson and Gerald, 1977; Norwood et al., 1976). The PEG-treated cells were washed and allowed to grow for 24 h. On the following day,

the treated cells were plated in the HAT medium (hypoxanthine (5 x 10^{-5} M), methotrexate (1 x 10^{-6} M), thymidine (5 x 10^{-5} M)) containing 2 x 10^{-3} M ouabain. Since the various mutant lines which were investigated were ouabain-sensitive and the EOT line, due to its HGPRT deficiency, does not grow in this medium, only the hybrids formed between the two parents survive and proliferate in this growth medium (Gupta et al., 1978a). Hybrid clones were obtained in the above crosses at high frequencies ($\approx 10^{-2}$ to 10^{-3}). In contrast, no colonies were observed in the control self-crosses (EOT x EOT and mutant x mutant), even when up to 2 x 10^5 cells were plated in the above medium. Colonies which appeared in the selective medium were picked and subsequently cultured in the same medium. A minimum of six colonies from each cross were examined for their degree of drug resistance. The hybrid nature of the selected clones were also ascertained by the number of chromosomes.

2.2.5 Chromosomal Analysis

For chromosomal analysis of various cell lines, about 1-2 x 10^5 cells were added to the chamber of a Lab-Tek dish (9 cm^2 area). After 2-3 days of growth at 37°C, cells growing on dishes were treated with colcemid (0.3 µg/ml) for about 2 h. Following colcemid treatment, cells were allowed to swell in hypotonic medium (0.075 M KCl) for 30 min, fixed with a methanol-acetic acid (3:1, v/v) solution and then stained with Giemsa. A minimum of 20 representative chromosome spreads were counted for every cell line to ascertain its ploidy.

2.2.6 Chromatography

The metabolites of formycin B were initially separated by ascending chromatography on Whatman #3 paper using n-propanol:water (nProH:H_2O; 6:4, v/v) as solvent (R_f values: formycin A or formycin B, 0.50; FoA-MP or FoB-MP, 0.22). The chromatograms were cut into 1 cm segments and analyzed for radioactivity. FoA-MP and FoB-MP were separated from each other by thin layer chromatography on polyethyleneimine-impregnated MN-300 cellulose sheets using n-propanol:ammonium hydroxide:water (nProH:NH_4OH:H_2O; 6:3:1, v/v/v) as solvents (R_f values: FoA-MP, 0.12; FoB-MP, 0.25).

2.2.7 Metabolism of Formycin B

Exponentially growing CHO cells were harvested by centrifugation (1000 x g for 10 min) and resuspended at a density of 2-5 x 10^7 cells per ml in fresh medium. The cells were incubated with the indicated concentrations of [^{3}H] formycin B at 37°C. After the specified period of incubation, cells were harvested by centrifugation and washed with 4 ml of ice-cold PBS. The cell pellet was resuspended in 1 ml of cold 5% perchloric acid (PCA). After 5 min at 0-4°C, the suspension was centrifuged to give the acid-soluble and acid-insoluble fractions. The acid-soluble portion was immediately neutralized with 1 N potassium hydroxide (KOH). An aliquot of the neutralized extract was centrifuged at 12,000 g x for 3 min, and the supernatant was analyzed by paper and thin-layer chromatography as described. The neutralized PCA-soluble fraction was also subjected to alkaline phosphatase treatment. The treatment was carried out in a total volume of 50 µl which contained 1 mM $ZnCl_2$, 1 mM $MgCl_2$, 0.1 M glycine buffer, pH 10.5,

10 µl of neutralized PCA-soluble fraction and 0.02 U alkaline phosphatase. Incubation was carried out for 30 min at 37°C. Subsequently, the sample was analyzed by paper chromatography using nProH:H_2O (6:4, v/v) solvent system.

The PCA-insoluble fraction was used to study the incorporation of the drug into nucleic acids, as described earlier (Jayaram et al., 1982b). For this purpose, the PCA-insoluble pellets were washed four times with cold 5% PCA. The pellets were dissolved in 100 µl of 40% KOH at 95° for 30 min , cooled and centrifuged at 12,000 x g for 3 min. The radioactivity in the pellet was determined by counting in toluene based scintillation fluid containing 2,5-diphenyloxazole (PPO) and 1,4-bis-2-(5-phenyloxazolyl) benzene (POPOP) and the radioactivity in the supernatant fractions was determined by counting in aqueous scintillant solution (ACS). The KOH hydrolysate was also analyzed by paper chromatography.

2.2.8 Effect of Nucleoside Analogs on Cellular Macromolecular Synthesis

For these studies, exponentially growing CHO cells were suspended in the appropriate growth medium at a concentration of about 5 x 10^5 cells/ml. After removing the zero time samples which were pulse labelled in the manner described below, the various analogs were added to separate cultures to give final concentrations of 0.2 µg/ml (toyocamycin), 2.0 µg/ml (tubercidin), 2 µg/ml (sangivamycin) and 5 µg/ml (TCN). After various times, thereafter, 2 ml aliquots of the cultures were removed and separately pulse labelled with either

[^{3}H]leucine (5 μCi/ml), [^{3}H]uridine (5 μCi/ml) or [^{3}H]thymidine (5 μCi/ml). After 30 min of labelling, an equal volume of cold 10% trichloroacetic acid was added to the cultures and the acid precipitable counts were determined in each case. Assuming the incorporation values obtained in the zero time samples which were not treated with the drugs as 100%, the radioactivity incorporated into the drug-treated samples were converted into percent of control values.

2.2.9 Cellular Uptake and Incorporation of Radiolabelled Nucleosides and Nucleoside Analogs

For studying the cellular uptake, about 5 x 10^4 cells were seeded (in duplicate for each time period) into the wells of 24-well tissue culture dishes. After approximately 2 days, when the wells were nearly confluent, the medium was carefully aspirated, and 0.25 ml of the solutions containing the desired concentrations of the radiolabelled nucleoside in growth medium (supplemented with 10 μg of ADA inhibitor EHNA per ml to prevent deamination of adenosine and 5 x 10^{-5} M uridine) were added to each well. At different time intervals, the labelled medium was removed and the cells were rinsed three times with PBS. The cells from each well were dissolved in 0.5 ml of a solution of 0.4% deoxycholic acid in 0.1 N sodium hydroxide. One half of the cell lysate was counted directly after the addition of ACS to determine the total cellular uptake of radioactivity. The remaining cell lysate was precipitated by the addition of cold 10% trichloroacetic acid and then filtered using GF/C filters. Filters were washed, and counted to obtain a measure of the amount of

radioactivity which had been incorporated into cellular macromolecules. At the same time, the total number of cells in two parallel control wells of each cell line was determined by trypsinization and counting samples in a Coulter Electronic Counter. The cellular uptake and incorporation observed in different cell lines was normalized for an equal number of cells (5×10^5).

2.2.10 Measurement of Protein

Protein concentration was measured spectrophotometrically according to the method of Lowry et. al. (1951) using BSA as the standard.

2.2.11 Assay of Adenosine Kinase (EC 2.7.1.20)

The cell extracts for measurement of AK activity were generally prepared by trypsinizing cells from three to four nearly confluent P-100 dishes and then washing them twice with PBS by centrifugation and resuspension. The washed cells were suspended at a concentration of 10^7 cells per ml in 0.1 M potassium phosphate buffer, pH 7.0, and were disrupted by sonication for two 30-s-interval bursts in a Bronson sonicator. The resulting crude cell extract was centrifuged for 30 min at 30,000 x g at 4°C. The supernatant from this run was dialyzed for 16 h at 4°C against 200 volumes of 0.01 M potassium phosphate buffer and then was used for measurement of AK activity.

The assay of AK activity in cell extracts was carried out as described before (Chan et al., 1973; Gupta and Siminovitch, 1978b). The reaction mixture (in a final volume of 250 µl) contained 50 mM potassium phosphate buffer, pH 7.0, 2.5 mM ATP, 0.25 mM $MgCl_2$, 4 x

10^{-5} M [^{3}H]adenosine (50 mCi/mmol) and between 25 and 50 µl of cell extracts. The reaction which was carried out at 37°C was initiated by the addition of cell extracts. At 5-min intervals, 50 µl of the reaction mixture was removed and added to 1.0 ml of an ice-cooled solution of 1.0 M $LaCl_3$, which precipitates AMP. The precipitated AMP was collected on GF/C filters, which were washed five to six times with cold water (6 to 7 ml each time). The filter papers were then dried and counted in a toluene-based scintillation fluid containing PPO and POPOP. The background radioactivity which was bound to the filter paper in a parallel control experiment lacking cell extract was subtracted from all experiments.

Under the conditions used, conversion of [^{3}H]adenosine into [^{3}H]AMP by WT cell extracts was linear for at least the first 15 min, and the duplicate samples generally showed less than 5% variation. The amount of [^{3}H]AMP produced by any cell extract was converted into nanomoles of [^{3}H]AMP. The specific activity of AK in various cell extracts was calculated by dividing AK activity by the concentration of proteins in the cell extracts.

2.2.12 Competition between Adenosine and Adenosine Analogs for AK

Competition studies were carried out with radiochemical methods which quantitated the formation of the nucleotides from labelled nucleosides using the method previously described with little modification (Hurley et al., 1983). The reaction mixture in a total volume of 40 µl contained: 50 mM Tris-HCl, pH 7.4, 1.6 mM $MgCl_2$, 1.2 mM ATP, 50 mM 2-mercaptoethanol (2-MSH), 50 mM KCl, 1 mg BSA/ml, 50 µM

EHNA, 10 μM [^{3}H]adenosine and 5 μl of drug or an equivalent volume of water. The reaction was initiated by addition of 10 μl of cell extracts prepared by the method described above. Control experiments which lacked ATP were carried out in parallel. Assays were incubated at 37°C for 20 min. Reactions were stopped by heating for 2 min at 85°C, and 25 μl aliquots of reaction mixtures were spotted onto DE81 filter paper. The discs were then washed two times for 5 min with ammonium formate to remove unreacted substrate. The discs were subsequently washed two times with water and once with ethanol. The dried discs were counted in a Beckman scintillation counter using a toluene-based scintillation fluid containing PPO and POPOP.

2.2.13 Assay of Purine Nucleoside Phosphorylase (EC 2.4.2.1)

PNP activity was measured by conversion of [8-^{14}C]inosine to [8-^{14}C]hypoxanthine as described by Thompson et. al. (1978). The assay mixture consisted of (in the order of addition) 25 μl of 0.4 M potassium phosphate buffer, pH 7.4, 5 μl of cell extract, 20 μl of inhibitor solution or water, and 5 μl of [8-^{14}C]inosine (2 mCi/mmol). The assays were performed at 37°C, started by the addition of the isotope to prewarmed tubes containing all other components, and terminated by the addition of 10 μl of 8 M formic acid; and the tubes were maintained on ice. The assay mixtures were clarified by centrifugation at 5000 x g for 5 min. The product hypoxanthine was separated from inosine by chromatography of 20 μl reaction mixture with 5 μl of marker solution (0.5 mg hypoxanthine/ml, 1.0 mg inosine/ml) on cellulose sheets. The plates were developed to 10 cm above the origin

with 1.6 M lithium chloride. The spots identified as inosine or hypoxanthine were cut out and counted in 3 ml of toluene-based scintillation fluid containing PPO and POPOP.

2.2.14 Assay of Adenylosuccinate Synthetase (EC 6.3.4.4)

The cell extracts for measurement of adenylosuccinate synthetase (ASS) activity were prepared by procedures similar to those described earlier (Anandaraj et al., 1980; Tyagi and Cooney, 1980). For these studies exponentially growing CHO cells were harvested by centrifugation (1000 x g for 10 min), washed twice with PBS and then suspended at a concentration of about 5 x 10^7 cells/ml in 0.1 M Tris-HCl buffer, pH 7.0, containing 1 mM dithiothreitol. The cell suspension was homogenized in a tight-fitting Dounce homogenizer and the homogenate was centrifuged at 12000 x g for 3 min. The supernatant was dialyzed at 4°C against three changes of the homogenization buffer for 6 h.

The assay of ASS activity in cell extracts was carried out essentially as described by Tyagi and Cooney (1980). The reaction mixture in a total volume of 175 µl contained: 20 µl of L-[4-^{14}C] aspartic acid (25-48 nmol, 0.5 µCi), 0.6 µmol each of GTP and $MgCl_2$; 35 nmol of IMP; 25 µl of drug or an equivalent volume of water and 35 µl of 0.1 M Tris-HCl buffer, pH 7.4. The reaction was initiated by the addition of 35 µl of the cell extract. The control experiments which lacked IMP were carried out in parallel. The samples were incubated for 30 min at 37°C after which the reaction was stopped by heating the tubes for 2 min in a 95°C water bath. Unreacted L-aspartic acid was

then decarboxylated by the addition of 250 µl of decarboxylation reagent consisting of 0.005 M α-ketoglutarate, 0.03 M zinc sulphate in 0.66 M sodium acetate buffer, pH 5.0 and 40 IU/ml of L-glutamate-oxaloacetate transaminase. After 3 h at 37°, the radioactivity remaining in the reaction vessel was counted in ACS. Under the conditions employed here, the ASS reaction proceeded linearly both with respect to time and the amount of cell extract.

2.2.15 Purification of Adenosine Kinase

The purification of AK from CHO cells was carried out by a scheme similar to that used by Andres and Fox (1979) for purification of AK from human placenta. Exponentially growing CHO cells were harvested by centrifugation (1000 x g for 10 min), washed twice with PBS and then suspended at a concentration of about 5 x 10^7 cells/ml in 0.1 M Tris-HCl buffer, pH 7.0. The cell suspension was homogenized in a tight-fitting Dounce homogenizer and the homogenate was centrifuged at 28,000 x g for 60 min. The supernatant was removed and stored at -80°C. CM- and DEAE-celluloses were treated with 0.5 M sodium acetate, pH 6.0 for one hour and then adjusted to pH 6.0 in 10 mM sodium acetate with HCl. The supernatant was also adjusted to pH 6.0 with HCl and mixed with an equal settled volume of CM-cellulose. After stirring for 90 min, the preparation was filtered on Buchner funnel, washed once with 10 mM sodium acetate, pH 6.0, and the eluate was then mixed with a volume of settled DEAE-cellulose equal to the initial volume of supernatant. This was stirred for 2 1/2 h, then filtered. The eluate was stored at -80°C.

The post-ion exchange solution was applied to a 10 ml 5'-AMP-Sepharose 4B column equilibrated in 10 mM sodium acetate, pH 6.0. The initial washing was carried out with a 70 ml wash of 10 mM sodium acetate, pH 6.0, containing 1.0 M KCl and 20 ml of 10 mM Tris-HCl, pH 7.4, containing 1.0 M KCl. The column was then washed with 15 ml of 0.1 M Tris-HCl, pH 7.4, 5 mM ATP followed by 0.1 M Tris-HCl, pH 7.4, 5 mM ATP, 5 mM $MgCl_2$, and 5 mM adenosine. AK activity eluted in a sharp peak with the final wash and was detected by the AK assay described earlier. The final preparation was stored at $-80^{\circ}C$.

2.2.16 SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide electrophoresis was performed as described by Laemmli (1970). All electrophoretic runs were done in slab gels utilizing the discontinous buffer system. Separating gels were made by mixing the following ingredients so that their final concentrations were 10% acrylamide (30%:0.8% acrylamide:bis acrylamide, w/w) 0.375 M Tris-HCl, pH 8.8, 0.1% (w/v) glycerol and 0.025% TEMED, 0.075% (w/v) ammonium persulphate and 0.10% (w/v) SDS. The solution was degassed prior to the addition of freshly prepared ammonium persulphate and SDS. The stacking gel was made by mixing the following ingredients so that their final concentrations were 5% acrylamide (30:0.8 acrylamide::bis acrylamide, w/w), 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 0.05% glycerol, 0.075% TEMED and 0.064% ammonium persulphate.

Samples were prepared for electrophoresis by mixing with an equal volume of twice-concentrated electrophoresis sample buffer (0.125 M

Tris-HCl, pH 6.8), 2% SDS, 20% glycerol, 2.5% (v/v) 2-MSH, 0.002% (w/v) bromophenol blue and proteins in them were denatured by boiling for 2 min. Electrophoresis running buffer consisted of 0.30% (w/v) Tris base, 1.44% (w/v) glycine and 0.1% (w/v) SDS. After loading the samples, a low constant voltage of 125 volts (V) was applied initially followed by high voltage (150 to 175 V) for approximately 5 h.

For staining proteins after electrophoresis, the gel was placed in staining solution (0.25% Coomassie blue, 50% methanol, 10% glacial acetic acid, v/v) for 1 h at room temperature with shaking followed by overnight destaining in 20% ethanol, 10% glacial acetic acid, v/v. Gels were dried on a piece of Whatman #1 chromatography paper using a Bio-Rad gel dryer. For detection of [^{35}S]radioactivity the dried gel was exposed to Kodak X-Omat XAR5 film.

2.2.17 Preparation of Antisera

Antibodies to the affinity purified adenosine kinase, which was more than 95% pure as estimated by the staining pattern of SDS-polyacrylamide gels, were raised in rabbit. Aliquots containing 250 µg of antigen were emulsified with an equal volume of complete Freund's adjuvant and injected both intramuscularly and subcutaneously into a rabbit two times during one week intervals and then alone (i.e. without any Freund's adjuvant) after two weeks. The rabbit was bled 8 days after the third injection. The activity of the antisera were assayed by immunoblotting (Towbin et al., 1979).

2.2.18 Immunoblotting

Immunoblotting of proteins on nitrocellulose was performed by

the method of Towbin et. al. (1979). Initially samples were electrophoresed on 10% SDS-polyacrylamide gel or on a two-dimensional (2-D) gel. When the electrophoretic run was complete, the gel was removed and placed on a sheet of nitrocellulose membrane. Polyacrylamide gel-nitrocellulose membrane was sandwiched in between the sheets of Whatman #3 chromatography paper and enclosed in plastic grids with nitrocellulose membrane facing the positive electrode. The whole assembly was transferred to Bio-Rad transblot chamber. After filling the chamber with transfer buffer (25 mM Tris base, 192 mM glycine, pH 8.3, 20% methanol) constant current of 0.2 ampere was applied for 3-4 h. Following transfer, unless stated otherwise, the blot was removed and soaked in saline (0.9% sodium chloride, in 10 mM Tris-HCl, pH 7.4) containing 3% BSA for 1 h at 37°C. The blot was subsequently rinsed with saline and incubated for 4 h at room temperature while shaking with antiserum appropriately diluted into saline containing 3% BSA. The blot was then washed with 4 changes of saline over a period of 2 h to remove any unbound antibody. Washed blot was then incubated overnight at room temperature on a rocker platform with goat [^{125}I]anti-rabbit IgG diluted to 2 x 10^5 cpm per ml in 3% BSA-saline solution. After washing the blots extensively (5 times over 2 h), it was dried and exposed to Kodak XAR-5 film for an appropriate length of time.

2.2.19 Immunoprecipitation

Immunoprecipitation was carried out by a procedure described before (Chien and Freeman, 1984). Cells (1 x 10^7) labelled with

[^{35}S] methionine, for an indicated period of time, were washed, suspended in 0.5 ml of 50 mM Tris-HCl, pH 7.2 and were allowed to swell for 20 min. Swollen cells were then homogenized in a tight-fitting Dounce homogenizer. The homogenate was centrifuged at 100,000 x g for 30 min at 4°C. 0.1 ml of the supernatant was mixed with 0.9 ml of the adjusting buffer which contained 0.3 M KCl, 1% (v/v) Triton X-100, 5 mM ethylenediaminetetraacetic acid, and 10 mM Tris-HCl, pH 7.2, 50 µg each of phenylmethylsulfonyl fluoride and a soyabean trypsin inhibitor. This was followed by the addition of 20 µl of anti-adenosine kinase or preimmune serum. After incubation at 4°C for 16 h under continuous rotation, 20 µl of swollen protein A-Sepharose was added and incubated for an additional 3 h. Protein A-Sepharose was recovered using an Eppendorf micro-centrifuge and washed six times with adjusting buffer and finally one time with 10 mM Tris-HCl, pH 7.2. The antibody-antigen protein A-Sepharose complexes were released by the addition of 100 µl of a solution containing 8% SDS, 6.5% 2-MSH, and 20 mM Tris-HCl, pH 6.8. The beads were removed by centrifugation and the supernatant was subjected to SDS gel electrophoresis by a procedure described earlier. In cases where immunoprecipitation was carried out under denaturing condition, SDS was added to supernatant (final concentration 1%) before the addition of adjusting buffer.

2.2.20 Two-Dimensional Gel Electrophoretic Analysis of [^{35}S]Methionine-Labelled Proteins

A. Labelling of total cellular proteins

Cells were seeded into the wells of a 24-well tissue culture

dish (Linbro, Flow Laboratories, 2-cm^2 area/well) at a concentration of about 1-2 x 10^4 cells/well. After 24 - 48 h, when cells had become nearly confluent, growth medium was removed and cells were rinsed with 2 ml of medium minus methionine. The labelling of cells was carried out in 0.2 ml of minus methionine medium containing 80 µCi of [^{35}S]methionine. After 2 h of labelling at 37°C, cells were rinsed with 2 ml of PBS at room temperature, and then 100 µl of lysis buffer (9.5 M urea, 1.6% Ampholine, pH 4 - 6, 0.4% Ampholine, pH 3 - 10, 5% 2-MSH, and 2% (w/v) Nonidet P-40) was added. After allowing 10 min for lysis and solubilization, the cell lysate was centrifuged at 8000 x g for 3 min and then directly layered onto prerun isoelectrofocusing (IEF) gels.

B. Two-dimensional gel electrophoresis

The protocol employed for running 2-D gel electrophoresis was essentially that of O'Farrell (1975) and O'Farrell et. al. (1977) with minor variations as described previously (Gupta et al., 1982). Ten ml of solution for IEF gels were made by mixing the following ingredients: 5.5 gm urea, 1.3 ml acrylamide solution (28.38% acrylamide, 1.62% bis acrylamide, filtered), 2.0 ml of Nonidet P-40 (10%), 0.6 ml ampholyte pH 4-6, 0.12 ml ampholyte pH 3-10. The solution was degassed carefully and then 7 µl of TEMED and 10 µl of 10% ammonium persulfate were added to the IEF solution. The solution was then immediately loaded into thoroughly washed tubes to a specified mark. The gel mixture was overlayed with 10 µl of H_2O and allowed to polymerize for atleast 2 h. After gels had polymerized, tubes were loaded into a standard gel

electrophoresis chamber, the lower reservoir being filled with 10 mM phosphoric acid. The solution above the gels was removed by aspiration and the top of the gels were overlayed with 10 µl of lysis buffer (9.8 M urea, 1% Nonidet P-40, 1.6% ampholyte pH 4-6, 0.4% ampholyte pH 3-10, 5% 2-MSH). The upper chamber was filled carefully with 40 mM sodium hydroxide and 20 mM calcium hydroxide, previously degassed, so as not to disturb the layers of solutions over the gel. Gels were prerun at 200 V for 15 min, 300 V for 30 min and at 400 V for 60 min. After the prerun, the upper chamber buffer was removed and discarded. The liquid above the gels was aspirated, and the top of the gels was washed twice with lysis buffer . At this point, samples were loaded and were covered with 10 µl of overlay buffer (9 M urea, 0.8% ampholyte pH 4-6, 0.2% ampholyte pH 3-10). The tubes were filled with upper chamber buffer and the upper chamber was replenished and the gels were run at 400 V for 10 h and 800 V for 1 h. After the electrophoretic run, gels were removed from the tubes, and equilibrated in equilibrium buffer (0.06 M Tris-HCl, pH 6.8, 2% SDS, 5% 2-MSH, 10% glycerol) for 1 h and are then frozen in a -80°C freezer. For running the second dimension gel, the slab gel techniques already described earlier were used. For application of IEF gels on slab gels, IEF gels were thawed, removed from the equilibration buffer, and carefully laid on top of the plates. The melted 1% agarose solution was used to keep the first dimension gel in place. Gels were run overnight at constant voltage (90 V). After electrophoresis, gel was removed, dried and exposed to Kodak X-Omat XAR-5 film at room temperature for appropriate lengths of time.

3. RESULTS AND DISCUSSION

3.1 GENETIC AND BIOCHEMICAL STUDIES ON SECOND-STEP MUTANTS OF CHO CELLS RESISTANT TO 7-DEAZAPURINE NUCLEOSIDES

As described in the Introduction, the pyrrolopyrimidine ribosides toyocamycin, tubercidin and sangivamycin which are structurally related to adenosine are readily phosphorylated to mono-, di- and triphosphates in cells (Acs et al., 1964; Hardesty et al., 1969; 1974; Parks and Brown, 1973; Suhadolnik, 1967). Further, because of the close structural similarity between their phosphorylated derivatives and adenine nucleotides (AMP, ADP, ATP), these derivatives have been reported to take the place of, or interfere with the metabolism of adenine nucleotides in a wide variety of cellular reactions. However, from the earlier studies it is not clear whether the cytotoxicity of the above drugs is due to inhibition of, or interference with one particular reaction, or whether it is a cummulative effect of interference with a large number of cellular reactions. It is also unclear at present whether the cytotoxic effects of all three of these analogs are produced by the same or different mechanisms.

Previously, single-step mutants of CHO cells resistant to toyocamycin and tubercidin have been isolated and it was observed that all such mutants contained greatly reduced amounts (< 1%) of the purine salvage enzyme, adenosine kinase (AK) which phosphorylates these

adenosine analogs before they become toxic to the cells (Gupta and Siminovitch, 1978b). This suggested that the most common mechanism for cellular resistance to these analogs involves an alteration in the very first biochemical step which is required for the synthesis of lethal metabolites. Furthermore, these AK$^-$ mutants selected for resistance to toyocamycin or tubercidin also exhibited cross-resistance to other adenosine analogs such as pyrazofurin and formycin A (Section 3.2) which require initial phosphorylation but differ in their mechanisms of cellular toxicity. Because these first-step mutants were affected in the initial phosphorylation step which is common to nearly all adenosine analogs, they provided no further information regarding the mechanisms of cellular toxicity of toyocamycin and tubercidin.

Though the single-step mutants of CHO cells obtained for resistance to adenosine analogs contained less than 1% of the AK activity in their cell extracts, such mutants can however phosphorylate adenosine and tubercidin at a rate between 5 to 15% of that seen with the parental sensitive cells (Section 3.2). It was not clear whether the observed phosphorylation in mutant cells was carried out by the residual AK activity in them or by some other enzymatic activity present in CHO cells. It was therefore decided to obtain second-step mutants using toxic concentration of toyocamycin and/or tubercidin to find out whether further resistance to these analogs results from an alteration in the residual phosphorylation activity or due to an alteration in some other cellular step. Isolation of second-step mutants from first-step mutants became possible because these analogs

are toxic to sensitive cells at very low concentration (in the ng/ml range) and first-step mutants were thousand fold resistant to them. As a result these drugs were toxic to the mutant cells in the μg/ml range.

The present studies describe the isolation and some characteristics of second-step mutants resistant to toyocamycin (Toy^{rII} mutants). These studies suggest that the further resistance to these drugs develops due to an alteration in some cellular step other than phosphorylation. The cross-resistance and biochemical studies with these mutants provide valuable information regarding the similarity and the differences in the mechanism of action of various pyrrolopyrimidine nucleosides.

3.1.1 RESULTS

3.1.1.1 Selection of Second-Step mutants from First-Step Mutants Using Increasing Concentrations of Toyocamycin

Fig. 5A shows the dose-response curves toward toyocamycin of the parental sensitive cells (Gat^-WT) and the first-step toyocamycin resistant mutant ($Toy^{rI}16$). As can be seen, the $Toy^{rI}16$ mutant is resistant up to about 50 ng/ml (other Toy^{rI} mutants behave similarly) and at higher concentrations their plating efficiency decreases sharply. The selection of second-step mutants was carried out by plating a mutagen-treated (300 μg EMS/ml for 20 h; plated on the 7th day after the mutagen treatment) culture of the $Toy^{rI}16$ cells in the presence of 0.4 μg toyocamycin/ml. The resistant clones were obtained at a frequency of about 1 in 10^6 in these experiments. Five of the

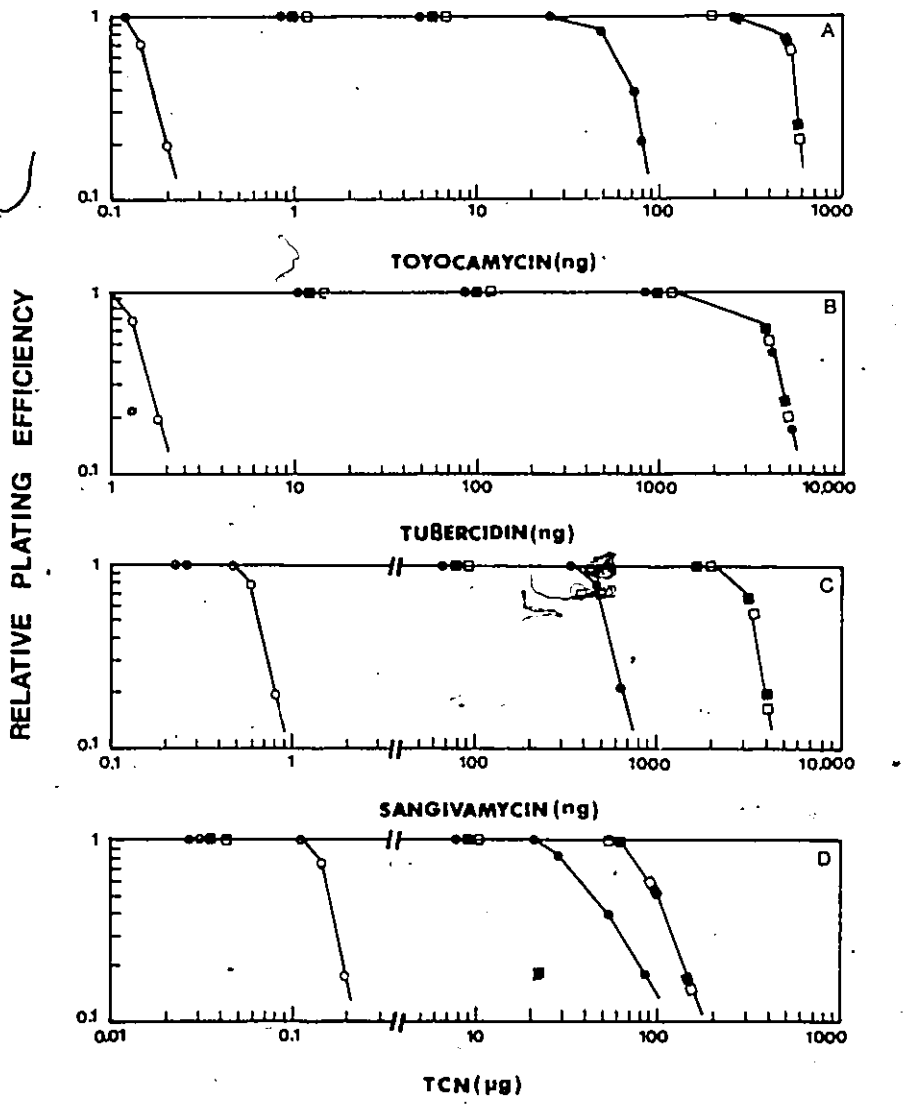


Figure 5: Dose-response curves of the parental sensitive cells and the first- and second-step toyocamycin resistant mutants in the presence of increasing concentrations of various 7-deazapurine nucleoside analogs. (A) toyocamycin, (B) tubercidin, (C) sangivamycin, (D) TCN. ○——○, $Gat^{-}WT$; ●——●, $Toy^{rII}16$; □——□, $Toy^{rII}1$; ■——■, $Toy^{rII}2$.

resistant clones were picked in a non-selective medium and subsequently their degree of resistance towards toyocamycin was determined. The dose-response curves of two of the clones, $Toy^{rII}1$ and $Toy^{rII}2$ towards toyocamycin are also shown in Fig. 5A (the other clones examined behaved similarly). Based upon their D_{10} values, these mutants are about 9- to 10-fold more resistant to toyocamycin as compared with the $Toy^{rI}16$ line. The drug-resistant phenotype of these mutants has remained completely stable upon growth in a non-selective medium for more than two years.

3.1.1.2 Cross-Resistance Studies

To check the specificity of the second-step mutants, their cross-resistance towards other 7-deazapurine nucleosides which included tubercidin and sangivamycin was determined. Results of these studies for the parental sensitive cell ($Gat^{-}WT$), first-step mutant ($Gat^{-}Toy^{rI}16$) and two second-step mutants ($Gat^{-}Toy^{rII}1$ and $Gat^{-}Toy^{rII}2$) are shown in Fig. 5B and C. As can be seen, the $Toy^{rI}16$ mutant which lacks adenosine kinase activity showed greatly increased resistance to tubercidin and sangivamycin. This result, as expected, indicated that the phosphorylation of all these analogs is an essential first-step in their toxic action and that the phosphorylation is carried out by the enzyme AK. Similar inferences regarding the toxicity and phosphorylation of toyocamycin, tubercidin and sangivamycin have been reached in earlier studies (Bennett et al., 1966; Gupta and Siminovitch, 1978b; Hardesty et al., 1974; Rabin and Gottesman, 1979). Very interestingly, in contrast to the first-step mutants which showed

a corresponding increase to all of these analogs, the second-step mutants showed no further increase in their resistance for tubercidin but were about 8-fold more resistant to sangivamycin. Similar results with these mutants have been obtained in a number of independent experiments. The increased resistance of the Toy^{rII} mutants to toyocamycin but not to tubercidin provided strong evidence that the mechanism of cellular toxicity of these two analogs is different from each other and suggested that these mutants may be affected in a cellular component which is specifically involved in the toxicity of toyocamycin. Furthermore, the cross-resistance of the Toy^{rII} to sangivamycin indicated that the mechanisms of cellular toxicity of this analog may be similar to that of toyocamycin.

In view of the above results, it was considered of interest to examine the cross-resistance of Toy^{rII} mutants towards another 7-deazapurine ribonucleoside, TCN (3-amino-1,5-dihydro-5-methyl-1-β-D-ribofuranosyl-1,4,5,6,8-pentaazaacenaphthylene) (for structure, see Fig. 6). In earlier studies, TCN was synthesized by chemical modification of toyocamycin (Schram and Townsend, 1971) and recently has been shown to possess useful anticancer activity (Mittleman et al., 1983). Studies on TCN have shown that it is phosphorylated to its 5'-monophosphate by AK in mammalian cells (Plagemann, 1976). Dose-response curves of various cell lines towards TCN are shown in Fig. 5D. It is evident from this figure that like other pyrrolopyrimidine nucleosides, $Toy^{rI}16$ mutant shows high degree of cross-resistance to TCN, an observation in agreement with earlier

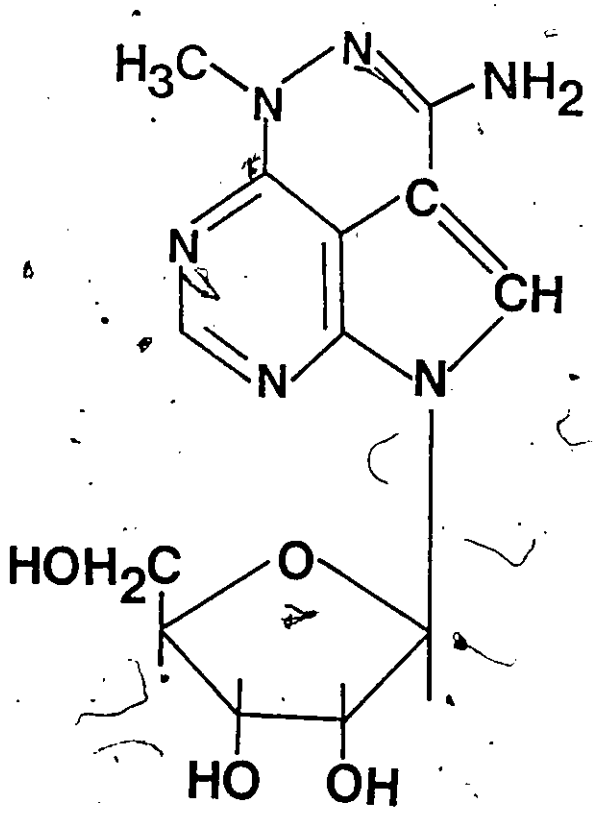


Figure 6: Structure of tricyclic nucleoside pentaaza-acenaphthylene.

studies. Interestingly, second-step mutants also show two fold resistance to TCN, indicating that the mechanism of cellular toxicity of TCN may be similar to that of toyocamycin and sangivamycin.

3.1.1.3 Measurements of Levels of AK

As mentioned before, the mutants of CHO cells resistant to various adenosine analogs which are obtained after a single-step selection contain less than 1% of the AK activity in their cell extracts (Gupta and Siminovitch, 1978b; Gupta and Singh, 1983; Rabin and Gottesman, 1979). However, such mutants can phosphorylate adenosine and tubercidin at a rate between 5-15% of that seen with the parental sensitive cells (Section 3.2). Therefore, it was not clear whether the observed phosphorylation in mutant cells is carried out by the residual AK activity in them or by some other enzymatic activity present in CHO cells. To find out whether the genetic lesion in Toy^{rII} mutants has affected this residual phosphorylation activity, the level of AK activity as well as the cellular uptake and phosphorylation of [^{3}H]adenosine and [^{3}H]tubercidin in the mutant cells was studied. AK activity was measured in the cell extracts of sensitive, first-step mutant and second-step mutant cell extracts at the same time by using the procedure described in Methods. Results of these studies are shown in Fig. 7. These results suggested that the Toy^{rII} mutants contained similar levels of residual AK activity (< 0.5% of WT cells) as was seen in the Toy^{rI} mutants. To increase the sensitivity of AK assay, higher specific activity adenosine was employed as substrate. However even then identical results were obtained. At that time, it was considered

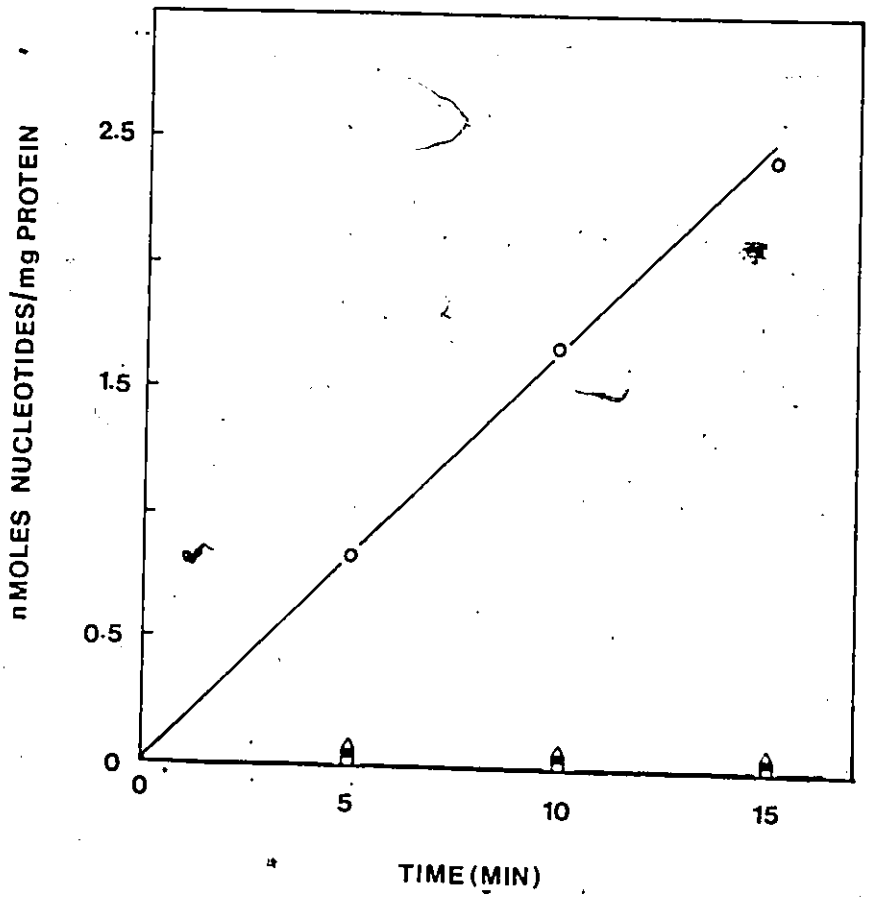


Figure 7: Adenosine kinase activity in the extracts of parental and mutant cells. Cell extracts were prepared for each cell line by sonication followed by centrifugation. AK activity was measured by the synthesis of lanthanum chloride precipitable AMP as described in Methods. The assay procedure for the enzymes is described in Methods. O——O, $Gat^{-}WT$; Δ——Δ, $Gat^{-}Toy^{rII}16$; ■——■, $Gat^{-}Toy^{rII}1$; □——□, $Gat^{-}Toy^{rII}2$.

possible that the enzyme AK may not carry out the phosphorylation of these drugs in first-step mutants but instead these analogs are phosphorylated by some other nonspecific cellular activity. Therefore, cellular uptake and phosphorylation of [^{3}H]adenosine in the first-step and second-step mutants were compared. As one can see in Table IV, the cellular uptake and phosphorylation of [^{3}H]adenosine in the Toy^{rII} mutants were also found to occur to a similar extent as seen in the first-step mutants. These results provided evidence that the genetic lesion in the second-step mutants does not further affect the level of phosphorylation of adenosine or its analogs.

3.1.1.4 Behaviour of Second-Step Mutants in Hybrid Cells

The behaviour of the Toy^{rII} mutation in somatic cell hybrids was examined by constructing hybrids between Toy^{rII} mutants and Toy^S (EOT) cells by a procedure described in the Methods section. As described in the Methods section, cell lines for crosses were chosen in such a way that while one of the parents was ouabain-resistant and unable to grow in HAT medium (containing hypoxanthine, aminopterin, and thymidine) (Oua^{R}HAT^S), the other parent had wild-type phenotype (i.e., Oua^{S}HAT^R) for these markers. Some of the clones which grew in the selective medium were picked and their hybrid nature was confirmed by chromosomal analysis. The degree of resistance of various hybrid and parental cell lines toward toyocamycin was examined. The complete dose-response curves of one representative hybrid clone is shown in Fig. 8. From the results, it is clear that the hybrid formed between

Table IV. Cellular Uptake of [^{3}H]Adenosine by the Parental and Mutant Cell Lines

Cell line	Duration of uptake (min)	[^{3}H]Adenosine* uptake/5x10^5 cells (cpm x 10^5)	Uptake relative to the sensitive line (%)
Gat$^-$WT	10	3.20 ± 0.01	100 ± 0.4
Gat$^-$Toy^{rI}16	10	0.32 ± 0.007	10 ± 0.12
Gat$^-$Toy^{rII}1	10	0.25 ± 0.006	7.8 ± 0.3
Gat$^-$Toy^{rII}2	10	0.28 ± 0.005	8.7 ± 0.2

The cellular uptake and incorporation of adenosine by various cell lines was studied as described in Methods. The cellular uptake of [^{3}H]tubercidin in various Toy^{rI} and Toy^{rII} mutants was also found to be reduced to similar extent, i.e., between 5 and 6% of the sensitive cells (results not shown).

* Mean ± S.D.

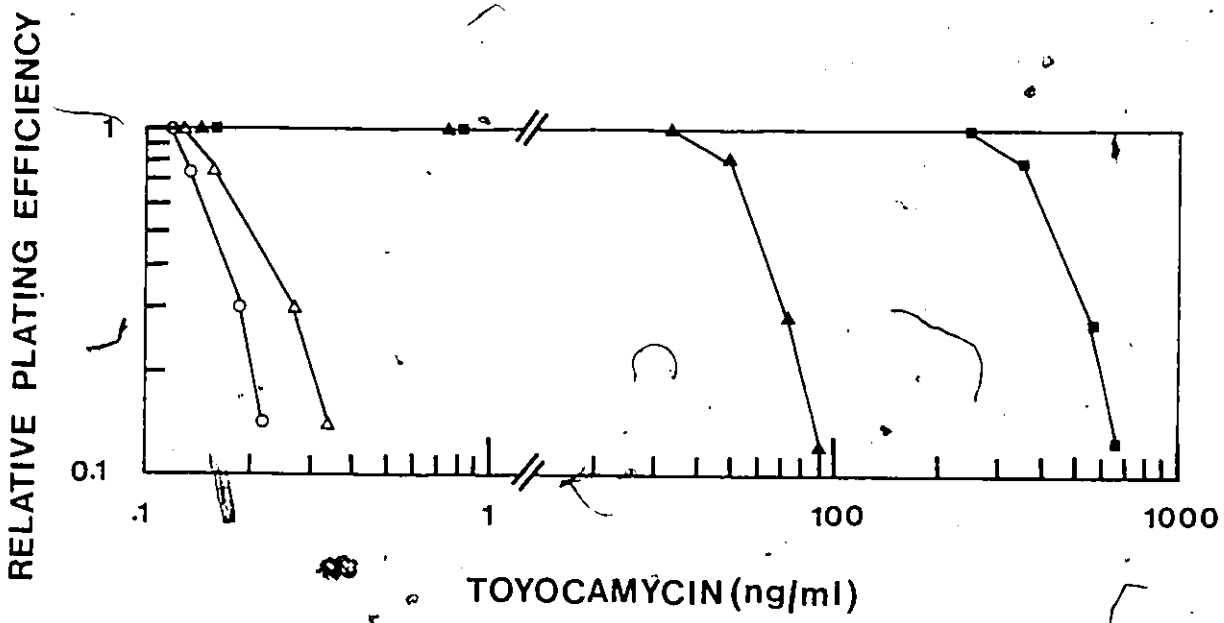


Figure 8: Dose-response curves for toyocamycin for the parental and hybrid ($Toy^{rII}2$ x Toy^{S}) cell lines. Somatic cell hybrids were constructed by using PEG-DMSO procedure and selected in HAT + ouabain medium as described in Methods. O——O, EOT; ▲——▲, $Gat^{-}Toy^{r}16$; ■——■, $Gat^{-}Toy^{rII}2$; △——△, EOT x $Gat^{-}Toy^{rII}2$ hybrid clone.

sensitive and resistant cells are sensitive to the drug. The sensitivity of hybrids, like toyocamycin sensitive cells, show that the Toy^{rII} mutation behaves recessively in somatic cell hybrids.

3.1.1.5 Effect of Adenosine Analogs on Macromolecular Sythesis

In earlier studies, it has been shown that in suspension culture of CHO cells, cellular DNA, RNA and protein synthesis were affected differently by toyocamycin and tubercidin (Gupta and Siminovitch, 1978b). For example, toyocamycin affected RNA synthesis first and then DNA and protein synthesis, whereas in the case of tubercidin, protein and DNA synthesis were inhibited before any effect on RNA synthesis was observed. The increased cross-resistance of Toy^{rII} mutants to sangivamycin and TCN indicated that the mechanism of cellular toxicity of these analogs may be similar to toyocamycin. To obtain further evidence in this regard, the effects of treatment of the above 7-deazapurine nucleosides on the macromolecular incorporation of [^{3}H]uridine, [^{3}H]thymidine and [^{3}H]leuicine, in suspension culture of CHO cells were examined. The experiments were carried out as described in the Methods. Drugs and their final concentrations were, toyocamycin (0.2 µg/ml), tubercidin (2.0 µg/ml), sangivamycin (2.0 µg/ml) and TCN (5.0 µg/ml). The results of these studies are shown in Fig. 9. As can be seen, the effects of sangivamycin and TCN on macromolecular synthesis in CHO cells were analogous to those observed with toyocamycin, i.e. RNA synthesis was inhibited first and then DNA and protein synthesis were affected. In contrast, both protein and DNA synthesis were inhibited very rapidly with tubercidin, whereas RNA

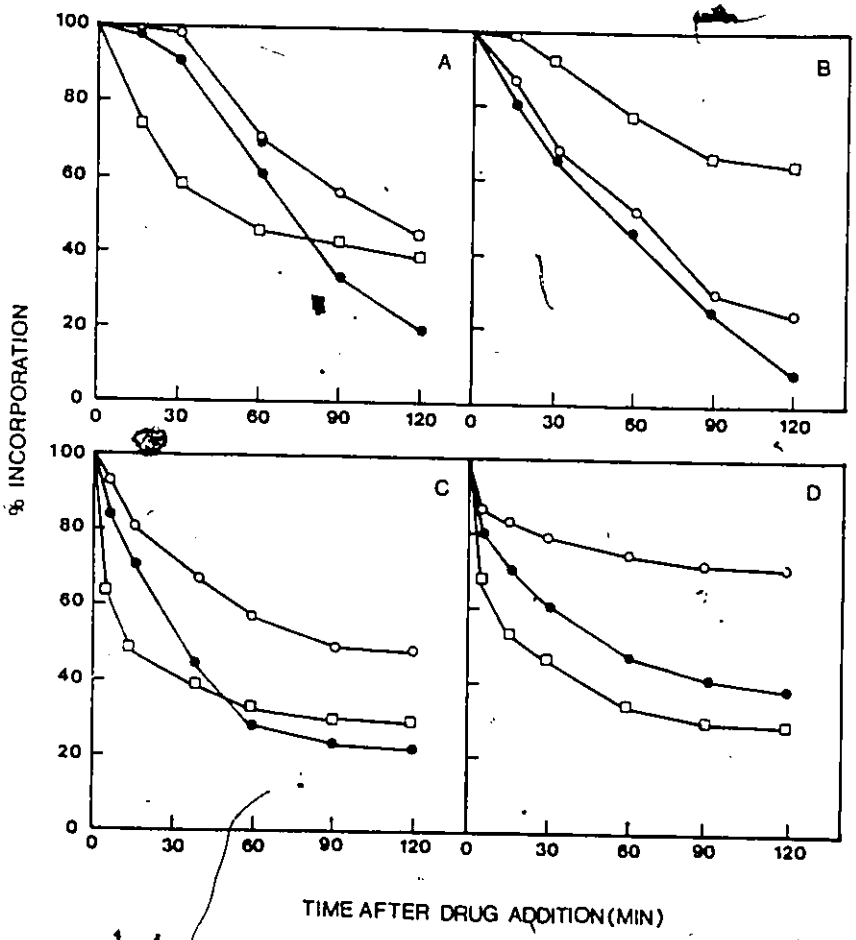


Figure 9: Effects of various 7-deazapurine nucleosides on the macromolecular incorporation of [^{3}H]uridine, [^{3}H]thymidine and [^{3}H]leucine in CHO cells. Kinetics of inhibition of DNA, RNA and protein synthesis by 7-deazapurine nucleosides in suspension culture of CHO cells were carried out as described in Methods. (A) toyocamycin, (B) tubercidin, (C) TCN, (D) sangivamycin. O——O, [^{3}H]leuicine; ●——●, [^{3}H]thymidine; □——□, [^{3}H]uridine.

synthesis was affected only at later times (Fig. 9). Thus toyocamycin, sangivamycin and TCN inhibited macromolecular synthesis in a manner different from that observed for tubercidin.

3.1.2 DISCUSSION

This section describes the selection and some biochemical characteristics of second-step mutants of CHO cells which display specific increase in their resistance towards a number of 7-deazapurine ribonucleosides. Although the biochemical function which is affected in the second-step mutants has not yet been identified, our results show that the lesion in these mutants does not further affect the level of phosphorylation of adenosine or its derivatives. Since mutants affected in the phosphorylation step are expected to exhibit cross-resistance to all of the adenosine derivatives, the lack of increased resistance of the second-step mutants to the related 7-deazapurine nucleoside, tubercidin, also supports this inference. The above observations indicate that the molecular lesion in the second-step mutants is very specific and may occur at the cellular site which is inhibited by the phosphorylated derivatives of toyocamycin.

In earlier studies with emetine and podophyllotoxin resistant mutants, it has been shown that the cross-resistance pattern of mutant cells can provide valuable information regarding other compounds which act in the same manner (Gupta, 1983a,c; Gupta et al., 1980). In this context, the lack of cross-resistance of the Toy^{rII} mutants to tubercidin provides strong evidence that the mechanisms of cellular

toxicity of toyocamycin and tubercidin are not identical. At the same time, the proportionally increased cross-resistance of the Toy^{rI} and Toy^{rII} mutants to sangivamycin and TCN provides suggestive evidence that the mechanism of cellular toxicity of these two adenosine analogs should be similar to that of toyocamycin. These inferences are supported by the inhibitory effects of the above nucleoside analogs on cellular macromolecular synthesis. Whereas toyocamycin, sangivamycin and TCN all showed greater inhibition of RNA synthesis initially, in the case of tubercidin, DNA and protein synthesis were inhibited first in comparison to the effect on RNA synthesis. These results show that the mechanism of cellular toxicity of toyocamycin, sangivamycin and TCN is different from that of tubercidin.

In addition to toyocamycin, several attempts have been made to obtain second-step mutants exhibiting increased resistance to tubercidin (Tub^{rII}). However, thus far all attempts to obtain any Tub^{rII} mutants have been unsuccessful. Failure to obtain any Tub^{rII} mutants provide a further support to the inference that the target sites of tubercidin and toyocamycin are different.

As shown earlier, Toy^{rII} mutants can be obtained easily at a mutation frequency of 10^{-6}. Therefore, recessive behaviour of second-step mutants in somatic cell hybrids (Toy^{S} x Toy^{rII}) raise the possibility that the genetic locus affected in second-step mutants may be functionally hemizygous as has been observed earlier for other loci in CHO cells (Campbell and Worton, 1978; Gupta et al., 1978a; Gupta et al., 1978b; Gupta and Siminovitch, 1978b; Siminovitch, 1976). At the

same time, failure to obtain Tub^{rII} mutants indicate that either the both copies of the cellular target site are functional in CHO cells or the cellular toxicity of tubercidin results from the inhibition of or incorporation into more than one cellular reaction by its metabolite(s). In either case, development of resistance to higher concentration of tubercidin would require occurrence of two or more independent mutations at the same time which normally occurs at a very low frequency in cells and as a result Tub^{rII} mutants cannot be readily isolated in single step.

Earlier studies with toyocamycin and sangivamycin have shown that both these analogs are preferentially incorporated into RNA (Hadjiolova et al., 1981; Ritch and Glazer, 1982a; Tavitian et al., 1968). On the other hand, in the case of TCN, its phosphorylation has been observed only to the 5'-monophosphate form and no incorporation into either RNA or DNA has been observed (Bennett et al., 1978; Plagemann, 1976; Schweinsberg et al., 1981). These results indicate that the active form of TCN is its 5'-monophosphate which most likely inhibits an essential cellular reaction. Since the second-step toyocamycin resistant mutants are affected in a function which leads to increased resistance to toyocamycin, sangivamycin as well as TCN, it is expected that the resistance mechanism (i.e., affected function) should not involve either the higher phosphorylated forms of these analogs or their incorporation into RNA. If this is true then the cellular toxicity of all three of these analogs (viz. toyocamycin, sangivamycin and TCN) should most likely be due to inhibition of essential cellular

reaction by their 5'-monophosphate derivatives.

It is interesting to note in this regard that for an adenosine analog formycin A, the mechanism of cellular toxicity was considered to be due to its phosphorylation and subsequent incorporation into macromolecules (Crabtree et al., 1981; Glazer and Lloyd, 1982; Majima et al., 1977). However, recently mutants which exhibit resistance to formycin B and formycin A have been reported in Leishmania, and these mutants show similar levels of cellular uptake as well as incorporation of [^{3}H]formycin A into macromolecules as parental cell line. This result has led to the suggestion that the incorporation of nucleotides of formycin A into macromolecules is not responsible for its cellular toxicity (Robinson et al., 1984). It has been suggested that the nucleotides of formycin A inhibit some cellular reactions which leads to a depletion of the essential metabolite(s) in treated cells. In view of this observation and above results, it is likely that the cellular toxicity of pyrrolopyrimidine ribosides may result due to inhibition of essential cellular reactions by their nucleotides, instead of their incorporation into RNA.

In order to better understand the mechanism of cellular toxicity and resistance to toyocamycin in mammalian cells, further studies on the identification of the cellular component affected in Toy^{rII} mutants were planned. Future studies were designed to test the possibility that 5'-monophosphate of toyocamycin is responsible for its cellular toxicity, by studying the incorporation of radiolabelled toyocamycin into macromolecules of first as well as second-step

mutants. Radiolabelled toyocamycin was not commercially available, therefore it was decided to obtain it by the custom labelling service of New England Nuclear Corp., Boston, MA. Radiolabelled toyocamycin was purified by paper chromatography to a single spot and its specific activity was determined. It was found that even on longer incubation, no appreciable incorporation of it into nucleic acids was observed for any of the cell lines, probably because of the low specific activity of the purified toyocamycin. As a result, no successful experiments could be performed to understand more about the mechanisms of actions of pyrrolopyrimidine nucleosides as well as the phenotype of the $Toy^{r}II$ mutants.

3.2 FORMYCIN A-RESISTANT MUTANTS OF CHINESE HAMSTER OVARY CELLS: NOVEL GENETIC AND BIOCHEMICAL PHENOTYPE AFFECTING ADENOSINE KINASE

The pyrazolopyrimidine ribosides, formycin A and formycin B constitute an important class of nucleoside analogs (C-nucleoside) which rather than having the usual C-N bond in the ribosidic linkage, are linked by a C-C bond (Daves and Cheng, 1976; Gutowski et al., 1973; Prusiner et al., 1973; Suhadolnik, 1979; Ward and Reich, 1968). As a result of this structural modification, these antibiotics possess very useful biochemical and medicinal properties, including antileishmanial, antiviral, anticancer and immunosuppressive activity (Carson and Chang, 1981; Ishida et al., 1967; Ishizuka et al., 1968; Kunimoto et al., 1968; Spermulli et al., 1983; Willemot, 1979). As discussed in the introduction, earlier biochemical studies with these analogs have indicated that formycin A like other adenosine analogs (e.g., toyocamycin and tubercidin), is phosphorylated in mammalian cells (Umezawa et al., 1967). Furthermore, the phosphorylated derivatives of formycin A, being structurally similar to adenine nucleotides have been reported to substitute or interfere with the metabolism of adenosine in a wide variety of cellular reactions. In view of their novel chemical structures, mutants resistant to formycin A have been isolated in CHO cells to investigate the similarity and differences in the mechanism of cellular resistance to this C-nucleoside with other adenosine analogs. This section describes the

various genetic and biochemical characteristics of one class of mutants resistant to formycin A that contain a novel type of mutation which affects AK in such a way that the phosphorylation of C-nucloeosides is affected without affecting the phosphorylation of N-nucleosides.

3.2.1 RESULTS

3.2.1.1 Selection of Mutants Resistant to Formycin A/Formycin B

While the cross-resistance of toyocamycin-resistant (Toy^r) mutants of CHO cells to various adenosine analogs was being examined, it was observed that in contrast to the other adenosine analogs such as toyocamycin, tubercidin, or 6-MeMPR, to which the Toy^r mutants were highly resistant (>100-fold), these mutants exhibited only very slight (approximately two fold) cross-resistance to formycin A (see Fig. 10). These experiments suggested that the toxicity of formycin A was mediated by a mechanism different from that of other adenosine analogs.

To understand the mechanism of cellular resistance to formycin A, mutants which exhibited increased resistance to it were obtained in CHO cells. The dose-response curve of WT (CHO) cells in the presence of different concentrations of formycin A showed that the plating efficiency of WT cells was reduced sharply at drug concentrations between 1 and 5 µg/ml (Fig. 10), and at 10 µg/ml, no colonies were obtained, even when 5×10^5 cells were plated in a dish. The selection of mutants resistant to formycin A was carried out in the presence of 10 µg of the drug per ml in both control and mutagen-treated cultures. In the control culture, no colonies were obtained from a total of 2.5×10^6 viable cells which were plated in presence of the drug in these

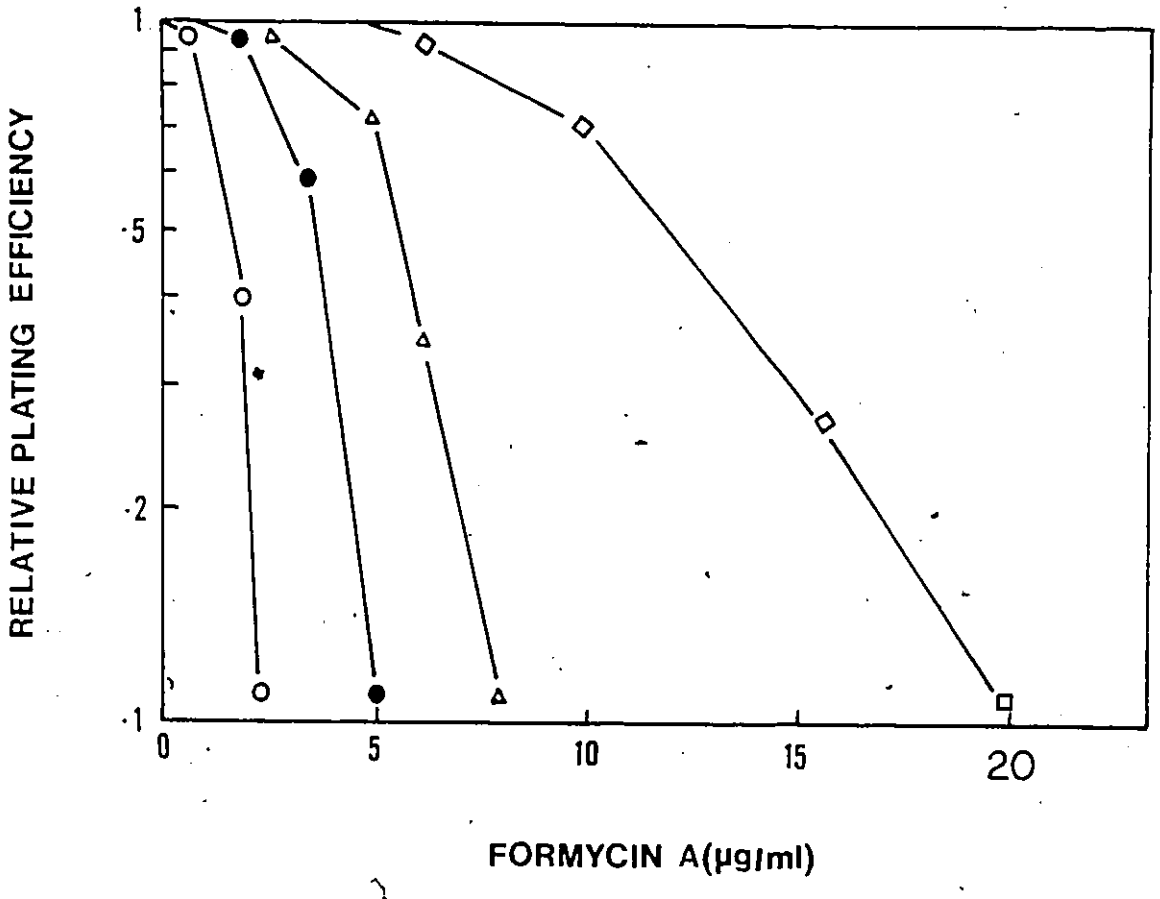


Figure 10: Survival curves of parental and the Fom^R and Toy^r mutants cell lines in the presence of increasing concentrations of formycin A (in the absence of EHNA). O——O, WT; Δ——Δ, Fom^R2; □——□, Fom^R4; ●——●, Toy^r4.

experiments. However, in the culture which was pretreated with the mutagen EMS, a number of resistant colonies were observed in various dishes, and the mutant frequency was estimated to be about 1.0×10^{-5}. Some of the resistant colonies which appeared in different dishes in these experiments were picked and grown in nonselective medium; then their degree of resistance towards formycin A was determined. All six colonies which were examined proved more resistant to formycin A as compared with the parental WT cells. The dose-response curves of two mutant clones, Fom^R2 and Fom^R4, which were chosen for further studies, are shown in Fig. 10. Based upon their D_{10} values, these mutants are about 3-(Fom^R2) and 8-(Fom^R4) fold more resistant to formycin A as compared with the WT cells. The different levels of resistance of these mutants to formycin A indicate that these mutants arose from independent genetic lesions. The drug-resistant phenotype of both these mutants has remained completely stable upon subcloning in the absence of formycin A and upon growth in nonselective medium for more than one year.

After obtaining the mutants described above, it was realized that formycin A, which is an adenosine analog, is a good substrate for the enzyme ADA, which is present in large amounts both in CHO cells as well as in fetal calf serum (Ishii and Green, 1973). Because of this, formycin A, under the conditions used in these experiments, was most probably rapidly deaminated into formycin B, which may have been the actual selective agent. This was consistent with the fact that all of the mutants selected for resistance to formycin A exhibited

cross-resistance to formycin B, and the concentration responses of various cell lines towards these two analogs were virtually identical (Fig. 11). Further evidence supporting this possibility was provided by experiments in which the toxicity of formycin A was determined in the presence of EHNA, which is a very potent inhibitor of the enzyme ADA (Agarwal et al., 1977). In the presence of 10 µg of EHNA per ml in the growth medium, a concentration which completely inhibits ADA activity but has no observable effect on the growth of cells, the toxicity of formycin A towards WT CHO cells was greatly enhanced (Fig. 12). Interestingly, the Toy^r4 mutant which showed only slight cross-resistance to formycin A in the absence of EHNA became highly resistant to this drug when EHNA was present. This result indicated that in the presence of EHNA, when deamination of formycin A was prevented, it behaved like other adenosine analogs such as toyocamycin or 6-MeMPR, which are phosphorylated by the enzyme AK. Furthermore, in accordance with earlier observations with toyocamycin and tubercidin (Gupta and Siminovitch, 1978b; Rabin and Gottesman, 1979), mutants resistant to formycin A (in the presence of EHNA) can be obtained in both control and mutagen-treated CHO cell cultures at very high frequencies (10^{-3} to 10^{-4}; data not shown). All of these observations strongly suggested that formycin A under the mutant selection conditions employed was largely converted into formycin B, and that similar mutants may also be obtained by directly selecting for formycin B.

In the presence of EHNA, sensitivity of WT cells towards

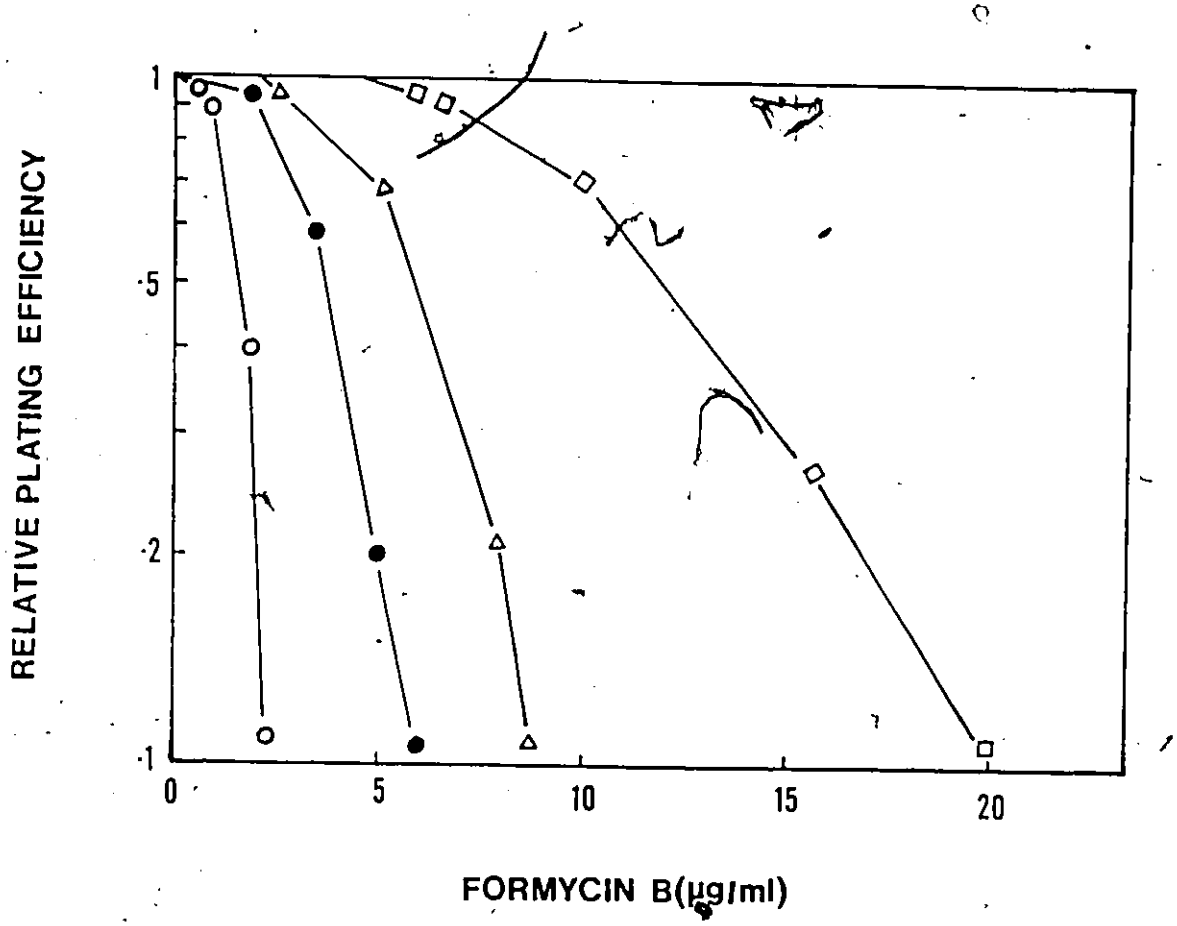


Figure 11: Survival curves of different cell lines for formycin B. O—O, WT; Δ—Δ, Fom^R2; □—□, Fom^r4; ●—●, Toy^r4.

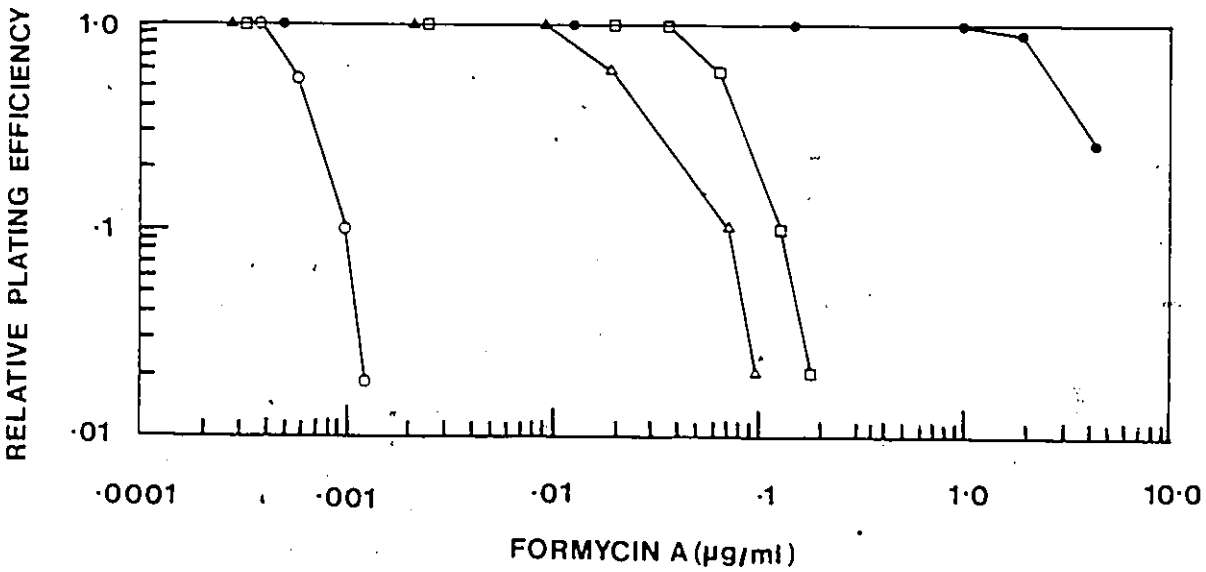
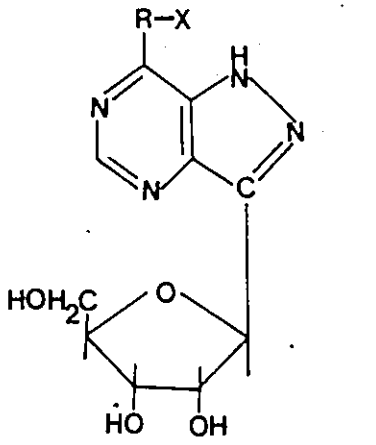


Figure 12: Dose-response curves of different cell lines for formycin A in medium containing EHNA (10 µg/ml). O——O, WT; Δ——Δ, Fom^R2; □——□, Fom^R4; ●——●, Toy^r4.

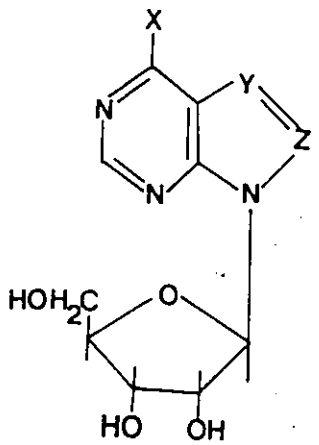
formycin A was greatly enhanced and the degree (i.e. fold) of resistance of the two Fom^R mutants was appreciably altered (Fig. 12). In comparison to WT cells, the two Fom^R mutants, Fom^R2 and Fom^R4, were found to be about 70-and 150-fold resistant to formycin A in the presence of EHNA. The resistance of Fom^R mutants to formycin B as well as to formycin A (+EHNA) suggested that in these mutants, a step which is common in the action of both these analogs is very likely affected.

3.2.1.2 Cross-Resistance Pattern of the Fom^R and Toy^r Mutants Towards Various Nucleoside Analogs

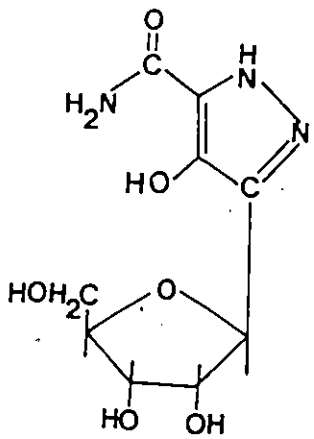
To characterize the Fom^R and Toy^r mutants further, their cross-resistance pattern towards a number of different nucleoside analogs was examined. In these experiments, cellular toxicity of different nucleoside analogs towards various cell lines was determined in parallel in medium containing 10 µg EHNA/ml. From the D_{10} values of the nucleoside analogs for different cell lines, the degree of resistance of the mutant cell lines in comparison to the parental sensitive cell line was calculated. Results of these studies for the parental CHO cells (WT), the two formycin A-resistant mutants (Fom^R2, Fom^R4) and two toyocamycin-resistant mutants (Toy^r4 and Toy^r5) are summarized in Table V. Similar results for these nucleoside analogs with these cell lines have been obtained in at least two independent experiments. As can be seen, the two Toy^r mutants examined showed high degree of cross-resistance to various adenosine analogs (see Fig. 13 for their chemical structures) which included both N-nucleosides viz. toyocamycin (2c), tubercidin (2b), 6-MeAPR (2d), 6-MeMPR (2e),



1 a, R=O , X=H
b, R=NH, X=H
c, R=NH, $X=CH_2C_6H_5$
d, R=NH,
$X=CH_2-CH=CH<^{CH_3}_{CH_3}$



2 a, $X=NH_2$, Y=N, Z=CH
b, $X=NH_2$, Y=CH, Z=CH
c, $X=NH_2$, $Y=C-C\equiv N$, Z=CH
d, $X=NH-CH_3$, Y=N, Z=CH
e, $X=S-CH_3$, Y=N, Z=CH
f, $X=NH_2$, Y=N, Z=N



PYRAZOFURIN

Figure 13: Structural formulae of various nucleoside analogs used in the present study.

Table V. Cross Resistance Patterns of Toy^r and Fom^R Mutants Towards Various Nucleoside Analogs

Nucleoside Analog	D_{10} value of the[a] analog for the WT cells	Relative resistance of[b] the mutant cell lines			
		Fom^R2	Fom^R4	Toy^r4	Toy^r5
C-Nucleosides					
Formycin A	(1.0 ng/ml) 1	70	150	>500	>500
Formycin B	(2.5 µg/ml) 1	3	8	2	2
Bbb-73	(2.0 µg/ml) 1	>50	>50	50	50
Bbb-85	(0.02 µg/ml) 1	100	350	1250	>1000
Pyrazofurin	(15.0 ng/ml) 1	20	45	1000	1200
N-Nucleosides					
Toyocamycin	(0.4 ng/ml) 1	1	2	500	400
Tubercidin	(1.0 ng/ml) 1	2	3	1000	1000
8-Azaadenosine	(3.0 ng/ml) 1	1	1	>1000	>1000
6-MeAPR	(0.2 µg/ml) 1	2	2	>500	>500
6-MeMPR	(0.25 µg/ml) 1	3	5	>400	>400

[a] The D_{10} value of a nucleoside analog towards the WT cells represent the concentration of the analog which reduces plating efficiency of the cells to 10% of that observed in the absence of any drug.

[b] Assuming the D_{10} value of an analog towards WT cells as 1, the relative degrees of resistance of the mutant cell lines were determined from the ratios of the D_{10} values of the mutant cell lines compared to the WT cell lines.

8-azaadenosine (2f), as well as C-nucleosides viz. formycin A (1b), Bbb-73 (1c), Bbb-85 (1d) and the structurally distinct nucleoside pyrazofurin which is also phosphorylated via adenosine kinase (Dix et al., 1979). These mutants also exhibited slight cross-resistance to formycin B (1a), which is an inosine analog. Very interestingly, in contrast to the Toy^r mutants, the two Fom^R mutants showed no appreciable cross-resistance to the different N-nucleosides examined viz. toyocamycin, tubercidin, 6-MeAPR, 6-MeMPR and 8-azaadenosine, but were highly resistant to various C-nucleosides which included formycin A, formycin B, Bbb-73, Bbb-85 and the structurally distinct nucleoside, pyrazofurin.

The above results suggested that the genetic lesion in the Fom^R mutants is such that it confers resistance to only C-nucleosides but not to N-nucleosides. This possibility was tested by examining the cross-resistance of the Fom^R and Toy^r mutants towards a newly synthesized adenosine analog, 9-deazaadenosine, in which the N atom at the 9 position is replaced by a C atom, resulting in a C - C glycosidic linkage (Lim and Klein, 1981). The dose-response curves of the Fom^R and Toy^r mutants towards 9-deazaadenosine are shown in Fig. 14. As can be seen, the Toy^r mutants are highly resistant to this analog, indicating that like other adenosine analogs, 9-deazaadenosine is also phosphorylated via AK (Zimmerman et al., 1983). Interestingly, the two Fom^R mutants also exhibited high degree of resistance to this compound, providing further support to our inference that these mutants exhibit resistance specifically to C-nucleosides.

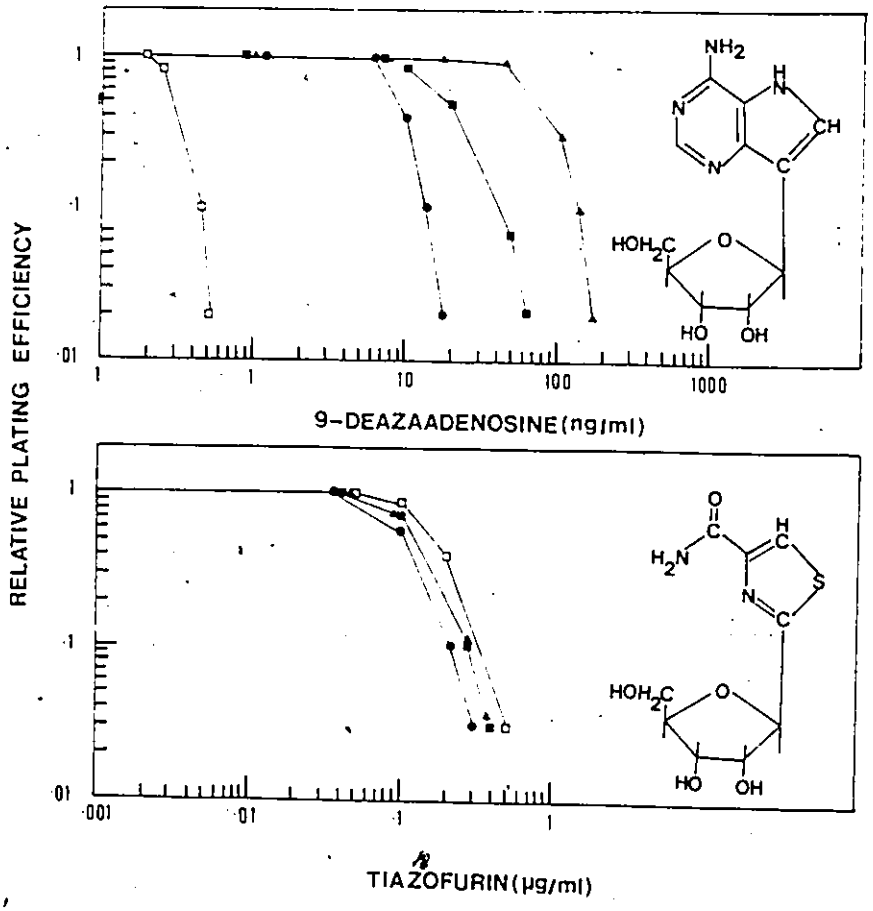


Figure 14: Dose-response curves of various cell lines towards 9-deazaadenosine and tiazofurin. The relative plating efficiency of various cell lines in medium containing different concentrations of the drugs were determined as described in Methods. ○——○, WT; ●——●, Fam^R2; ■——■, Fam^R4; △——△, Toy^r4; ▲——▲, Toy^r5.

The cross-resistance of the Toy^r and Fom^R mutants to another recently synthesized C-nucleoside tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide) was also examined (Srivastava et al., 1977). Tiazofurin (see Fig. 14) is structurally related to ribavirin and pyrazofurin. The major difference between pyrazofurin and tiazofurin is that the former contains a 4-OH pyrazole ring, instead of a thiazole ring in tiazofurin. Recent studies on tiazofurin have shown that this analog is phosphorylated in mammalian cells and cellular mutants lacking AK activity are not resistant to this compound, indicating that AK is not involved in its phosphorylation (Saunders et al., 1983). Since tiazofurin is a C-nucleoside, it was of much interest to determine whether the Fom^R mutants which exhibited resistance to other C-nucleosides would show resistance to this compound. Results of these studies which are presented in Fig. 14 showed that neither the Toy^r nor the Fom^R mutants exhibited any cross-resistance towards tiazofurin. The lack of cross-resistance of the Toy^r mutants to tiazofurin supports the inference from earlier studies that this compound is not phosphorylated via AK. These studies also demonstrate that the Fom^R mutants are specifically resistant to only those C-nucleosides which are phosphorylated via AK.

3.2.1.3 Deficiency of AK in the Extracts of Fom^R Mutants

Earlier studies have shown that most of the nucleoside analogs require initial phosphorylation before they become toxic to cells, and that the vast majority of mutants resistant to various nucleoside

analogs are affected in the phosphorylating enzymes (Bennett et al., 1966; Gupta and Siminovitch, 1978b; McBurney and Whitmore, 1974a; Rabin and Gottesman, 1979; Thacker, 1980). We therefore examined whether these mutants were affected in any of the purine nucleoside phosphorylating enzymes. Currently, no enzyme which specifically phosphorylates inosine or inosine analogs is known in mammalian cells. However, the major purine nucleoside phosphorylating activity that is present in CHO cells is of AK; therefore, initially the activity of AK and the protein concentration in the parental and various mutant cell extracts were determined. The assay of AK activity in various cell extracts was carried out as described in Methods, and the results of these studies are shown in Fig. 15. As can be seen, extracts from the WT cells phosphorylated [^{3}H]adenosine in a linear time-dependent manner. In contrast to the WT cell extract, extracts from both the Fom^R mutants could not synthesize any lanthanum chloride precipitable adenine nucleotides and therefore contained no measurable AK activity. In this regard, these mutants were very similar to the Toy^r mutants, which also lacked this enzyme. Similar results with these mutants were obtained in a number of independent experiments.

In view of the sensitivity of the Fom^R mutants to N-nucleosides, the absence of AK activity in cell extracts from such mutants was surprising. Since our routine procedure for preparation of cell extracts involved trypsinization and sonication of cells, the possibility that such treatments may cause inactivation of the mutant enzyme was considered. To exclude this and related possibilities, cell

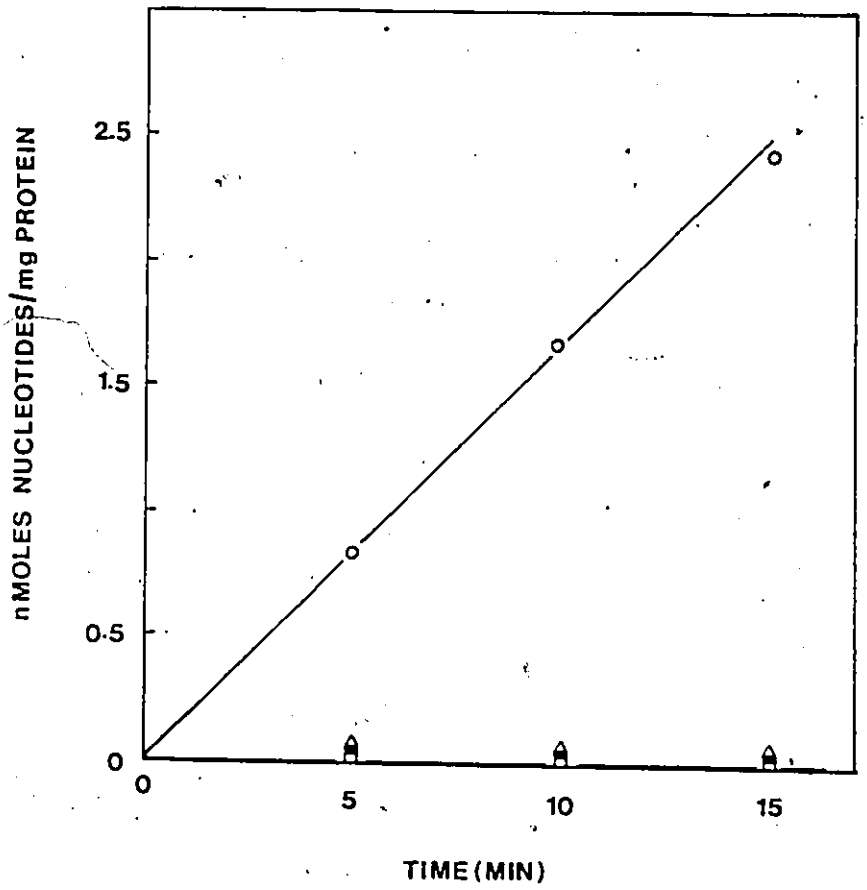


Figure 15: Adenosine kinase activity in the extracts of wild type and mutant cells. The assay for AK activity and estimation of protein in cell extracts were carried out as described in Methods. O——O , WT; Δ——Δ, Fom^R2; ■——■, Fom^R4; □——□, Toy^r4.

extracts from various cell lines were prepared in a number of different ways, including (i) growth of cells in suspension culture followed by sonication, (ii) growth of cells in suspension culture and the preparation of cell extracts by swelling in hypertonic buffer followed by Dounce homogenization, (iii) examination of AK activity in crude cell extracts before centrifugation and dialysis, (iv) measurement of AK activity in presence of varying concentrations of both [^{3}H]adenosine (10 μM to 1.0 mM) and ATP (10 μM to 12.5 mM) and also using substrates other than adenosine, (v) examination of AK activity in the presence of of 1% SDS, 1% Triton X-100, and 1% Nonidet P-40 as well as at different temperatures, and (vi) AK activity in the presence of 1 M 2-MSH as well as in the presence of 1 M and 2 M urea were also measured. Results from these studies showed that no AK activity could be detected in the extracts from Fom^R mutants under any of the conditions described above.

The possibility that the cell extracts from Fom^R mutants contain an inhibitor of AK activity was also investigated. This was done by mixing cell extracts from the WT and the mutant cells in different proportions and then measuring AK activity. Results from these studies showed that AK activity in such mixtures was exactly as much as may be expected from the relative proportion of the WT cell extract. These studies exclude the presence of an inhibitor of AK activity in mutant cell extracts, as well as activation of the mutant cell enzyme by the WT cell extracts.

3.2.1.4 Behaviour of the Fom^R Mutants in Somatic Cell Hybrids

The cross-resistance pattern of the Fom^R mutants and their lack

of AK activity in cell extracts indicated that these mutants were affected in AK in a manner different from the Toy^r mutants. It was, therefore, of interest to determine the behaviour of Fom^R mutants in somatic cell hybrids (Fom^R x Fom^S). This was carried out by constructing cell hybrids between the two Fom^R mutants and a Fom^S cell line EOT, which contained a number of genetic markers that are very useful for the selection of hybrid clones. The cell lines were fused, using PEG and DMSO, by a procedure as described in Methods. Subsequently, the selection for hybrid clones was carried out in HAT plus ouabain medium in which none of the parental cell lines survive but the hybrids between the two cell lines are able to grow. As expected, the hybrid clones were obtained in these crosses at high frequency ($\approx 10^{-2}$), whereas in control crosses (e.g., EOT x EOT or Fom^R x Fom^R cells), the frequency of cells which were able to grow in the selective medium was found to be less than 1 in 10^5.

Many of the hybrid clones from the crosses of Fom^R2 and Fom^R4 mutants with the EOT cell line were picked and grown in nonselective medium. The hybrid nature of these clones was ascertained by chromosomal analysis and all of the clones examined were found to be pseudotetraploid as may be expected for hybrids. Results with one of the hybrid (Fom^S x Fom^R) clone and parental cells are shown in Fig. 16. Subsequently, the degree of resistance of these hybrid clones towards formycin A and formycin B was determined. The dose-response curves towards formycin B of two representative hybrid clones from these crosses are shown in Fig. 17. All of the other hybrid clones examined

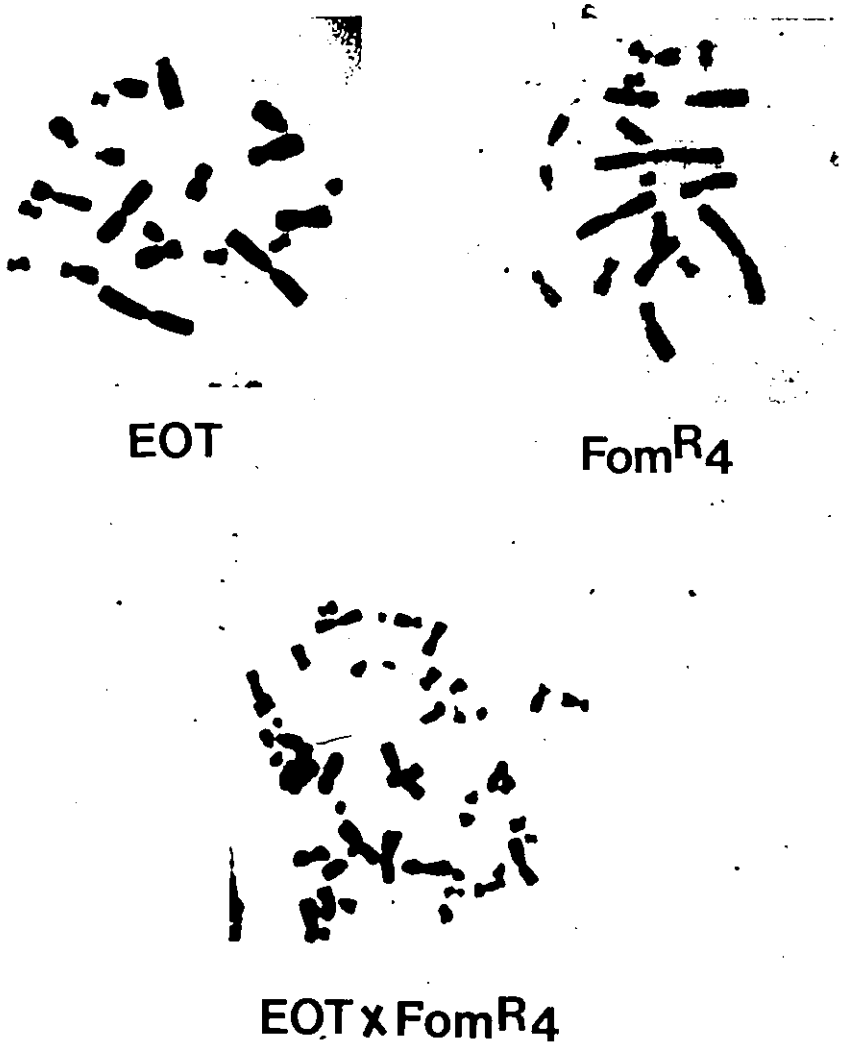


Figure 16: Metaphase preparations of the parental cells, EOT and Fom$^{R}_{4}$ and a hybrid cell, EOT x Fom$^{R}_{4}$ formed between them. These preparations were made according to procedure described in Methods.

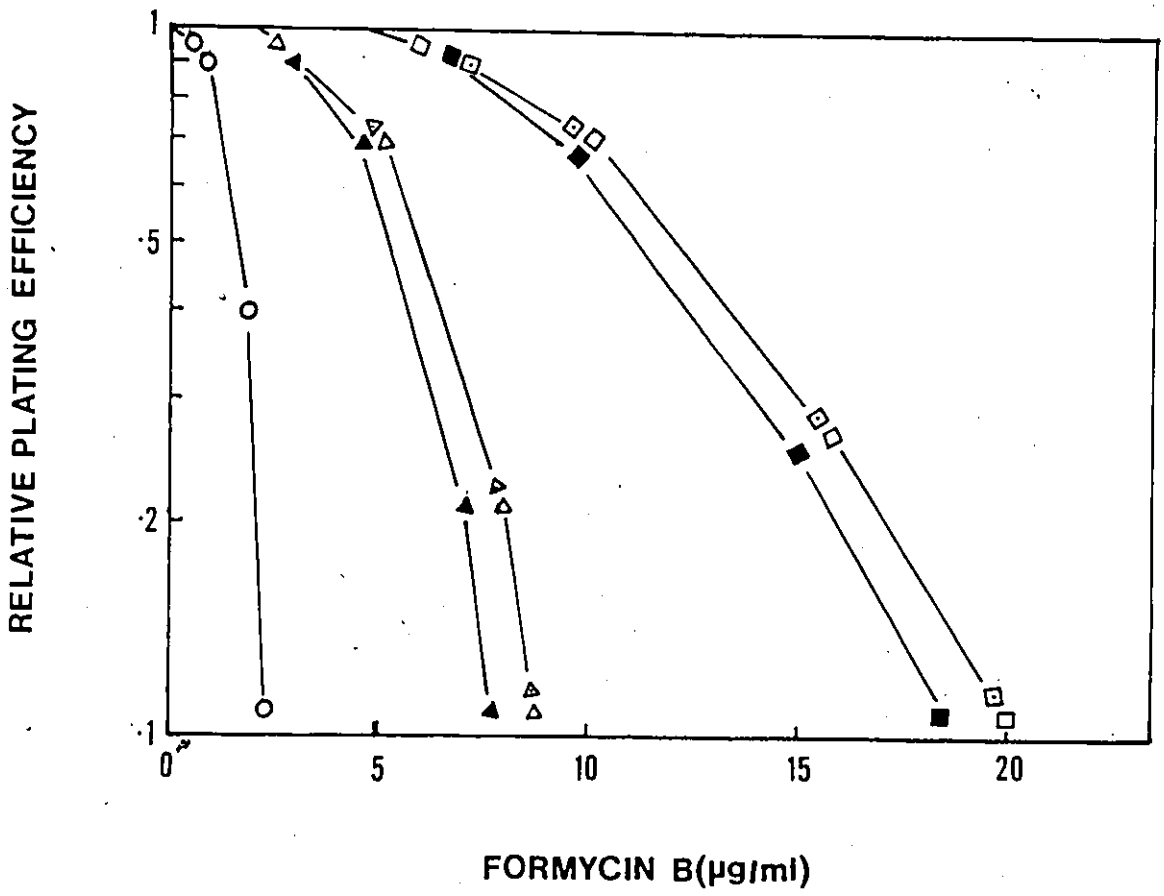


Figure 17: Survival curves in the presence of increasing concentrations of formycin B for the parental EOT and Fom^R cell lines and the Fom^R x Fom^S hybrid cell lines. The cells were fused using the PEG-DMSO procedure and selected in HAT medium supplemented with ouabain as described in methods. O——O , EOT; ▲——▲, Fom^R2, △——△ and △——△, EOT x Fom^R2 hybrid clones; □——□, Fom^R4; □——□ and ⊡——⊡, EOT x Fom^R4 hybrid clones.

(at least six from each cross) showed very similar results. As can be seen, the hybrids formed between the two Fom^R mutants and the EOT cell line were nearly as resistant to formycin B as the resistant parent (Fig. 17). These results indicated that the Fom^R phenotype of these mutants expresses itself in a dominant/codominant manner.

In view of the behaviour of Fom^R mutants in hybrids, it was of obvious interest to know if the hybrids formed between the Fom^R x Fom^S cells contained any AK activity or not. For such purposes, cell extracts were prepared in parallel from the parental cell lines and a number of hybrid clones, and the AK activity in such extracts was measured. The results of such studies are shown in Fig. 18. As can be seen, the specific activity of AK in cell hybrids formed between Fom^R x Fom^S lines was about 50% of that observed for the WT cells. These results indicated that the AK activity from the mutant cells was not being expressed in the hybrids and that this characteristic of the Fom^R mutant was not transdominant as has been observed for mutants affected in PNP (Hoffee et al., 1983) (therefore referred to as codominant expression). These results also provide further evidence against the presence of an inhibitor of AK activity in the mutant cells.

I next examined whether the AK deficiency of the Fom^R mutants can be complemented upon hybridization with an AK^-Toy^r mutant. The cell hybrids between Fom^R4 and a Toy^r mutant of EOT line (EOT Toy^r2) were constructed as before, and subsequently the AK activity in the extracts of such hybrids was measured. The results of these studies

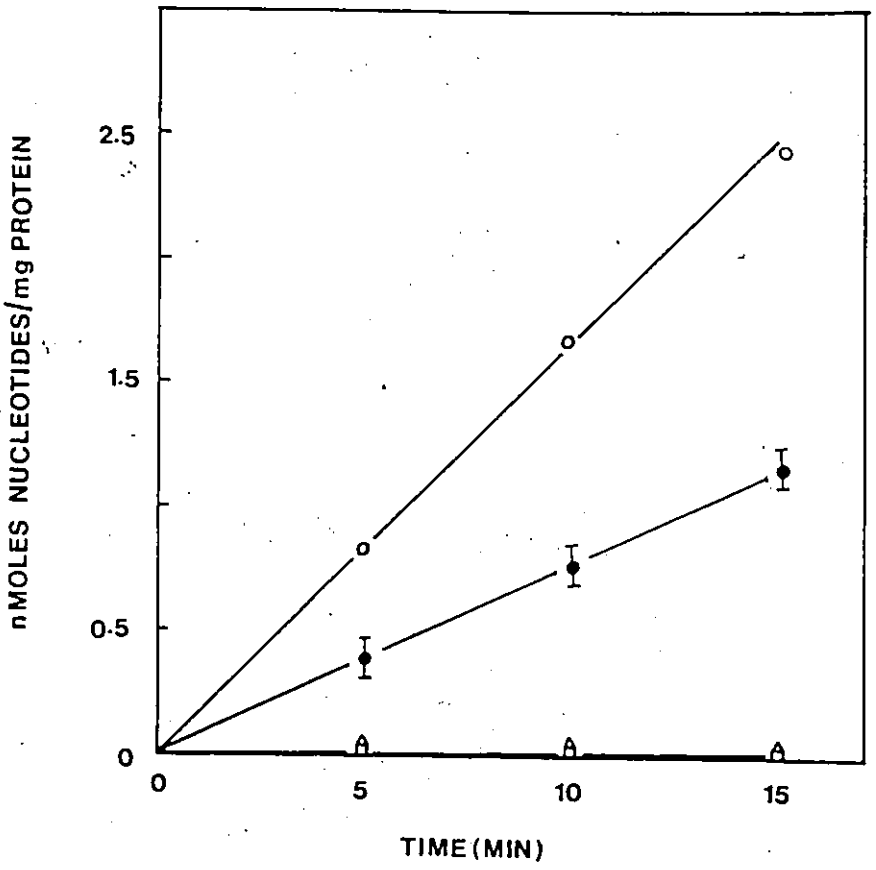


Figure 18: Adenosine kinase activity in the extracts of parental and the Fom^R x Fom^S hybrid cell lines. AK activity and proteins in various cell extracts were measured by procedures as described in Methods. O—O , EOT, Δ—Δ , Fom^R4; ●—● , EOT x Fom^R4 hybrid; □—□ , EOT Toy^r2 x Fom^R4 hybrid.

showed that none of the hybrids formed between Fom^R_4 and EOT Toy^r_2 contained any measurable AK activity (Fig. 18). The sensitivity of these hybrid cell lines towards formycin B and toyocamycin was also determined, and the representative results with two hybrid clones are shown in Figs. 19 and 20. As can be seen, the hybrids formed between cell lines were nearly as resistant to formycin B as the Fom^R parent, which again shows the codominant nature of the Fom^R phenotype. However, very interestingly the above hybrid cell lines were found to be as sensitive to toyocamycin as the WT cells, even though these hybrids showed no activity of AK in cell extracts.

3.2.1.5 Cellular Uptake and Phosphorylation of [^{3}H]Adenosine, [^{3}H]Tubercidin and [^{3}H]-Formycin A by Various Mutant Lines

Earlier studies on purine and pyrimidine nucleoside analogs have shown that the most common mechanism by which cells could become resistant to nucleoside analogs involves an alteration or deficiency in the phosphorylating step which is required for the lethal conversion of these analogs (Bennett et al., 1966; Chan and Juranka, 1981; Gupta and Siminovitch, 1978b; McBurney and Whitmore, 1974a; Rabin and Gottesman, 1979; Thacker, 1980). Therefore, the observed resistance of the Fom^R mutants to only C-nucleosides suggested that these mutants may be specifically affected in the cellular uptake and phosphorylation of C-nucleosides but not N-nucleosides. This possibility was tested by studying the cellular uptake and phosphorylation of two radiolabelled N-nucleosides, namely [^{3}H]adenosine, [^{3}H]tubercidin and a radiolabelled C-nucleoside, (viz. [^{3}H]formycin A) in various cell lines. Results of

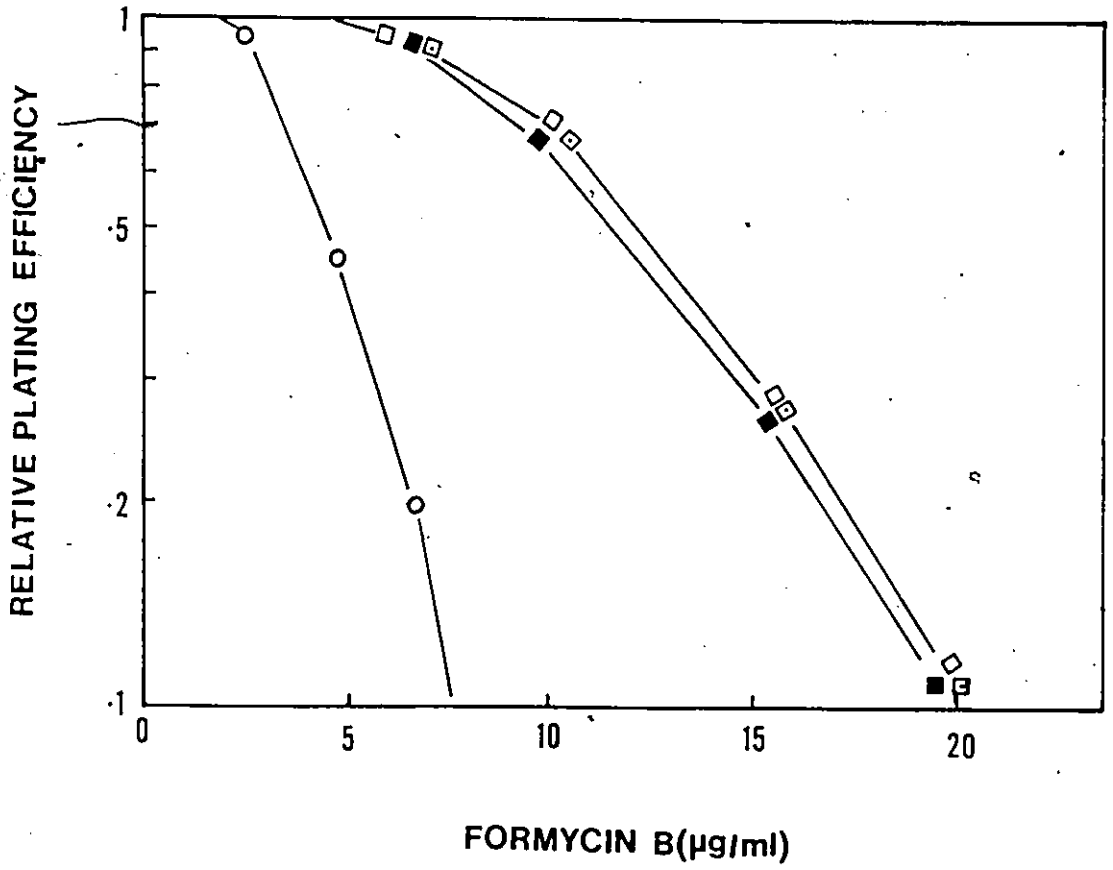


Figure 19: Dose-resonse curves in increasing concentrations of formycin B for the parental Fom^R and Toy^r cell lines and for the hybrids formed between them. O—O, EOT $Toy^r 2$; ■—■, $Fom^R 4$; □—□ and ⊡—⊡, EOT $Toy^r 2$ x $Fom^R 4$ hybrid clones.

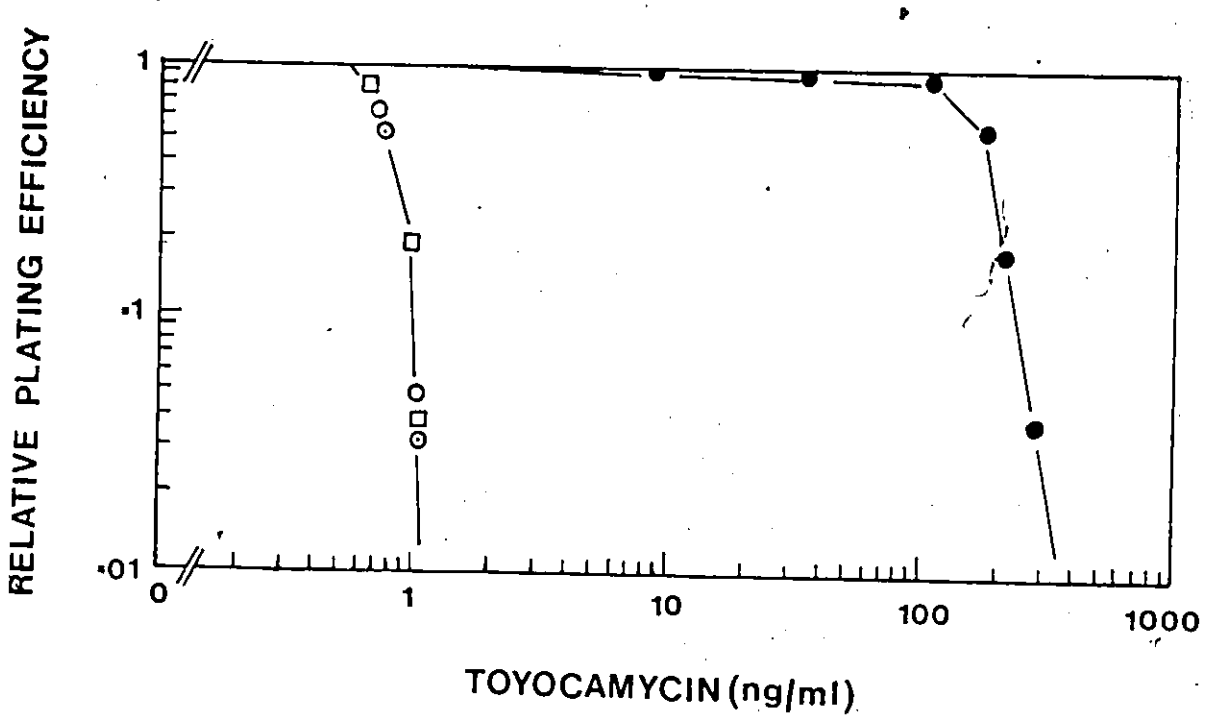


Figure 20: Dose-response curves for toyocamycin for the parental Fom^R and Toy^r cell lines and for the hybrids formed between them. Somatic cell hybrids were constructed as described in Methods. ●—● , EOT Toy^r2; ○—○ , Fom^R4; ⊙—⊙ and □—□, EOT Toy^r2 x Fom^R4 hybrid clones.

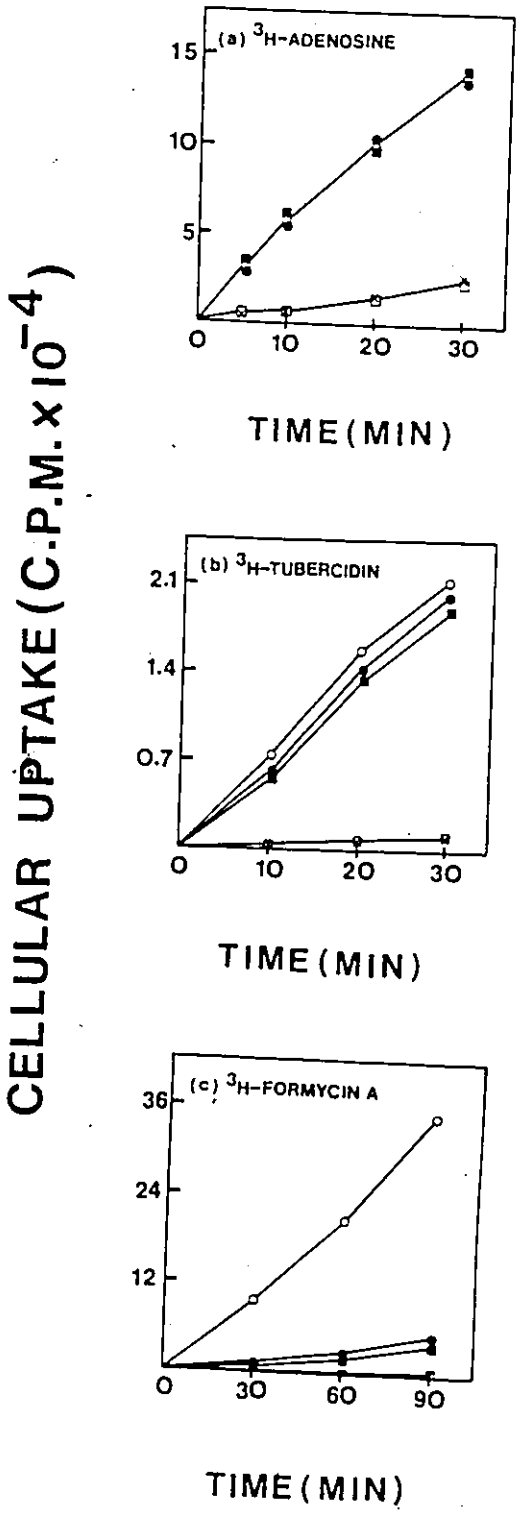
these studies are shown in Fig. 21. As can be seen, in the case of Toy^r mutants, cellular uptake of all adenosine analogs was greatly reduced. In them, cellular uptake of [^{3}H]adenosine, [^{3}H]tubercidin and [^{3}H]formycin A was found to be 12%, 6% and <1% respectively than that of parental WT cells. In contrast to the Toy^r mutants, both Fom^R2 and Fom^R4 lines showed nearly normal uptake (>90% of the parental WT cells) of the two N-nucleosides i.e. [^{3}H]adenosine and [^{3}H]tubercidin, however, very interestingly, in both these mutants the cellular uptake of [^{3}H]formycin A was found to be greatly reduced (10% of the parental WT cell line). Similar results with these cell lines have been obtained in a number of independent experiments.

In a separate experiment, two hours after the addition of [^{3}H]formycin A to various mutant lines, the amount of radioactivity which was present in the acid-soluble and acid-insoluble fractions was analyzed. Results of these studies showed that approximately 90% of total radioactivity which was taken up by different cell lines was present in the form of phosphorylated derivatives, and of this, about 10-15% was in an acid-insoluble form, indicating incorporation into nucleic acid.

3.2.2 DISCUSSION

Results presented in this section show that the mutants of CHO cells selected for resistance to the nucleoside analog formycin A (-EHNA) exhibit a novel genetic and biochemical phenotype. Although the biochemical phenotype of the Fom^R mutants (i.e., lack of any measureable AK activity in cell extracts) is similar to that of the

Figure 21: Cellular uptake of [^{3}H]adenosine, [^{3}H]tubercidin and [^{3}H]formycin A by various cell lines. The final concentration of [^{3}H]adenosine, [^{3}H]tubercidin and [^{3}H]formycin A in the growth medium were 10 μCi/ml, 0.2 μCi/ml and 2 μCi/ml, respectively. The experiment was carried out as described in Methods. (a) [^{3}H]adenosine, (b) [^{3}H]tubercidin, (c) [^{3}H]formycin A. O—O, WT; ●—●, Fom^R2; ■—■, Fom^R4; □—□, Toy^r4; ×—×, Toy^r5.


CELLULAR UPTAKE (C.P.M. x 10^{-4})
(a) ^{3}H-ADENOSINE
15
10
5
0
10
20
30
TIME (MIN)
(b) ^{3}H-TUBERCIDIN
2.1
1.4
0.7
0
10
20
30
TIME (MIN)
(c) ^{3}H-FORMYCIN A
36
24
12
0
30
60
90
TIME (MIN)

toyocamycin or tubercidin resistant (Toy^r) mutants described earlier (Bennett et al., 1966; Gupta and Siminovitch, 1978b; Rabin and Gottesman, 1979; Thacker, 1980), several different lines of evidence show that these two types of mutants involve different kinds of genetic lesions. First, in contrast to the Toy^r mutants which are highly resistant to various N- as well as C-nucleosides including toyocmycin, tubercidin, 6-MeMPR, formycin A, pyrazofurin, etc., the Fom^R mutants exhibit increase resistance only to various C-nucleosides, namely formycin A, formycin B, Bbb-73, Bbb-85, 9-deazaadenosine and pyrazofurin but do not exhibit appreciable cross-resistance to various N-nucleosides (viz. toyocamycin, tubercidin, 6-MeMPR, 6-MeAPR and 8-azaadenosine) that are examined. At the same time, the Fom^R mutants are much more resistant to formycin A (-EHNA) or formycin B in comparison with the Toy^r mutants. Second, the drug-resistant phenotype of the Fom^R mutants is expressed in a codominant manner in cell hybrids constructed between Fom^R x Fom^S or Fom^R x Toy^r cell lines. The drug-resistant phenotype of the Toy^r mutants, on the other hand, behaves recessively under these conditions (Gupta and Siminovitch, 1978b). Third, whereas the Toy^r mutants show a severe deficiency in the cellular uptake, phosphorylation and incorporation of all adenosine derivatives examined (viz. [^{3}H]adenosine, [^{3}H]tubercidin and [^{3}H]formycin A), the Fom^R mutants, in accordance with their cross-resistance pattern, show reduced cellular uptake, phosphorylation and incorporation of [^{3}H]formycin A but nearly normal capacity to phosphorylate and incorporate [^{3}H]adenosine and [^{3}H]tubercidin.

Finally, in contrast to the Toy^r mutants which are obtained spontaneously in CHO cells at very high frequencies (10^{-3}-10^{-4}, Gupta and Siminovitch, 1978b), the spontaneous frequency of Fom^R mutants is less than 10^{-6}, and the two Fom^R mutants which have been investigated are obtained from EMS treated cultures at a frequency of about 10^{-5}. The various genetic and biochemical differences in the properties of Toy^r and Fom^R mutants also exclude the possibility that the Fom^R mutants arise from a second mutation in a Toy^r mutant. In fact, the observation that two independent Fom^R mutants which exhibit different degrees of resistance to formycin B behave similarly provide strong evidence that the phenotype described above results from a single genetic lesion. Furthermore, the above observations strongly indicate that the genetic lesion in the Fom^R mutants is highly specific and it seems to affect the phosphorylation of only C-nucleosides. The lack of cross-resistance of the Fom^R mutants to tiazofurin, a C-nucleoside which is not phosphorylated via AK, further indicates that the lesion in these mutants is specific for only those C-nucleosides which are substrates for adenosine kinase.

The two Fom^R mutants which have been studied have both been found to contain no measurable activity of the enzyme AK in cell extracts. However, results presented here indicate that the mutant cells may in fact contain normal amounts of AK activity in vivo. Some of the observations which support this view are: (i) the Fom^R mutants are as proficient as WT cells in the cellular uptake, phosphorylation, and incorporation into macromolecules of [^{3}H]adenosine and

[^{3}H]tubercidin; (ii) the Fom^R mutants show normal sensitivity to various toxic adenosine analogs (e.g., toyocamycin and tubercidin) to which other AK$^-$ mutants reported earlier, exhibit a high degree of cross-resistance. These results taken together provide strong evidence that the Fom^R mutants contain AK activity in vivo but that this activity is somehow not observed in the cell extracts. All the efforts to find AK activity in the mutant cell extracts using various conditions viz. preparation of cell extracts by different means, e.g., sonication, detergent solubilization, hypotonic swelling and dounce homogenization; assay of AK activity at different temperatures; varying the concentrations of both [^{3}H]adenosine and ATP in the reaction mixture and using substrates other than adenosine, as well as measurement of AK activity in presence of various denaturing agents to exclude the possibility that AK forms a complex with other protein which makes it inactive, have so far failed. This has precluded further biochemical investigations with these mutants. Nonetheless, the lack of AK activity in the mutant cell extracts together with the observed reduced phosphorylation of [^{3}H]formycin A and the specific cross-resistance of mutant cells to only C-nucleosides which are substrates for AK, provide strong evidence that the genetic lesion in the Fom^R mutants is of novel nature and involve AK but in a novel manner.

At present the nature of the biochemical lesion in the Fom^R mutants and the basis of its observed specificity remains unclear. However, one observation that is perhaps of interest in this regard is

that in contrast to various N-adenosine derivatives which normally exist in anti-conformation, the C-nucleosides such as formycin A and pyrazofurin are predominantly present in syn-conformation (Daves and Cheng, 1976; Gutowski et al., 1973; Prusiner et al., 1973; Suhadolnik, 1979; Ward and Reich, 1968). Because of this difference in their preferred conformations, it is quite possible that C-nucleosides interact with certain specific regions or domains of AK which is not important in interaction with N-nucleosides. If the above presumption is correct, then it is possible that the genetic lesion in the Fom^R mutants may be affecting this specific domain of AK which is important in interaction of C-nucleosides, thus accounting for the altered response of the Fom^R mutants towards C-nucleosides. Although this possibility is attractive, at this stage other possibilities such as the genetic lesion in the Fom^R mutants may be affecting AK indirectly, could not be entirely excluded. Further investigation of the nature of genetic lesion in these mutants was hampered due to our lack of knowledge regarding metabolism of formycin B in mammalian cells.

3.3 GENETIC AND BIOCHEMICAL CHARACTERIZATION OF ANOTHER CLASS OF MUTANTS AFFECTED IN ADENOSINE KINASE AND SELECTION FOR RESISTANCE TO FORMYCIN B

In section 3.2, I have described the characteristics of CHO cell mutants selected for resistance to an adenosine analog, formycin A. These mutants differed in their genetic and biochemical properties from mutants resistant to other adenosine analogs such as toyocamycin and tubercidin, and these differences have been indicated previously. The presence of a large amount of ADA activity both in the CHO cells and in the fetal calf serum raised the possibility that formycin A under the selection conditions employed was rapidly deaminated to formycin B. As a result, instead of formycin A, formycin B may have been the actual selective agent for mutants isolation. To investigate this possibility, I have selected mutants of CHO cells which have become resistant to toxic concentrations of formycin B.

Studies presented in this section describe the genetic and biochemical phenotypes of the mutants obtained for resistance to formycin B (Fom^r mutants). These studies strongly indicate that the genetic lesion in Fom^r mutants affects AK directly in a novel manner. They also show that, similar to the situation with adenosine analogs, cellular resistance to formycin B can develop due to an alteration in the enzyme AK. In addition, present studies provide evidence that the Fom^r mutants differ from earlier described Toy^r as well as Fom^R mutants, in a number of genetic and biochemical characteristics.

3.3.1 RESULTS

3.3.1.1 Selection of Mutants Resistant to Formycin B and their Cross-Resistance to Various Nucleoside Analogs

Fig. 22 shows the dose-response curve of WT (CHO) cells towards formycin B. As may be seen, plating efficiency of WT cells was reduced sharply at drug concentrations between 1 and 5 µg/ml, and at 10 µg/ml, no colonies were obtained, even when 5 x 10^5 cells were plated in a P-100 dish. However, upon plating a large number of cells in medium containing 10 µg formycin B/ml, discrete colonies of resistant cells on a light background of giant single cells appeared on dishes. The frequencies of such colonies in nonmutagenized cultures was found to be in the range of 0.2 to 0.5 x 10^{-5}. It was observed that prior treatment of WT cells with the mutagen EMS led to a 10-fold increase in the frequencies of resistant colonies, as compared to the parallel untreated cultures. When some clones were picked, grown in nonselective medium, and examined for their degree of resistance to formycin B, all clones were found to be resistant to formycin B. The dose-response curves of two representative mutants, Fom^r10 and Fom^r12, which have been chosen for further studies are shown in Fig. 22. Based upon their D_{10} values, these mutants are approximately 5-(Fom^r10) and 7-(Fom^r12) fold more resistant to formycin B as compared to the parental WT cells. The different levels of resistance of these mutants to formycin B indicate that these mutants arose from independent genetic lesions. The drug resistant phenotype of both of these mutants has remained completely stable upon subcloning in the absence of

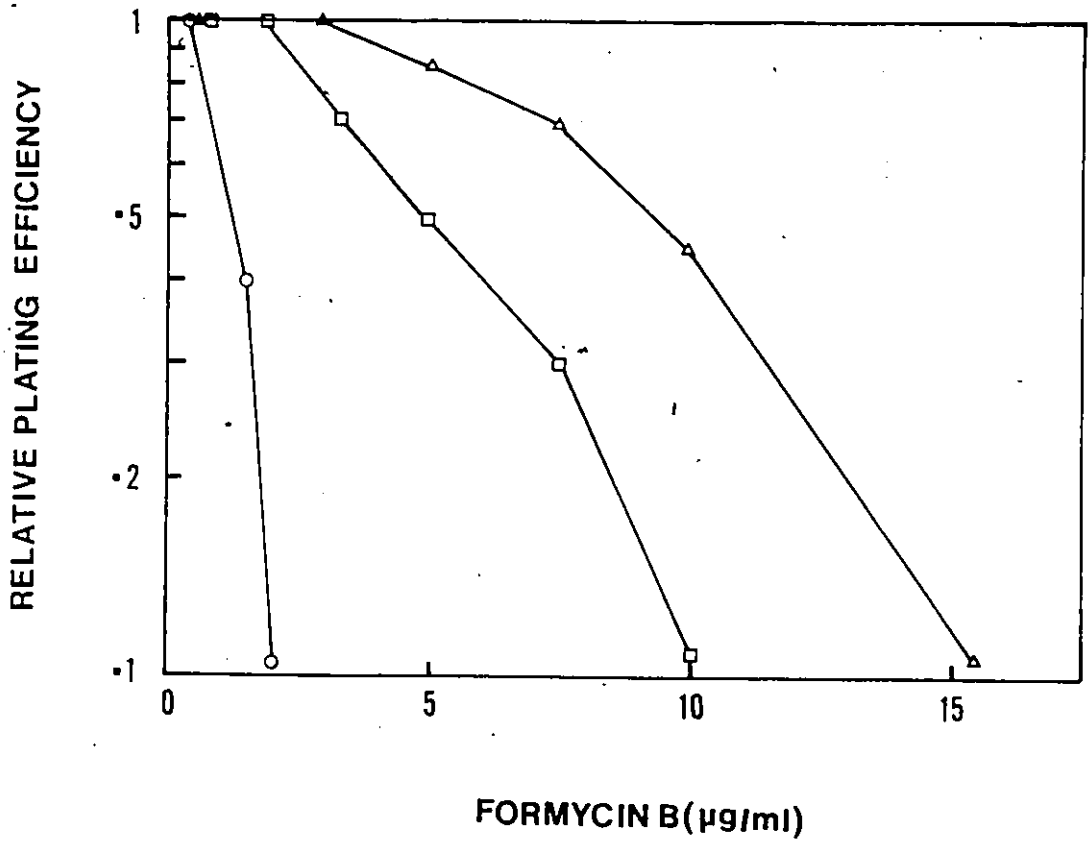


Figure 22: Survival curves of parental and the Fom^r mutant cell lines in the presence of increasing concentrations of formycin B. ○—○, WT; □—□, Fom^r10; △—△, Fom^r12.

formycin B and upon growth in nonselective medium for over one year.

After obtaining the above mutants, their cross-resistance towards a number of different nucleoside analogs was determined. The objective of these studies was to determine whether these mutants were similar or different from the Fom^R mutants isolated previously. In these experiments, cellular toxicity of different nucleoside analogs towards various cell lines was determined in parallel as described in the Methods section. From the D_{10} values of the nucleoside analogs for different cell lines, the degree of resistance of the mutant cell lines in comparison to the parental sensitive cell line was calculated. Results of these studies for the parental CHO cells (WT) and two formycin B resistant mutants (Fom^r10 and Fom^r12) are summarized in Table VI. Results with Fom^R mutant (Fom^R4) as well as Toy^r mutant (Toy^r4) were included for the sake of comparison. Similar results for these nucleoside analogs with the above set of cell lines have been obtained in at least two independent experiments. As can be seen, both Fom^r mutants showed a high degree of cross-resistance to various adenosine analogs which are known to be phosphorylated by enzyme AK including the N-nucleosides viz. tubercidin, 6-MeMPR and 6-MeAPR, as well as the C-nucleosides viz. formycin A, Bbb-73, Bbb-85 and 9-deazaadenosine and structurally distinct nucleoside analog, pyrazofurin. These mutants differed from the Toy^r mutants which also showed cross-resistance to both N- and C-nucleosides, in that their degree of resistance was lower in comparison to the Toy^r mutants. The only exception in this regard were formycin B and Bbb-85 to which Fom^r

Table VI. Cross-Resistance Patterns of Mutants Resistant to Formycin A, Formycin B and Toyocamycin Towards Various Nucleoside Analogs

Nucleoside Analog	D_{10} value of the[a] analog for the WT cells	Relative resistance of[a] the mutant cell lines Fom^R10	Fom^r12	Fom^R4	Toy^r4
C-Nucleosides					
Formycin A	(1.0 ng/ml) 1	15	20	150	>500
Bbb-73	(2.0 µg/ml) 1	10	55	>50	50
Bbb-85	(0.02 µg/ml) 1	25	70	350	1250
9-Deazaadenosine	(0.5 µg/ml) 1	5	15	130	400
Pyrazofurin	(15.0 ng/ml) 1	5	10	45	1000
Tiazofurin	(0.3 µg/ml) 1	1	1	1	1
N-Nucleosides					
Toyocamycin	(0.4 ng/ml) 1	3	5	2.5	500
Tubercidin	(1.0 ng/ml) 1	2	3	3	1000
6-MeAPR	(0.2 µg/ml) 1	50	70	2	>500
6-MeMPR	(0.25 µg/ml) 1	15	30	5	>500

[a] The D_{10} values of nucleoside analogs towards the WT cells and the mutant cell lines were determined as described in the legend of Table V.

mutants exhibited higher resistance in comparison to the Toy^r mutants. The Fom^r mutants also differed from Fom^R mutants, which showed no appreciable cross-resistance to different N-nucleosides examined, but were highly resistant to various C-nucleosides.

3.3.1.2 AK Activity in the Cell Extracts of Fom^r Mutants

Work presented in section the 3.2 on the development of resistance to adenosine analogs has shown that the mammalian cells can develop resistance to them due to an alteration or deficiency of AK, the enzyme responsible for the conversion of the nucleosides to the phosphorylated forms. In view of these results, it seemed probable that the Fom^r mutants were affected in AK and therefore AK activity as well as amounts of protein were measured in the cell extracts of parental and mutant cell lines by a procedure described in the Methods section. Fig. 23 shows the average results of three experiments performed to find out the specific activity of AK in various cell extracts. As can be seen, extracts from both Fom^r mutants were found to contain different levels of AK activity than that present in WT cell extracts. In different experiments, cell extracts of Fom^r10 was always found to contain slightly higher levels of AK activity ($\approx$110%) and Fom^r12 mutant was found to have lesser activity ($\approx$70%) than that present in WT cell extract. Thus Fom^r mutants differ from parental WT cells in terms of the levels of AK activity in their cell extracts. Further differences can be seen if the kinetics of synthesis of the lanthanum chloride precipitable AMP is compared. It is also evident from Fig. 23, that for AK in WT cell extract, synthesis of adenine

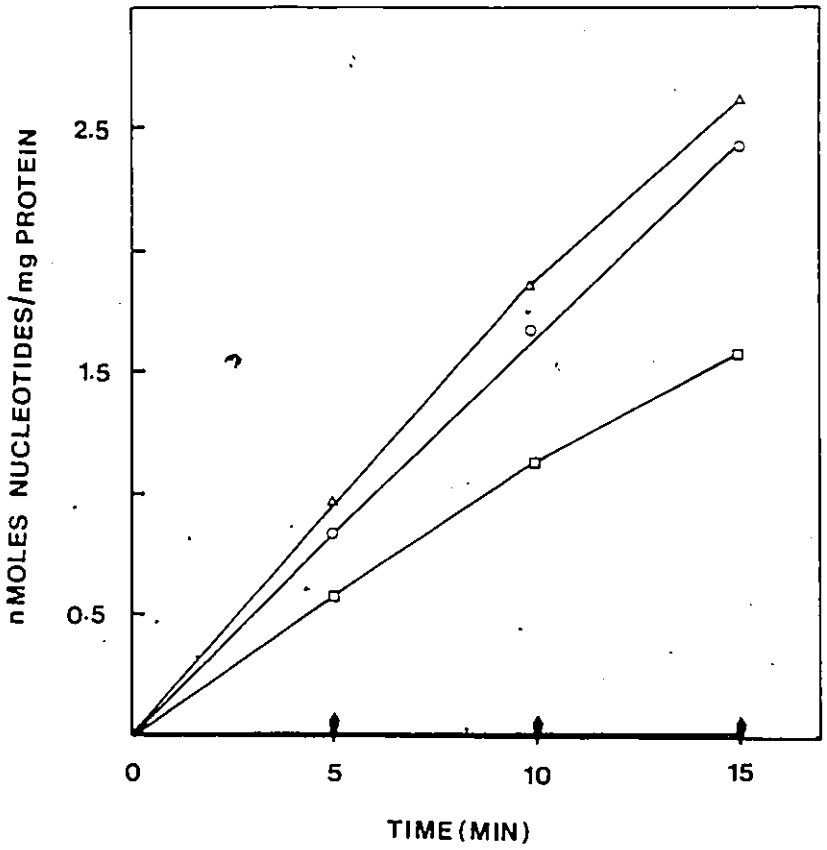


Figure 23: AK activity in the extracts of WT and mutant cells. The assay procedure for the enzymes is described in Methods. ○—○, WT; △—△, Fom^r10; □—□, Fom^r12.

nucleotides continued at a linear rate for 15 min but in contrast for AK present in Fom^r12 mutant linear behaviour was observed for the first 10 min followed by a saturation kinetics.

3.3.1.3 Biochemical Properties of AK Present in Cell Extracts of Various Cell Lines

In view of the cross-resistance of Fom^r mutants to both N- as well as C-nucleosides, presence of AK activity in mutant cell extracts was somewhat surprising. It is known from earlier studies, that mutation in an enzyme could often change its biochemical and kinetic characteristics (Fenwick et al., 1977; Gupta et al., 1977; McBurney and Whitmore, 1974a), therefore the possibility that resistance to various adenosine analogs in Fom^r mutants may result from a structural alteration in AK was investigated. In this regard, various biochemical and kinetic properties of the AK activity present in the cell extracts of both Fom^r mutants were compared with the AK activity of the parental WT cells.

(A) Km values

The Km values for AK present in cell extracts of various cell lines was determined by using the standard radioactive assay with radiolabelled adenosine as substrate. The adenosine concentration was varied between 1 μM and 100 μM for each experiment and Km values of adenosine for AK present in various cell extracts were determined from Lineweaver-Burk plots. The Km value of adenosine for AK present in WT, Fom^r10 and Fom^r12 cell extracts was found to be 20 μM, 32 μM and 40 μM, respectively. This indicated that there is very little difference

in the affinities of the enzyme AK from different cell extracts for adenosine.

(B) Thermal inactivation

I next examined the heat inactivation kinetics of AK from the parental and mutant cell extracts. Initially, AK activity present in WT cell extract was determined at different temperatures. From the above experiments it was observed that 50°C is the most suitable temperature for comparison of heat sensitivity of AK, because the decrease in AK activity was found to be linear with time. Fig. 24 represents data on the thermal inactivation at 50°C of the AK activity present in the WT and Fom^r mutants. As one can see, the heat-inactivation of the AK activity present in WT cells as well as in both the Fom^r mutants occurs at similar rates indicating that the AK present in parental and mutant cells are very similar in terms of their heat sensitivity.

(C) Competition between adenosine and an adenosine analog for AK from different cell lines

It has been shown by earlier studies that cellular resistance to purine nucleoside analogs develops by an alteration in the first biochemical step which leads to the synthesis of lethal metabolites (Section 3.2). In view of these results, the possibility that AK present in the Fom^r mutants may show reduced affinity for phosphorylation of adenosine analogs to which the Fom^r mutants exhibited cross-resistance was examined. To examine this aspect, competition experiments were carried out between adenosine and an

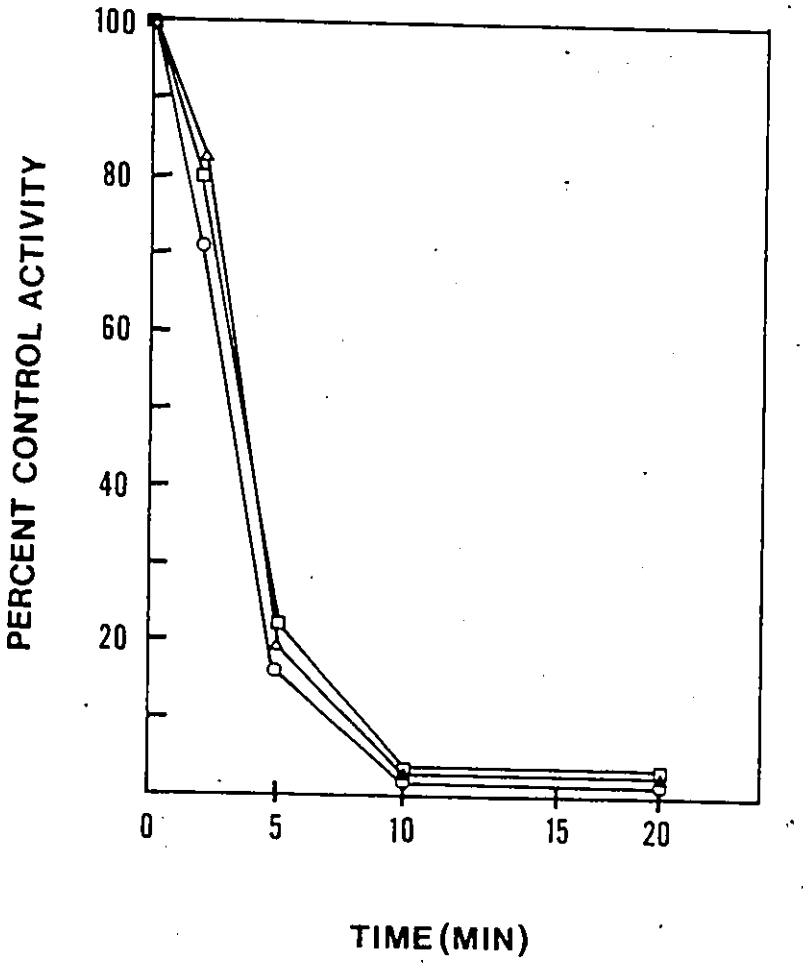


Figure 24: Thermal inactivation of adenosine kinase from different cell lines. The kinetics of thermal inactivation of the AK were determined by diluting the crude extracts of the various cell lines to a fixed activity per millilitre in 10 mM potassium phosphate buffer, pH 7.0. Samples were then taken for zero time assays, and the tubes were placed in a 50°C water bath. Samples were removed at appropriate intervals, chilled in ice, and then assayed for residual AK activity by using the procedure as described in Methods. O—O, WT; Δ——Δ, Fom^r10; □——□, Fom^r12.

adenosine analog, Bbb-85 using AK present in cell extracts of WT and both Fom^r mutants. The adenosine analog, Bbb-85 was selected for competition experiments because Fom^r mutants show highest degree of cross-resistance to it among all adenosine analogs examined (Table VI). Therefore, it was considered that there is a good possibility of detecting any differences in the phosphorylation of Bbb-85 using AK from WT and mutant cells. The experiments were performed by incubating the equal amounts of AK activity from either the WT or the Fom^r mutants cell extract with constant concentration of radiolabelled adenosine and varying amounts of adenosine analog, Bbb-85. From the results of such study, which are shown in Fig. 25, it is evident that the WT and mutant enzymes show some interesting differences in terms of their ability to phosphorylate Bbb-85. As one can see, for 50% competition, the concentration of Bbb-85 required for AK from WT cells is about 0.8 mM but on the otherhand, for AK present in Fom^r10 and Fom^r12 cell extracts are 4 mM and 6 mM respectively. These values suggest that the enzyme AK present in both Fom^r mutants have less affinity for phosphorylation of Bbb-85 in comparison to the enzyme from WT cells.

3.4.1.4 Behaviour of Fom^r Mutants in Somatic Cell Hybrids

To examine the behaviour of Fom^r mutants in a hybrid Fom^r x Fom^S situation, somatic cell hybrids were constructed between EOT x Fom^r cells. As described before, the cell lines for crosses were chosen in such a way that while one of the parents was ouabain-resistant and unable to grow in HAT medium (Oua^RHAT^S), the other parent had WT phenotype (i.e., Oua^SHAT^R) for these markers. The

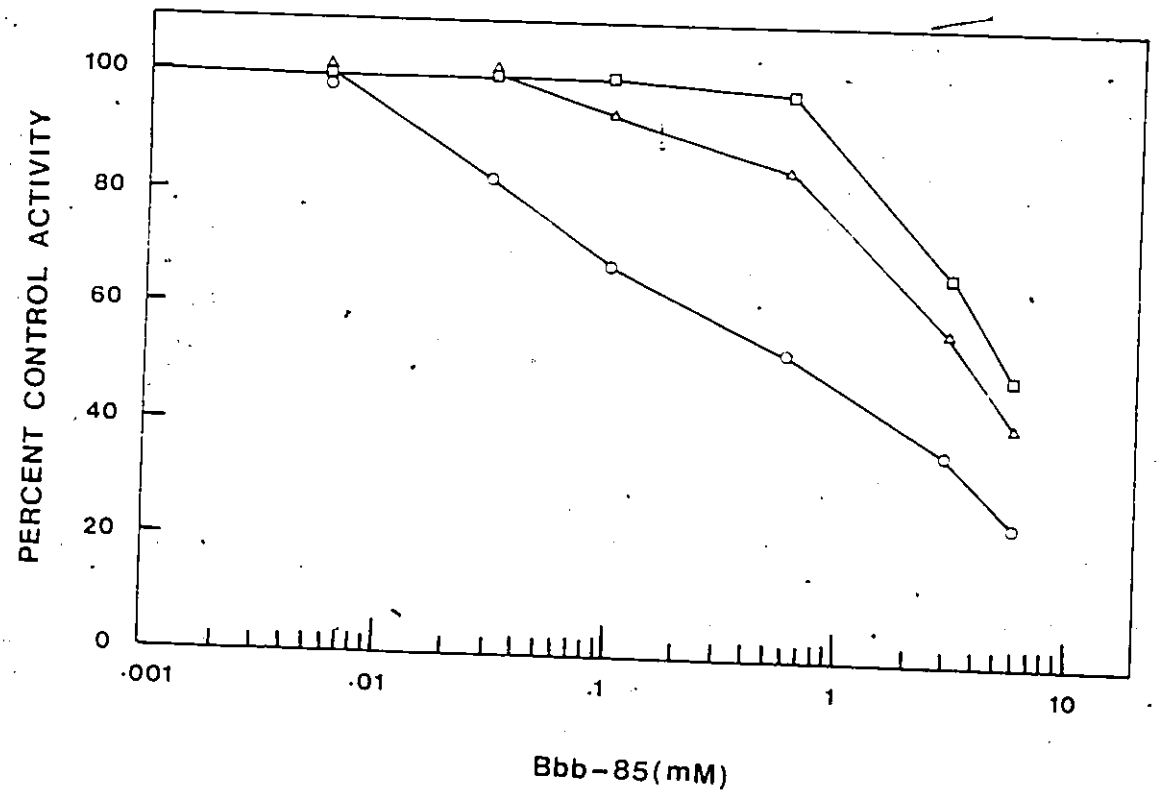


Figure 25: Competition between adenosine and an adenosine analog, Bbb-85. Approximately equal amounts of AK activity from wild-type or resistant cells were assayed with increasing amounts of adenosine analog Bbb-73 and using constant concentration of [^{3}H]adenosine as substrate as described under Methods. The residual activity in presence of adenosine analog is expressed as a percentage of the activity in the absence of the drug. O—O, WT; Δ—Δ, Fam^{r}10; □—□, Fam^{r}12.

cells were fused using PEG-DMSO and subsequently hybrid clones were selected by procedure as described in the Methods. Some of the clones (6-8 from each cross) which grew in the selective medium in different crosses were picked and their hybrid nature, i.e., pseudotetraploid was ascertained by chromosomal analysis. The degree of resistance of various hybrid and parental cell lines towards formycin B was examined by semiquantitative analysis in 24 well dishes and results are shown in Fig. 26. The hybrids formed between the Fom^r mutants and the EOT cell line were nearly as sensitive as the sensitive parental cell line. These results indicates that the Fom^r phenotype of these mutants expresses itself in a recessive manner. In view of the recessive behaviour of the formycin B resistant phenotype, resistance in these mutants was indicated by "r" instead of "R" which had been used to represent the codominance/dominance behaviour in somatic cell hybrids of drug resistance phenotype of formycin A resistant mutants.

3.4.1.5 Cellular Uptake and Phosphorylation of Nucleoside and Various Nucleoside Analogs in Fom^r Mutants

From studies described in section 3.2 on Toy^r and Fom^R mutants, it was evident that in accordance with their cross-resistance pattern, they showed reduced cellular uptake as well as decreased phosphorylation of various nucleoside analogs. Therefore, it was of interest to determine whether the cellular uptake of Fom^r mutants parallels their behaviour in cross-resistance studies. Cellular uptake of nucleoside and various nucleoside analogs were carried out as described in the Methods and results of such studies are summarized in

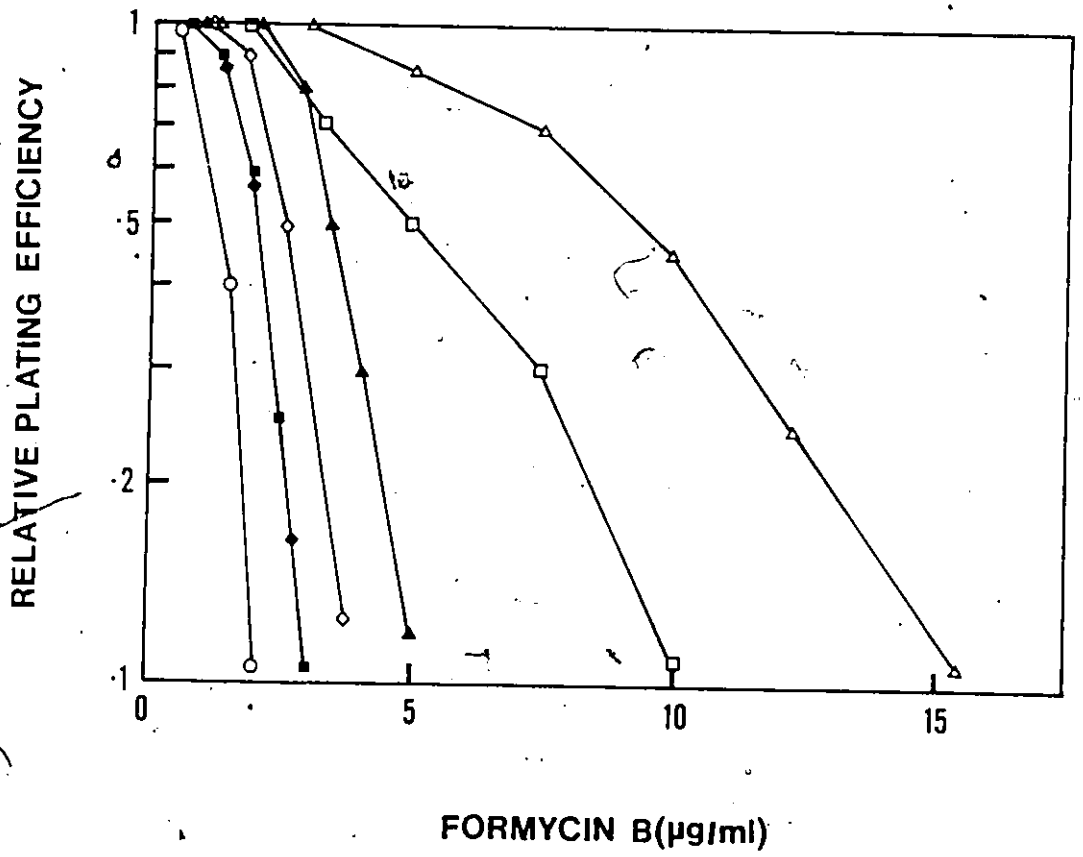


Figure 26: Dose-response curves towards formcyin B for the parental and hybrid cell lines. ○—○, EOT; □—□, Fom^{r}10; ◆—◆ and ■—■, EOT x Fom^{r}10 hybrid clones; △—△, Fom^{r}12; ◇—◇ and ▲—▲, EOT x Fom^{r}12 hybrid clones.

Table VII. Results with Toy^r mutant was included for comparison. As one can see, Fom^r mutants show reduced cellular uptake of both [^{3}H]tubercidin and [^{3}H]formycin A but nearly normal uptake of [^{3}H]adenosine in comparison to the WT cells. On the otherhand, Toy^r mutants show reduced cellular uptake of [^{3}H]adenosine, [^{3}H]tubercidin as well as of [^{3}H]formycin A. Since Fom^r mutants exhibited cross-resistance to both N- as well as C-nucleosides, the reduced cellular uptake of [^{3}H]tubercidin (N-nucleoside) and [^{3}H]formycin A (C-nucleoside) is in agreement with their cross-resistance pattern.

3.3.2 DISCUSSION

This section deals with the isolation and genetic and biochemical characterization of novel types of mutants selected for resistance to formycin B. Results presented in this section provide evidence that the genetic lesion in the Fom^r mutants affect AK directly. Some of the observations which support this inference are: (i) Both the Fom^r mutants exhibit cross-resistance to only those adenosine analogs which require initial phosphorylation by AK for cellular toxicity. (ii) In accordance with their cross-resistance pattern, both the Fom^r mutants showed reduced cellular uptake of various adenosine analogs. (iii) Cell extracts from both the Fom^r mutants contain altered levels of AK activity in comparison to the WT cell extract. In addition, cell extract from one of the Fom^r mutants, Fom^r10 does not show a linear time course for the synthesis of AMP as observed with WT cell extract. (iv) The competition for AK activity present in WT and the mutant cell extracts by adenosine analog, Bbb-85

Table VII. Cellular Uptake of Radiolabelled N- and C-Nucleosides by the Parent and Resistant Cell Lines

Cell line	Relative cellular uptake of nucleoside or nucleoside analogs[1]		
	[^{3}H]-Adenosine (%)	[^{3}H]-Tubercidin (%)	[^{3}H]-Formycin A (%)
WT	100 ± 1	100 ± 0.5	100 ± 1.5
Fom^{r}10	100 ± 1.5	22 ± 0.3	21.5 ± 1
Fom^{r}12	92.8 ± 1	20 ± 0.1	19.6 ± 0.5
Toy^{r}4	9.5 ± 2	3.7 ± 0.1	1.4 ± 0.1

[1] The experiment was carried out by incubating 5 x 10^5 cells in the presence of [^{3}H]adenosine (10 μCi/ml, 20 min), [^{3}H]tubercidin (0.2 μCi/ml, 50 min) and [^{3}H]formycin A (2 μCi/ml, 50 min). The cellular uptake values obtained for WT cells have been taken as 100% for each experiment for calculating relative uptake in mutant cells. The 100% values are give in parenthesis, [^{3}H]adenosine (1.4 x 10^5 cpm), [^{3}H]tubercidin (1.5 x 10^4 cpm), [^{3}H]formycin A (5.1 x 10^4 cpm).

show that the mutants enzyme has less affinity for phosphorylation of adenosine analogs in comparison to the enzyme of the WT cells.

On the basis of the biochemical characteristics of AK present in Fom^r mutants, the behaviour of both the Fom^r mutants in cellular uptake studies can easily be explained. For example, both the Fom^r mutants showed nearly similar levels of uptake of adenosine but reduced cellular uptake of adenosine analogs, tubercidin and formycin A. This suggest that the AK from the mutant cells show normal affinity for adenosine but its affinity for the other adenosine analogs such as tubercidin and formycin A have been altered. This is consistent with the results of the competition experiment.

The Fom^r mutants represent a novel type of mutants as they differ from all the other mutants previously selected for resistance to the purine nucleoside analogs, which contain no detectable level of the AK activity in cell extracts (Bennett et al., 1966; Chan and Juranka, 1981; Dix et al., 1979; Gupta, 1981; Gupta and Siminovitch, 1978b; Gupta and Singh, 1983; Rabin and Gottesman, 1979; Thacker et al., 1980). The mutants which show resistance to the various adenosine analogs and at the same time contain large amounts of AK activity in cell extracts have not been observed before and therefore, the Fom^r mutants are the first of its kind. The presence of biochemically altered AK activity in Fom^r mutants strongly indicate that the genetic lesion in these mutants is in the structural gene for AK. Based on their cross-resistance and reduced cellular uptake for both N- and C-purine nucleoside analogs, the biochemical lesion in these mutants.

appear very similar to that seen in the Toy^r mutants. However, while in the Toy^r mutants the genetic lesion which occurs at a high frequency causes complete loss of AK activity, but on the otherhand, in Fom^r mutants a different type of mutation which occurs at much lower frequency specifically alters AK in its biochemical properties.

3.4 METABOLISM AND THE MECHANISM OF ACTION OF FORMYCIN B IN CHINESE HAMSTER OVARY CELLS: INVOLVEMENT OF ADENOSINE KINASE IN DRUG PHOSPHORYLATION

It had been reported earlier that formycin B is not metabolized by mammalian cells and is toxic to cells without initial phosphorylation (Umezawa et al., 1967). This report about the inability of cellular kinases to phosphorylate formycin B has led investigators to study the cellular processes that are affected by this inosine analog. Formycin B has been reported to be a potent inhibitor of the growth of several species of Leishmania and recently it has been suggested (Carson and Chang, 1981; Nelson et al., 1982; Rainey and Santi, 1983) that the antileishmanial activity of formycin B is due to its phosphorylation by Leishmania sp. but not by mammalian cells. In view of the reported lack of phosphorylation of formycin B in mammalian cells, alteration in AK, which is the major purine nucleoside phosphorylating activity, in mutants selected for resistance to formycin A and formycin B (Fom^R as well as Fom^r mutants) was quite surprising. This encouraged us to investigate the metabolism of formycin B in CHO cells in detail and therefore the present studies were undertaken. This section deals with the metabolism and mechanism of action of formycin B in CHO cells by using genetic and biochemical approaches. These studies show that in CHO cells formycin B is phosphorylated and metabolized as in Leishmania and provide strong suggestive evidence that the AK is involved in the phosphorylation of

formycin B.

3.4.1 RESULTS

3.4.1.1 Effect of Formycin B on the Growth of Mammalian Cells

Formycin B has been reported to be a potent inhibitor of the growth of several species of Leishmania and it has been suggested that the formycin B inhibits the growth of cultured mammalian cells at much higher concentration range than that observed for Leishmania (Carson and Chang, 1981). In order to examine the effect of formycin B on mammalian cells, the growth rate of CHO and HeLa cells was studied at different concentrations of formycin B.

Fig. 27 shows the effects of two different concentrations of formycin B on the growth of HeLa and CHO cell lines. Formycin B at 5 µg/ml reduced the growth rate of both CHO and HeLa cells but the cells kept growing exponentially. However, at 20 µg/ml concentration, cell growth stopped completely by 48-72 hours, and at later time some cell killing as indicated by decrease in cell numbers was observed.

The toxicity of formycin B towards the above cell lines was also determined by another method in which the plating or cloning efficiencies of the above cell lines in medium containing different concentrations of formycin B was examined. Results of these studies are presented in Fig. 28. As can be seen, the plating efficiencies of both HeLa and WT (CHO) cells were sharply decreased between 1 to 2 µg/ml (i.e. 4-8 µM) formycin B. These concentrations are very similar to the concentration of formycin B (1 to 10 µM) which have been reported to inhibit the in vitro growth of promastigotes and

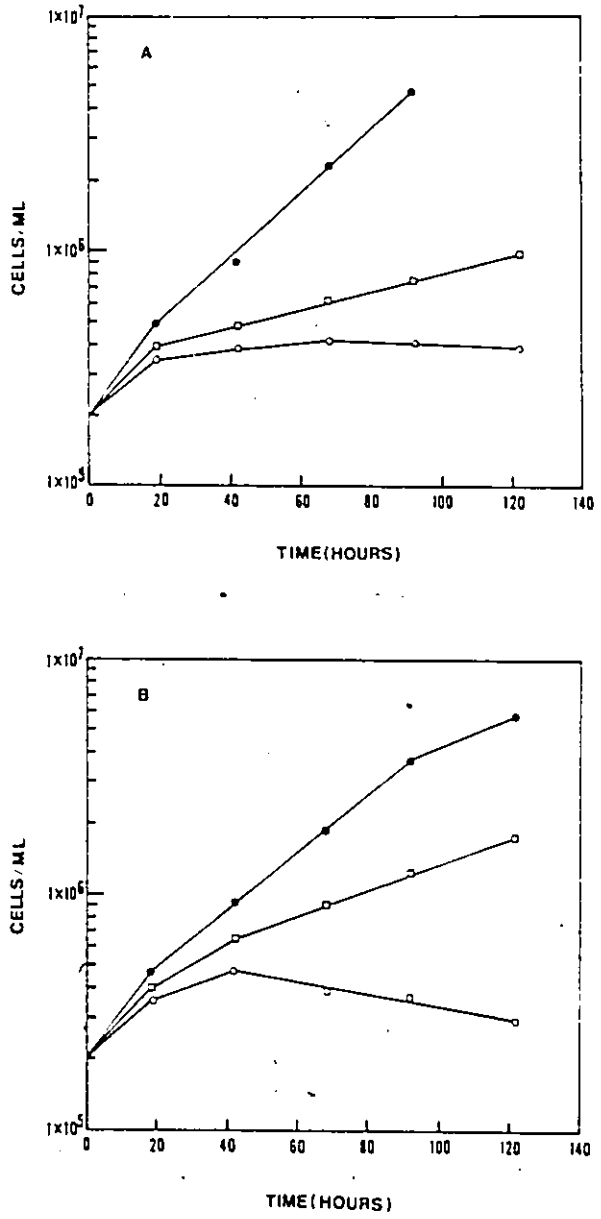


Figure 27: Effect of formycin B on the growth of CHO (A) and HeLa (B) cells in culture. ●—●, no addition; □—□, 5.0 µg/ml formycin B; ○—○, 20.0 µg/ml.

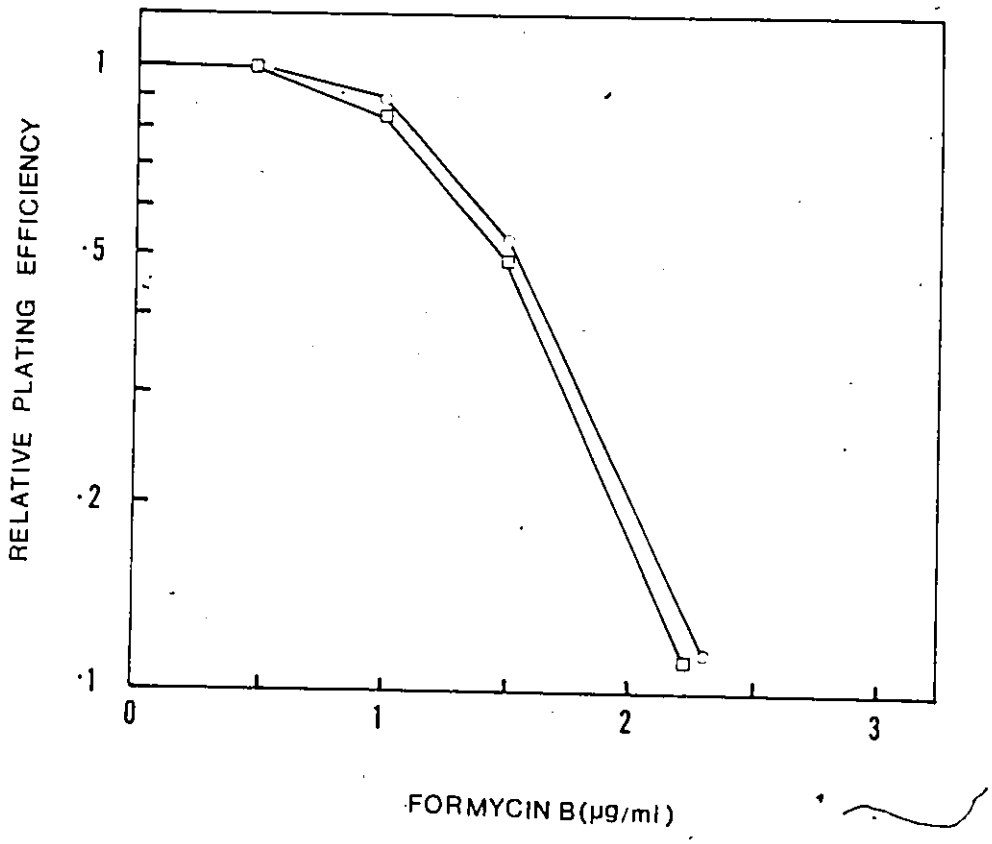


Figure 28: Relative plating efficiencies of HeLa and CHO (WT) cells in the presence of increasing concentrations of formycin B. O—O, WT; □—□, HeLa.

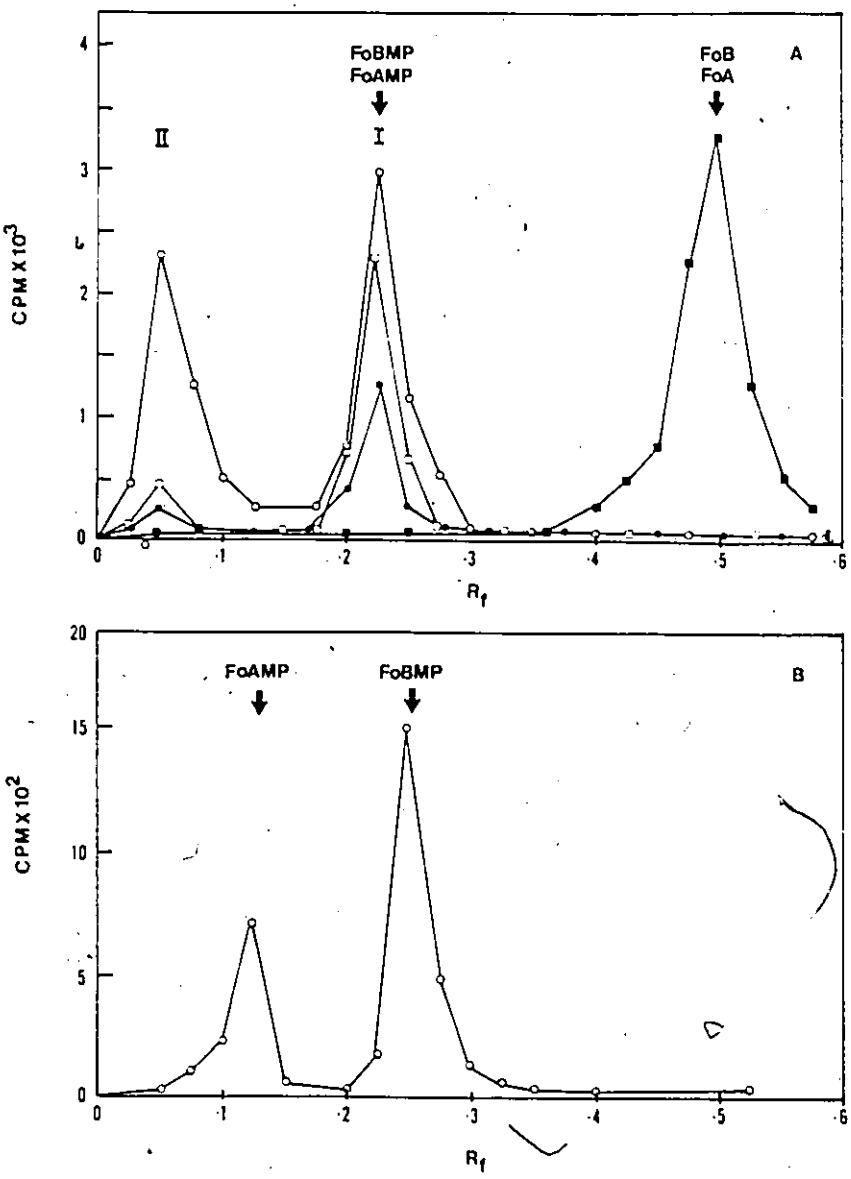
amastigotes of Leishmania (Carson and Chang, 1981; Nelson et al., 1982).

3.4.1.2 Metabolism of Formycin B in Mammalian Cells

To determine the fate of formycin B in mammalian cells, CHO cells were incubated with [^{3}H]formycin B (5 μM) for different periods of time and a portion of the acid-soluble extract was subjected to paper chromatography along with the appropriate markers, viz. formycin B, formycin A, FoB-MP and FoA-MP. Results of these studies are shown in Fig. 29A. As can be seen, upon incubation of [^{3}H]formycin B with WT cells, two main peaks of radioactivity were observed. The amount of radioactivity in both peaks was found to increase with time. Based on its chromatographic position the peak I corresponded to the 5'-monophophates of either formycin B or formycin A (which do not separate in this solvent system). To resolve these two, the material from this peak was eluted and run in a second solvent system which separate FoB-MP from FoA-MP (Fig. 29B). Results of these studies indicated that although the bulk of the radioactivity in peak I corresponded to FoB-MP, a small amount of FoA-MP was also present. The second peak (peak II) which was present in only small amounts at shorter incubation periods (Fig. 29A) most likely represented higher phosphates of either formycin B or formycin A. It is also clear from Fig. 29A that no free [^{3}H]formycin B/formycin A was observed in the acid extracted material which indicate that phosphorylation of the drug is essential for its entry into the cells.

Further evidence that the peaks I and II correspond to the

Figure 29: Chromatographic analysis of the products of [^{3}H]formycin B metabolism in WT$_3$(CHO) cells. WT cells (2×10^8) were incubated with [^{3}H]formycin B (5 μM) for the indicated times at 37°C. After the specified periods of incubation, cells were washed with PBS and then extracted with PCA. (A) The acid-soluble fraction was analyzed by paper chromatography (solvent, nPrOH:H_2O;6:4) as described in Methods. ●—● , 30 min; □—□ , 60 min; ○—○ , 120 min; ■—■, 120 min sample subjected to alkaline phosphatase treatment. (B) Chromatographic analysis of peak I material (Fig. 29A) using solvent system nProH:NH_4OH: H_2O;6:3:1.


FoBMP
FoAMP
FoB
FoA
A
II
I
CPM X 10^3
R_f
FoAMP
FoBMP
B
CPM X 10^2
R_f

phosphorylated derivatives of formycin B/formycin A was provided by experiments in which the PCA-soluble fraction was treated with the enzyme alkaline phosphatase prior to its chromatography. Results of these experiments which are also presented in Fig. 29B showed that upon treatment with alkaline phosphatase radioactivity in both peaks I and II completely disappeared and at the same time a new radioactivity peak which migrated in the same position as formycin B/formycin A was now observed.

3.4.1.3 Incorporation of [^{3}H] Formycin B into Cellular Macromolecules

Upon incubations of [^{3}H]formycin B with WT cells it was observed that a fraction of the radioactivity was progressively converted into a PCA precipitable form. In two different experiments, it was found that after 60 min of incubation about 7% of the total [^{3}H]formycin B which was taken up by cells was in a PCA-insoluble form (Table VIII). To find out whether the PCA-insoluble radioactivity was incorporated into DNA or RNA, the precipitated material was treated with KOH under conditions which should hydrolyze cellular RNA. Results of these studies which are presented in Table VIII showed that nearly all (between 90-100%) of the PCA-insoluble radioactivity was hydrolyzed by KOH, and hence it was most likely incorporated into RNA. The nature of the incorporated material was examined by chromatographing the KOH hydrolysate. Results of these studies showed that all of the incorporated radioactivity after KOH hydrolysis migrated in the same position as FoA-MP. These results provided strong evidence that only the phosphorylated derivatives of formycin A, but not formycin B, are

Table VIII. Cellular Uptake and Incorporation of [^{3}H]Formycin B in CHO Cells

Experiment number	Amount of radioactivity in different fractions (cpm x 10^5)			
	Total cellular uptake	PCA soluble	PCA insoluble	KOH soluble
1	15	13.2	1.1	1
2	15.2	14.5	1.3	1.3
3	16	14.8	1.3	1.2

The experiment was carried out by incubating 2 x 10^8 WT cells in the presence of [^{3}H]formycin B (6 μM) for 60 min at 37°C. The uptake and incorporation of [^{3}H]formycin B in various fraction was measured as described in Methods. The KOH soluble counts were measured for the PCA-insoluble fraction.

incorporated into RNA. Similar metabolism of [^{3}H]formycin B in other species have been reported by other investigators (Carson and Chang, 1981; Nelson et al., 1982; Rainey and Santi, 1983; Berman et al., 1983; Spector et al., 1984).

3.4.1.4 Involvement of Adenosine Kinase in the Phosphorylation of Formycin B

In view of the observed phosphorylation of formycin B, it was of interest to obtain information regarding the enzyme(s) which may be responsible for carrying out its phosphorylation. In mammalian cells, one of the major purine nucleoside phosphorylating activity corresponds to the enzyme AK, which phosphorylates adenosine and its various other analogs. Although formycin B is an inosine derivative, the first indication that AK may be involved in its phorphorylation was provided by the studies which showed that in mutants of CHO cells selected for resistance to formycin B, resistance to it is associated with the alteration in AK (Section 3.3). Furthermore, in mutants obtained for resistance to formycin A, which exhibit cross-resistance to formycin B, AK was found to be affected in a novel manner (Section 3.2). If AK is involved in the phosphorylation of formycin B, then all mutants lacking AK, selected using various adenosine analogs, should exhibit cross-resistance to formycin B. In earlier studies it has been observed that toyocamycin-resistant (Toy^r) mutants of CHO cells exhibited about 2 to 2.5 fold cross-resistance to formycin B (Section 3.2), which is consistent with the above inference. However, in view of the relative low degree of cross-resistance of these mutants to

formycin B, it was considered necessary to confirm the cross-resistance pattern and to show that all AK^- mutants showed similar behaviour. This was done by selecting a number of independent mutants (see Table IX) resistant to nucleoside analogs toyocamycin, tubercidin, 6-MeMPR and pyrazofurin. To ensure that the mutants obtained were completely independent, all mutant clones were obtained from independent cultures, each of which was grown up from a single clone (see Table IX). Some of the results of the studies with these mutants are presented in Table IX. As can be seen, all the mutants obtained using any of the above selective agents exhibited high degree of cross-resistance to toyocamycin (Table IX), tubercidin, 6-MeMPR and pyrazofurin (results not shown). All of these mutants were also found to contain no measurable activity of AK in cell extracts (Table IX). However, most interestingly, all the AK^- mutants also exhibited between 2 to 3.0 fold cross-resistance to formycin B, as observed with Toy^r mutants in earlier studies (see Section 3.2). These results clearly showed that the AK^- mutants exhibit cross-resistance to formycin B, and provided further support to the inference that formycin B may be phosphorylated via AK.

Further evidence that AK is involved in the phosphorylation of formycin B was provided by experiments in which cellular uptake and incorporation of [^{3}H]formycin B in WT and various mutant cell lines affected in AK were examined. Results of these studies (Table X) showed that in comparison to WT (i.e. AK^+) cells all Toy^r, Fom^R and Fom^r mutants showed reduced cellular uptake and phosphorylation of

Table IX. Cross-Resistance of Independently Isolated AK^- Mutants Towards Formycin B

Cell line	Degree of resistance[1] of mutants to toyocamycin	% AK activity[2]	Degree of resistance[1] of mutants to formycin B
WT	1	100	1
Toy^r4	> 200	< 0.5	2.5
Toy^r10	> 200	< 0.5	2.5
Toy^r11	> 200	< 0.5	2.5
Tub^r12	> 200	< 0.5	2.2
Tub^r22	> 200	< 0.5	2.8
Mpr^r16	> 200	< 0.5	2.5
Mpr^r20	> 200	< 0.5	2.3
Pyr^r13	> 200	< 0.5	2.5
Pyr^r19	> 200	< 0.5	2

1 The degree of resistance of mutants was determined by plating different CHO cell lines in medium containing increasing concentrations of toyocamycin or formycin B. From dose response curves, D_{10} values were calculated for different cell lines. Assuming D_{10} value of the drugs for the WT cells as 1, the relative degree of resistance of mutant cell lines was calculated.

2 The activity of AK in cell extracts was determined by measuring the synthesis of lanthanum chloride precipitable nucleotides by cell extracts as described in Methods.

Table X. Cellular Uptake of [^{3}H]Formycin B in Different CHO cell lines

Cell line	Degree of resistance towards formycin B	Total cellular uptake (cpm x 10^4)	Uptake relative to the WT cells (%)
WT	1	1.86	100
Toy^{r}4	2.5	1.10	59.1
Fom^{R}2	3	1.06	56.9
Fom^{R}4	8	0.78	41.9
Fom^{r}10	5	0.93	50
Fom^{r}12	7.5	0.83	44.6

The experiment was carried out by incubating 5 x 10^6 cells in the presence of [^{3}H]formycin B (2 μCi/ml) for 24 h at 37°C. The uptake and incorporation of [^{3}H]formycin B in various fraction was measured as described in Methods.

[^{3}H]formycin B. The cellular uptake of [^{3}H]formycin B in Toy^r mutant was reduced to about 60% of WT level, whereas in Fom^{R}2 and Fom^{R}4 mutant, which was about 3- and 8-fold resistant to formycin B, only about 57% and 42% uptake of [^{3}H]formycin B was observed respectively. Similarily in both Fom^r mutants, cellular uptake was found to be reduced in comparison to WT cells. In separate experiments with WT and mutant cells, it was observed that nearly all (> 90%) of [^{3}H]formycin B which was taken up by cells was in the form of phosphorylated derivatives (Table VIII). In view of this, the reduced uptake of [^{3}H]formycin B by mutant cell lines which lack AK and exhibit increased resistance to formycin B, provided strong evidence that the formycin B in CHO cells is phosphorylated via AK, and like other nucleoside analogs the phosphorylation step is essential for the cellular toxicity of formycin B.

In view of the above results, it was of much interest to determine if [^{3}H]formycin B was also phosphorylated in cell extracts. These experiments were carried out by incubating [^{3}H]formycin B with cell extracts from either WT or the mutant (both Toy^r and Fom^R) cells, under conditions similar to those used in assay for AK. However, in these experiments no conversion of [^{3}H]formycin B into its phosphorylated derivatives was observed (results not shown). Recently, Carson and Chang (1981) have reported that in cell extracts of Leishmania, phosphorylation of formycin B is carried out by a novel nucleoside phosphotransferase activity which uses p-nitrophenol phosphate as the donor of the phosphate group. However, when CHO cell

extracts were incubated with p-nitrophenol phosphate under conditions used by these authors (Carson and Chang, 1981), no phosphorylation of [^{3}H]formycin B was observed.

3.4.1.5 Inhibition of Adenylosuccinate Synthetase by Formycin B-5'-Monophosphate and Formycin A-5'-Monophosphate

Since monophosphates of formycin B and formycin A are analogs of monophosphates of inosine and adenosine, which are the substrate and product, respectively, of the reaction catalyzed by the enzyme ASS (see Fig. 3), it was of interest to examine whether they had any effect on the activity of ASS. Results of these studies are shown in Fig. 30. As one can see, addition of monophosphates of formycin B and formycin A to IMP containing reaction mixtures inhibited adenylosuccinate formation in a dose-dependent manner. At an IMP concentration of 0.2 mM, FoB-MP and FoA-MP inhibited the ASS activity by 50% at 0.27 mM and 0.14 mM, respectively.

To gain insight into the mechanism of inhibition of ASS by FoB-MP and FoA-MP, studies on the kinetics of inhibition of ASS by these compounds were carried out. The Lineweaver-Burke plots at two different concentrations of these analogs are presented in Fig. 31. As can be seen, while the inhibition of ASS by FoB-MP was competitive, FoA-MP appears to be a non-competitive inhibitor of the enzyme. From the plots, Km value for IMP was determined to be 0.17 mM, whereas Ki values for FoB-MP and FoA-MP were found to be 0.25 mM and 0.05 mM, respectively. The Km and Ki value for IMP and FoB-MP are very similar to those reported by Spector et al., 1984 for the partially purified

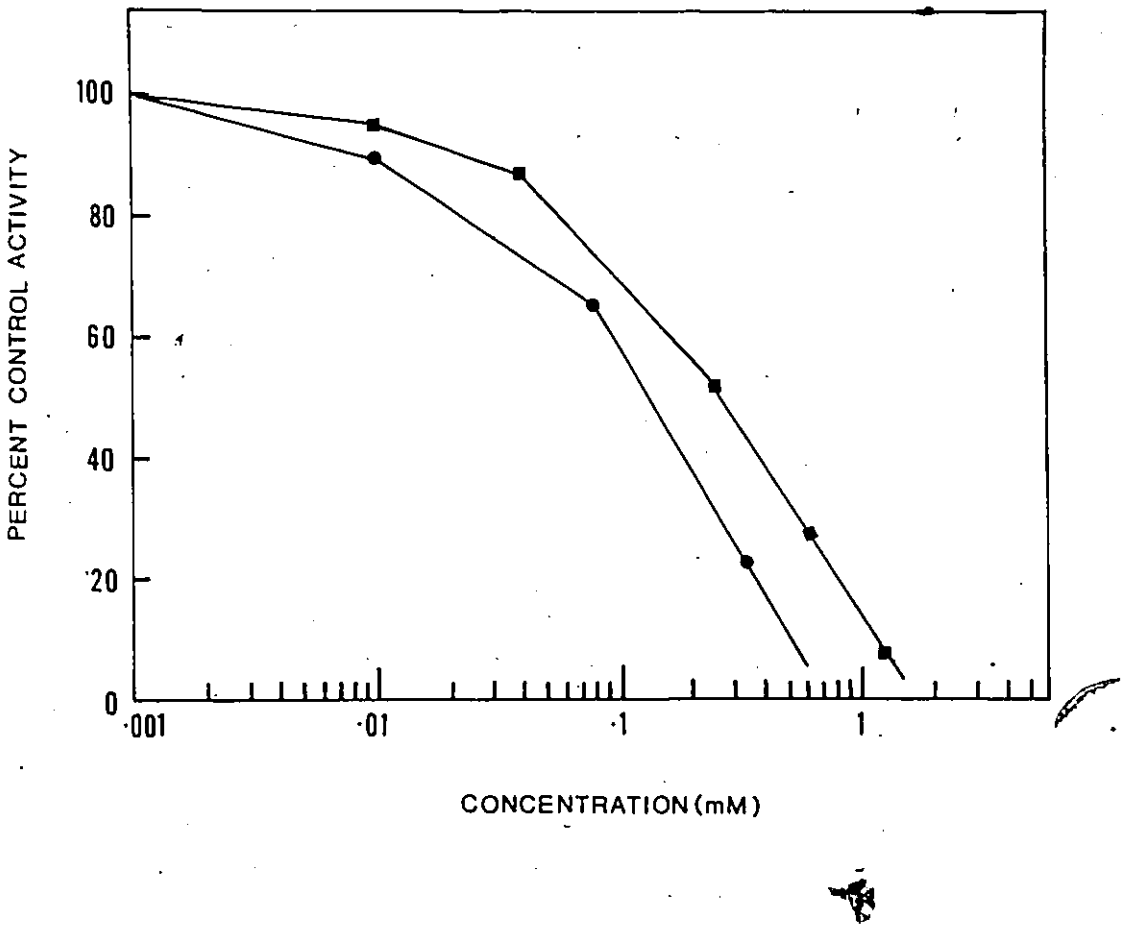


Figure 30: Inhibition of adenylosuccinate synthetase in CHO cell extract by formycin A-5'-monophosphate (●——●) and formycin B-5'-monophosphate (■——■). The assay of ASS activity in presence of 0.2 mM IMP was carried out as described in the Methods Section.

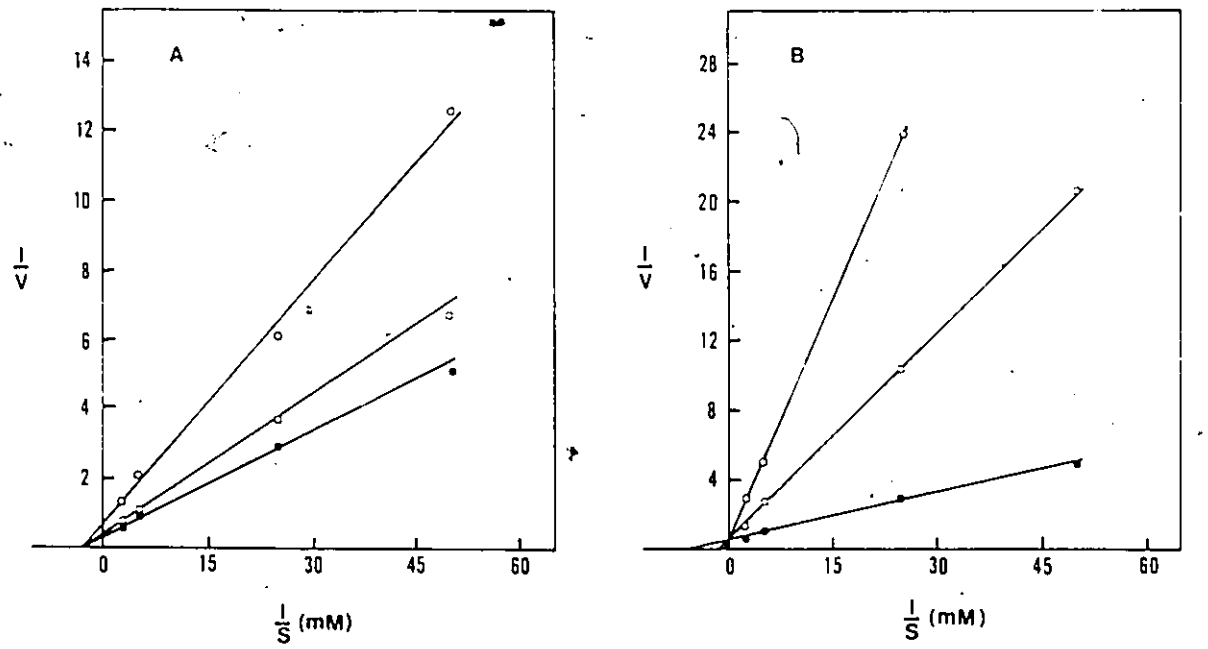


Figure 31: Kinetics of inhibition of adenylosuccinate synthetase by formycin A-5'-monophosphate (A) and formycin B-5'-monophosphate (B). (A) ●—● , control in the absence of FoA-MP; □—□ , 0.01 mM of FoA-MP; ○—○ , 0.08 mM of FoA-MP; (B) ●—● , control in the absence of FoB-MP; □—□ , 0.25 mM of FoB-MP; ○—○ , 0.6 mM of FoB-MP. Velocity is expressed as nmol AMP/mg protein/30 min.

enzyme from rabbit muscle. Under the assay conditions used in the present studies, FoB-MP is not a substrate for ASS. Similar results have been obtained by Carson and Chang (1981).

If phosphates of formycin B and formycin A also inhibit ASS in vivo, then treatment of CHO cells with formycin B or formycin A should inhibit hypoxanthine metabolism and uptake with a concomitant accumulation of IMP. The results of pretreatment of CHO cells with formycin B on the cellular uptake of [^{14}C]hypoxanthine are shown in Table XI. As one can see, pretreatment of CHO cells with formycin B reduced hypoxanthine uptake in cells by 40 to 50%. The observed decrease in [^{14}C]hypoxanthine uptake was similar to that seen with alanosine which upon metabolism specifically inhibits ASS (Gale and Schmidt, 1968; Graff and Plagemann, 1976). When cells were incubated with [^{14}C]hypoxanthine for 1 h in the presence of either 50 µg formycin B/ml or 2 µg alanosine/ml, a significant increase of up to 3-fold in the IMP pool was observed in the both cases, as determined by the paper chromatographic analysis of the PCA soluble fraction using ammonium acetate:ethanol (30:70, v/v) as solvent.

In view of the sensitivity of ASS to FoA-MP and FoB-MP, the activity of this enzyme in cell extracts of mutant cells resistant to formycin A (Fom^R mutants) as well as to formycin B (Fom^r mutants) were examined. Results of these studies indicated that both the Fom^R and Fom^r mutants contain normal levels of activity of this enzyme, which shows similar sensitivity to inhibition by monophosphates of formycin A and formycin B as the enzyme from WT cells.

Table XI. Effect of Formycin B and Alanosine on the Cellular Uptake and Incorporation of [^{14}C]Hypoxanthine in CHO Cells

Drug	Concentration	Total cellular uptake ($cpm \times 10^4$)	Incorporation into TCA-precipitable fraction ($cpm \times 10^4$)
Control	no drug	6.5	2.10
Formycin B	30 µg/ml	4.8	1.69
	80 µg/ml	2.9	1.06
Alanosine	1 µg/ml	4.5	1.70
	5 µg/ml	2.6	0.88

The drugs were added at the indicated concentrations to the WT cell culture 15 min before addition of [^{14}C]hypoxanthine. The cellular uptake and incorporation into macromolecules was determined as described in the Methods Section. Assuming the values obtained in control cells as 100%, the relative uptake or incorporation in drug-treated cells was calculated.

3.4.1.6 Effect of Other Bases and Nucleosides on the Cellular Toxicity of Formycin B

In earlier studies it had been observed that guanine exerted a synergistic effect on the cellular toxicity of alanosine while the presence of adenine or adenosine in the growth medium reversed the toxic effect of this drug (Gupta, 1980). Since the studies described above indicated that formycin B, like alanosine, had an inhibitory affect on ASS activity, it was of interest to determine the effect of various purine bases and nucleosides on its cellular toxicity. The results of these studies for both formycin B and alanosine are summarized in Table XII. As can be seen, addition of adenosine to the growth medium reduced the toxicity of formycin B in a concentration-dependent manner. A similar but smaller effect was also observed with adenine. Furthermore, as in the case of alanosine, when guanine was present in the growth medium it increased the toxicity of formycin B towards WT cells. Other purine and pyrimidine bases and nucleosides (viz. hypoxanthine, cytidine, thymidine; each at 5×10^{-5} M concentration) which were examined had no effect on the toxicity of these drugs. The observed similarities in the effects of various purine bases and nucleosides on the toxicity of both formycin B and alanosine provide support to the view that formycin B metabolites exert an inhibitory effect on ASS in vivo.

3.4.2 DISCUSSION

Studies presented in this section reveal a number of interesting aspects regarding the metabolism and the mechanism of

Table XII. Effect of Different Bases and Nucleosides on the Toxicity of Formycin B and Alanosine Towards CHO Cells

Supplement	Concentration [M]	Relative Cellular Toxicity (D_{10} values of the drug, µg/ml) Formycin B	Alanosine
No addition	-	2 (1.0)	0.01 (1.0)
Adenine	5×10^{-6}	3 (1.5)	10.0 (10,000)
	5×10^{-5}	5 (2.5)	N.D.
Adenosine	1×10^{-5}	5 (2.5)	10.0 (10,000)
	2×10^{-5}	9 (4.5)	10.0 (10,000)
	5×10^{-5}	15 (7.5)	N.D.
Cytidine	5×10^{-5}	2 (1.0)	0.01 (1.0)
Guanine	5×10^{-5}	1.5 (0.75)	0.003 (0.3)
	5×10^{-4}	0.4 (0.2)	N.D.
Hypoxanthine	5×10^{-5}	2 (1.0)	0.01 (1.0)
Thymidine	5×10^{-5}	2 (1.0)	0.01 (1.0)

The experiment was carried out by determining the plating efficiency of WT (CHO) cells in medium containing increasing concentrations of either formycin B or alanosine. The various bases and nucleosides were added to the growth medium at the indicated final concentrations. The growth medium also contained 5 µg adenosine deaminase inhibitor EHNA/ml and 5×10^{-5} M uridine to prevent cellular toxicity of adenine nucleotide. From the dose response curves, concentrations of the drugs which were required to reduce the plating efficiency of the cells to 10% of that obtained in the absence of any drug (D_{10} values) were determined. Assuming the D_{10} value of the drug in the absence of any addition as 1, the relative cellular toxicities in presence of various additions were determined from the ratios of the D_{10} values (given in parenthesis).

N.D. - not determined

action of formycin B in mammalian cells. These studies show that when CHO cells are incubated with [^{3}H]formycin B, it is phosphorylated to FoB-MP. The other phosphorylated derivatives which are detected include FoA-MP and its higher phosphorylated forms which have been found to be incorporated into cellular RNA. After this work was completed, similar metabolism of formycin B has been reported in the mouse L cells (Lafon et al., 1983; Spector et al., 1984). The formation of FoA-MP from formycin B could possibly occur by one of the two routes: First, formycin B could initially be phosphorylated into FoB-MP, and the latter metabolite which is an analog of IMP is then converted into FoA-MP by the enzymes adenylosuccinate synthetase and lyase. Second, it is also conceivable that formycin B is first converted into formycin A by a yet unidentified cellular enzyme and the latter is then phosphorylated by the enzyme AK. However, our failure to observe any intracellular pool of either formycin A or formycin B favors the former possibility. This possibility is also supported by recent studies in Leishmania (Nelson et al., 1982; Rainey and Santi, 1983).

Studies presented here also strongly indicate that the purine salvage pathway enzyme AK is involved in the phosphorylation of formycin B in CHO cells. Some of the observations which have led to this inference are: (i) A large number of independent CHO cell mutants selected for resistance to various adenosine analogs and which lack AK activity (<0.5%), all consistently exhibited two to three-fold resistance to formycin B. (ii) The Fom^R mutants of CHO cells which

exhibit specific cross-resistance towards C-nucleosides are also affected in AK as seen by loss of AK activity in cell extracts. (iii) Resistance in mutant cells selected for resistance to formycin B was found to be associated with the alteration in AK. (iv) All the three types of mutants, i.e. Toy^r, Fom^R and Fom^r cells exhibit reduced cellular uptake of [^{3}H]formycin B in comparison to WT (AK^+) cells. A good correlation was observed in these studies between the degree of resistance of the mutant cells to formycin B and the decreased uptake of [^{3}H]formycin B by these cell lines. (v) The cellular toxicity of formycin B is decreased in presence of adenosine in a concentration dependent manner. Since adenine or other purine bases were not as effective as adenosine, part of this effect may result from competition between formycin B and adenosine for binding to AK. (vi) It has recently been reported that 5-iodotubercidin which is a specific inhibitor of AK, blocks the phosphorylation of formycin B in mouse L cells (Lafon et al., 1983). All of these observations together provide strong evidence that like other nucleoside analogs, formycin B also needs to be phosphorylated before it becomes toxic to cells and that AK is involved in the phosphorylation of this drug. Willis et. al. (1980) have previously reported that AK deficiency has no effect on the toxicity of a human lymphoblast line towards formycin B. However, the concentration of formycin B which was employed in their experiment was at least 100-fold higher than the minimum concentration which had an observable effect on cell growth. If the AK^- mutants of lymphoblast line also exhibited only 2 to 3-fold resistance to formycin B, as seen

here with CHO cell mutants, then the effect of AK deficiency on formycin B toxicity would not be observed at the high drug concentration employed.

In view of the high degree of resistance of AK^- mutants to other adenosine analogs (i.e. 100- to 1000-fold) the 2.0 to 3.0-fold resistance of AK^- mutants to formycin B at first does not appear very significant. However, the low degree of resistance of mutants to formycin B could be understood if one considers the fact that in contrast to adenosine analogs such as toyocamycin and tubercidin which are toxic to CHO cells at ng/ml concentrations, formycin B shows similar toxicity at µg/ml range. The fact that AK^- mutants are killed in the presence of µg/ml concentrations of the former analogs indicates that at such high concentrations, these nucleoside analogs are perhaps phosphorylated by other cellular enzyme(s) for which they may have low substrate affinity. Since formycin B, which is an inosine analog, is toxic to cells at µg/ml range (this is expected because substrate efficiency of inosine for AK is about 1000-fold less than that of adenosine; Miller et al., 1979b), its affinity for AK may not differ very much from that for other non-specific phosphorylating enzyme present in mammalian cells, which have yet to be characterized. If this view is correct then mutants lacking AK are not expected to show very high level of resistance to formycin B. The presence of other non-specific purine nucleoside phosphorylating enzyme could also account for the fact that AK^- mutants which contain no detectable activity of AK in cell extracts are still able to phosphorylate

[^{3}H]adenosine and [^{3}H]tubercidin to about 10 and 15% of WT level (Section 3.1 and 3.2). This view is also consistent with our observation that in various AK$^-$ mutants, the cellular uptake and incorporation of [^{3}H]formycin B is reduced by only about 40 to 50%.

At present the mechanism of cellular toxicity of formycin B is not known. Results presented in this section provide evidence that one of the cellular reactions which is inhibited by formycin B/formycin A metabolites is the purine-nucleotide biosynthetic enzyme ASS. This is indicated by the following observations: (i) In cell extracts, FoB-MP and FoA-MP are found to inhibit ASS activity in competitive and non-competitive manners, respectively. Inhibition of ASS by FoB-MP in Leishmania has previously been also observed by Carson and Chang (1981). However, recently it has been shown (Nelson et al., 1982; Spector et al., 1984) that FoB-MP is also a substrate for ASS, but its substrate efficiency was lower in comparison to IMP. This observation may account for the competitive inhibition of ASS by FoB-MP. The observation that Ki of FoA-MP is much lower than that of FoB-MP, suggests that the inhibition of ASS may be caused by the former metabolite. (ii) The cellular toxicity of formycin B is partly overcome by the addition of adenine or adenosine (which are converted into AMP which is the end product of the pathway involving ASS), but other bases such as hypoxanthine have no effect on the toxicity of formycin B. (iii) Addition of formycin B to CHO cell cultures leads to inhibition of hypoxanthine uptake and enlargement of intracellular IMP pools. These effects are analogous to the effects of alanosine, which is a

known specific inhibitor of the enzyme ASS (Graff and Plagemann, 1976). Furthermore, similar to the earlier reported synergistic effect of guanine on the toxicity of alanosine (Gupta, 1980), the toxicity of formycin B towards CHO cells is also enhanced in presence of guanine. The above observations indicated that, like alanosine, toxicity of formycin B may result from inhibition of ASS by its metabolites, resulting in cellular starvation for adenine nucleotides. However, this inhibition alone could not account for the toxicity of formycin B at higher concentrations. Unlike alanosine, whose toxicity is completely reversed by adenine or adenosine (as may be expected if a drug only inhibits ASS; toxicity of alanosine is reduced by 10,000-fold in presence of adenine or adenosine) the cellular toxicity of formycin B is only slightly overcome by adenine (Table XII). The somewhat larger effect of adenosine in these studies in comparison to adenine is perhaps caused by its competition with formycin B for cellular uptake. The failure of adenine or adenosine to overcome toxicity of high concentrations of formycin B indicate that at higher concentrations, formycin B metabolites inhibit some other cellular reaction(s) or it may cause depletion of some other essential cellular metabolites as suggested by Robinson et. al. (1984).

3.5 GEL ELECTROPHORETIC ANALYSIS OF GENETIC LESION IN DIFFERENT TYPES OF MUTANTS AFFECTED IN ADENOSINE KINASE

Previous studies on the development of resistance to purine base analogs, 6-thioguanine and 8-azaadenine have shown that the resistance to these agents is developed in mammalian cells due to an alteration in the purine salvage enzyme HGPRT and APRT respectively (Brockman, 1963; Jones and Sargent, 1974; Taylor et al., 1977). On the basis of the detailed analysis of a large number of mutants selected for resistance to the above drugs, different classes of mutants affected in enzyme HGPRT and APRT have been identified. Each class show interesting differences in their genetic and biochemical characteristics (Bradley, 1979, 1983; Caskey and Kruh, 1979; Fenwick et al., 1977, 1984; Fuscoe et al., 1983; Simon et al., 1982, 1983; Simon and Taylor, 1983; Turker et al., 1984). Recently, studies utilizing immunological and molecular biology approaches, have shown that the phenotypes of the different types of mutants can be explained on the basis of the molecular nature of the genetic lesion involved (Capecchi et al., 1977; Fenwick et al., 1977; Milmann et al., 1976; 1977; Simon et al., 1982, 1983; Simon and Taylor, 1983; Taylor et al., 1977). In addition, such an analysis has proved very useful in establishing that the altered genetic and biochemical phenotype of mutant cells is a direct consequence of mutation in the DNA and does not represent some secondary effect.

In earlier sections, studies with mutants obtained for resistance to toyocamycin, formycin A and formycin B are described. All the mutants examined were found to be affected in the same protein i.e., AK, but exhibited interesting differences in their genetic and biochemical phenotypes. To gain further insight into the nature of the genetic lesions in different types of mutants affected in AK, these mutants have been further investigated using immunological and biochemical techniques.

To investigate the possible alteration in AK by immunological techniques, it was necessary to purify AK from CHO cells so that antibodies could be raised against it. The present section describes the purification of AK from CHO cells to homogeneity, raising of specific antibodies against purified AK, as well as immunoblot analyses of the cross-reacting material (CRM) in different types of mutants affected in AK. Using these antibodies, region (or spots) on 2-D gels that very likely correspond to the AK protein have been identified. Comparison of the 2-D gel electrophoretic patterns of total cellular proteins from different mutant cell lines indicate that some of the mutants contain a specific alteration in this region. These and other results presented in this section provide an evidence that the genetic lesion in many of the AK mutants studied here involves a missense type of alteration in the structural gene of AK.

3.5.1 RESULTS

3.5.1.1 Purification of Adenosine Kinase from CHO Cells

AK was purified from CHO cells via a procedure used earlier by

Andres and Fox (1979) for isolation of AK from human placenta. The scheme of purification is summarized in the Methods Section. Basically, after DEAE- and CM-cellulose batch elution of the crude extract, the post-ion exchange solution was applied to an affinity column consisting of AMP-Sepharose beads. After washing the non-specifically bound proteins the bound enzyme was eluted using buffer containing adenosine, $MgCl_2$ and ATP. Measurement of the AK activity and a protein estimation were carried out after each step of purification. It was observed during the purification studies that AK present in CHO cell extracts did not bind to any of the ion-exchange resins at pH 6.0, indicating that the isoelectric pH (pI) of AK is in the range of pH 6.0. This pI value is similar to the pI value reported earlier for AK from human placenta (Andres and Fox, 1979). Ion exchange batch elution resulted in 7-9 fold purification with a 70% overally recovery. The main step for the purification of AK was the affinity chromatography, where the AK activity displayed a sharp distinct single peak on elution with adenosine. The affinity chromatography step resulted in more than 500-fold purification. The specific activity of the final purified preparation was 5.0 μmole/min/mg. This preparation showed only one major band on SDS gel electrophoresis (Fig. 32, lane 7 and 8) in the relative molecular mass range of 38,000 after staining with Coomassie brilliant blue.

3.5.1.2 Specific Reactions of Antibodies with Adenosine Kinase

Antiserum against AK was raised by injecting purified preparation of AK from CHO cells into a rabbit. Serum from the rabbit

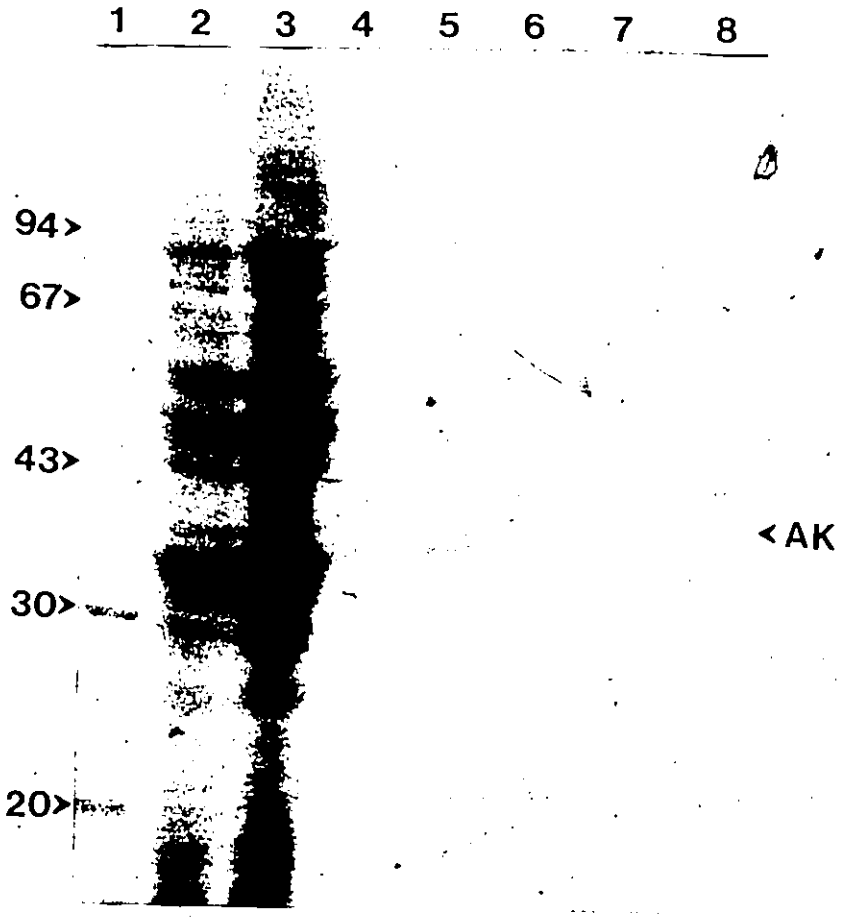


Figure 32: SDS-polyacrylamide slab gel analysis of the proteins in CHO (WT) cell extracts after ion-exchange and affinity chromatography. Proteins on the gel were stained with Coomassie brilliant blue. Lane 1, relative molecular mass marker X 10^{-3}; Lane 2, 20 µl of crude cell extract of CHO cells; Lane 3, 50 µl of crude cell extract CHO cells; Lane 4, 20 µl of post-ion exchange solution; Lane 5, 50 µl of post-ion exchange solution; Lane 6, 20 µl of the final purified preparation after affinity chromatography; Lane 7, 100 µl of the above preparation; Lane 8, 150 µl of the final preparation.

was tested regularly after three injections of proteins by using the immunoblot technique, as described in the Methods. Results obtained with the preimmune serum and immunized serum are shown in Fig. 33. As expected when using the preimmune serum, no CRM in the M_r region of 38,000 was observed, but, contrastingly with the immunized serum, (even after one-hundred and two-hundred times dilution of the immunized serum in 3% BSA) a major band at the M_r corresponding to AK was detected. The specificity of the above antibody was tested by examining the effect of the antiserum treatment on the activity of AK after incubation with the a crude cell extract of CHO cells. This was done or incubating the preimmune or antiserum with crude cell extract of CHO cells for 16 h followed by treatment with protein A-Sepharose for 3 h at $4^{o}C$. After centrifugation and washing of the protein A-Sepharose beads, the AK activity was determined both in the supernatant and with the sepharose beads. Results of these studies are presented in Table XIII. As can be seen, treatment with antiserum resulted in the decrease of AK activity in the supernatant and at the same time caused an increase in AK activity associated with the sepharose beads. Treatment with the preimmune serum under similar conditions, caused neither an appreciable decrease in AK activity in the supernatant nor an increase in AK activity associated with the beads. These results provided a strong indication that the antibodies were reacting with the AK present in the cell extracts of CHO cells. Furthermore, in order to demonstrate that the decrease in AK activity of the supernatant was associated with the removal of AK protein, immunoprecipitate i.e.,

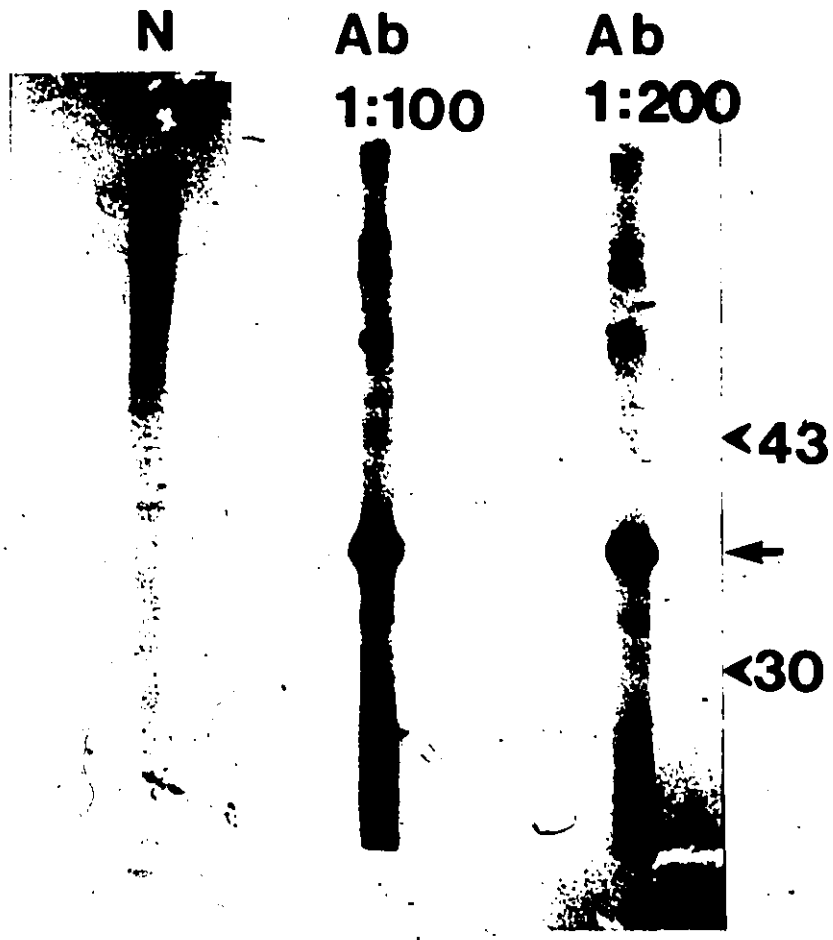


Figure 33: Immunoblotting of protein extract from WT (CHO) cells by using preimmune and antiserum to purified AK to detect their cross-reactivity. Whole cell proteins from 5 X 10^5 WT cells were subjected to SDS polyacrylamide slab gel electrophoresis. The separated proteins were transferred onto a nitrocellulose sheet and probed for CRM with the preimmune serum as well as with the diluted antiserum raised against purified AK by a procedure as described in Methods. > , indicates $M_r = 10^{-3}$ of the marker proteins; ➞, indicates position of major CRM.

Table XIII. Immunoprecipitation of AK by Using Preimmune Serum and Antibodies Raised Against Purified AK

Experiment	Type of serum[1]	AK activity[2] in the supernatant ($cpm \times 10^3$)	% of total activity	AK activity[2] associated with the sepharose beads ($cpm \times 10^3$)	% of total activity
I	P	17	100	0.05	<0.5
	A	3.1	18.2	8	47
II	P	18	100	0.05	<0.5
	A	2.5	13.8	9	50

Immunoprecipitation was carried out by incubating with rotation 500 µl of crude cell extract of CHO cells with 50 µl of preimmune serum or antiserum against AK for 16 h followed by treatment with 50 µl of swollen protein A-Sepharose CL-4B for 16 h at 4°C. Sepharose beads were removed by centrifugation. AK activity in the supernatant was determined by using 25 µl of it in the assay for AK and for determination of AK activity associated with the beads. Washed beads (three times with 10 mM phosphate buffer, pH 7.0) were incubated with the reaction mixture for AK.

[1] P denotes preimmune serum and A denotes antiserum raised against AK.

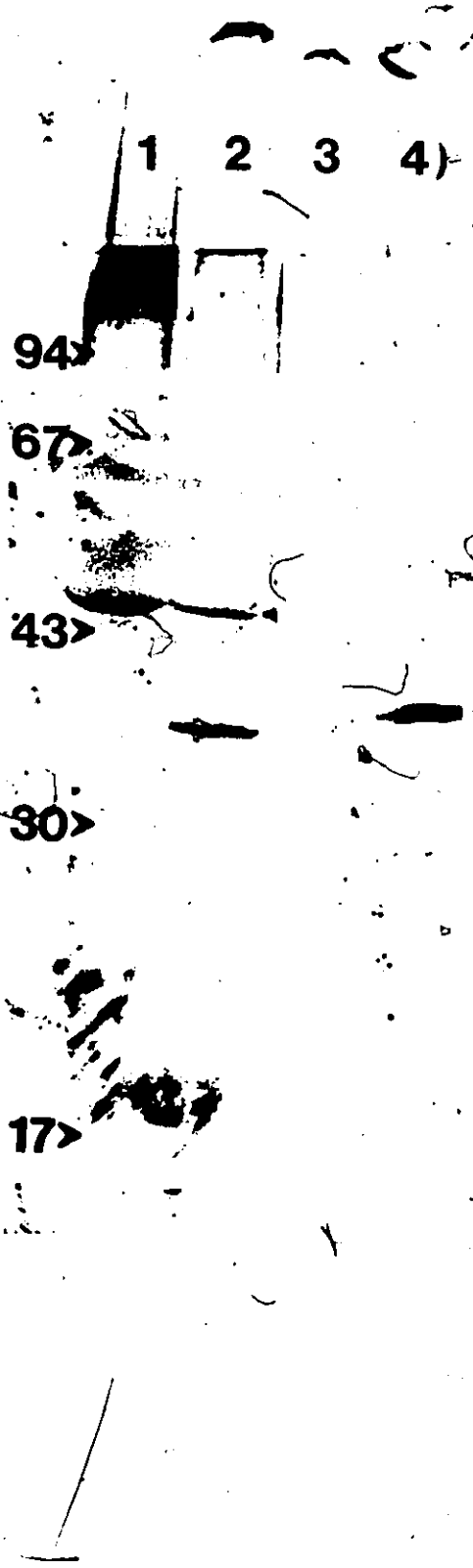
[2] AK activity was determined as described in Methods and it is represented in this table by the counts of adenine nucleotides precipitable by lanthanum chloride after a 20 min assay by using the volumes mentioned above.

protein(s) associated with the protein A-Sepharose beads, obtained both under native and denaturing (+SDS) conditions were analyzed on SDS-polyacrylamide gels. Results of these studies are presented in Fig. 34. As demonstrated in Fig. 34, the antiserum raised against AK specifically pulled out a protein from the cell extracts which is of the same relative molecular mass as AK (Fig. 34, lane 2 and 4). The preimmune serum on the otherhand did not immunoprecipitate any protein in the same M_r range as AK, and only some faint non-specifically binding protein was observed in the high M_r range (Fig. 34, lane 1 and 3). The demonstration that the antiserum immunoprecipitate a protein of a relative molecular mass of 38,000 and such a treatment results in removal of AK activity from cell extracts, provide strong suggestive evidence that the antiserum raised is specifically cross-reacting with AK present in CHO cell extract.

2.5.1.3 Immunoblot Analyses of the Cross-Reacting Material in the Wild Type and Various Mutant Cell Extracts

Having established the specificity of the antibody, cell extracts of various cell lines were examined for the presence of CRM by the immunoblot technique. Results of these studies are shown in Fig. 35. As can be seen, cell extracts of WT (Fig. 35A, lane 1 or Fig. 35B, lane 1) as well as various mutants, viz., Fom^R2 (Fig. 35A, lane 2), Fom^R4 (Fig. 35A, lane 3), Fom^r10 (Fig. 35A, lane 4) Fom^r10 (Fig. 35B, lane 2), Toy^r4 (Fig. 35B, lane 3) and Pyr^r1 (Fig. 35B, lane 4), contain the major cross-reacting protein that has a relative molecular mass of approximately 38,000 and most likely corresponds to AK. In addition to

Figure 34: SDS-polyacrylamide slab gel analysis of the immunoprecipitates obtained from labelled CHO cell extract both under native as well as denaturing conditions. Immunoprecipitation was carried out by incubating cell extract from [^{35}S] methionine (200 µCi, 4 h) labelled WT cells with 20 µl of antiserum raised against purified AK from CHO cells for 16 h. This was followed by 3 h incubation with 20 µl of swollen protein A-Sepharose beads. Proteins associated with the sepharose beads were analyzed as described in Methods. For using denaturing conditions, SDS was added to a final concentration of 1% before incubation with antiserum. Lane 1 and 3, immunoprecipitates using preimmune serum under native and denaturing conditions, respectively; Lane 2 and 4, immunoprecipitates using antiserum against AK under native and denaturing condition respectively. ➡, indicates position of AK.


1
2
3
4
94>
67>
43>
30>
17>

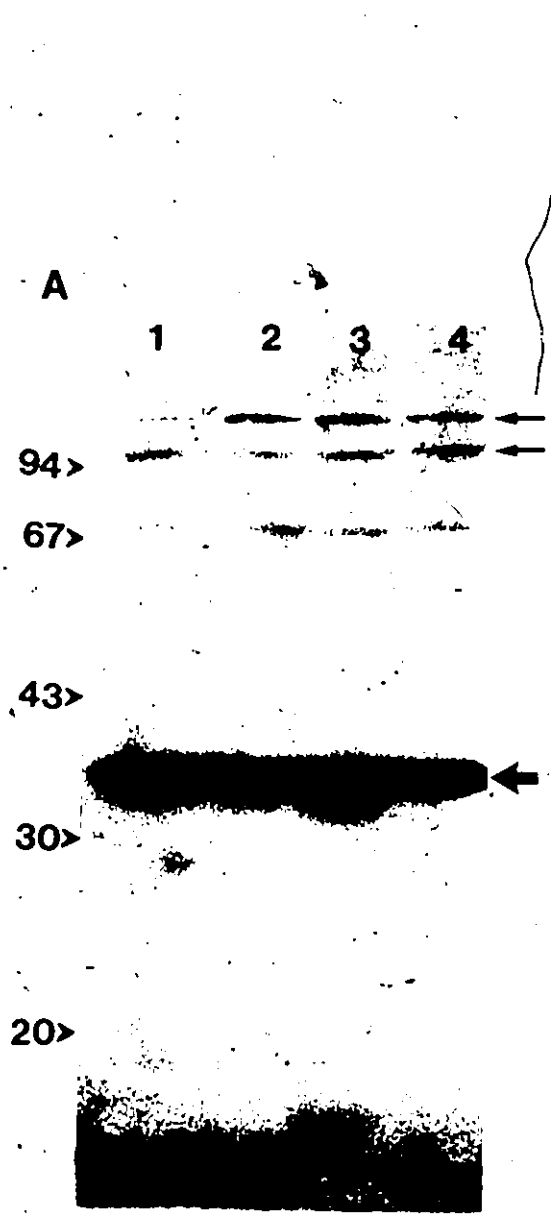


Figure 35: Immunoblotting of proteins from the whole cell extracts of parental WT cells and the different mutant cell lines affected in AK. Following SDS-polyacrylamide gel electrophoresis of the protein extract of 5 X 10^5 cells of WT and different mutant lines, proteins were electrophoretically transferred onto a sheet of nitrocellulose membrane. The membrane was reacted with one-hundred times diluted AK antibodies and then [^{125}I]IgG as described in Methods.
A) Immunoblots of WT (lane 1), Fom^{R}2 (lane 2), Fom^{R}4 (lane 3) and Fom^{r}10 (lane 4). ⟶ , indicates positions of CRM in the high M_r range; ➡ , indicates position of AK.

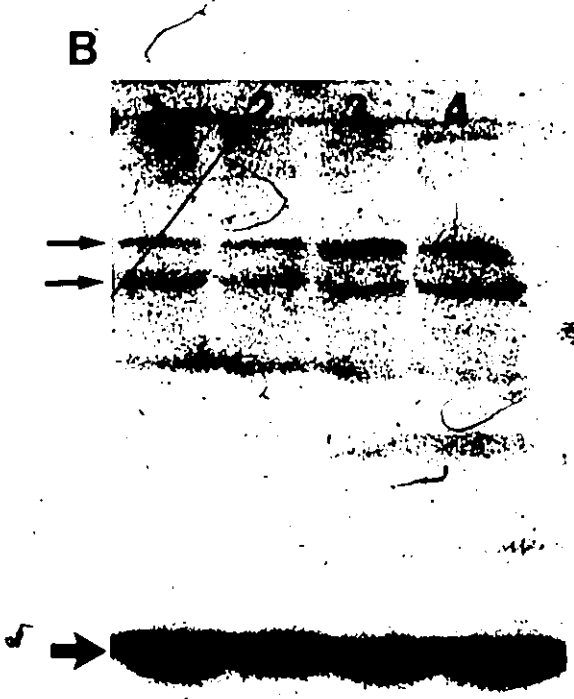


Figure 35: B) Immunoblots of WT (lane 1), Fom^r12 (lane 2), Tgy^r4 (lane 3) and Pyr^r (lane 4). Big arrow indicates the position of protein corresponding to AK and small arrows show the high relative molecular mass to which antibodies to AK cross-react.

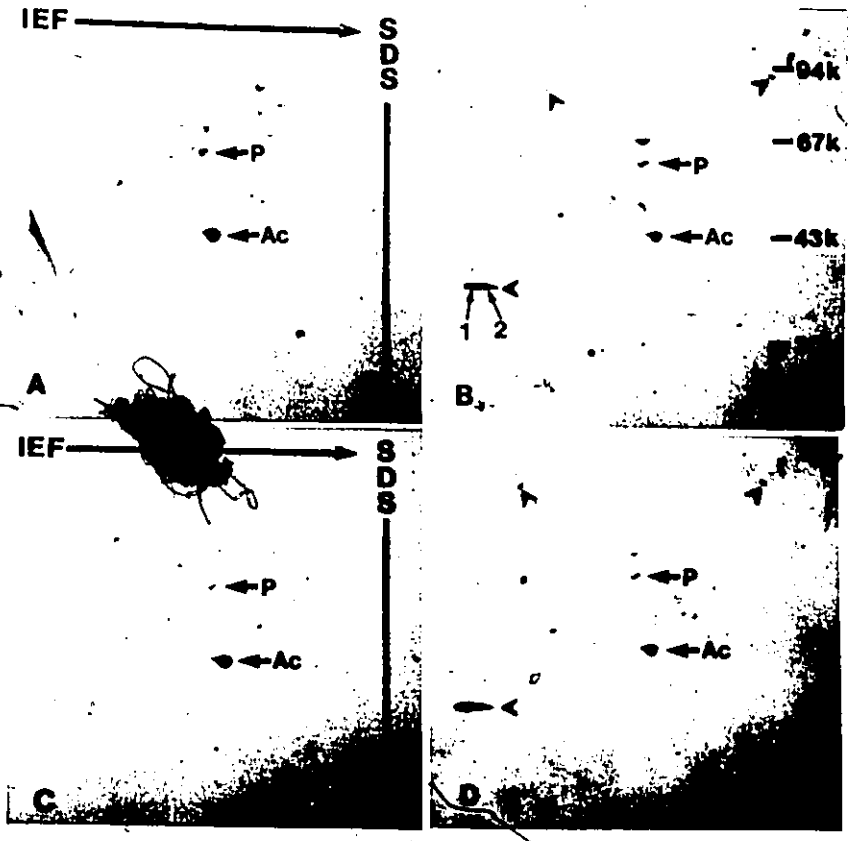
this band a few minor bands in the higher relative molecular mass region which cross-reacted with AK antibody were observed. The reason for the appearance of bands in the high relative molecular mass region is not clear at present but they could either be the precursors for AK or may just represent some non-specific binding proteins. Very interestingly, if the immunoblots of various mutant lines are compared with the WT cells, it is evident that all the mutant cell lines contain the major cross-reacting protein i.e., it has the same electrophoretic mobility in SDS-gels as the AK from the parental WT cells. These results indicate that the mutant protein is not altered in its size. Furthermore, the intensity of the major CRM (i.e., M_r = 38,000) in immunoblots of various mutant lines were almost similar to that of WT cells. The presence of a similar extent of CRM in different mutant lines provide strong suggestive evidence that the genetic lesion in these mutants does not involve either the deletion or the extinction of expression of the AK gene. It is important to mention here that because of the limitation of sensitivity, small differences in the intensity of the CRM observed with different cell lines cannot be ruled out.

2.5.1.4 Two-Dimensional Gel Electrophoresis Analyses of the Mutant Cells

In view of the above results which suggested that there is no alteration in the amount or size of AK protein in different mutant cells, the possibility that the various mutants may contain a missense type of alteration in the structural gene of AK was tested. Since

about 30% of the missense mutations in proteins have been estimated to lead to a charge alteration, proteins from the parental and mutant cells were analyzed by 2-D gel electrophoresis. Initially, to identify spot(s)/region corresponding to enzyme AK on 2D-gels, the proteins from the WT cells were labelled with low concentration of [^{35}S]methionine and then separated by 2-D gel electrophoresis. The separated proteins were then electrophoretically transferred onto a sheet of nitrocellulose membrane. The blot was then exposed to Kodak X-Omat XAR 5 films for 1 day to locate the position of the major protein spots on 2-D gel. Subsequently, the blot was treated with antibodies specific for AK followed by [^{125}I]-labelled goat anti-rabbit IgG. The antibody treated blot was then again exposed to Kodak X-Omat XAR 5 films, to identify the protein spots which had reacted with the AK antibodies. Such an approach has not only identified the spot(s) corresponding to AK but has also dictated information concerning the position of the AK spot(s) in relation to the other major protein spots, which can serve as markers. Results of such studies are shown in Fig. 36. Fig. 36 also includes results obtained with Fam^{r}12 mutants. On comparison of the autoradiograph of the 2-D immunoblots of WT cells, both before and after antibody treatment, three spots can be identified which became prominently labelled after antibody treatment (spots are represented by symbol ➤). Of these three spots, two spots are in the high M_r range and may represent the high M_r bands visible in the one-dimension immunoblots of WT cells (see Fig. 35). The third protein spot which is in the relative molecular mass range of AK is a smear and may be due to

Figure 36: Immunoblotting of the two-dimensional gels of the whole cell extracts of parental WT cells and Fom^r12 mutant cells. WT cells (1×10^6) labelled with [^{35}S]methionine (2 µCi) for 1 h were lysed and subjected to isoelectrofocussing followed by SDS-polyacrylamide gel electrophoresis in the second dimension. Separated proteins were transferred onto a sheet of nitrocellulose membrane as described in the Methods. Blots were probed for the spot(s) for AK protein by using one-hundred times diluted antibodies against AK. Blots were then exposed to photographic film both before and after treatment of the AK antibodies. (A) immunoblot of 2-D gel of WT cells before treatment of antibodies against AK, (B) above immunoblot after antibody treatment, (C) immunoblot of 2-D gel of Fom^r12 mutants before treatment with antibodies against AK, (D) immunoblot of Fom^r12 mutants after antibody treatment. The positions of molecular weight markers as obtained after running a parallel gel are shown in B. The molecular weight markers employed were albumin (M_r = 67,000), ovalbumin (M_r = 43,000) and carbonic anhydrase (M_r = 30,000). The arrows indicate the position of actin (Ac), the P protein (P) and > indicates the protein spots which become prominently labelled after antibody treatment. Spots 1 and 2 represent protein(s) for AK. The direction of IEF and SDS is indicated.

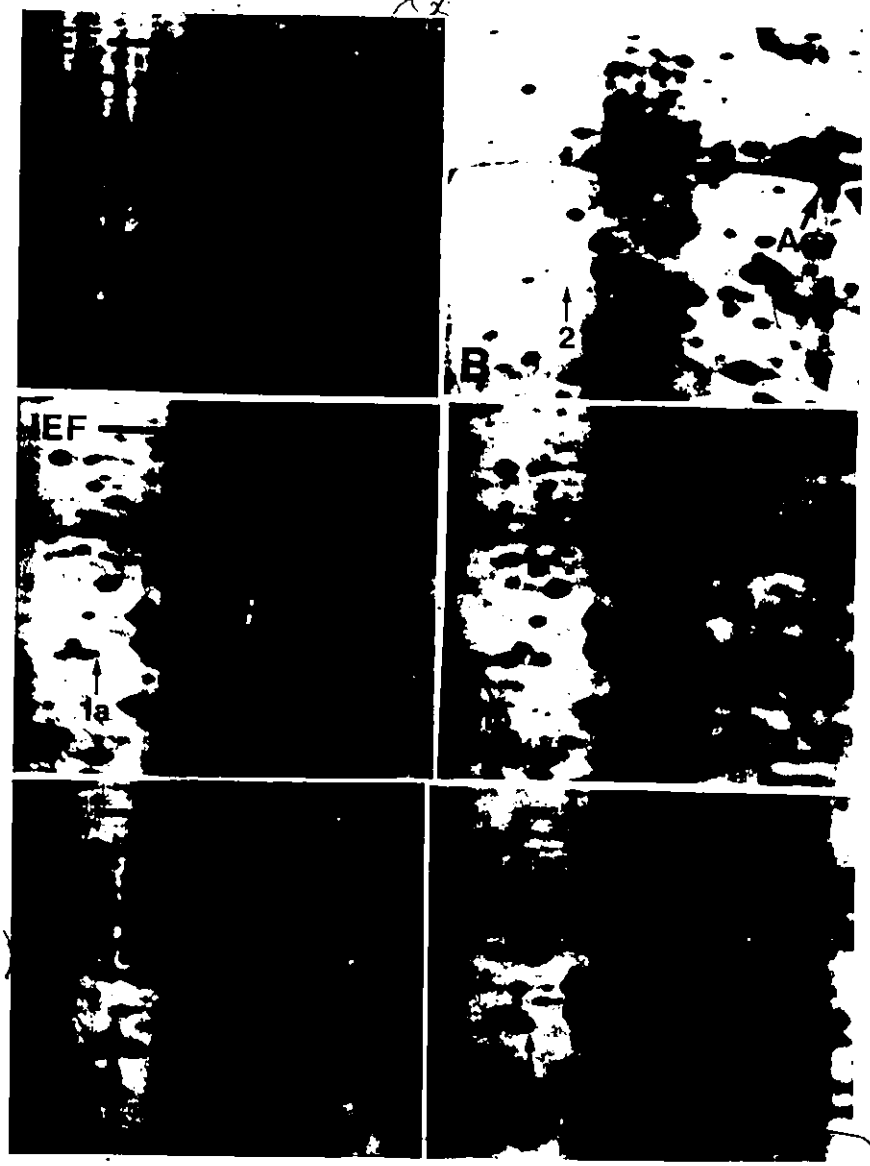

IEF
SDS
P
Ac
A
94k
67k
43k
1
2
B
IEF
SDS
P
Ac
C
P
Ac
D

the presence of two or more isozymic and electrophoretically distinct forms of the AK protein. The possibility that they may represent subunits of AK is unlikely since AK is known to be monomeric (Andres and Fox, 1979; Chang et al., 1980; Miller et al., 1979a).

This smear was also faintly visible in the Coomassie brilliant blue stained gels of WT CHO cells. When purified AK from CHO cells was added to WT cell extract, the smear corresponding to AK became larger in size, supporting the conclusion that these spots most likely correspond to that of AK. In view of the uncertainty of the exact location of AK protein spot(s) on 2-D gels, spots 1 and 2 (Fig. 36A) have been assumed to represent the protein corresponding to AK.

The region containing the spots of AK were compared on 2-D gels of parental WT cells and different mutant cells. The 2-D gel electrophoretic patterns of protein present in cell extract of sensitive (WT) and a number of mutants affected in AK are shown in Fig. 37. Comparison of the region containing the AK spots in 2-D gels of WT and various mutant cells revealed some differences on spots 1 and 2. On comparison of the intensity of both spots between WT and Toy^r mutants, it is evident that in the case of Toy^r mutants, (Fig. 37B) the intensity of spot 2 is highly reduced in comparison to spot 2 present in 2-D gels of WT cells (Fig. 37A). On the otherhand, in Pyr^r mutants, an additional spot designated as 1a (Fig. 37C) seems to have appeared adjacent to the spot 2 and at the same time, the intensity of the spot 1 has reduced. Unlike the Pyr^r mutants, in the case of both the Fam^R mutants, an additional spot is present on the left hand side of spot 1

Figure 37: Two-dimensional gel electrophoretic patterns of the total cellular proteins from different cell types. 2-D gels were run as described in the Methods section. (A) WT, (B) $Toy^{r}4$, (C) $Pyr^{r}1$, (D) $Fom^{R}2$, (E) $Fom^{R}4$, (F) $Fom^{r}10$. The big arrow indicates the position of the marker protein, Actin (Ac) and small arrows indicate the positions of spots 1, 2 and other additional spots. Additional spots are designated as 1a and 1b. The direction of IEF and SDS is indicated.


A
2
B
IEF
1a

and this spot has been designated as 1b (Fig. 37D and E). The appearance of this new spot in the 2-D gels of both mutants neither changed the intensity of spots 1 and 2 nor their arrangement pattern in comparison to the other spots. However, 2-D gels of Fom^r10 mutants (Fig. 37F), like the Pyr^r mutants, show an increased intensity of spot 2, which could have arisen due to appearance of an additional spot in close proximity to spot 2.

3.5.2 DISCUSSION

The results put forth in this section have provided insight into the nature of the molecular lesion in different types of mutants affected in AK. Analyses of the mutant cells have been carried out at the protein level, by using immunological and biochemical techniques.

The relative molecular mass of AK purified from CHO cells by affinity chromatography in the above studies, is about 38,000, which is very close to that obtained for the enzyme from brewer's yeast (Leibach et al., 1971), rat heart (De Jong, 1977), mouse erythrocyte (Schmidt et al., 1974), human placenta (Andres and Fox, 1979) and rabbit liver (Miller et al., 1979a), but differs from the value of 23,000 for rabbit liver AK (Lindberg et al., 1967) and 56,000 for L1210 murine leukemia cells (Chang et al., 1980).

From comparison of the immunoblots of the parental and different mutant cells, three inferences can be made. First, since all cell lines contain material which cross-reacts with antibodies to AK, this strongly indicates that AK is expressed in all the mutant cells. These results provide evidence that the resistance to purine nucleoside

analogs is not due to "gene inactivation" or loss of the corresponding genetic material. Secondly, the amount of AK expressed in all mutant cell lines is in the same range which suggests that these mutants do not involve either the gene amplification or the regulatory type of genetic alterations. Thirdly, the similar electrophoretic mobility in SDS gels of the major band which cross-reacts with the AK antibody provides an evidence that the AK in all the mutant cells has the same relative molecular mass as the AK present in WT cells. This result strongly argues against the possibility of a large deletion within the AK gene or a nonsense type of premature chain termination. All these results, together with the electrophoretic charge alteration seen in 2-D gels of various mutants in the region corresponding to AK, provide strong suggestive evidence that the most of these mutants may contain a missense mutation within the structural gene for AK. This inference is also supported by the results of the genetic and biochemical studies which showed that both the Fom^R as well as Fom^r mutants contained biochemically altered forms of AK within them.

A major paradox in somatic cell genetics is the ease with which large number of phenotypically recessive mutants affected at the autosomal loci can be isolated (Caskey and Kruh, 1979; Simon and Taylor, 1983; Turker et al., 1984). This paradox becomes more puzzling by the report that the recessive mutants affected at the AK locus (an autosomal loci) can be obtained at high frequencies (10^{-3}-10^{-4}, Gupta and Siminovitch, 1978b). These results have led to the suggestion that the high frequency of mutations at this locus may involve a chromosomal

loss or inactivation type of genetic event, rather than the usual base substitution type of mutation that generally occurs at lower frequency (Bradley, 1983; Eves and Farber, 1981; Meuth and Arrand, 1981; Siminovitch, 1976; Turker et al., 1984; Wilson et al., 1983). However, the above results which show that these mutants contain equivalent amounts of CRM provide strong evidence that the Toy^r mutants do not involve either loss or repression of the AK gene. Much of the available results, indicate that the Toy^r mutants which are obtained at high frequency contain a missense type of genetic alteration in AK and it is, therefore, suggested that the high frequency of mutation at this locus may be due to the presence of a mutational hot spot within the AK locus. The occurrence of single point mutation at high frequency has previously been reported in the structural gene of APRT (Simon and Taylor, 1983) as well as in the structural gene of HGPRT (Caskey and Kruh, 1979).

4. CONCLUSIONS AND FUTURE PROSPECTS

Studies presented in this thesis show that, in CHO cells, three different classes of mutants affected in the purine nucleoside phosphorylating enzyme AK can be obtained. The genetic and biochemical characteristics of these mutants are summarized in Table XIV.

Class I mutants which are represented by the Toy^r mutants could be selected at a high frequency (approximately 10^{-3}) by using any of a large number of N-nucleosides such as toyocamycin, tubercidin, 6-MeMPR, pyrazofurin etc. (Gupta and Siminovitch, 1978b; Gupta and Singh, 1983; Rabin and Gottesman, 1979; Thacker et al., 1980). This class of mutants exhibited a high degree of cross-resistance to various N- as well as C-nucleosides including toyocamycin, tubercidin, 6-MeAPR, 6-MeMPR, 8-azaadenosine, formycin A, formycin B, Bbb-73, Bbb-85, 9-deazaadenosine and pyrazofurin as well as showed greatly reduced cellular uptake and phosphorylation of all adenosine analogs examined. In cell extract, class I mutants contained no measurable activity of enzyme AK, which is consistent with their high degree of cross-resistance and reduced cellular uptake of all adenosine analogs. These results suggest that the genetic lesion in this class of mutants directly affects AK in a manner which alters its ability to phosphorylate both N- as well as C-nucleosides.

An unusual and very interesting feature of this class of mutants is the relatively high frequency with which such mutants can be

TABLE XIV. Summary of Genetic and Biochemical Characteristics of Different Types of Mutants Affected in AK

Class	Selective agent	Mutation* frequency	AK activity in cell extracts	Cross-resistance to adenosine analogs	In vivo AK activity	Behaviour in hybrids ($Drug^R$ x $Drug^S$)	Presence of CRM	Alteration in 2-D gel
I (Toy^r)	Toyocamycin or Pyrazofurin	10^{-3}-10^{-4}	<0.5%	High degree of cross-resistance to both N- and C-nucleosides	Greatly reduced ability for phosphorylation of both N- and C-nucleosides	Recessive	yes	Specific alteration in AK protein
II (Fom^R)	Formycin A	10^{-5}	<0.5%	Cross-resistance to only C-nucleosides	Reduced phosphorylation of only C-nucleosides	Codominant	yes	Specific alteration in region of AK
III (Fom^r)	Formycin B	5×10^{-5}	Contain 60 to 110% of biochemically altered AK activity	Cross-resistance to both N- and C-nucleosides	Reduced phosphorylation of both N- and C-nucleosides	Recessive	yes	Probable alteration

* Frequency in mutagen treated CHO cells.

obtained in CHO cultures. Although at present, the genetic basis of increased mutant frequency at AK locus remains unclear, there are two possibilities which could account for this observation. The first possibility is that the mutants are affected in a cis-acting positive regulatory gene whose function or product is essential for the expression of the AK gene and which is either lost or inactivated in these cells at high frequency. The possibility of mutations occurring in a trans-acting positive regulatory gene is excluded due to the recessive behaviour of AK^- mutants in somatic cell hybrids (Section 3.2). Alternatively, there may be a mutational hot spot present within the AK gene such that the mutations at this particular site occur with greater frequency compared to the rest of the genome; an idea which is supported by the data presently available. The presence of a mutational hot spot may readily explain why all AK^- mutants behaved very similarly and why a wide spectrum of mutants are not obtained. In addition, according to the present studies, Toy^r mutants contain CRM (M_r = 38,000) that represents inactive enzyme of approximately the same size and in an amount similar to that present in parental cells. These results strongly suggest that the high frequency of class I mutants is not due to a regulatory type of mutation, but in contrast, suggest that a mutation may lie in the structural gene of AK in the form of a missense alteration as indicated by their 2-D gel pattern.

The second class of mutants, Fom^R, which have been obtained at a frequency of 10^{-5} after selection in presence of the C-nucleoside, formycin A (an adenosine analog), exhibit a very novel genetic and

biochemical phenotype. Unlike class I mutants, which show cross-resistance to both N- as well as C-nucleosides, class II mutants exhibited increased resistance to any C-nucleosides but did not show any appreciable cross-resistance to various N-nucleosides. In accordance with their cross-resistance pattern, the Fom^R mutants showed reduced cellular uptake and phosphorylation of only C-nucleosides and normal levels of phosphorylation of N-nucleosides. The above results suggest that the genetic lesion in Fom^R mutants is highly specific, affecting the cellular uptake and phosphorylation of C-nucleosides only. Furthermore, the lack of cross-resistance of the Fom^R mutants to tiazofurin, a C-nucleoside which is not phosphorylated via AK, further indicate that the genetic lesion in class II mutants is specific for only those C-nucleosides which are a substrate for AK. Very interestingly, both Fom^R mutants contained no measurable activity in cell extracts but otherwise possess normal AK activity as indicated by the cellular uptake of N-nucleosides. This characteristic of class II mutants is different from class I mutants which lacked adenosine kinase activity both in cell extracts as well as within the cell. The lack of AK activity in the mutant cell extracts, together with the observation of both reduced phosphorylation of C-nucleosides and specific cross-resistance of mutant cells to only those C-nucleosides which are phosphorylated via AK, provides strong suggestive evidence that the genetic lesion in this class of mutants also involves AK but in a novel manner. Analysis of class II mutants by immunoblot techniques using specific antibodies to AK suggests that since these mutants contain CRM

of same size and in amount as present in parental WT cells, the resistance to C-nucleosides probably is the consequence of missense type of alteration in the structural gene of AK. This type of alteration in these mutants is to be expected because Fom^R mutants possess AK activity in cells and can phosphorylate N-nucleosides nearly normally. 2-D gel electrophoretic patterns of class II mutants show one additional protein spot adjacent to the AK spots, which may represent a mutationally altered form of AK. However, further studies are required to establish this fact.

The third class of mutants (Fom^r mutants) can be obtained by selection for resistance to formycin B at a mutant frequency of 5×10^{-5}. These mutants also exhibit a novel phenotype which has not been observed previously. The class III mutants like the class I mutants exhibited cross-resistance to both N- as well as C-nucleosides but in comparison to the class I mutants they showed lower degree of cross-resistance to various nucleoside analogs. In accordance with their cross-resistance pattern, the class III mutants show reduced phosphorylation of all nucleoside analogs which are known to be phosphorylated by AK. Very interestingly, class III mutants show cross-resistance to adenosine analogs and they contain AK activity in cell extracts. In this respect, class III mutants differ from class I mutants which lack AK activity in cell extracts and cells, as well as from class II mutants which have no measurable AK activity in cell extract but possess normal ability to phosphorylate N-nucleosides. Furthermore, AK activity present in Fom^r mutants differs from the AK

activity present in parental WT cells in two respects. First, the levels of AK activity in class III mutants is different from the level of AK activity in WT cells. Secondly, AK activity present in Fom^r mutants differ from the parental cell AK activity in terms of its ability to phosphorylate adenosine analogs to which class III mutants show resistance. It was found that higher concentrations of the adenosine analog Bbb-85 were required to compete with adenosine using AK activity from class III mutants than from WT cells. The decreased affinity of the mutant enzyme for adenosine analogs, as suggested by these experiments can account for their observed genetic and biochemical phenotype. The presence of biochemically altered AK activity in class III mutants provides strong suggestive evidence that the phenotype exhibited by these mutants involves a structural gene mutation in AK. This inference is also supported by the immunoblot analysis of these mutants which indicates that they contain similar amounts of CRM in comparison to WT cells. 2-D gel electrophoresis of Fom^r10 mutant cells show that the relative intensities of the two spots corresponding to AK is altered. The increase in intensity of spot 2 could arise due to the appearance of a new spot in close proximity of spot 2.

In view of the above results, important follow up experiments would be to investigate the nature of the changes observed on 2-D gels from different mutants by using immunoblots of 2-D gels of both parental and mutant cells. The relationship between the new spots which were seen in 2-D gels of different AK mutants could then be

established by using tryptic peptide analyses of the the protein isolated from those spots. For further studies, a useful approach would be to clone the AK gene and analyze the genetic alteration in these mutants at the DNA level. In this regard, availability of specific antibodies to AK should prove useful in immunological screening of complementary DNA libraries using specific antibodies to AK (Young and Davis, 1983). The cloned AK gene would be of great value in providing a better understanding of phenotypes of these three classes of mutants as well as for investigating related unsolved problems. Southern blot analyses of the restriction fragments obtained after treatment with different restriction endonucleases could identify the specific regions of AK gene which are altered in the different mutant classes. From a DNA sequence analysis of those regions, it should be possible to identify specific changes in nucleotides which are responsible for the characteristic phenotypes of different classes of mutants. Recently, an analogous approach has been used to demonstrate the presence of a single point mutation in the structural gene of APRT in mutant cells resistant to 2,6-diaminopurine (Simon and Taylor, 1983; Simon et al., 1983).

Knowledge of the molecular alteration at the nucleotide level would make it possible to predict the changes in amino acid sequences that have occurred in the mutant proteins. Since the enzymatic activity of a protein is determined by its three-dimensional conformation, which in turn is dependent upon its amino acid sequence, it would be of great interest to investigate how specific amino acid

alterations in different mutants affect the overall conformation of AK and its possible relationship to the functioning of the enzyme. For example, identification of the mutation in the class I mutants should dictate information concerning the site on the AK protein which recognizes both N- as well as C-nucleosides for phosphorylation. Furthermore, X-ray analysis can be used to compare the three-dimensional structure of the AK protein present in both the parental and mutant cells. Such an approach would correlate the changes in the amino acid sequence with its three-dimensional structure. Similar characterization of class II mutants would be indispensable in identifying the regions of the AK protein which are specifically involved in the recognition of C-nucleosides. In addition, the above approach can also be used to investigate the unsolved problem of different mutation frequencies for Toy^r resistance in various Chinese hamster cell lines. It has been reported earlier that the frequency of AK^- mutants in other Chinese hamster cell lines, e.g., M3-1, CHO-K1, V-79, CHW, etc. (Gupta and Siminovitch, 1978b; Thacker, 1980) and other mammalian cell lines (Gupta and Hodgson, 1981) is from 10 to more than 1000 times lower than that observed in CHO cells. To account for the differences in mutation frequencies, Gupta and Siminovitch (1978b) have suggested that, similar to a number of other genetic loci in CHO cells (Campbell and Worton, 1978; Gupta et al., 1978a; Gupta et al., 1978b; Siminovitch, 1976), the AK locus in this cell line is also present in a functionally hemizygous form. Such a hypothesis can easily be tested by using the specific antibodies to

AK along with the cloned AK gene. In addition, the cloned gene can also be used to determine the underlying mechanism(s) responsible for higher mutation frequency at AK locus in CHO cells.

Recently, the AK locus has also been found to be a valuable marker for quantitative mutagenesis studies in CHO cells (Gupta and Singh, 1983). It has been shown that the frequency of AK^- mutants in cell cultures increases in a nearly linear concentration-dependent manner upon treatment with a number of direct acting mutagens, e.g., ethyl methanesulfonate, ultraviolet light, and indirect acting mutagens, e.g., benzo[a]pyrene. These studies presented show that this mutant selection system, because it involves loss or inactivation of a function that is not required for normal growth, is capable of detecting both base substitution as well as frameshift and deletion types of genetic changes. The availability of a specific antibody to AK and a cloned AK gene would provide a means of directly demonstrating the type of lesion in the mutants obtained after treatment with different types of mutagens.

In addition, the above mutants have been used to investigate the metabolism and mechanism of action of formycin B. Studies presented show that in contrast to the results reported earlier, formycin B is metabolized by CHO cells into FoB-MP, FoA-MP and higher phosphorylated derivatives of formycin A which are incorporated into RNA. In addition, these studies show that the enzyme AK is invovled in the phosphorylation of formycin B in CHO cells. In earlier studies, it has been suggested that the antileishmanial activity of formycin B is

due to its phosphorylation by Leishmania and not by mammalian cells (Carson and Chang, 1981; Rainey and Santi, 1983). However, the above studies show that, not only is formycin B toxic to both cultured human and CHO cells in the same range as reported for Leishmania, but formycin B is metabolized by mammalian cells in a manner similar to that for Leishmania as well (Carson and Chang, 1981; Nelson et al., 1982; Rainey and Santi, 1983). In view of these observations, the mechanism of specific antileishmanial activity of formycin B is unclear at present. Further studies on the metabolism and mechanism of action of both formycin B and formycin A in both mammalian cells and Leishmania would prove useful.

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