Title

The effects of inhibitors of ornithine decarboxylase on proliferation, intercellular polyamine content and cell cycle progression of 9L rat brain tumor cells

Author

Seidenfeld, Jerome,

Publication Date

1980

Peer reviewed|Thesis/dissertation

THE EFFECTS OF INHIBITORS OF ORNITHINE DECARBOXYLASE ON PROLIFERATION, INTRACELLULAR POLYAMINE CONTENT AND CELL CYCLE PROGRESSION OF 9L RAT BRAIN TUMOR CELLS

by

JEROME SEIDENFELD

B.A. (YESHIVA UNIVERSITY) 1967
M.S. (UNIVERSITY OF CHICAGO) 1969

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PHARMACEUTICAL CHEMISTRY

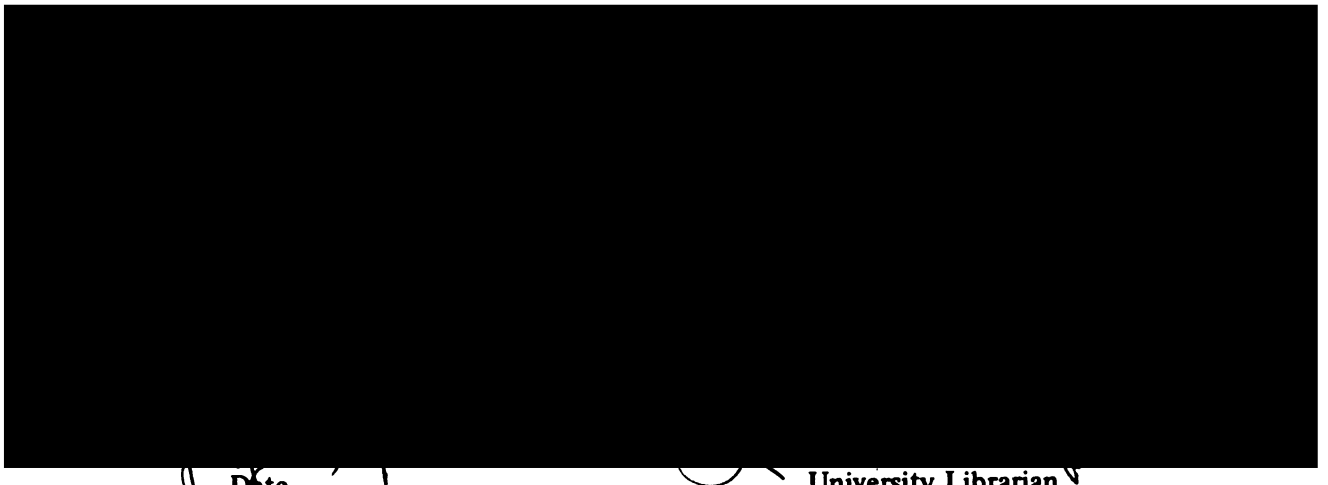
in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



Date University Librarian

Degree Conferred: JAN 1 1980

ACKNOWLEDGEMENTS

Much thanks is owed to so many people. To my dissertation committee for their guidance in preparing this manuscript. To Bob Weinkam, my research director, for both the advice and the independence he gave me. To Larry Marton, in whose laboratory these experiments were performed, for re-awakening my interest in and excitement with scientific investigation, for guiding the research described in this dissertation, for exposing me to the questions of polyamine research, and for treating me as a collaborator rather than an employee from the very beginning.

The personnel of the Brain Tumor Research Center taught me many valuable skills and made my four years there very enjoyable. Warren Lubich taught me, among other things, the care and feeding of the D-500, helped with much of the polyamine analysis, and was the ideal laboratory partner. Kathy Knebel showed me how to grow 9L, how to avoid growing "bugs", and had the patience necessary to help with my ever-escalating "definative" experiments. Dennis Deen and Mary Williams taught me the CFE assay, helped carry out the experiments using it, and gave invaluable advice on tissue culture techniques. Victor Levin instructed me in the use of computers for data analysis and presentation. Takao Hoshino, Kabo Nomura, and Isao Muraoka helped with the autoradiographic study. The office staff, Pam Vestneys, Lynn Harney, Marilyn Minaar, and Lee Banks, kept everything running smoothly and typed innumerable versions of many manuscripts. To these and all the other

folks at the BTRC I owe a great deal.

Assistance was also forthcoming from sources outside the BTRC. The faculty of the Department of Pharmaceutical Chemistry contibuted significantly to my scientific education. My fellow graduate students helped lighten the load of the past three years. Flow cytometry for the work described here was done in the laboratory of Dr. Joe W. Gray at the Lawrence Livermore Laboratories. His helpful discussion of the results was greatly appreciated. Mary Look and Janet Bieber helped keep the FCM in tune. The αMO and DFMO used in these studies was a generous gift from the Centre de Recherche Merrell International in Strasbourg, France. The final manuscript of this dissertation was valiantly typed by Julie Kuzera.

Having saved the best for last, I now thank my wife Linda, for everything.

ABSTRACT

The effects of inhibitors of ornithine decarboxylase (ODC) on 9L rat brain tumor cells in monolayer culture was investigated. DL-α-methyl-ornithine (αMO), a competitive inhibitor, produced cytostasis at 10 mM when initial cell density was 5 x 10^5/flask but not 1 x 10^6. Fifty millimolar caused cytostasis at both initial densities, but more effectively at the lower one. Cytocidal effects, measured by a colony forming efficiency assay, were observed at 100 mM but not at 75 mM or less. Ten millimolar αMO did not alter the DNA histograms of treated 9L cultures from those of controls over four days; 50 mM produced a partial G_1-S block and elongation of G_2, as shown by flow cytometric and autoradiographic techniques. Both concentrations of αMO at both initial cell densities depleted intracellular putrescine content to <5% of control cells by 12 hours and spermidine content to <20% of control by 48 hours post treatment. Intracellular spermine content of treated cells increased between 1.5 and 2 fold over controls under all treatment conditions. The cytostatic effect of 10 mM αMO on 9L cultures of lower initial cell density appeared to be a specific result of polyamine depletion, since it was reversible by addition of exogenous putrescine 24 hours after treatment. The effects of 50 mM αMO were not reversible by exogenous putrescine or pyridoxal added simultaneously or after 24 hours. These effects are thus not attributable to polyamine depletion. Early rates of decline in intracellular putrescine and spermidine content were greater for higher concentrations of αMO but did not seem to vary with initial cell density. Even with 50 mM αMO total polyamine content per culture continued to increase slightly, indicating some

continued putrescine synthesis in its presence.

A similar series of experiments was performed with DL-α-difluoromethylornithine (DFMO), an irreversible inactivator of ODC. This drug was a more effective cytostatic agent than was αMO. Again, reduction of the initial cell density by half resulted in more effective cytostasis. Concentrations as low as 1 mM inhibited 9L proliferation to about 25% of control growth when initial cell density was 5 x 10^5/flask. DFMO was also more effective in depleting 9L polyamine content, reducing spermidine content to <5% of control by 48 hours post treatment. Cytostasis due to treatment with 10 mM DFMO could both be reversed and blocked by addition of exogenous putrescine. Replating of cells pretreated with DFMO for 48 hours showed that polyamine depleted cells could not enter exponential tissue culture growth until after replenishment of polyamine content. Addition of exogenous putrescine at the time of replating allowed pretreated cells to resume exponential growth at the same time as controls. Flow cytometry of cells incubated with DFMO showed that polyamine depletion prevented the substantial increase in cells in S and G_2M observed at about 24 hours with control cultures. Flow cytometry of cells pretreated with DFMO and replated in its absence revealed that the fraction of cells in G_1 increased until polyamine accumulation resumed, implying the presence of a G_1-S block. Control cells had decreases in the G_1 fraction occurring within six hours of replating. These observations support the hypothesis that entry of 9L cells into S phase is dependent upon an adequate intracellular pool of polyamines.

A final series of experiments tested the hypothesis that polyamine depletion might alter intracellular DNA structure in a manner that

might sensitize 9L to the lethal effects of X rays. Treatment with αMO and DFMO, as well as with methylglyoxal-bis-guanylhydrazone (an inhibitor of S-adenosyl-L-methionine decarboxylase) alone and in combination with DFMO, was used to achieve polyamine depletion prior to irradiation. None of the treatments used altered the X-ray dose-response curves of treated cells from those of controls.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

αHO : α-hydrazinoornithine

αMO : α-methylornithine

BME : Eagle's basal medium

CFE : colony forming efficiency

Con-A : Concanavalin-A

CV : coefficient of variation

DFMO : difluoromethylornithine

FCM : flow cytometry

FCS : fetal calf serum

HBSS : Hanks' balanced salt solution

HS : horse serum

HTC : hepatoma tissue culture

MBAG : 1,1'-[(methylethanediylidene)-dinitrilo]-bis-(3-aminoguanidine)

MGBG : methylglyoxal-bis-guanylhydrazone

MI : mitotic index

ODC : ornithine decarboxylase

PBS : phosphate buffered saline

PHA : phytohemagglutinin

PLM : percent labelled mitoses

PLP : pyridoxal phosphate

Pu : putrescine

SAH : S-adenosylhomocysteine

SAM : S-adenosylmethionine

SAMDC : S-adenosylmethionine decarboxylase

SD : standard deviation

Sd : spermidine

SEM : standard error of the mean

SHU : sample holder unit

Sp : spermine

SSA : 5-sulfosalicylic acid

CHAPTER I: INTRODUCTION AND LITERATURE REVIEW

The polyamines spermidine (Sd) {$H_2N(CH_2)_3NH(CH_2)_4NH_2$} and spermine (Sp) {$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$} and their diamine precursor putrescine (Pu) {$NH_2(CH_2)_4NH_2$} are intracellular constituents of virtually all living organisms. At the intracellular pH of mammalian cells, to which all discussion in this dissertation is restricted, all amine groups are protonated and the polyamines exist as organic polycations. Attempts to elucidate the intracellular role(s) of these biomolecules have followed several approaches; one of these, treatment of cultured mammalian cells with inhibitors of polyamine biosynthetic enzymes, is the subject of this dissertation.

I-A: Polyamine Biosynthesis and Impinging Pathways

Ornithine is the only precursor of Pu in mammals (1-3) although other biosynthetic pathways to Pu are known in some nonmammalian organisms (1,2). The enzyme responsible for Pu biosynthesis, ornithine decarboxylase (ODC), uses pyridoxal phosphate (PLP) as its cofactor (4). The mechanism of this decarboxylation initially involves Schiff's base formation between the aldehyde group of the PLP and the α-amino group of ornithine. After loss of the carboxyl group as CO_2, with the pyridine ring of PLP acting as an electron sink, the Schiff's base is hydrolyzed and free Pu is released as the divalent cation (Fig. I-1).

The aminopropyl moeity of both Sd and Sp derives from S-adenosyl-L-methionine (SAM) (1-3). This amino acid-nucleoside adduct is first

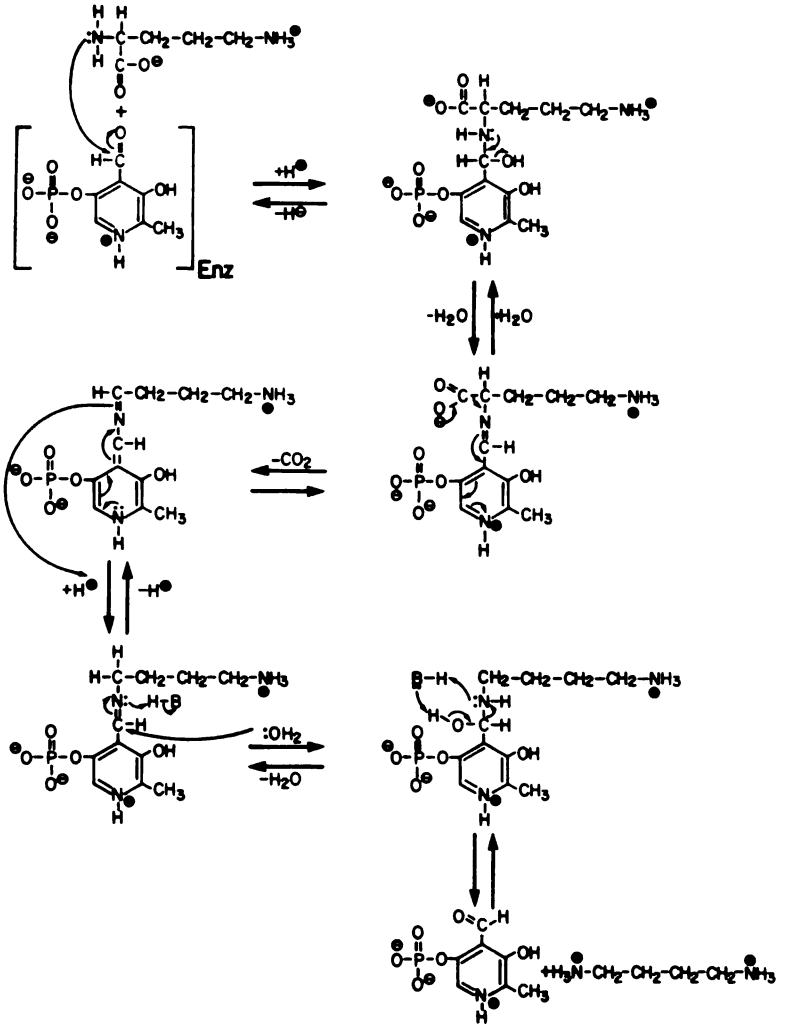


FIGURE I-1

Molecular Mechanism of Ornithine Decarboxylase

decarboxylated by S-adenosyl-L-methionine decarboxylase (SAMDC) (5,6) which has as its cofactor a covalently bound pyruvate group (7,8). The mammalian enzyme is activated by both Pu and Sd. The product of this decarboxylation, S-(5'-adenosyl)-3-methyl-thiopropylamine, provides an aminopropyl group for Sd and Sp synthesis (Fig. I-2). There is as yet no evidence available regarding the mechanism of SAMDC.

Conversion of Pu to Sd is catalyzed by spermidine synthase (9). This enzyme forms a covalent bond between one of the nitrogen atoms of Pu and the sulfur-bound carbon atom at the end of the aminopropyl moiety of S-(5'-adenosyl)-3-methylthio-propylamine (Fig. I-2). In a similar reaction, spermine synthase forms a covalent bond between the carbon atom at the end of a second aminopropyl group and the nitrogen at the four-carbon end of Sd (9). The two distinct synthase enzymes have been chromatographically separated from each other and from SAMDC (9-11). In addition, they differ from each other in sensitivity to inhibition by various substrate and product analogs (12). No cofactor requirement has as yet been demonstrated for either of these enzymes (3) and nothing is known regarding their mechanisms.

Arginase, one of the enzymes of the urea cycle, is the major source of ornithine in mammalian systems (13). This enzyme catalyzes the hydrolysis of arginine to produce urea and ornithine (Rxn. 1, Fig. I-3). There are also two enzymes that utilize ornithine as substrates: ornithine carbamoyltransferase (Rxn. 2, Fig. I-3), a urea cycle enzyme that forms citrulline from ornithine and carbamoyl phosphate (14); and ornithine aminotransferase (Rxn. 3, Fig I-3), which transfers the δ-amino group of ornithine to α-ketoglutarate to produce glutamic-γ-semialdehyde and glutamate, and is involved in the metabolism of proline

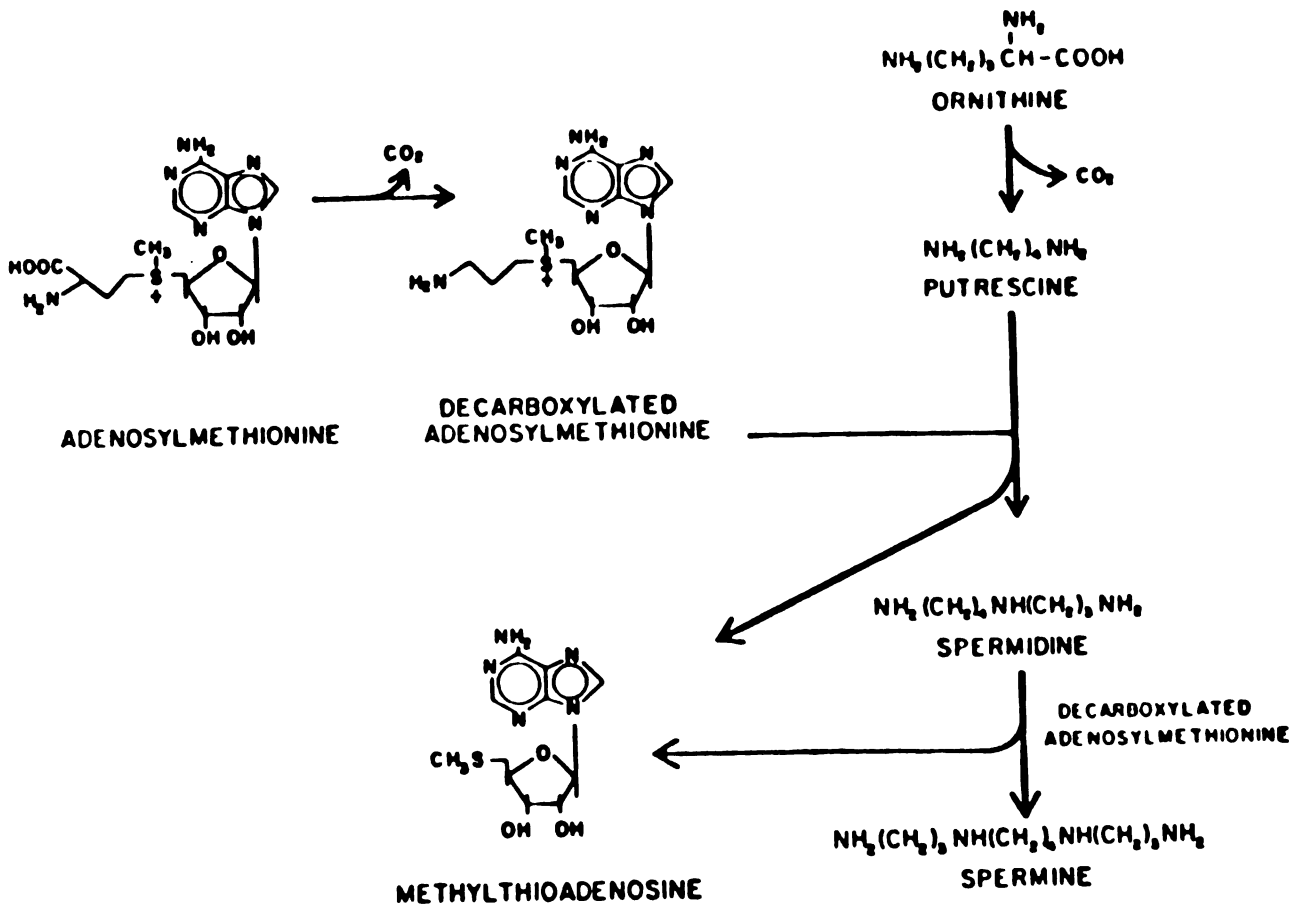


FIGURE I-2

Polyamine Biosynthetic Pathway

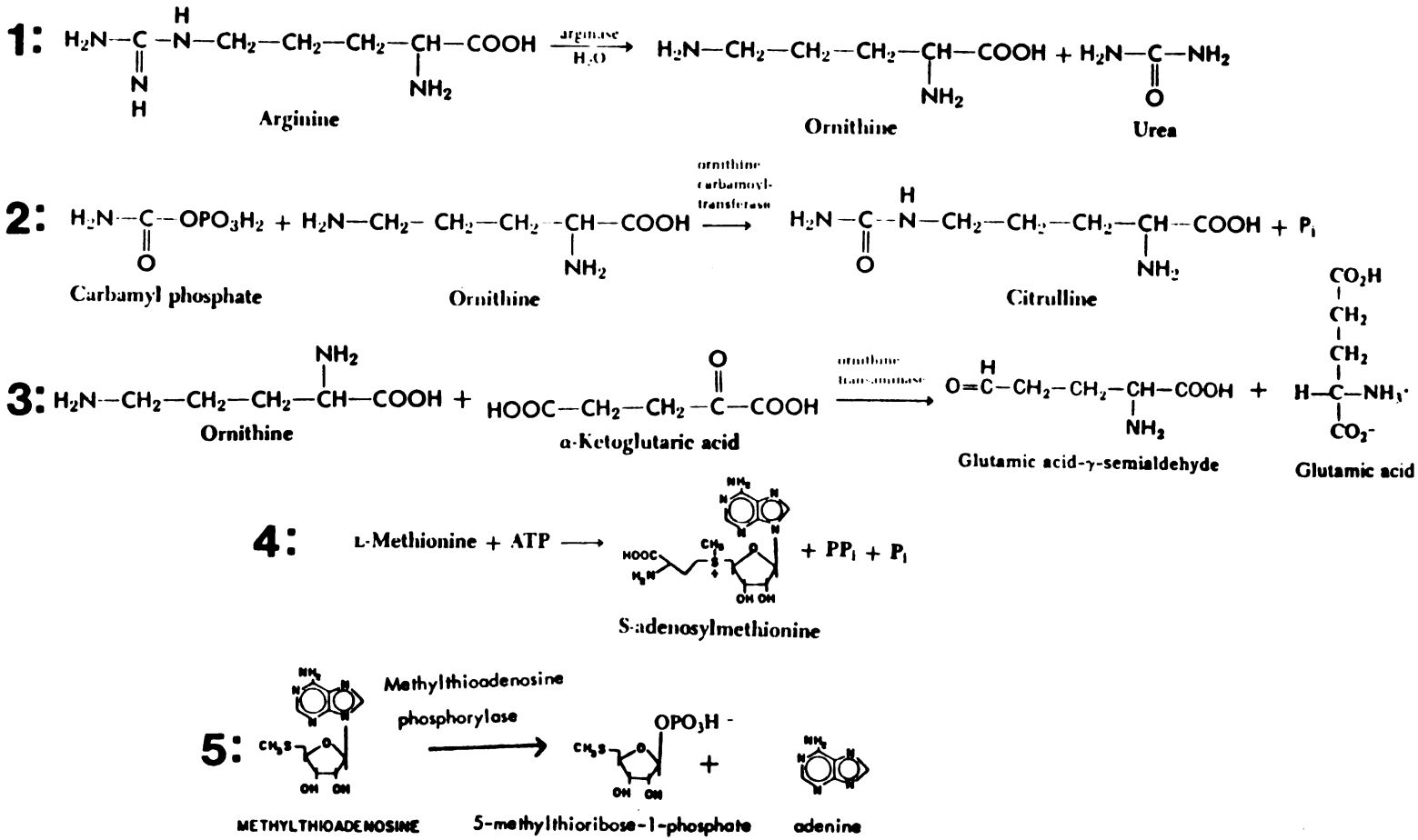


FIGURE I-3
Enzymatic Reactions Impinging on the Polyamine Biosynthetic Pathway

(15). Although these three enzymes could play some role in regulating intracellular polyamine concentration by varying the availability of ornithine, ODC is generally thought to be the rate limiting enzyme in polyamine biosynthesis (1-3).

Methionine adenosyl transferase is the enzyme responsible for production of SAM (Rxn. 4, Fig. I-3) from methionine and ATP (16). In addition to its use in polyamine biosynthesis after decarboxylation, this product is a substrate for a wide variety of methyl transferase enzymes, all of which have as their common product S-adenosylhomocysteine (SAH) (17). After transfer of the aminopropyl group in the spermidine or spermine synthase reactions, methylthioadenosine is released (Fig. I-2). This is cleaved to adenine and 5-methylthioribose-1-phosphate by a phosphorylase (Rxn. 5, Fig. I-3) that is different from purine nucleoside phosphorylase (18). The adenine is then recycled via the purine nucleotide "salvage" pathway while a methylthiolase enzyme cleaves methylthio groups from 5-methylthioribose-1-phosphate to libereate ribose-1-phosphate (19).

I-B: Polyamine Bio-transformation

The polyamines are substrates for several mammalian oxidative enzymes with varying substrate specificity (Fig. I-4). The end result of these enzymatic reactions is conversion of polyamines to a variety of aminoaldehydes via oxidative deamination. Thus Pu is converted to γ-aminobutyraldehyde (Rxn. 1, Fig. I-4) by a number of diamine oxidases present in kidney, liver, placenta, small intestine, lung, and in the circulation of various mammalian species (2,20). Oxidation of Sd and Sp can occur in two different ways by two polyamine oxidases. The first

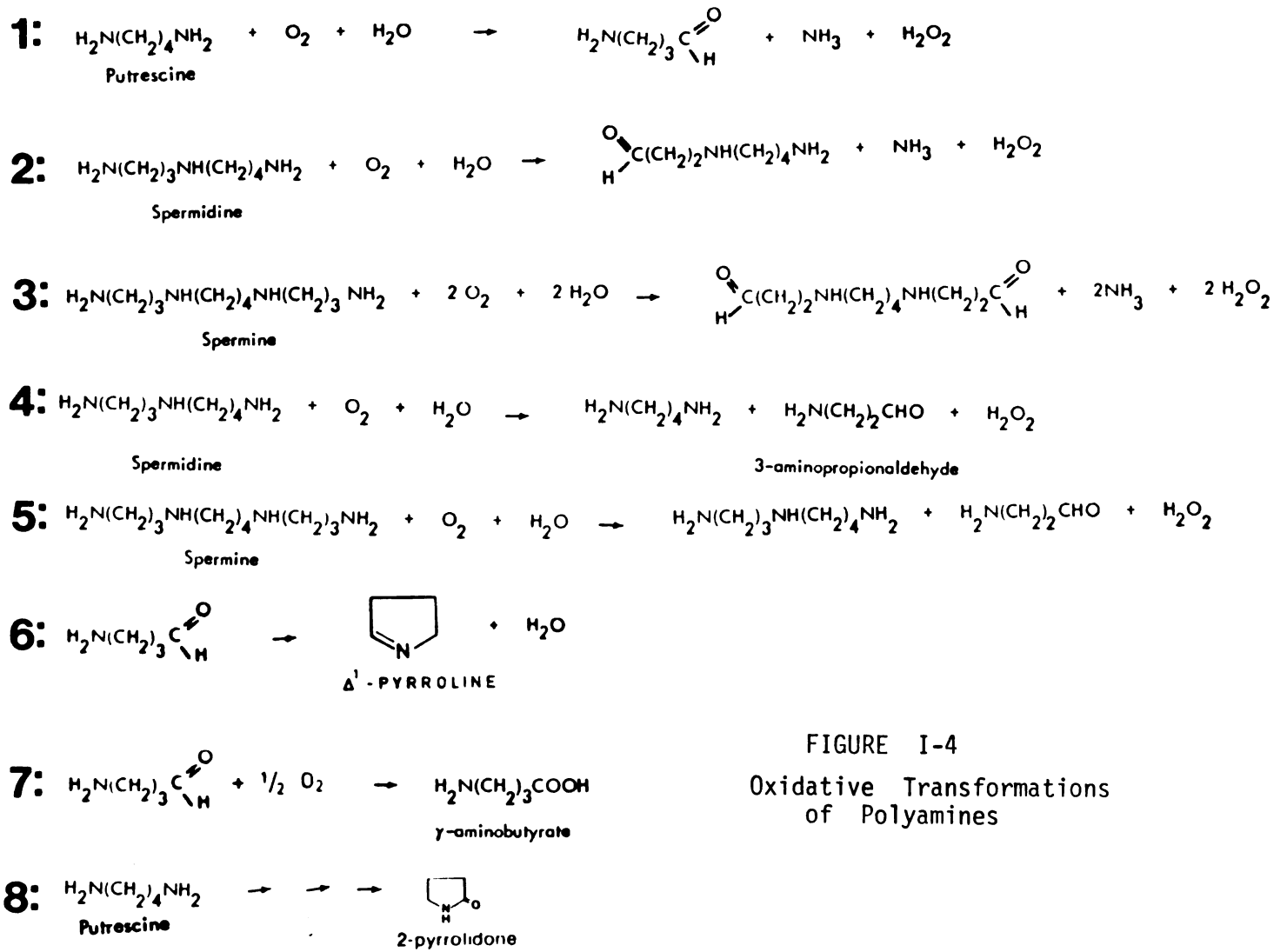


FIGURE I-4

Oxidative Transformations of Polyamines

of these, present in blood plasma of ruminants (21), removes the primary nitrogen at the end of the amino-propyl group of Sd (Rxn. 2, Fig. I-4) or Sp (Rxn. 3, Fig. I-4), converting these polyamines into highly toxic amino-aldehydes (22,23). The second of these enzymes, isolated from liver peroxisomes, catalyzes oxidative deamination at the secondary nitrogen of the aminopropyl moeity of Sd or Sp (24). This results in formation of 3-aminopropionaldehyde and Pu from Sd (Rxn. 4, Fig. I-4) or Sd from Sp (Rxn. 5, Fig. I-4).

The other major metabolic transformation of polyamines in mammals is acetylation (Fig. I-5). An acetyl-CoA dependent N-acetyl transferase is present in the cell nuclei of various tissues (25,26). This enzyme forms mono-N-acetyl Pu, Sd, and Sp from the corresponding di- and polyamines. Both monoacetyl Sd and monoacetyl Sp are de-acetylated by cytoplasmic enzymes present in a number of rat tissues (27,28). While the presence of both N^1- and N^8-monoacetyl-Sd has been demonstrated in the urine of humans and other mammalian species (29,30), monoacetyl Pu is the only acetylated polyamine which has been reported to be an intracellular constituent of mammalian cells (31).

The γ-aminobutyraldehyde formed by oxidative de-amination of Pu can be further biotransformed in one of two ways. It can cyclyze to form Δ^1-pyrroline (Rxn. 6, Fig. I-4) or it can be further oxidized (Rxn. 7, Fig. I-4) to form γ-aminobutyric acid (32,33). It has also been recently shown (34) that a major metabolic product resulting from incubation of Pu with rat liver, as well as spleen and lung, is 2-pyrrolidone (Rxn. 8, Fig. I-4), the lactam of γ-aminobutyric acid. The aminoaldehydes formed from Sd and Sp by plasma polyamine oxidase (which oxidizes at the primary nitrogen of the aminopropyl group) are quite

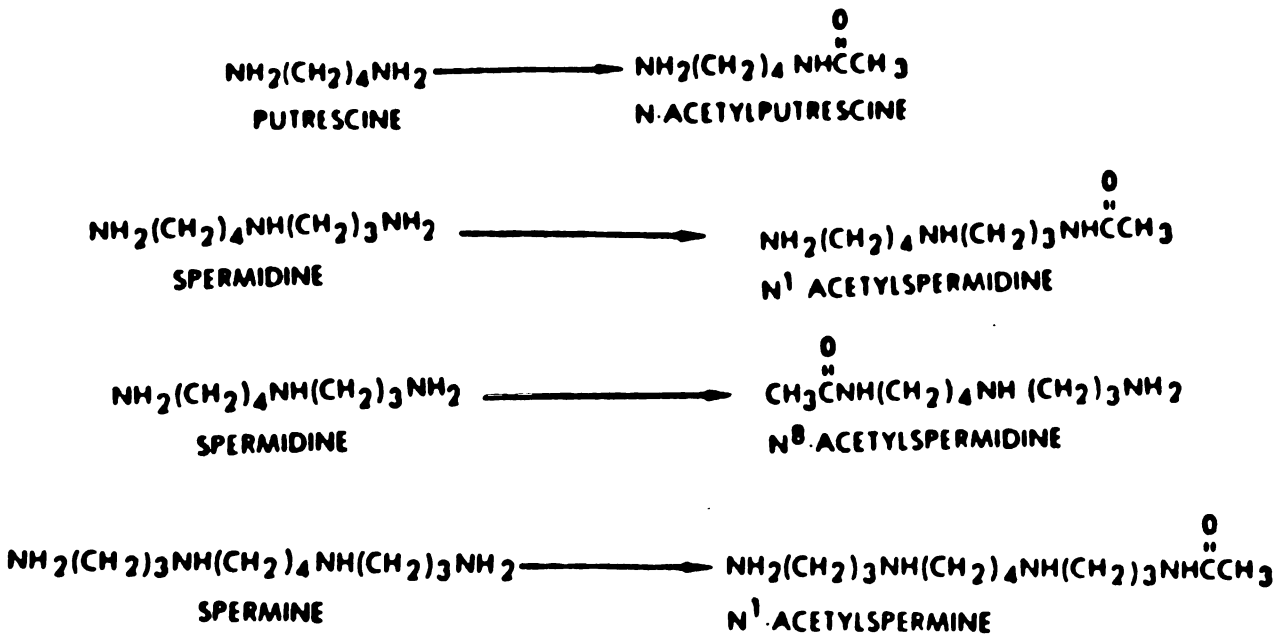


FIGURE I - 5

Acetylation of Polyamines

reactive and rapidly form covalent bonds to free amino groups of a variety of nucleic acids and proteins (23). This reactivity is thought to be responsible for the toxicity of polyamines to cells in culture media containing fetal calf serum. The ultimate fate of the 3-aminopropionaldehyde from polyamines oxidized by the liver peroxisome enzyme is not as yet known. Monoacetyl Pu is a substrate for monoamine oxidase, resulting in formation of N-acetyl-γ-aminobutyric acid which can then be de-acetylated (35).

A number of reports have recently appeared of peptide conjugates of polyamines in various physiological fluids (36,37). The structure and nature of these conjugates have not yet been elucidated. There is also no information available regarding the possible functions of such conjugates.

It is obvious that in whole mammalian organisms in vivo the enzymes responsible for polyamine biotransformation play an important role in controlling the total pool of polyamines. It is less well understood, however, what part, if any, these enzymes play in determining the intracellular concentration of polyamines in cells grown in tissue culture.

I-C: Regulation of Polyamine Metabolism

The majority of resting adult mammalian tissues have rather low levels of ODC activity (1-3). Based on measurements of the decline of ODC activity after treatment of cells with protein synthesis inhibitors such as cycloheximide, intracellular ODC appears to be very rapidly turned over with a half-life on the order of twenty minutes (38). It has also been reported that different forms of ODC, with varying

affinities for the PLP cofactor, can be isolated from the same tissues (39). These multiple forms of ODC do not differ from each other in molecular size. There is some speculation, but little firm proof, that regulation of intracellular ODC activity is, at least in part, achieved by interconversion between these alternate forms.

A wide variety of stimuli that result in increased rates of cell replication also produce many-fold elevations of intracellular ODC activity. Examples include partial hepatectomy (40,41), thioacetamide injection (42), addition of serum to serum-depleted cells in culture (43), treatment of animals with tumor promotion phorbol esters (44) or growth hormone (45), and addition of mitogens to lymphocytes in culture (46). Experiments with simultaneous application of the stimulus and cycloheximide have suggested that at least part of this induction of ODC activity is dependent upon synthesis of new enzyme (38). Elevations of ODC activity are almost invariably followed by increases in intracellular polyamine concentrations with lag times of varying duration (1-3). For this reason, ODC is often considered the rate-limiting enzyme in polyamine biosynthesis and the major point of control of intracellular polyamine concentration.

An additional mechanism for regulation of intracellular ODC activity recently has been reported (47). Addition of Pu or other diamines to tissue culture media was found to prevent the induction of ODC that normally occurred upon serum stimulation of serum-depleted cells. It was shown that this was the result of the putrescine-mediated release of a polypeptide ODC inhibitor that was non-competitive with respect to both substrate and cofactor; this inhibitor was named ODC antizyme. The ODC-antizyme complex is formed via non-covalent interactions and

can be dissociated at high salt concentrations (48). Induction of ODC antizyme by exogenous Pu can be partially blocked by cycloheximide (47) and thus appears at least partially dependent on new protein synthesis. There is also some evidence suggesting that addition of exogenous polyamines affects levels of ODC activity by altering rates of enzyme synthesis as well as by inducing the antizyme (49,50). As McCann has pointed out in his recent revue of ODC regulation in eurkaryotes (51), intracellular ODC activity appears to be regulated by the rate of transcription, the rate of new enzyme synthesis, antizyme induction or release, alteration of turnover rates, and changes between a more and a less active form. These factors influencing intracellular ODC activity then, in turn, affect the intracellular polyamine content.

In many cases, stimuli that cause dramatic elevations of ODC activity produce only small increases in SAMDC activity (52). There are, however, examples of more substantial elevations of SAMDC, particularly in the prostate of castrated rats after androgen treatment (53). Since SAMDC is activated by physiological concentrations of Pu and Sd (54), and the activation by Pu can be inhibited by Sp (55), intracellular polyamine concentrations undoubtedly play some role in regulating this aspect of polyamine metabolism. The half-life of SAMDC measured by loss of activity after cycloheximide treatment is about forty minutes (46,56). While this is longer than that of ODC, it is sufficiently short for the turnover rate to be an important factor in regulation of intracellular SAMDC activity. Intracellular concentrations of SAM also must play a role in regulating this pathway.

It seems likely that the Sd and Sp synthase enzymes do not play

an important regulatory role in polyamine metabolism. Resting tissue levels of these two enzymes are much higher than those of ODC and SAMDC (9,57). Their half lives following cycloheximide are on the order of 12 hours (58), as opposed to the much shorter half lives of ODC and SAMDC. Increases in tissue levels of the two aminopropyltransferases as responses to growth stimulation have not been reported; the only exception to this is the increase in Sd synthase observed by Oka et. al. (59) in mouse mammary gland after hormonal stimulation.

I-D: Possible Functions for Polyamines

Central to this dissertation is the hypothesis that polyamines play a vital role in the growth, differentiation, and replication of all living cells. This section will explore some of the evidence on which this hypothesis rests. One approach that has been followed is to look for *in vitro* effects of polyamines on the structure and function of various macromolecules and subcellular particles. Although this approach has the obvious limitation that one can never tell which effects observed *in vitro* are of importance to cellular functioning *in vivo*, the fact that most of these effects are found using physiological concentrations of polyamines is encouraging.

Many effects of polyamines on replication, post-synthetic covalent modification, and degradation of DNA have been described (2,60). Polyamines also bind reversibly to native DNA in an interaction which stabilizes the double helix to thermal denaturation as measured by the elevation of melting temperature (61) to enzymatic degradation (62) and to denaturation by X-rays (63). Binding of polyamines has also been shown to protect native DNA from photochemical formation of psoralen

monoadducts and interstrand crosslinks (64), and from methylation of the N^7 and O^6 positions of guanine residues and N^3 positions of adenine residues by N-methyl-N-nitrosourea (65). The thermal and radioprotective effects have been shown to be a function of carbon chain length for a series of diamines (63,66) and for a series of Sp analogs of varying internal chain length (i.e. varying n in $H_2N(CH_2)_3NH(CH_2)_n NH(CH_2)_3NH_2$) (67). The absence of a change in the UV spectrum (66) or in the ORD curve (68) of native DNA subsequent to binding of polyamines has been taken as evidence that such binding is to the native conformation and does not induce a conformational change in DNA. Sd and Sp bind more strongly to DNA than do the histones F-1 (lysine rich) and F-3 (arginine rich) (69). The X-ray studies of Liquore (70-72) have been interpreted as indicating that the two nitrogens on either end of the aminopropyl groups of Sd and Sp can bind to adjacent phosphate groups on one strand of double helical DNA while the four-carbon chain stretches across the minor groove of the double helix forming a cross-bridge between phosphate groups on opposite strands. Support for this model, which explains the stabilization of DNA structure by polyamines as arising from these non-covalent cross-bridges and the charge neutralization they bring about, comes also from the X-ray studies of Tsuboi (61).

Polyamines have also been shown to bind to and affect the functions of all three forms of RNA, as well as to stimulate the rate of DNA-dependent RNA synthesis (transcription) *in vitro* (2,60). Binding of polyamines to RNA stabilizes the macromolecule to thermal denaturation and enzymatic degradation (73-75) and facilitates the formation of double stranded RNA copolymers from single stranded polynucleotide

chains (76). Endogenous polyamines have been found non-covalently bound to tRNA (77); it is thought they serve to form or help stabilize the looped structure of tRNA by holding together double helical regions of the single strand (78). Binding of polyamines to RNA differs from binding to DNA in that there is simultaneous binding to the 3'-phosphate and 2'-hydroxyl groups of RNA nucleotides as shown by the NMR data of Bolton and Kearns (79).

It is obvious from the stimulation of enzymes involved in nucleic acid metabolism mentioned earlier that polyamines interact with proteins in vitro. They have also been shown to stimulate the rate of in vitro protein synthesis by cell free systems (80) and the phosphorylation of non-histone nuclear proteins (81). Evidence that polyamines increase the binding of N-formylmethionyl-tRNA to 30S ribosomal subunits (82) implies that their stimulation of protein synthesis may be the result of increased rates of chain initiation. Among other observations, the facts that polyamines can induce the polymerization of globular actin monomers to fibrous active polymeric thin filaments (83), and that they can be used in cross-linking polypeptide chains by transglutaminase enzymes (84) point to a possible role for polyamines in protein structure.

On a level of organization one step higher than biological macromolecules, polyamines have been shown to increase the aggregation of subunits into functioning ribosomes (85). Polyamines have also been found as natural constituents of ribosomes (86). Isolated mitochondria (87) and nuclei (88) from a variety of organisms are stabilized when polyamines are added to the medium in which they are suspended. Viral assembly (89) and compaction and packaging of phage DNA into the phage

heads (90) are aided by the presence of polyamines. Finally, a host of changes in membrane permeability and transport have been noted (2), but these have been in so many different directions (i.e. both increased and decreased permeability into and out of cells) for different solutes as to present a very confusing picture of the role of polyamines in determining membrane permeability.

A second source of support for the hypothesis that polyamines play an important role in cell growth and replication comes from temporal correlations of changes in intracellular activities of polyamine biosynthetic enzymes and intracellular polyamine content with stimulated cell or tissue growth. The evidence obtained by this approach is somewhat stronger than the in vitro effects discussed above in that, as noted previously, one can never tell which in vitro interactions do play a part in in vivo regulation and which do not. An early observation belonging to this second approach is that addition of polyamines to the culture medium will stimulate the growth of some (but not all) cells (91). Of course, this observation is only true in the absence of ruminant serum and the diamine and polyamine oxidase activities it contains.

One of the best studied stimulated growth systems is rat liver following partial hepatectomy. Intracellular polyamine content begins to increase by four hours following surgery (92). The peak levels of polyamine content coincide with maximal RNA content for both rat (93) and mouse (94) liver after partial hepatectomy and for rat mammary gland with onset of lactation (95). Maximal increases in intracellular ODC activity also correlate temporally with peak RNA synthesis in rat liver after partial hepatectomy (96). For chicks (97) and

amphibians (98) it has been observed that increases in polyamine content and ODC and SAMDC activity occur upon incubation, coincide with maximal nucleic acid content, and correlate with embryonic growth rate. It has also been reported (98) that polyamines are not detectable in unincubated chick eggs. Many hormones that elicit proliferative responses in their target tissues cause increases in ODC activity and in polyamine content. Examples include growth hormone in liver (99), testosterone in rat prostate (53), and estradiol in rat uterus (100).

In many tissue culture systems and experimental tumors, it has been observed that ODC activity and intracellular polyamine content are low before and shortly after cell growth starts (the lag phase of tissue culture growth), increase as growth accelerates (the logarithmic phase), and decrease as the growth rate declines. Cells in the plateau phase of tissue culture growth, or serum-depleted cells, also have low levels of both ODC activity and polyamine content. Tissue culture systems for which some or all of these observations have been made include mouse neuroblastoma (101), BHK 21 (102), L1210 (103), rat embryo fibroblasts (104), Ehrlich ascites (105), 9L rat brain tumor (106) and WI38 (43). Bovine lymphocytes, which will not grow in culture unless stimulated with a mitogen such as Concanavalin-A, undergo marked increases in ODC and SAMDC activity and intracellular polyamine content subsequent to such stimulation (46,107).

One of the most interesting observations of a correlation between cell growth rates and ODC activity was made in a series of Morris hepatoma cells. These are all neoplastic liver cells that vary in their rate of growth, their degree of malignancy, and their degree of differentiation. It has been reported (108-110) that, while ODC activity is higher

in all hepatoma cell lines than in normal liver, the magnitude of this elevation correlated with the growth rate of the different lines (i.e. the fastest growing lines had the greatest increases in ODC activity). These differences were accompanied by variations in intracellular activities of the two other ornithine utilizing enzymes: ornithine carbamoyl transferase and ornithine transaminase. The fastest growing hepatomas had decreased levels of these two enzymes, ostensibly channeling more of the cells' ornithine into polyamine biosynthesis.

One final system that bears mentioning is the lytic infection of primary cultures of mouse kidney cells with polyoma virus (111). This oncogenic virus causes a biphasic induction of ODC and SAMDC, accompanied by a biphasic accumulation of intracellular polyamines. The earlier and smaller peak co-incides with the time of synthesis of viral mRNA. The larger and later peak occurs at the same time as virus-induced systhesis of host cell DNA.

A third approach, directed mainly at establishing a role for the polyamines in traverse of the mammalian cell cycle, has been the study of variations in polyamine biosynthetic enzyme activity and intracellular content in synchronized cell populations as they traverse the cell cycle. For Chinese hamster ovary cells, synchronized by selective detachment of mitotic cells, it has been reported that ODC activity increases beginning in mid G_1 with a peak just before the start of S phase (112). During S phase ODC activity decreases but rises once again in G_2. These changes in ODC activity are also accompanied by corresponding changes in intracellular polyamine content with peaks just before start of S and again just prior to initiation of mitosis (113). It has also been observed for baby hamster kidney cells synchronized by

excess thymidine treatment that ODC activity increases in early S, decreases through G_2 and mitosis, and increases once again in late G_1 and the second round of early S (114).

The temporal correlations of increases in polyamine biosynthetic enzyme activities and intracellular content as responses to stimulated growth do not, of course, prove a causal relationship between ODC induction and initiation of cell replication. The same is true for the variations observed in synchronized cells as they traverse the cell cycle. It is possible that ODC is induced as a result of a general stimulation of enzyme synthesis subsequent to triggering the cell's replicative machinery. The universality of ODC induction in stimulated growth systems is, however, suggestive of an important role for the polyamines in intracellular processes. Proof of an absolute requirement for a minimum intracellular polyamine content needed to support cell replication remains to be found.

I-E: Inhibition of Ornithine Decarboxylase *in vitro*

The largest group of ODC inhibitors are structural modifications of the normal substrate for this enzyme, ornithine (Table I-1). The first analog studied, canaline (α-amino-γ-aminooxybutyric acid) produces 50% inhibition of ODC *in vitro* at 1×10^{-4} M (115). This inhibition could, however, be reversed by addition of excess PLP to the incubation medium, but not by addition of excess ornithine. Canaline was also found to inhibit a number of other PLP dependent enzymes and to react non-enzymaticly with PLP. Based on these observations, it was concluded that inhibition of ODC by canaline was a result of inactivation of the cofactor rather than by prevention of binding of

TABLE I - 1

Compounds Tested as Inhibitors of Ornithine Decarboxylase

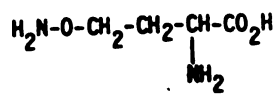
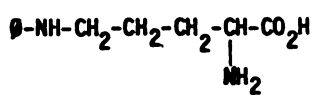
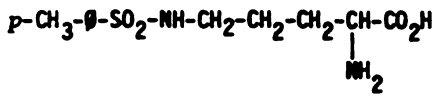
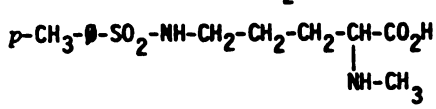
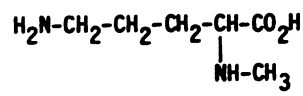
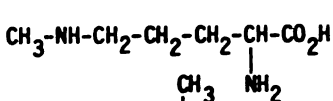
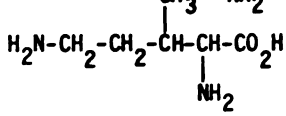
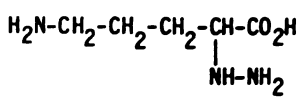
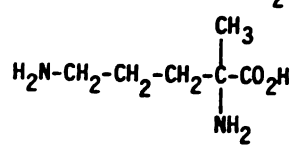
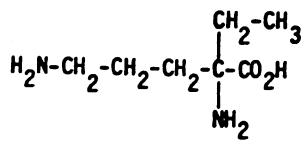
NAME	STRUCTURE	K_i
L-Canaline		a
N^5-phenylornithine		x
N^5-tosylornithine		x
N^5-tosyl-N^1-methylornithine		x
N^1-methylornithine		x
N^5-methylornithine		x
β-methylornithine		x
α-hydrazinoornithine		2×10^{-6} M
α-methylornithine		4×10^{-5} M (d,l) 2×10^{-5} M (+) 1×10^{-3} M (-)
(±)α-ethylornithine		5.3×10^{-3} M

TABLE I - 1 (cont.)

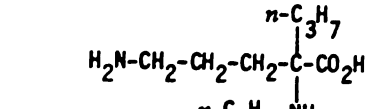
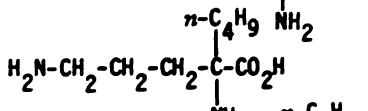
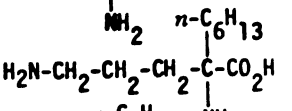
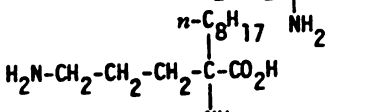
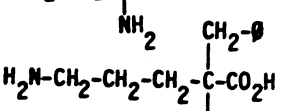
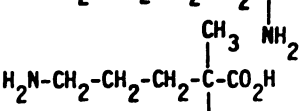
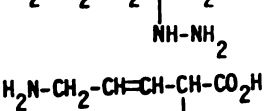
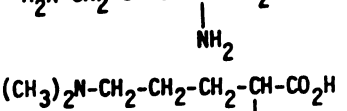
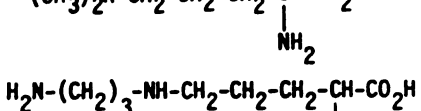
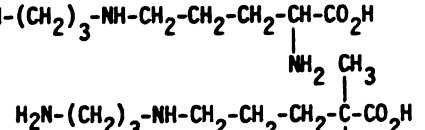
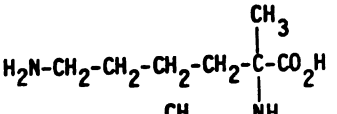
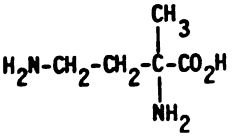
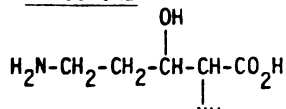
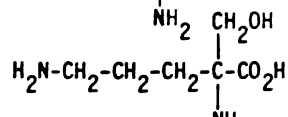
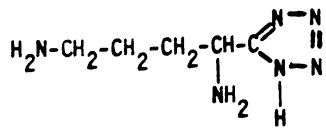
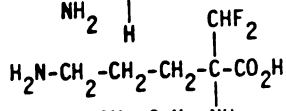
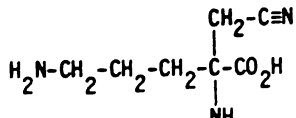
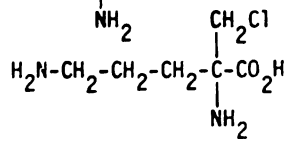
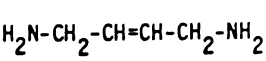
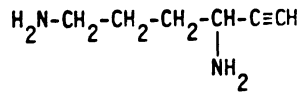
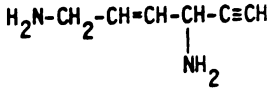
NAME	STRUCTURE	K_i
(±)α-n-propylornithine		7.8×10^{-3} M
(±)α-n-butylornithine		1.1×10^{-2} M
(±)α-n-hexylornithine		4.7×10^{-3} M
(±)α-n-octylornithine		2.3×10^{-3} M
(±)α-benzylornithine		5.3×10^{-3} M
(±)α-methyl-α-hydrazino-ornithine		3.0×10^{-6} M
trans-3-dehydroornithine		4.4×10^{-6} M
N^5-dimethylornithine		x
N^5-aminopropylornithine		x
N^5-aminopropyl-α-methyl-ornithine		x
trans-1,4-diaminocyclohexane-1-carboxylic acid		7.0×10^{-5} M
cis-1,4-diaminocyclohexane-1-carboxylic acid		x
α-methyllysine		x
α-methyl-2,4-diaminobutyric acid		x

TABLE I - 1 (cont.)

NAME	STRUCTURE	K_i
β-hydroxyornithine		x
α-hydroxymethylornithine		x
tetrazolylornithine		7.0×10^{-5} M
α-difluoromethylornithine		3.9×10^{-5} M[b]
α-cyanomethylornithine		8.7×10^{-3} M[b]
α-chloromethylornithine		a,b
trans-1,4-diamino-2-butene		2.0×10^{-6} M
5-hexyne-1,4-diamine		2.3×10^{-6} M[b]
trans-2-hexene-5-yne-1,4-diamine		1.0×10^{-6} M[b]

---

[a] inhibits ODC, but K_i was not measured or reported

[b] irreversible inactivator of ODC

[x] does not inhibit ODC

ornithine to the ODC active site (115).

A series of N-substituted ornithine analogs, including N^5-phenyl-, N^5-tosyl-, N^5-tosyl-N^1-methyl-, N^1-methyl-, and N^5-methylornithine, as well as β-methylornithine (Table I-1), synthesized by Skinner and Johansson (116) all failed to inhibit rat liver ODC in vitro. Conversion of the α-nitrogen to a hydrazino group to form α-hydrazinoornithine (αHO, Table I-1) does produce a potent inhibitor of ODC (117, 118). This analog has a K_i for rat ventral prostate ODC of $2x10^{-6}$ M, and inhibition of ODC by αHO is reversible and competitive with respect to substrate (117). Inhibition of ODC by αHO could, however, be overcome by large concentrations of PLP (118).

Abdel-Monem and his co-workers (119,120) have prepared an extensive series of α-substituted ornithines including methyl, ethyl, n-propyl, n-butyl, n-hexyl, n-octyl and benzyl. All compounds in the series were competitive inhibitors of mammalian ODC. The K_i values of these analogs as inhibitors of rat prostate ODC varied greatly (Table I-1). The most potent was α-methyl ornithine (αMO). Inhibition of ODC by αMO could not be reversed by high concentrations of PLP. The next best inhibitor, α-n-octylornithine, was only one sixtieth as potent as αMO. The least active was the n-butyl compound, which was 270-fold less active than αMO. It is apparent from this work that an α-substituent larger than methyl impairs binding of the ornithine analog to the active site of the enzyme.

These workers also prepared 1-^{14}C-αMO and incubated it with ODC to determine if this inhibitor was a substrate for ODC (120). No $^{14}CO_2$ production was observed over a one hour incubation even at a 1-^{14}C-αMO concentration of 10^{-2} M. It was thus concluded that αMO is not a

substrate for ODC. Later work by O'Leary and Herreid (121) showed this conclusion to be incorrect and the result of too short an incubation. They showed, for bacterial ODC, that αMO is decarboxylated, albeit at a 6000-fold slower rate than is ornithine. It was also shown that in a significant fraction of the αMO decarboxylations a subsequent transamination takes place which results in the production of 5-amino-2-pentanone and pyridoxine. Conversion of the cofactor to an inactive form is responsible for the observed inactivation of the enzyme in vitro after long incubations with αMO. The lost activity can be restored by addition of more pyridoxal phosphate. There are as yet no reports regarding the ability of mammalian ODC to produce a similar decarboxylation and transamination of αMO.

Abdel-Monem's group has also synthesized α-methyl-α-hydrazinoornithine (122). This competitive inhibitor (Table I-1) has a K_i for rat prostate ODC of $3x10^{-6}$ M, but this inhibition could be abolished at high PLP concentrations. Relyea and Rando (123) prepared trans-3-dehydroornithine (Table I-1) a reversible competitive inhibitor of ODC. This analog has a $K_i = 4.4x10^{-6}$ M for ODC from liver of thioacetamide-treated rats. The synthesis of this compound, however, has not as yet been published. Bey et. al. reported the synthesis of a series of fourteen analogs of ornithine (124). They tested the ability of these compounds (Table I-1) to inhibit ODC from rat liver, hepatoma tissue culture cells, and bull prostate. The most potent inhibitor in their series was (+)-αMO. This had exactly half the K_i of the racemic mixture, while the (-)-αMO isomer was 70-fold less active an ODC inhibitor. In agreement with the results of Skinner and Johansson (116), substitution of one or two methyl groups on the terminal amino group

of αMO abolished the ODC inhibitory activity of this analog. This was true as well for an aminopropyl substituent on the terminal nitrogen of either αMO or ornithine. These observations point to the requirement of a primary terminal amino group for binding to the enzyme's active site.

Two cyclic analogs, cis- and trans-1, 4-diaminocyclohexane-1-carboxylic acid were also examined in this study (124). The trans isomer had a $K_i = 7x10^{-5}$ while the cis isomer does not inhibit ODC at all. The lower and higher homologues of αMO, α-methyllysine and α-methyl-2, 4-diaminobutyric acid also do not inhibit ODC. These observations have been interpreted to suggest that ornithine is in a fully stretched conformation with the protonated amine groups as far from each other as possible when bound to the enzyme's active site. A hydroxyl substituent on the β carbon of ornithine or on the α-methyl carbon of αMO produced an ornithine analog with no ODC inhibitory activity. The last analog studied, in which the carboxyl group of ornithine was replaced by a tetrazolyl ring, had a $K_i = 7x10^{-5}$ M for the racemic mixture. All compounds in this series that showed ODC inhibitory activity were reversible, competitive inhibitors.

Recently the same group of investigator has reported the synthesis of three ornithine analogs that are enzyme-activated irreversible inhibitors of ODC (125). These compounds were α-difluoromethylornithine (DFMO), α-chloromethylornithine, and α-cyanomethylornithine (Table I-1). Incubation of ODC from liver of thioacetamide-treated rats with any of these compounds resulted in inactivation of the enzyme that could not be reversed by dialysis. Ornithine, αMO, or putrescine could protect the enzyme from inactivation by any one of the above compounds, showing

that they do act at the enzyme's active site. These investigators proposed a mechanism for ODC inactivation by these three analogs (Fig. I-6) which involves formation of a Schiff's base with PLP, decarboxylation, and then nucleophyllic substitution at the α-methyl group resulting in alkylation of the enzyme. This substitution is possible only as a result of activation of the halogen due to resonance with the pyridoxal ring. No evidence was presented in support of this mechanism. Of the three analogs, DFMO has the highest affinity for ODC. Abdel-Monem and Mikhail have also recently reported the synthesis of α-cyanomethyl ornithine, but observed no inhibition of rat prostate ODC by this analog at concentrations up to $1x10^{-3}$ M (126).

Several analogs of Pu, the product of ODC, have also been prepared and tested as inhibitors of ODC. Relyea and Rando reported (123) that trans-1,4-diamino-2-butene is a reversible competitive inhibitor of rat liver ODC with $K_i = 2x10^{-6}$ M. This inhibition was reversible by excess PLP. Metcalf, et. al. reported the synthesis of 5-hexyne-1,4-diamine and trans-2-hexene-5-yne-1,4-diamine (125), both of which are irreversible inactivators of ODC in vitro. The enzyme could be protected from inactivation by these product analogs with ornithine or Pu, and could not be reactivated after dialysis. The reported K_i values of these two inhibitors for rat liver ODC are $2.3x10^{-6}$ M and $1x10^{-6}$ M respectively.

One last ODC inhibitor that has been reported (127) is a reduced Schiff's base adduct of ornithine and pyridoxal phosphate, N-(5'-phosphopyridoxyl)-ornithine. This transition state analog is a reversible rat liver ODC inhibitor that is non-competitive with respect to both substrate and co-factor. Although this adduct is a potent inhibitor of ODC in vitro, its use as a tool for studying the intracellular

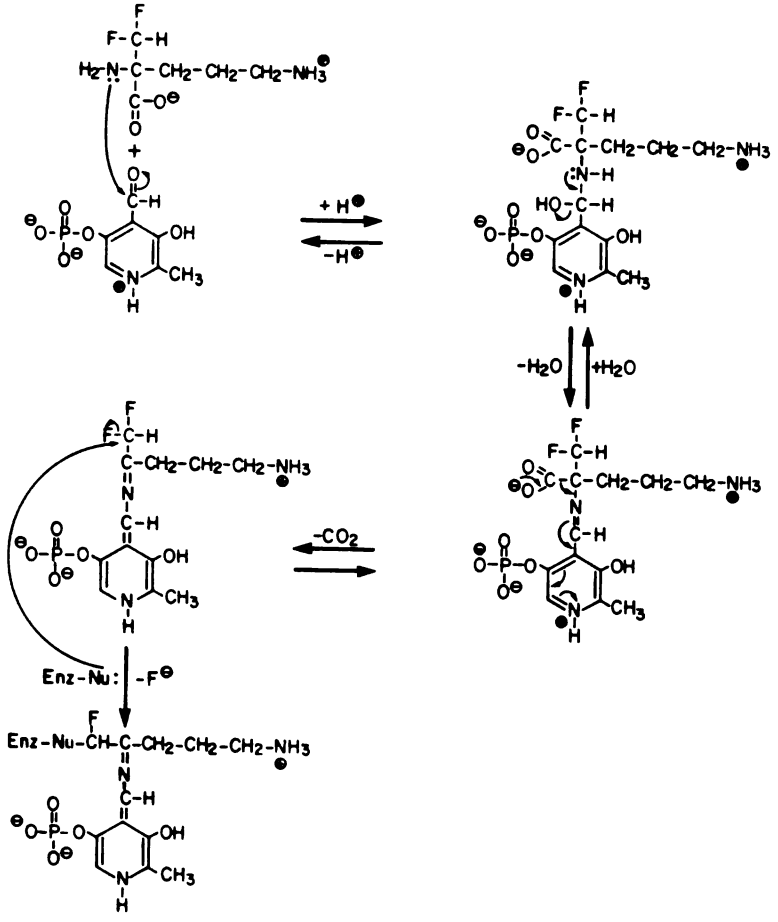


FIGURE I-6

Proposed Mechanism of Inactivation of

ODC by DFMO

functions of polyamines by producing polyamine depletion is limited by its inability to cross cell membranes. This is probably a result of the charge conferred on this molecule by the 5'-phosphate group.

Since the effects of αMO and DFMO on 9L cells is the central topic of this dissertation, their effects on other cell systems will be discussed at length in section I-G. The only other ODC inhibitor which has been studied in cultured mammalian cells and whole mammalian organisms is αHO. There also are numerous studies on 1,3-diaminopropane and various other α,ω-diamines as inhibitors of the induction of ODC, but as this has more bearing on the regulatory mechanisms for ODC than on the physiological role of the polyamines, such studies will not be reviewed. None of these diamines inhibit ODC in vitro (128).

Treatment of rat hepatoma cells in culture, intact rats, or partially hepatectomized rats with αHO results in depletion of intracellular Pu levels but does not effect the incorporation of ^{3}H -Uridine into RNA or ^{3}H -Thymidine into DNA (129). The degree of intracellular Pu depletion in this study, was, however, far from complete and Sd and Sp content were not measured. In addition, rates of macromolecular synthesis were only measured for the first 24 hours following addition of αHO. One can not reach any valid conclusions regarding the need for polyamines for macromolecular synthesis from these data. When isoproterenol-stimulated mice were treated with αHO (130), Pu contents in parotid gland tissue declined and DNA synthesis and cell proliferation in these glands also declined. Content of Sd and RNA synthesis rates were unaffected. The effect of αHO on DNA synthesis in mouse parotid gland could be blocked by simultaneous administration of Pu. Similar effects of αHO were observed on the growth of sarcoma 180 inoculated

into the axillary region of mice (131), and on rat liver after partial hepatectomy (132). Rupniak and Paul have also reported (133) similar effects on intracellular polyamine content by αHO treatment of fetal rat liver cells and rat embryo fibroblasts in culture. It appears, though, from the data presented in all of the above reports that αHO does not produce sufficient polyamine depletion to be a useful tool for the study of polyamine function.

One of the irreversible ODC inhibitors that is a product analog, 5-hexyne-1,4-diamine, was recently reported to cause a rapid, dose-dependent decrease of ODC activity in rat prostate, thymus, and testis when injected intraperitoneally (134). This decreased enzyme activity was accompanied by large decreases in Pu content in all three tissues but a decline in Sd content only in prostate. This inhibitor is, however, a substrate for monoamine oxidase which metabolizes it to 4-aminohex-5-ynoic acid, an irreversible inhibitor of 4-aminobutyrate: 2-oxoglutarate aminotransferase and of glutamate decarboxylase (135). The changes in ODC activity and Pu and Sd content of rat tissues observed upon administration of 5-hexyne-1,4-diamine to rats are thus accompanied by five-fold increases in brain γ-aminobutyrate concentration and large declines in activities of the above two enzymes (135). Pretreatment of animals with pargyline, an MAO inhibitor, prevents the oxidation of this ODC inhibitor, thus potentiating its effects on ODC activity and polyamine content and preventing the interference with γ-aminobutyrate metabolism (135).

I-F: Other Polyamine Antimetabolites

The earliest attempts to inhibit SAMDC examined the effects of administration of ethionine on chick embryos (97) and rats (136). In

both cases, such treatment resulted in decreased tissue levels of Sd. Later in vitro work showed that S-adenosyl ethionine, (Fig. I-7), which was not utilized for Sd synthesis, inhibited the production of Sd from SAM and Pu (137). Adenosyl ethionine likewise inhibits conversion of Sd to Sp, and is decarboxylated by rat prostate SAMDC at an 85-90% slower rate than is SAM (137). As a tool for investigating the consequences of polyamine depletion, however, neither ethionine nor S-adenosylethionine are very useful. Both are potentially able to interfere with a wide variety of enzymes from pathways other than polyamine biosynthesis.

The SAMDC inhibitor that has received the most attention is methylglyoxal-bis-guanylhydrazone (MGBG, Fig. I-7, 1,1'-{(methylethanediylidine)-dinitrilo} diguanidine). Rat ventral prostate SAMDC activated by Pu is inhibited by MGBG with 50% inhibition at 1.5×10^{-6} M MGBG (138). Rat kidney SAMDC was 50% inhibited at 2.5×10^{-7} MGBG (139). Twenty-fold higher concentrations of MGBG are required to inhibit the much slower rate of SAM decarboxylation by rat prostate enzyme that occurs in the absence of Pu activation (138). These and other observations were interpreted as evidence for binding of MGBG at the Pu binding site that is responsible for activation of the rat prostate enzyme, rather than at the substrate binding site (138). Inhibition of rat prostate SAMDC by MGBG was also shown to be reversible and competitive with respect to substrate (139). The bis(guanylhydrazones) of both dimethylglyoxal and ethylglyoxal (Fig. I-7) inhibit rat prostate SAMDC to the same extent as does MGBG, but propane and pentane dialdehyde-bis (guanylhydrazones) (Fig. I-7) are poor inhibitors of this enzyme (139). The three other enzymes of polyamine biosynthesis, ODC, Sd synthase, and Sp synthase,

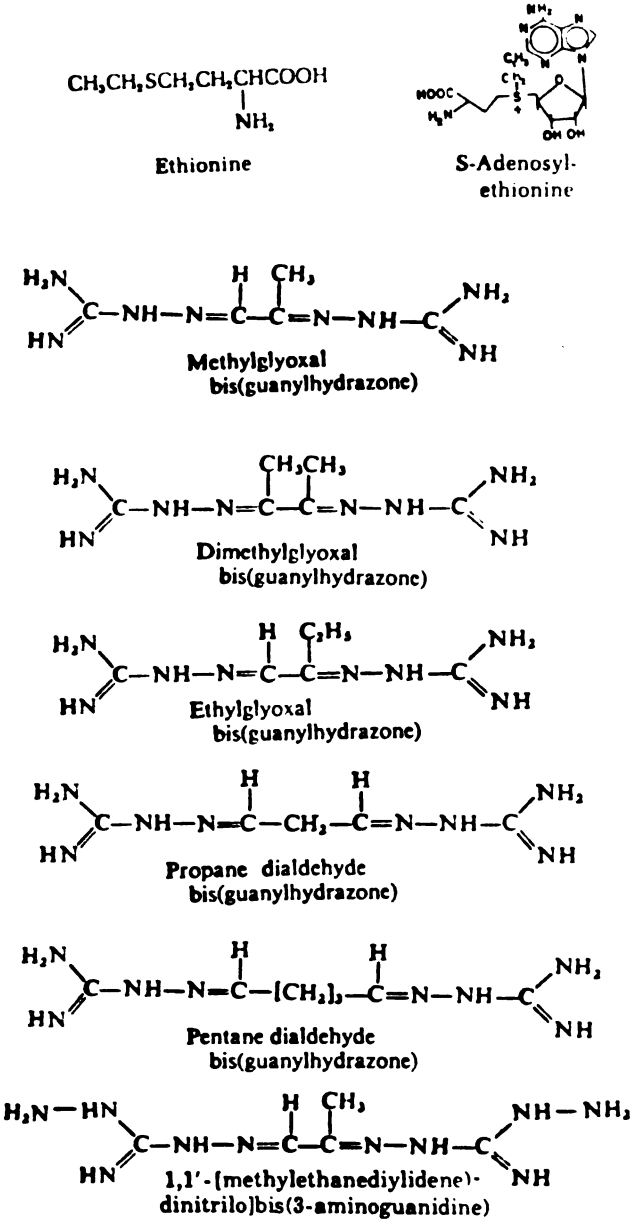


FIGURE I-7

Structures of Inhibitors of SAMDC

are unaffected by MGBG (139).

Recently, Pegg reported that 1,1'-{(methylethanediylidene)-dinitrilo}-bis-(3-aminoguanidine) (MBAG, Fig. I-7) is an irreversible inactivator of rat liver SAMDC (140). This inactivation occurs more rapidly in the presence of Pu and is slowed by both substrate and MGBG. Kinetic evidence was presented that is consistent with a reversible binding of MBAG to the enzyme followed by covalent bond formation resulting in inactivation. Presumably this occurs at the carbonyl group of the covalently bound pyruvate cofactor of SAMDC (140). Administration of MBAG to rats resulted in significant but not complete decreases in tissue levels of SAMDC activity in kidney, liver, and prostate (140). Content of Sd and Sp were slightly reduced in kidney but not in liver, while Pu content was elevated in both. In regenerating rat liver, MBAG given in combination with diaminopropane (an inhibitor of ODC induction) one hour after partial hepatectomy completely inhibits DNA synthesis (141). Neither compound has such an effect when given alone. Similar effects on the elevated Sd levels normally observed subsequent to partial hepatectomy were also reported (141).

Only one report has appeared regarding inhibition of the Sd and Sp synthase enzymes (142). Both 5'-methylthioadenosine and SAH can inhibit Sp synthase from rat ventral prostate at low concentrations which do not affect rat prostate Sd synthase. The two enzymes are also inhibited by α,ω-diamines with from three to twelve carbon atoms (except, of course, Pu), with Sp synthase more susceptible to such inhibition than Sd synthase. Both enzymes were, however, inhibited by S-adenosylethionine to the same extent.

It was initially thought that MGBG would be a useful tool for

producing polyamine depletion in tissues of intact animals. Early reports showed that kidney and liver SAMDC were inhibited by MGBG administration to intact rats (143). Incorporation of ^{14}C Pu into Sd was also inhibited by such treatment. While Pu content was increased, tissue levels of Sd and Sp were unaffected by MGBG administration. Later studies showed that if tissue extracts were dialyzed to remove the MGBG, the level of SAMDC activity in treated animals was actually increased over controls (144,145). Experiments with both intact animals (144, 145) and with perfused rat liver (146) showed that this increase was a result of a lengthened half life for SAMDC as measured after cycloheximide treatment. This increased half life was also not the result of the increased Pu content since addition of Pu to the perfusion fluid did not increase the half life of SAMDC in isolated perfused rat liver even though it did produce elevated tissue levels of Pu (146). It thus appears that MGBG stabilizes SAMDC to proteolytic degradation. The inability of MGBG to produce significant polyamine depletion in intact animals, regardless of mechanism, prevented its use for elucidating the biochemical and physiological consequences of such depletion in that system.

Hormonal stimulation of tissue explants or organ cultures is a system in which MGBG has been used successfully to demonstrate a requirement for polyamines in the normal response to such stimulation. Mouse mammary explants can be stimulated by a combination of insulin, hydrocortisone, and prolactin to synthesize casein and α-lactalbumin (147). Addition of MGBG to the culture medium can inhibit this milk protein synthesis, and this inhibition can be overcome by simultaneous addition of Sd (147). Similar results have been reported for the

effects of MGBG on progesterone-stimulated glycogen synthesis by organ-cultured endometrial fragments from guinea pig uteri (148). A later report on insulin-stimulated DNA synthesis in cultured mouse mammary explants showed that not only Sd but also Sp, Pu and cadaverine, can reverse the MGBG-produced inhibition of DNA synthesis in this system (149). Only Sd, however, could do so at physiological concentrations.

Morris and coworkers have carried out an extensive series of investigations on the effect of MGBG on Concanavalin-A (Con-A) stimulated proliferation of bovine lymphocytes. In the absence of mitogen, these lymphocytes do not undergo DNA synthesis, nor do they enter mitosis (107). Con-A stimulation causes a 3 to 5 fold increase in the rate of RNA synthesis at about 60 minutes following stimulation (107). Accumulation of intracellular Pu to 10-15 times unstimulated levels begins about 8 hours after addition of Con-A, with increased content of Sd (5-7 fold) and Sp (2-2.5 fold) following at 10 hours (107). Treatment of Con-A stimulated bovine lymphocytes with MGBG results in a 2500 fold increase in intracellular SAMDC activity after removal of MGBG by dialysis, compared to a 20 to 25 fold increase normally observed subsequent to mitogen stimulation (150). Measurements of SAMDC half life after cycloheximide treatment showed that, as in rat liver, MGBG increased the half-life of SAMDC from 40 minutes to greater than 20 hours (150).

Later reports from this group indicated that 2 μM MGBG or more, added to the culture medium either along with or up to 9 hours after Con-A, resulted in a 50-60% inhibition of new DNA synthesis and a 60% decrease of the rate of entry into mitosis (151). No effects on the rates of RNA or protein synthesis were observed (107,151). Addition of

MGBG to Con-A stimulated lymphocytes also completely prevented the accumulation of intracellular Sd and Sp normally observed, but resulted in greatly increased intracellular levels of Pu (107). Both the decrease in rate of DNA synthesis and the inhibition of cell entry into mitosis can be reversed by addition of Sd or Sp to the cultures 12 hours after addition of MGBG (151). This observation, together with the absence of an effect by MGBG added after polyamine accumulation has taken place (151) indicates that the inhibition of DNA synthesis and mitosis produced by MGBG is a consequence of insufficient intracellular polyamine concentration. Completely analogous results were reported for the rates of in vitro DNA synthesis by nuclei isolated from Con-A stimulated lymphocytes cultured in the presence or absence of MGBG (152).

Other investigators have examined the effects of MGBG on phytohemaglutinin (PHA) stimulated human (153,154) and guinea pig (155) lymphocytes. Essentially similar results were observed regarding inhibition of DNA synthesis and polyamine accumulation, and reversibility of the DNA synthesis inhibition by exogenous polyamines. The only difference reported for this system is that MGBG produced inhibition of protein synthesis as well (153,154). This effect was also reversible by exogenous polyamines (154).

Many other cell lines have been treated with MGBG, resulting in similar observations regarding intracellular polyamine levels and cellular proliferation. Rat embryo fibroblasts (104,133), Ehrlich ascites (156), HeLa (157), BHK-21/C13 (158), human fibroblast PA2 (159), mouse 3T3 (159), and CHO cells (159) all show increased intracellular Pu content, decreased levels of Sd and Sp, and inhibition of cellular proliferation. In the case of rat embryo fibroblasts this inhibition of

proliferation did not occur until after an initial doubling of the cell population, indicating that the cells' polyamine content after 24 hours of MGBG treatment was sufficient to support one round of replication (104). After 24 hours the cells became growth arrested in the G_1 phase of the cell cycle, and this arrest could be reversed by addition of Sd or Sp to the culture medium (104). With Ehrlich ascites cells it was reported that MGBG produced an accumulation of cells in the S phase of the cell cycle (156). In contrast to the reports of MGBG treatment of lymphocytes, HeLa calls treated with MGBG were inhibited in DNA, RNA, and protein synthesis (157). This inhibition was observed for both whole cells and isolated nuclei, but could be reversed by addition of exogenous polyamines only for whole cells (157). It was also reported that MGBG treatment of CHO, HeLa, PA2, and 3T3 cells resulted in a greatly increased incidence of binucleate cells, implying a dependence of normal cytokinesis on presence of minimal intracellular polyamine content (160). Addition of Sd or Sp to these cells along with MGBG reduced the frequency of binucleate cells to control levels.

Some of the effects of MGBG on cells grown in culture may not be the result of depletion of intracellular pools of Sd and Sp. It was recently reported that treatment of L1210 cells with from 1 to 10 μM MGBG resulted in extensive damage to mitochondria (161). This is within the range of concentrations used to inhibit SAMDC in cultured cells. Since L1210 mitochondrial fractions do not have significant SAMDC activity, it appears that this effect is probably not a result of SAMDC inhibition by MGBG, unless mitochondrial integrity depends upon the cytoplasmic pool of polyamines. It was also observed that MGBG treatment of L1210 cells produced a number of chromosomal

aberrations and extensive presence of cytoplasmic vacuoles (162). While it is important to be aware of the possibility of non-polyamine related effects of this drug, it seems likely that those effects discussed above which are reversible by addition of exogenous polyamines are specific consequences of polyamine depletion.

I-G: Effects of αMO and DFMO on Cell Systems Other than 9L

The effects of αMO and DFMO have been studied in a variety of biological systems. The largest body of work has been reported by the group at Centre de Recherche Merrell International using rat hepatoma tissue culture (HTC) cells. It was first observed that 5 μM αMO inhibits HTC cell proliferation by 75% over a four day growth period (163). This inhibition was, however, only manifested after an initial lag of one day during which treated and untreated cultures grew at the same rate. Pu content was depleted by four hours and Sd content by 11-12 hours after treatment with 5 μM αMO; Sp content, however, was slightly increased. Incorporation of ^{3}H-Thymidine into DNA in whole HTC cells was also inhibited by 5 μM αMO after the initial 24 hour lag. The inhibition of proliferation produced by 5 μM αMO could be reversed by addition of exogenous polyamines. A later report (164) showed that the (-) enantiomer of αMO, which does not inhibit ODC in vitro (124), does not inhibit HTC cell proliferation, while the (+) enantiomer does. Apparently, however, 24 hours are required to reduce intracellular polyamine content sufficiently to produce inhibition of proliferation even after synthesis of Pu is stopped by αMO.

It was subsequently shown (165) that treatment of HTC cells with αMO causes a 3-fold increase in ODC activity in cell extracts washed to remove the αMO. This increase was only observed for serum-depleted

cells that were stimulated by addition of fresh serum. Addition of αMO to depleted cells without simultaneous addition of serum produced no increase in HTC cell ODC activity, implying that αMO only stabilized newly synthesized ODC. Experiments utilizing cycloheximide to block new protein synthesis showed that 5 μM αMO increased the half-life of newly synthesized ODC three-fold (165). Increased levels of SAMDC activity were also observed in HTC cells incubated with αMO (164), possibly the cell's response to polyamine depletion.

Treatment of HTC cells with 0.1 mM or more of DFMO completely blocks the biphasic stimulation of ODC activity normally observed when fresh serum is added to serum-depleted cultures (103). The ODC activity of cell extracts from DFMO treated HTC cells is not restored by extensive dialysis (103) indicating irreversible inactivation of the intracellular enzyme. Treatment with DFMO does, however, increase HTC intracellular SAMDC activity (164). Content of Pu in treated cells is depleted to undetectable levels by six hours post DFMO treatment, and Sd is depleted to less than 50% of control levels by 8 hours (103). Content of Sp, however, is unaffected even after eight days of incubation with DFMO (103). In addition, while 5 mM DFMO inhibited incorporation of ^{3}H from ^{3}H-Ornithine into Pu and Sd to <2% of control, incorporation of ^{3}H-ornithine into Sp was the same for DFMO treated and control cells (164). These observations imply some continued Pu synthesis followed by rapid conversion to Sd and Sp even in the presence of DFMO. After the same 24 hour lag observed with αMO, HTC cell proliferation is significantly inhibited by 5 mM DFMO (103,164). This inhibition is reversible by addition of exogenous polyamines to HTC cultures 24 hours after treatment with 5 mM DFMO (103,164). Addition of polyamines to DFMO

treated HTC cells also partially reduces the elevated SAMDC activity produced by incubation with DFMO (164).

Long term incubation of HTC cells with 5 mM αMO was used to produce a new clone of this cell line, designated HMO_A, whose growth is unaffected for at least 3 days by incubation with 5 mM αMO (166). This clone has a 3 fold higher basal level of ODC activity, but has about the same level of SAMDC activity as wild-type HTC cells. The higher basal ODC activity of HMO_A was maintained over 100 successive generations when cultured in the absence of αMO. Both HTC-ODC and HMO_A-ODC had the same K_M for ornithine and the same K_i for αMO. Initial intracellular Pu content was 7-fold higher, and Sd content was 2-fold higher for HMO_A than for HTC (166). Treatment of HTC cells with 5 mM αMO or DFMO results in depletion of intracellular Pu and Sd within one day of treatment, while it requires 3 days of treatment to achieve Pu and Sd depletion for HMO_A as a result of its higher basal levels (166). It is thus no surprise that while HTC cells proliferation is inhibited after one day of incubation with 5 mM αMO or DFMO, HMO_A continues to proliferate at control rates for 3 days and then is inhibited (166).

The same group of investigators has studied the effects of αMO and DFMO on several other cell systems. After an initial lag of 24 hours, 5 mM αMO or DFMO inhibits proliferation of L1210 leukemic cells in suspension culture to less than 0.5% of the growth observed for untreated controls (103). Intracellular Sd was completely depleted by 1.5 generation times by either treatment, while Pu was only depleted to about 40% of control levels. Complete depletion of Pu was observed after 5 doublings. Prostate adenoma MA160 cells were inhibited by 5 mM

DFMO to about 10% of control growth rate, but 5 mM αMO had no effect on MA160 proliferation (103). They have also shown that 200 mg/kg of DFMO administered intraperitoneally to mice inoculated with $1x10^6$ L1210 leukemic cells prolongs the survival of treated mice from a mean of 7.5 days to a mean of 9.4 days (167). Normal mice treated with the same dose of DFMO showed no effect on spleen ODC activity, had increased spleen SAMDC activity, and decreased spleen Pu and Sd (but not Sp) content. With L1210 inoculated mice, the above dose of DFMO prevented 75% of the rise in spleen ODC normally observed after inoculation, lowered spleen Pu content by 58% and Sd content by 30%, but had no effect on spleen Sp content. The minimal effects of tissue polyamine contents observed in this study are probably a result of rapid clearance of DFMO from the treated mice. Incomplete depletion of polyamine content could thus be responsible for the minimal effect on life span observed with the treated rumor-bearing animals.

Morris and his coworkers have used αMO, primarily in combination with MGBG, to prevent the accumulation of intracellular polyamines normally observed when bovine lymphocytes in culture are treated with Con-A (168). When 5 mM αMO was used without MGBG, Pu content remained at the same level as in unstimulated cells, while only 75% of the Sd accumulation occurring in untreated but stimulated lymphocytes was prevented. No effect on Sp accumulation was observed. This treatment did not affect the rate of incorporation of radiolabelled uridine into RNA and labelled amino acids into protein, and only inhibited incorporation of ^{3}H-thymidine into DNA by 10% (168). When αMO and MGBG were used in combination, the abnormal elevations of Pu content observed when MGBG is used alone was prevented (168). The inhibition of DNA synthesis

produced by MGBG alone was increased from 50% to 75% of control rates when 5 mM αMO was used in combination with 8 μM MGBG (168), while rates of RNA and protein synthesis were unaffected. Addition of exogenous polyamines to stimulated lymphocyte cultures treated with MGBG and αMO reversed the inhibition of DNA synthesis (168). Furthermore, addition of MGBG and αMO to stimulated cultures after polyamine accumulation had taken place did not affect the rates of DNA synthesis (168). Identical results were reported for DNA synthesis by nuclei isolated from lymphocytes treated with αMO alone, MGBG alone, or the two inhibitors in combination (152). In contrast to the above results, it was recently reported that 0.5 mM DFMO treatment of PHA stimulated cultured human lymphocytes prevented accumulation of Sp as well as Sd and Pu (154). Such treatment not only inhibited DNA synthesis, but also protein synthesis and, to a lesser and variable extent, RNA synthesis as well. The inhibition of all three types of macromolecular synthesis was reversible by addition of exogenous polyamines to the PHA-stimulated lymphocytes along with or after treatment with DFMO (154).

Abdel-Monem and his coworkers reported that treatment of L1210 leukemic cells with αMO over two generations resulted in a 50% decrease in intracellular Sd content, a decline in Pu to nondetectable levels, and a slight increase in Sp levels (169). The DNA content of the cell suspension was unaltered, leading these investigators to conclude that loss of all intracellular Pu and 50% of intracellular Sd did not affect L1210 DNA synthesis. The results reported by Mamont et. al. (103,164) discussed above showed that this conclusion is incorrect and that inhibition of L1210 DNA synthesis did occur after αMO treatment if the incubation was carried out over a longer time. Abdel-Monem and his colleagues

also showed that, in contrast to the results discussed earlier for DFMO (167), treatment of L1210 inoculated mice with up to 300 mg/kg αMO had no effect on the survival time of the leukemic mice or on the spleen content of polyamines (170).

Flow cytometric measurements of Ehrlich ascites cells grown in the peritoneal cavity of mice treated with αMO showed an accumulation of cells in the S and G_2M phases of the cell cycle (156). As discussed earlier for HTC cells, serum-starved human fibroblasts treated with DFMO at the same time as addition of serum showed a lag in inhibition of proliferation and of DNA synthesis (171). Addition of exogenous polyamines reversed both of these inhibitions. Finally, the exogenous polyamine-reversible effects on cytokinesis of a variety of cultured mammalian cells reported by Sunkara et. al. (160) as a result of MGBG treatment were also observed subsequent to αMO treatment.

I-H: Previous Studies on Polyamines in 9L Cells

Several lines of evidence have implicated a role for the polyamines in 9L cell proliferation. It was first reported (172) that when 9L cells are grown as a solid subcutaneous tumor in the flank of rats, activities of ODC and SAMDC and content of Pu, Sd, and Sp were higher in the faster growing periphery of the solid tumor than in the slower growing center. In addition, while whole tumor and central portion polyamine contents declined from day 22 to day 29 after tumor cell inoculation, no such decline was observed in the peripheral portion. That the growth rate and growth fraction of the peripheral portion was markedly higher than that of the central portion was indicated by a significant amount of necrosis and a markedly reduced mitotic index in microscopic sections of central portions over peripheral portions of the tumor.

Subsequently, it was shown for 9L cells grown in tissue culture over a seven day period that spermidine content was greatest during the exponential phase of growth and declined when cell proliferation ceased (173). The Sd/Sp ratio as well as the intracellular Sd content showed a high positive correlation with the specific growth rate of the 9L cells. This evidence indicates that Sd accumulation is probably a requirement for 9L cell replication. A later extension of this study showed that the intracellular activities of both ODC and SAMDC also exhibited high positive correlations with the 9L specific growth rate (106). A low positive correlation for Pu content, and a negative correlation for Sp content, with specific growth rate were also reported in this study. The high positive correlations obtained for Sd content, ODC activity, and SAMDC activity support the hypothesis that polyamines are necessary for 9L cell proliferation.

Treatment of exponentially growing 9L cells in tissue culture with MGBG resulted in inhibition of cellular proliferation after a lag of one doubling time (174). This inhibition, however, was not reversed by resuspension of MGBG-treated cells in fresh growth medium or in fresh medium containing 2 mM Sd. Flow cytometry performed on MGBG-treated 9L cells and on untreated controls demonstrated a marked accumulation of cells in the G_1 phase of the cell cycle in the treated samples. This G_1 block could not be reversed by resuspension of cells in fresh medium with or without Sd. The 9L intracellular Sd and Sp contents of MGBG treated 9L cells began to decline immediately after addition of the drug, reaching 35% of control levels for Sd and 57% of control levels for Sp at their lowest. In contrast, intracellular Pu content increased due to decreased utilization for Sd and Sp synthesis, reaching 20% of

control levels 10 hours after treatment. These data suggest a possible requirement for Sd and Sp in traverse of the cell cycle by 9L cells which can not be met by increases in Pu.

While the above discussed studies do provide suggestive evidence for a role for polyamines in 9L cell proliferation and in traverse of its cell cycle, conclusive data supporting this hypothesis have not as yet been obtained. It would be most desirable to produce complete depletion of 9L intracellular polyamine content and study the effects of such depletion on continued cell proliferation and cell cycle distribution of polyamine depleted cells. The above-mentioned study of Heby et. al. (174) showed that MGBG could only produce partial depletion of Sd and Sp, along with increased levels of Pu. Furthermore, the effects on cell proliferation and cell cycle traverse observed following MGBG treatment of 9L cells could not be reversed by exogenous Sd and thus might not be the result of Sd and Sp depletion. Inhibitors of ODC, which interfere with polyamine biosynthesis at an earlier step in the pathway, could conceivably produce greater degrees of polyamine depletion in 9L cells than does MGBG. In addition, ODC inhibitors would deplete Pu as well as the higher polyamines. Accordingly, the studies to be described in the remainder of this dissertation were undertaken to investigate the effects of αMO and DFMO, a reversible and an irreversible ODC inhibitor, on 9L cell proliferation, intracellular polyamine content, and progression of the cells through the mammalian cell cycle.

CHAPTER II: EXPERIMENTAL METHODS

II-A: Tissue Culture Techniques

Rat 9L brain tumor cells were grown in monolayer tissue culture as previously described (175). The origin and characteristics of the 9L cell line have been described by Benda et. al. (176) and by Barker et. al. (175). The desired number of cells ($5x10^5$ or $1x10^6$) suspended in growth medium (to be described below) were seeded into plastic Falcon flasks with 75 cm^2 surface area. The total volume of growth medium was then adjusted to 13.5 ml and the flasks were incubated at 37° C in a National CO_2-incubator in a 5% CO_2-95% air humidified environment. The growth medium consisted of Eagle's basal medium (BME) supplemented with L-glutamine (292 mg/l) fetal calf serum (FCS, 10%) BME vitamin mixture (1%), BME essential amino acid mixture (1%), and penicillin-streptomycin mixture (1%). All growth medium supplies were purchased from Grand Island Biological Co.

Cells were allowed to attach to the growth surface and to begin replicating for 24 hours before treatment with drugs. This insured that cells would be in the exponential phase of growth at the time of treatment (106,174). Cells were treated with 1.5 ml of a 10-fold concentrated solution of the appropriate polyamine antimetabolite in Hank's balanced salt solution (HBSS) at pH 7.2. When added to the culture flask this brought the total volume of medium to 15 ml and diluted the drug to the appropriate concentration. Control flasks were treated with 1.5 ml of HBSS. Solutions of DFMO in HBSS, which were initially quite

acidic, were adjusted to pH 7.2 with concentrated NaOH.

At the times shown in the growth curves, 3 culture flasks treated at each concentration of drug and 3 control flasks were removed from the incubator and the cells harvested by trypsinization. The medium present in the flask was poured off and the cells were washed with 2 ml of a solution containing 0.25% trypsin and 0.02% Na_2EDTA in Ca^{+2}- and Mg^{+2}-free HBSS. After decanting this wash solution a second 2 ml aliquot of the trypsin solution was pipetted into the flasks and they were then incubated for 5 min. at 37° C to allow cells to detach from the plastic growth surface.

Several variations on the above techniques were employed. After effects of polyamine antimetabolites were observed the specificity of such effects were tested by addition of exogenous Pu to the culture medium to counteract the polyamine depletion produced by the antimetabolites. Since FCS contains diamine oxidase activity, addition of Pu to the culture medium with FCS resulted in oxidation products toxic to 9L cells. These experiments were thus carried out in medium containing 10% horse serum (HS) in place of 10% FCS. Three methods of addition were used in such tests for specificity. In the first, Pu was added at the same time as the polyamine antimetabolites. Reversal of effects by this method could be due to interference of the exogenous polyamine with antimetabolite uptake. Therefore, reversal of effects 24 hours after their production was tested by addition of Pu to treated cultures one day after addition of the antimetabolite. The third method tested the ability of exogenous pyridoxal (50 mg/l), the unphosphorylated form of the cofactor of ODC, to reverse the effects of ODC inhibitors. One final variation was employed in only one experiment with DFMO. This

involved addition of DFMO at the same time as seeding of the cells. In all of the above variations all other techniques for both culturing and harvesting 9L cells were identical to those described above.

II-B: Measurement of Cellular Proliferation

The loosened cells were washed out of the tissue culture flasks into 15 ml conical plastic centrifuge tubes using a first aliquot of 6 ml and a second aliquot of 4 ml of ice cold 0.02% Na_2EDTA in HBSS. This final 12 ml of cell suspension was then vigorously shaken to disperse the cells into a single cell suspension. All samples were kept on ice from this point through all further preparatory steps.

The total number of cells harvested from each flask was measured by counting an aliquot of the cell suspension using a Royco Cell-Crit model 927-TC cell counter, as previously described (177). This instrument uses electronic resistance detection to count each cell as it passes through a 100μm orifice in an aperture tube containing two electrodes. A 1:100 dilution of the cell suspension to be counted was made using a Royco Model 365-A automatic dilutor which withdraws 0.1 ml of cell suspension and then delivers this volume plus 9.9 ml of isotonic saline diluent into a plastic cup. The 927-TC counter then measures the number of cells in 0.5 ml of this diluted cell suspension and displays the measured value on a digital LED readout. Each diluted cell suspension was counted three times and the three values averaged and multiplied by 2,000 to obtain the number of cells/ml of the original cell suspension. Multiplication by the volume of the original cell suspension (12 ml in most cases) then gave the total number of cells harvested from each flask. The results obtained for 3 replicates at each time point under

each treatment condition were averaged and the mean values plotted as a function of time after treatment. Error bars indicate standard deviations.

II-C: Polyamine Analysis

After counting the cells, a 6 ml aliquot of the cell suspension in HBSS was removed to a new 15 ml plastic conical centrifuge tube. The cells were pelleted by centrifugation for 10 minutes (1000 rpm, 900xg, at $4^{o}C$) in a refrigerated centrifuge. The supernatant was then decanted and the inside of the tube dried with a Kimwipe wrapped around forceps. The cell pellet was next homogenized by sonication in 8% 5-sulfosalicylic acid (SSA) (approximately 250µl/10^{6} cells) using a Lab-Line Ultratip Labsonic system. This homogenate was kept on ice for one hour to insure complete precipitation of protein and then centrifuged to 10 minutes (8000xg, at $4^{o}C$). The supernatant was tranferred to a 450µl Brinkman polystyrene microfuge tube and stored at $-20^{o}C$ until polyamine analysis was carried out.

A Dionex Instruments, Inc. Model D-500 Automated Amino Acid Analyzer was used for measurement of polyamines, as previously described (179,180). A 50µl aliquot of the supernatant from the cell pellet homogenate was loaded into a Sample Holder Unit (SHU) and then up to 22 of these SHU's were placed into the sample feed chute of the analyzer at one time. Separation of the polyamines was accomplished by high pressure cation exchange chromatography using an 11.5 cm, 1.75 mm internal diameter stainless steel column maintained at $50^{o}C$ packed with a 16% cross linked, 10±1µm diameter, sulfonated polystyrene polymer cation exchange resin (Durrum Chemical Co.). Four buffers used for sequential

elution of the polyamines were prepared as follows. A stock buffer (Buffer IV), 2.40N KCL and 0.09N potassium citrate (pH 5.56), to which 12 ml/l thiodiglycol and 0.5 ml/l liquified phenol were added and which was filtered through 47 mm diameter 0.45μm pore size Millipore filters was diluted to prepare Buffers I-III as follows (stock: deionized distilled water): I,1:4; II,1:1; III,4:1. Sample injection, buffer changes, and starting and stopping of the recorder, as well as all other operations in each analytical run were controlled by a Digital PDP8/M mini-computer. Buffer and reagent flow were both maintained at 18.4 ml per hour.

Each chromatographic run was performed as follows. The column was first washed by pumping 0.2N KOH for 2 minutes. The column was then re-equillibrated with Buffer I for 7 minutes, at which time the SHU containing the sample to be analyzed was loaded into place. The sample was then injected onto the column. At 26 minutes after start of the procedure, pumping of the o-phthalaldehyde reagent solution (to be described below) was begun. Flow of Buffer I, which elutes all of the amino acids from the column, was continued until 29 minutes into the sequence, at which time pumping of Buffer II was begun and the recorder was turned on. Putrescine elutes from the column with Buffer II beginning at 7 minutes after start of Buffer II (36 minutes into the run) and ending 3.5 minutes later with the peak occurring at 8-8.5 minutes (37-37.5 minutes of entire sequence). Pumping of Buffer II is continued until 42 minutes after the start of the procedure, at which time flow of Buffer III, which elutes spermidine from the column, is started. Elution of spermidine begins at 49 minutes into each run (7 minutes after start of Buffer II), continues until 53 minutes, with the peak occurring at 50

minutes. Flow of Buffer IV starts at 54 minutes and continues until the end of the run at 66 minutes. Spermine elutes from the column with this last buffer beginning at 57.5 minutes, ending at 62.0 minutes and peaking at 59.5 minutes of the sequence. Figure II-1 shows typical chromatograms obtained for standard solutions and for cell homogenates.

Quantitation of the three polyamines was achieved by fluorescence detection of the chromophore produced by reaction with o-phthalaldehyde. The eluent from the cation exchange column was mixed with a reagent solution, prepared as follows. A stock buffer was prepared by dissolving 100 g KOH and 120 g H_3BO_3 in 3.6 l deionized distilled water, adjusting pH to 10.4 with H_3BO_3 crystals, and adjusting final volume to 4 l. The final reagent solution was prepared by adding 4.5 ml of β-mercaptoethanol, 3.0 ml BRIJ (a detergent), 5.8 g KSCN and 0.800 g o-phthalaldehyde dissolved in 20 ml anhydrous methanol to one liter of the above stock buffer. This reagent solution was then filtered through 47 mm diameter 0.45μm pore size Millipore filters. The combined flows of reagent and column eluate passed through the 70μl flow cell of an Aminco Fluoromonitor operated at an excitation wavelength of 340 nm and an emission wavelength of 455 nm. For most analyses the fluorometer was operated at a sensitivity setting of 30, although in some cases higher (10 or 3) or lower (100) sensitivity settings were used. The output from the fluorometer was fed into a strip chart recorder operating at a speed of 0.2 inches per minute.

To test for the linearity and reproducibility of the assay, a series of standard solutions of all three polyamines in 0.1 M HCL were analyzed at sensitivity setting 30 (125-1000 pmols of each polyamine per

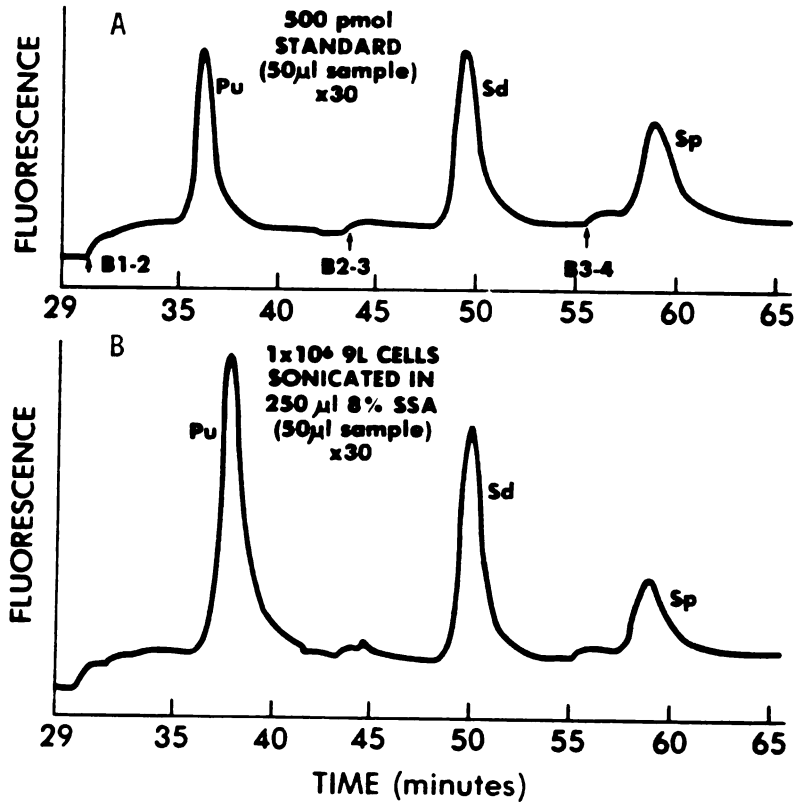


FIGURE II - 1

Chromatograms of Polyamine Standard Solution (A) and 9L Cell Homogenate (B) run on the Dionex D-500 Amino Acid Analyzer. B_{1-2}, B_{2-3}, and B_{3-4} are buffer change artifacts which appear slightly later than the actual changes due to the time necessary for the buffer change to pass through the column and arrive at the fluorometer.

50µl) and at sensitivity setting 10 (50-500 pmols of each polyamine per 50µl). The heights of each peak were measured and a linear correlation was performed on the peak height vs. concentration (in pmol/50µl) data. The linear correlation coefficients for each of the three polyamines at each of the two sensitivity settings were all greater than 0.99. At each setting, four standard solutions of increasing polyamine concentration were used, and each standard solution was analyzed three times, averaged, and coefficients of variation (CV) calculated. At sensitivity setting 30 the CV ranged from 4.1% to 8.8% for Pu, from 1.9% to 5.2% for Sd, and from 2.4% to 8.2% for Sp. At sensitivity setting 10, the CV ranged from 0.8% to 2.6% for Pu, 1.9% to 3.4% for Sd, and 6.4% to 13.2% for Sp. Other workers in this laboratory have shown (179) that the day to day CV over a five month period of this assay, used to measure polyamine concentrations in pooled cerebrospinal fluid samples, was 10.7% for Pu and 8.2% for Sd. This work involved a different method of sample preparation with greater losses than simple homogenization.

Given the high linearity and reproducibility of the assay, polyamine content of the 50µl samples from each cell homogenate supernatant was calculated from the ratio of sample peak height to standard peak height. Standards containing 500 pmols of each of the three polyamines per 50µl were run after each six samples analyzed. From the total volume of SSA used for homogenization of the cell pellet, and from the total number of cells homogenized in each sample, the Pu, Sd, and Sp content of each sample harvested were calculated in nanomoles/10^6 cells. The results obtained for each of three samples harvested for every treatment condition at each time point were averaged. These results

were then plotted as a function of time, with error bars indicating standard deviations.

II-D: Cell Cycle Analysis Using Flow Cytometry

Cells harvested for flow cytometry (FCM) were taken up following trypsinization in ice cold growth medium with 10% FCS in place of the 0.02% Na_2EDTA in HBSS. In these experiments the growth medium originally present in the flask was combined with the trypsinized and resuspended cells. This avoided selective loss of mitotic cells due to their weaker attachment to the growth surface. The cells were dispersed by pipetting the suspension out of and back into the tube several times. The cells were then pelleted by gentle centrifugation for 10 minutes (1000 rpm, 900xg, at 4^oC) in a refrigerated centrifuge. The supernatant was decanted, 10 ml of ice cold phosphate buffered saline solution (PBS) were added, and the cells were resuspended by gentle vortexing. These more gentle techniques were necessary for cells prepared for flow cytometry to avoid damaging the cells and broadening the DNA distributions obtained.

Cells suspended in ice cold PBS were pelleted by centrifugation (1000 rpm, 900xg, for 10 minutes at 4^oC) in a refrigerated centrifuge. The supernatant was decanted and the cells were fixed by resuspending them in ice cold 70% aqueous ethanol with gentle vortexing. Cell samples were stored in this manner in a refrigerator at least overnight and could remain fixed in the ethanol suspension for up to 2 months. The cells were then stained by pelleting (as described above), decanting the aqueous ethanol supernatant, and adding 3 ml/10^6 cells of stain solution. This stain solution was prepared by dissolving 10 mg of

Chromomycin A_3 (Calbiochem) and 1.5g $MgCl_2 \cdot 6H_2O$ in 500 ml distilled water. Chromomycin A_3 is a fluorescent antibiotic that binds tightly, but not covalently, to double helical DNA but not to RNA (180). The cells were resuspended by gentle vortexing in this stain solution, allowed to sit for 20 minutes at room temperature in the dark, and then stored in the refrigerator until analyzed on the Livermore bicolor flow cytometer (181-183), usually within 1-2 days after staining.

With this technique stained cells in single cell suspension flow in a narrow stream at uniform velocities through an intense argon ion laser beam of exciting 457 nm light at rates up to 1000 cells/sec. Each cell is illuminated for the same period of time so that the fluorescence emitted by each cell is proportional to the amount of stain taken up by that cell. This in turn is directly proportional to the amount of DNA present in the cell, so that measurement of fluorescence intensity is also a measurement of cellular DNA content (181-183). Fluorescence from each cell was detected by a photomultiplier which converted the light pulse to a number proportional to pulse intensity. Each of these numbers, representing the DNA content of one cell, was stored in the memory of a multichannel pulse-height analyzer. Analysis of each sample continued until 3×10^5 cells had accumulated in the modal channel of the largest peak in the DNA distribution (almost invariably the G_1 peak). All of the acquired data is then output to the screen of an oscilloscope as a histogram of number of cells in each channel on the ordinate axis versus channel number (i.e. fluorescence intensity which is proportional to DNA content) on the abscissa. A coefficient of variation for each DNA distribution was calculated by computer from the full width at half height of the highest peak in the histogram, and was

also displayed on the oscilloscope screen. The DNA histogram was recorded by taking a Polaroid picture of the oscilloscope screen, and the data (number of cells in each channel) was also recorded on magnetic tape for subsequent computer analysis of fraction of the total cell population in each phase of the cell cycle.

Cells in the G_1 phase of the cell cycle, which have not yet begun replicating their DNA, have a 2C complement of DNA. Cells in G_2, as well as cells in M, have twice this amount, or 4C. Cells which are in the process of synthesizing DNA, S phase cells, have DNA content between 2C and 4C. Computer analysis of the cell cycle distribution of samples obtained in the re-seeding study with DFMO pre-treatment (Section IV-D) was performed as previously described (184). This analysis assumes a Gaussian distribution for DNA content of cells in G_1, measures the area under the G_1 peak of the DNA histogram, and computes the ratio of this area to the total area under the histogram. A similar but broader Gaussian distribution is assumed for the G_2M peak, its area is computed, and the ratio of this area to the total gives the fraction of cells in the G_2 and M phases. These two peaks are then subtracted from the total, a polynomial is computer-fit to the remainder, and the area under this polynomial is divided by the total area to give the fraction of cells in S. In addition, the coefficient of variation for this computer fit to the data is calculated. Since the program used is less reliable for distinguishing cells in S from those in G_2M, attention was focused solely on the fraction of cells in G_1.

II-E: Cell Cycle Analysis Using ^{3}H-Thymidine Autoradiography

A single experiment was performed using this technique to investigate

the effects of 50 mM αMO on the cell cycle progression of 9L cells in tissue culture. The pulse chase method of obtaining a percent labeled mitoses (PLM) curve was used as previously described (185,186). Falcon flasks into which $5x10^5$ 9L cells in 10 ml of the previously described growth medium had been seeded, were treated with 1 μCi of ^{3}H-TdR (specific activity 0.36 Ci/mmol; Schwartz Bio-Research, Inc.). The flasks were incubated for 30 minutes at 37°C, the medium decanted, and the cells washed twice with cold Ca^{+2}- and Mg^{+2}-free HBSS. Fresh medium containing 50 mM αMO and pre-equillibrated to 37°C, pH 7.3 was added to each flask and the flasks were returned to the incubator. This procedure selectively labels a cohort of cells actively engaged in DNA synthesis (S phase cells) at the time of treatment with ^{3}H-TdR.

Progression of this labeled cohort of cells through the cell cycle was followed by harvesting 3 flasks every 1.5 hours following treatment with 50 mM αMO, for a total of 46.5 hours. Harvesting procedure was as previously described (Section II-A) except that the medium from each culture, which sometimes contains detached single cells, was combined with the trypsinized cells from each flask. This change was made since a substantial fraction of the mitotic cells, which tend to round up and loosen from the plastic growing surface, can be lost if the growth medium is discarded. After pelleting the cells by centrifugation (221xg at 4°C for 5 min.) and decanting the supernatant, the cells were treated for 5 minutes with a hypotonic solution of 2.8 g NaCl/l of 0.002 M phosphate buffer at pH 7.0. The cells were again pelleted, the hypotonic saline decanted, the cells fixed in a 3:1 absolute ethanol: glacial acetic acid fixative for five minutes, and spread on a slide according to the method of Puck and Steffen (187). The slides were air-dried, dipped

in Kodak NTB-2 nuclear emulsion and exposed at 4°C in light-tight dessicated boxes for 10-14 days. The autoradiographs were then developed in Kodak D-19 Developer for 4 minutes at 18°C, fixed in Kodak Rapid Fixer, rinsed, dried, and stained with hematoxylin. Each slide was examined under a light microscope and 50-100 mitotic cells were counted on each slide. The fraction of all mitoses counted that were labeled with exposed grains of photographic emulsion was computed for each of the three replicates at every time point. The mean values at each time point, with error bars indicating standard deviations, was used to construct a PLM curve for 50mM αMO-treated 9L cells. In addition, a mitotic index (MI) curve was obtained by measuring the number of mitotic cells in 1000-3000 cells on each slide and averaging the results for the three replicates at each time point. The results obtained from this experiment were compared to the PLM and MI curves obtained with untreated 9L cells in previous experiments (186).

II-F: Colony Formation Efficiency Assay

The colony formation efficiency (CFE) assay was used in three types of experiments. The first of these was designed to test for cytocidal effects of αMO on 9L cells. Cultures of 9L cells were prepared as described earlier (Section II-A) seeding $1x10^6$ cells/flask. After 24 hours of incubation cells were treated with concentrations of αMO ranging from 0 to 100mM and returned to the incubator for a further 24 hours. The cells were harvested by trypsinization as described for FCM samples (Section II-D) except that medium from the culture flask was discarded. The resulting single cell suspensions were used to assess survival of cells after a 24 hour exposure to various concentrations of αMO by means

of the CFE assay, as described below.

The second type of experiment using the CFE assay investigated the effects of polyamine depletion (subsequent to treatment of 9L cells in vitro with polyamine antimetabolites) on the X-ray dose-response curve for survival of cells after irradiation. Cell cultures were prepared as in the previous paragraph and, after an initial 24 hour incubation, were treated in separate experiments with 10mM αMO, 25mM DFMO, 40μM MGBG, or a combination of 25mM DFMO plus 40μM MGBG. In each experiment an equal number of control flasks, treated with HBSS, were prepared. The flasks were then returned to the incubator for an additional 48 hours, at which time both treated and control flasks were irradiated with X-rays as previously described (188) at doses of 0-2000 rads. The tissue culture flasks were placed on a styrofoam support with the growth surface 50 cm from the target of a Westinghouse Quadrocondex X-ray tube (230 kVp, 15mA, half value layer ≈ 1.6 mm Cu, and dose rate ≈ 360 rad/min). The cells were maintained at 37°C until immediately before irradiation, and were returned to 37°C immediately after irradiation. At the highest X-ray dose (2000 rads) the exposure time was 5.6 minutes and the temperature of cultures irradiated at this dose did not drop below 33°C. Two treated and two untreated cultures in each experiment were not irradiated and served as controls. After irradiation of all cultures, the cells from each flask were harvested by trypsinization as described in section II-D, dispersed into single cell suspensions by shaking, counted as described in Section II-B, and these cell suspensions were then used to assess survival of treated and untreated cells after various doses of X-ray by means of the CFE assay.

The third experiment that utilized the CFE assay investigated the

effect of addition of 0.1 to 25 mM DFMO to the Petri dishes used in the assay on the CFE of untreated, unirradiated 9L cells. A series of cell suspensions in growth medium were prepared so that from 50 to $1x10^5$ cells could be added to the plates in 0.5 ml. Solutions of DFMO in HBSS were prepared at ten-fold higher concentrations than ultimately desired. Half the plates were treated with 0.5 ml of these solutions at the same time as seeding the cells, while the remainder had the drug added 24 hours later. These plates were then used to assess the ability of 9L cells to form colonies of 50 or more cells in the presence of DFMO.

The CFE assay was performed as previously described (189). One day prior to the start of the assay, separate cultures of 9L cells were heavily irradiated (4000 rads), harvested, counted, and suspended in complete medium to a cell density of $5x10^4$ cells/4 ml of medium. Four ml of this suspension of feeder cells were pipetted into each 60 mm plastic Petri dish (Lux Scientific Corp.) to be used in the CFE assay. The Petri dishes were then incubated for 24 hours at 37^oC in a humidified 5% CO_2-95% air environment. A series of dilutions of the cell suspensions to be assayed were prepared and one ml of each dilution was pipetted into each of five replicate Petri dishes. Dilutions were chosen to insure that at least one set of five plates would be countable (i.e. -- would produce from 20 to 60 colonies). The Petri dishes were then returned to the incubator for 12-14 days, at which time the medium was decanted, the colonies were fixed in absolute methanol, and stained with 2% Geimsa (Harleco Chemical Co.). The number of colonies of 50 or more cells were counted and the fraction of surviving cells calculated from the ratio of number of colonies obtained to number of cells seeded. The data obtained from 5 (if only one dilution was countable) or more replicate plates were

CHAPTER III: STUDIES WITH α-METHYLORNITHINE

Part One: Results

III-A: Effects on 9L Cell Proliferation and Intracellular Polyamine Content

Preliminary experiments with 9L cell cultures seeded at $1x10^6$ cells/flask showed that rather high concentrations of αMO were required to produce significant inhibition of proliferation. Over a 96 hour treatment period 2.5 to 10 mM αMO did not significantly inhibit 9L cell growth. Cells treated with 25 mM αMO reached 60% of the number of cells present in untreated controls, while cells treated with 40 or 50 mM αMO only grew to 40% and 20% of the control cell number. Only 50 mM αMO produced a continuous decline in the ratio of treated cells to control cells. These results were confirmed in a subsequent experiment utilizing 1, 10, and 50 mM αMO (Fig. III-1). By day four the highest concentration (50 mM) inhibited cell growth to 55% of that in untreated controls (Fig. III-1A). Treatment with either 10 mM or 1 mM αMO resulted in cell growth equal to or greater than controls.

By 12 hours after treatment, both 50 mM and 10 mM αMO depleted intracellular Pu content to below the limit of detection of the preparative and analytical methods used (0.1 nmol/10^6 cells), while cells treated with 1.0 mM αMO had low but measurable levels of Pu through four days post treatment (Fig. III-1B).

The intracellular Sd content decreased to approximately 1/3 of control in cells incubated with 50 mM αMO by 12 hours after treatment, while

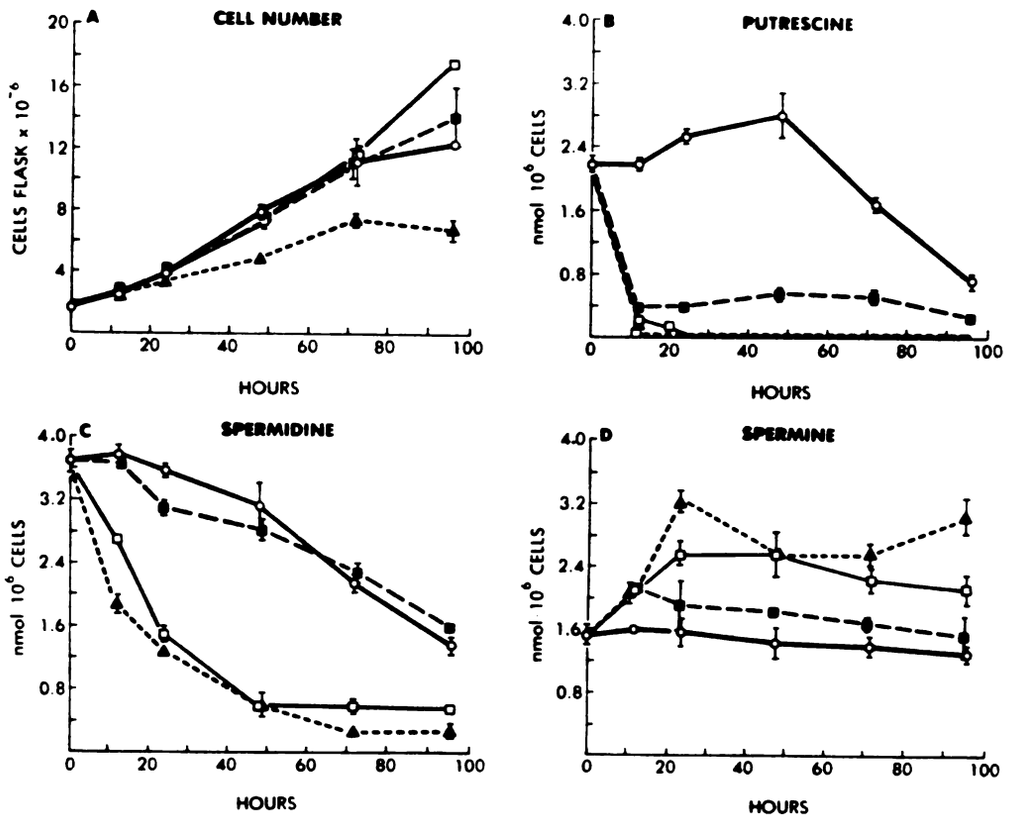


FIGURE III - 1

The Effect of αMO on 9L Cell Proliferation (A), Pu Content (B), Sd Content (C), and Sp Content (D). Cells were incubated for 96 hours following treatment (at time 0) 24 hours after seeding 1 x 10^6 cells/flask with 0 (○), 1.0 (■), 10.0 (□), or 50 mM (▲) αMO. Points represent the mean (±SD) of three determinations.

cells treated with 10 mM αMO required 24 hours post treatment to achieve the same degree of depletion (Fig. III-1C). Except for a slight difference at 24 hours, no depletion of intracellular Sd was observed for cells treated with 1 mM αMO.

Treatment of 9L cells with αMO resulted in increased rather than decreased intracellular Sp levels (Fig. III-1D). For cells incubated with 50 mM and 10 mM αMO this increase was two and 1.5-fold, respectively, by 24 hours post treatment. This increase never exceeded 120% of control levels for cells incubated with 1 mM αMO.

If the initial cell density was reduced to $5x10^5$ cells/flask 50 mM αMO caused an even greater inhibition of 9L cell proliferation (Fig. III-2A). Under these conditions 10 mM αMO also significantly inhibited cell growth. As in the previous experiment, 1 mM αMO had no effect on 9L cell proliferation. This reduction in initial cell density resulted in a lengthening of the exponential growth period of the untreated controls and thus allowed extension of the experiment for one day beyond the previous study. The effects on intracellular Pu (Fig. III-2B), Sd (Fig. III-2C), and Sp (Fig. III-2D) content observed upon αMO treatment of cells seeded at $5x10^5$/flask were similar to those found previously at the higher initial density.

III-B: Kinetics of Putrescine Depletion as a Function of αMO Concentration

In a series of shorter experiments the decline in intracellular Pu subsequent to treatment with αMO was investigated at concentrations of from 0.1 to 100 mM. As a result of variations in initial Pu content of untreated cells due, most likely, to differences in number of passages before start of the experiment and in lots of fetal calf serum, these data are presented as fraction of control Pu content in the treated cells

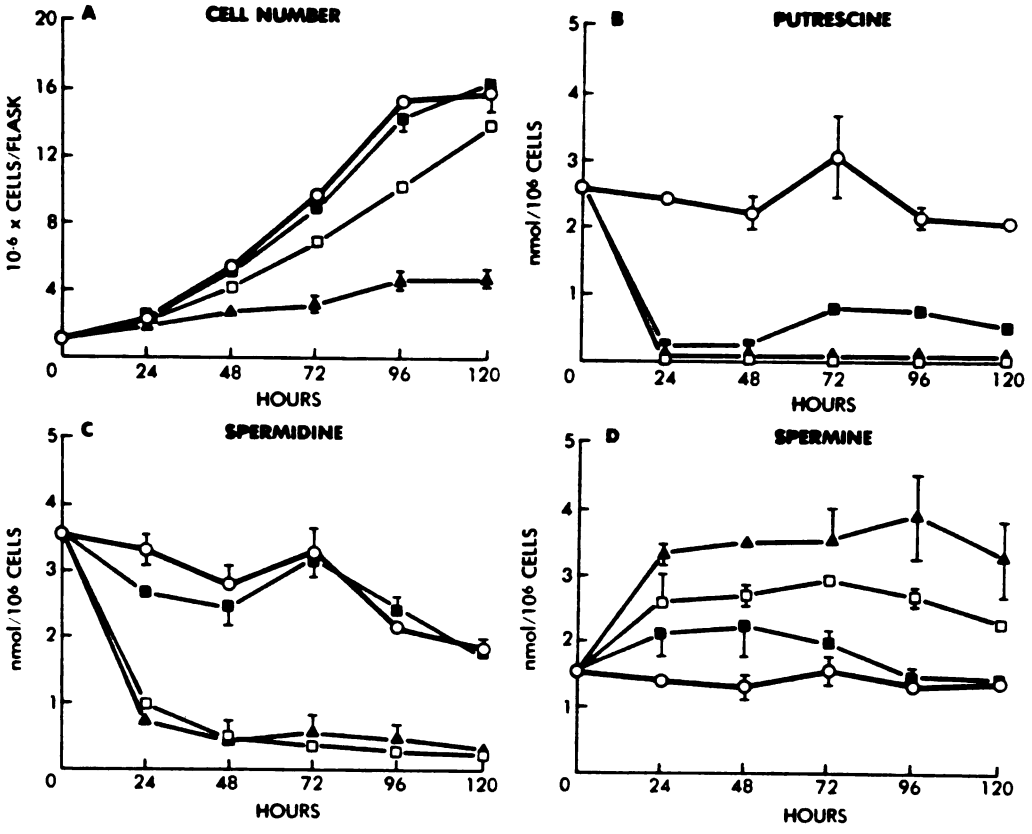


FIGURE III - 2

The Effect of αMO on 9L Cell Proliferation (A), Pu Content (B), Sd Content (C), and Sp Content (D). Cells were incubated for 120 hours following treatment (at time 0) 24 hours after seeding 5 x 10^5 cells/flask with 0 (○), 1.0 (■), 10.0 (□), or 50 mM (▲) αMO. Points represent the mean (±SD) of three determinations.

(Fig. III-3). Treatment with 0.1 mM αMO did not deplete 9L intracellular Pu content by more than 10% over the first 12 hours of incubation, although a decline to 75% of control was observed after 36 hours of treatment. Increasing concentrations of αMO increased the rate of depletion of intracellular Pu with 100 mM αMO achieving 90% depletion by 4 hours post-treatment. This degree of depletion was not achieved with 50 mM αMO until 6 hours after treatment and with 10 mM αMO until 12 hours.

III-C: Recovery and Blocking Studies

As discussed in Chapter II, attempts to block or reverse the cytostatic effect of 50 mM αMO on 9L cell proliferation by addition of Pu to the culture medium necessitated replacing the 10% FCS with 10% HS to avoid oxidation of Pu to toxic products by amine oxidases present in FCS. This change did not affect the growth curve of 9L cells. Reversal of the cytostatic effect of 50 mM αMO on cells seeded at $1x10^6$/flask was attempted by one of three treatments. One group of flasks (Group C) was treated with 50 mM αMO and 24 hours later received fresh medium with no αMO. A second set of flasks (Group D) was treated with 50 mM αMO and 24 hours later received fresh medium with 3 mM Pu. This concentration of Pu produced a three-fold increase in intracellular Pu content of untreated 9L cells. The third group of flasks (Group E) had 3 mM Pu added to the medium already in the flask 24 hours after treatment with 50 mM αMO. One set of control flasks (Group B) were treated with 50 mM αMO only, and a second set of controls (Group A) received no treatment at all. As shown in Table III-1 none of the above treatments could reverse the inhibition of 9L cell proliferation produced by 50 mM αMO.

The results of an attempt to block the cytostatic effect of 50 mM

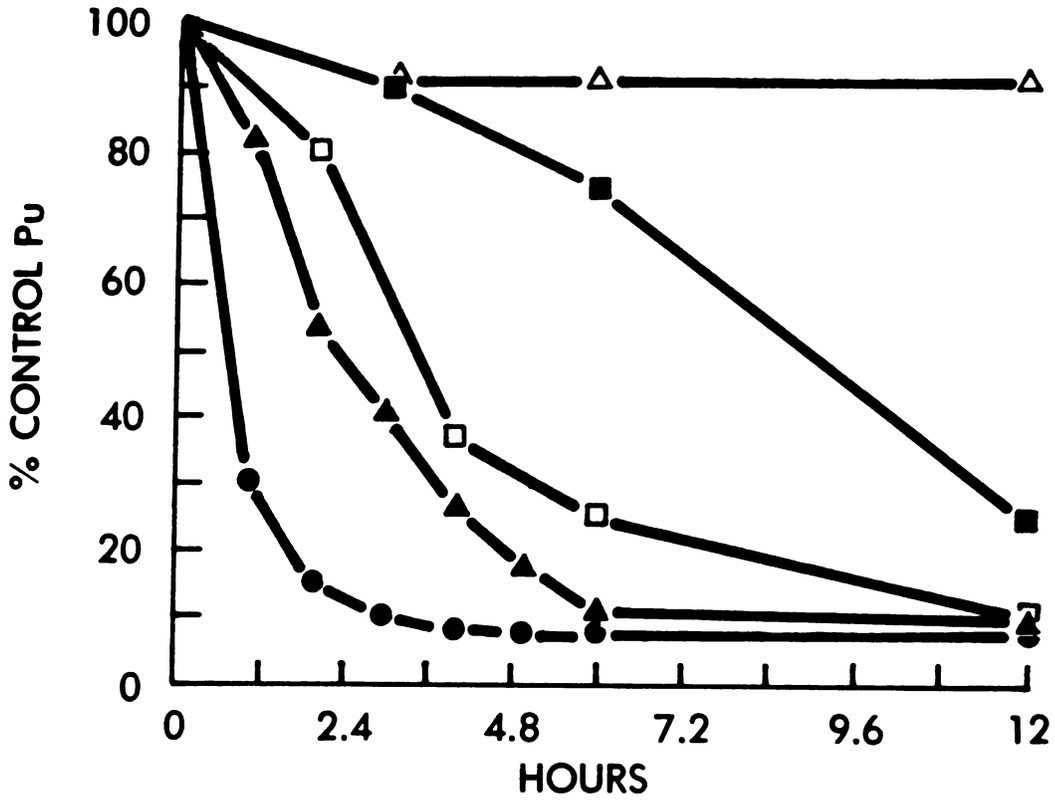


FIGURE III - 3

Kinetics of Depletion of 9L Intracellular Pu by αMO.
Cells were treated with 100 (●), 50 (▲), 10 (□), 1 (■), or 0.1 mM (△) αMO 24 hours after seeding 1 x 10^6 cells/flask. Intracellular Pu of treated cells was determined and expressed as % of untreated control Pu content at the same time point.

TABLE III - 1

Attempted Reversal of Cytostatic Effect of 50 mM αMO on 9L Cells with Putrescine

GROUP	TREATMENT	10^{-6} x CELLS/FLASK 48 HOURS POST TREATMENT
A	none (control)	5.16 ± 0.37
B	50 mM αMO alone	2.78 ± 0.05
C	50 mM αMO for 24 hrs then fresh medium	2.56 ± 0.28
D	50 mM αMO for 24 hrs then fresh medium with 3 mM Pu	2.96 ± 0.18
E	50 mM αMO for 24 hrs then 3 mM Pu added to flask	3.10 ± 0.47

9L rat brain tumor cells seeded at 1 x 10^6/flask were incubated in the presence or absence of 50 mM αMO. At 24 hr after treatment fresh medium, fresh medium + 3 mM Pu, or 3 mM Pu alone was added and cells were incubated for a further 24 hr. Cells were then harvested, counted, and results from three replicates at each condition were averaged. Results are expressed as mean ± SD.

αMO by adding 3 mM Pu to the incubation medium at the same time as the ODC inhibitor are shown in Figure III-4. This experiment used four groups of flasks: untreated controls (group A), cells treated with 50 mM αMO only (group B), cells treated with 3 mM Pu only (group C), and cells treated with both 3 mM Pu and 50 mM αMO (group D). Addition of 3 mM Pu to the incubation medium had no effect on the proliferation of 9L cells whether they were treated with αMO or not (Fig. III-4A). In addition, 50 mM αMO inhibited 9L cell proliferation to the same extent in the presence of 3 mM Pu as in its absence. The intracellular Pu content of cells in group D, while not as high as that of group C, was higher than that of both group A and group B (Fig. III-4B). The cells in group D also showed a smaller decrease in Sd content (Fig. III-4C) and a smaller increase in Sp content (Fig. III-4D) than those in group B.

An attempt was also made to reverse or block the cytostatic effect of 50 mM αMO on 9L cells seeded at $1x10^6$ cells/flask by adding 50 mg/l pyridoxal, the unphosphorylated form of the cofactor of ODC, to the culture medium either 24 hours after or simultaneous with the addition of αMO. This concentration represents a 50-fold increase in pyridoxal over that normally present in the culture medium. As shown in Table III-2, 50 mg/l pyridoxal neither reversed nor blocked the inhibition of 9L cell proliferation produced by incubation with 50 mM αMO.

As a result of the inability to reverse or block the growth inhibition of 9L cells by 50 mM αMO, an additional control experiment was performed. The effect on 9L cell proliferation of the addition to the culture medium of either 50 mM L-ornithine or 50 mM DL-ornithine is shown in Table III-3. Flasks treated with 50 mM L-ornithine had only 60% as many cells 48 hours after treatment as did untreated controls. Flasks

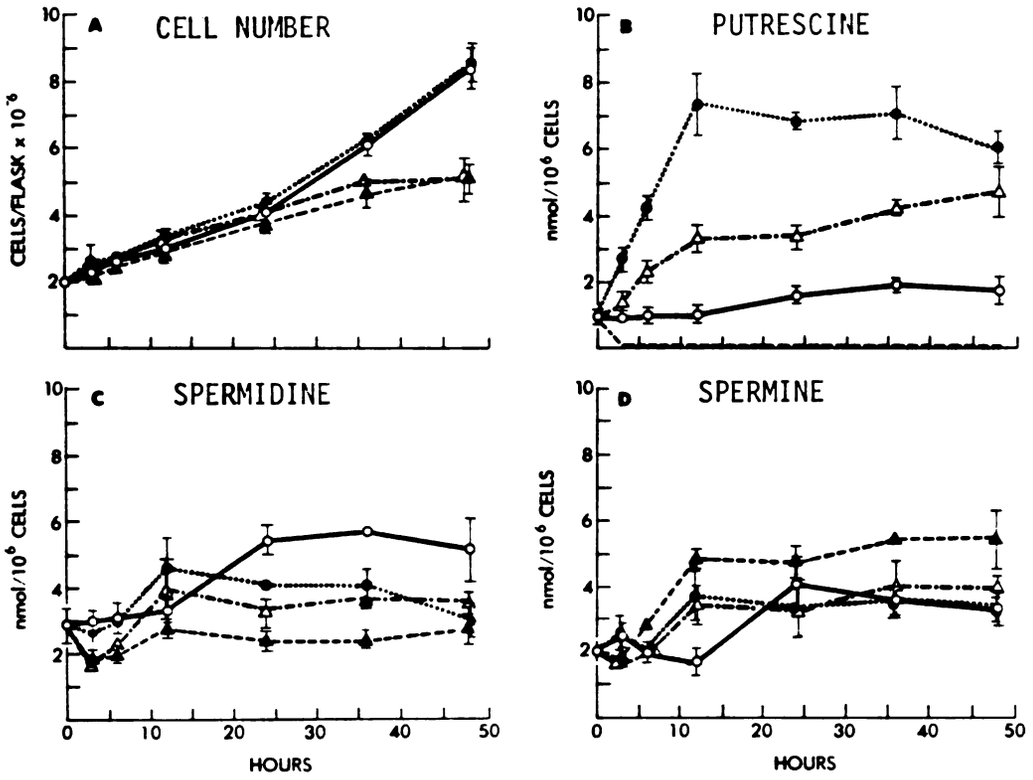


FIGURE III-4

Attempted Blocking of the Cytostatic Effect of 50 mM αMO on 9L Cells by Exogenous Pu. Cells were incubated for 48 hours following treatment (at time 0) 24 hours after seeding 1 x 10^6 cells/flask with HBSS (○), 50 mM αMO (▲), 3 mM Pu (●), or 50 mM αMO + 3 mM Pu (△). Effects on cell proliferation (A), Pu content (B), Sd content (C), and Sp content (D). Points represent the mean (±SD) of three determinations.

TABLE III - 2

Attempted Reversal and Blocking of Cytostatic Effect of 50 mM αMO on 9L Cells with Exogenous Pyridoxal

TREATMENT	10^{-6} x CELLS/FLASK 24 HOURS POST TREATMENT	10^{-6} x CELLS/FLASK 48 HOURS POST TREATMENT
none (control)	3.92 ± 0.26	7.66 ± 0.36
50 mM αMO	3.02 ± 0.26	4.30 ± 0.44
50 mg/l pyridoxal	3.40 ± 0.13	6.58 ± 0.58
50 mM αMO + 50 mg/l pyridoxal	2.67 ± 0.05	3.33 ± 0.12
50 mM αMO for 24 hr then fresh medium with 50 mg/l pyridoxal	-	3.89 ± 0.25
50 mM αMO for 24 hr then 50 mg/l pyridoxal added to flask	-	3.90 ± 0.48

9L rat brain tumor cells seeded at 1 x 10^6/flask were incubated in the absence or presence of 50 mM αMO, 50 mg/l pyridoxal, or both. At 24 hr after treatment 3 flasks from each condition were harvested and counted. Additional flasks treated with αMO alone had either fresh medium with 50 mg/l pyridoxal or pyridoxal with no change of medium. At 48 hr 3 flasks from each condition were again harvested and counted. Results are expressed as mean ± SD.

TABLE III - 3

Effect of Incubation of 9L Cells with 50 mM DL- or L-ornithine on Cell Proliferation

TREATMENT	10^{-6} x CELLS/FLASK 24 HOURS POST TREATMENT	10^{-6} x CELLS/FLASK 48 HOURS POST TREATMENT
none (control)	4.42 ± 0.06	8.62 ± 0.52
50 mM DL-ornithine	3.74 ± 0.16	4.90 ± 0.42
50 mM L-ornithine	3.78 ± 0.50	5.15 ± 0.34

9L rat brain tumor cells seeded at 1 x 10^6/flask were incubated in the absence or presence of 50 mM DL- or L-ornithine. Three flasks at each condition were harvested and counted at both 24 and 48 hr post treatment. Results are expressed as mean ± SD.

treated with 50 mM DL-ornithine had only 57% of the number of cells in control flasks. In comparison with these data, flasks treated with 50 mM αMO in the experiment shown in Figure III-1 had only 61% of the number of cells in control flasks 48 hours after treatment. In addition, neither 50 mM L-ornithine nor 50 mM DL-ornithine produced any effect on 9L intracellular polyamine content.

Since 10 mM αMO did inhibit 9L cell proliferation when the initial cell density was $5x10^5$ cells/flask (Fig. III-2), the ability of exogenous Pu to reverse this inhibition was also investigated. In this study 1 mM Pu was added 24 hours after treatment with 10 mM αMO. The ODC inhibitor was added 24 hours after seeding $5x10^5$ cells/flask. As shown in Figure III-5A, 1 mM Pu was able to reverse the effects of 10 mM αMO in this study. This concentration of exogenous Pu reversed the depletion of intracellular Pu (Fig. III-5B) and Sd (Fig. III-5C) produced by 10 mM αMO. The increased intracellular Sp content caused by 10 mM αMO was also reversed by 1 mM exogenous Pu (Fig. III-5D).

III-D: Effect of αMO on Colony Forming Efficiency of 9L Cells

The viability of 9L cells after treatment with αMO was studied using the CFE assay as described in Section II-F. Cultures of 9L cells were seeded at an initial density of $1x10^6$ cells/flask and after 24 hours were treated with αMO at concentrations ranging from 5 to 100 mM. After a further 24 hour incubation in the presence of αMO, the clonogenicity of these cells was investigated. As shown by the data in Table III-4, the CFE of treated cells did not decline appreciably, relative to that of untreated controls, until concentrations of αMO as high as 100 mM were used.

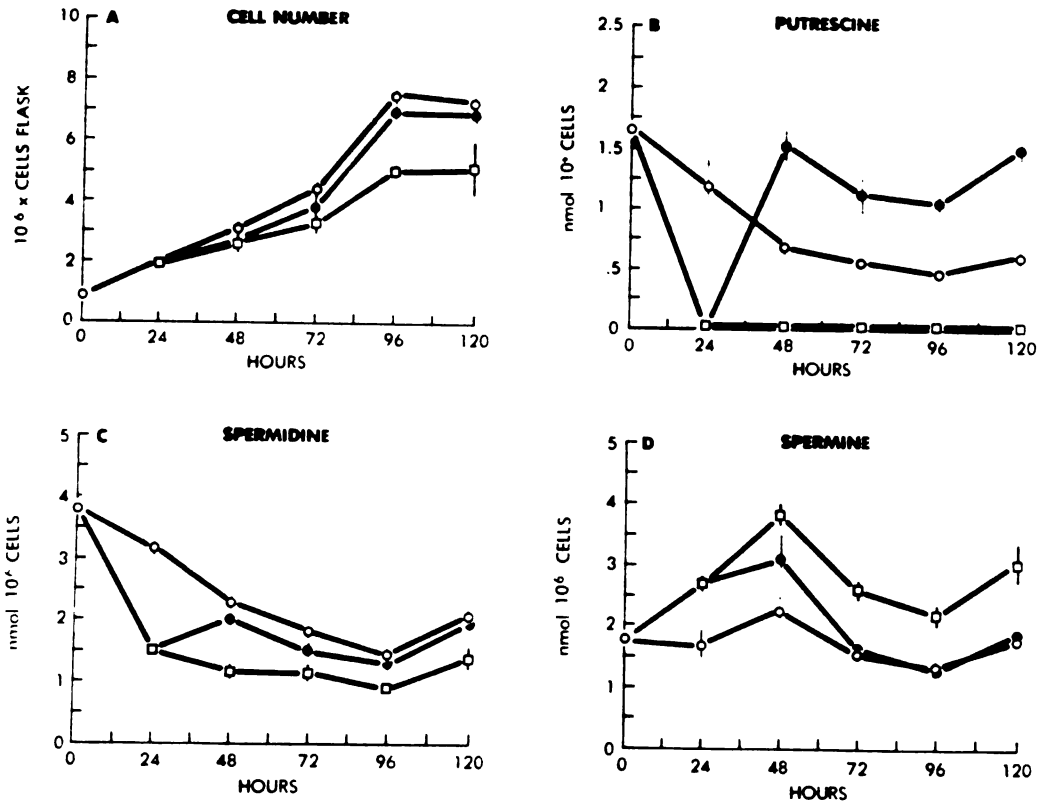


FIGURE III - 5

Reversal of Cytostatic Effect of 10 mM αMO on 9L Cells by Exogenous Pu. Cells were incubated for 120 hours following treatment (at time 0) 24 hours after seeding 5 x 10^5 cells/flask with HBSS (○), or 10 mM αMO (□). Half of the treated cultures had 1 mM Pu added 24 hours after treatment (●). Effects on cell proliferation (A), Pu content (B), Sd content (C), and Sp content (D). Points represent the mean (±SD) of three determinations.

TABLE III - 4

Effect of αMO on CFE of 9L Cells

CONCENTRATION OF αMO	PERCENT SURVIVAL	SEM
0	61.4	3.0
5 mM	55.1	2.0
10 mM	58.6	3.2
25 mM	56.4	4.5
50 mM	58.5	3.9
75 mM	56.5	2.9
100 mM	38.5	2.0

9L rat brain tumor cells were incubated with various concentrations of αMO for 24 hr. The effect of this treatment on the clonogenicity of the cells was assayed using an *in vitro* colony forming efficiency assay. Results are expressed as mean of five replicate determinations ± SEM.

III-E: Flow Cytometric Studies with αMO Treated Cells

The cell cycle distribution of 9L cells seeded at an initial cell density of $1x10^6$ cells/flask and then treated 24 hours later with 50 mM αMO was studied by flow cytometry for 44.5 hours after treatment. Figure III-6 shows representative examples of the DNA histograms obtained for the treated cells and for untreated controls harvested at the same time points. The results obtained show that from about 6 to 18 hours following treatment with 50 mM αMO there is marked decrease in the fraction of cells in the S phase of the cell cycle when compared to untreated controls. This is accompanied by an increase in the number of cells in the G_2 and M phases of the cell cycle in the treated samples. From about 24 hours, these differences between treated and control cell populations begin to disappear. By 44 hours, there are no significant differences between the cell cycle distributions of treated 9L cells and controls. Lower concentrations of αMO, which did not cause inhibition of 9L cell proliferation when initial cell density was $1x10^6$ cells/flask, did not alter the cell cycle distribution of treated cells from that of untreated controls.

Since 10 mM αMO did inhibit 9L cell proliferation when the initial cell density was $5x10^5$ cells/flask, the effect of this treatment on 9L cell cycle distribution was also investigated. Representative examples of the DNA histograms are shown in Figure III-7. There do not appear to be any differences in the cell cycle distributions of controls and of treated 9L cells. This observation is in spite of the fact that a significant inhibition of 9L cell proliferation did occur in this experiment.

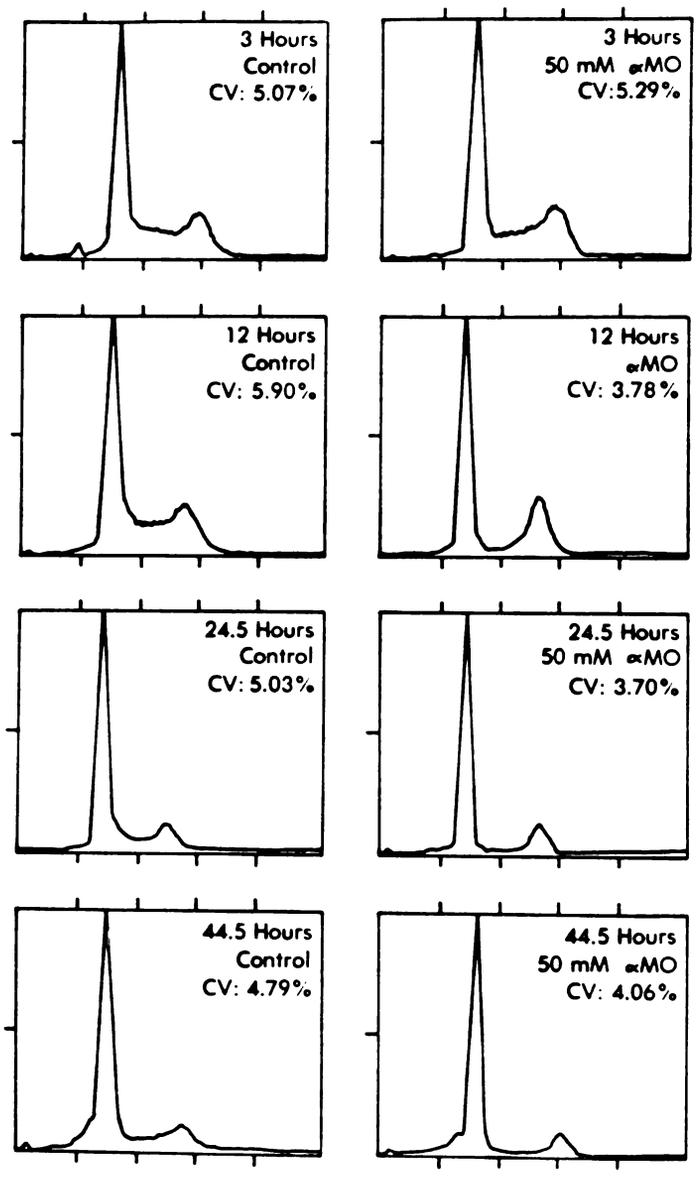


FIGURE III - 6

Flow Cytometric DNA Histograms of Untreated 9L Cells and of Cells Treated at time 0 with 50 mM αMO 24 hours after Seeding 1 x 10^6 cells/flask. Cells were harvested, washed with PBS, fixed in 70% aqueous ethanol, and stained with chromomycin A_3.

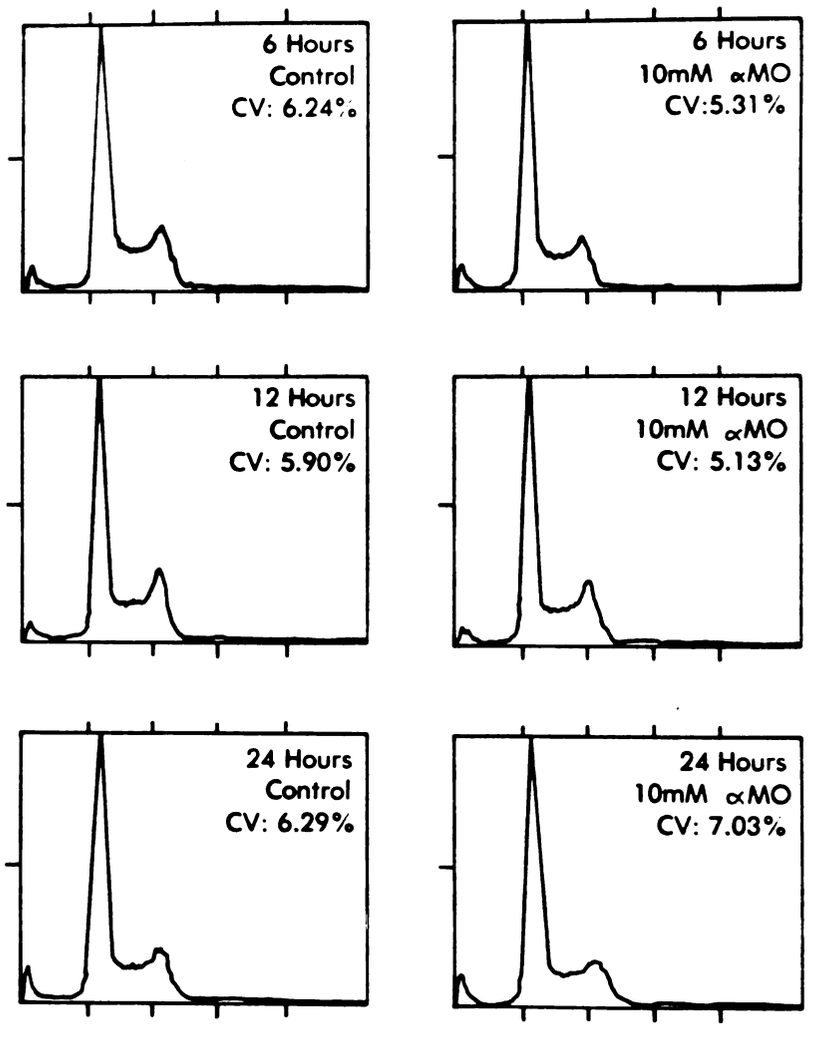


FIGURE III - 7

Flow Cytometric DNA Histograms of Untreated 9L Cells and of Cells Treated (at time 0) with 10 mM αMO 24 hours after Seeding 5 x 10^5 cells/flask. Cells were harvested, washed with PBS, fixed in 70% aqueous ethanol, and stained with Chromomycin A_3.

III-F: Autoradiographic Study with 50 mM αMO Treated 9L Cells

The effect of 50 mM αMO treatment 24 hours after seeding $5x10^5$ cells/flask on the cell cycle progression of 9L cells was also studied by ^{3}H-thymidine autoradiography, as described in Section II-E. The cells used in this study were from the same pool and were harvested at the same (plus some additional) time points as those in the FCM study shown in Fig. III-6. Figure III-8 shows the PLM (A) and MI (B) curves obtained for treated cells and, for comparison, shows the PLM curve of untreated 9L cells published previously (186) by other workers in this laboratory. From the PLM curve of untreated 9L cells, one can calculate a total cell cycle time of 19.5-20 hours with G_1 = 8.5 hours, S = 8.2 hours, G_2 = 3.2 hours, and mitosis = 0.5 hours. The PLM curve of 50 mM αMO treated cells shows a prolongation of G_2 to about 9 hours, the point at which labelled mitosis first appear. In addition, instead of the normally observed second wave of labelled mitoses a gently descending limb of the first wave was observed, implying a block in the cell cycle between G_1 and S.

The mitotic index of 9L cells treated with 50 mM αMO decreased from about 2% to 1% over the first six hours of treatment. This was followed by an increase to nearly 3% at about 18 hours. From this time on, the fraction of treated cells in mitosis gradually declined, eventually reaching 0.5% of the total population.

Part Two: Discussion

An important consideration in evaluating an ODC inhibitor is the question of complete vs. partial cessation of new Pu synthesis in its presence. This question can not be answered by examination of the total intracellular polyamine content on a per cell basis since despite the

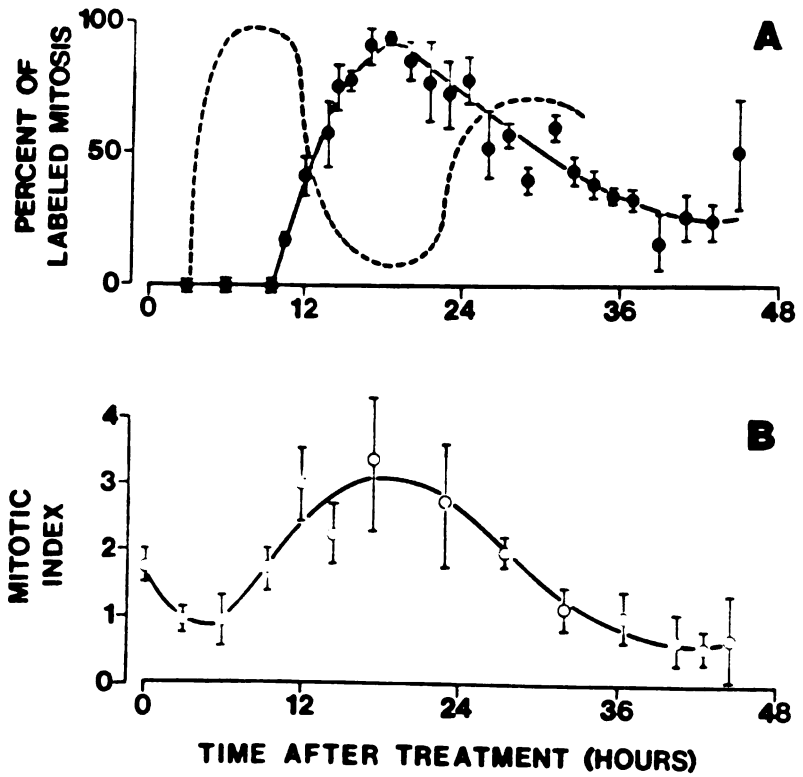


FIGURE III - 8

Percent Labelled Mitoses (A) and Mitotic Index (B) Curves Obtained by ^{3}H-thymidine Autoradiography of 9L Cells Seeded at 5 x 10^{5} cells/flask 24 hours prior to treatment with 50 mM αMO. (The dashed curve in the upper pannel is the PLM curve of untreated 9L cells.)

fact that some synthesis occurs, if the rate of cell proliferation is faster than the rate of synthesis, a decline in total intracellular polyamine content is observed.

To determine if Pu synthesis is completely shut off one must calculate the total amount of polyamine content present in each culture flask as the product of mean intracellular polyamine content multiplied by the total number of cells and plot this as a function of time after treatment. The results obtained from such computations for cells seeded at $1x10^6$/flask, using the data of Figure III-1 are shown in Figure III-9A, while similar calculations for cells seeded at $5x10^5$/flask, using the data of Figure III-2, are shown in Figure III-9B.

Control cultures appear to slightly more than double their total polyamine content every 24 hours during exponential growth and then reach a plateau or slight decline as the cells' growth rate decreases. Cultures treated with 50 mM αMO have the major portion of this increase supressed. However, even with this high concentration of αMO some continued Pu synthesis must occur since the total polyamine content of these cultures more than doubles over the course of both experiments. This is not too surprising since αMO is a competitive inhibitor of ODC. This allows some binding of ornithine to the enzyme even in its presence and thus some continued Pu synthesis, albeit at a drastically reduced rate. It is somewhat surprising, however, that 10 mM αMO is more effective in inhibiting Pu synthesis, and in inhibiting 9L cell proliferation, when initial cell density is $5x10^5$ cells/flask than when initial density is $1x10^6$ (Figs. III-1,2 and 9). Presumably, the amount of αMO entering into the cells should only depend upon concentration in the medium. One rationale for this observation could be a result of a lengthened lag

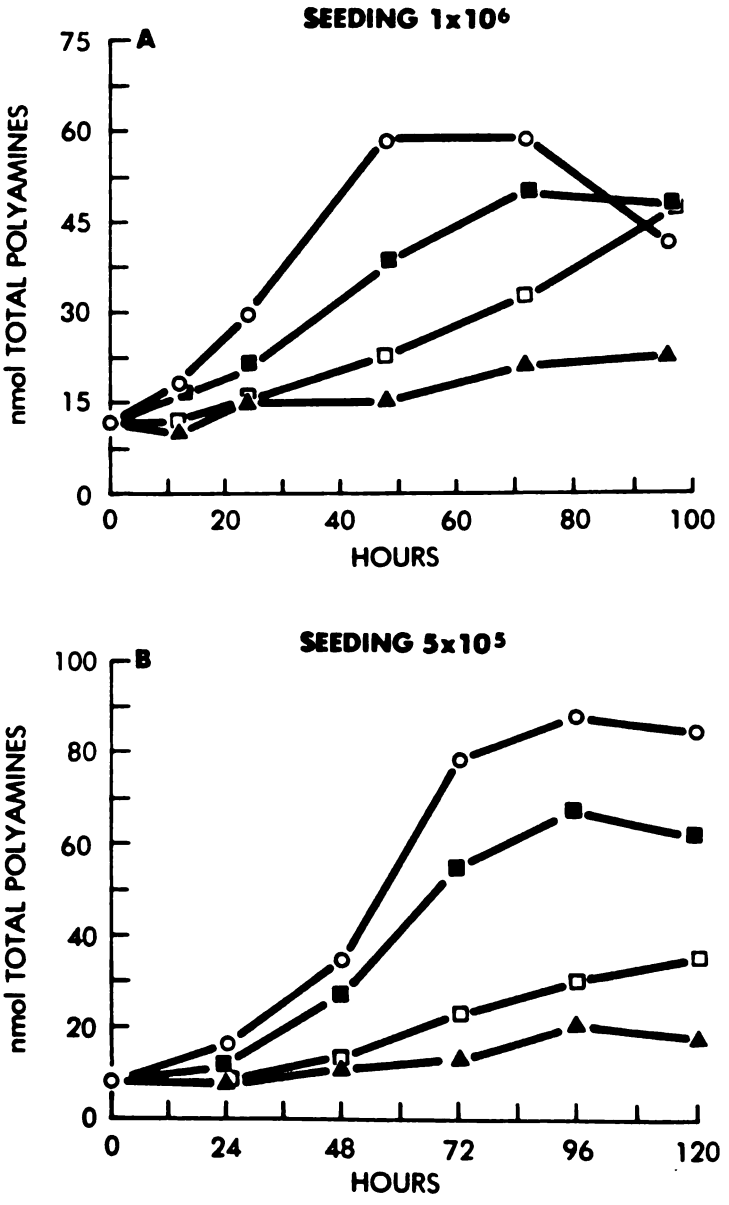


FIGURE III - 9

Total Polyamine Content of 9L Cell Cultures Seeded at 1 x 10^6 (A) or 5 x 10^5 (B) cells/flask and Treated 24 hours later (at time 0) with 0 (○), 1 (■), 10 (□), or 50 mM (▲) αMO.

phase of growth when cells are seeded at the lower density. If polyamines are needed to achieve a normal exponential rate of proliferation, lengthening the initial lag phase could result in fewer cells, relative to controls. This does not, however, satisfactorily explain the apparent greater effectiveness in inhibition of new Pu synthesis at the lower initial cell density.

The data shown in Figure III-3 support the conclusion that some Pu synthesis does take place even in the presence of 50 mM αMO. If Pu synthesis were completely blocked increasing the αMO concentration to 100 mM should not result in more rapid Pu depletion. The data from such short incubation experiments can also be used to gain information regarding the fate of intracellular Pu after inhibiting its synthesis. It has been assumed that all Pu present at the time of treatment with ODC inhibitors is converted to Sd and then to Sp. One can calculate the total intracellular Pu content per culture, as discussed above, and then compute the total amount lost at each time point from the start of the experiment (i.e.: $\{(\text{nmol}/10^6\text{cells})(\#\text{cells}/10^6)\}_{\text{time=o}} - \{(\text{nmol}/10^6\text{cells})(\#\text{cells}/10^6)\}_{\text{time=t}}$). A similar computation can be done for the increase in total Sd + Sp. If all intracellular Pu lost after treatment with αMO is converted to Sd and Sp, then at each time point the amount of Pu lost should equal the amount of Sd + Sp gained. Figure III-10 shows the results obtained from such computations using the data for the first 6 hours of treatment with 50 mM αMO from the experiment shown in Figure III-3. These calculations demonstrate that at each time point more nanomoles of Pu are lost than the total increase in Sd + Sp. This implies that some Pu loss occurs by other means than conversion to Sd + Sp. One possible route of loss is

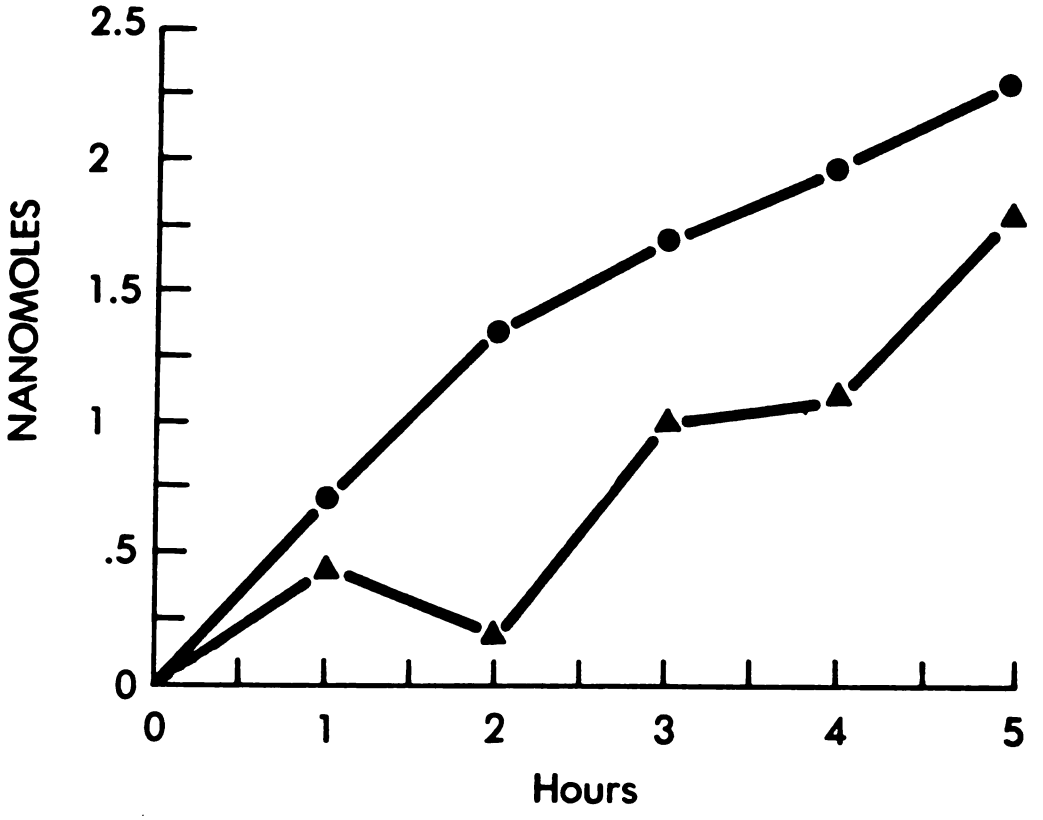


FIGURE III - 10

Loss of Intracellular Pu (●) and Gain of Intracellular Sd + Sp (▲) from 9L Cell Cultures as a Function of Time After Treatment with 50 mM αMO.

leakage from the treated cell. It hardly makes sense, though, for inhibition of Pu synthesis to result in increasing the ability of Pu to leak out of the cell. Since normal untreated 9L cells can be washed a number of times with no loss of intracellular polyamine content, leakage is probably not responsible for the unaccounted portion of Pu depletion. This leaves biotransformation via oxidation and/or acetylation of all three polyamines as the probably cause of Pu loss. It would thus be of interest to study the levels of these enzyme activities in 9L cells in culture.

The results presented in Figures III-1 and 2 show that while αMO does cause almost complete depletion of intracellular Pu and a significant reduction in Sd levels, it results in an increase in Sp levels. In the unperturbed cell, there is a competition between Pu and Sd for the aminopropyl moeity of decarboxylated S-adenosyl-L-methionine. When cells are treated with αMO, leading to a drastic decline in intracellular Pu, this competition is removed, with a resultant increase in the rate of Sp production and intracellular Sp increases. Support for this explanation comes from the observation that when 9L cells are treated with 1 mM αMO, resulting in depletion of intracellular Pu to ca. 20% of that in controls, slight but significant elevation of Sp is observed even though no real depletion of Sd occurs (Figs. III-1 and 2). Moreover, there is no significant increase in intracellular Sp when cells are simultaneously incubated with αMO and Pu (Figs. III-4D and 5D).

A number of observations lead to the conclusion that the cytostatic action of 50 mM αMO on 9L cells is not the result of depletion of intracellular Pu and Sd. First, 10 mM and 50 mM αMO produce the same degree of depletion of Pu and Sd but only 50 mM αMO causes inhibition of 9L

cell proliferation when cells are seeded at $1x10^6$/flask (Fig. III-1). Second, the cytostatic effect of 50 mM αMO could neither be reversed (Table III-1) nor blocked (Fig. III-4) by addition of 3 mM Pu to the culture medium. Since this extracellular concentration of Pu prevented the depletion of intracellular Pu (Fig. III-4B) and Sd (Fig. III-4C) by 50 mM αMO, but did not either block or reverse the inhibition of 9L cell proliferation, some mechanism other than depletion of intracellular Pu and Sd must be responsible for the cytostatic action of 50 mM αMO.

The first possibility considered was that concentrations of 50 mM αMO or greater were cytocidal to 9L cells. However, the results of the CFE study (Table III-4) showed that both 50 mM and 75 mM αMO did not decrease the viability of 9L cells relative to that of untreated controls. Second, O'Leary and Herreid (121) showed that bacterial ODC accepts αMO as a substrate and that in some fraction of the enzyme-αMO interactions, αMO undergoes a decarboxylation-dependent transamination, resulting in the production of 5-amino-2-pentanone and pyridoxamine phosphate. If the observations of O'Leary and Herreid are true for both mammalian and bacterial ODC, 50 mM αMO could possibly deplete intracellular levels of pyridoxal and thus inhibit 9L cell replication. However, a 50-fold increase in pyridoxal concentration in the culture medium did not reverse or block the cytostatic effect of 50 mM αMO (Table III-2). (Pyridoxal was used rather than pyridoxal phosphate in this experiment because the ionized cofactor does not cross cell membranes.)

A third hypothesis is that 50 mM αMO is cytostatic as a result of the increased osmolality or ionic strength of the medium. While this possibility is not proven by the data of Table III-3, the observation that both 50 mM L-ornithine and 50 mM DL-ornithine produce about the

same degree of inhibition of 9L cell proliferation as does 50 mM αMO suggests that this hypothesis is worthy of further exploration. In addition, this observation provides further support for the conclusion that the cytostatic effect of 50 mM αMO on 9L cells is not the result of depletion of intracellular Pu and Sd. These data also point to the importance of such controls when effects of ornithine analogs are observed that are not reversible by exogenous polyamines.

The inhibition of 9L cell proliferation produced by treatment of cultures seeded at $5x10^5$ cells/flask with 10 mM αMO (Fig. III-2) does appear to be a result of polyamine depletion. Addition of 1 mM exogenous Pu to the culture medium 24 hours after treatment with 10 mM αMO did reverse the growth arrest (Fig. III-5A). Furthermore, this concentration of exogenous Pu prevented the depletion of intracellular polyamines produced by the αMO (Fig. III-5B-D). The specificity of this growth inhibition is, in turn, support for the hypothesis that polyamines do play a crucial role in 9L cell proliferation.

There does remain, however, an important question regarding the above hypothesis. If it is correct, why can cells seeded at $1x10^6$ per flask and then treated after 24 hours with 10 mM αMO continue to proliferate at the same rate as untreated controls for 96 hours in spite of significant depletion of intracellular Pu and Sd by 12 hours post-treatment (Fig. III-1B & C)? One possible answer may be the observation that while the Pu and Sd content of these cells did decline, the Sp content increased (Fig. III-1). This increase could compensate for the loss of Pu and Sd in one of two ways. It is possible that Sp may have a higher binding affinity than Pu or Sd for some polyamine-requiring sites that are involved in control of cell replication. A

small increase in concentration of the higher affinity ligand could thus compensate for a larger decrease in concentration of the weaker ligand. A second compensatory mechanism could be a result of the polycationic nature of the polyamines. Any possible intracellular role for these biomolecules may be a result of two or more centers of positive charge separated by a neutral carbon chain. If this is the case it is possible that the cells' requirement for polyamines is a need for a constant level of positive charge in the form of organic polycations, rather than a specific amount of each polyamine. The increase in intracellular Sp (Fig. III-1D), which has four equivalents of positive charge per molecule, could possibly compensate for the depletion of Pu and Sd, which only have two and three equivalents, respectively.

To test this possibility, the data shown in Figure III-1B-D were used to calculate the total number of neq/10^6 cells of polyamines at each concentration of αMO in this study. As shown in Figure III-11A, both 50 mM and 10 mM αMO resulted in significant decreases in total neq/10^6 cells of polyamines, while only the cells treated with 10 mM αMO continued to proliferate at the same rate as untreated controls. Under normal conditions, however, Pu may serve only as a precursor for Sd and Sp. It could then be the total neq/10^6 cells of Sd plus Sp that would determine the cells' ability to proliferate. As shown in Figure III-11B,there were no statistically significant differences between cells seeded at 1x10^6/flask and treated with αMO and controls in neq/10^6 cells of Sd plus Sp. There was also no difference between controls and cells treated with 50 mM αMO, but as already discussed, the growth arrest caused by 50 mM αMO is most likely due to factors

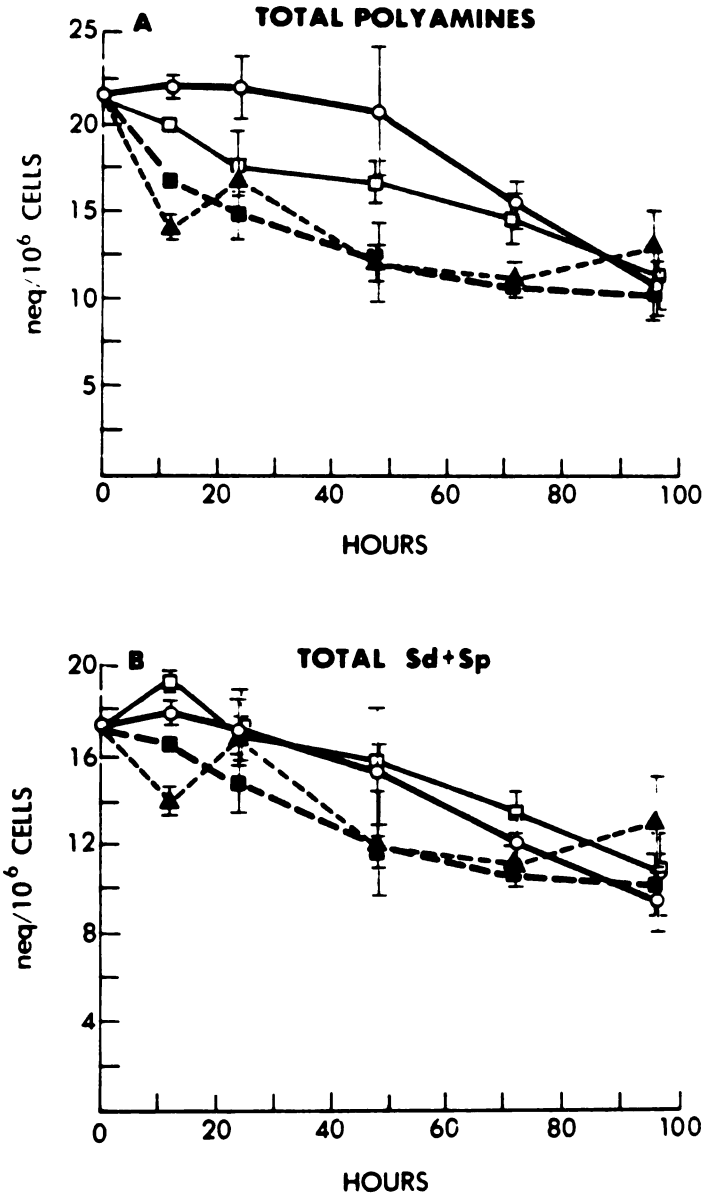


FIGURE III - 11

Total Intracellular Polyamine Content (A) and Intracellular Sd + Sp Content (B) of 9L Cells Seeded at 1×10^6/flask and treated 24 hours Later (at time 0) with 0 (○), 1 (■), 10 (□), or 50 mM (▲) αMO. Points represent the mean (±SD) of three determinations.

other than polyamine depletion. When cells were seeded at $5x10^5$/ flask (Figure III-2) and 10 mM αMO did produce a small degree of cytostasis, there was a slight decline in neq/10^6 cells of Sd + Sp.

When 9L cells seeded at $1x10^6$/flask were treated with 50 mM αMO flow cytometry revealed (Fig. III-6) a decreased rate of entry of treated cells into S phase, implying a decrease in the cells' ability to initiate DNA synthesis. Since exogenous Pu was unable to reverse (Table III-1) or block (Fig. III-4) the cytostatic effect of 50 mM αMO, one cannot attribute this inhibition of DNA replication to polyamine depletion. This applies equally well to the accumulation of cells under the G_2M peak of the DNA histograms (Fig. III-6) and to the prolongation of G_2 and the block between G_1 and S implied by the PLM curve of Fig. III-8. Both of these studies do agree, however, in the effects they note of 50 mM αMO on 9L cell cycle progression. The G_1-S block implied by the PLM curve can account for the decreased fraction of cells in early S observed in the FCM study. Similarly, the lengthening of G_2 seen in the PLM experiment could be responsible for the increased fraction of cells in the G_2M peak of the DNA histograms.

Treatment of 9L cells seeded at $5x10^5$/flask with 10 mM αMO, which does inhibit proliferation (Fig. III-2) by a Pu-reversible mechanism (Fig. III-5), does not alter the cell cycle distribution (Fig. III-7) of treated cells. Such observations could result from a decreased growth fraction, but the CFE study (Table III-4) showed no effect of 10 mM αMO on the growth fraction of 9L. These observations thus imply a generalized slowing of the entire cell cycle. These results are in agreement with the reports of Mamont, et. al. (163) and Morris, et. al. (168) who observed decreased rates of DNA synthesis

in cells treated with αMO. In addition, neither of these previous studies reported a complete cessation of DNA synthesis in the presence of αMO. The flow cytometric results showing the absence of a G_1-S block with 10 mM αMO (Fig. III-7) are in agreement with such observations.

The implications of all these results is that treatment of cells with an ODC inhibitor does not prevent the cells from initiating DNA synthesis but does prevent them from maintaining optimal rates of DNA replication. The increases in ODC activity and in intracellular polyamine content observed in stimulated cell growth thus do not appear to be the signal for initiating DNA synthesis. This does not mean, however, that the polyamines do not play an important role in cell replication. The fact that polyamine depletion decreases the rate of DNA synthesis (163,168) and of cellular proliferation argues against such a conclusion.

A question which none of the previously reported studies has addressed is: does the basal polyamine content present at the start of an experiment with an antimetabolite play a permissive role in allowing the cell to respond to whatever is the signal for initiating DNA synthesis? The approached used in all of the experiments described in this chapter and in the studies reviewed in Chapter I are unsatisfactory for answering this question. This is a result of all such experiments beginning with cells containing a normal basal complement of polyamines and then attempting to deplete them of polyamines. At the time of application of the initial growth stimulus, the normal basal content of polyamines is present in the cell. The answer to this question can only be found by beginning an experiment with cells that are

already depleted of polyamines and testing to see if they can initiate DNA synthesis. In addition, the data reported here and by others (163,164,169) show that substantial depletion of Sd must occur before DNA synthesis and cellular proliferation are inhibited. During the first 24 hours of all the experiments reported here, control and αMO treated cells grew at the same rate. For this reason, αMO which does not deplete Sd below 20% of control levels, is not satisfactory for obtaining an answer to the question at hand. The next chapter will explore the effects of DFMO, an irreversible inactivator of ODC, on 9L proliferation and intracellular polyamine content and will introduce a new approach to experiments with polyamine antimetabolites that will shed some light on the postulated requirement for intracellular polyamines in initiation of DNA synthesis by mammalian cells.

CHAPTER IV: STUDIES WITH DIFLUOROMETHYLORNITHINE

Part One: Results

IV-A: Effects of DFMO on 9L Cell Proliferation and Intracellular Polyamine Content

The results obtained when 9L cells were treated 24 hours after seeding $1x10^6$ cells/flask with 1, 10, or 25 mM DFMO are shown in Figure IV-1. After the initial 24 hours of treatment, during which treated cells grew at the same rate as untreated controls, 25 mM DFMO caused a marked inhibition of 9L cell proliferation (Fig. IV-1A). Cells treated with 10 or 1 mM DFMO replicated at a slower rate than controls from 24 to 48 hours, but at about the same rate as controls from 48 to 72 hours. Cells treated with the two lower concentrations also reached the same end point as controls after 96 hours of treatment. All three concentrations depleted intracellular Pu to <0.1 nmol/10^6 cells by 6 hours after treatment (Fig. IV-1B) and decreased Sd content to <0.1 nmol/10^6 cells by 48 hours after treatment (Fig. IV-1C). No effect on Sp content was observed by any of the three concentrations used (Fig. IV-1D).

A similar experiment was performed in which the drug was added at the same time as seeding $1x10^6$ cells/flask (Fig. IV-2). A much more dramatic effect of 25 mM DFMO on cell proliferation was observed in this study (Fig. IV-2A). In addition, both 1 and 10 mM DFMO were markedly cytostatic. The effects of all three concentrations on Pu, Sd, and Sp contents were similar to those observed in the initial study (Fig. IV-2B-D), except that Sd depletion by 10 and 1 mM DFMO occurred earlier than when treatment took place 24 hours after seeding.

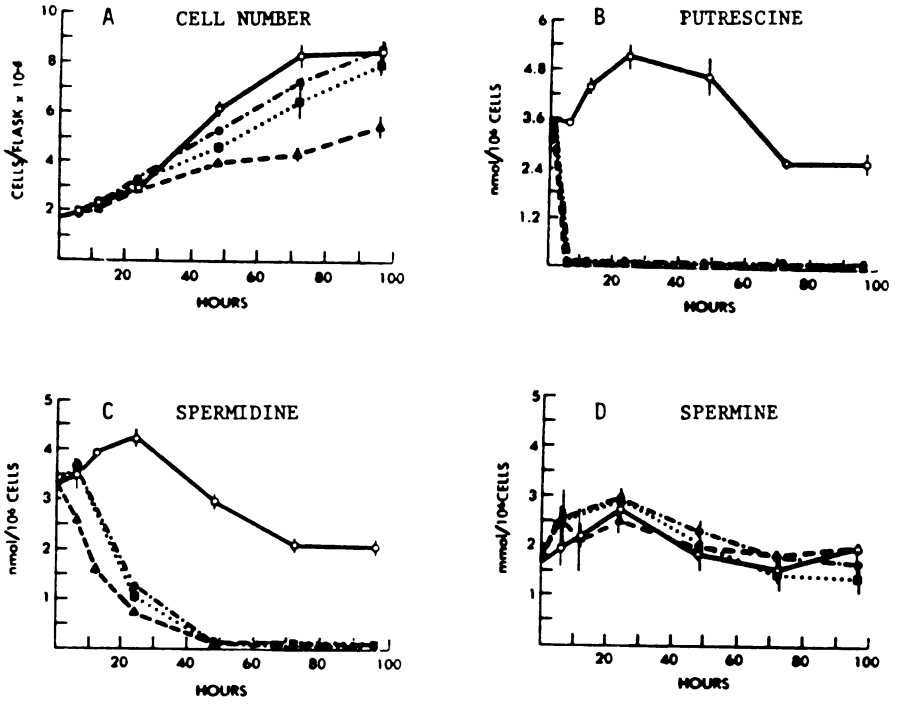


FIGURE IV - 1

Effect of DFMO on 9L Cell Proliferation (A), Pu Content (B), Sd Content (C), and Sp Content (D). Cells were incubated for 96 hours following treatment (at time 0) 24 hours after seeding 1 x 10^6 cells/flask with 0 (○), 1 (●), 10 (■), or 25 mM (▲) DFMO. Points represent the mean (±SD) of three determinations.

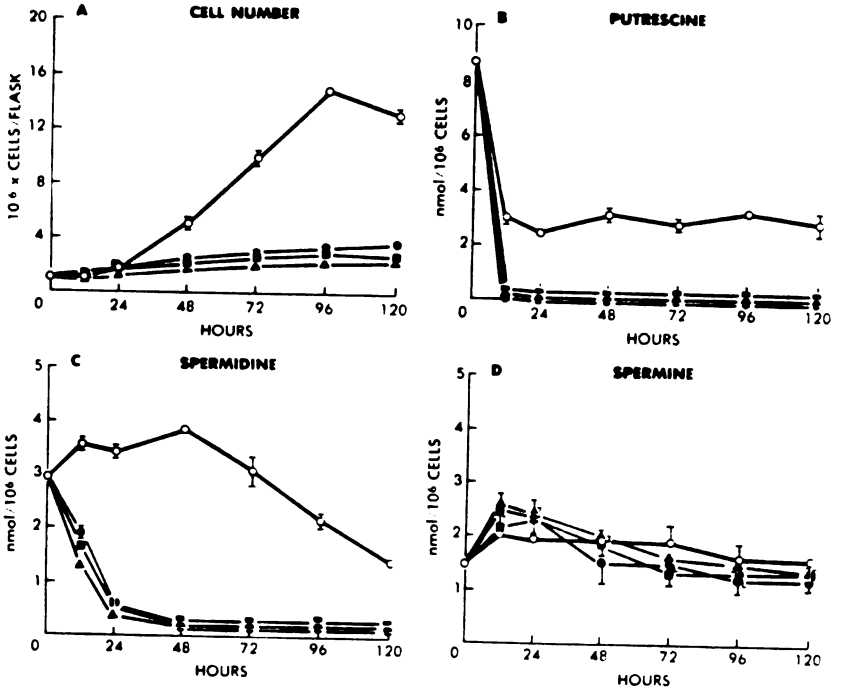


FIGURE IV - 2

Effect of DFMO on 9L Cell Proliferation (A), Pu Content (B), Sd Content (C), and Sp Content (D). Cells were incubated for 120 hours following treatment (at time 0) at the same time as seeding 1 x 10^6 cells/flask with 0 (○), 1 (●), 10 (■), or 25 mM (▲) DFMO. Points represent the mean (±SD) of three determinations.

A third study investigated the effects of the same three concentrations of DFMO on 9L cells seeded at $5x10^5$/flask. As in the first experiment, treatment took place 24 hours after seeding. Once again a more pronounced cytostatic effect was observed subsequent to the initial 24 hours of treatment (Fig. IV-3A), although not as dramatic as when treatment was simultaneous with seeding (Fig. IV-2A). Once again Pu and Sd were depleted to <0.1 nmol/10^6 cells by 12 and 48 hours (Fig. IV-3B&C) while Sd was slightly elevated during the first 48 hours of treatment (Fig. IV-3D).

IV-B: Kinetics of Pu Depletion as a Function of DFMO Concentration

In two separate experiments the decrease in intracellular Pu content following treatment with 1 or 25 mM DFMO was investigated. As with similar experiments using αMO, and for the same reasons (see Section III-B), the results shown in Figure IV-4 are expressed as fraction of Pu content of untreated controls measured at the same time points. Except for the first hour of treatment, no difference was observed in the rate of Pu loss between cells treated with 1 or 25 mM DFMO (Fig. IV-4).

IV-C: Recovery and Blocking Study

Exogenous Pu added to the culture medium either 24 hours after or simultaneous with DFMO treatment was able to both reverse and block the inhibition of cell proliferation (Fig. IV-5A). In this experiment 9L cells were seeded at an initial density of $5x10^5$/flask in medium containing 10% H.S. and treated with 10 mM DFMO, 3 mM Pu, or 10 mM DFMO + 3 mM Pu 24 hours after seeding. Half of the flasks initially treated with DFMO alone had 3 mM Pu added to the culture medium 24 hours after treatment. This concentration of exogenous Pu reversed or prevented

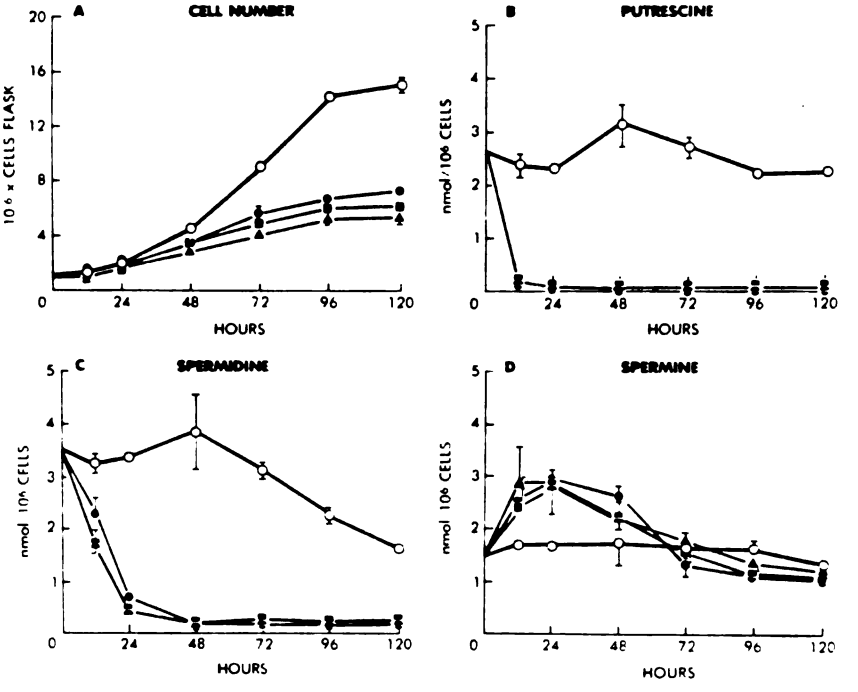


FIGURE IV - 3

Effect of DFMO on 9L Cell Proliferation (A), Pu Content (B), Sd Content (C), and Sp Content (D). Cells were incubated for 120 hours following treatment (at time 0) 24 hours after seeding 5 x 10^5 cells/flask with 0 (○), 1 (●), 10 (■), or 25 mM (▲) DFMO. Points represent the mean (±SD) of three determinations.

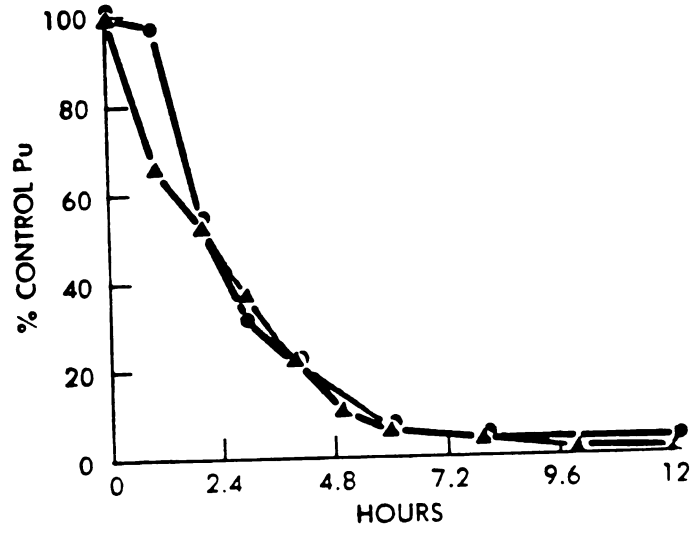


FIGURE IV - 4

Kinetics of Pu Depletion by 1.0 (●) and 25 mM (▲) DFMO. Cultures of 9L cells seeded at 1 x 10^6 cells/flask were treated 24 hours after seeding and intracellular Pu content was determined. Points represent the ratio of the mean of three determinations for treated cells to the mean of three determinations for control cells harvested at the same time.

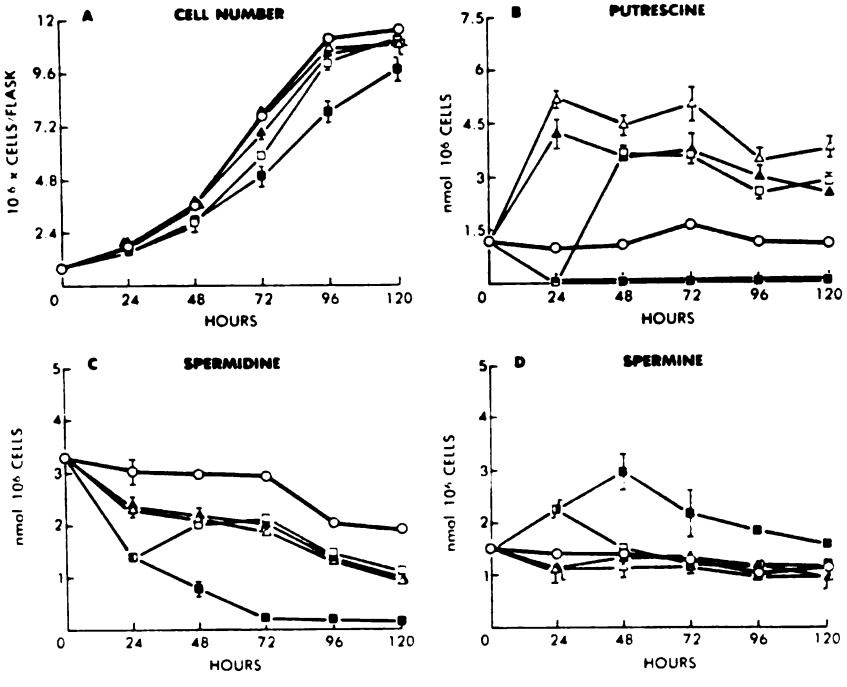


FIGURE IV - 5

Reversal of Effects of 10 mM DFMO on 9L Cell Proliferation (A), Pu Content (B), Sd Content (C), and Sp Content (D) by 3 mM Exogenous Pu. Cells were incubated for 120 hours following treatment (at time 0) 24 hours after seeding 5 x 10^5 cells/flask with HBSS (○), 10 mM DFMO (■), 3 mM Pu (△), or 10 mM DFMO + 3 mM Pu (▲). Half of the flasks initially treated with DFMO alone had 3 mM Pu added 24 hours after treatment (□). Points represent the mean (±SD) of three determinations.

the depletion of intracellular Pu content of treated cells and produced a three-fold elevation of Pu content of cells not treated with DFMO (Fig. IV-5B). Exogenous Pu also reversed and prevented the depletion of intracellular Sd produced by 10 mM DFMO (Fig. IV-5C), although the Sd content of cells incubated with 3 mM Pu was somewhat less than that of untreated controls. The elevation of intracellular Sp content resulting from treatment with 10 mM DFMO also was reversed and blocked by exogenous Pu (Fig. IV-5D).

IV-D: Replating Studies Using 9L Cells Pre-treated with DFMO

The ability of polyamine-depleted cells to resume replicating and to replenish their intracellular pools of Pu and Sd was investigated by replating cells treated with DFMO in the absence of the inhibitor. Cultures of 9L cells seeded at $1x10^6$ cells/flask were treated 24 hours after seeding with 0, 1, 10, or 25 mM DFMO and incubated for a further 48 hours. These cultures were then harvested, counted, and $1x10^6$ cells/flask were replated in the absence of DFMO. Control cultures went through the usual lag of 12-24 hours before entering exponential growth (Fig. IV-6A). Cells pretreated with 1 mM DFMO had this lag period extended to between 24 and 48 hours. Cells pretreated with 10 or 25 mM DFMO were still in the lag phase of tissue culture growth at the end of the 120 hour experiment. The intracellular content of Pu (Fig. IV-6B) and Sd (Fig. IV-6C) of cells pretreated with 1 mM DFMO began to increase 48 hours after replating, the same time point at which these cells entered exponential growth. Cells pretreated with 10 or 25 mM DFMO never replenished their Pu and Sd pools. Except for an increase in Sp content of cells pretreated with 10 mM DFMO during the final 24 hours of this study, no significant differences in Sp content were observed

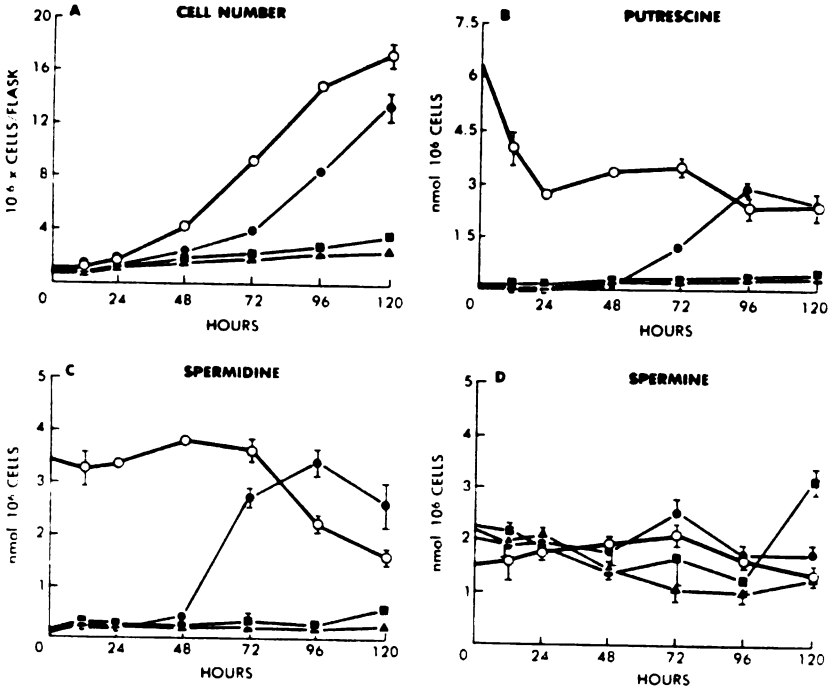


FIGURE IV - 6

9L Cell Proliferation (A), Pu Content (B), Sd Content (D), and Sp Content (D) of Cultures Seeded at 1 x 10^6 cells/flask Following a 48 hour Pretreatment with 0 (○), 1 (●), 10 (■) or 25 mM (▲) DFMO. Points represent the mean (±SD) of three determinations.

between control and pretreated cells (Fig. IV-6D).

A similar but more extended study was performed with 9L cells pretreated 24 hours after seeding $1x10^6$ cells/flask with 0, 1, or 10 mM DFMO for 48 hours and then replated at $5x10^5$ cells/flask in the absence of the inhibitor (Fig. IV-7). Control cells required slightly more than 24 hours before entering exponential growth (Fig. IV-7A). Cells pretreated with 1 mM DFMO did not enter exponential growth until 72 hours after seeding, and cells pretreated with 10 mM DFMO remained in the lag phase for approximately 144 hours. The intracellular Pu and Sd content of cells pretreated with 1 and 10 mM DFMO began to increase at 48 and 120 hours respectively (Fig. IV-7B&C). Pretreated cells also showed increased Sp content at about the same time as replenishment of their Pu and Sd pools (Fig. IV-7D).

A third experiment tested the ability of 3 mM exogenous Pu to reverse the delayed entry of cells pretreated for 48 hours with 1 or 10 mM DFMO into exponential growth. Cells pretreated with 1 or 10 mM DFMO and replated in the absence of exogenous Pu showed a delayed entry into exponential growth (Fig. IV-8A) which was reversible by 3 mM exogenous Pu (Fig. IV-8B) as shown by the absence of any differences between the growth curves for control cells replated in the absence of exogenous Pu and those for pretreated cells replated in the presence of Pu. Figure IV-8C shows that 3 mM Pu added to control cells caused a slight inhibition of growth. As in previous experiments involving addition of Pu to the culture medium, 10% H.S. replaced the 10% FCS normally used. This resulted in an altered intracellular polyamine content for pretreated cells replated in the absence of added Pu (Fig. IV-9). Exogenous Pu replenished intracellular Pu and Sd pools by 12 hours after replating

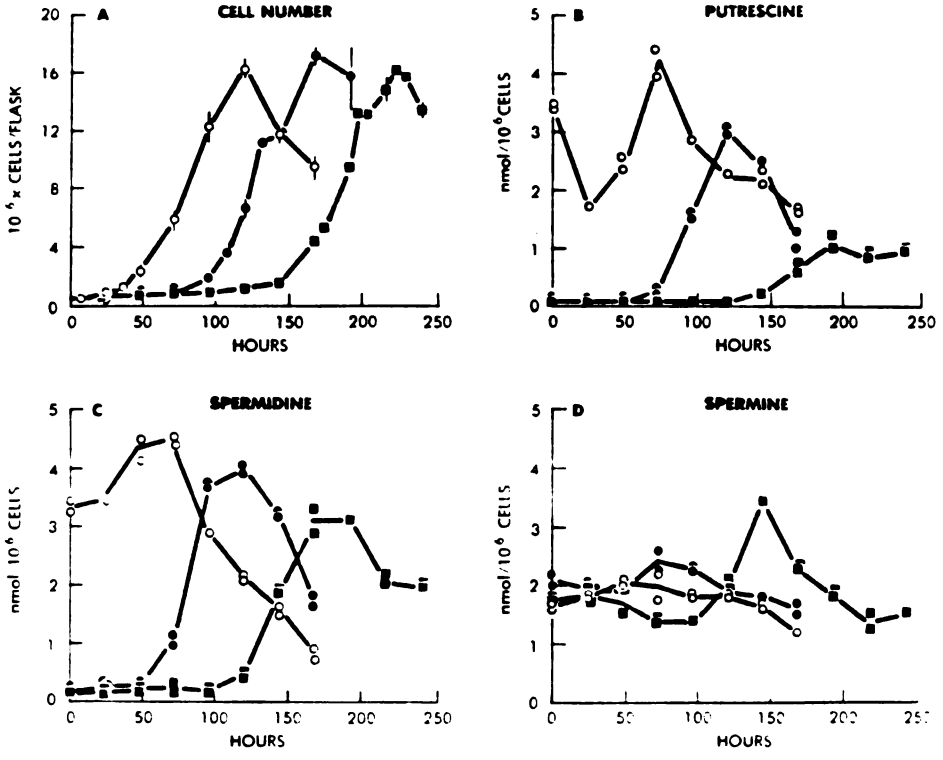


FIGURE IV - 7

9L Cell Proliferation (A), Pu Content (B), Sd Content (C), and Sp Content (D) of Cultures Seeded at 5 x 10^5 cells/flask Following a 48 hour Pretreatment with 0 (○), 1 (●) or 10 mM (■) DFMO. Points in Panel A represent the mean (±SD) of three determinations. Lines in Panels B,C,& D pass through the means of two determinations while points represent the actual data obtained.

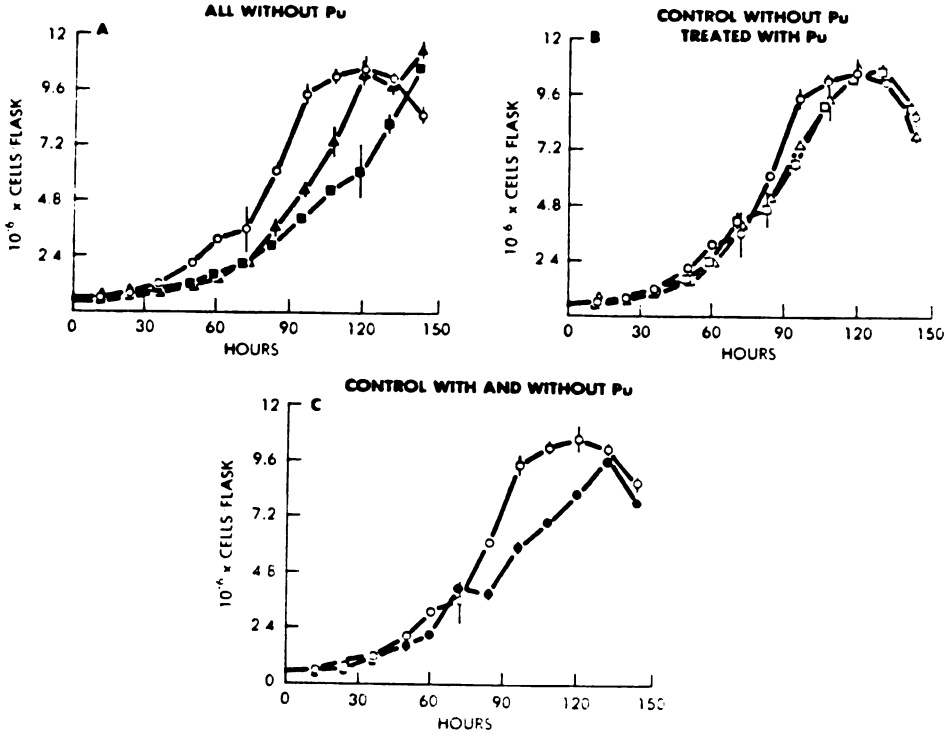


FIGURE IV - 8

Proliferation of 9L Cells Pretreated with DFMO and Replated in the Absence or Presence of Pu. A) Untreated controls (○) and cells pretreated with 1 (▲) or 10 mM (■) DFMO all replated in the absence of exogenous Pu. B) Control cells (○) replated in the absence of Pu and cells treated with 1 (△) or 10 mM (□) DFMO replated in the presence of Pu. C) Control cells replated in the absence of (○) and in the presence of (●) Pu. Points represent the mean (±SD) of three determinations.

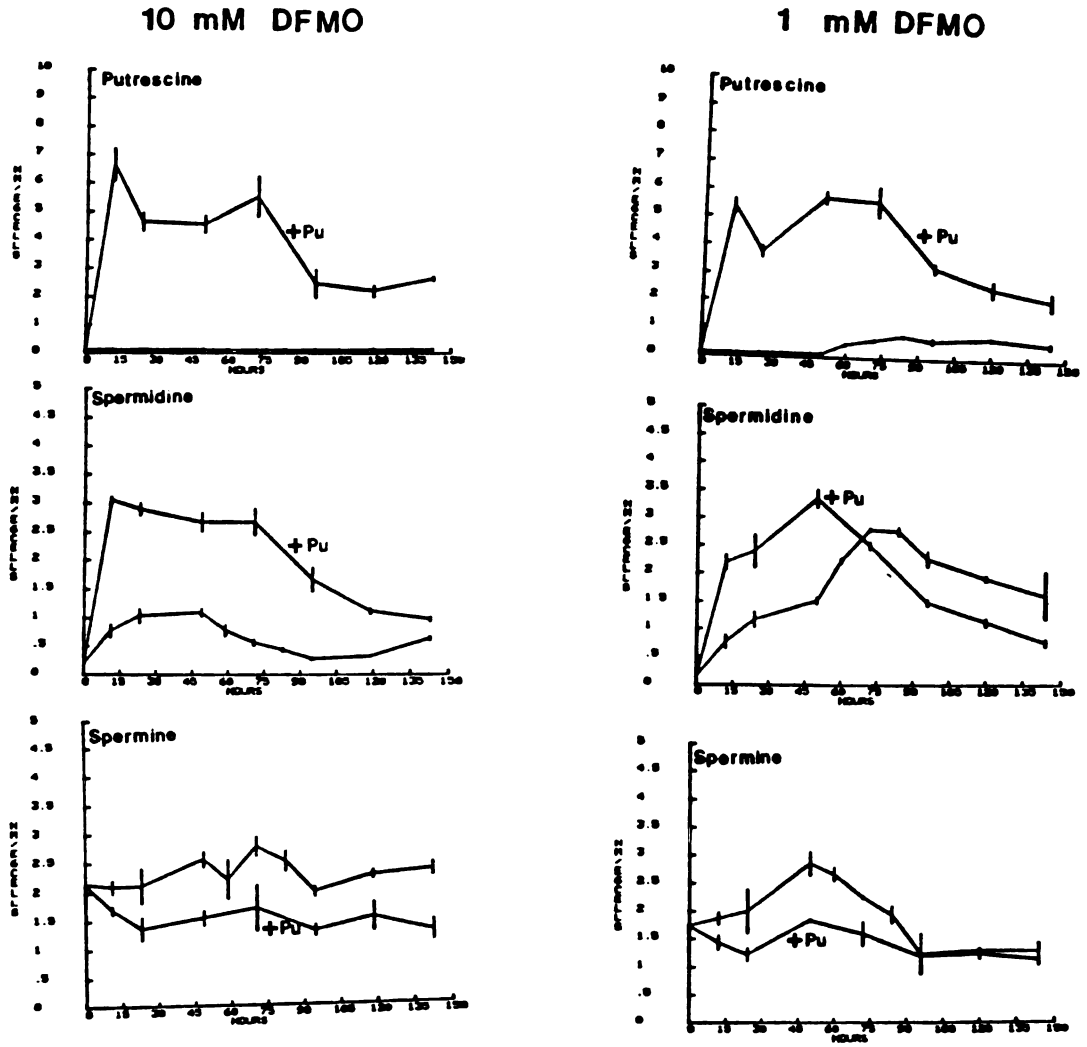


FIGURE IV - 9

Intracellular Pu (top panel), Sd (middle panel), and Sp (bottom panel) Contents of 9L Cells Pretreated for 48 hours with 10 (left) or 1 mM (right) DFMO and Replated in the Presence or Absence of 3 mM Pu. Points represent the mean (±SD) of three determinations.

and caused a slight decline in Sp content. The cells replated in the absence of Pu in this study had increased Sd content much earlier than was observed when the growth medium for replating contained 10% FCS (Fig. IV-6&IV-7). For cells pretreated with 10 mM DFMO, this early increase in Sd content was followed by a decline at about 48 hours, while for cells pretreated with 1 mM DFMO a further increase in Sd content occurred at the same time.

IV-E: Flow Cytometric Studies with DFMO Treated Cells

The effect of incubation of 9L cells seeded at $1x10^6$/flask with 1, 10, or 25 mM DFMO on the cell cycle distribution of the cell population was investigated using cells harvested in the study shown in Fig. IV-1. The DNA histograms obtained are shown in Figure IV-10. At the time of treatment, a substantial fraction of the cells were in the S and G_2M phases of the cell cycle. In the control cultures, this fraction of actively cycling cells increased for the first 24 hours of the experiment and then declined as the cells neared confluency and the growth rate declined. During the first 6 hours of treatment no substantial effect on the cell cycle distribution of treated cells was noted. By 24 hours after treatment, a distinct change from the control histograms was observed. The substantial increase in the fraction of cells in S and G_2M occurring at this time with controls was prevented by 10 or 25 mM DFMO, while 1 mM DFMO had a lesser effect. By 48 hours after treatment the DNA histograms of control and treated cells appeared the same. Histograms obtained at additional time points in this study but not shown here demonstrated that treated cells never underwent the increase in fraction of cells in S and G_2M found with controls.

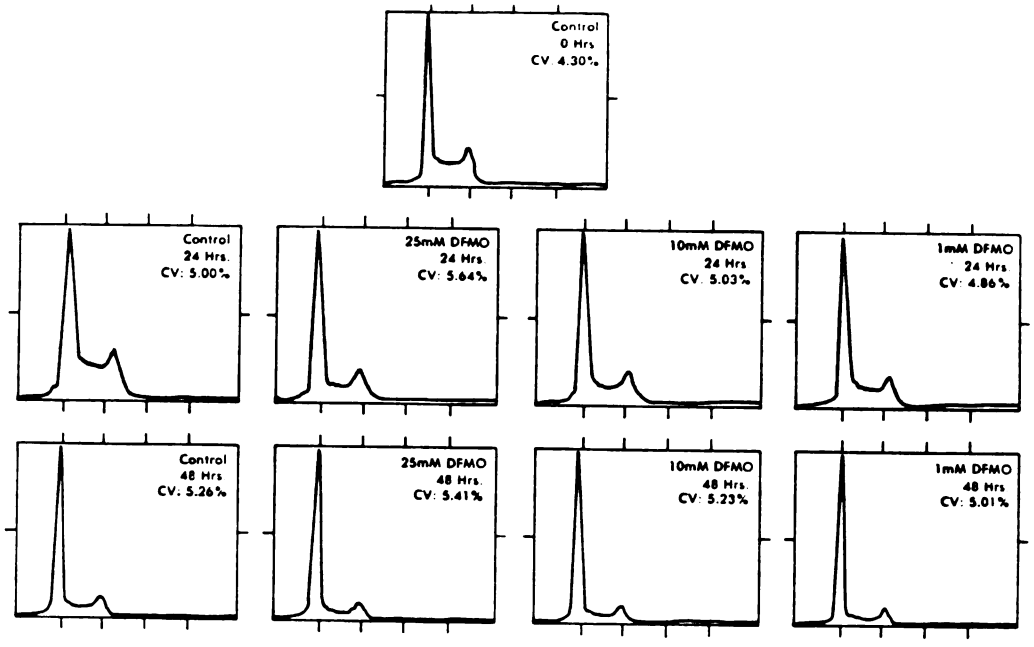


FIGURE IV - 10

DNA Histograms Obtained by Flow Cytometry of 9L Cells Incubated with 0,1, 10, or 25 mM DFMO. Cultures seeded at 1 x 10^6 cells/flask were treated after 24 hours (at time 0) and then harvested, washed with PBS, fixed in 70% aqueous ethanol, stained with Chromomycin A_3, and run on the Livermore flow cytometer.

Flow cytometry was also used to follow the cell cycle progression of cells pretreated for 48 hours with 1 or 10 mM DFMO and then harvested and replated at $5x10^5$/flask in the absence of the inhibitor. Figure IV-11 shows that control cells were able to enter S phase within 6 hours of replating. The fraction of cells actively cycling as shown by the S and G_2M portions of the DNA histograms of control cells continued to increase over the first 48 hours, after which a decline was observed. For cells pretreated with DFMO, the early increase in the cycling fraction of the population was absent. Instead, it appeared that a decrease in the fraction of cells in S and G_2M occurred. For cells pretreated with 1 mM DFMO this decline continued until about 48 hours after replating, at which time an increase in the S and G_2M portions of the DNA histograms was observed. For cells pretreated with 10 mM DFMO the fraction of cells in S and G_2M did not begin to increase until about 108-120 hours following replating.

To confirm these visual impressions of the DNA histograms, computer fits to selected samples of the flow cytometric data were used to calculate the fraction of cells in each of the three portions of the cell cycle. This program is less than reliable for accurate distinction of cells in S phase from those in the G_2M peak. Accordingly, attention was focused solely on the fraction of cells in the G_1 peak, for which the coefficients of variation were all either 4 or 5%. The results of these computations are shown in Figure IV-12 and are in fairly good agreement with the visual impressions presented above. Control cells showed an immediate decline in the G_1 fraction, although it did not fall below 50% until between 6 and 12 hours after replating. This decline continued until 24 hours, after which a gradual increase was

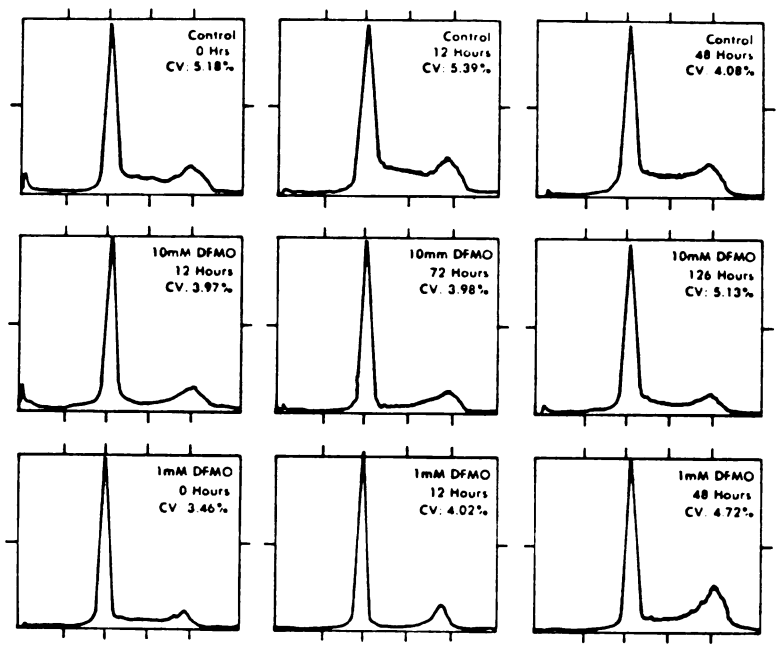


FIGURE IV - 11

DNA Histograms Obtained by Flow Cytometry of 9L Cells Pretreated with 0, 1, or 10 mM DFMO for 48 hours and then Replated at 5×10^5 cells/flask. Cultures were harvested, cells were washed with PBS, stained with Chromomycin A_3, and run on the Livermore Flow Cytometer.

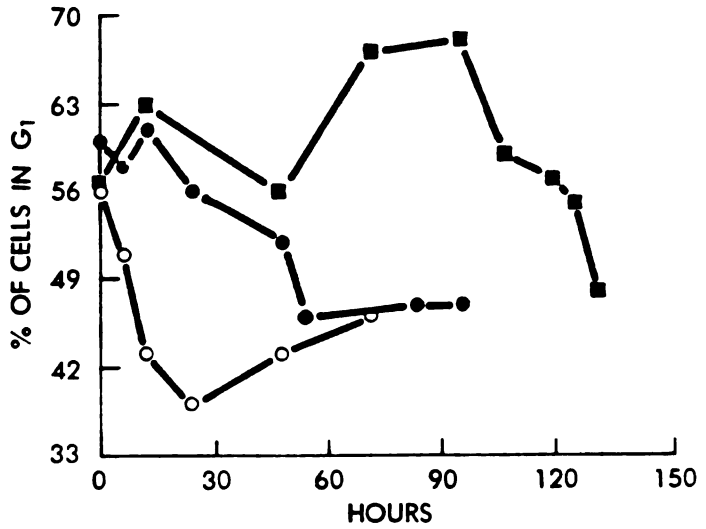


FIGURE IV - 12

Fraction of Total Cell Population in the G_1 Phase of the Cell Cycle for 9L Cells Pretreated with 0 (○) 1 (●) or 10 mM (■) DFMO and Replated at 5 x 10^5 cells/flask in the Absence of Inhibitor. Computer Analysis of selected DNA histograms obtained in the experiment of Figures IV-7 and IV-11 was used to estimate the fraction of cells in G_1. Coefficients of variation were all 4 to 5%.

observed. The fraction of cells in G_1 did remain below 50%, though, for at least 72 hours. Cells pretreated with 1 mM DFMO showed considerable fluctuation in their G_1 fraction for the first 48 hours after replating. While this value never decreased as low as with control cells, it did fall below 50% of the total population sometime after 48 hours, and remained below 50% until at least 96 hours. Cells pretreated with 10 mM DFMO also had a variable G_1 fraction for the first 72 hours, but appeared to gradually accumulate cells in the G_1 peak until a maximum of 68% of the population at 96 hours. By 108 hours this declined to 59%, but did not really fall below 50% until 132 hours.

Part Two: Discussion

In contrast to αMO, DFMO appears to completely turn off new Pu synthesis. When the total polyamine content per culture flask is computed from the data of Figures IV-1&3 as described in the discussion of Chapter III, it appears that even 1 mM DFMO prevents any increase in total polyamine content per culture over the entire incubation period. This is true for both seeding $1x10^6$ cells/flask and $5x10^5$ cells/flask (Fig. IV-13A&B). Of course this is not absolute proof that no Pu synthesis is taking place in these cells. There is always the possibility that some polyamines are being excreted into the medium or are being biotransformed. One could, however, determine if any Pu synthesis is taking place in cells treated with DFMO. Incubation of treated cells with trace amounts of ornithine radiolabeled at some position other than the carboxyl group would lead to incorporation of this label into the polyamines if Pu synthesis was taking place. Mamont et. al. have reported the results of such experiments with HTC cells treated with 5 mM DFMO

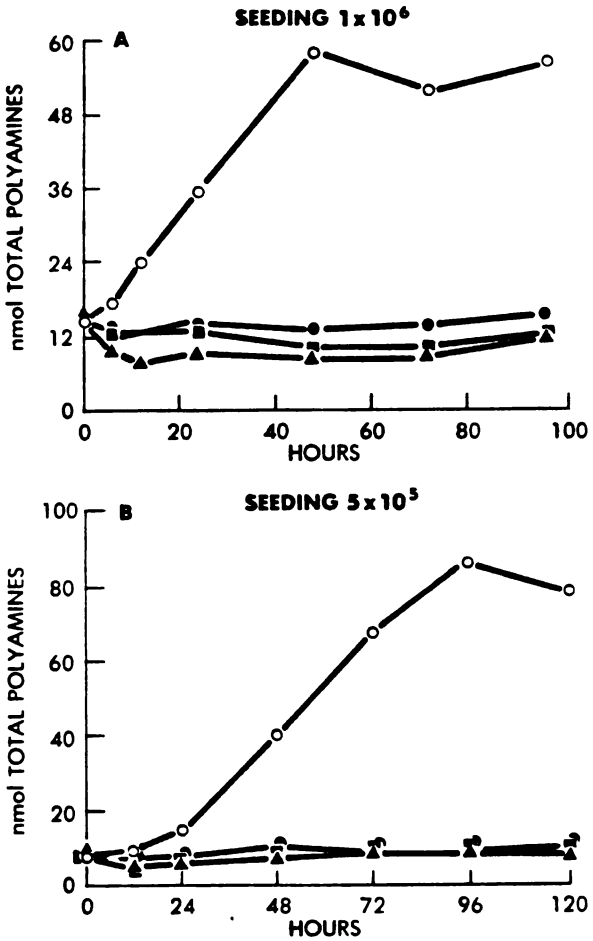


FIGURE IV - 13

Total Polyamine Content of 9L Cell Cultures Seeded at 1×10^6 (A) or 5×10^5 (B) cells/flask as a Function of Time After Treatment with 0 (○), 1 (●), 10 (■), or 25 mM (▲) DFMO.

(164). They observed a 98-99% reduction of incorporation of label by treated cells into Pu and Sd, but no difference in incorporation of label into Sp by treated and control cells. It thus appears that, at least for HTC cells, some small amount of Pu synthesis does take place even in the presence of 5 mM DFMO. It would thus be of interest to use this approach to determine if 9L cells can also convert ornithine to putrescine in the presence of DFMO.

As was observed for αMO, there is a definite dependence of the ability of DFMO to inhibit 9L cell proliferation on the initial cell density. When cells were seeded at $1x10^6$/flask, 1 and 10 mM DFMO added 24 hours after seeding produced only a slight degree of cytostasis (Fig. IV-1A). While cultures treated with these two concentrations grew at about the same rate as control cultures between 48 and 72 hours, control cultures were nearing confluency during this period and thus had already experienced a decline in their growth rate (106,173). These treated cultures did, however, reach the same endpoint as controls. In contrast, when cells were seeded at $5x10^5$/flask and treated 24 hours later, both 1 and 10 mM DFMO caused a much greated inhibition of 9L cell replication (Fig. IV-3A). This dependence upon initial cell density remains to be explained but, as discussed earlier (Chapter III, Part Two) may be related to the extended lag period of cultures seeded at the lower density.

When cultures are treated with DFMO at the same time as seeding an even greater degree of cytostasis is observed (Fig. IV-2A). This may support the hypothesis that entry of cells into exponential growth is dependent upon an adequate supply of polyamines. There is, however, an alternative explanation for this observation. When 9L cells are first

seeded into culture, they must recover from the damage done to the cell membrane during the trypsinization used to harvest them. They must then attach to the new growing surface before proliferation can begin. Addition of DFMO at the same time as seeding, or the polyamine depletion it produces, may interfere with either or both of these processes and this may be responsible for the greater degree of cytostasis observed in the experiment of Figure IV-2. Support for this rationale comes from data to be presented in Chapter V in which DFMO was added to the Petri dishes used in the CFE assay with untreated 9L cells. Addition of DFMO at the same time as seeding the cells reduced the CFE of 9L cells by substantially more than an equal concentration added 24 hours later (Table V-1).

Except for the first 1-2 hours of treatment, there were no significant differences in the rate of Pu depletion between cells treated with 1 or 25 mM DFMO (Fig. IV-4). This adds some support to the possibility that, at least in 9L cells, all three concentrations of DFMO used were equally effective in shutting off further Pu synthesis. The difference observed for the first two hours of treatment could be the result of different rates of entry of DFMO into 9L cells. A 25-fold decrease in extracellular concentration would produce a decreased rate of uptake of the drug into the cells. This would, in turn, lead to a slower rate of inactivation of ODC. It is apparent, however, that by 2 hours after treatment sufficient inhibitor has entered 9L cells treated with 1 mM DFMO to produce the same rate of Pu depletion from this time on as with 25 mM.

The results of the recovery and blocking study (Fig. IV-5) show that the cytostatic effect of 10 mM DFMO is most likely a specific result of polyamine depletion produced by ODC inhibition rather than due to some

nonspecific effect of the drug. Additional support for this hypothesis comes from the observation that cells incubated with DFMO grew at the same rate as controls for the first 24 hours of treatment (Fig. IV-1&3). It was not until after this time that almost complete depletion of Sd was observed. The ability of exogenous Pu to reverse the inhibition of proliferation when added after 24 hours of treatment is, of course, stronger support for this explanation than is the blocking of cytostasis by addition of Pu at the same time as DFMO. This is due to the possibility that when the two are added simultaneously, Pu could interfere with DFMO uptake and thus prevent its action. The fact that Pu added 24 hours after DFMO reversed not only Pu depletion but Sd depletion and Sp elevation supports the contention that cytostasis resulted from the altered polyamine content of treated cells.

The replating studies presented in Section IV-D are a new approach to the use of ODC inhibitors in elucidating the intracellular role of the polyamines. These experiments were designed to address the question, presented at the end of Chapter III, of a possible permissive role for the basal level of polyamines in allowing cells to initiate DNA synthesis. The results presented in Figures IV-6 and IV-7 show that cells pretreated with DFMO and then replated in its absence are unable to resume proliferation until after they replenish their supply of polyamines. This slower rate of growth of the pretreated cells could have been due to a lower growth fraction in the treated population. However, results obtained in the X-ray dose-response studies to be presented in Chapter V showed that the CFE of 9L cells incubated with 25 mM DFMO and then replated in its absence was the same as that of untreated controls at all X-ray doses including 0 (Fig. V-2). Furthermore, when exogenous Pu was

added to the pretreated cells, the rate of cell growth was not significantly different from that of controls, while in the absence of added Pu pretreated cells grew at a slower rate than controls (Fig. IV-8A&B). This was not the result of a general growth stimulation by Pu since exogenous Pu actually inhibited the growth of control cells to some degree (Fig. IV-8C). Since addition of exogenous Pu to pretreated cells replenished the intracellular pools of both Pu and Sd (Fig. IV-9), it is reasonable to attribute the inhibition of proliferation to polyamine depletion.

One major difference was observed between the two replating studies using medium with 10% FCS and the replating study in medium with 10% H.S. In the experiments shown in Figures IV-6 and IV-7 cells pretreated with 1 mM DFMO required 48-72 hours to replenish their intracellular polyamine content as well as to enter exponential growth. Cells pretreated with 10 mM DFMO did not begin exponential proliferation until 132 hours after replating and did not replenish their polyamine content until 120 hours. In contrast, in the study with 10% H.S. shown in Figures IV-8 and IV-9, pretreated cells replated in the absence of exogenous polyamines did show a somewhat faster early rate of proliferation, and those pretreated with 10 mM DFMO entered exponential growth by 72 hours after reseeding. Furthermore, the pretreated cells replated in the absence of added Pu showed much earlier increases in Sd content than when medium with 10% FCS was used. It seems reasonable to attribute the earlier resumption of proliferation to the early increase in Sd content. The source of this Sd, however, remains unexplained. Measurements of polyamine concentration in the two media did not show any significant differences between them, thus ruling out differences in polyamine uptake from

extracellular pools as the cause of the differences in early Sd content.

The DNA histograms obtained by flow cytometry of cells incubated with DFMO showed that treated cells experienced an inhibition of entry into S phase (Fig. IV-10). Support for the hypothesis that this partial G_1-S block was the specific result of polyamine depletion rather than due to some nonspecific effect of DFMO comes from the histograms obtained for cells pretreated with DFMO and replated in its absence (Fig. IV-11). These results showed that polyamine depleted cells did not initiate DNA synthesis (i.e., enter S phase) as readily as did controls, until they replenished their polyamine content. The results of computer analysis of the DNA histograms also support this hypothesis. Control cells showed a decline to less than 50% of the population in G_1 within 12 hours of reseeding while pretreated cells did not have less than half the population in G_1 until about the time of renewed Pu synthesis and Sd accumulation (Fig. IV-12). It should be noted that this preceeds the time of increasing cell density for the pretreated cells by about one cell cycle time. These results imply that the presence of some minimal polyamine content is necessary for initiation of DNA synthesis, which is then followed by a lag of about one cell cycle time before increases in the number of cells are observed. This replating approach thus fairly convincingly demonstrates a permissive role for the basal level of polyamines in initiation of DNA replication.

One last point that should be mentioned is the fact that with pretreated cells replated in the absence of added Pu, although declines in the fraction of cells in G_1 are noted at the times of polyamine accumulation, the decline was never as great as for untreated

controls. This implies some irreversible damage to the cells which does not allow them to initiate DNA synthesis at their normal rate. It is possible that this damage occurs during the trypsinization preceeding replating and that polyamine depleted cells are more fragile than controls. A second possibility is that the damage occurs after reseeding and results from the cells' attempts to continue proliferating under less than optimal conditions. Since it appears that the increases in polyamine content in early tissue culture growth are probably not the signal for initiating replication, polyamine depletion probably does not turn off this signal. The pretreated cells would thus be programmed to begin replication but unable to do so due to insufficient polyamine content. This could lead to some irreversible damage to the cells' replicative machinery. It would therefore be of interest to determine if addition of exogenous Pu to pretreated cells would result in decreases in the fraction of cells in G_1 equal to and at the same time as those observed for controls. Unfortunately, the altered Sd content of pretreated cells during the early part of the experiment shown in Figures IV-8 and IV-9 imply that the results of such an experiment would be less than conclusive. These early increases in Sd content of pretreated cells replated in medium with 10% H.S. in the absence of added Pu and the earlier entry of these cells into exponential growth seem to indicate that the G_1-S block observed in the experiment of Figures IV-7, IV-11, and IV-12 would not be as evident when medium with H.S. was used.

CHAPTER V: EFFECT OF POLYAMINE DEPLETION ON THE X-RAY DOSE-RESPONSE OF 9L CELLS

Part One: Rationale

As discussed in Section I-D, numerous studies have shown that polyamines bind to DNA *in vitro* (60, 66-72), and stabilize DNA to enzymatic degradation (62), denaturation by X-rays (63), and thermal denaturation (61). Gosule and Schellman have reported that polyamines aid in packaging of DNA into phage heads (90) and lead to the condensation and compaction of DNA in solution (191,192). One could thus hypothesize that depletion of intracellular polyamine content could alter the structure of nuclear DNA in such a way as to produce a more open, less compact form.

It is generally accepted that the mechanism by which X-irradiation leads to cell death involves damage to the cell's DNA (193). This damage results from reaction of hydroxyl radicals, produced by collision of X-ray photons with intracellular water molecules, with the DNA bases, rather than from direct interaction of X-rays and DNA (193,194). This leads to the possibility that depletion of intracellular polyamine content by treatment with polyamine antimetabolites could alter intracellular DNA structure in a manner that would sensitize the cell to lethal damage resulting from X-irradiation. Sensitization could result from an increase in the fragility of the DNA, or from an increase in the accessibility of the bases in the interior of the double helix to reactive intermediates produced from

interaction of X-rays and intracellular water. Support for this hypothesis comes from the recent reports of Rosiek, et. al. (195,196) that $1x10^5$ M exogenous Sd reduced the frequency of chromatid breaks and stimulated repair of chromosome breaks in X-irradiated cultured human lymphocytes. Accordingly, the following studies were undertaken to assess the ability of polyamine antimetabolites to sensitize 9L cells to lethal damage by X-rays.

Part Two: Results

Figure V-1 presents the results of an X-ray dose response study using the CFE assay (see Section II-F) with untreated 9L cells and with cells treated for 24 hours with 10 mM αMO 24 hours after seeding $1x10^6$ cells/flask. The polyamine content of untreated 9L cells after irradiation did not vary with X-ray dose and was 3.42 ± 0.49 nmol/10^6 cells for Pu, 4.25 ± 0.61 nmol/10^6 cells for Sd, and 1.53 ± 0.28 nmol/10^6 cells for Sp. Similarly, X-ray dose did not affect the polyamine content of αMO-treated cells which was <0.1 nmol/10^6 cells for Pu, 1.83 ± 0.07 nmol/10^6 cells for Sd, and 2.83 ± 0.34 nmol/10^6 cells for Sp. Treatment with αMO thus reduced Pu content by more than 97%, Sd content by 67%, and total polyamine content by 50%, but did not increase the sensitivity of 9L cells to killing by X-rays.

A similar experiment was performed with 9L cells incubated for 48 hours in the presence or absence of 25 mM DFMO. Treatment with DFMO occurred 24 hours after seeding $1x10^6$ cells/flask. The mean polyamine content of untreated controls harvested after irradiation was 2.62 ± 0.15 nmol/10^6 cells for Pu, 3.62 ± 0.11 nmol/10^6 cells for Sd and 1.54 ± 0.14 nmol/10^6 cells for Sp. The DFMO-treated cells had mean polyamine contents of <0.1 nmol/10^6 cells for both Pu and Sd, and 1.92 ±

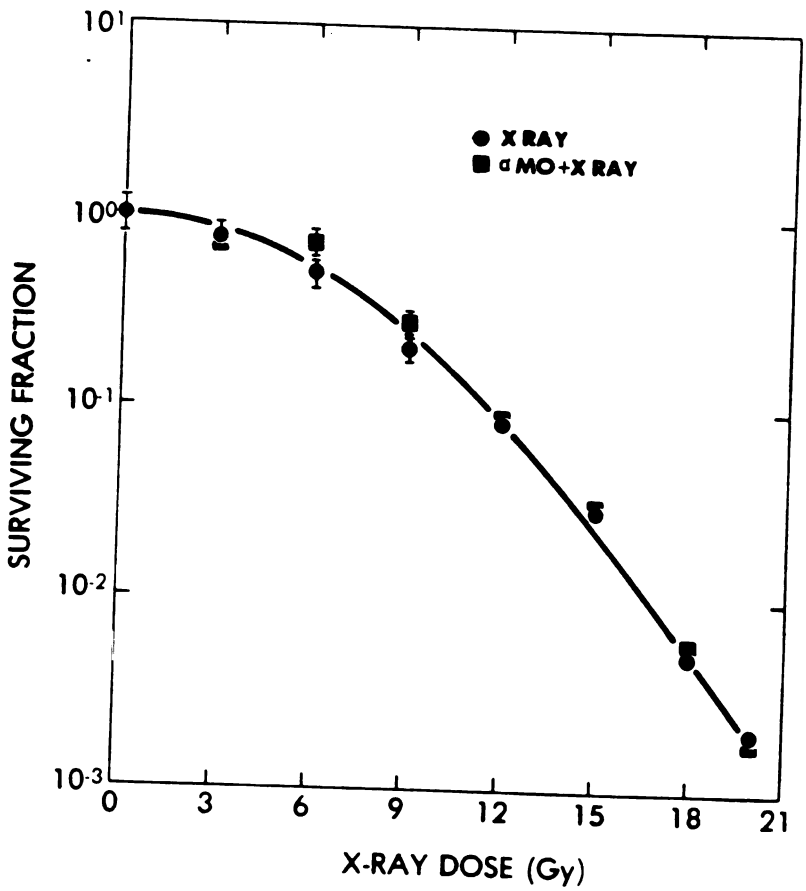


FIGURE V - 1

X-ray Dose - Response Curves of Untreated 9L Cells (●) and of 9L Cells Incubated for 24 Hours with 10 mM αMO (■).

0.17 nmol/10^6 cells for Sp. As shown in Figure V-2, this treatment which reduced total polyamine content by 75%, did not affect the X-ray dose-response curve of 9L cells.

A third experiment tested the ability of a 48 hour pre-treatment with 40 μM MGBG (see Section I-F) to increase the sensitivity of 9L cells seeded at 1x10^6/flask 24 hours before treatment to killing by X-rays. In this study, untreated 9L cells harvested after irradiation had mean polyamine contents of 4.19 ± 0.37 nmol/10^6 cells for Pu, 3.83 ± 0.27 nmol/10^6 cells for Sd and 1.68 ± 0.16 nmol/10^6 cells for Sp. Cells treated for 48 hours with MGBG and then irradiated had mean polyamine contents of 7.95 ± 0.18 nmol/10^6 cells for Pu, 1.12 ± 0.03 nmol/10^6 cells for Sd and 0.56 ± 0.02 nmol/10^6 cells for Sp. Figure V-3 shows that reduction of Sd by 70% and of Sp by 66% also did not affect the X-ray dose-response curve of cultured 9L cells. The total polyamine content of treated cells, however, did not differ from that of controls.

A fourth study explored the possibility that the combination of an ODC inhibitor and a SAMDC inhibitor should achieve a greater degree of polyamine depletion than either inhibitor alone and thus increase the sensitivity of 9L cells to lethal damage by X-rays. Cultures of 9L cells were incubated for 48 hours in the presence or absence of 40 μM MGBG and 25 mM DFMO, added 24 hours after seeding 1x10^6 cells/flask. The mean polyamine content of untreated cells after irradiation was 9.73 ± 0.71 nmol/10^6 cells for Pu, 2.58 ± 0.10 nmol/10^6 cells for Sd and 1.29 ± 0.11 nmol/10^6 cells for Sp. Cells treated with 40 μM MGBG + 25 mM DFMO had mean polyamine contents after irradiation of <0.1 nmol/10^6 cells for Pu, 0.76 ± 0.02 nmol/10^6 cells for Sd, and

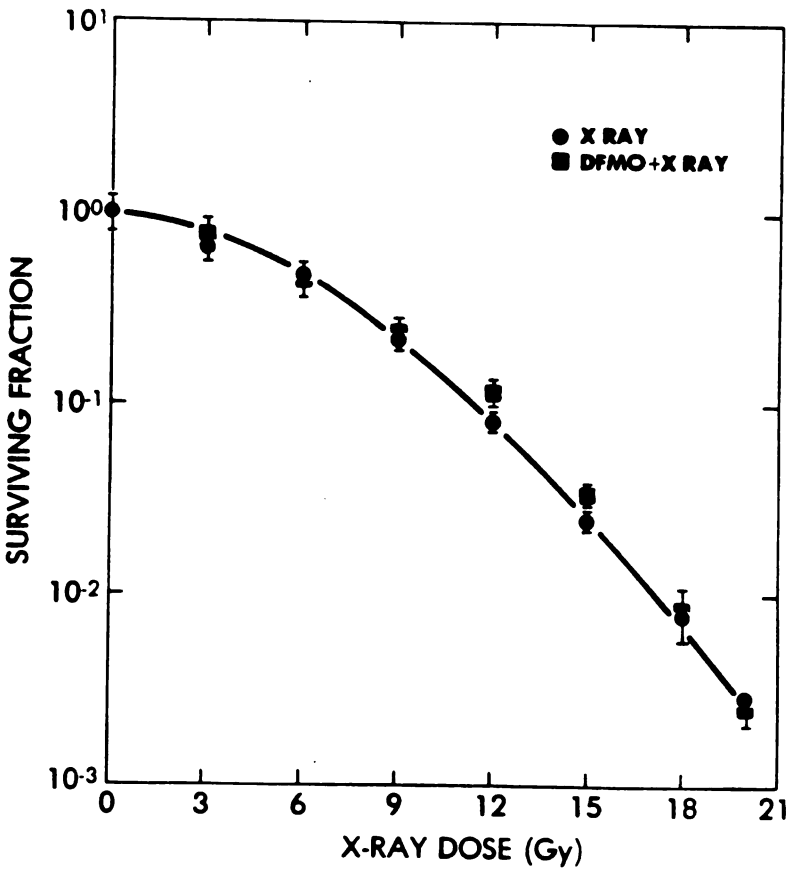


FIGURE V - 2

X-ray Dose - Response Curves of Untreated 9L Cells (●) and of 9L Cells Incubated for 48 Hours with 25 mM DFMO (■).

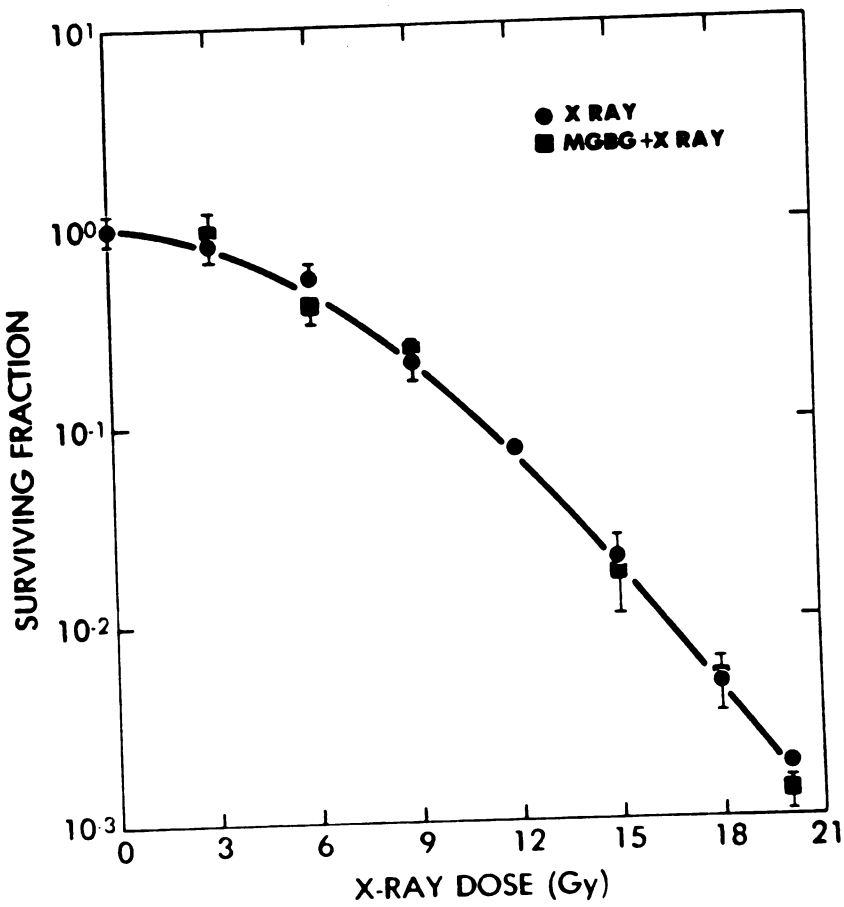


FIGURE V - 3

X-ray Dose - Response Curves of Untreated 9L Cells (●) and of 9L Cells Incubated for 48 Hours with 40 μM MGBG (■).

1.01 ± 0.11 nmol/10^6 cells for Sp. While this is an 87% reduction in total polyamine content from controls, Sd content of cells treated with DFMO alone was lower than that of cells treated with DFMO plus MGBG. As shown in Figure V-4, the combination of MGBG with DFMO did not increase the susceptibility of 9L cells to killing by X-irradiation.

A final experiment examined the effect of addition of DFMO to the Petri dishes used in the CFE assay on the ability of untreated, unirradiated 9L cells to form colonies of 50 or more cells. The range of concentrations of DFMO used was 0.1 to 25 mM. The drug was added at the same time as seeding of the cells in half of the plates, and 24 hours after seeding of the cells in the remaining half. Table V-1 shows that 0.1 mM DFMO did not affect the CFE of untreated unirradiated 9L cells. Addition of 1 mM DFMO reduced the CFE of 9L cells by 80% when added at the same time as seeding the cells and by 66% when added 24 hours later. There was no difference between the effects of 10 and 25 mM DFMO on the CFE of 9L cells. Both of these concentrations reduced the CFE by 99.95% when added at the same time as seeding the cells and by 99.8% when added 24 hours later.

Part Three: Discussion

The results obtained in these studies indicate that the degree of polyamine depletion achieved with αMO, DFMO, MGBG, or the combination of DFMO+MGBG does not sensitize 9L cells to killing by X-irradiation. Several alternative explanations for these observations are possible. The first and most obvious one is that while the presence or absence of polyamines does affect DNA structure in cell-free solution (60-63, 66-72), it is possible that the presence of histones and

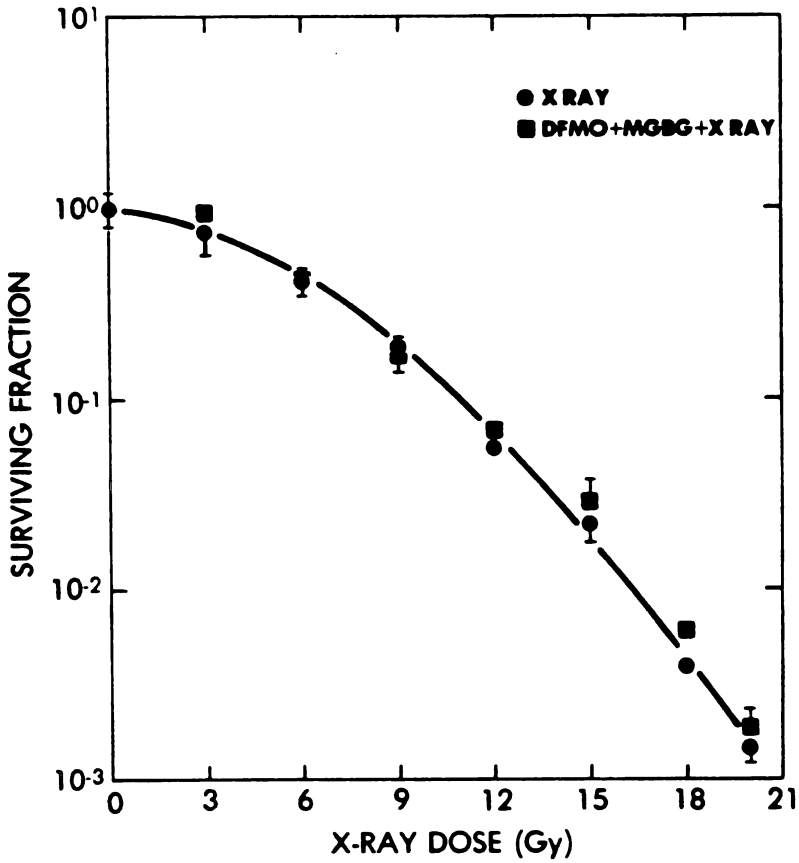


FIGURE V - 4

X-ray Dose - Response Curves of Untreated 9L Cells (●) and of 9L Cells Incubated for 48 Hours with 40 μM MGBG + 25 mM DFMO (■).

TABLE V - 1

Effect of Addition of DFMO to the Petri Dishes on the CFE of Untreated 9L Cells

	% of Plated Cells Forming Colonies of 50 or More Cells	
DFMO Concentration	Treatment With Plating	Treatment 24 hrs Post Plating
0 mM	61.1 ± 9.0	60.8 ± 6.1
0.1 mM	58.6 ± 4.5	57.8 ± 5.6
1 mM	12.2 ± 3.1	20.2 ± 5.4
10 mM	0.034 ± 0.015	0.122 ± 0.020
25 mM	0.038 ± 0.010	0.152 ± 0.037

other nuclear proteins, as well as divalent metal cations, would prevent depletion of intracellular polyamine content from having an effect on the structure of intracellular DNA. A second rationale could be that a greater degree of polyamine depletion than was achieved here is necessary before intracellular DNA structure is effectively altered. A third possibility is that some or all of the treatments used did produce changes in DNA structure, but these alterations did not increase the accessibility of the DNA base pairs to hydroxyl radicals produced by X-rays from intracellular water molecules. Regardless of which rationale is true, the data presented here indicate that the polyamine antimetabolites tested in this study will probably not be useful as radiosensitizers.

Several reports have implied that Sd may stimulate the enzymes involved in DNA repair (196,197). The results of the replating studies discussed in Chapter IV show that 9L cells treated with an irreversible ODC inhibitor for 48 hours and then harvested and replated in its absence do regain the ability to synthesize new polyamines. If an effect of polyamine depletion on the 9L X-ray dose-response curve had been observed, this effect could have been due to inhibition of DNA repair rather than to potentiation of damage. Similarly, the lack of an effect could have been the result of replenishment of intracellular polyamine content when the irradiated cells were replated in the absence of polyamine antimetabolites. This possibility could have been tested by addition of polyamine antimetabolites to the Petri dishes used in the CFE assay, thus preventing the replated cells from replenishing their intracellular polyamine content. The data of Table V-1 show, however, that addition of DFMO to the CFE plates reduces the CFE

of untreated unirradiated 9L cells. This observation means that one could not obtain appropriate control results in testing the possibility discussed above.

One final point that should be mentioned is that the results obtained here do not speak to the question of the effect of polyamine depletion on intracellular DNA structure. Several additional approaches are possible to gain information relevant to this issue. One such approach would be to compare physical measurements, such as sedimentation rates in a density gradient or viscosity, of DNA isolated from control cells to that from cells treated with polyamine antimetabolites. A second approach could be investigation of the effects of polyamine depletion on the 9L dose-response to cancer chemotherapeutic drugs, such as the chloroethylnitrosoureas, known to act by alkylating DNA. It is possible that polyamine depletion could alter DNA structure in a manner that would not increase accessibility of the base pairs to hydroxyl radicals but would increase their accessibility to larger molecules such as the alkylating agents. The report by Rajalakshmi et. al. (65) of a protective effect of exogenous polyamines on methylation of DNA by N-methyl-N-nitrosourea supports this possibility. Experiments evaluating this possibility are planned for the near future.

CHAPTER VI: CONCLUSIONS

The results of the studies with αMO presented in Chapter III show that αMO is not as effective an inhibitor of 9L intracellular Pu biosynthesis as it is an inhibitor of partially purified ODC. It is thus not surprising that αMO is not very effective as a cytostatic agent. Several new observations come out of the work outlined in Chapter III. The first is the dependence of the degree of inhibition observed on the initial cell density. This unexplained phenomenon has not been reported in any of the studies with polyamine antimetabolites. The second is the importance of control experiments using equal concentrations of ornithine when effects of ornithine analogs that are not reversible by exogenous Pu are observed. Such control experiments have not been performed in any of the studies published on effects of ODC inhibition on cell growth in culture. High concentrations of αMO do appear to have nonspecific effects on 9L cell proliferation, while lower concentrations are only weakly cytostatic. It thus seems apparent that αMO will not be sufficiently cytostatic to be useful chemotherapeutically. The studies with 10 mM αMO treatment of cells seeded at $5x10^5$ cells/flask did show however that blocking the early increase in ODC activity and in polyamine accumulation did not hamper initiation of DNA synthesis, but did prevent cells from maintaining optimal rates of DNA synthesis. These observations are in agreement with the findings of Mamont et. al. (163) and Morris et. al. (168) published while this work was in progress.

Experiments outlined in Chapter IV show that DFMO is much more effective in inhibiting 9L intracellular Pu synthesis and proliferation than is αMO. The replating of pre-treated cells is a new approach to the use of irreversible ODC inhibitors to study the intracellular role of the polyamines. Results obtained with this approach convincingly demonstrate a requirement for intracellular Sd to allow cells to enter S phase. Furthermore, the cytostatic effect of DFMO and the delayed entry of pre-treated cells into exponential growth appear to be specifically related to polyamine depletion since they are readily reversible by exogenous Pu. DFMO thus shows some promise as a tool for elucidating the intracellular role of the polyamines, and deserves further study as an in vivo cytostatic agent for a variety of diseases including cancer.

It was initially hoped that polyamine depletion would be a useful new approach to radiosensitization. This seemed to be a reasonable expectation based on previously published reports of the radioprotective effects of exogenous polyamines (195,196). The studies discussed in Chapter V, however, seem to rule out this possibility at least for 9L cells. Further work is needed to confirm and extend these findings to other cell lines. In addition the possibility that polyamine depletion may sensitize cells to the cytotoxic effects of alkylating agents is worthy of further investigation.

At least two potential uses for DFMO in cancer chemotherapy can be envisioned. Current modes of cancer chemotherapy rely on highly toxic drugs. For example, the chloroethylnitrosoureas are the most effective agents being used to treat patients with intracerebral tumors. These are highly cytotoxic agents which can only be administered

in succeedingly longer intervals and at succeedingly lower doses to allow the non-tumor proliferating populations of cells in the host's body to recover from the toxic effects. During the intervals between chemotherapy, tumor cells that have not been killed begin to proliferate. There are also some indications that the growth rate of the tumor following chemotherapy may be faster than prior to treatment. This tumor regrowth during the recovery interval decreases the effectiveness of chemotherapy and makes complete cure difficult if not impossible. A non-toxic cytostatic agent, such as DFMO appears to be (J. Koch-Weser, personal communication), would find great utility in slowing tumor regrowth between courses of chemotherapy. This would greatly increase the effectiveness of current methods of treatment for tumors.

A second potential use for DFMO grows out of the observations presented in Chapter IV that polyamine depletion subsequent to DFMO treatment leads to at least a partial G_1-S block. While these observations have only been made for cells grown in tissue culture, it is possible that similar effects will occur in vivo. If this is the case, then withdrawal of the drug, or administration of exogenous polyamines some time after treatment, may lead to a synchronous wave of tumor cells entering into S phase. Administration of a cell cycle specific cancer chemotherapeutic agent at this time might lead to enhanced effectiveness of this mode of tumor therapy.

REFERENCES

1) Cohen, S.S.: "Introduction to the Polyamines". Englewood Cliffs, New Jersey: Prentice Hall. (1971).

2) Bachrach, U.: "Function of Naturally Occurring Polyamines". New York: Academic Press. (1973).

3) Williams-Ashman, H.G. and Canellakis, Z.N. (1979). Perspectives in Biology and Medicine, 22: 421-453.

4) Janne, J. and Williams-Ashman, H.G. (1971). J. Biol. Chem., 246: 1725-1732.

5) Pegg, A.E. and Williams-Ashman, H.G. (1970). Arch. Biochem. Biophys., 137: 156-165.

6) Coppoc, G.L., Kallio, P. and Williams-Ashman, H.G. (1971). Int. J. Biochem., 2: 673-681.

7) Pegg, A.E. (1977). FEBS Letters, 84: 33-36.

8) Demetriou, A.A., Cohn, M.S., Tabor, C.W. and Tabor, H. (1978). J. Biol. Chem., 253: 1684-1686.

9) Hannonen,P., Janne, J. and Raina, A. (1972). Biochem. Biophys. Res. Commun., 46: 341-348.

10) Janne, J., Schenone, A. and Williams-Ashman, H.G. (1971). Biochem. Biophys. Res. Commun., 42: 758-764.

11) Janne, J. and Williams-Ashman, H.G. (1971). Biochem. Biophys. Res. Commun., 42: 222-229.

12) Hibasami, H. and Pegg, A.E. (1978). Biochem. Biophys. Res. Commun., 81: 1398-1405.

13) Greengard, O., Sahib, M.K. and Knox, W.E. (1970). Arch. Biochem. Biophys., 137: 477-482.

14) Jones, M.E., Anderson, A.D., Anderson, C. and Hodes, S. (1961). Arch. Biochem. Biophys., 95: 499-507.

15) Strecker, H.J. (1965). J. Biol. Chem., 240: 1225-1230.

16) Cantoni, G.L. and Durell, J. (1957). J. Biol. Chem., 225: 1033-1048.

17) Salvatore, F., Borek, E., Zappia, V., Williams-Ashman, H.G. and Schlenk, F. (eds.): "The Biochemistry of S-Adenosylmethionine". New York: Columbia University Press. (1977).

18) Pegg, A.E. and Williams-Ashman, H.G. (1969). Biochem. J., 115: 241-247.

19) Toohey, J.I. (1978). Biochem. Biophys. Res. Commun., 83: 27-35.

20) Buffoni, F. (1966). Pharmacol. Rev., 18: 1163-1199.

21) Tabor, C.W., Tabor, H. and Bachrach, U. (1964). J. Biol. Chem., 239: 2194-2203.

22) Bachrach, U., Abzug, S. and Bekierkunst, A. (1967). Biochim. Biophys. Acta, 134: 174-181.

23) Bachrach, U. (1970). Ann N.Y. Acad. Sci., 171: 939-956.

24) Holtta, E. (1977). Biochemistry, 16: 91-100.

25) Seiler, N. and Al-Therib, M.J. (1974). Biochim. Biophys. Acta, 354: 206-212.

26) Blankenship, J. and Walle, T. (1977). Arch. Biochem. Biophys., 179: 235-242.

27) Blankenship, J. (1978). Arch. Biochem. Biophys., 189: 20-27.

28) Libby, P.R. (1978). Arch. Biochem. Biophys., 188: 360-363.

29) Walle, T. (1973). In: Polyamines in Normal and Neoplastic Growth (D.H. Russell, ed.) pp. 355-365. New York: Raven Press.

30) Abdel-Monem, M.M. and Ohno, K. (1977). J. Pharm. Sci., 66: 1195-1197.

31) Seiler, N., Al-Therib, M.J. and Knodgen, B. (1973). Hoppe-Seyler's Z. Physiol. Chem., 354: 589-590.

32) Okuyama, T. and Kobayashi, Y. (1961). Arch. Biochem. Biophys., 95: 242-250.

33) Kremzner, L.T., Hiller, J.M. and Simon, E.J. (1975). J. Neurochem., 25: 889-894.

34) Lundgren, D.W. and Hankins, J. (1978). J. Biol. Chem., 253: 7130-7133.

35) Seiler, N. and Al-Therib, M.J. (1974). Biochem. J., 144: 29-35.

36) Rosenblum, M.G. and Russell, D.H. (1977). Cancer Res., 37: 47-51.

37) Rosenblum, M.G., Durie, B.G.M., Salmon, S.E. and Russell, D.H. (1978). Cancer Res., 38: 3161-3163.

38) Russell, D.H. and Snyder, S.H. (1969). Mol. Pharmacol., 5: 253-262.

39) Clark, J.L. and Fuller, J.L. (1976). Eur. J. Biochem., 67: 303-314.

40) Janne, J. and Raina, A. (1968). Acta Chem. Scand., 22: 1349-1351.

41) Russell, D.H. and Snyder, S.H. (1968). Proc. Natl. Acad. Sci. USA, 60: 1420.

42) Ono, M., Inoue, H., Suzuki, F. and Takeda, Y. (1972). Biochim. Biophys. Acta, 284: 285-297.

43) Heby, O., Marton, L.J., Zardi, L., Russell, D.H. and Baserga, R. (1975). Exp. Cell Res., 90: 8-14.

44) O'Brien, T.G., Simsimian, R.C. and Boutwell, R.K. (1975). Cancer Res., 35: 1662-1670.

45) Russell, D.H. and Snyder, S.H. (1969). Endocrinology, 84: 223-228.

46) Kay, J.E. and Lindsay, V.J. (1973). Exp. Cell Res., 77: 428-436.

47) Fong, W.F., Heller, J.S. and Canellakis, E.S. (1976). Biochim. Biophys. Acta, 428: 456-465.

48) Heller, J.S., Kyriakidis, D., Fong, W.F. and Canellakis, E.S. (1977). Eur. J. Biochem., 81: 545-550.

49) Clark, J.L. and Fuller, J.L. (1975). Biochemistry, 14: 4403-4409.

50) Canellakis, Z.N. and Theoharides, T.C. (1976). J. Biol. Chem., 251: 4436-4441.

51) McCann, P.P. (1980). In: Polyamines in Biomedical Research (J.M. Gaugas, ed.) in press. London: J. Wiley and Sons.

52) Morris, D.R. and Fillingame, R.H. (1974). Ann. Rev. Biochem., 43: 303-325.

53) Pegg, A.E., Lockwood, D.H. and Williams-Ashman, H.G. (1970). Biochem. J., 117: 17-31.

54) Pegg, A.E. and Williams-Ashman, H.G. (1969). J. Biol. Chem., 244: 682-693.

55) Oka, T., Kano, K. and Perry, J.W. (1978). In: Advances in Polyamine Research, vol. 1 (R.A. Campbell et. al., eds.) pp. 59-67. New York: Raven Press.

56) Russell, D.H. and Taylor, R.L. (1971). Endocrinology, 88: 1397-1403.

57) Hannonen, P., Janne, J. and Raina, A. (1972). Biochim. Biophys. Acta, 289: 225-231.

58) Hannonen, P., Raina, A. and Janne, J. (1972). Biochim. Biophys. Acta, 273: 84-90.

59) Oka, T., Perry, J.W. and Kano, K. (1977). Biochem. Biophys. Res. Commun., 79: 979-986.

60) Stevens, L. (1970). Biol. Rev. Cambridge Philos. Soc., 45: 1-27.

61) Tsuboi, M. (1964). Bull. Chem. Soc. Japan, 37: 1514-1522.

62) Bachrach, U. and Eilon, G. (1969). Biochim. Biophys. Acta, 179: 494-496.

63) Brown, P.E. (1968). Radiat. Res., 34: 24-35.

64) Ben-Hur, E. (1975). Israel J. Chem., 13: 221-228.

65) Rajalakshmi, S., Rao, P.M. and Sarma, D.S.R. (1978). Biochem. Biophys. Res. Commun., 81: 936-940.

66) Mahler, H.R. and Mehrotra, B.D. (1963). Biochim. Biophys. Acta, 68: 211-233.

67) Stevens, L. (1967). Biochem. J., 103: 811-815.

68) Maestre, M.F. and Tinoco, I. (1967). J. Molec. Biol., 23: 323-335.

69) Agrell, I. and Heby, O. (1971). Hoppe-Seyler's Z. Physiol. Chem., 352: 39-42.

70) Damiani, A., Giglio, E., et. al. (1965). J. Molec. Biol., 11: 441-442.

71) Damiani, A., Liquori, A.M., et. al. (1965). J. Molec. Biol., 11: 438-440.

72) Liquori, A.M., Constantino, L., et. al. (1967). J. Molec. Biol., 24: 113-122.

73) Herbst, E.J. and Doctor, B.P. (1959). J. Biol. Chem., 234: 1497-1500.

74) Schlenk, F. and Dainko. (1966). Arch. Biochem. Biophys., 113: 127-133.

75) Brakke, M.K. and Van Pelt, N. (1969). Virology, 39: 516-533.

76) Felsenfeld, G. and Huang, S. (1960). Biochim. Biophys. Acta, 37: 425-433.

77) Cohen, S.S., Morgan, S. and Streibel, E. (1969). Proc. Natl. Acad. Sci. USA, 64: 669-676.

78) Cohen, S.S. (1970). Ann. N.Y. Acad. Sci., 171: 869-881.

79) Bolton, P.H. and Kearns, D.R. (1978). Nucleic Acids Res., 5: 1315-1324.

80) Igarashi, K., Sugawara, K., et. al. (1974). Eur. J. Biochem., 48: 495-502.

81) Farron-Furstenthal, F. and Lightholder, J.R. (1978). Biochem. Biophys. Res. Commun., 83: 94-100.

82) Igarashi, K., Watanabe, Y., et. al. (1978). Biochem. Biophys. Res. Commun., 83: 806-813.

83) Oriol-Audit, C. (1978). Eur. J. Biochem., 87: 371-376.

84) Schrode, J. and Folk, J.E. (1978). J. Biol. Chem., 253: 4837-4840.

85) Siekevitz, P. and Palade, G.E. (1962). J. Cell. Biol., 13: 217-232.

86) Cohen, S.S. and Lichtenstein, J. (1960). J. Biol. Chem., 235: 2112-2116.

87) Tabor, C.W. (1960). Biochem. Biophys. Res. Commun., 2: 117-120.

88) Busch, H., Narayan, K.S. and Hamilton, J. (1967). Exp. Cell Res., 47: 329-336.

89) Hiebert, E. and Bancroft, J.B. (1969). Virology, 39: 296-311.

90) Gosule,L.C. and Schellman, J.A. (1976). Nature, 259: 333-335.

91) Ham, R.G. (1965). Proc. Natl. Acad. Sci. USA, 53: 288-293.

92) Russell, D.H., Medina, V.J. and Snyder, S.H. (1970). J. Biol. Chem., 245: 6732-6738.

93) Dykstra, W.J. and Herbst, E.J. (1965). Science, 149: 428-429.

94) Heby, O. and Lewan, L. (1971). Virchows. Arch. {Zellpathol.}, 8: 58-66.

95) Russell, D.H. and McVicker, T.A. (1972). Biochem. J., 130: 71-76.

96) Fausto, N. (1969). Biochim. Biophys. Acta, 190: 193-201.

97) Raina, A. (1963). Acta Physiol. Scand., 60(Suppl. 218): 7-81.

98) Caldarera, C.M. and Moruzzi, G. (1970). Ann. N.Y. Acad. Sci., 171: 709-722.

99) Janne, J. and Raina, A. (1969). Biochim. Biophys. Acta, 174: 769-772.

100) Kaye, A.M., Icekson, I. and Lindner, H.R. (1971). Biochim. Biophys. Acta, 252: 150-159.

101) Bachrach, U. (1976). FEBS Letters, 68: 63-67.

102) Hibasami, H., Tanaka, M., Nagai, J. and Ikeda, T. (1978). Australian J. Exp. Biol. Med. Sci., 56: 279-285.

103) Mamont, P.S., Duchesne, M.C., Grove, J. and Bey, P. (1978). Biochem. Biophys. Res. Commun., 81: 58-66.

104) Rupniak, H.T. and Paul, D. (1978). J. Cell. Physiol., 94: 161-170.

105) Andersson, G. and Heby, O. (1977). Cancer Res., 37: 4361-4366.

106) Heby, O., Marton, L.J., Wilson, C.B. and Martinez, H.M. (1975). J. Cell. Physiol., 86: 511-522.

107) Fillingame, R.H. and Morris, D.R. (1973). Biochemistry, 12: 4479-4487.

108) Williams-Ashman, H.G., Coppoc, G.L. and Weber, G. (1972). Cancer Res., 32: 1924-1932.

109) Tomino, I., Katunuma, N. and Morris, H.P. (1974). Cancer Res., 34: 627-636.

110) Weber, G., Queener, S.F. and Morris, H.P. (1972). Cancer Res., 32: 1933-1940.

111) Goldstein, D.A., Heby, O. and Marton, L.J. (1976). Proc. Natl. Acad. Sci. USA, 73: 4022-4026.

112) Heby, O., Gray, J.W., Lindl, P.A., Marton, L.J. and Wilson, C.B. (1976). Biochem. Biophys. Res. Commun., 71: 99-105.

113) Heby, O., Marton, L.J., Gray, J.W., Lindl, P.A. and Wilson, C.B. (1976). In: Proceedings of the 9th Congress of the Nordic Society for Cell Biology (F. Bierring, ed.,) pp. 155-164. Odense, Denmark: Odense University Press.

114) Hibasami, H., Tanaka, M., Nagai, J. and Ikeda, T. (1977). Australian J. Exp. Biol. Med. Sci., 55: 379-383.

115) Rahiala, E.L., Kekomaki, M., Janne, J., Raina, A. and Raiha, N.C.R. (1971). Biochim. Biophys. Acta, 227: 337-343.

116) Skinner, W.A. and Johansson, J.G. (1972). J. Med. Chem., 15: 427-428.

117) Harik, S.I. and Snyder, S.H. (1973). Biochim. Biophys. Acta, 327: 501-509.

118) Harik, S.I., Pasternak, G.W. and Snyder, S.H. (1973). In: Polyamines in Normal and Neoplastic Growth (D.H. Russell, ed.) New York: Raven Press.

119) Abdel-Monem, M.M., Newton, N.E. and Weeks, C.E. (1974). J. Med. Chem., 17: 447-451.

120) Abdel-Monem, M.M., Newton, N.E., Ho, B.C. and Weeks, C.E. (1975). J. Med. Chem., 18: 600-604.

121) O'Leary, M.H. and Herreid, R.M. (1978). Biochemistry, 17: 1010-1014.

122) Abdel-Monem, M.M., Newton, N.E. and Weeks, C.E. (1975). J. Med. Chem., 18: 945-948.

123) Relyea, N. and Rando, R.R. (1975). Biochem. Biophys. Res. Commun., 67: 392-402.

124) Bey, P., Danzin, C., Van Dorsselear, V., et. al. (1978). J. Med. Chem., 21: 50-55.

125) Metcalf, B.W., Bey, P., Danzin, C., et. al. (1978). J. Am. Chem. Soc., 100: 2551-2553.

126) Abdel-Monem, M.M. and Mikhail, E.A. (1978). J. Pharm. Sci., 67: 1174-1175.

127) Heller, J.S., Canellakis, E.S., Bussolotti, D.L. and Coward, J.K. (1975). Biochim. Biophys. Acta, 403: 197-207.

128) Poso, H. and Janne, J. (1976). Biochem. Biophys. Res. Commun., 69: 885-892.

129) Harik, S.I., Hollenberg, M.D. and Snyder, S.H. (1974). Nature, 249: 250-251.

130) Inoue, H., Kato, Y., Takigawa, M., et. al. (1975). J. Biochem. (Tokyo), 77: 879-893.

131) Kato, Y., Inoue, H., Gohda, E., et. al. (1976). Gann, 67: 569-576.

132) Kato, Y., Nishihara, H., Inoue, H., et. al. (1978). J. Biochem. (Tokyo), 84: 1617-1623.

133) Rupniak, H.T. and Paul, D. (1978). J. Cell. Physiol., 96: 261-264.

134) Danzin, C., Jung, M.J., Metcalf, B.W., et. al. (1979). Biochem. Pharmacol., 28: 627-631.

135) Danzin, C., Jung, M.J., Seiler, N. and Metcalfe, B.W. (1979). Biochem. Pharmacol., 28: 633-639.

136) Raina, A., Janne, J. and Siimes, M. (1964). Acta Chem. Scand., 18: 1804-1806.

137) Pegg, A.E. (1969). Biochim. Biophys. Acta, 177: 361-364.

138) Williams-Ashman, H.G. and Schenone, A. (1972). Biochem. Biophys. Res. Commun., 46: 288-295.

139) Corti, A., Dave, C., Williams-Ashman, H.G., et. al. (1974). Biochem. J., 139: 351-357.

140) Pegg, A.E. (1978). J. Biol. Chem., 253: 539-542.

141) Wiegand, L. and Pegg, A.E. (1978). Biochim. Biophys. Acta, 517: 169-180.

142) Hibasami, H. and Pegg, A.E. (1978). Biochem. Biophys. Res. Commun., 81: 1398-1405.

143) Pegg, A.E. (1973). Biochem. J., 132: 537-540.

144) Pegg, A.E., Corti, A. and Williams-Ashman, H.G. (1973). Biochem. Biophys. Res. Commun., 52: 696-701.

145) Holtta, E., Hannonen, P., Pispa, J. and Janne, J. (1973). Biochem. J., 136: 669-676.

146) Pegg, A.E. and Jefferson, L.S. (1974). FEBS Letters, 40: 321-324.

147) Oka, T. and Perry, J.W. (1974). J. Biol. Chem., 249: 7647-7652.

148) Feil, P.D., Pegg, A.E., Demers, L.M. and Bardin, C.W. (1977). Biochem. Biophys. Res. Commun., 75: 1-6.

149) Sakai, T., Lundgren, D.W. and Oka, T. (1978). J. Cell. Physiol., 95: 259-268.

150) Fillingame, R.H. and Morris, D.R. (1973). Biochem. Biophys. Res. Commun., 52: 1020-1025.

151) Fillingame, R.H., Jorstad, C.M. and Morris, D.R. (1975). Proc. Natl. Acad. Sci. USA, 72: 4042-4045.

152) Knutson, J.C. and Morris, D.R. (1978). Biochim. Biophys. Acta, 520: 291-301.

153) Kay, J.E. and Pegg, A.E. (1973). FEBS Letters, 29: 301-304.

154) Holtta, E., Janne, J. and Hovi, T. (1979). Biochem. J., 178: 109-117.

155) Otani, S., Mizoguchi, Y., Matsui, I. and Morisawa, S. (1974). Molec. Biol. Rep., 1: 431-436.

156) Heby, O., Andersson, G. and Gray, J.W. (1978). Exp. Cell Res., 111: 461-464.

157) Krokan, H. and Erikson, A. (1977). Eur. J. Biochem., 72: 501-508.

158) Melvin, M.A.L. and Keir, H.M. (1978). Biochem. J., 174: 349-352.

159) Sunkara, P.S., Pargac, M.B., Nishioka, K. and Rao, P.N. (1979). J. Cell. Physiol., 98: 451-458.

160) Sunkara, P.S., Rao, P.N., Nishioka, K. and Brinkley, B.R. (1979). Exp. Cell Res., 119: 63-68.

161) Pathak, S.N., Porter, C.W. and Dave, C. (1977). Cancer Res., 37: 2246-2250.

162) Dave, C., Pathak, S.N. and Porter, C.W. (1978). In: Advances in Polyamine Research (R.A. Campbell, et. al., eds.) pp. 153-171. New York: Raven Press.

163) Mamont, P.S., Bohlen, P., McCann, P.P., et. al. (1976). Proc. Natl. Acad. Sci. USA, 73: 1626-1630.

164) Mamont, P.S., Duchesne, M.C., Joder-Ohlenbusch, A.M. and Grove, J. (1978). In: Enzyme Activated Irreversible Inhibitors (N. Seiler, et. al., eds.) pp. 43-54. Amsterdam: Elsevier/North Holland Biomedical Press.

165) McCann, P.P., Tardif, C., Duchesne, M.C. and Mamont, P.S. (1977). Biochem. Biophys. Res. Commun., 76: 893-899.

166) Mamont, P.S., Duchesne, M.C., Grove, J. and Tardif, C. (1978). Exp. Cell Res., 115: 387-393.

167) Prakash, N.J., Schechter, P.J., Grove, J. and Koch-Weser, J. (1978). Cancer Res., 38: 3059-3062.

168) Morris, D.R., Jorstad, C.M. and Seyfried, C.E. (1977). Cancer Res., 37: 3169-3172.

169) Newton, N.E. and Abdel-Monem, M.M. (1977). J. Med. Chem., 20: 249-253.

170) Weeks, C.E. and Abdel-Monem, M.M. (1977). J. Pharm. Sci., 66: 1586-1589.

171) Holtta, E., Pohjanpelto, P. and Janne, J. (1979). FEBS Letters, 97 : 9-14.

172) Marton, L.J. and Heby, O. (1974). Int. J. Cancer, 13: 619-628.

173) Heby, O., Marton, L.J., Wilson, C.B. and Martinez, H.M. (1975). FEBS Letters, 50: 1-4.

174) Heby, O., Marton, L.J., Wilson, C.B. and Gray, J.W. (1977). Europ. J. Cancer, 13: 1009-1017.

175) Barker, M., Hoshino, T., Gurcay, O., et. al. (1973). Cancer Res., 33: 976-986.

176) Benda, P., Someda, K., Messer, J. and Sweet, W.H. (1971). J. Neurosurg., 34: 310-323.

177) Deen, D.F. and Hoshino, T. (1978). American Laboratory, 10: 115-119.

178) Marton, L.J. and Lee, P.L.Y. (1975). Clin. Chem., 21: 1721-1724.

179) Marton, L.J., Edwards, M.S., Levin, V.A., et. al. (1979). Cancer Res., 39: 993-997.

180) Crissman, H.A. and Tobey, R.A. (1974). Science, 184: 1297-1298.

181) Van Dilla, M.A., Trujillo, T.T., Mullaney, P.F. and Coulter, J.R. (1969). Science, 163: 1213-1214.

182) Van Dilla, M.A., Steinmetz, L.L., Davis, D.T., et. al. (1974). IEEE Trans. Nucl. Sci., NS-21: 714-721.

183) Gray, J.W., Carrano, A.V., Steinmetz, L.L., et. al. (1975). Proc. Natl. Acad. Sci. USA, 72: 1231-1234.

184) Gray, J.W. (1974). J. Histochem. Cytochem., 22: 642-650.

185) Nomura, K., Hoshino, T., Knebel, K. and Barker, M. (1978). In Vitro, 14: 174-179.

186) Nomura, K., Hoshino, T., Knebel, K., et. al. (1978). Cancer Treat. Rep., 62: 747-754.

187) Puck, T.T. and Steffen. (1963). Biophys. J., 3: 379-397.

188) Wheeler, K.T., Deen, D.F., Wilson, C.B., et. al. (1977). Int. J. Radiation Oncology Biol. Phys., 2: 79-88.

189) Rosenblum, M.L., Knebel, K.D., Wheeler, K.T., et. al. (1975). In Vitro, 11: 264-273.

190) Pegg, A.E. and Williams-Ashman, H.G. (1968). Biochem. J., 108: 533-539.

191) Gosule, L.C. and Schellman, J.A. (1978). J. Mol. Biol., 121: 311-326.

192) Chattoraj, D.K., Gosule, L.C. and Schellman, J.A. (1978). J. Mol. Biol., 121: 327-337.

193) Cember, H. (1969). Introduction to Health Physics . Oxford, England: Pergamon Press. pp. 179-181.

194) Johns,H.E. and Cunningham, J.R. (1969). The Physics of Radiology (3rd ed.). Springfield, Ill: Charles C. Thomas. pp. 680-686.

195) Rosiek, O., Bocian, E. and Ziemba-Zak, B. (1975). Studia Biophys., 53: 153-154.

196) Bocian, E., Rosiek, O. and Ziemba-Zoltowska, B. (1978). Bull. Acad. Pol. Sci., 26: 639-643.

197) Schwimmer, S. (1968). Biochim. Biophys. Acta, 166: 251-254.