EFFECTS OF SPERMINE ON LETTRE H ASCITES TUMOR CELLS

By

James Phillip Rupp

Bachelor of Arts

Western State College

Gunnison, Colorado

1962

Submitted to the faculty of the Graduate School
of the Oklahoma State University
in partial fulfillment of the requirements
for the degree of
MASTER OF SCIENCE
August, 1965

EFFECTS OF SPERMINE ON LETTRE H

ASCITES TUMOR CELLS

Thesis Approved:

Franklin R. Leach
Thesis Advisor

Kurt E. Ebner

George V. Odell

Dean of the Graduate School

ACKNOWLEDGEMENTS

Thanks are due to Dr. Franklin R. Leach for guidance, assistance and counsel given me in the writing of this thesis and in the research preliminary to it.

I would also like to thank Dr. Kurt E. Ebner and George V. Odell for advice and encouragement in preparing the manuscript.

Special plaudits are due to my wife, Margaret for encouragement, moral support and technical assistance during the course of these studies.

This study was made possible through the facilities of the Department of Biochemistry and financial aid provided by the Agricultural Experiment Station and the American Cancer Society.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

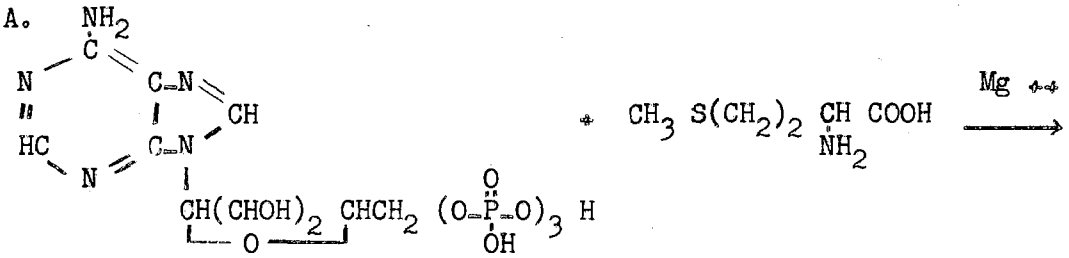
CHAPTER I

INTRODUCTION

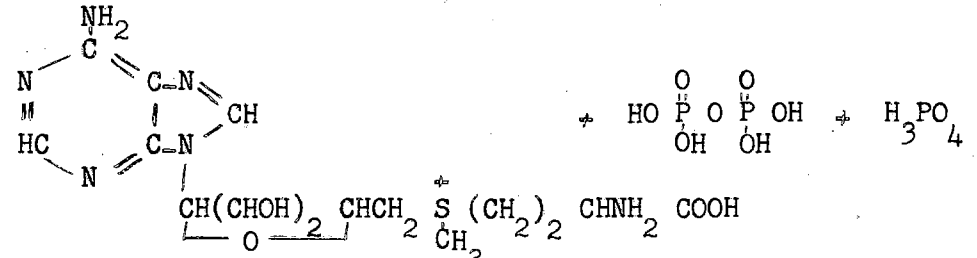
The polyamines, spermine and spermidine, are widely distributed. Either or both appear in some bacteria, bacteriophages, yeasts, silkworm pupae, sea urchins and certain plant alkaloids (1). They are found in various tissues of small laboratory animals, apparently of endogenous origin (2). Spermine is widely distributed in both male and female human tissue, being highest in semen, prostrate and pancreas (3). Despite their wide distribution, specific metabolic functions of these polyamines remain obscure.

Biosynthesis of spermidine in microorganisms has been shown to occur by the following series of reactions:

A.

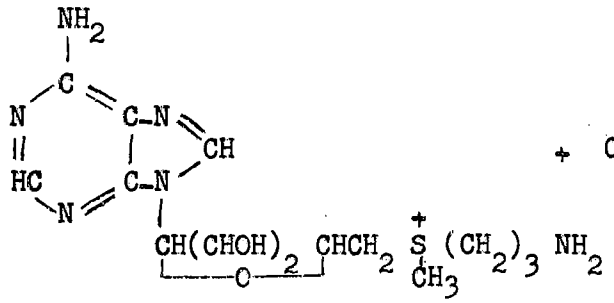


Adenosine triphosphate L-methionine



S-adenosyl methionine (I) Pyrophosphoric acid

B. (I) $\xrightarrow{Mg^{++}}$

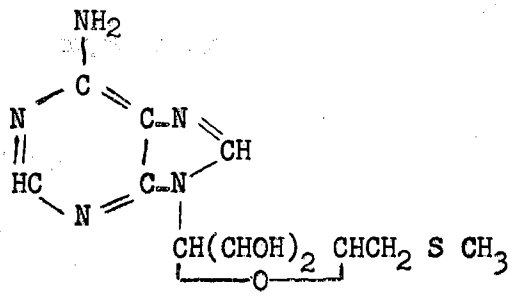


+ CO_2

5'-deoxyadenosyl-(5'), 3 aminopropyl-(1), methylsulfonium salt (II)

C. (II) + $H_2N(CH_2)_4NH_2$ $\longrightarrow$

1,4-butanediamine (putrescine)



+ $H_2N(CH_2)_4NH(CH_2)_3NH_2$

methylthioadenosine spermidine

Tabor, Tabor and Rosenthal (1, 4, 6) and Green (5), have shown that ^{14}C and ^{15}N labeled putrescine is incorporated into both spermidine and spermine in several microorganisms and in cell free extracts. Spermine biosynthesis presumably occurs by way of a reaction similar to C, with spermidine in place of putrescine.

Weaver and Herbst have reported degradation of spermine and spermidine to 1,3-propanediamine in Hemophilus parainfluenzae, Neisseria perflava, and Pasteurella tularensis (7). Spermine and spermidine are converted to monoacetyl and diacetyl products by Escherichia coli B. Essentially no degradation of these acetylated products occurs (8). Enzymatic degradation of spermine and spermidine by a plasma amine

oxidase has been demonstrated. This enzyme, termed spermine oxidase, has been purified some 200-fold (9, 10). Spermine oxidase activity appears to be confined to the plasma of ruminants and to be associated with rumen activity (11, 12). This enzyme is distinct from intracellular monoamine oxidase which also possesses activity toward spermine and spermidine (13). Purified beef serum spermine oxidase oxidizes spermine and spermidine according to:

$$\text{Spermine} \xrightarrow{O_2} H_2NCH_2(CH_2)_2NH(CH_2)_4NH(CH_2)_2CHO \text{ (IV)} \xrightarrow{O_2}$$
$$OHC(CH_2)_2NH(CH_2)_4NH(CH_2)_2CHO \text{ (V)} + 2NH_3 + 2H_2O_2$$
$$\text{Spermidine} \xrightarrow{O_2} OHC(CH_2)_2NH(CH_2)_4NH_2 \text{ (VI)} + NH_3 + H_2O_2$$

with stoichiometric formation of the corresponding aldehydes, ammonia and H_2O_2. The compounds (IV), (V) and (VI) should easily undergo β-elimination, and indeed incubation mixtures containing (V) or (VI) heated to 100° do give putrescine. When (IV) is present, spermidine is formed. Neither spermidine nor putrescine appear in incubation mixtures after six hours at 37° (10, 14, 15).

Polyamines are required as a growth factor in H. parainfluenzae (16, 17) a mutant of Aspergillus nidulans (18), N. perflava (19), P. tularensis (20) and Chinese hamster cells grown in a protein free synthetic nutrient mixture (21). They promote growth in Lactobacilli (22) and some other microorganisms (1), while exhibiting a bacteriostatic or bactericidal effect in Staphylococcus aureus (23), tubercle bacilli (24, 25), E. coli (26) and various other organisms (27).

A product isolated from beef heart or skeletal muscle, ultimately identified as spermine, was found to inhibit growth of several mammalian cell lines grown in tissue culture when calf serum was present in the

medium. Inhibition did not occur when the cultures were grown with horse serum in place of the calf serum. Spermine oxidase, present in calf serum but not in horse serum, was found to enzymatically modify spermine to produce inhibition (28). Inhibition in this case was reported to be due to a combination of effects of H_2O_2 and acrolein, which both are presumed to be enzymatic degradation products of spermine (29, 30). In several cases, inhibitory properties of spermine are attributed to enzymatic breakdown products of spermine itself (23, 24, 25, 26).

Recent work by Goldstein (33), using the Walker carcinosarcoma and by Ochoa and Weinstein (34), using a subcellular system from L 1210 mouse ascites leukemia, has shown inhibition of protein synthesis by spermine which is apparently not due to the activity of spermine oxidase. The inhibition appears to be due to interference by spermine at the level of the ribosome-m-RNA-s-RNA complex[1], by a mechanism similar to the interference by streptomycin (35). Spermine, spermidine and putrescine also show an antagonistic effect to streptomycin in cell free extracts of E. coli, indicating the possibility of the action of these polyamines and of streptomycin at a common site (36).

In vivo studies with small laboratory animals showed marked kidney damage when spermine was injected intraperitoneally. This damage could be largely reversed by concurrent injections of purified spermine ox-

---

[1]Abbreviations used are: m-RNA for messenger ribonucleic acid; s-RNA for soluble ribonucleic acid; RNase for ribonuclease; DNA for deoxyribonucleic acid; DNase for deoxyribonuclease; I for inosinic acid; C for cytidylic acid; A for adenylic acid; U for uridylic acid; RNA for ribonucleic adid.

idase (2, 31). Spermine and spermidine have been shown to bind with cellular nucleic acid in H. parainfluenzae and spermine has been shown to be a potent RNase and DNase inhibitor (37). Polyamines are also known to act as a stabilizer for certain bacteriophages by way of nucleic acid stabilization (38). Conductimetric titration studies have shown that polyamines, particularly spermine, react with the phosphate groups of the backbone of DNA, causing release of base bound protons into solution. Spermine shows a type of selective "site binding" with polyribonucleotides, since it forms a soluble, ordered structure with poly(I + C) between pH's 6.8 and 4.5[2], but under similar conditions, forms a precipitate with poly(A + U). The solubility of poly(I + C) with spermine resembles that of s-RNA, which has a high percentage of guanine and cytosine (39). Spermine causes bunching of chromosomes at anaphase in certain plant cells (40) and has been shown to be essential to transformation in a human cell line, possibly because it protects the transforming DNA from nucleases (40, 41). A wide variety of polyamines are incorporated into protein by an enzyme from the soluble fraction of guinea pig liver (42). Spermine has recently been shown to produce a 4-fold stimulation of the rate of formyltetrahydrofolate formation by the enzyme tetrahydrofolate formylase in preparations purified 100 to 200-fold from Lactobacilli (43).

The objective of this study was to clarify further the role of the polyamine, spermine, in the life processes of Lettre H ascites tumor cells, in order to provide conditions which would protect DNA in trans-

---

[2]Spermine carries four positive charges throughout this pH range.

formation experiments. The approach was to investigate the effects of spermine administration on cells growing in tissue culture. Variations in the time of administration of inhibitor to the cells and in growth conditions were carried out. Isolation and assay of the cell components DNA, RNA and protein from cells under normal and inhibitory conditions were conducted. Microautoradiography using tritium labeled thymidine was undertaken to elucidate the portion of the cell life cycle at which spermine is involved, and the mode of inhibition.

CHAPTER II

METHODS AND MATERIALS

A. Cell line used

The Lettre H line of cells, developed for suspension culture by R. M. Humphrey at the M. D. Anderson Hospital and Tumor Institute, Houston, Texas, was used in these studies. This line is characterized cytologically by 44 chromosomes per cell and 20% polyploidy. Generation time in suspension is around 12-14 hours under Houston conditions. This line was isolated from mouse ascites tumors in July, 1960. Samples were graciously provided by Dr. Humphrey in March, 1961, from which time they have been cultured routinely in this laboratory.

B. Culture techniques

The cells were serially cultured in McCoy's 5a, 10% pooled adult calf serum (44). Serum was purchased from Microbiological Associates. Cells were grown at 37° in a 30 ml volume in sterile, stoppered 125 ml Erlenmeyer flasks, using standard tissue culture techniques for their handling (45). Suspension was maintained by rotary shaking at 50 oscillations per minute on a New Brunswick Scientific Co. rotary shaker, Model G 10. Spermine and spermidine were purchased from California Corporation for Biochemical Research, and were sterilized by filtration of aqueous solutions through Millipore filters (Millipore Filter Corp., Bedford, Mass.).

C. Cell counts

Cell populations were determined by counting on a Model B electronic counter from the Coulter Electronics Co. After pipetting the suspension cultures up and down two or three times in a 10 ml pipette in order to seperate cells which might be clumped together, 0.2 ml samples were removed and diluted in 19.8 ml of 0.9% aqueous NaCl. These dilutions were made under a sterile hood, with care being taken to avoid contamination of the cultures. Dilutions were mixed thoroughly by pipetting up and down three or four times. Each sample was then counted four times. Averaging these four counts and correcting for dilution gave the cells per ml in suspension.

D. Preparation of conditioned medium

Approximately $3x10^5$ Lettre H cells per ml were suspended in 30 ml suspensions of freshly prepared McCoy's 5a, 10% pooled adult calf serum. Ten of these suspensions were incubated with shaking, at 37° for 48 hours. The suspensions were then removed under sterile conditions to capped centrifuge tubes and centrifuged at 1000 rpm on a clinical centrifuge (International Equipment Co., Boston) for 7 minutes. The supernatant solutions were pooled and combined with an equal volume of freshly prepared sterile McCoy's 5a, 10% pooled adult calf serum.

E. DNA, RNA and protein determinations

1. Isolation

Cells were counted and cultures were harvested at 0, 12, 24, 48, and 96 hours after subculture. Harvesting was by centrifugation at 1000 rpm in a clinical centrifuge (International Equipment Co., Boston). After washing the cells twice with Hanks' balanced salt solution (46),

RNA was extracted by addition of 5 ml of cold 1.0 N perchloric acid, allowing this to stand overnight at 4°, and centrifuging in the cold at 1000 rpm for 10 minutes. The residue was then washed twice with 5 ml portions of cold 1.0 N perchloric acid. These three perchloric acid fractions were combined into fraction A for RNA assays. This fraction also contains the soluble nucleotides which would be detected by the assays used. To the residue, 5 ml of 0.5 N perchloric acid were added. This mixture was heated to 70° for 20 minutes and centrifuged at 1000 rpm for 10 minutes. After repeating this hot perchloric acid extraction procedure with another 5 ml of 0.5 N perchloric acid, the two hot perchloric acid fractions were combined into fraction B for DNA assays. The residue C was saved for protein assay.

2. Assay

The RNA and nucleotide content of fraction A was determined by measuring the absorbance at 260 mμ, compared to a standard RNA solution prepared from commercial grade yeast RNA from the California Corporation for Biochemical Research. The RNA of fraction A was also determined by a colorimetric assay for RNA using orcinol reagent (47), compared to the same standard. Orcinol was purchased from Mann Laboratories, Inc. Feric chloride was reagent grade from the W. H. Curtin Co., and hydrochloric and acetic acids were both reagent grade from the J. T. Baker Chemical Co.

The DNA content of fraction B was determined by measuring the absorbance at 260 mμ compared to a DNA standard prepared from commercial grade herring sperm DNA from the California Corporation for Biochemical Research. The DNA content of fraction B was also determined by a color-

imetric assay for DNA using diphenylamine (48), compared to the same standard. Reagent grade diphenylamine was obtained from the Fischer Scientific Co. and acetic and sulfuric acids were both reagent grade from the J. T. Baker Chemical Co.

The residue C was solubilized in 0.5 ml of 1.0 N NaOH and assayed for protein according to the method of Lowry, Rosebrough, Farr and Randall (49). Bovine serum albumin (Armour Laboratories, Chicago) was used as a protein standard.

F. Spermine oxidase[3] assay

Incubation mixtures of McCoy's 5a, 20% calf serum, with and without cells, were assayed for spermine oxidase activity. These mixtures were incubated at 37° with shaking and 5 ml samples were removed at 0, 1, 2, 6, 18, 24, and 48 hours after subculture. The samples were centrifuged at 1000 rpm on a clinical centrifuge (International Equipment Co., Boston) for 10 minutes. The supernatant solution was then carefully decanted for assay of the enzyme. Enzymatic activity was followed spectrophotometrically on a Beckman DU-Gilford Multiple Sample Absorbance system by the method of Tabor, Tabor and Rosenthal (50). This assay depends on the increase of absorbance at 250 mμ which accompanies conversion of the substrate, benzylamine, to benzaldehyde. The assay mixture consisted of 1 ml of 0.2 M potassium phosphate, pH 7.2, 1 ml of enzyme containing solution, 0.1 ml of 0.1 M benzylamine sulfate and H_2O to a final 3 ml volume. The blank was the same without benzylamine. Readings were made in Quartz cuvettes at 250 mμ each minute for 5 min-

---

[3]Classification of the Commission on Enzymes of the International Union of Biochemistry, 1961, is 1.5.3.3 and the systematic name is Spermine: O_2 oxidoreductase.

utes. Benzylamine was from the Kodak Co. and all other chemicals for this assay were reagent grade from the J. T. Baker Chemical Co.

G. Reversal studies

Agents added to incubations in an effort to reverse spermine inhibition included DNA, RNA, polyphosphate and carboxymethylcellulose. DNA was from Lettre H cells, isolated in this laboratory by Dr. M. L. Higgins (51). Purified yeast RNA was prepared from commercial bakers yeast in this laboratory by Mr. S. Erhan (52). Polyphosphate was synthesized by fusing sodium dihydrogen phosphate at 500° and cooling rapidly (53).

H. Studies with tritium labeled thymidine

Analysis of the position in the cell life cycle at which inhibition occured was undertaken by preparation of incubation mixtures containing 0.25 µg per ml of colcimid, 20 µg per ml of spermine, 5.6 µg per ml of acrolein, 0.25 µg per ml of colcimid and 5.6 µg per ml of acrolein, and 0.25 µg of colcimid and 20 µg per ml of spermine. Colcimid was a gift from Ciba Chemical Co. Acrolein was synthesized according to the method of Adkins and Hartung (54). Acrolein was used immediately after synthesis, so that hydroquinone stabilizer could be omitted from the final distillate. The 53-55° boiling fraction was taken. Glycerol and acid potassium sulfate for this synthesis were obtained from the J. T. Baker Chemical Co.

One hundred microcuries of tritiated thymidine, obtained from Schwarz Bioresearch Inc., were added to each of the above incubation mixtures. From each mixture, 2 ml samples were removed at 0, 2, 4, 6, 10, 20, 30 and 50 hours after subculture, and slides were prepared by way of a modification of the method of Puck and Steffan (55). Puck's

trypsin treatment was omitted since the cells were already in suspension. The cells were centrifuged in cold tubes for 10 minutes at 1000 rpm on a clinical centrifuge. The supernatant solution was discarded and 2 ml of 0.1 M citric acid were added to the residue. After warming at 37° for 30 seconds and centrifuging, the supernatant solution was again discarded. The residue was then treated with 1 ml of a 3:1::ethanol: glacial acetic acid mixture for four minutes, after which the cells were suspended in this same mixture and 2 drops of the suspension were placed on a slide and spread by gently blowing on the drop. Following air drying the slides were stained with 1% orcein in 50% acetic acid. Emulsions were imposed on the stained slides with Kodak AR-10 stripping plates, according to the suggested procedure of the Kodak Co. accompanying the emulsion preparations. After 6 days exposure, the emulsions were developed in Kodak D-19 developer for 8 minutes, fixed, washed and dried.

CHAPTER III

RESULTS

A. Effects of polyamine concentration on inhibition

Spermine was found to produce total growth inhibition at concentrations of 10 µg per ml of medium and above, with lesser amounts causing intermediate degrees of inhibition, as may be seen from Figure 1. Approximately 50% inhibition is observed at 3 µg of spermine per ml. In order to obtain equivalent inhibition with spermidine, approximately 3 times as much spermidine was required on a weight basis, or nearly 4 times the molar concentration, as may be seen from Figure 2. The inhibitors were added to the suspensions immediately after subculture into fresh medium.

Considerable variation in the rate of growth of different subcultures was observed although duplicate samples from the same subculture grew at a fairly uniform rate. Some variation in the degree of inhibition in different subcultures from a given concentration of inhibitor was also observed. This may have been due to variations in the amount of spermine oxidase in different lots of the serum used or to the percentage of cells which were in some particular stage of the life cycle more susceptible to inhibition at the time of subculture. At no time were cells used which had previously been exposed to the inhibitors.

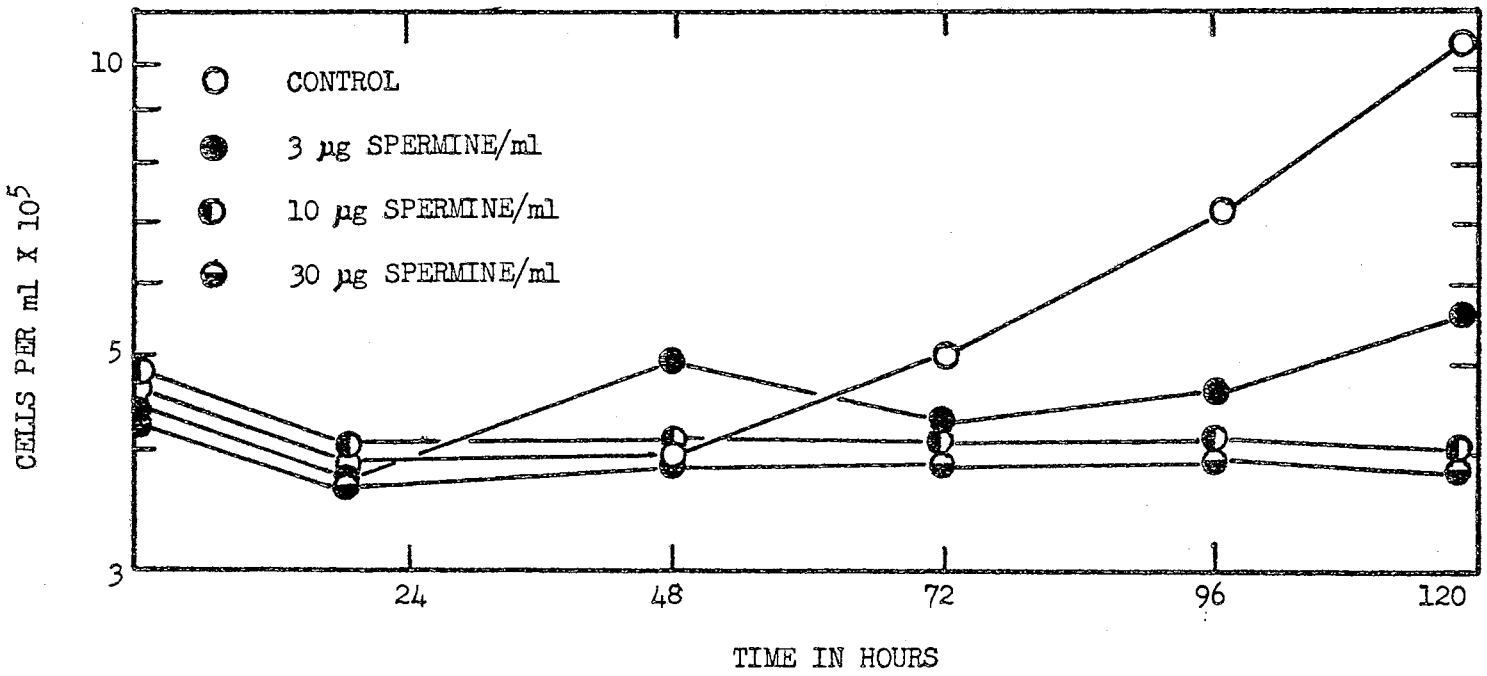


Figure 1. The effect of spermine concentrations on growth of Lettre H ascites tumor cells. Spermine was added at 0 hours after subculture and cells were grown for 5 days in McCoy's 5a, 10% pooled adult calf serum.

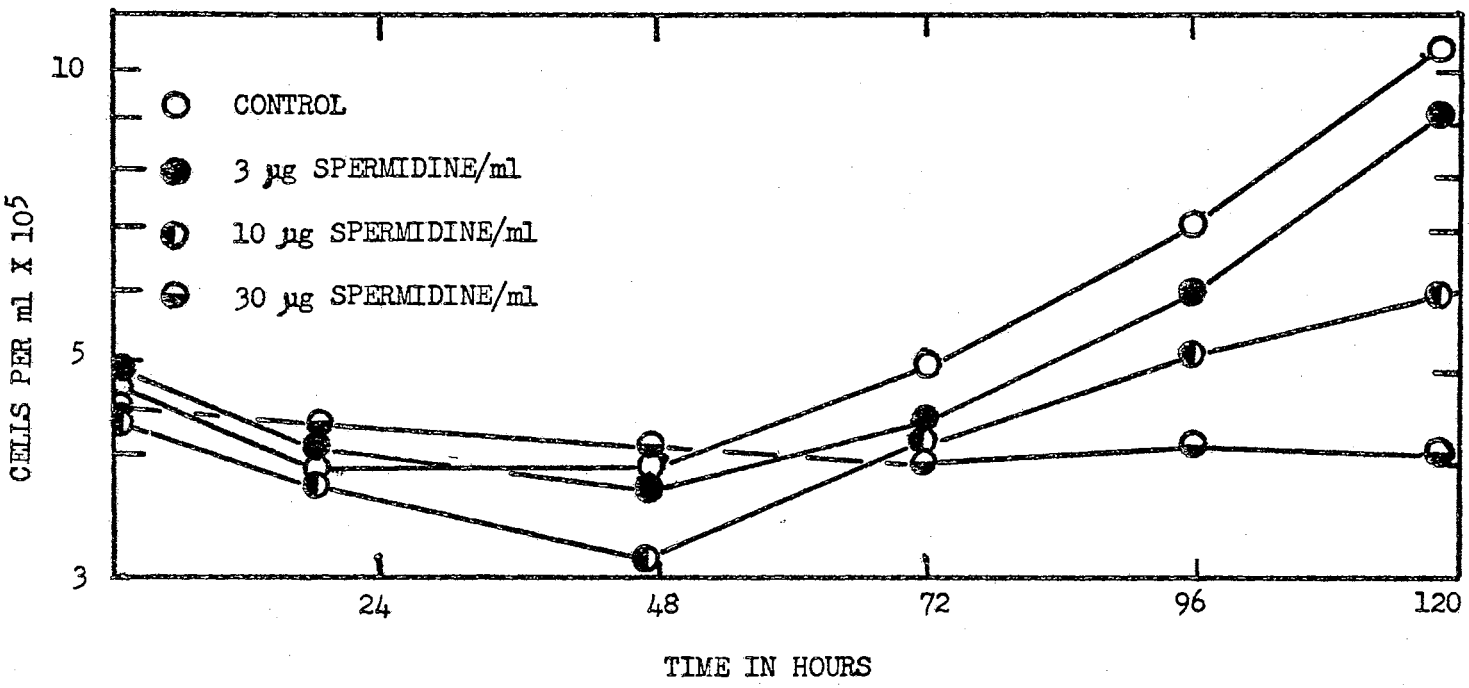


Figure 2. Effect of spermidine concentration on growth of Lettre H ascites tumor cells. Spermidine was added at 0 hours after subculture and cells were grown for 5 days in the same medium; McCoy's 5a, 10% pooled adult calf serum.

B. Inhibition reversal studies

1. Time of addition

Since considerable variation in the rate of growth of different subcultures and in the lag period prior to the beginning of growth was observed, difficulties arose in establishing levels of inhibition. Cells innoculated from rapidly growing cultures frequently did not begin to increase in number for 24 to 48 hours after subculture into new medium. In an effort to use established cells for inhibition studies, the inhibitors were not added until after growth had begun. This revealed that growth inhibition occured only if the inhibitor was added immediately after subculture. Figure 3 shows a typical example of the effect of prior incubation on spermine and spermidine inhibition.

2. Conditioned medium

When cells were subcultured in conditioned medium, no inhibition of growth by spermine or spermidine was observed, as may be seen from Figure 4. This lack of inhibition was independent of the time at which the inhibitor was added.

3. DNA, RNA, carboxymethylcellulose and polyphosphate reversal

In an effort to discover the nature of the factor in the medium which reversed spermine inhibition, Lettre H cell DNA, purified yeast RNA, carboxymethylcellulose or polyphosphate was added to incubation mixtures. If any of these substances reversed inhibition, they might indicate a mechanism of inhibition. Figure 5 shows the effect of the addition of DNA. No reversal of inhibition was obtained with DNA, although the DNA itself causes some retardation of growth in higher concentrations. A similar situation is observed with RNA, as may be seen from Figure 6. Considerable difficulty in obtaining accurate counts

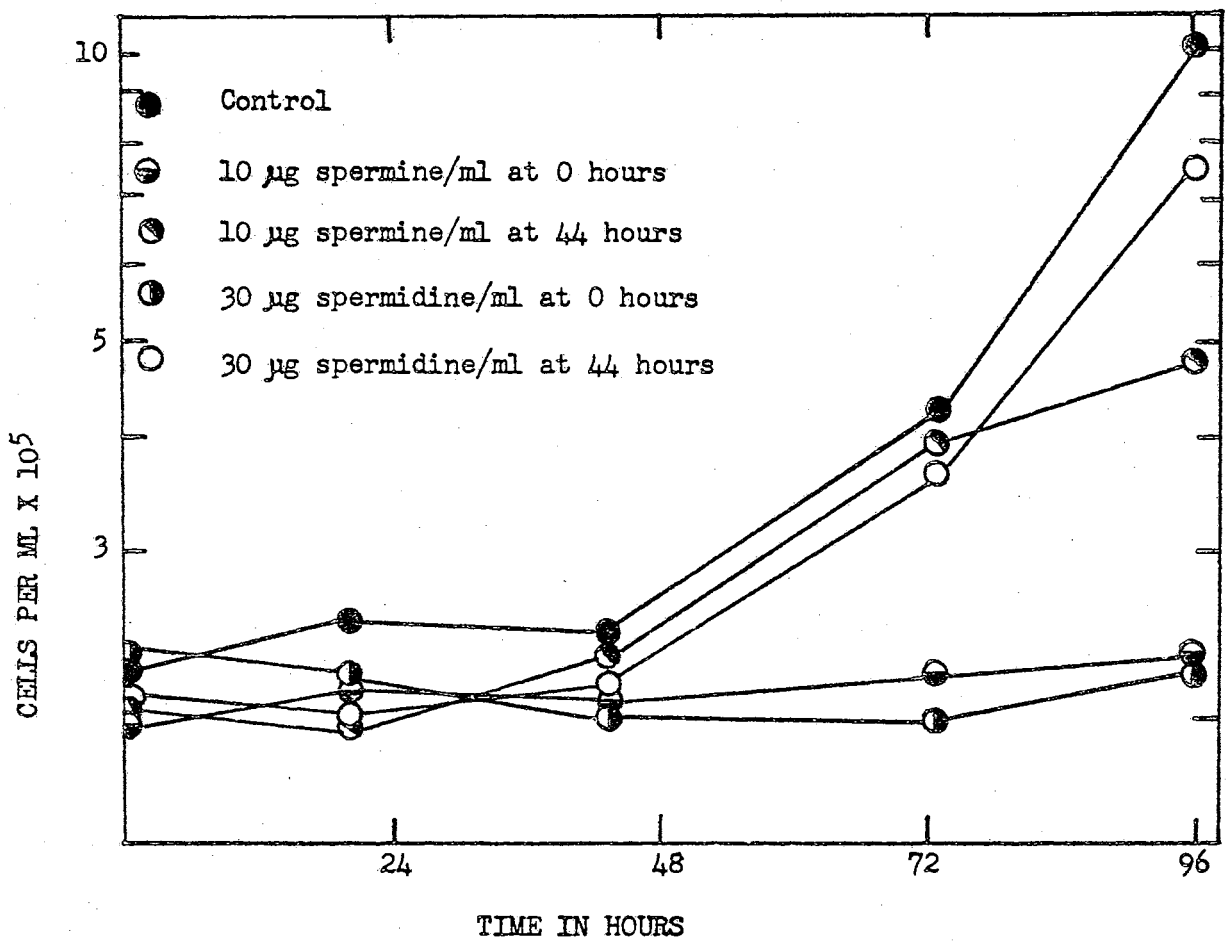


Figure 3. Effect of time of addition on inhibition of Lettre H ascites tumor cells by spermine and spermidine. Cells were grown in McCoy's 5a, 10% pooled adult calf serum for 4 days. Inhibitiors were added at 0 and 44 hours after subculture.

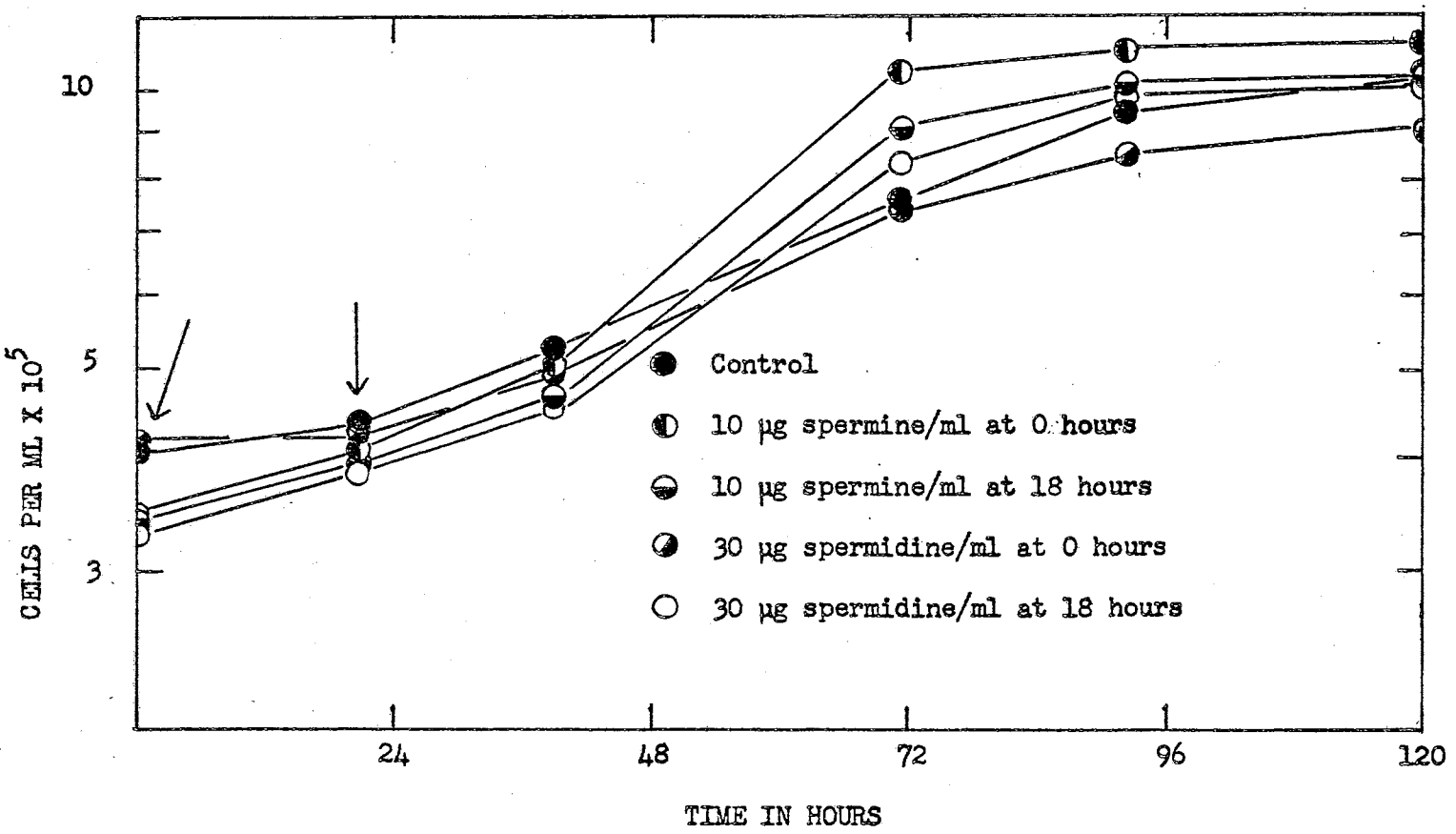


Figure 4. Effect of conditioned medium on inhibition of Lettre H ascites tumor cells by spermine and spermidine. Cells were grown in the same medium for 5 days. Inhibitors were added at 0 or 18 hours.

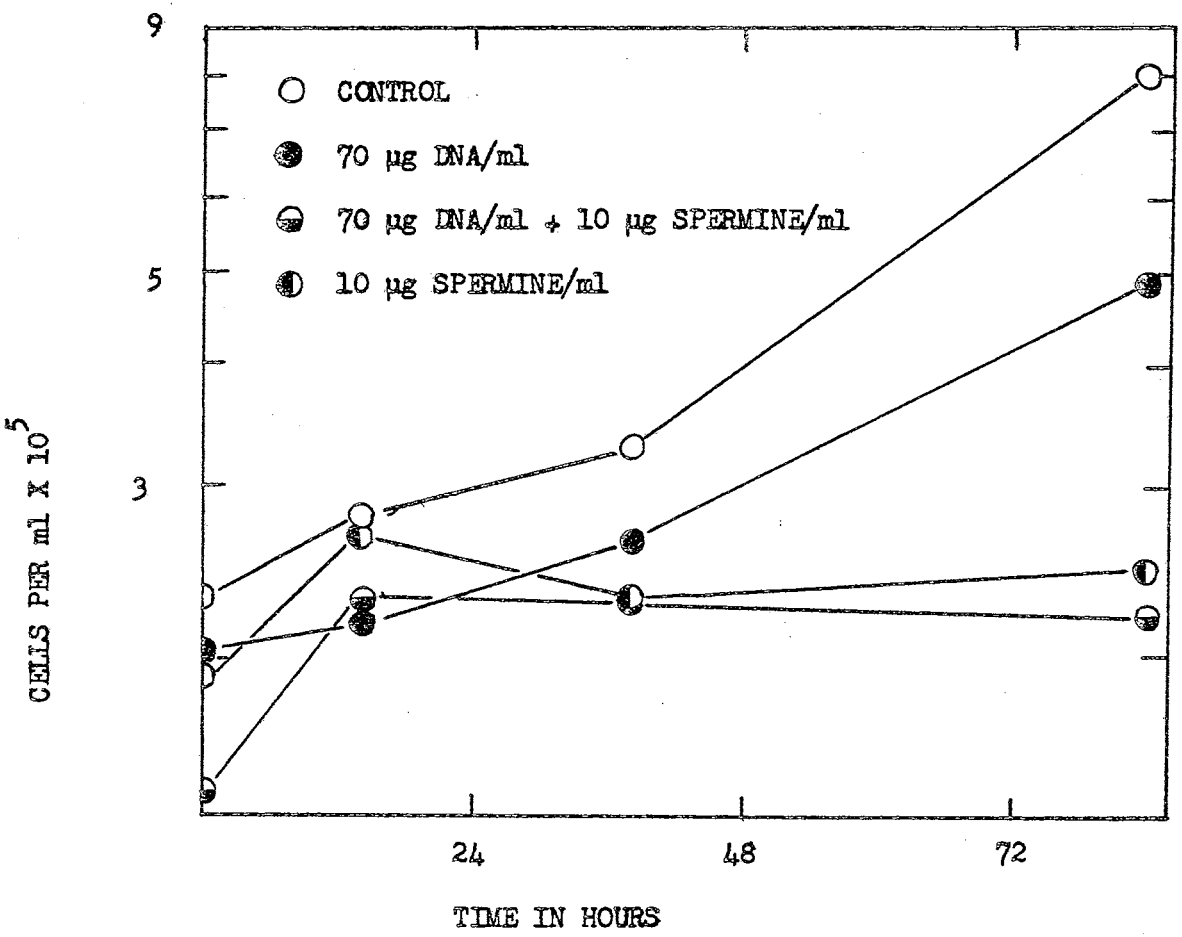


Figure 5. Effect of added DNA on inhibition of Lettre H ascites tumor cells by spermine. Cells were grown in McCoy's 5a, 10% pooled adult calf serum for 3½ days. DNA and spermine were added at 0 hours after subculture.

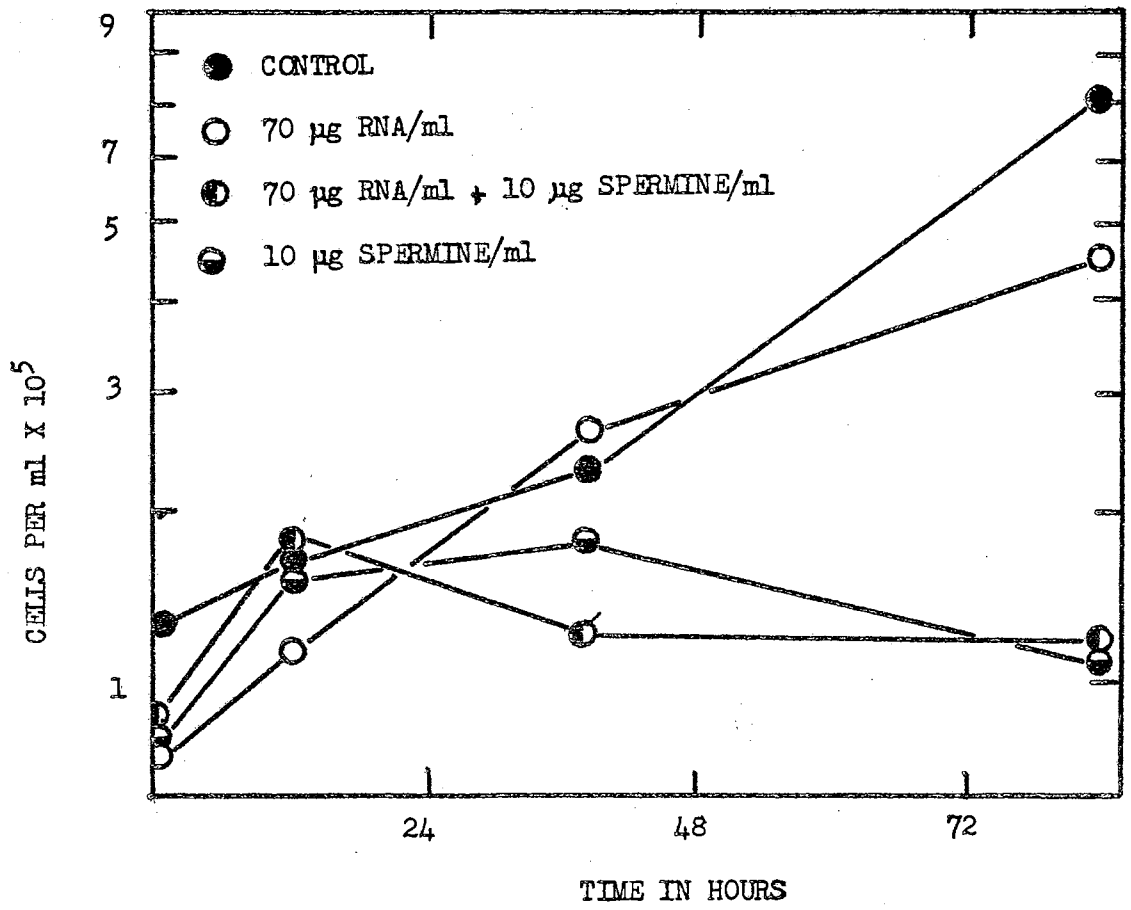


Figure 6. The effect of added RNA on inhibition of Lettre H ascites tumor cells by spermine. Cells were grown in McCoy's 5a, 10% pooled adult calf serum for $3\frac{1}{2}$ days. RNA and spermine were added at 0 hours after subculture.

with carboxymethylcellulose was encountered since this compound approximates the size of the cells themselves, thus causing an exceptionally high count. Figures 7 and 8 show the results of incubations with carboxymethylcellulose and polyphosphate, respectively. No reversal of inhibition was apparent by either carboxymethylcellulose or polyphosphate.

C. Spermine oxidase in the medium

Table I shows the levels of spermine oxidase in McCoy's 5a, 20% pooled adult calf serum. An appreciable amount of activity remained even after 48 hours of incubation at 37° with shaking at 50 oscillations per minute. The presence of cells in the medium appears to have a slight stabilizing effect on the enzyme, or perhaps the cells produce a small amount of this enzyme. The notable stability of this enzyme under the conditions of cell growth prevent its deterioration being used as an explanation for loss of inhibition in cultures which have been incubated prior to the addition of the inhibitor.

D. DNA, RNA and protein levels of cells

Figure 9 shows the levels of DNA, RNA and protein concentrations of cultures at various time intervals under normal and inhibitory conditions. Inhibitor was added immediately after subculture in all cases. Table II shows DNA, RNA and protein concentrations on a per 10^5 cells basis. In either case, spermine produced drastic reductions in the level of intracellular DNA, RNA and protein, with the most severe reduction appearing at the protein level. Some growth was observed by 96 hours (data not shown), apparently due to the outgrowth of a resistant strain of cells.

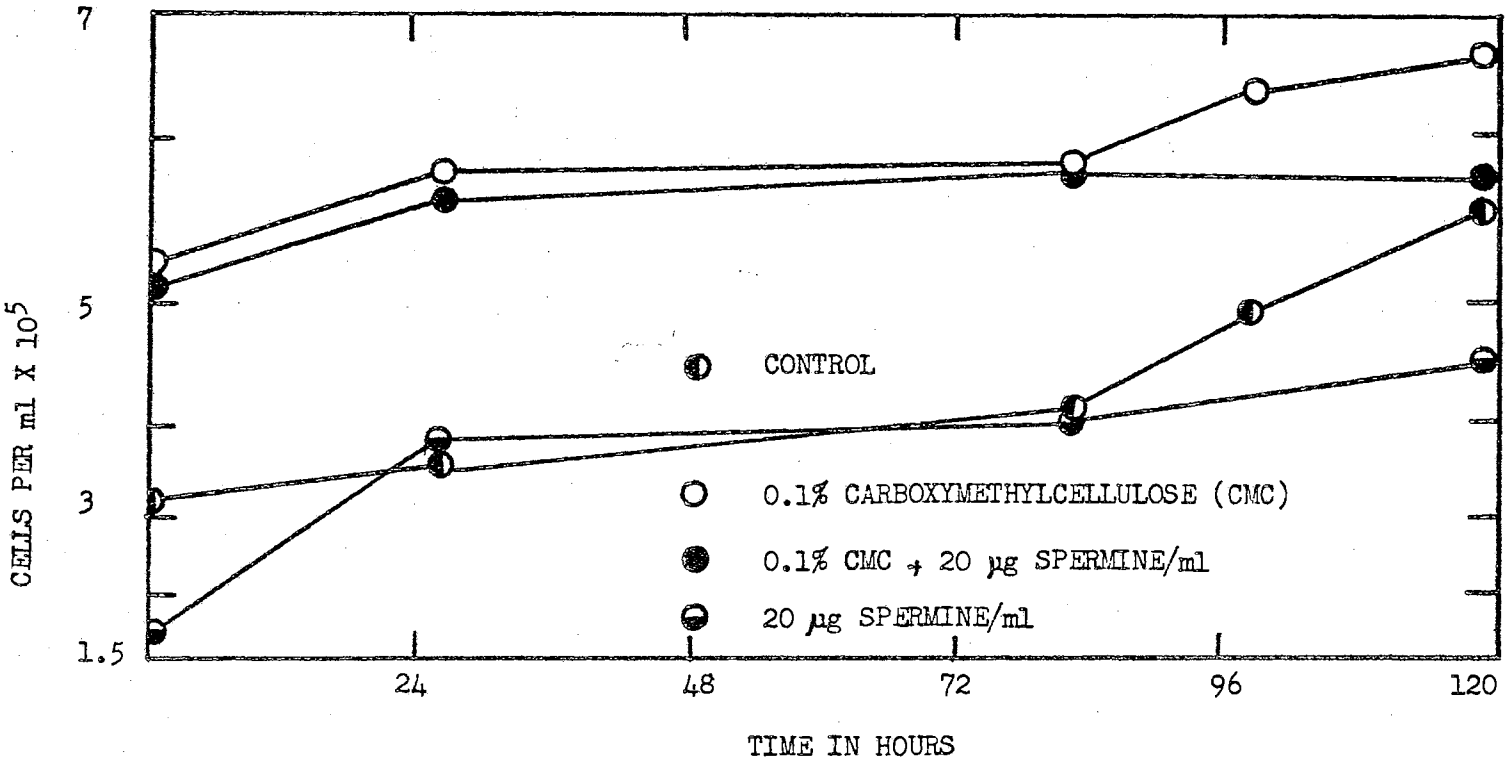


Figure 7. Effect of added carboxymethylcellulose on inhibition of Lettre H ascites tumor cells by spermine. Cells were grown in McCoy's 5a, 10% pooled adult calf serum for 5 days. Carboxymethylcellulose and spermine were added at 0 hours after subculture.

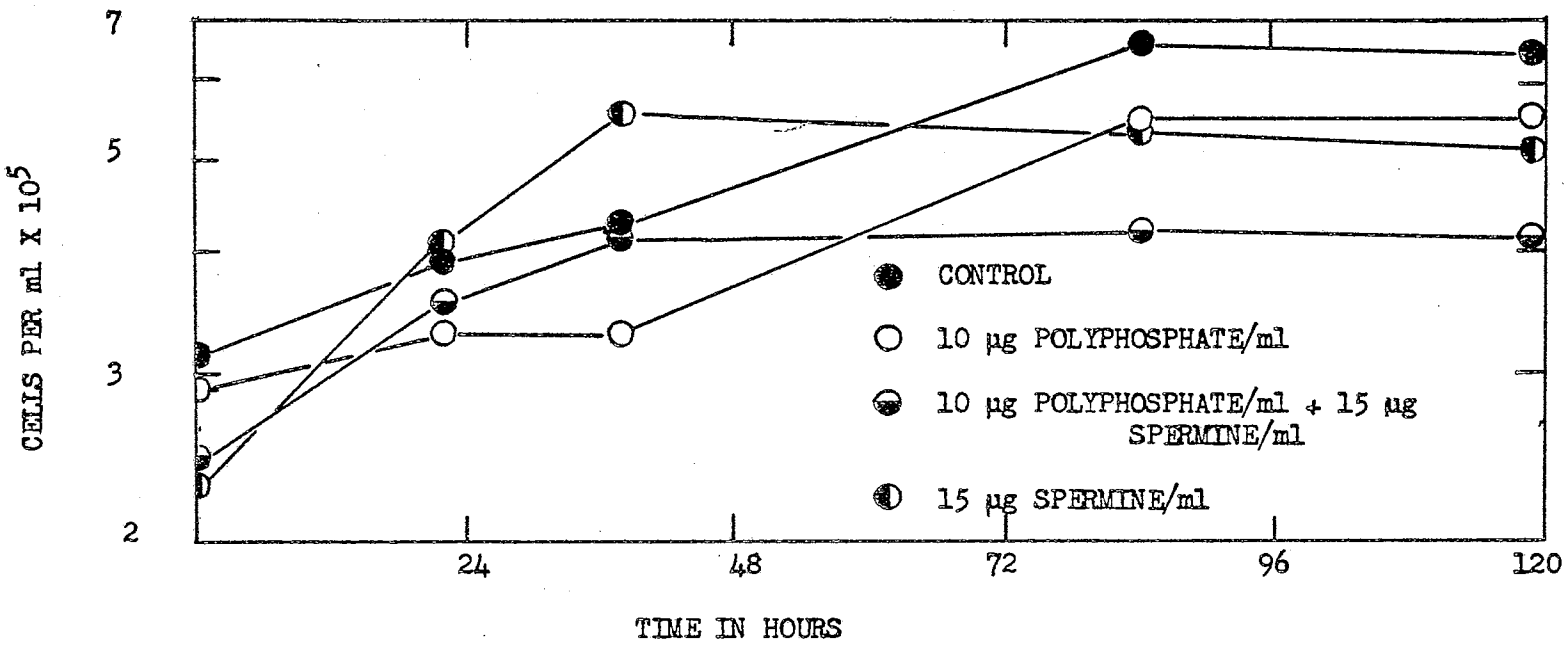


Figure 8. Effect of added polyphosphate on inhibition of Lettre H ascites tumor cells by spermine. Cells were grown in McCoy's 5a, 10% pooled adult calf serum for 5 days. Spermine and polyphosphate were added at 0 hours after subculture.

TABLE I

LEVELS OF SPERMINE OXIDASE ACTIVITY IN McCOY'S 5a MEDIUM AFTER INCUBATION AT 37° WITH ROTARY SHAKING AT 50 OSCILLATIONS PER MINUTE

Time after transfer, hours	Spectrophotometric units[1]/ml medium		Percent of activity retained	
	with cells	without cells	with cells	without cells
0	16	16	100	100
1	16	16	100	100
2	16	16	100	100
6	16	16	100	100
18	12	12	75	75
24	12	11	75	69
48	5	3	31.2	18.8

[1]A spectrophotometric unit is defined as the amount of enzyme which produces an absorbance change of 0.001 per minute at 30°.

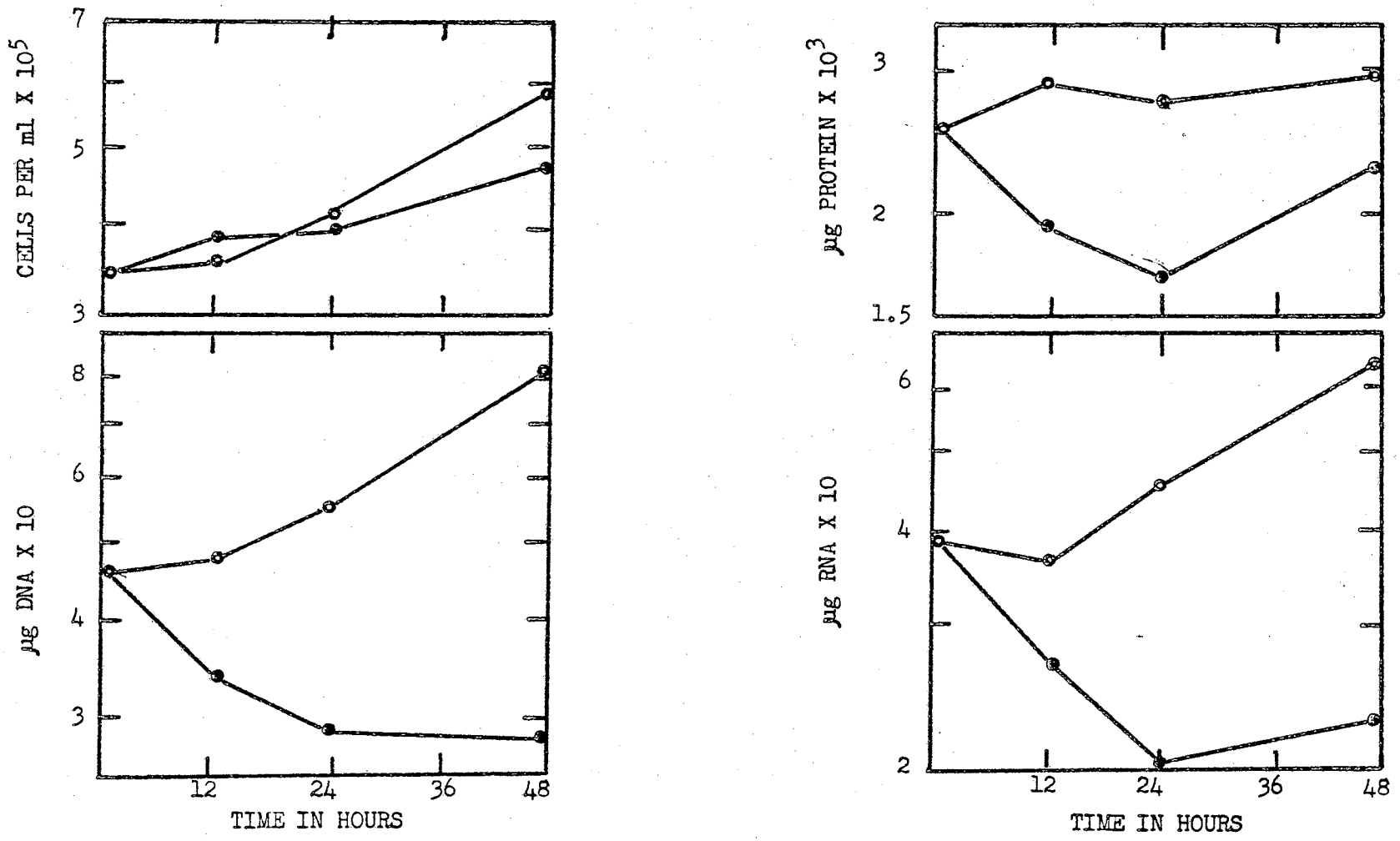


Figure 9. DNA, RNA, protein and cell concentrations for Lettre H ascites tumor cells under normal and inhibitory conditions of 10 ug spermine per ml. ○ for control, ● for spermine. Cells were grown in McCoy's 5a, 10% pooled adult calf serum. Inhibitor was added at 0 hours after subculture.

TABLE II

LEVELS OF DNA, RNA, AND PROTEIN CONCENTRATION IN LETTRE H CELLS UNDER THE INFLUENCE OF 10 μg PER ml SPERMINE. AVERAGES OF SIX DETERMINATIONS

Time after transfer, hours	ug DNA/10^5 cells		ug RNA/10^5 cells		ug protein/10^5 cells	
	control	spermine	control	spermine	control	spermine
0	13.2	--	11.2	--	129	--
12	13.1	9.0	10.3	7.1	111	33.8
24	13.4	7.4	11.0	5.2	76	12.8
48	14.0	5.8	11.0	4.8	64	18.8

E. Labeling studies with tritiated thymidine

Table III shows the relative number of labeled and non-labeled cells under the influence of spermine or acrolein. The acrolein was present in concentrations 3 to 4 times as great as the dosage reported by Alarcon (30) to cause 50% inhibition in S-180 cells and was in 4-fold excess of the amount of acrolein which reportedly would be isolated from 20 µg of spermine per ml. Colcimid was included in some of the incubation mixtures in the hope that dividing cells, arrested at metaphase by the colcimid, could be scored also, thus allowing identification of the position in the cell life cycle at which inhibition occured using the method of Puck and Steffan (55). However the staining procedure used was inadequate to allow accurate identification of dividing cells. Cells treated with acrolein seemed to show no inhibition of thymidine uptake, while in those under the influence of spermine, thymidine uptake was completely blocked, even after 50 hours incubation.

TABLE III

UPTAKE OF TRITIATED THYMIDINE BY LETTRE H ASCITES TUMOR CELLS UNDER THE INFLUENCE OF INHIBITORY DOSES OF SPERMINE OR ACROLEIN

Time after transfer, hours		controls	10^{-4} millimoles spermine/ml	10^{-4} millimoles acrolein/ml
2	%labeled	3	0	3
	%non-labeled	97	100	97
4	%labeled	-	0	6
	%non-labeled	-	100	94
6	%labeled	42	0	44
	%non-labeled	58	100	66
10	%labeled	49	0	46
	%non-labeled	51	100	64
20	%labeled	49	0	50
	%non-labeled	51	100	50
24	%labeled	50	0	51
	%non-labeled	50	100	49
30	%labeled	-	0	65
	%non-labeled	-	100	35

Cells were randomly selected from various points in the slides. At least 225 cells were counted for each value.

CHAPTER IV

DISCUSSION

Because all experiments were conducted in the presence of calf serum, and hence in the presence of spermine oxidase, the presence of spermine in the cultures was temporary. The concentration of spermine oxidase initially present in the cell culture medium was sufficient to degrade almost completely the amount of spermine used for inhibition within two hours, and the spermidine within 5 hours (56).

The question of whether inhibition was due to spermine itself or to enzymatic oxidation products naturally arises. It appears that inhibition of Lettre H ascites cells by spermine is not due to production of acrolein by spermine oxidase as is reported by Alarcon (30) to be the case with several cell lines. Acrolein produced no inhibition of uptake of tritiated thymidine, while spermine completely blocked such uptake. The amount of acrolein used was well in excess of the amount which should have been formed from the amount of spermine used. The report of Tabor, Tabor, McEwen and Kellogg (15) on the stability of the enzymatic oxidation products of spermine and spermidine suggest that acrolein is formed by isolation procedures and is not present in the cell cultures; however, this does not eliminate the possibility of inhibition being due to the oxidation products formed from spermine and spermidine by spermine oxidase. The observation that inhibition appeared to be more complete

in subcultures which showed rapid initial growth than in those with a fairly long lag period would suggest that inhibition was due to spermine itself. In more slowly growing cultures, the rate of spermine uptake might be reduced, thus allowing for more of its enzymatic degredation prior to uptake, and thus less inhibition.

The attractive possibility of explaining inhibition as due to oxidative degradation products of spermine or spermidine, and then explaining loss of inhibitory activity as a result of deterioration of spermine oxidase in medium subjected to prior incubation, is eliminated since this enzyme remains fairly stable under incubation conditions for as long as 48 hours. Also, conditioned medium is comprised of 50% freshly prepared medium which has had no chance to lose enzymatic activity. Loss of inhibition in conditioned medium is apparently due to accumulation of some unknown reversing factor in the medium. Whether this factor binds with the spermine itself or is a cell metabolite not present in freshly prepared medium but for which spermine or spermidine can substitute is unknown.

Although spermine inhibits protein production most severely, a decrease in DNA and RNA concentrations was also observed. No RNA accumulation was observed in the presence of spermine as was found by Goldstein (33) but rather a general decrease in each of the cell components DNA, RNA and protein. Goldstein was conducting much shorter term experiments, however, and a transitory RNA accumulation could have occurred at some time prior to the removal of the first sample, and thus not have been detected. Inhibition would appear to be due to spermine itself and to have as its primary site of inhibition, the inhibition of protein syn-

thesis. This is in agreement with the observations of Goldstein (33) and Mager, Benedict, and Artman (36), who report spermine inhibition to occur at the ribosome-s-RNA-m-RNA complex level.

SUMMARY

Spermine and spermidine were found to produce inhibition of growth in Lettre H ascites tumor cells grown in tissue culture. A concurrent depression of levels of DNA, RNA and protein in cells under inhibitory conditions was observed. Inhibition did not occur with medium in which cells had been incubated prior to addition of the inhibitor. Spermine oxidase, present in the medium, was found to be quite stable under growth conditions and therefore its disappearance is not responsible for this lack of inhibition. Spermine inhibition was not reversed by added DNA, RNA, polyphosphate or carboxymethylcellulose. Acrolein, reported to be an enzymatic oxidation product of spermine responsible for spermine inhibition in some cell lines, did not appear to have the same effect as spermine in this line of cells.

A SELECTED BIBLIOGRAPHY

1. Tabor, H., Tabor, C. W. and Rosenthal, S. M., Ann. Rev. Biochem., 30, 579 (1961).

2. Rosenthal, S. M. and Tabor, C. W., J. Pharmacol. Exptl. Therap., 116, 131 (1956).

3. Harrison, G. A., Biochem. J., 25, 1885 (1931).

4. Tabor, H., Rosenthal, S. M. and Tabor, C. W., Federation Proc., 15, 367 (1956).

5. Green, R. C., J. Am. Chem. Soc., 79, 3929 (1957).

6. Tabor, H., Rosenthal, S. M. and Tabor, C. W., J. Am. Chem. Soc., 79, 2978 (1957).

7. Weaver, R. H. and Herbst, E. J., J. Biol. Chem., 231, 637 (1958).

8. Dubin, D. T. and Rosenthal, S. M., J. Biol. Chem., 235, 776 (1960).

9. Hirsch, J. G., J. Exptl. Med., 97, 345 (1953).

10. Tabor, C. W., Tabor, H. and Rosenthal, S. M., J. Biol. Chem., 208, 645 (1954).

11. Blashko, H. and Hawes, R., Biochem. J., 69, 8P (1958).

12. Blashko, H. and Hawes, R., J. Physiol., 145, 124 (1959).

13. Blashko, H., J. Physiol., 153, 17P (1960).

14. Tabor, C. W., Tabor, H. and Bachrach U., J. Biol. Chem., 239, 2194 (1964).

15. Tabor, C. W., Tabor, H., McEwen, C. M., Jr. and Kellogg, P. D., Federation Proc., 23, (2 pt. 1) 385 (1964).

16. Herbst, E. J. and Snell, E. E., J. Biol. Chem., 176, 989 (1948).

17. Herbst, E. J. and Snell, E. E., J. Biol. Chem., 181, 47 (1949).

18. Sneath, P. H. A., Nature, 175, 818 (1955).

19. Martin, W. H., Pelczar, M. J., Jr. and Hansen, P. A., Science, 116, 483 (1952).

20. Traub, A., Mager, J. and Grossewicz, N., J. Bacteriol., 70, 60 (1955).

21. Ham, R. G., Biochem. Biophys. Res. Commun., 14, 34 (1964).

22. Guirard, B. M. and Snell, E. E., J. Bacteriol., 88, 72 (1964).

23. Razin, S. and Rozansky, R., J. Lab. Clin. Med., 49, 877 (1957).

24. Hirsch, J. G. and Dubos, R. J., J. Exptl. Med., 95, 191 (1952).

25. Hirsch, J. G., J. Exptl. Med., 97, 323 (1953).

26. Razin, S. and Rozansky, R., Arch. Biochem. Biophys., 81, 36 (1959).

27. Rozansky, R., Bachrach, U. and Grossowicz, N., J. Gen. Microbiol., 10, 11 (1954).

28. Alarcon, R. A., Foley, G. E. and Modest, E. J., Arch. Biochem. Biophys., 94, 540 (1961).

29. Alarcon, R. A., Proc. Am. Assoc. Cancer Res., 3, 300 (1962).

30. Alarcon, R. A., Arch. Biochem. Biophys., 106, 240 (1964).

31. Tabor, C. W. and Rosenthal, S. M., J. Pharmacol. Exptl. Therap., 116, 139 (1956).

32. Hirsch, J. G., J. Exptl. Med., 97, 327 (1953).

33. Goldstein, J., Exptl. Cell Res., 37, 494 (1965).

34. Ochoa, M. and Weinstein, I. B., Biochim. Biophys. Acta, 95, 176 (1965).

35. Davies, J., Gilbert W. and Gorini, L., Proc. Natl. Acad. Sci. U. S., 51, 883 (1964).

36. Mager, J., Benedict, M. and Artman, M., Biochim. Biophys. Acta, 62, 202 (1962).

37. Keister, D. L., Federation Proc., 17, 84 (1958).

38. Ames, B. N. and Dubin, D. T., J. Biol. Chem., 235, 769 (1960).

39. Felsenfeld, G. in Symp. Fundamental Cancer Res., M. D. Anderson Hospital and Tumor Institute, Texas, 1961, Univ. of Texas Press, Austin, Texas, 1962, p. 104.

40. Szybalski, W. in Fourth Josiah Macy, Jr. Conference on Genetics, Princeton, New Jersey, 1962, Univ. of Michigan Press, Ann Arbor, Michigan, 1964, p. 226.

41. Szybalska, E. H. and Szybalski, W., Proc. Natl. Acad. Sci. U. S., 48, 2026 (1962).

42. Clarke, D. D., Mycek, M. J., Neidle, A. and Waelsch, H., Arch. Biochem. Biophys., 79, 338 (1959).

43. Lansford, E. M., Jr., J. Biol. Chem., 239, 497 (1964).

44. McCoy, T. A., Maxwell, M. and Kruse, P. F., Jr., Proc. Soc. Exptl. Biol. Med., 100, 115 (1959).

45. Paul, J., Cell and Tissue Culture, 2nd Ed., The Williams and Wilkins Co., Baltimore, Md., 1960.

46. Hanks, J. H. and Wallace, R. E., Proc. Soc. Exptl. Biol., 71, 196 (1949).

47. Mejbaum, W., Z. Physiol. Chem., 258, 117 (1939).

48. Burton, K., Biochem. J., 62, 315 (1956).

49. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., J. Biol. Chem., 193, 265 (1951).

50. Tabor, C. W., Tabor, H. and Rosenthal, S. M. in Methods in Enzymology, II, S. P. Colowick and N. O. Kaplan, editors, Academic Press, N. Y., 1955, p. 390.

51. Zamenhof, S. in Biochemical Preparations, Vol. 6, John Wiley and Sons, Inc., N. Y., 1958, p. 8.

52. Crestfield, A. M., Smith, K. C. and Allen, F. W., J. Biol. Chem., 216, 185 (1955).

53. Audrieth, L. F., editor, Inorganic Syntheses, Vol. 3, McGraw-Hill Book Co., N. Y., 1950, p. 103.

54. Adkins, H. and Hartung, W. H. in Organic Syntheses, Vol. 1, John Wiley and Sons, N. Y., 1941, p. 15.

55. Puck, T. T. and Steffan, J., Biophys. J., 3, 379 (1963).

56. Bachrach, U. and Bar-Or, R., Biochim. Biophys. Acta, 40, 545 (1960).

VITA

James Phillip Rupp

Candidate for the Degree of

Master of Science

Subject of Thesis: EFFECTS OF SPERMINE ON LETTRE H ASCITES TUMOR CELLS

Major Field: Chemistry (Biochemistry)

Biographical Information:

Personal Data: Born in Grand Junction, Colorado, March 14, 1940, the son of Dr. and Mrs. G. A. Rupp.

Education: Attended Hawthorne and Tope Elementary Schools, Grand Junction Junior High School, and graduated from Grand Junction Senior High School, Grand Junction, Colorado; received the Associate in Science degree from Mesa Junior College, Grand Junction, Colorado, in 1960; received the Bachelor of Arts degree from Western State College, Gunnison, Colorado, in 1962 with majors in Chemistry and Biology; completed requirements for the Master of Science degree in Biochemistry at Oklahoma State University in August, 1965.

Professional Experience: Laboratory assistant, Mesa Junior College and Western State College, N. S. F. Research Grant Recipient, Western State College, Graduate Research Assistant, Oklahoma State University.