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POLYAMINE METABOLISM AND ITS RELATIONSHIP TO MULTIPLICATION
AND DIFFERENTIATION OF ACANTHAMOEBA CASTELLANII

The Ohio State University

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POLYAMINE METABOLISM AND ITS RELATIONSHIP
TO MULTIPLICATION AND DIFFERENTIATION
OF ACANTHAMOEBA CASTELLANII

DISSERTATION

Presented in Partial Fulfillment of the Requirement for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By

Byeong Gee Kim, B.S., M.S.

The Ohio State University
1986

Dissertation Committee: Approved by
T.J. Byers
B. Oakley
R.M. Pfister
W.R. Strohl

Adviser
Department of Microbiology
.....with memories of

my father
ACKNOWLEDGEMENTS

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VITA

August 26, 1953-------------------------Born, Busan, Korea

1977-------------------------------------B.S., Department of Biology, Busan National University

1979-1981-------------------------------M.S., Department of Zoology, Research and Teaching Associate, Iowa State University

1981-1986-----------------------------Department of Microbiology, Research and Teaching Associate, The Ohio State University
PUBLICATIONS

Abstracts:


FIELDS OF STUDY

Major Field: Microbiology
Specialty: Microbiol developmental biology
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>VITA</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xi</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>xiv</td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1. Polyamine biosynthesis</td>
<td>3</td>
</tr>
<tr>
<td>1. Polyamine biosynthesis in microorganisms</td>
<td>7</td>
</tr>
<tr>
<td>2. Polyamine biosynthesis in higher animal cells</td>
<td>15</td>
</tr>
<tr>
<td>3. Polyamine biosynthesis in plants</td>
<td>17</td>
</tr>
<tr>
<td>1.2. Study of polyamine biosynthesis by using specific enzyme inhibitors</td>
<td>18</td>
</tr>
<tr>
<td>1. Ornithine decarboxylase and its specific inhibitors</td>
<td>18</td>
</tr>
<tr>
<td>2. Inhibitors for S-adenosylmethionine decarboxylase</td>
<td>22</td>
</tr>
<tr>
<td>3. Inhibitors of spermidine synthase</td>
<td>25</td>
</tr>
<tr>
<td>1.3. Role of polyamines in cell proliferation</td>
<td>26</td>
</tr>
<tr>
<td>1. Polyamine metabolism during the cell cycle</td>
<td>27</td>
</tr>
<tr>
<td>2. Role of polyamines in DNA synthesis</td>
<td>29</td>
</tr>
<tr>
<td>3. Role of polyamines in RNA synthesis</td>
<td>31</td>
</tr>
<tr>
<td>4. Role of polyamines in protein synthesis</td>
<td>32</td>
</tr>
<tr>
<td>1.4. Role of polyamines in cell differentiation</td>
<td>33</td>
</tr>
<tr>
<td>1.5. Polyamine studies in Acanthamoeba</td>
<td>36</td>
</tr>
<tr>
<td>2. MATERIALS AND METHODS</td>
<td>40</td>
</tr>
<tr>
<td>2.1. Cell cultures and cloning</td>
<td>40</td>
</tr>
</tbody>
</table>
2.2. Inhibition of multiplication .................. 41
2.3. Induction of encystment ........................ 41
2.4. Homogenization of amebas for polyamine and enzyme assay .................. 42
2.5. Separation and quantitation of polyamines ..... 43
2.6. In vitro enzyme assays .......................... 44
   1. Arginine decarboxylase ........................ 44
   2. Arginase ........................................ 45
   3. Arginine deiminase ............................ 47
   4. Citrulline hydrolase/Citrullinase ........... 48
   5. Urease .......................................... 49
   6. Ornithine decarboxylase ........................ 49
   7. Diaminopropane synthetase ........................ 50
2.7. In vivo assays for enzyme activity ............. 52
   1. Arginine decarboxylase and arginine deiminase ........................ 52
   2. Diaminopropane synthetase ........................ 53
2.8. Thin layer chromatography ....................... 53
2.9. Autoradiography .................................. 54
2.10. Protein assay .................................... 55
2.11. Chemicals ......................................... 55
3. RESULTS ....................................... 57
3.1. Polyamine contents of postexponential phase amebas .................................. 57
   1. Cultures grown in broth ........................ 57
   2. Cultures grown in defined growth medium ..... 62
3.2. Enzymes of polyamine metabolism in postexponential phase DGM-11 cultures ......... 63
   1. Arginase ........................................ 63
   2. The conversion of citrulline to ornithine 70
   3. The conversion of arginine to citrulline ... 70
   4. The conversion of spermidine to diaminopropane ........................ 76
   5. Ornithine decarboxylase ........................ 76
3.3. Changes in polyamine levels and ornithine decarboxylase activity during cell multiplication and starvation-induced
encystment.............................................. 88
1. Cultures grown in OGM............................. 88
2. Cultures grown in DGM-11......................... 90

3.4. Effects of ornithine, putrescine, and spermidine analogues on cell multiplication and differentiation................................. 95
1. Arrest of multiplication by DFMO, Δ-MFMOme, R,R-MAP in OGM and DGM-11 cultures............... 95
2. Inhibition of ornithine decarboxylase by DFMO, Δ-MFMOme, and R,R-MAP in OGM and DGM-11 cultures.......................... 109
3. Induction of differentiation by DFMO supplemented with CaCl2 or MgSO4 in OGM cultures................................. 121
4. Effects of MGBG, Berenil, hydroxystilbamidine, pentamidine, amicarbalide, Antycide, and ethidium bromide on multiplication and differentiation in OGM and DGM-11 cultures.. 122
5. Changes in polyamine levels and ornithine decarboxylase activity during Berenil-induced encystment in OGM and DGM-11............... 142

3.5. Arrest of multiplication and induction of differentiation by DFMA......................................... 146

4. DISCUSSION.................................................. 150
4.1. Polyamine content of *Acanthamoeba castellanii* 150
4.2. Enzymes of polyamine metabolism in *A. castellanii*.............................. 154
4.3. Effects of ornithine and polyamine antagonists on multiplication and differentiation in *A. castellanii*.......................... 160

5. SUMMARY OF RESULTS........................................... 171

APPENDIX.................................................... 174

REFERENCES.................................................. 176
LIST OF TABLES

Table                                                                 Page

1. Polyamine contents of postexponential-early postexponential phase A. castellanii grown in OGM and DGM-11 ........................................ 60

2. Retention times of benzoylated polyamines separated by reverse phase high performance liquid chromatography ................................ 61

3. In vitro arginase activity measured by urea nitrogen production .................................. 69

4. Pyridoxal 5-phosphate and dithiothreitol requirements for ornithine decarboxylase activity ............................................... 89

5. Effect of starvation on levels of polyamine........ 96

6. Effect of starvation on ornithine decarboxylase activity ............................................... 97

7. Change in levels of the ornithine decarboxylase activity in the presence of its irreversible inhibitor in OGM cultures ...................... 116

8. Effect of DFMO on ornithine decarboxylase activity and polyamine levels in vivo .............. 117

9. In vitro inhibition of ornithine decarboxylase activity by DFMO in OGM-11 cultures ........... 118
10. Effect of Berenil on polyamine levels and ornithine decarboxylase activity................. 145

11. Polyamines in various lower eukaryotes............. 151

12. The kinetic parameters of ornithine decarboxylase in various protozoa.................. 158

13. Components of culture medium......................... 175
<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Composite of biosynthetic pathways for polyamines in animal, plant, or bacterial cells...</td>
<td>4</td>
</tr>
<tr>
<td>2. Diagram of arginine-ornithine metabolism in <em>Neurospora crassa</em></td>
<td>11</td>
</tr>
<tr>
<td>3. A composite of biosynthesis for putrescine, spermidine, and decarboxylated SAM</td>
<td>20</td>
</tr>
<tr>
<td>4. Separation of benzoylated polyamines by HPLC</td>
<td>58</td>
</tr>
<tr>
<td>5. <em>In vitro</em> production of $^{14}$C-ornithine from L-(U-$^{14}$C)-arginine by arginase</td>
<td>64</td>
</tr>
<tr>
<td>6. Kinetics of the <em>in vitro</em> production of $^{14}$C-ornithine from L-(U-$^{14}$C)-arginine by arginase</td>
<td>67</td>
</tr>
<tr>
<td>7. <em>In vitro</em> production of ornithine from citrulline</td>
<td>71</td>
</tr>
<tr>
<td>8. Fate of $^{14}$C-citrulline from L-(U-$^{14}$C)-arginine <em>in vivo</em></td>
<td>74</td>
</tr>
<tr>
<td>9. Fate of (U-$^{3}$H)-spermidine <em>in vivo</em></td>
<td>77</td>
</tr>
<tr>
<td>10. <em>In vitro</em> production of $^{14}$C-putrescine from DL-(5-$^{14}$C)-ornithine by ornithine decarboxylase</td>
<td>79</td>
</tr>
<tr>
<td>11. <em>In vitro</em> CO$_2$ production from DL-(1-$^{14}$C)-ornithine by ornithine decarboxylase in OGM-11 cultures</td>
<td>82</td>
</tr>
</tbody>
</table>
12. Double reciprocal plot of enzyme kinetic for OrnDC from amebas grown in OGM .................. 84

13. Double reciprocal plot of enzyme kinetic for OrnDC from amebas grown in DGM-11 .............. 86

14. Intracellular polyamine levels during postexponential growth and starvation-induced encystment ............................................ 91

15. Intracellular levels of polyamines and ornithine decarboxylase activity in DGM-11 cultures .......................................................... 93

16. Inhibition of multiplication in OGM by antagonists of polyamine synthesis ...................... 99

17. Arrest of growth in OGM by antagonists of polyamine synthesis and its reversal by 0.8 mM putrescine ........................................... 101

18. Arrest of growth in OGM by antagonists of polyamine synthesis and its reversal by 0.8 mM spermidine ............................................ 103

19. Arrest of growth in OGM by antagonists of polyamine synthesis and its reversal by 8 mM diaminopropane ........................................ 105

20. Arrest of growth in OGM by antagonists of polyamine synthesis and its reversal by 0.8 mM cadaverine ............................................. 107

21. Inhibition of multiplication in DGM-11 by inhibitors of ornithine decarboxylase .............. 110

22. Arrest of growth in DGM-11 by inhibitors of ornithine decarboxylase and its reversal by 0.8 mM putrescine ........................................ 112
23. Arrest of growth in DGM-11 by inhibitors of ornithine decarboxylase and its reversal by 0.8 mM spermidine.............................. 114

24. Kinetics of encystment in 40 mM DFMO plus CaCl₂ or MgSO₄ in DGM cultures.................. 119

25. "Structural analogues" of spermidine................. 123

26. Structures of ethidium bromide and Antrycide as "structural analogues" of spermidine......... 125

27. Induction of encystment in DGM by spermidine analogues.............................................. 128

28. Berenil-induced encystment in 55 subclone of PH28................................................... 131

29. Induction of encystment by Berenil and hydroxystilbamidine and its reversal by polyamines..................................................... 133

30. Inhibition of multiplication in DGM-11 by spermidine analogues................................. 136

31. Induction of encystment in DGM-11 by spermidine analogues........................................ 138

32. Induction of encystment by spermidine analogues and its inhibition by polyamines........ 140

33. Intracellular polyamine levels during postexponential growth and Berenil-induced encystment..................................................... 143

34. In vitro decomposition of DFMA...................... 147

xiii
ABBREVIATIONS

agm: agmatine
ala: alanine
AMI: amicarbalide
ANT: Antrycide
AR: autoradiogram
Arg: arginine
ArgDC: arginine decarboxylase
Ber: Berenil
BUN: blood urea nitrogen
CHA: cyclohexylamine
Cit: citrulline
CP: carbamyl phosphate
Dap: diaminopropane
DCHA: dicyclohexylamine
DFMA: $\alpha$-difloromethylarginine
DFMO: $\alpha$-difloromethylornithine
DGM-11: defined growth medium
EB: ethidium bromide
EEA: encystment enhancing activity
Gln: glutamine
Glu: glutamate
HPLC: high performance liquid chromatography
HS: hydroxystilbamidine
LysDC: lysine decarboxylase
R,R-MAP: (R,R)-£-methyl-α-acetylenicputrescine
Δ-MFMO: α-monofluoromethyldehydroornithine
Δ-MFMOme: α-monofluoromethyldehydroornithine methyl ester
MGBG: methylglyoxal bis(guanylhydrazone)
MTA: 5'-methylthioadenosine
NEM: Neff's encystment medium
Norspd: norspermidine
OGM: optimal growth medium
Orn: ornithine
OrnDC: ornithine decarboxylase
PA: polyamine
PENT: pentamidine
Put: putrescine
SADTO: S-adenosyl-1,8-diamino-3-thiooctane
SAM: S-adenosylmethionine
dcSAM: decarboxylated S-adenosylmethionine
SAMDC: S-adenosylmethionine decarboxylase
Spd: spermidine
Spm: spermine
TLC: thin layer chromatography
Acanthamoeba castellanii, a small free-living amoeba, has attracted researchers in recent years because it is a good model system for studies of cell differentiation (Byers, 1979; Akins et al., 1985) and especially because it is a possible human pathogen (Griffin, 1978; Ludwig et al., 1986). This amoeba easily can be cultured axenically and can exist in two morphological forms, an actively growing trophozoite and a dormant cyst. Formation of cysts (encystment) and hatching (excystment) can be induced synchronously. Encystment is associated with adverse environmental conditions including starvation (Neff et al., 1964), desication or high salt concentration (Band, 1963), and decreased oxygen tension (Byers et al., 1969). Encystment also can be induced by many different metabolic inhibitors. Some inhibitors of DNA synthesis are especially effective under certain conditions (Neff and Neff, 1972; Byers, 1979). The induction of encystment by different conditions does not necessarily result from stimulation of the same response system. When the adverse environmental condition is removed, the excystment process can be started synchronously (Chambers and Thompson, 1974).
Neff and Neff's findings on the induction of encystment by antimetabolites were modified and expanded by Akins and Byers (1980). According to the latter workers, antimitochondrial drugs (Berenil, ethidium bromide, chloramphenicol, and erythromycin) were the best encystment inducers. Berenil and ethidium bromide are known to block mitochondrial DNA replication and transcription, and chloramphenicol and erythromycin are translation inhibitors that are specific for the large ribosomal subunit of organelles in eukaryotes. Thus, mitochondria may play a direct role in differentiation. Recently, Akins et al. (1985) found that encystment induced by antimitochondrial drugs was preceeded by the extracellular accumulation of an encystment enhancing activity (EEA). The EEA could not induce encystment by itself, but it was required for a maximum response during drug- and starvation-induced encystment. Sufficient EEA titers for maximum cyst formation were produced by high density cultures (around 300 amebas/mm²), but not by low density cultures (around 20-50 amebas/mm²). The activity of EEA was associated with a low molecular weight compound (about 500 daltons) which could be inactivated by 5'-nucleotidase and snake venom phosphodiesterase (Akins, 1981). However, identification of EEA requires further study.
1.1. Polyamine biosynthesis. The polyamines, putrescine (1,4-diaminobutane), spermidine, and spermine are widely distributed in both prokaryotic and eukaryotic cells. They are involved in a wide range of biological processes including stabilization of whole cells, organelles, membranes, and nucleic acids; modulation of protein synthesis; cell differentiation; and induction of pharmacological effects in whole animals (Heby, 1981; Porter and Sufrin, 1986; Smith, 1985a). Although a large number of papers have been published on the subject during the last decade, the role of polyamines is still unknown.

Both prokaryotic and eukaryotic cells produce putrescine and spermidine but spermine is confined to eukaryotic cells. Prokaryotes have a higher concentration of putrescine than spermidine, whereas, eukaryotic cells have higher levels of spermidine and spermine than putrescine (Heby, 1981). Besides those, several other unusual polyamines also are found in some biological materials, these include diaminopropane, cadaverine, norspermidine, norspermine, and sym-homospermidine in both prokaryotes and eukaryotes (Tabor and Tabor, 1984, 1985).

The major biosynthetic pathway of polyamines begins with the production of putrescine from ornithine (Fig. 1). In microorganisms and plant cells, however, putrescine also can be synthesized from arginine through agmatine (Smith, 1985; Tabor and Tabor, 1985). A probable new pathway for
Fig. 1. Composite of biosynthetic pathways for polyamines in animal, plant or bacterial cells. Enzymes that typically catalyze each step: 1, arginase; 2, arginine decarboxylase; 3, arginine deiminase; 4, citrulline hydrolase or citrullinase; 5, ornithine decarboxylase; 6, spermidine synthetase; 7, spermine synthetase; 8, S-adenosylmethionine decarboxylase; 9,10, polyamine oxidase or spermidine (spermine) dehydrogenase; 11, agmatinase; 12, acetylglutamate kinase.
putrescine synthesis was proposed in *E. coli*. Putrescine could be formed not from ornithine or arginine but from glucose or glutamate (Cataldi and Algranati, 1986). The synthesis of spermidine and spermine requires decarboxylated S-adenosylmethionine (dcSAM) as a principal precursor besides putrescine. Thus, the principal precursors of polyamines are ornithine and dcSAM. However, the formation of ornithine from arginine by arginase, which can be found in many tissues, indicates the possible importance of arginase in polyamine biosynthesis. Ornithine also can be synthesized from acetylglutamate by N-acetylglutamate kinase in *Neurospora* (Goodman and Weiss, 1980) and from citrulline in certain bacteria (Oglinsky and Gehrig, 1953) and trypanosomatids (Yoshida et al., 1978) (Section 1.1.1).

Putrescine is formed from ornithine through decarboxylation. This step is catalyzed by ornithine decarboxylase (OrnDC) which is a rate-limiting enzyme in polyamine biosynthesis in all organisms (Section 1.2.1).

Synthesis of spermidine and spermine is catalyzed by spermidine and spermine synthases, respectively. Both transfer an aminopropyl group derived from decarboxylated SAM (dcSAM) to their substrates (Fig. 1). Decarboxylation of SAM is catalyzed by SAM decarboxylase (SAMDC) which is another rate-limiting enzyme in polyamine biosynthesis (1.2.2).
1.1.1 **Polyamine biosynthesis in microorganisms.** The number of kinds and the contents of polyamines in microorganisms are quite different from those of higher animal cells. The isolation of mutants, which had defects in the polyamine biosynthetic pathways, made polyamine research much easier in *Escherichia coli*, *Saccharomyces cervisiae*, and *Neurospora crassa* (Tabor and Tabor, 1984; 1985).

In *E. coli*, putrescine is synthesized by two alternative pathways; either by OrnDC or by the combination of ArgDC (arginine decarboxylase) and agmatine ureohydrolase (Fig. 1). Agmatine, which is produced from arginine by ArgDC, is converted to putrescine and urea by agmatine ureohydrolase (Morris and Koffron, 1967; Morris and Pardee, 1966). Since urease is absent from *E. coli*, urea is accumulated in the medium. The relative degree of use of each putrescine synthetic pathway is dependent on the growth conditions. The relative utilization of OrnDC or ArgDC is controlled by the intracellular ornithine concentration; putrescine is synthesized from ornithine rather than from agmatine under normal growth conditions in minimal medium. If the intracellular concentration of ornithine is lower than that of arginine for any reason, the ArgDC mediated pathway is preferred. Even though the concentration of arginine is higher than ornithine,
arginine cannot be converted to ornithine because of the lack of arginase in *E. coli*. The unusual polyamine cadaverine is found in the medium when putrescine biosynthesis is blocked by mutation (Tabor et al., 1980; Boeker and Fischer, 1983). Cadaverine is probably formed from lysine by lysine decarboxylase (LysDC) which is formed under the inducing condition: depletion of OrnDC or of polyamine synthesis.

OrnDC is present in a biodegradative form (induced) or a biosynthetic form (constitutive) in *E. coli* (Applebaum et al., 1977; Morris and Boeker, 1983). These two different forms have different pH optima but both require pyridoxal phosphate for activity. The biosynthetic enzyme is found in all tested *E. coli* strains, is inhibited by putrescine and spermidine, and is activated by guanosine triphosphate (Applebaum et al., 1977). Interestingly, OrnDC in *E. coli* is not inhibited by the mechanism-based specific inhibitor α-difluoromethylornithine (DFMO) in vitro even though the activity decreased a little bit and the normal growth was inhibited by DFMO in vivo (Kallio and McCann, 1981). The different response seen in vivo might be due to an effect at the level of enzyme synthesis, or other regulatory mechanism, i.e., enzyme turnover, induction of antizyme, etc. ArgDC also exists in biodegradative and biosynthetic forms with different pH optima in *E. coli*, and both forms require pyridoxal phosphate for activity (Morris and
Putrescine and spermidine inhibit the biosynthetic enzyme and the arginine analog α-difluromethylarginine (DFMA) irreversibly blocks its activity (Kallio et al., 1981). SAMDC in E. coli requires divalent cations but not pyridoxal phosphate, and usually is not stimulated by putrescine (Markham et al., 1982).

In S. cerevisiae, neither citrulline hydrolase nor ArgDC have been found. Therefore, the only source for putrescine is ornithine, as in most animal cells. Aminopropyl transfer to putrescine and then to spermidine is catalyzed by spermidine and spermine synthases, respectively. The yeast OrnDC requires pyridoxal phosphate and is stabilized by thiols. This enzyme is stable for at least 10 weeks at -80°C (Tyagi et al., 1983). The activity is competitively inhibited by putrescine; spermidine and spermine have only a slight inhibitory effect on the activity in vitro. The loss of activity is known to be associated with posttranslational modification of this enzyme (Tyagi et al., 1981). SAMDC has covalently linked pyruvate which is required for activity. Pyridoxal phosphate is not present in the enzyme. The enzyme is activated by putrescine but not by Mg²⁺ and is inhibited by methylglyoxal bis(guanylhydrazone) (MGBG) and dcsSAM (Tabor and Tabor, 1985).
Polyamine synthesis in *Neurospora crassa* is particularly interesting. The sole source of putrescine is ornithine, but almost all ornithine is localized in vacuoles (Fig. 2). Ornithine first is synthesized in mitochondria from acetylglutamate and converted to citrulline in this organelle by the reaction with carbamyl phosphate (Davis and Ristow, 1983). Citrulline is converted to argininosuccinate in the cytoplasm by argininosuccinate synthetase which provides arginine for protein synthesis and a large storage pool in vacuoles. During growth in medium free of arginine, over 95% of the amino acids is sequestered in the vacuoles, thus, cytosolic arginine concentration is very low. When the cytosolic concentration of ornithine is high, partly due to the cytosolic arginase activity and partly due to the flux of ornithine from mitochondria, most ornithine is stored in vacuoles. The stored ornithine cannot be freely exchanged with cytosolic ornithine. Moreover, the influx and the efflux of ornithine across the mitochondrial membrane are faster than the normal rate of ornithine synthesis from arginine (Bowman and Davis, 1977; Karlin et al., 1976). OrnDC activity is found only in the cytoplasm (Weiss and Davis, 1973). Thus, some of the cytosolic ornithine is converted to putrescine and spermidine. About 60-80% of the putrescine and spermidine formed in the cytoplasm is sequestered in vacuoles and is not readily available for
Fig. 2. Diagram of arginine-ornithine metabolism in *Neurospora crassa*. Abbreviations are: At-Glu, acetylglutamate; Gln, glutamine; Cit, citrulline; CP, carbamyl phosphate; Arg, arginine; Orn, ornithine; Put, putrescine; Spd, spermidine; Spm, spermine. Figure modified from Davis and Ristow (1983).
the formation of spermidine and spermine in the cytosol. Only 10-20% of the cellular content of these compounds is freely available as pathway intermediates (Paulus and Davis, 1982; Paulus et al., 1983; Cramer and Davis, 1984). Cadaverine is not found in wild type N. crassa. However, an arginase deficient aga mutant can synthesize it when arginine is present in the growth medium. The synthesis of cadaverine also is observed in this mutant in the presence of exogenously added lysine, which derepresses OrnDC activity. Thus, it is likely that the continued growth is due to the presence of cadaverine and that OrnDC is responsible for cadaverine synthesis from lysine. As discussed above, ornithine is formed from either arginine in the cytosol or acetylglutamate within the mitochondria. The synthesis of mitochondrial ornithine is catalyzed by acetylglutamate kinase which is controlled by feedback inhibition by cytosolic arginine (Davis and Ristow, 1983). In the aga mutant, arginine added to the medium caused inactivation of acetylglutamate kinase, but stimulated OrnDC 75-fold relative to controls. When protein synthesis was blocked by cycloheximide, the activity of OrnDC was inhibited by putrescine but not by spermidine. The result indicated translational regulation of OrnDC by putrescine. Spermidine did not inactivate OrnDC, but it blocked the synthesis of the enzyme (Davis et al., 1985).
Knowledge about polyamine metabolism in protozoa is limited to a few species. *Euglena gracilis* (Aleksijevic et al., 1979) and *E. viridis* (Hamana and Matsuzaki, 1985) have the following unusual polyamines besides the three most common ones: diaminopropane, cadaverine, norspermidine, homospermidine, and norspermine. Norspermidine and norspermine were proposed to be synthesized from diaminopropane and norspermidine, respectively, by aminopropyl transferases in *E. gracilis*. The synthesis of norspermidine from diaminopropane also was found in bacterial cells recently (Yamamoto et al., 1986). The algal OrnDC required dithiothreitol and pyridoxal phosphate for maximal activity. SAMDC was stimulated by putrescine but not by divalent ions (Aleksijevic et al, 1979).

Enzymes involved in arginine-ornithine metabolism were extensively studied in 21 species of 5 different genera of trypanosomatids (Camargo et al., 1978; Figueiredo et al., 1978; Yoshida et al., 1978). Arginase activity was found in *Crithidia, Leishmania*, and *Leptomonas*, but not in *Herpetomonas* or *Trypanosoma*. Citrulline can be split into ornithine, CO$_2$, and NH$_3$ in two different ways. One is in a single step reaction which is catalyzed by citrulline hydrolase, another is a two step reaction catalyzed by ornithine transcarbamylase and carbamate kinase which collectively are called citrullinase (Oglinsky and Gehrig, 1953) (Fig. 1). Citrulline hydrolase activity
was first described in the ciliate *Tetrahymena* (Hill and van Eys, 1965) and has only been found in protozoans. This single step reaction activity was found in *Leptomonas*, *Herpetomonas*, and *Crithidia*. Arginine deiminase could not be detected in *Leishmania*, *Crithidia*, *Trypanosoma*, or *Leptomonas* (except one species), but was present in *Herpetomonas*. Ornithine transcarbamylase, which catalyses the synthesis of citrulline from ornithine and carbamyl phosphate only was found in one species of *Leptomonas* and two species of *Crithidia*. Thus, considerable variation in pathways exists among the different trypanosomatid genera.

*Trichomonas* had three major polyamines, putrescine, spermidine, and spermine (North et al., 1986). Arginine decarboxylase was absent from all three tested species of *Trichomonas*. OrnDC was found in all three but SAMDC was absent in *T. batrachorum*. Since *T. batrachorum* had spermidine and spermine, the absence of SAMDC might be due to activity below the limit of detection (North et al., 1986).

### 1.1.2. Polyamine biosynthesis in higher animal cells.

The rate-limiting enzymes of polyamine biosynthesis in higher animal cells are OrnDC and SAMDC. OrnDC of these cells has an extraordinarily short half-life of about 15 minutes in rat liver (Russell and Sneider, 1969) and 8 minutes in chicken liver (Grillo and Bedino, 1977). It has a rapid
response to a variety of hormonal and other stimuli, for example, androgen or thioacetamide injection (Hayashi and Kameji, 1983; Isomaa et al., 1983). It can be completely inhibited by an OrnDC-antizyme (Grillo et al., 1980, McCann, 1980; Murakami and Hayashi, 1985) which can be induced by putrescine or diaminopropane in chicken tissue (Grillo et al., 1980) or rat hepatoma cells (McCann, 1980). OrnDC has been reported in the nuclei of mammalian cells, but most of the enzyme was found in the cytoplasm based on immunofluoroscence and autoradiography studies (Persson et al., 1983; Zagon et al., 1983). But these studies were conducted after enzyme induction. A recent experiment with uninduced cells showed an equal distribution between nucleus and cytoplasm (Bartholeynsa, 1983). Thus, localization of OrnDC is still unclear. The proportion of this enzyme to total protein is very low. It can be induced to increase one thousand-fold by hormones, drugs, tissue regeneration, and growth factors. However, OrnDC still represents a very small fraction of the total protein in mammalian cells: < 0.01% (Pegg and McCann, 1982). SAMDC of rat liver does not need pyridoxal phosphate but requires pyruvate (Pegg, 1977) and is activated by putrescine. The formation of spermidine and spermine are by the usual pathways (Fig. 1). Both compounds inhibit spermine synthase activity.
1.1.3. **Polyamine biosynthesis in plants.** Relatively little is known about the characteristics of OrnDC in higher plants. In barley seedlings, 75% of the activity was found in association with nuclear chromatin and the remaining activity was in the cytoplasm. OrnDC activity was suppressed by putrescine and spermidine through inhibition of protein synthesis (Kiriakidis, 1983; Kiriakidis et al., 1983). ArgDC is widespread in higher plants. This enzyme requires a thiol compound and pyridoxal phosphate for its activity and stability (Choudhuri and Ghosh, 1982). Spermine can be a strong inhibitor in some cases (Ramakrishna and Adiga, 1975). The ratio of OrnDC to ArgDC varies in different species, tissues, and physiological states (Smith, 1985a); it is not known which pathway is the main source of putrescine. SAMDC requires Mg\(^{+2}\) and is not stimulated by putrescine. Lysine decarboxylase has been detected in many plants (Smith, 1985a), and has an absolute requirement for Mn\(^{+2}\) or Fe\(^{+2}\). Spermidine and spermine synthases also are found in plants. Spermidine synthase is strongly inhibited by cyclohexylamine (CHA) (Sindhu and Cohen, 1983); this compound sold by the Sigma Chemical Co. is incorrectly identified by them and by workers publishing results with it as dicyclohexylamine (DCHA) (Pegg, 1986; Porter and Sufrin, 1986).
1.2. Study of polyamine biosynthesis by using specific enzyme inhibitors. The role of polyamines is not yet known in spite of extensive research in several different areas. Recently developed inhibitors specific for enzymes of polyamine biosynthesis should help with the effort to characterize and identify the roles of polyamines. Many inhibitors are "structural analogues" of either ornithine, putrescine, or spermidine. In this section, inhibitors which block OrnDC, SAMDC and spermidine synthase are discussed.

1.2.1. Ornithine decarboxylase and its specific inhibitors. Purified OrnDC has variety in its size and characteristics. The enzyme is a dimer with a subunit of about 80K in E. coli (Morris and Boeker, 1983) and about 50K in rat liver (Kameji et al., 1982). Saccharomyces contains only one subunit of about 70K (Tyagi et al., 1981). OrnDC is of special importance because it is a rate-limiting enzyme in polyamine synthesis. Mechanism based specific inhibitors of OrnDC can be very useful in studies of possible roles of the enzyme and polyamines in cell growth and differentiation. Such inhibitors are especially useful where it is difficult to obtain suitable genetic mutations.
There are several highly specific, irreversible inhibitors for OrnDC (Fig. 3) (Bitonti et al., 1985; Sjoerdsmra and Schechter, 1984).

$\alpha$-Difluoromethylornithine (DFMO) is the most widely and extensively studied suicide inhibitor. It is highly specific for OrnDC and does not inhibit any other enzymes in ornithine metabolism (Seiler et al., 1978). DFMO covalently binds to OrnDC, and binding and inhibition can be prevented by the presence of ornithine (Pritchard et al., 1981). After administration of DFMO, the levels of putrescine and spermidine, but not spermine, usually drop rapidly and there is a considerable growth inhibition in rapidly replicating cells (Tabor and Tabor, 1984). Since DFMO inhibits cell growth, it has proved to be a useful pharmacological agent. There are a large number of studies on the relationship between cell proliferation and polyamine function that used DFMO, for example, in human cancer, in the treatment of parasitic infections, and in embryonic development as an abortifacient (Pegg and McCann, 1982; Porter and Sufrin, 1986). Especially, DFMO and other related OrnDC inhibitors are widely used to cure infections of parasitic protozoans in animals and humans (McCann et al., 1981; Sjoerdsmra and Schechter, 1984). The levels of OrnDC and polyamines increase in neoplastic tumor cells in the central nervous system. OrnDC activity can be a useful biochemical indicator of a tumor's presence and OrnDC
Fig. 3. A composite of biosynthesis for putrescine, spermidine, and decarboxylated SAM. A diagram indicating biosynthetic pathways and primary sites of inhibitor action that have been found in animals, plants, or bacterial cells. Enzymes: 1, ornithine decarboxylase; 2, SAM decarboxylase; 3, spermidine synthase. Inhibitors are indicated in boxes. Abbreviations are: DFMO, \( \alpha \)-difluoromethylornithine; \( \Delta \)-MFMOme, \( \alpha \)-monofluoromethyldehydroornithine methyl ester; R,R-MAP, (R,R)-\( \delta \)-methyl-\( \alpha \)-acyetylenic putrescine; MGBG, methylglyoxal bis(guanylhydrazone); BER, berenil; AMI, amicarbalide; HS, hydroxystilbamidine; PENT, pentamidine; ANT, Antrycide; EB, ethidium bromide; CHA, cyclohexylamine.
inhibitors can be useful chemotherapeutic agents (Scalabrino et al., 1985).

Along with DFMO, several other inhibitors of OrnDC have been synthesized and their effects examined; for example, $\alpha$-monofluoromethyldehydroornithine ($\Delta$-MFMO), $\alpha$-monofluoromethyldehydroornithine methyl ester ($\Delta$-MFMOme), and (R,R)-$\delta$-methyl-$\alpha$-acetylenicputrescine (R,R-MAP) (Bitonti et al., 1985; Sjoerdsma and Schechter, 1984). DFMO, $\Delta$-MFMO, and $\Delta$-MFMOme are analogues of ornithine, and R,R-MAP of putrescine. All these agents are irreversible (suicide) inhibitors--specific for OrnDC. The inhibitory effects of these drugs varies among different cells (Pegg, 1986; Pera et al., 1986).

1.2.2. Inhibitors for S-adenosylmethionine decarboxylase.

SAMDC is another rate-limiting enzyme in polyamine biosynthesis, but has not been as extensively studied as OrnDC. However, it also has a relatively short turnover time and its activity can be changed under the effect of several stimuli (Grillo, 1985). Many "structural analogues" of polyamines are known to inhibit SAMDC. A widely known inhibitor for SAMDC is methylglyoxal bis(guanylhydrazone) (MGBG). MGBG blocks spermidine and spermine biosynthesis and cell proliferation. It is not specific for SAMDC, however, and has numerous other effects. For example, it inhibits diamine oxidase (Pegg
and McGill, 1978), stimulates OrnDC (Nikula et al., 1985a),
gives severe damage to mitochondria (Nikula et al., 1985b),
or interferes with polyamine transport (Kramer et al.,
1985). Furthermore, although MGBG is an SAMDC inhibitor in
vitro, it reduces its own effectiveness as an inhibitor in
vivo because MGBG substantially increases SAMDC activity;
(Pegg, 1986). In mouse leukemia cells, MGBG induces a
massive accumulation of putrescine and strikingly
stimulates ODC activity by stabilizing the enzyme. MGBG
also counteracts the inhibitory effects of DFMO by unknown
mechanisms (Holm et al., 1985). MGBG is a reversible
inhibitor. Its therapeutic application is limited, but it
can be used in combination with OrnDC inhibitors (McCann et
al., 1981). MGBG has an effect on mitochondrial structure
causing extensive swelling, loss of internal structure and
increase in matrix density, and rapid aggregation of the
organelles in mouse leukemia cells (Pathak et al., 1977;
Porter et al., 1979), various human cell lines
(Mikles-Robertson et al., 1979), yeast cells (Diala et al.,
1980), and rat liver (Byszkowski et al., 1981). It also
induces structural alteration of the kinetoplast complex in
Trypanosoma (Chang et al., 1978). It causes depletion of
adenosine triphosphate pools and a marked reduction of
overall adenylate energy charge in mouse leukemia cells
(Porter et al., 1979). It causes extensive mitochondrial
DNA deletion in yeast cells (Diala et al., 1980). The
antiproliferative action of MGBG could be more directly related to mitochondrial damage than to inhibition of polyamine biosynthesis (Diala et al., 1980). The reason why mitochondria are targets is not known. However, MGBG is a "structural analogues" of spermidine and addition of spermidine can counteract the mitochondrial damage and cell growth inhibition induced by MGBG (Chang et al., 1978). Thus, the cytotoxicity of MGBG also might be caused by its interference with normal spermidine function.

MGBG is an aliphatic "structural analogue" of spermidine having amidine groups at both ends. Amicarbalide, hydroxystilbamidine, pentamidine, and Berenil are aromatic diamidines also related to spermidine. Diamidines can inhibit nucleic acid and protein synthesis, disrupt ribosomal structure, and inhibit thymidylate synthetase (Bacchi et al., 1981). Hydroxystilbamidine and pentamidine release Mg^{2+} and polyamines from trypanosomal ribosomal preparations (Wallis, 1966). Pentamidine may compete with polyamines for binding to mitochondrial or kinetoplast DNA, especially, at adenosine-thymidine rich regions (Sands et al., 1985). In Leishmania, pentamidine even caused severe disruption of mitochondrial cristae (Croft and Brazil, 1982). Amicarbalide as well as Berenil inhibited DNA synthesis in calf thymus (Marcus et al., 1985). Berenil and pentamidine irreversibly inhibited SAMDC activity in rat liver, yeast,
and *E. coli* (Karvonen et al., 1985). Berenil also was an irreversible inhibitor of SAMDC in *Trypanosoma brucei* (Bitonti et al., 1986). Antrycide and ethidium bromide (EB) are not diamidines, but are more distantly related "structural analogues" of spermidine with the same backbone structures (Fig. 26). Antrycide induces an intracellular aggregation of ribosomes which makes ribosomes biologically inactive (Newton, 1966). It also leads to release of Mg$^{2+}$ and polyamines from isolated *Crithidia oncopelti* ribosomal preparations (Newton, 1966). EB damages mitochondria in mouse leukemia cells causing depletion of ATP pools, reduction in adenylate energy charge, and formation of numerous electron densities within the matrix (Porter et al., 1979). The damage by MGBG can be repaired by removing that agent, but the damage by EB cannot be recovered. All these "spermidine analogues" including diamidines are cations and, therefore, can compete with polyamines. They all have the potential to inhibit SAMDC reversibly or irreversibly and to subsequently block cell proliferation.

1.2.3. **Inhibitors of spermidine synthase.** Spermidine synthase carries out the synthesis of spermidine and 5'-methylthioadenosine (MTA) from putrescine and dcsAM (Fig. 1). The enzyme is inhibited by several different drugs, S-adenosyl-1,8-diamino-3-thio-octane (SADTO),
cyclohexylamine (CHA) and by its by-product MTA. SADTO was the most potent specific inhibitor (Pegg, 1986; Pegg et al., 1983), but CHA (DCHA) also was a strong inhibitor of spermidine synthase in rat brain tumor cells (Feurstein et al., 1985) and rat hepatoma HTC cells (Mitchell et al., 1985). In rat brain tumor 9L cells, 1 mM CHA (DCHA) blocked cell growth, depleted spermidine to less than 10% without affecting the spermine level, and caused the accumulation of putrescine to more than 350%. However, growth recovered within 2 days without any change in the spermidine or putrescine levels even though the treatment was continued. The high level of putrescine probably could replace the function of the depleted spermidine. Similar results were found in CHA treated HTC cells. Besides the severe changes in levels of putrescine and spermidine, however, CHA also enhanced the conversion of the preexisting spermidine to spermine. The depression of spermidine was accompanied by a 4-fold increase in the stability of OrnDC.

1.3. **Role of polyamines in cell proliferation.** It has been reported for a long time that the concentrations of polyamines and the activities of polyamine biosynthesis are elevated in rapidly growing cells and tissues. The onset of the cell cycle and of cell division in quiescent or
cycling cells is preceded by increases in the rates of DNA, RNA, and protein synthesis, and these changes are correlated with changes in polyamine metabolism.

1.3.1. Polyamine metabolism during the cell cycle. Polyamines are required for optimal growth in all cells that have been tested. In general, when cells are in the regular division cycle or quiescent cells are stimulated to multiply, the levels of OrnDC and polyamines increase during G1 phase (Tabor and Tabor, 1984). The synthesis of polyamines is initiated in mid-G1 and they start to accumulate toward the end of this phase in Chinese hamster ovary (CHO) fibroblasts (Heby and Andersson, 1980). The highest rate of synthesis is at the beginning of S phase. The levels of polyamines and OrnDC decrease during mid-S phase, but start to increase again at the end of this phase and reach a second maximum right before cell division. This biphasic increase of polyamines has been noted in other experimental cell systems as well (McCann et al, 1975). These results suggest that polyamines might be involved in the cell's preparations for DNA synthesis. The second surge in OrnDC and polyamine in G2/M may influence the chromosome condensation of nuclei prior to mitosis. Therefore, the biphasic peaks of polyamines may be part of the cell's preparation for DNA synthesis and cell division.
DFMO can completely inhibit cell growth in transformed mouse fibroblasts (Pegg et al., 1981), in *E. coli* and *Pseudomonas aeruginosa* (Bitonti et al., 1982), and in the fungus *Helminthosporium mydis* (Birecka et al., 1986). The cultures of *E. coli* and *P. aeruginosa* showed 40% and 70% increases, respectively, in generation time by treatment with DFMO. Decreased polyamine levels were correlated with increase of generation time. Even though both putrescine and spermidine restored the growth inhibited by DFMO, putrescine was much more effective than spermidine in *P. aeruginosa* (Bitonti et al., 1984). MGBG did not completely inhibit, but increased the generation time in yeast cells (Brawley and Ferro, 1979). DFMO with or without MGBG specifically inhibited DNA replication in activated lymphocytes and caused an the expansion of S phase (Rupniak and Paul, 1978). Likewise, the increase of generation time caused by treatment with inhibitors of polyamine biosynthesis was thought to be the result of the expansion of G1 and S phase, but not G2 and M phase, in CHO cells (Harada & Morris, 1981). However, according to more recent results, the prolongation of S and G2 phase is more directly related to the increase of doubling time in CHO cells (Anehus et al., 1984). Those two different findings may result from different cell lines, culture conditions, and analytical methods.
1.3.2. **Role of polyamines in DNA synthesis.** Polyamines are strong cations and stabilize DNA molecules. They can protect DNA from denaturation by heat or from damage by shearing and can precipitate DNA to form DNA-polyamine complexes (Tabor and Tabor, 1984). When dilute solutions of DNA are treated with spermidine or spermine under controlled conditions, they increase the compaction of double strands in DNA molecules rather than causing gross aggregation. Polyamines may be associated with the whole chromatin. Spermine was able to increase the ability of isolated rat liver nuclei to synthesize RNA and the transcription of isolated liver chromatin while other polycations were ineffective (Barbiroli et al., 1978). Moreover, isolated chromatin from rat liver or kidney cells had polyamine acetyltransferase activity which could acetylate putrescine, spermidine, and spermine (Blankenship and Walle, 1978). Interestingly, histones contained the same N-acetyltransferase activity. Thus, the acetylation of histones and/or polyamines may affect chromatin structure through effects on binding of histones and/or polyamines to DNA.

Polyamines also are known to interact with various enzymes concerned with DNA synthesis or metabolism, but there is no definite proof that this occurs *in vivo*. However, there is evidence that polyamines are involved in
either DNA chain initiation or movement of DNA replication forks. DFMO inhibited incorporation of $^3$H-thymidine into DNA of bovine lymphocytes (Seyfried and Morris, 1979) and the inhibition was correlated with depressed intracellular polyamine levels (Holtta et al., 1979). In experiments with rat brain tumor (Heby et al., 1977), human WI-38 and mouse BAU3/3T3 cells (Bey et al., 1978), it was found that MGBG reduced or stopped the flow of cells into S phase. Polyamine-dependent cells in the absence of polyamines showed a decrease of DNA chain initiation without alteration of chain elongation (Sunkara et al., 1979). However, Geiger and Morris (1980) found that polyamine depletion resulted in reduction of the rate of DNA replication fork movement in E. coli. DFMO prolonged the progression of S phase without affecting the entry into S in bovine lymphocytes, indicating a role of polyamines in DNA elongation (Seyfried and Morris, 1979). MGBG inhibited the synthesis of not only nuclear DNA but also mitochondrial DNA (Feuerstein et al. 1979; Diala et al., 1980). In yeast cells, MGBG selectively inhibited mitochondrial DNA synthesis, thus suggesting some selective interaction with DNA based on nucleotide sequence or overall structure (Diala et al., 1980). Some irreversible inhibitors of SAMDC also inhibit DNA synthesis. For example, both pentamidine (Sands et al., 1985) and Berenil (Newton, 1972) inhibited kinetoplast DNA synthesis. Also,
Berenil and amicarbalide inhibited Rausher leukemia virus DNA polymerase DNA-directed DNA synthesis (Marcus et al., 1985). In contrast, Antrycide stimulated the enzyme at concentrations that inhibit eukaryotic DNA polymerase.

Work with the polyamine analogues clearly indicates that they can influence DNA synthesis and may mean that polyamines are important for this process. The role of the polyamines could be related to DNA structure or to regulation of enzymes and other proteins required for DNA replication.

1.3.3. Role of polyamines in RNA synthesis. RNA synthesis is not significantly affected by inhibitors of polyamine biosynthesis according to most studies. However, there seems to be some kind of correlation between RNA synthesis and intracellular levels of polyamines and OrnDC. For example, androgen-stimulated mouse kidney cells in which RNA synthesis was stimulated, also had an almost 1,000-fold increase in OrnDC activity (Henningsson et al., 1978). In another example, injection of OrnDC into Xenopus oocytes induced stimulation of ribosomal RNA synthesis (Russell, 1983). The injection of OrnDC resulted in a four-fold increase in incorporation of guanosine into 45S rRNA and a two-fold increase in 18S/28S RNA. DFMO reduced the guanosine incorporation to about 10% of that in control-injected oocytes. Incubation of oocytes in 10 mM
DFMO resulted in a 92% inhibition of OrnDC activity and decrease in putrescine concentration. Externally added putrescine could not remove the inhibitory effect of DFMO, except that the highest guanosine incorporation occurred after DFMO was washed out in the presence of putrescine. This result indicated both OrnDC and putrescine were necessary for the initiation of rRNA synthesis.

1.3.4. **Role of polyamines in protein synthesis.** A polyamine-depleted mutant of *E. coli* which could not synthesize putrescine grew slowly in minimal medium. The addition of spermidine induced immediate stimulation of DNA, RNA, and protein synthesis and rapid cell division (Young and Srivasan, 1972). The result suggests the possibility that polyamines are important in protein synthesis. However, in most cases studied, the effects are not specific in that they can be duplicated by larger amounts of divalent cations. For example, putrescine or Mg$^{2+}$, but not spermidine, stabilize ribosomes of *Pseudomonas* species in an *in vitro* protein synthesizing system (Rasano et al., 1983). Similar results were found in *E. coli* (Algranati and Goldemberg, 1977) and *B. subtilis* (Igarashi et al., 1982) where spermidine or Mg$^{2+}$ stabilized ribosomes *in vitro*. However, there is some evidence for specific effects of polyamines on protein synthesis. Ribosomes of polyamine-depleted *E. coli* were
less active in protein synthesis compared to normal ribosomes, even in the presence of Mg\(^+2\) (Igarashi et al., 1981). The defect was related to a decrease in the methylation of 16S rRNA and in the amount of S1 protein in 30S ribosomes. Polyamines also may be involved in peptide chain elongation by reducing misincorporation of amino acids into peptide chains and by preventing premature peptide termination. In an experiment using polyamine-depleted *E. coli*, erroneous incorporation of amino acids into bacteriophage MS2 increased two-fold in the absence of polyamines (McMurry and Algranati, 1986).

1.4. Role of polyamines in cell differentiation. There is a large amount of experimental data which shows a close correlation between cell differentiation, OrnDC activity and polyamine accumulation. In the water mold, *Blastocladiella emersonii*, the activity of OrnDC increased 20-fold during sporulation even while RNA and protein were broken down. The level of spermidine was not significantly changed, but the putrescine level increased 2-fold (Mennucci et al., 1975). During the yeast-hypha transition of a dimorphic fungus *Mucor racemosus*, the activity of OrnDC increased 30 to 50-fold. The maximum activity was reached right before conversion and the activity dropped rapidly thereafter (Inderlied et al., 1980). Both exogenous
putrescine and spermidine blocked the increase in enzyme activity, which suggests that the reason for the transitory increase in OrnDC activity was the production of putrescine or spermidine. In contrast, the rise in OrnDC activity and accumulation of putrescine and spermidine during differentiation was not essential in another water mold Achlya ambisexualis (Wright et al., 1982). OrnDC activity increased 5.2-fold during hormone-induced sexual differentiation of mycelia in an enriched medium, and the increase of OrnDC activity was correlated with an increase in putrescine and spermidine. However, sexual differentiation in Achlya also occurred without the rise of OrnDC activity or polyamine levels in a minimal medium.

Treatment with DFMO blocked differentiation in mouse fibroblasts (Bethell and Pegg, 1981) and LG myoblasts (Erwin et al., 1983). The process was restored to control values by the addition of putrescine or spermidine along with the inhibitor. The opposite results were found in several different embryonal carcinoma cell lines (Schindler et al., 1983; 1985). The inhibition of OrnDC by DFMO induced differentiation in murine embryonal carcinoma cells. The activity of OrnDC dropped over 95% in some cell lines during differentiation. DFMO also reduced levels of putrescine and spermidine, but had less effect on spermine levels. The addition of putrescine along with DFMO blocked differentiation. A similar result was found in the
induction of transformation from long and slender to short and stumpy forms in *Trypanosoma* by DFMO (Giffin et al., 1986). MGBG induced differentiation in Friend erythroleukemia, but this could be blocked by dimethyl sulfoxide and hexamethylene bisacetamide, which stimulate OrnDC activity (Gazitt and Friend, 1980). Clearly, the activity of OrnDC in cell differentiation needs more study.

Spermidine may have a role in the differentiation of some experimental systems. Epithelial cells in mouse mammary glands start functional differentiation upon initiation of pregnancy or by hormone-induction with prolactin, insulin, or cortisol (Oka et al., 1981). The cells respond to these stimuli by increasing the synthesis of milk proteins, casein, and \( \alpha \)-lactalbumin and proliferating. An increase of spermidine and spermine levels in functionally differentiated cells precedes the onset of DNA synthesis. If spermidine synthesis is blocked by MGBG treatment, the initiation of DNA synthesis is prevented. The addition of spermidine along with MGBG restores the spermidine concentration to the control level and DNA synthesis resumes. The effect of MGBG can be reversed totally by spermidine, but only partially by putrescine, cadaverine, or spermine. Therefore, it was proposed that spermidine might be specifically required for the functional differentiation of mammary gland cells (Oka et al., 1981).
1.5. Polyamine studies in Acanthamoeba. There are no known specific mutants of polyamine biosynthesis in *A. castellanii*, but the ameba still has several features that make it a good model system for polyamine studies: a) it can be grown axenically in a chemically defined medium with a reasonable generation time (12-13 hours); b) synchronous differentiation can be induced by several different methods, for example, total nutrient starvation, glucose starvation, and the addition of certain metabolic inhibitors; and c) the differentiation that occurs is unambiguous and can be monitored at the morphological or biochemical levels. The fact that inhibitors such as Berenil and ethidium bromide, which had been associated with polyamine metabolism in one way or another, were very effective inducers of encystment, suggested that a more thorough study of the relationship between polyamine metabolism and differentiation in *Acanthamoeba* was warranted. Since previous experience in the laboratory suggested that it would be very difficult to obtain mutants in the polyamine biosynthetic pathway (I believe that *Acanthamoeba* is polyploid), the availability of inhibitors thought to be specific for various enzymes of polyamine metabolism offered a way of studying relationships between the synthesis and accumulation of polyamines and
encystment. This approach was stimulated by observations reported above that inhibition of OrnDC induced differentiation in mammalian embryonic carcinoma cells (Schindler et al., 1983; 1985) and trypanosomes (Giffin et al., 1986). The work that had been published on Acanthamoeba at the beginning of these studies suggested that polyamine metabolism in the ameba might be somewhat unusual. Preliminary experiments indicated that DFMO neither induced encystment nor inhibited cell multiplication in Acanthamoeba (Schuster, 1982). Moreover, Acanthamoeba would have a very unusual polyamine character if several studies were correct. Only putrescine and spermidine were detected in A. culbertsoni (Gupta et al., 1984; Srivastava and Shulka, 1982) and putrescine was the major polyamine. Putrescine, spermidine, and spermine were undetectable by TLC and HPLC in A. castellani, but a very high level of the unusual polyamine diaminopropane and trace amounts of a norspermidine-like polyamine were reported (Poulin et al., 1984). The difference between the results for the two species may be due to strain differences, but both results are rather unexpected based on results with other eukaryotes; eukaryotic cells usually have high amounts of spermidine and spermine but little putrescine, whereas prokaryotes have higher concentrations of putrescine than spermidine, but lack spermine.
Since so little was known about polyamine metabolism in the ameba, I first decided to reexamine the polyamine content of the Neff strain and then to identify the major pathways of polyamine biosynthesis. I then planned to begin studying the relationship of polyamines to growth and differentiation by using specific enzyme inhibitors. I began with inhibitors of OrnDC because these agents had been the most thoroughly tested on other systems, because some of the inhibitors had been shown to be useful chemotherapeutic agents in other organisms, and because OrnDC was a key enzyme in the polyamine biosynthetic scheme. I next examined a group of inhibitors that shared some structural relationship to spermidine because some members of the group were known to inhibit SAMDC, another key enzyme, because a couple of these inhibitors were known to induce differentiation, and because similar compounds had been effective chemotherapeutic agents against amebic keratitis. I did not expect these to be specific inhibitors, but was interested in whether their effects on polyamine metabolism might correlate with differentiation. Future studies were anticipated that would look at some of the other specific enzyme inhibitors.

My study had the potential of providing information about the possibility of using polyamine metabolism as a target for chemotherapy of amebic infections as well as providing information about the more general problem of how
polyamines are associated with multiplication and differentiation.
2. MATERIALS AND METHODS

2.1. Cell cultures and cloning. The Neff strain of Acanthamoeba castellani was grown in an optimal growth medium (OGM) or in a chemically defined medium (DGM-11) prepared as described in Byers et al. (1980) (Table 13). Amebas were grown at 30°C in unagitated monolayers in plastic tissue culture flasks, multiwell plates, or petri dishes in culture volumes of 1.2 ml/1000 mm², or in Roux bottles in culture volumes of 5 ml/1000 mm² bottom surface. Cell numbers were determined by using a grid and an inverted microscope or with a hemacytometer. Early postexponential phase cultures of about 1200 amebas/mm² in Roux bottles were used for the majority of experiments. Multiwell plates with 24 wells were used for determining the concentrations of drugs that affected cell multiplication or differentiation.

Subclones were begun in 96-well plates. I seeded 0.5-1 amebas/well in OGM cultures or 5 amebas/well in DGM-11 cultures. Viability was relatively low, thus, the surviving progeny should be clones. Five days (OGM) or nine days (DGM-11) later, amebas from wells containing fast growing cultures were transferred to 9 cm² petri dishes.
dishes. After the second transfer, amebas were transferred to 25 cm² plastic tissue culture flasks for further growth, or were induced to encyst by starvation. Cysts were stored in silica gel (Seilhamer, 1979). Subclones used for the experiments in OGM and DGM-11 were BK 35 and BK 92-2, respectively. BK 35 was subcloned from PH 28 which was cloned from the Neff strain. BK 92-2 was a subclone of BK 92 which was subcloned from BK 35. Both BK 35 and BK 92-2 were selected for high encystment in berenil.

2.2. Inhibition of multiplication. In studies on the inhibition of multiplication by polyamine antagonists, inhibitors were added during early exponential phase at about 80 amebas/mm² in 25 cm² plastic tissue culture flasks. All antagonists were dissolved in water, except ethidium bromide and dicyclohexylamine which were dissolved in 70 % ethanol. Cell growth was checked twice a day after inhibitor addition until each culture reached a stationary phase.

2.3. Induction of encystment. Starvation induced encystment was initiated in late exponential phase OGM or DGM-11 cultures at 800–1000 amebas/mm² by pouring off
the medium and replacing it with Neff's encystment medium (NEM) (Martin, 1973). Cyst counts were made daily and were maximal by the fourth day. Drug-induced encystment was initiated by adding inhibitors to cultures in early to midexponential phase. To obtain the best encystment, cultures were treated at densities of about 300 amebas/mm² (Akins, 1981). Encystment was maximal by the fourth day when cultures were fixed with 0.5 % formaldehyde and examined in a hemacytometer for cyst counts.

2.4. Homogenization of amebas for polyamine and enzyme assays. The cells were harvested by scraping them from bottom surfaces of culture flasks or bottles with a rubber policeman. They were washed twice in 0.15 M KCl and then resuspended in this salt solution. Trophozoites were homogenized by sonication (Branson Sonic Power, Branson Instruments Inc., Danbury, CN) for 10 seconds at 10 amperes with probe No. 3235. Cysts were disrupted in a French press (SLM Instruments Inc., Urbana, IL) at 2,000 psi. In both homogenization procedures, the total volume was confined to less than 1.5 ml. Homogenate supernatants were prepared by centrifugation for 20 minutes at 38,000 g.
2.5. **Separation and quantitation of polyamines.** Most of the identification and quantitation of polyamines was done by methods adapted from Flores and Galston (1982). Approximately $1.2 \times 10^8$ amebas were harvested when putrescine was measured, or $4 \times 10^7$ when only the more abundant polyamines were assayed. Polyamines were extracted from ameba homogenates for 30 minutes in 6 % ice-cold $\text{HClO}_4$. Extracts were centrifuged 20 minutes at 10,000 g. The supernatant containing free polyamines was stored frozen at $-20^\circ\text{C}$ in a plastic vial, or was used immediately. Cell extracts or standards were benzoylated according to Redmond and Tseng (1979). Three ml of 2 N NaOH were added to 1.5 ml of the $\text{HClO}_4$ extract in a plastic centrifuge tube. Thirty ul of benzoylchloride was added, the mixture vortexed for 10 seconds, and the samples then incubated for 20 minutes at room temperature. The reaction was terminated by adding 6 ml of saturated NaCl. Benzoylated polyamines were extracted in 5 ml anhydrous diethyl ether. The phases were separated by centrifugation at 1,500 g for 5 minutes. The ether layer was collected, evaporated to dryness, and redissolved in 100-200 ul of solvent buffer consisting of 64 % HPLC grade methanol and 36 % water deionized with the Nanopure II deionization system (Barnstead, Boston, MA). Samples were filtered through 0.2 um or 0.4 um membrane filters and then used immediately or stored at $4^\circ\text{C}$.
Stored samples were used within one five days. A 20 μl inoculation loop was used for each analysis with a programmable Altex HPLC system connected to an Hewlett Packard 3390A integrator. The sample was run isocratically at the flow rate of 1 ml/minute. An Alltech C18 reversed phase column (25 cm x 4.6 mm, 5 μm diameter resin) was used for all analyses and the polyamines were detected at 254 nm. Separation of putrescine and diaminopropane was best with a fresh column.

2.6. Invitro enzyme assays. Enzyme assays used either crude cell homogenates or homogenate supernatant fluid obtained after 20 minutes centrifugation of the homogenate at 38,000 g. Whole homogenates were prepared by resuspending 4-8 x 10⁷ amebas/ml in a proper reaction buffer, and then disrupting them by sonication or with a French press as described above (Section 2.4).

2.6.1. Arginine decarboxylase. Arginine decarboxylase activity was searched for by looking for the release of 14CO₂ from DL-(1-14C)-arginine or L-(U-14C)-arginine. The assay mixture, modified from the method of Smith (1983a), contained 0.8 ml of 0.1 M Tris-HCl (pH 7.5), 1 mM freshly prepared pyridoxal phosphate, 25 mM freshly prepared dithiothreitol, 31.3 mM
DL-arginine or L-arginine, 2 uCi of DL-(1-14C)-arginine or L-(U-14C)-arginine, and 0.2 ml of whole homogenates or supernatants of 0.8-1.6 x 10^7 amebas. The reaction was run for up to 1.5 hours at 30°C in a tightly stoppered 15 ml scintillation vial, and then was terminated by adding 0.2 ml of 6 N HCl. 14CO₂ was trapped on a 1 x 1.5 cm² piece of filter paper saturated with 50 ul of 2 N KOH and suspended on a pin from the rubber stopper. After termination of the reaction, the vial was incubated for 30 minutes more to capture any further 14CO₂ released. The thirty minutes of additional incubation was enough because 40 or 50 minutes of additional incubation did not increase the level of 14CO₂ captured. The filters were then dried and counted by liquid scintillation. Controls were prepared by premixing supernatants or homogenates with HCl.

2.6.2. Arginase. Arginase activity was detected by assaying for urea and ornithine production from arginine using the reaction conditions of Schimke (1970). Amebas washed in 0.15 M KCl were resuspended to 0.01 M MnCl₂ to a concentration of 8 x 10^7 amebas/ml. The cells were homogenized and 38,000 g supernatants prepared (Section 2.4). The homogenates or supernatants were heated at 55°C for 5 minutes to activate the enzyme and then equilibrated for 10 minutes at 37°C. One tenth ml of
50 mM L-arginine plus 1 uCi L-(U-\(^{14}\)C)-arginine at pH 9.7 was mixed with 0.1 ml of homogenates or supernatants. The reaction was incubated at 37°C in a water bath. Samples were taken at 15, 30, 60, and 90 minutes.

The following assay was used for detection of ornithine production from arginine. One tenth ml of the reaction mixture was transferred to a test tube containing 10 ul of 6 N HCl and incubated for 5 minutes at room temperature to stop the reaction. Zero time samples were boiled or acid-treated prior to the addition of substrate. The samples were centrifuged for 5 minutes at 650 g to remove the acid precipitate. Then, 5 ul of the supernatant was applied to a TLC plate for the separation of \(^{14}\)C-ornithine from \(^{14}\)C-arginine. Arginine and ornithine on the TLC plate were separated by solvent system I (Section 2.8), and visualized by ninhydrin staining. Autoradiograms were prepared from the TLC plates (Section 2.9).

For the measurement of arginase activity by urea production, 25 ul of 30 % TCA was added to the remaining 0.1 ml of the above reaction mixture to stop the reaction. The sample was incubated for 5 minutes at room temperature and then was mixed with 0.1 ml of water. After centrifugation for 5 minutes at 650 g, 0.2 ml of the reaction mixture supernatant was used for a Blood Urea Nitrogen (BUN) colorimetric test (Kit No. 353-B, Sigma
Chemical Co., St. Louis, MO). To each sample, 2.8 ml of BUN acid reagent and 2.0 ml of BUN color reagent were added, and mixed thoroughly. All samples were simultaneously placed in a boiling water bath for exactly 10 minutes, then quickly removed and placed in cold tap water for 5 minutes. The amount of urea nitrogen was measured with a spectrophotometer (Spectronic 2000, Bausch and Lomb Inc., Rochester, NY) at 535 nm, and readings were completed within 20 minutes. Standard curves were prepared for each test using a standard provided by Sigma (urea at a urea N level of 53.5 mmol/L). The range of the standard urea N concentrations was from 0 to 26.8 umol/ml.

2.6.3. Arginine deiminase. I attempted to assay arginine deiminase by the production of citrulline from arginine. Ameba homogenates were prepared as described above (Section 2.4) in a 0.1 M potassium phosphate buffer (pH 6.5) (Oglinsky, 1955), or in a 40 mM Na citrate buffer (pH 6.4) (Yoshida et al., 1978). For the detection of $^{14}$C-citrulline production from L-(U-$^{14}$C)-arginine, 0.1 ml of supernatant (8 x 10^7 amebas/ml) was mixed with 0.1 ml of 50 mM L-arginine plus 1 uCi L-(U-$^{14}$C)-arginine. The reaction was incubated up to 60 minutes at 37°C and terminated by adding 20 ul of 6 N HCl. The mixtures were centrifuged for 5 minutes at 650 g. Zero time controls used boiled or acid-fixed enzyme
preparations. Five µl of the resulting supernatant was applied to a TLC plate and separated by solvent system II (Section 2.8).

NH₃ measurements also were used in an effort to detect arginine deiminase activity (Searcy et al., 1961). The reaction conditions were the same as those above except that unlabeled arginine was used as substrate. After 30 minutes of reaction at 37°C, the reaction was stopped by adding 5 ml of phenol color reagent and 5 ml alkaline hypochloride solution. Color was developed by 15 minutes incubation at 37°C and the amount of NH₃ was colorimetrically determined at 630 nm. The calibration curve was prepared with a nitrogen standard (35.35 mg ammonium sulfate in 100 ml of Nanopure water) in the concentration range of 0 to 7 ug NH₃/reaction tube.

Phenol color reagent contained 5 g phenol and 25 mg nitroprusside in a final volume of 500 ml of Nanopure water stored at 4°C in glass. Alkaline hypochlorite reagent contained 2.5 g NaOH and 0.21 g Na hypochlorite per 500 ml of Nanopure water. The hypochlorite was prepared from Chlorox containing 5.25 % (w/v) of Na hypochlorite.

2.6.4. Citrulline hydrolase/Citrullinase. Supernatants of ameba homogenates were prepared using 4 x 10⁷ amebas/ml in 40 mM Na citrate buffer (pH 6.4) (Yoshida et al., 1978). Reactions were carried out in 20 ml scintillation vials by
mixing 0.2 ml of supernatant with 0.8 ml of 12.5 mM L-citrulline plus 1 uCi $^{14}$C-citrulline prepared in the reaction buffer. The preparation of zero time controls, termination of the reaction, and detection of $^{14}$CO$_2$ production from L-(ureido-$^{14}$C)-citrulline used the same methods as in the measurement of arginine decarboxylase activity (Section 2.6.1). Unlabeled 0.1 M citrulline, instead of labeled citrulline, was used in a 0.2 ml total reaction volume to visualize the production of ornithine from citrulline. The reaction was incubated for 30 minutes and terminated by adding 20 μl of 6 N HCl. The development of thin layer chromatography was the same as for of arginase (Section 2.6.2).

2.6.5. Urease. Urease was assayed in whole homogenates or homogenate supernatants by the method of Yoshida et al. (1978). The reaction mixture contained 0.25 M sodium phosphate (pH 6.4), 0.85 mM or 0.04 mM urea plus 1 uCi $^{14}$C-urea, and enzyme preparations from 8 x 10$^6$ amebas. Assay conditions, measurements of $^{14}$CO$_2$ released from $^{14}$C-urea, and preparations for controls were the same as those in arginine decarboxylase (Section 2.6.1).

2.6.6. Ornithine decarboxylase. Ornithine decarboxylase activity was determined by measuring the rate of formation
of $^{14}\text{C}_2\text{O}_2$ from DL-(1-$^{14}$C)-ornithine and by measuring $^{14}$C-putrescine production from DL-(5-$^{14}$C)-ornithine. Supernatants from ameba homogenates were prepared as described above (Section 2.4) using 4 x $10^7$ amebas/ml of the same buffer as used for the assay of arginine decarboxylase (Section 2.6.1). The reaction was incubated up to 140 minutes at 37°C. All the other assay conditions and the $^{14}$CO$_2$ detection method were the same as those for arginine decarboxylase except that the substrate was 10 mM DL-ornithine plus 1 uCi DL-(1-$^{14}$C)-ornithine.

For the detection of putrescine production from ornithine, 0.1 ml of ameba homogenate supernatant was mixed with 0.1 ml of 20 mM DL-ornithine plus 1 uCi DL-(5-$^{14}$C)-ornithine and incubated at 37°C in a water bath. Reactions were terminated by adding 20 ul of 6 N HCl and 5 ul aliquots prepared as in the arginase assay (Section 2.6.2) were used for TLC using solvent system I (Section 2.8).

2.6.7. Diaminopropane synthetase. I attempted to detect diaminopropane synthesis by assaying for polyamine oxidase and spermidine dehydrogenase. The polyamine oxidase assay was a modification of the method of Smith (1983b). The reaction mixture included 0.25 ml of 0.1 M Tris-HCl (pH 6.5), 50 ug/ml of horseradish peroxidase (Type II), 15 mM
spermidine plus 5 uCi (UL-^H)-spermidine, and ameba homogenate fractions prepared from 8 x 10^6 amebas at either 30°C or 37°C. Reactions were stopped by adding 20 ul of 6 N HCl after 30 or 60 minutes incubation. The samples were centrifuged for 5 minutes at 650 g to remove the acid precipitate. Then 5 ul of each supernatant were applied to a TLC plate for the separation of ^H-diaminopropane from ^H-spermidine using solvent system I (Section 2.8). The enzyme preparations used were whole homogenates or 38,000 g supernatants and were dialyzed or non-dialyzed. Each preparation represented 4 x 10^7 amebas/ml. Homogenates or supernatants were dialyzed in cellulose dialysis tubing with molecular weight cutoff of 3,500 (Spectrapor Medical Ind. Inc., Los angeles, CA) for 24 hours in 0.1 M Tris-HCl buffer (pH 6.5).

The spermidine dehydrogenase assay was a modification of the method of Campello et al. (1965). The incubation mixture consisted of 0.1 ml of 0.1 M potassium phosphate (pH 6.5), 0.1 mM phenazine methosulphate or 1 mM potassium ferricyanide as an electron carrier, 10 mM spermidine plus 10 uCi (UL-^H)-spermidine, and 0.1 ml of each enzyme preparation. The reaction was incubated for 30 minutes at 37°C, and terminated as described above. Methods for enzyme preparations and preparation for TLC were the same as those in polyamine oxidase assay, except that potassium buffer was used instead of Tris-HCl buffer.
2.7. In vivo assays for enzyme activity. Arginine decarboxylase, arginine deiminase, and diaminopropane synthetase could not be detected by standard in vitro assay methods in A. castellanii, indicating the absence of these enzymes or unfavorable reaction conditions. As a check for the possible presence of other activities that could convert arginine to agmatine or citrulline, or spermidine to diaminopropane, in vivo labeling experiments were performed.

2.7.1. Arginine decarboxylase and arginine deiminase. For the measurement of arginine deiminase, amebas grown 3 days in Roux bottles in DGM-10 (DGM-11 without arginine) were harvested and washed twice with DGM-10 and then resuspended in 0.4 ml of DGM-10 supplemented with 0.47 mM L-arginine plus 10 uCi L-(U-14C)-arginine at 37°C. DGM-11 has 4.7 mM arginine. One tenth ml samples were taken after 0, 2.5, 5, and 9 hours incubation. Amebas were washed twice with 1.5 ml of 0.15 M KCl by centrifugation at 10,000 g for 7 seconds. After the second wash, the supernatant was removed and the pellet was frozen and thawed twice to break the intact amebas. The disrupted pellet was resuspended in 50 ul of 0.2 N HCl and centrifuged for 20 minutes at 10,000 g. Five ul of the aliquot was applied to a TLC plate for
the separation of $^{14}$C-arginine or $^{14}$C-citrulline from $^{14}$C-arginine using solvent system I or II (Section 2.8), respectively.

2.7.2. Diaminopropane synthetase. For in vivo experiments, a total of $2 \times 10^7$ amebas were washed twice with DGM-11 and resuspended in 0.4 ml of DGM-11 with 60 uCi of 0.1 mM spermidine plus 60 uCi (UL-$^3$H)-spermidine in a 5 ml glass vial at 30°C. A 0.1 ml sample was removed after 0, 1, 3, and 7 hours incubation and mixed with 1.5 ml of 0.15 M KCl. Amebas were collected by centrifugation for 7 seconds at 10,000 g. After a second wash with 0.15 M KCl, the pellet was frozen and thawed twice and resuspended in 50 ul of 0.2 N HCl. The mixture was centrifuged for 20 minutes at 10,000 g. Five ul of the supernatant were applied to a TLC plate. Solvent system I was used to separate $^3$H-diaminopropane from $^3$H-spermidine (Section 2.8).

2.8. Thin layer chromatography. One dimensional ascending thin layer chromatography of polyamines was carried out on Cellulose MN 300 TLC plates (Analtech Inc., Newark, DE). A solvent containing l-butanol, acetic acid, pyridine, and water (4:1:1:2) (solvent system I) was used for the separation of arginine, ornithine, putrescine,
spermidine, spermine, diaminopropane, and agmatine (Morris and Pardee, 1966). The separation of citrulline from arginine used a solvent system containing 1-butanol, acetic acid, and water (7:1.5:1.5) (solvent system II). For all separations, 5 ul of each sample was spotted at 2 cm from the bottom of the plate. The distance between two neighboring spots was at least 1.5 cm. The sizes of spots ranged up to 5 mm in diameter at the origin. Separations were stopped when the solvent reached within 1-1.5 cm from the top of the plate. After development, the plate was dried in a hood at room temperature, sprayed with 0.5 % ninhydrin in 70 % ethanol, and heated at 100°C for 5 minutes. The cellulose was then scraped off for measurement of radioactivity or the intact plates were used for the preparation of autoradiograms. For quantitation of activity, areas of interest were scraped off and transferred to a scintillation vial, and counted in 10 ml of Scinti Verse (Fisher Scientific, Fair Lawn, NJ). The activity was measured by a Beckman LS 6800 liquid scintillation counter (Beckman, Irvine, CA).

2.9. Autoradiography. Each completely developed TLC plate was sprayed with either EN³HANCE (New England Nuclear, Boston, MA) or 20 % 2,5-diphenyloxazole in DMSO to enhance the fluorescent activity. The plates were then dried in a
hood. The enhancer sprayed plate was exposed to 8 x 10 inch Kodak XAR-5 film (Eastman Kodak, Rochester, NY) in Kodak X-ray film holders containing Coronex Quanta-III intensifying screens. Exposure time varied widely, from 1-20 days at -20°C. Film was developed 5 minutes with Kodak developer No. 1900984, development was stopped by 1.3 % acetic acid (0.5 minutes), and the film was fixed with Kodak fixer No. 1902485 (2.5 min). Standard procedures were used.

2.10. Protein assay. Ameba protein contents were measured with the Comassie Brilliant Blue technique using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA). Standards were γ-globulin bovine Cohn fraction II purchased from Sigma.

2.11. Chemicals. Putrescine dihydrochloride, diaminopropane dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, cadavarine dihydrochloride, horseradish peroxidase (Type II), pyridoxal 5-phosphate, DL-dithiothreitol, ethidium bromide, CHA (cyclohexylamine), isethionate, L-arginine, DL-arginine, L-citrulline, DL-ornithine, and agmatine were obtained from Sigma. MGBG (methylglyoxal-bis
(guanlyhydrazone) and norspermidine were from Aldrich Chemical Co. Inc. (Milwaukee, WI). Berenil was from Calbiochem-Behring Corp. (La Jolla, CA). DFMO
difluoromethylornithine), DFMA (difluoromethylarginine),
$\Delta$-MFMOme ($\alpha$-monofluoromethyldehydroornithine methyl ester), R,R-MAP ((R,R)-$\delta$-methyl-$\alpha$-acetylenic putrescine),
and hydroxystilbamidine were gifts from the Merrell Dow Research Institute, Cincinnati, OH. USA. Amicarbalide,
Antrycide, and pentamidine isethionate were gifts from Dr. C. J. Bacchi (Pace University, NY, NY).
L-(ureido-$^{14}$C)-citrulline (50.5 mCi/mmol) and
$^3$H-spermidine (24.3 Ci/mmol) were obtained from New England Nuclear Corp. DL-(1-$^{14}$C)-arginine (40-50 mCi/mmol), L-(U-$^{14}$C)-arginine (250-300 mCi/mmol),
DL-(1-$^{14}$C)-ornithine (40-50 mCi/mmol), and
DL-(5-$^{14}$C)-ornithine (40-55 mCi/mmol) were purchased from Research Products Intl. (Mt. Prospect, IL).
3. RESULTS

3.1. Polyamine contents of postexponential phase amebas. Polyamines have been found in all tested bacteria, animals, and plants (Smith, 1985a; Tabor and Tabor, 1985). The numbers of kinds of polyamines present vary from organism to organism and can even be different in the same organism in different culture conditions. Therefore, I examined polyamines in amebas grown in both broth (OGM) and defined growth medium (DGM-11).

3.1.1. Cultures grown in broth. All three of the most commonly found polyamines including putrescine, spermidine, and spermine were detected by high performance liquid chromatography (Fig. 4). Besides these three major polyamines, 1,3-diaminopropane and norspermidine, which are not so commonly found, also were detected. The level of diaminopropane was very high compared to putrescine (Table 1) and the retention times of these two compounds were very close (Table 2). Thus, the putrescine peak was easily obscured by the large peak of diaminopropane. The lack of good separation became worse when the column got old. All tested polyamines eluted within 20 minutes in experiments
Fig. 4. Separation of benzoylated polyamines by HPLC. A. A standard mixture containing 0.2 nmoles of each polyamine. B, C. Extracts of postexponential phase amebas grown in either DGM-11 (B) or OGM (C). The peaks are 1, putrescine; 2, diaminopropane; 3, norspermidine; 4, spermidine; 5, spermine; and 6, unknown. The solvent system used was 64% methanol.
Table 1. Polyamine contents of postexponential-early postexponential phase A. castellanii grown in OGM and DGM-11.

<table>
<thead>
<tr>
<th>Polyamine</th>
<th>Intracellular content</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>fmol/ameba&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>OGM</td>
</tr>
<tr>
<td>Putrescine</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>(0.01)</td>
</tr>
<tr>
<td>Diaminopropane</td>
<td>39.10</td>
</tr>
<tr>
<td></td>
<td>(12.30)</td>
</tr>
<tr>
<td>Norspermidine</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>(0.03)</td>
</tr>
<tr>
<td>Spermidine</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>(0.05)</td>
</tr>
<tr>
<td>Spermine</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>(0.23)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Average plus SD in parentheses for three replica experiments.

<sup>b</sup>Estimate based on a cell water content of 1.58 ng/ameba for the Neff strain grown in OGM. However, possible differences in growth conditions and substrains used may result in a large error when this estimate is applied to the clone used in our experiments.

<sup>c</sup>Based on an average cell protein content for postexponential phase cells of 226 +/- 66 pg/ameba in OGM and 435 +/- 87 pg/ameba in DGM-11 cultures.

<sup>d</sup>Not detected.
Table 2. Retention times of benzoylated polyamines separated by reverse phase high performance liquid chromatography.

<table>
<thead>
<tr>
<th>Polyamine</th>
<th>Retention time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putrescine</td>
<td>6.48 (0.04)</td>
</tr>
<tr>
<td>Diaminopropane</td>
<td>6.90 (0.04)</td>
</tr>
<tr>
<td>Cadaverine</td>
<td>7.47 (0.05)</td>
</tr>
<tr>
<td>Norspermidine</td>
<td>9.61 (0.07)</td>
</tr>
<tr>
<td>Spermidine</td>
<td>10.77 (0.09)</td>
</tr>
<tr>
<td>Norspermine</td>
<td>15.17 (0.09)</td>
</tr>
<tr>
<td>Spermine</td>
<td>18.55 (0.09)</td>
</tr>
</tbody>
</table>

Samples were detected at 254 nm, and separations were carried out at room temperature with a flow rate of 1 ml/minute. The solvent system consisted of methanol : water (64 : 36). Values in parentheses represent SD for three replicas.
reported here (Table 2). However, the retention time could extend up to 35 minutes in columns used for more than 200 sample injections.

In order to check the identification of putrescine, samples were sent to Drs. Peter McCann and Alan Bitonti (Merrell Dow Research Institute, Cincinnati, OH) who used cation exchange chromatography and confirmed my observation. In my assays, norspermidine was only detected in 55% of the samples. Thus, this compound may be produced at very variable rates, or may be readily converted to a different product, or may be excreted.

3.1.2. Cultures grown in defined growth medium. The contents of polyamines in perchloric acid extracts of amebas grown in defined growth medium were similar to those of amebas grown in optimal growth medium; the most significant difference was the consistent absence of spermine in DGM-11 (Fig. 4). Postexponential phase cell contents (fmoles/ameba) are in Table 1. Cadaverine and norspermine could be separated well by the same column, but the former was not detected in either OGM or DGM-11 cultures. The ameba extracts from both growth media showed one unidentified peak with a retention time of 12.52 ± 0.07 minutes (Fig. 4). Either an unidentified polyamine or an amino acid derivative containing amine groups could be a candidate for the unknown peak.
3.2. Enzymes of polyamine metabolism in postexponential phase DGM-11 cultures. Nothing previously was known about the biosynthetic pathways of polyamines in Acanthamoeba castellanii. However, it is almost impossible to explore the relationship between polyamines and amebic encystment without knowing the biosynthetic pathways and enzymes involved. Therefore, I have attempted to determine which enzymes are present and, therefore, which pathways are possible.

3.2.1. Arginase. The sole source of putrescine in most animal cells is ornithine. The latter can be derived from arginine by arginase, or citrulline by citrulline hydrolase or citrullinase, or more rarely from glutamate by glutamate kinase. The activity of arginase can be measured by the detection of urea or ornithine produced from the substrate arginine (Fig. 1). Arginase was found in 38,000 g supernatants of cell homogenates. Ornithine was detected in the reaction mixture by TLC and ninhydrin staining after 30 minutes of reaction using L-(U-14C)-arginine as a substrate (Fig. 5). Autoradiography proved that the ornithine was produced from arginine. Separated radioactive compounds were scraped off the TLC plate and counted in a liquid scintillation counter. The rate of
Fig. 5. *In vitro* production of $^{14}$C-ornithine from L-(U-$^{14}$C)-arginine by arginase. Ameba homogenate supernatants prepared as described in Section 2.4. were incubated for 60 min with 25 mM L-arginine plus 1 uCi L-(U-$^{14}$C)-arginine in arginase buffer. A. Ninhydrin stained thin layer chromatogram of reaction mixture at 0, 30, and 60 min. S, Standard mixture containing 50 nmol each of putrescine (put), arginine (arg), and ornithine (orn); T0, 0 min reaction; T30, 30 min reaction; T60, 60 min reaction. B. Autoradiogram of TLC plate.
ornithine production was 2.1 umoles/30 minutes/mg protein, and the increase of $^{14}$C-ornithine produced was proportional to the reaction time (Fig. 6), indicating that the reaction rate was at Vmax.

Arginase also was detected by the measurement of urea nitrogen produced (Table 3). The activity of the whole homogenate was the same as that of the 38,000 g supernatant indicating that arginase is a soluble enzyme. When DFMO, the specific inhibitor of ornithine decarboxylase, was present the activity of arginase dropped to 70-80% of the control. This probably was due to the accumulation of ornithine (estimated to reach 8-10mM) as indicated by the inhibition caused by adding ornithine directly. The rate of urea production was 2.34 +/- 0.33 umoles urea/30 minutes/mg protein in 3 replicas. Thus urea and ornithine appeared in near stoichiometric amounts. Of course, urea can be broken down to CO$_2$ and NH$_3$ by urease in some cells. An assay performed with $^{14}$C-urea as a substrate gave very low urease activities of 1.23 +/- 0.28 pmoles $^{14}$CO$_2$ released/30 minutes/mg protein using the whole homogenates, or 0.67 pmoles/30 minutes/mg protein using the 38,000 g supernatants. These low activities probably would not degrade urea sufficiently to have any significant effect on the measurement of arginase activity by urea production.
Fig. 6. Kinetics of the in vitro production of 14C-ornithine from L-(U-14C)-arginine by arginase. Reaction conditions were the same as in Fig. 5. Homogenate supernatants were from cultures grown in DGM-11. Arginase specific activity was based on total cell protein. The activity was determined by scintillation counting of material scraped off a TLC plate. Data represent a single experiment.
umol 14C-ornithine/mg protein vs Incubation Time (min)
Table 3. *In vitro* arginase activity measured by urea nitrogen production.

<table>
<thead>
<tr>
<th>Reaction Mixture</th>
<th>pg Urea N/ameba/ 15 min</th>
<th>Percent activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole homogenate + 0.25 M arginine</td>
<td>6.48 (0.97)</td>
<td>90.3</td>
</tr>
<tr>
<td>38K supernatant + 0.25 M arginine</td>
<td>7.18 (1.02)</td>
<td>100.0</td>
</tr>
<tr>
<td>+ 10 mM DPMO</td>
<td>5.14 (0.85)</td>
<td>71.6</td>
</tr>
<tr>
<td>+ 40 mM DPMO</td>
<td>5.77 (0.59)</td>
<td>80.3</td>
</tr>
<tr>
<td>+ 50 mM Ornithine</td>
<td>2.18 (0.89)</td>
<td>30.3</td>
</tr>
<tr>
<td>+ 100 mM Ornithine</td>
<td>0.01 (0.004)</td>
<td>0.1</td>
</tr>
<tr>
<td>+ 10 mM Putrescine</td>
<td>7.18 (0.25)</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Whole homogenates and 38 K supernatants were prepared from 8 x 10⁶ amebas in 0.01 M MnCl₂ buffer as described in Section 2.4. The reaction was incubated for 15 min at 37°C. Urea N was detected colorimetrically by BUN reagents (Sigma). SD for 3 replicas in parentheses. One pg urea nitrogen is equivalent to 35.7 fmol urea.
3.2.2. The conversion of citrulline to ornithine.
Citrulline can be converted to ornithine by two mechanisms: citrulline hydrolase or citrullinase (Section 1.1.1). Both mechanisms produce NH$_3$ and CO$_2$ besides ornithine (Fig. 1). Thus, activity can be assayed by the detection of NH$_3$, CO$_2$, or ornithine production. The most specific assays would be the detection of $^{14}$CO$_2$ or $^{14}$C-ornithine production from appropriately labeled $^{14}$C-citrulline. However, only L-(ureido-$^{14}$C)-citrulline is commercially available. This releases $^{14}$CO$_2$ but not $^{14}$C-ornithine. Therefore, I looked for the production of $^{14}$CO$_2$ and unlabeled ornithine using ameba homogenate supernatants. Ornithine could be separated from the substrate citrulline by TLC and was detected by ninhydrin after 30 minutes of reaction (Fig. 7). Ornithine production was only useful as a qualitative indication that citrulline hydrolase was present. $^{14}$CO$_2$ release was used for quantitation of the activity. The rate determined was 19.6 nmoles CO$_2$/30 minutes/mg protein. In this study, the specific enzyme responsible for ornithine production was not determined.

3.2.3. The conversion of arginine to citrulline.
Citrulline can be synthesized from arginine by arginine deiminase. The activity of arginine deiminase can be
Fig. 7. In vitro production of ornithine from citrulline. Ameba homogenate supernatants were incubated in Na citrate buffer with 10 mM unlabeled L-citrulline. Samples were taken at 0 and 30 min and chromatographed by TLC. The figure is a ninhydrin stained TLC plate. S, Standard mixture containing 50 nmol each of citrulline (cit) and ornithine (orn).
measured by the detection of either NH$_3$ production, or the production of $^{14}$C-citrulline from $^{14}$C-arginine.

Standard reaction conditions (Yoshida et al., 1978) that were tried produced negligible amounts of NH$_3$ or $^{14}$C-citrulline from $^{14}$C-arginine with either whole cell homogenates or 38,000 g supernatants. However, production of $^{14}$C-citrulline occurred when amebas were incubated in DL-$(U-^{14}$C$)$-arginine in vivo. Following labeling, cell extracts were separated by TLC and the activity in citrulline and arginine was determined by scraping the labeled regions off the plates and counting in a liquid scintillation counter (Fig. 8). Arginine started to be taken up after one hour of incubation. Citrulline could not be detected until 2.5 hours, indicating a lag in the internal conversion of arginine to citrulline. Since Acanthamoeba has arginase (Section 3.2.1.), ornithine also was produced from the substrate arginine. More ornithine was produced than citrulline and the possibility that the label reaching citrulline first passed through ornithine cannot ruled out. At 9 hours incubation, the amount of ornithine produced was higher than either arginine or citrulline. The production of $^{14}$C-putrescine indicated the presence of ornithine decarboxylase activity which is mentioned in Section 3.2.5.
Fig. 8. Fate of $^{14}$C-citrulline from L-(U-$^{14}$C)-arginine in vivo. Exponential phase cultures were incubated in arginine-free DGM-11 supplemented with 0.47 mM L-arginine plus 10 uCi L-(U-$^{14}$C)-arginine. Extracts were prepared as described in Section 2.7.1. and aliquots were separated by TLC. Plates were stained with ninhydrin, the spots were scraped off and the activity determined. Radioactivity was only found in ornithine (●), arginine (▲), citrulline (▼), putrescine (■), and unidentified material at the origin. S, Standard mixture of 50 nmol each of citrulline (a), arginine (b), and ornithine (c) on a TLC plate. Data are from two replica experiments.
$^{14}$C-ARGININE INCORPORATION (cpm/8x10^6 cells) vs INCUBATION TIME (HR)
3.2.4. The conversion of spermidine to diaminopropane. Acanthamoeba has an unusually large amount of diaminopropane (Section 3.1.2.). This diaminopropane is uncommon in animal cells but it is found in other lower eukaryotes, bacteria, and higher plants. It can be formed from either spermidine or spermine by polyamine oxidase (plants), or spermidine dehydrogenase (bacteria). Spermidine is available in a tritiated form. Several standard in vitro methods for polyamine oxidase and spermidine dehydrogenase which used $^3$H-spermidine as a substrate failed to produce $^3$H-diaminopropane, and, therefore, any evidence of diaminopropane synthetase. The presence of a synthetase subsequently was demonstrated by an in vivo labeling experiment with $^3$H-spermidine. Spermidine uptake was almost linear up to 3 hours and then leveled off. $^3$H-diaminopropane appeared following a lag (Fig. 9) as would be expected for a product of spermidine metabolism. Of course, the in vivo method did not determine the kind of enzyme involved.

3.2.5. Ornithine decarboxylase. The presence of ornithine decarboxylase was assayed by the detection of $^{14}$CO$_2$ produced from DL-(5-$^{14}$C)-ornithine and by the production of $^{14}$C-putrescine from DL-(1-$^{14}$C)-ornithine in the 38,000 g supernatant fraction (Fig. 10). The amount of $^{14}$CO$_2$ production
Fig. 9. Fate of (U-³H)-spermidine in vivo.

Exponential phase cultures were incubated in 0.1 mM spermidine plus 60 uCi (UL-³H)-spermidine.

Preparations of extracts and determination of activity were as in Fig. 8. Activity was found in spermidine (○) and diaminopropane (▲) only. No activity was found at the origin. ●, Sum of spermidine and diaminopropane. S, Standard mixture of 50 nmol diaminopropane (a) and spermidine (b) on a TLC plate. Data are from a single experiment.
3H-SPD INCORPORATION (cpm/6x10^6 cells)

INCUBATION TIME (HR)
Fig. 10. In vitro production of $^{14}$C-putrescine from DL-(5-$^{14}$C)-ornithine by ornithine decarboxylase. Ameba homogenate supernatants prepared from $8 \times 10^6$ amebas as described in Section 2.4. were incubated for 60 min with 10 mM DL-ornithine plus 1 uCi DL-(5-$^{14}$C)-ornithine in ornithine decarboxylase buffer. A. Ninhydrin stained thin layer chromatogram of reaction mixture at 0 (T0), 30 (T30), and 60 (T60) min. S, Standard mixture containing 50 nmol each of agmatine (agm), alanine (ala), arginine (arg), ornithine (orn), putrescine (put), spermidine (spd), and spermine (spm). B. Autoradiogram of TLC plate. 30 min reaction was done with or without 5 mM DFMO to block ornithine decarboxylase activity.
increased linearly up to 30 minutes incubation, then slowed down (Fig. 11). The zero time sample contained some intracellular putrescine but this was not radioactive as indicated in the autoradiogram. As the reaction time increased, the ninhydrin staining putrescine spot became darker (Fig. 8 TLC), and \(^{14}\text{C}\)-putrescine production could be detected in the autoradiogram (Fig. 8 AR). DFMO, the irreversible inhibitor of ornithine decarboxylase, blocked putrescine production totally at 5 mM concentration. Further effects of inhibitors on ornithine decarboxylase activity are reported in Section 3.4.2. The activity of ornithine decarboxylase as a function of substrate concentration is shown in Figs. 12 and 13. The maximum reaction rate \((V_{\text{max}})\) per mg protein in DGM-11 cultures (19.0 nmol CO\(_2\)/30 min/mg protein) was about half of that in OGM cultures (46.1 nmol CO\(_2\)/30 min/mg protein) possibly because the amount of protein in DGM-11 cultures was almost twice as much as that in OGM cultures. The \(K_m\) values in OGM (Fig. 12) and DGM-11 (Fig. 13) cultures were 1.1 mM and 2.1 mM, respectively.

Ornithine decarboxylase activity is found in both cytoplasm and nucleus in cells of several organisms, but the majority is present in the cytoplasm (Persson et al., 1983; Zagon et al., 1983). The enzyme requires pyridoxal 5-phosphate as a cofactor in all cells that have been tested. In \textit{A. castellanii}, the 38,000 g supernatant
Fig. 11. *In vitro* $^{14}$CO$_2$ production from DL-(1-$^{14}$C)-ornithine by ornithine decarboxylase in DGM-11 cultures. Assay conditions were the same as in Fig. 10. The reaction was incubated for 140 min and $^{14}$CO$_2$ detection methods were as in Section 2.6.1.
Fig. 12. Double reciprocal plot of enzyme kinetic for OrnDC from amebas grown in OGM. Assay conditions were the same as in Fig. 10. The reaction rate was determined by $^{14}$CO$_2$ produced from DL-(1-$^{14}$C)-ornithine for 30 minutes at 30°C by the methods described in Section 2.6.1. Data are from two replica experiments.
Fig. 13. Double reciprocal plot of enzyme kinetic for OrnDC from amebas grown in DGM-11. Conditions are the same as in Fig. 12. Data is from a single experiment.
contained 52% of the whole cell protein and 58.8% of the decarboxylase activity. Dialyzed supernatants supplemented with pyridoxal 5-phosphate and dithiothreitol had 38% of the homogenate protein and 32.9% of the decarboxylase activity (Table 4). Thus, the enzyme specific activity was probably similar for cytosolic and non-cytosolic cell fractions. Activities were substantially depressed in the absence of either pyridoxal 5-phosphate or dithiothreitol.

3.3. Changes in polyamine levels and ornithine decarboxylase activity during cell multiplication and starvation-induced encystment. Actively growing cells usually contain high levels of polyamines and high ornithine decarboxylase activities compared to slow growing or quiescent cells (Grillo, 1985; Heby, 1981). The levels of ornithine decarboxylase activity also change during differentiation. Therefore, possible changes in polyamine levels and ornithine decarboxylase activity have been studied during multiplication and encystment of A. castellani.

3.3.1. Cultures grown in OGM. Changes in levels of five polyamines were followed during encystment induced by starvation in the nutrient-free encystment medium (Neff encystment medium (NEM)). Late exponential phase amebas
Table 4. Pyridoxal 5-phosphate and dithiothreitol requirements for ornithine decarboxylase activity.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>fmol CO₂/30 min/ameba</th>
<th>nmol CO₂/30 min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole homogenates</td>
<td>8.5 (100)</td>
<td>17.6</td>
</tr>
<tr>
<td>Non-dialyzed supernatants</td>
<td>5.0 (58.8)</td>
<td>19.9</td>
</tr>
<tr>
<td>(−) pyridoxal 5-phosphate</td>
<td>1.3 (15.3)</td>
<td>1.4 (16.5)</td>
</tr>
<tr>
<td>(−) dithiothreitol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dialyzed supernatants</td>
<td>2.8 (32.9)</td>
<td>14.9</td>
</tr>
<tr>
<td>(−) pyridoxal 5-phosphate</td>
<td>1.8 (21.2)</td>
<td></td>
</tr>
<tr>
<td>(−) dithiothreitol</td>
<td>0.0 (0.0)</td>
<td></td>
</tr>
</tbody>
</table>

Ameba homogenate supernatants were prepared as described in Section 2.4 and used for the enzyme assay. Dialysis was done in 0.1 M Tris-HCl (pH 7.5) at 4°C for 24 hours. The reaction mixture included 1 mM pyridoxal 5-phosphate and 25 mM dithiothreitol except where the omission of one of the agents is indicated. The protein contents of the 3 fractions were: 484 pg/ameba in whole homogenates, 251 pg/ameba in non-dialyzed supernatants, and 185 pg/ameba in dialyzed supernatants. Percent activity in whole homogenate is shown in parentheses. Data from a single experiment.
were used as starting cultures in order to have enough material to measure putrescine levels. In control cultures grown in OGM, cells were kept growing until they reached stationary phase. No encystment occurred (Fig. 14 A). Polyamine levels initially tended to increase or stay relatively constant. When cell numbers stopped increasing, polyamine concentrations began to decrease (Fig. 14 C). In contrast, cells immediately stopped multiplication and started to form cysts in the nutrient-free encystment medium (Fig. 14 B). The levels of putrescine, spermidine, and diaminopropane rapidly dropped to 50% within one day in the encystment medium; the levels of spermine and norspermidine decreased 50% over 4 days (Fig. 14 D). Externally added 0.8 mM putrescine, spermidine, spermine, cadavarine, or 8 mM diaminopropane did not block starvation-induced differentiation. It did not matter whether the polyamines were added at the beginning of starvation or up to 18 hours before starvation was initiated to avoid possible decreases of membrane permeability during encystment.

3.3.2. Cultures grown in DGM-11. Levels of diaminopropane and spermidine, and ornithine decarboxylase activity increased on average at least two-fold from midexponential phase to postexponential phase (Fig. 15). Norspermidine levels were relatively stable. When cell multiplication
Fig. 14. Intracellular polyamine levels during postexponential growth and starvation-induced encystment.  
A. Growth in OGM.  B. Encystment in encystment medium.  
C, D. Intracellular polyamine levels in (C) OGM or (D) encystment medium.  
A & B. •, amebas/mm²; ○, % cysts.  
C & D. •, putrescine; □, spermidine; ▲, spermine; ▼, norspermidine; ○, diaminopropane. Values are averages for three experiments.
Fig. 15. Intracellular levels of polyamine and ornithine decarboxylase activity in DGM-11 cultures. A. Growth in DGM-11. B. Changes in levels of spermidine (●), diaminopropane (○), and ornithine decarboxylase activity (▲) during growth.
was blocked and cells were induced to encyst by simple
starvation, levels of diaminopropane and spermidine dropped
slightly within a day while control levels were increasing
(Table 5). The level of norspermidine remained unchanged
in control and starved cultures. In contrast to polyamine
levels, ornithine decarboxylase activity dropped 91% within
2 days (Table 6). The percentage of cysts increased from 0
to 68.5 during this 2 day period.

3.4. Effects of ornithine, putrescine, and spermidine
analogues on cell multiplication and differentiation.
Polyamine biosynthesis or the function of polyamines are
blocked by many different inhibitors. I have concentrated
attention on two groups of inhibitors, analogues of
ornithine or putrescine and analogues of spermidine, to
evaluate their effects on cell multiplication and
encystment.

3.4.1. Arrest of multiplication by DFMO, Δ-MFM0me,
R,R-MAP in OGM and DGM-11. The most important rate
limiting enzyme of polyamine biosynthesis is ornithine
decarboxylase. The activity of this enzyme can be
irreversibly blocked by a group of specific inhibitors
which include: DFMO, Δ-MFM0me, and R,R-MAP. These are
mechanism based irreversible (suicide) inhibitors. When
Table 5. Effect of starvation on levels of polyamines.

<table>
<thead>
<tr>
<th>Culture age</th>
<th>Medium</th>
<th>Cells/mm²</th>
<th>Percent</th>
<th>Polyamines (fmol/ameba)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 days</td>
<td>DGM-11</td>
<td>1050</td>
<td>0</td>
<td>33.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1150</td>
<td>0</td>
<td>38.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>1 days</td>
<td>DGM-11</td>
<td>2000</td>
<td>0</td>
<td>48.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2080</td>
<td>0</td>
<td>54.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>1 days</td>
<td>NEM</td>
<td>930</td>
<td>48</td>
<td>21.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1070</td>
<td>36</td>
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<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.03</td>
</tr>
</tbody>
</table>

At day 0, DGM-11 was replaced by Neff encystment medium (NEM) to induce encystment. Amebas were harvested the next day. Data from two independent experiments.
Table 6. Effect of starvation on ornithine decarboxylase activity.

<table>
<thead>
<tr>
<th>Culture age</th>
<th>Medium</th>
<th>Cells/mm²</th>
<th>Percent cysts</th>
<th>nmol CO₂/30 min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 days</td>
<td>DGM-11</td>
<td>700</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>950</td>
<td>0</td>
<td>22</td>
</tr>
<tr>
<td>2 days</td>
<td>DGM-11</td>
<td>1500</td>
<td>0</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2000</td>
<td>0</td>
<td>48</td>
</tr>
<tr>
<td>2 days</td>
<td>NEM</td>
<td>700</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>900</td>
<td>77</td>
<td>2</td>
</tr>
</tbody>
</table>

At day 0, DGM-11 was replaced by Neff encystment medium (NEM) to induce encystment. Cells were harvested 2 days later. Ornithine decarboxylase activity was measured as in Fig. 10.
each drug was added to either early exponential phase cultures (50-80 amebas/mm²) or midexponential phase cultures (300-400 amebas/mm²) growing in OGM, it blocked cell multiplication. Population growth measured during a period of 2 days decreased in increasing concentrations of all three drugs (Fig. 16). At concentrations of 50 mM DFMO, 10 mM Δ-MFMOme, or 50 mM R,R-MAP, growth was limited to about one population doubling.

The inhibition of cell multiplication by these agents could be reversed by the addition of polyamines, but not by the inorganic cations Mg++ or Ca++. I used an 0.8 mM concentration of most of the polyamines because at this concentration they had an effect on multiplication in the presence of the inhibitors, but did not have any visible effect on cell growth by themselves (Figs. 17, 18). The intracellular concentration of diaminopropane was much higher than that of putrescine or spermidine (Table 1), thus, 8 mM diaminopropane was used for the reversal of the inhibition of multiplication (Fig. 19). Even the undetected polyamine, cadaverine, could reverse the growth inhibition at 0.8 mM (Fig. 20).

Cell multiplication in DGM-11 also could be blocked by DFMO, Δ-MFMOme, and R,R-MAP, the three irreversible inhibitors of ornithine decarboxylase. The inhibitory effect increased with the inhibitor concentration in all
Fig. 16. Inhibition of multiplication in OGM by antagonists of polyamine synthesis. Conditions are described in Section 2.2. Inhibitors were added to early exponential phase cultures at about 80 amebas/mm$^2$ and population growth was measured over two days. ■, DFMO; ○, MGBG (scale expanded in inset); ▲, ∆-MFMOme; △, R,R-MAP.
Fig. 17. Arrest of growth in OGM by antagonists of polyamine synthesis and its reversal by 0.8 mM putrescine. Conditions are described in Section 2.2. ○, control; □, 40 mM DFMO; ■, 10 mM Δ-MFMOme; Δ, 50 mM R,R-MAP; ○, 0.6 mM MGBG; □, 30 mM DFMA.
Add Putrescine
Add Inhibitor
Fig. 18. Arrest of growth in OGM by antagonists of polyamine synthesis and its reversal by 0.8 mM spermidine. Symbols same as in Fig. 17.
Add Spermidine
Add Inhibitor
Fig. 19. Arrest of growth in OGM by antagonists of polyamine synthesis and its reversal by 8 mM diaminopropane. Symbols same as in Fig. 17.
Fig. 20. Arrest of growth in OGM by antagonists of polyamine synthesis and its reversal by 0.8 mM cadaverine. Symbols same as in Fig. 17.
AMEBAS/mm²

O '  
O '  

Cadaverine
Add Inhibitor

TIME (hr)

AMEBAS/mm²

TIME (hr)
three drugs (Fig. 21). 30 mM DFMO, 3 mM Δ-MFMOMe, or 50 mM R,R-MAP permitted only 1 to 1.5 population doublings in 2 days compared to 4.5 doublings in controls. The effective concentrations of each inhibitor in DGM-11 cultures were almost the same as in broth cultures. The inhibition of cell multiplication could be reversed by the addition of 0.8 mM putrescine or spermidine (Figs. 22, 23). The reversal of growth inhibition by diaminopropane or cadaverine was not tested in these cultures.

3.4.2. Inhibition of ornithine decarboxylase by DFMO, Δ-MFMOMe, and R,R-MAP in OGM and DGM-11 cultures.

Ornithine decarboxylase activity was assayed in extracts of amebas that were treated in vitro with concentrations of inhibitors that arrested multiplication. The degree of inhibition of enzyme activity by DFMO, Δ-MFMOMe, and R,R-MAP at concentrations that gave the same degree of growth inhibition ranged from 68-97 % (Table 7). Two other polyamine-related inhibitors that arrest growth in Acanthamoeba also were tried. MGBG, a reversible inhibitor of S-adenosylmethionine decarboxylase, and DFMA, an irreversible inhibitor of arginine decarboxylase, did not have significant effects on the ornithine decarboxylase activity (Table 7). Although relatively high concentrations of inhibitors were required to arrest cell multiplication, inhibition of ornithine decarboxylase
Fig. 21. Inhibition of multiplication in DGM-11 by inhibitors of ornithine decarboxylase. Conditions are described in Section 2.2. Inhibitors were added to early exponential phase cultures at about 80 amebas/mm$^2$ and population growth was measured over two days. ■, DFMO; ▲, Δ-MFMOMe; Δ, R,R-MAP.
Fig. 22. Arrest of growth in DGM-11 by inhibitors of ornithine decarboxylase and its reversal by 0.8 mM putrescine. Conditions are described in Section 2.2. ●, control; ■, 30 mM DFMO; ▲, 3 mM Δ-MFMOme; and △, 50 mM R,R-MAP.
Fig. 23. Arrest of growth in DGM-11 by inhibitors of ornithine decarboxylase and its reversal by 0.8 mM spermidine. Conditions are described in Section 2.2. Symbols as in Fig. 22.
Add Spermidine

Add Inhibitor
Table 7. Change in levels of the ornithine decarboxylase activity in the presence of its irreversible inhibitor in OGM grown cultures.

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>nmol CO$_2$/30 min/mg protein</th>
<th>Percent activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant + Substrate</td>
<td>51.1</td>
<td>100</td>
</tr>
<tr>
<td>+ 40 mM DPMO</td>
<td>5.2</td>
<td>10.2</td>
</tr>
<tr>
<td>+ 10 mM Δ-MPMOme</td>
<td>16.4</td>
<td>32.1</td>
</tr>
<tr>
<td>+ 50 mM R,R-MAP</td>
<td>1.5</td>
<td>2.9</td>
</tr>
<tr>
<td>+ 0.6 mM MGBG</td>
<td>44.8</td>
<td>87.7</td>
</tr>
<tr>
<td>+ 30 mM DEMA</td>
<td>46.9</td>
<td>91.8</td>
</tr>
</tbody>
</table>

The measurement and assay conditions were the same as those in Fig. 8 except that 38,000 g supernatants were used instead of whole homogenates. Each inhibitor was mixed with substrate right before the start of each reaction. Data from a single experiment.
Table 8. Effect of DMO on ornithine decarboxylase activity and polyamine levels in vivo.

<table>
<thead>
<tr>
<th>DMO (mM)</th>
<th>Culture age (cells/mm²)</th>
<th>nmol CO₂/30 min/ mg protein</th>
<th>Polyamines (fmol/ameba)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dap</td>
</tr>
<tr>
<td>0</td>
<td>1167</td>
<td>23.2(4.7)</td>
<td>40.2(4.9)</td>
</tr>
<tr>
<td>1</td>
<td>1100</td>
<td>11.2(3.9)</td>
<td>31.2(6.1)</td>
</tr>
<tr>
<td>5</td>
<td>917</td>
<td>8.5(2.8)</td>
<td>15.6(1.7)</td>
</tr>
</tbody>
</table>

DMO was added to the DGM-11 cultures at 100 amebas/mm². Amebas were harvested 3 days later. Ornithine decarboxylase activity was measured using ameba homogenate supernatants as in Fig. 10. SD for 3 replicas in parentheses.
Table 9. *In vitro* inhibition of ornithine decarboxylase activity by DEMO in DGM-11 cultures.

<table>
<thead>
<tr>
<th>DEMO (mM)</th>
<th>nmol CO₂/30 min</th>
<th>mg protein</th>
<th>Percent activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>25.0</td>
<td></td>
<td>100.0</td>
</tr>
<tr>
<td>0.1</td>
<td>10.3</td>
<td></td>
<td>41.1</td>
</tr>
<tr>
<td>0.5</td>
<td>5.0</td>
<td></td>
<td>19.6</td>
</tr>
<tr>
<td>5.0</td>
<td>1.9</td>
<td></td>
<td>7.4</td>
</tr>
<tr>
<td>10.0</td>
<td>1.0</td>
<td></td>
<td>4.1</td>
</tr>
</tbody>
</table>

Ornithine decarboxylase activity was measured using ameba homogenate supernatants as in Fig. 8. DEMO was mixed with substrate right before the start of each reaction.
Fig. 24. Kinetics of encystment in 40 mM DFMO plus CaCl$_2$ or MgSO$_4$ in OGM cultures. 40 mM DFMO was added to exponential phase cultures at 80 amebas/mm$^2$. Two days after arrest of multiplication, CaCl$_2$ (●) or MgSO$_4$ (○) were added. ▲, control culture with DFMO, but no other additives. △, cultures with DFMO plus 0.8 mM putrescine only. Data from a single experiment.
activity by DFMO was demonstrated at much lower inhibitor concentrations both in vivo (Table 8) and in vitro (Table 9). Activity of the enzyme was inhibited 50% in vivo by 1 mM DFMO even though this concentration did not significantly block cell multiplication. Although only slight growth inhibition was detected in 5 mM DFMO (Figs. 16, 21, Table 8), ornithine decarboxylase was inhibited 63.3% at this concentration (Table 8). The inhibitory effect of DFMO was even greater in in vitro experiments where 0.1 mM DFMO was enough to inhibit the enzyme more than 50% (Table 9).

3.4.3. Induction of differentiation by DFMO supplemented with CaCl$_2$ or MgSO$_4$ in OGM cultures. DFMO arrested multiplication and induced differentiation in murine embryonal carcinoma cells (Schindler et al., 1983; 1985), and trypanosomes (Giffin et al., 1986), but it arrested multiplication without inducing differentiation in *A. castellanii*. Cell lysis occurred at the highest inhibitor concentrations tested. However, ameba cultures formed 21-24% cysts two days after growth was completely arrested with 40 mM DFMO if 30 mM CaCl$_2$ or MgSO$_4$ were present. The inorganic ions did not induce any encystment by themselves (Fig. 24).
3.4.4. Effects of MGBG, Berenil, hydroxystilbamidine, pentamidine, amicarbalide, Antrycide, and ethidium bromide on multiplication and differentiation in OGM and DGM-11 cultures. Besides ornithine decarboxylase, S-adenosylmethionine decarboxylase is a second rate limiting enzyme in polyamine synthesis. This enzyme catalyses the decarboxylation of S-adenosylmethionine (Fig. 1). The product of this reaction is the source of aminopropyl groups for spermidine, spermine and possibly norspermidine synthesis. MGBG, a reversible inhibitor of S-adenosylmethionine decarboxylase, also blocked cell multiplication (3.4.1). The effect could be fully bypassed by the addition of either 0.8 mM putrescine or spermidine in OGM cultures (Figs. 17, 18). However, the effect was not bypassed (reversed) by putrescine and only was partially bypassed by the same concentration of spermidine in DGM-11 cultures.

Amicarbalide, pentamidine, hydroxystilbamidine, Berenil, and ethidium bromide are known as polyamine antagonists (Bacchi, 1981; Bacchi et al., 1981). All these agents have some structural features in common with spermidine (Fig. 25, 26). Like MGBG, the first four inhibitors have two terminal amidine groups each containing one primary amine and one secondary amine. Spermidine is not a diamidine, but contains terminal amine groups. Ethidium bromide and Antrycide clearly are different
Fig. 25. "Structural analogues" of spermidine.
Spermidine

\[ \text{H}_2\text{N-CH}_2\text{-CH}_2\text{-CH}_2\text{-NH-CH}_2\text{-CH}_2\text{-CH}_2\text{-NH}_2 \]

Methylglyoxal Bis(guanylylhydrazone)

\[ \text{H}_2\text{N-C-NH-N=CH-NH-C-NH}_2 \]

Hydroxystilbamidine

\[ \text{H}_2\text{N-C-CH=CH-C-NH}_2 \]

Berenil

\[ \text{H}_2\text{N-C-NH=NH-C-NH}_2 \]

Pentamidine

\[ \text{H}_2\text{N-COCO-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CO-C-NH}_2 \]

Amicarbidol

\[ \text{H}_2\text{N-C=NHCONH-C-NH}_2 \]
Fig. 26. Structures of ethidium bromide and Antrycide as "structural analogues" of spermidine. The presence of structures resembling spermidine that are embedded in each analog are indicated by heavy lines.
Ethidium Bromide

Spermidine

Antrycide

Spermidine
structures, but as pointed out previously (Sakai et al., 1975; Lambros et al., 1977), a backbone structure resembling spermidine is clearly present in both (Fig. 26). Besides structural similarities Antrycide, ethidium bromide, and pentamidine cause the release of polyamines from ribosomes (Newton, 1966; Sakai et al., 1975; Sands et al., 1985; Wallis, 1966), and compete with polyamines for binding to \( \alpha \)-glycerophosphate dehydrogenase (Lambros et al., 1985). MGBG is a reversible inhibitor of S-adenosylmethionine decarboxylase. Berenil and pentamidine are inhibitors of S-adenosylmethionine decarboxylase in yeast, a bacterium and rat liver (Karvonen et al., 1985), and *T. b. brucii* (Bitonti et al., 1986). Therefore, it was reasoned that the related analogues also possibly are irreversible inhibitors of this enzyme. Like inhibitors of ornithine decarboxylase, all the spermidine analogues inhibited cell multiplication in OGM. In addition, all but MGBG induced differentiation to various degrees (Fig. 27). The data illustrated are for ameba clones BK 35 and BK 92. Both were selected for high encystment in 4 \( \mu \)M Berenil. Selection was necessary because there was considerable variability among cell clones in the degree of response to each of the drugs. Responsiveness did not seem to vary in a coordinate fashion for all of the drugs. Furthermore, the encystment response often was unstable. For example, clone PH 28 gave 65 %
Fig. 27. Induction of encystment in OGM by spermidine analogues. Conditions are described in Section 2.3.
Analogues were added at about 300 amebas/mm² and cysts were counted four days later. A. Amicarbalide; B. Antrycide; C. Pentamidine; D. Hydroxystilbamidine; E. Ethidium bromide; F. Berenil. ●, clone 35; ○, clone 92.
cysts in Berenil when first isolated and only 50% cysts four months later. Subclones examined at the later time were very heterogeneous in response to the drug; encystment varied from 0 to 74% in 55 different subclones (Fig. 28). Other isolates had more stable encystment responses. Thus, it was possible to select clones that gave over 80% encystment in optimal concentrations of hydroxystilbamidine or Berenil. The optimal encystment-inducing concentrations for the various analogues in OGM were 0.2 μM for amicarbalide, 1.5 μM for pentamidine, 5.6 μM for hydroxystilbamidine, 70 μM for ethidium bromide, and 4 μM for Berenil.

If the spermidine analogues inhibit S-adenosylmethionine decarboxylase activity, they should depress intracellular levels of polyamines, especially spermidine, spermine, and diaminopropane (Fig. 1). Since reduced levels of one or more of these compounds could be a signal for differentiation, I tested whether the differentiation-inducing effects of Berenil and hydroxystilbamidine could be blocked by the addition of polyamines. Both spermidine and putrescine had blocking effects at concentrations that had no visible effect on cell growth in the absence of drugs (Fig. 29). Spermidine was much more effective than putrescine for the inhibition of differentiation. Neither polyamine was able to restore multiplication, however, indicating that differentiation
Fig. 28. Berenil-induced encystment in 55 subclones of PH 28. Cultures were treated with 4 μM Berenil at 300 amebas/mm² and cysts were counted 4 days later.
Fig. 29. Induction of encystment by Berenil and hydroxystilbamidine and its reversal by polyamines.

Replicate cultures were treated at 300 amebas/mm$^2$ with 4 uM Berenil or 5.6 uM hydroxystilbamidine, and supplemented with polyamines. Cysts were counted four days later. ●, Berenil + putrescine; ○, Berenil + spermidine; ▲, hydroxystilbamidine + spermidine.
POLY AMINE CONC (mM)
had been initiated and was irreversible, or that the analogues might have more than one multiplication-inhibiting effect involving targets other than the polyamine metabolism system.

BK 92-2, which was another subclone selected for high Berenil-induced encystment in DGM-11, responded in defined growth medium to MGBG, Berenil, hydroxystilbamidine, pentamidine, and amicarbalide with very similar patterns of growth inhibition (Fig. 30). As in OGM, the inhibition of multiplication by the spermidine analogues was up to 10,000 times more effective than the inhibition by DFMO, Δ-MFMOme, or R,R-MAP. In all cases except MGBG, population growth was limited to about one doubling within the range of 1-30 μM. Likewise, the inhibition of multiplication by Berenil, pentamidine, amicarbalide, or hydroxystilbamidine could not be reversed by the addition of 0.8 mM putrescine or spermidine. Partial recovery was possible with 0.8 mM spermidine in MGBG treated cultures. All of these analogues except MGBG induced from 40 to 70% encystment (Fig. 31). The optimal conditions were 1.5 to 4 μM. Spermidine analogue-induced encystment was prevented by the addition of putrescine or spermidine. In all cases, spermidine was more effective than putrescine for the inhibition of encystment as was seen before in OGM cultures (Fig. 32).
Fig. 30. Inhibition of multiplication in DGM-11 by spermidine analogues. Concentration dependence of growth inhibition. ■, MGBG; ○, Berenil; ○, hydroxystilbamidine; ▲, amicarbalide; △, pentamidine. Conditions are as in Fig. 16.
Fig. 31. Induction of encystment in DGM-11 by spermidine analogues. Cultures were treated with each antagonist at 300 amebas/mm$^2$ and cysts were counted 4 days later. A. Amicarbalide; B. Berenil; C. Pentamidine; D. Hydroxystilbamidine.
Fig. 32. Induction of encystment by spermidine analogues and its inhibition by polyamines. Replicate cultures were treated at 300 amebas/mm² with each analogues plus putrescine (open symbols) or spermidine (closed symbols). Cysts were counted 4 days later. •,○, 3 μM Berenil; ▲, △, 4 μM hydroxystilbamidine; ▼, ▽, 8 μM Amicarbalide; ■, □, 1.6 μM pentamidine. Data from a single experiment.
3.4.5. Changes in polyamine levels and ornithine decarboxylase activity during Berenil-induced encystment in OGM and DGM-11. As a further test of the possibility that spermidine analogues induced encystment by depressing pools of polyamines that were dependent on S-adenosylmethionine decarboxylase for their synthesis, I examined spermidine and diaminopropane pools during Berenil induced encystment in DGM-11. In order to have maximum encystment, early to midexponential phase amebas (200-350 amebas/mm²) were used as starting cultures. In OGM, the levels of spermidine and diaminopropane increased until the stationary phase and then decreased (Fig. 33 C). When berenil was added to the cultures, cells ceased multiplication and started to form cysts two days later. The maximum encystment was achieved within three days of berenil treatment (Fig. 33 B). Although changes in levels of spermidine and diaminopropane were variable during encystment, the patterns of change resembled the drug-free control cultures, except that maximal levels were reached sooner. Neither spermidine nor diaminopropane levels dropped below those at the time of Berenil addition until after cysts formed (Fig. 33 D). Somewhat similar responses were found in amebas grown in DGM-11 (Table 10). The levels of diaminopropane and norspermidine were near the initial values after 2 days of Berenil treatment, although the polyamines were at higher levels in untreated controls.
Fig. 33. Intracellular polyamine levels during postexponential growth and Berenil-induced encystment. A. Growth in OGM. B. Encystment in OGM with 4 μM Berenil. C,D. Intracellular polyamine levels in (C) OGM or (D) OGM plus berenil. Symbols as for Fig. 14. Values are averages for three experiments.
Table 10. Effect of Berenil on polyamine levels and ornithine decarboxylase activity.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Cells/ age</th>
<th>Percent</th>
<th>PA (fmol/ameba)</th>
<th>OrnDC Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
<td>mm² cysts</td>
<td>(nmol CO₂/30 min/ mg protein)</td>
<td></td>
</tr>
<tr>
<td>0 days  - Ber</td>
<td>350</td>
<td>0</td>
<td>21.5 0.94 14.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>0</td>
<td>14.0 0.60 9.3</td>
<td></td>
</tr>
<tr>
<td>2 days  - Ber</td>
<td>1500</td>
<td>0</td>
<td>17.0 2.40 19.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1400</td>
<td>0</td>
<td>20.0 2.00 14.6</td>
<td></td>
</tr>
<tr>
<td>2 days  + Ber</td>
<td>420</td>
<td>32</td>
<td>16.5 1.00 4.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>615</td>
<td>13</td>
<td>15.5 1.00 2.9</td>
<td></td>
</tr>
</tbody>
</table>

Cultures were grown in DGM-11. At day 0, one half of the cultures received Berenil and the rest received none. Ornithine decarboxylase activity was measured using ameba homogenate supernatants as in Fig. 10. Data are from two independent experiments.
The activity of ornithine decarboxylase increased 45% in untreated controls, but dropped 70% below the initial value during the Berenil treatment (Table 10).

3.5. Arrest of multiplication and induction of differentiation by DFMA. In bacteria and plants, putrescine can be synthesized from agmatine. Arginine decarboxylase catalyses the production of agmatine from arginine, and its activity can be irreversibly inhibited by DFMA. This inhibitor blocked cell multiplication, and the effect at most was only partially reversed by the addition of 0.8 mM putrescine, spermidine (Figs. 17, 18), or 20 mM agmatine. The observation that reversal by polyamines of growth inhibition by DFMA was only partial probably was because encystment had been initiated by the inhibitor. At the optimal concentration of 30-50 mM, DFMA induced about 25% encystment. The induction of differentiation by DFMA was not caused by the inhibition of arginine decarboxylase because this enzyme could not be detected either in vivo or in vitro. Rather, DFMA probably was chemically modified to another form that was active in inducing differentiation. Evidence for modification was obtained from TLC of cell homogenates treated in vitro with DFMA (Fig. 34 lane E). It has been proposed that DFMA can be converted to DFMO.
Fig. 34. *In vitro* decomposition of DFMA. Ameba homogenates were incubated in Tris buffer with 50 or 250 mM arginine. Samples were taken at 0 and 15 min and chromatographed by TLC. The figure is a ninhydrin stained TLC plate. A, standard mixture containing 50 nmol each of agmatine (Agm), alanine (Ala), arginine (Arg), and ornithine (Orn); B, 0 min reaction with 50 mM arginine; C, 15 min reaction with 50 mM arginine; D, 15 min reaction with 250 mM arginine; E, 15 min reaction with 250 mM arginine plus 40 mM DFMA; F, standard mixture of 25 nmol each of DFMA and DFMO.
The modified form I saw did not correspond to DFMO by TLC, nor did it inhibit ornithine decarboxylase (Table 7).
4. DISCUSSION

4.1. Polyamine content of Acanthamoeba castellanii. Acid extracts of Acanthamoeba castellanii grown in OGM contained all three polyamines most commonly found in eukaryotes: putrescine, spermidine, and spermine. The relative amounts of the three compounds also were characteristic of eukaryotic cells; spermidine and spermine were higher than putrescine. Besides those three major polyamines, a very high level of the unusual diaminopropane also was detected. Diaminopropane has been found in bacteria (Campello et al., 1965; Okata et al., 1983), algae (Aleksijevic et al., 1979; Hamana and Matsuzak, 1985), and higher plants (Smith, 1970; 1983b; 1985b), but Dictyostelium (Mach et al., 1982) is the only lower eukaryote examined to date that has such high amounts of diaminopropane (Table 11). The level of diaminopropane measured in the present study agrees with that of Poulin et al. (1984) who observed 20-40 fmol/ameba in the same strain that I studied. The levels of putrescine were very low compared to diaminopropane and the retention times of putrescine and diaminopropane were too close for clear separation (Fig. 4, Table 2). Furthermore, the separation got worse as columns got older. These
Table 11. Polyamines in various lower eukaryotes

<table>
<thead>
<tr>
<th></th>
<th>Content (umoles/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Put</td>
</tr>
<tr>
<td>Protozoans/algae</td>
<td></td>
</tr>
<tr>
<td>Acanthamoeba castellanii</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>Entamoeba histolytica²</td>
<td>92.5</td>
</tr>
<tr>
<td>Euglena gracilis²</td>
<td>0.06</td>
</tr>
<tr>
<td>Euglena viridis³</td>
<td>12.2</td>
</tr>
<tr>
<td>Trychomonas vaginalis⁴</td>
<td>67.1</td>
</tr>
<tr>
<td>Slime mold</td>
<td></td>
</tr>
<tr>
<td>Dictyostelium discoideum</td>
<td></td>
</tr>
<tr>
<td>amoebas e</td>
<td>112.3</td>
</tr>
<tr>
<td>Filamentous fungus</td>
<td></td>
</tr>
<tr>
<td>Neurospora crassa</td>
<td>3.3</td>
</tr>
</tbody>
</table>

¹Gillin et al. (1984)
²Calculated from original data assuming 350 pg protein/cell
(Aleksijevic et al., 1979)
³Calculated from original data assuming a cell specific gravity of 1.05
and a protein content of 9% of wet weight (Hamana and Matsuzaki, 1985)
⁴Mach et al. (1982)
⁵Calculated from original values assuming that cell protein is 33%
of dry weight (Paulus et al., 1982)
probably were the reasons why putrescine was not found previously in *A. castellanii*.

Poulin et al. detected low levels of a norspermidine-like compound, but did not find spermidine or spermine in OGM cultures. However, the levels of spermidine and spermine were higher than norspermidine in the present study. Thus, it is surprising that Poulin and his colleagues could not find them since they used a very similar detection method (only the manufacturer of the HPLC column differed). One plausible explanation is that their results differed from mine because of different culture conditions, especially if they used an older culture age, since polyamine levels drop dramatically in old cultures (Fig. 14). The presence of putrescine, diaminopropane, spermidine, and spermine in *Acanthamoeba* was confirmed by Drs. Peter McCann and Alan Bitonti (Merrell Dow Research Institute, Cincinnati, OH) who used cation exchange chromatography, a different separation technique from mine. The presence of norspermidine was not checked by them.

In my study, spermine was found in OGM cultures, but was absent in DGM-11 grown cultures (Fig. 4, Table 1). The absence of spermine in DGM-11 could be due to an inactive spermine synthase. Alternatively, *Acanthamoeba* may not have a spermine synthase; the spermine in OGM cultures could have come from the medium because a benzoylated peak which comigrated with spermine was found in extracts of
OGM. However, clear proof that spermine was in the medium was not possible due to interference from many other benzoylated peaks in HPLC analysis of OGM. Labeled substrates could be used in the future to determine whether a spermine synthase is present. The levels of other polyamines also differed in the two media; putrescine, diaminopropane, and spermidine in DGM-11 cultures were 21, 38, and 144%, respectively, of OGM values. These differences, however, may not be very significant.

HPLC analysis revealed one unidentified peak at a retention time of 12.5 minutes in both OGM and DGM-11 cultures (Peak 6, Fig. 4). This peak may be either a polyamine derivative or one of the abundant amino acids. Even though A. castellanii has high levels of alanine, glutamate, and proline (Poulin et al., 1984), none of these can be a candidate for the unknown peak since I could not detect them by my HPLC analysis method.

Preliminary studies in our laboratory with A. palestinensis and A. rhysodes found patterns of polyamine content that were significantly different from A. castellanii, but both showed high levels of diaminopropane (Ware, K., Kim, B. G., and Byers, T. J., unpublished results). The high level of putrescine and absence of diaminopropane which was found in A. culbertsoni by using TLC separation (Gupta et al., 1984) may be a case of mistaken identity due to insensitivity of the techniques.
In my experience, putrescine almost completely comigrates with diaminopropane on TLC plates.

Although HPLC is generally accepted in practice as a sufficient method for the identification of polyamines (Abdel-Monem, 1978; Seiler, 1983), in reality it only provides tentative identification. There are many polyamine derivatives that are hard to separate by this method. Definitive identification requires mass spectrometry or infrared analysis (Tait, 1985). I have not used either of these methods. Therefore, I cannot be absolutely certain that the compounds identified in my studies are not unusual derivatives rather than the parental compounds.

4.2. Enzymes of polyamine metabolism in A. castellanii. The major source of putrescine in cells of most organisms that have been tested is ornithine. Ornithine can be derived from several different sources in different organisms; from arginine by arginase, from citrulline by citrulline hydrolase or citrullinase, or from acetylglutamate by acetylglutamate kinase. Arginine is broken down to ornithine and urea by arginase. The presence of arginase in A. castellanii was proven in this study by detection of the production of ornithine and urea in stoichiometric amounts. DFMO inhibited arginase
activity possibly by increasing the product ornithine level in the reaction mixture, but direct effects on the enzyme are not ruled out. The latter would be novel.

Citrulline can be decomposed to $\text{CO}_2$, $\text{NH}_3$, and ornithine by a single step reaction catalyzed by citrulline hydrolase (Yoshida et al., 1978) or by a two step reaction catalyzed by ornithine transcarbamylase and carbamate kinase which collectively are called citrullinase (Oglinsky and Gehrig, 1953) (Section 1.1.1). Both $\text{CO}_2$ and ornithine were produced from citrulline in *Acanthamoeba*, but I did not determine which enzyme(s) was (were) responsible. Citrullinase can be distinguished from citrulline hydrolase by its requirement for $\text{Mg}^{2+}$ (or $\text{Mn}^{2+}$), inorganic phosphate, and AMP. Consequently, it can be inactivated by dialysis or filtration to remove low molecular weight cofactors (Oglinsky and Gehrig, 1953).

The rate of $\text{CO}_2$ production in *Acanthamoeba* was only 12% of the rate measured for *Crithidia* (Figueiredo et al., 1978), and 7% of the rate for *Herpetomonas* (Yoshida et al., 1978). However, the activity in *Acanthamoeba* might increase if reaction conditions are optimized as was the case for the activities measured in the trypanosomatids.

Diaminopropane can be formed from either spermidine or spermine by polyamine oxidase in plants (Smith, 1970; 1983b; 1985b), or from spermidine by spermidine dehydrogenase in bacteria (Campello et al., 1965; Okada et
Spermidine dehydrogenase requires electron carriers to react with molecular oxygen. Efforts to use in vitro reaction conditions for polyamine oxidase or spermidine dehydrogenase, using phenazine methosulfate (Campello et al., 1965) or ferricyanide (Okada et al., 1983) as electron acceptors, did not reveal any enzyme activity in homogenates of *A. castellanii*. However, $^3$H-diaminopropane was produced in vivo from $^3$H-spermidine. Therefore, there should be some form of polyamine oxidase or spermidine dehydrogenase in the ameba. A recently developed inhibitor of polyamine oxidase, MDL 72521 (Seiler et al., 1985), may be useful in efforts to identify the unknown enzyme. In *Serratia*, the dehydrogenase requires flavin adenine dinucleotide (FAD) as a cofactor. The plant polyamine oxidases also require FAD, but the cofactor is tightly bound to the enzyme in some cases. In addition, the activity of plant enzymes greatly depends on pH (Smith, 1985b). Therefore, the standard methods for detection of diaminopropane synthesizing enzymes may not be sufficient for *Acanthamoeba*. Other cofactors and reaction conditions must be explored.

Another possibility is that the ameba enzyme is unstable. Putrescine is produced from ornithine by OrnDC in all animal cells including *Acanthamoeba*. It can be synthesized from arginine through the intermediate agmatine in bacteria or plants (Smith, 1985a; Tabor and Tabor, 1985), but my
results show that agmatine is not made from arginine in Acanthamoeba either in vitro or in vivo. A recent finding proposed a new pathway for putrescine synthesis from glucose or glutamate in E. coli (Cataldi and Algranati, 1986), but the responsible enzymes were not identified. I did not test this possibility in the ameba. The values for Vmax/mg protein for OrnDC in DGM-11 were about half of those in OGM, but the total activities per cell were about the same in the two different cultures because DGM-11 amebas had twice as much total protein. The values of Vmax and Km in Acanthamoeba are in the same range as in several other protozoans (Table 12). The location of OrnDC can vary in different cells and under different test conditions. In the ameba, the OrnDC activity in the 38,000 g supernatant was a little higher than that in the whole homogenate, indicating that the enzyme is soluble (Table 4). Hormone induced rabbit kidney cells had most of their OrnDC activity in the cytosol (Persson et al., 1983), whereas, normal uninduced rat liver cells had an equal distribution of the enzyme activity between the cytosol and the nucleus (Bartholeyns, 1983). When the enzyme of rat liver cells was induced by hepatectomy or by treatment with dexamethasone, cytosolic OrnDC was markedly induced, but the enzyme in the nuclear fraction was not induced. ³H-DFMO, which can localize the active enzyme because it is a mechanism based irreversible inhibitor, was found
Table 12. The kinetic parameters of ornithine decarboxylase in various protozoa.

<table>
<thead>
<tr>
<th>Species</th>
<th>Vmax (nmol/30 min/mg protein)</th>
<th>Km (mM)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acanthamoeba castellanii</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in OGM</td>
<td>46.1</td>
<td>1.1</td>
<td>a; Aleksijevic et al, 1979</td>
</tr>
<tr>
<td>in DGM-11</td>
<td>19.0</td>
<td>2.1</td>
<td>b; North et al, 1986</td>
</tr>
<tr>
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<td>4.8</td>
<td>a;</td>
</tr>
<tr>
<td>Trychomonas vaginalis</td>
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<td>9.6</td>
<td>b;</td>
</tr>
<tr>
<td>batrachorum</td>
<td>3.3</td>
<td></td>
<td>b;</td>
</tr>
<tr>
<td>Giardia lamblia</td>
<td>27.8</td>
<td></td>
<td>c; Gillin et al, 1984</td>
</tr>
<tr>
<td>Entamoeba histolytica</td>
<td>16.7</td>
<td></td>
<td>c;</td>
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only in the cytosol and was totally absent from the nuclear fraction. Since the activity of soluble OrnDC is much higher in tissues containing rapidly dividing cells than in organs in the resting state, the cytosolic enzyme may be more important for the biological function of polyamines. In Neurospora, OrnDC is a cytosolic enzyme (Weiss and Davis, 1973) and some of the cytosolic ornithine is used for polyamine biosynthesis even though most of the ornithine is synthesized in the mitochondria and is stored in vacuoles. The cytosolic presence of OrnDC in Acanthamoeba is not surprising, then, but it would be very interesting to discover whether any is localized in the nucleus, mitochondria, or vacuoles.

OrnDC requires pyridoxal phosphate for its activity in both prokaryotic and eukaryotic cells (Heby, 1981; Tabor and Tabor, 1985) and is stabilized by thiol compounds in yeast (Tyagi et al., 1981, 1983), Euglena (Aleksijievic et al., 1979), rat liver (Hayashi and Kameji, 1983), and barley seeds (Kyriakidis et al., 1983). Both pyridoxal phosphate and dithiothreitol were required for the activity in Acanthamoeba (Table 4). Thus, the enzyme is "normal" with respect to these requirements. The decrease of total OrnDC activity in dialyzed supernatants might result from loss of cofactors other than pyridoxal phosphate and thiol. The ionic requirements for OrnDC activity have not been studied.
4.3. Effects of ornithine and polyamine antagonists on multiplication and differentiation in A. castellanii. The levels of polyamines tend to be inversely related to multiplication. It is important to determine whether differences in levels between multiplying and nonmultiplying cells are critical, but that is difficult to prove. It would be especially interesting to determine whether the levels of one particular polyamine might be more important for regulation of multiplication and differentiation. In the absence of mutations, inhibitors of polyamine synthesis have been used to explore this question. To begin with, spermine probably is not essential for growth and/or differentiation in Acanthamoeba because it is not present in DGM-11 grown cultures. It is possible, however, that one of the other polyamines might be essential. The level of spermidine seemed to have special significance for cell multiplication in murine leukemia cell lines (Casero et al., 1984). Recovery from DFMO, which lowered both putrescine and spermidine levels and induced cytostasis, occurred without recovery of the depleted putrescine pool, but only after intracellular spermidine levels regained 30% of the untreated control levels. Thus, although spermidine levels could be important, 70% of the normal pool seemed to be in excess.
These results also indicated that the putrescine level was not critical for cell proliferation. The importance of spermidine was suggested by use of cyclohexylamine (CHA), another inhibitor of spermidine synthase. CHA (DCHA) caused depletion of spermidine and accumulation of putrescine (Davis et al., 1985; Feuerstein et al., 1985; Mitchell et al., 1985) and inhibited cell multiplication at as low as 1 mM concentration. In this case putrescine depletion was ruled out as a signal. CHA (DCHA) at up to 5 mM concentrations slowed down acanthameba multiplication without inducing differentiation in the present study, but the levels of polyamines were not measured. This should be examined.

The possible significance of OrnDC for cell multiplication was studied by using its irreversible inhibitors: DFMO, Δ-MFMOme, and R,R-MAP (Fig. 16, 21). All three inhibited cell multiplication and none of them induced encystment. Δ-MFMOme was the most effective and R,R-MAP the least effective in both OGM and DGM-11 cultures. However, when the in vitro inhibitory effect on OrnDC was measured at the concentrations giving the same range of growth inhibition, R,R-MAP was the most potent enzyme inhibitor (Table 7). In Trypanosoma brucei brucei; R,R-MAP was the most effective against OrnDC both in vitro and in vivo (Bitonti et al., 1985). The differences in growth inhibition that I observed among various inhibitors
might result from uptake differences. Putrescine, spermidine, spermine, diaminopropane, and cadaverine all reversed the growth inhibition in *Acanthamoeba* caused by the OrnDC inhibitors. Similar results were found in murine leukemia cell lines where all three OrnDC inhibitors blocked cell multiplication and the inhibition could be by-passed by addition of putrescine (Pera et al., 1986). These OrnDC inhibitors reduced intracellular levels of putrescine and spermidine but not spermine. In the presence of DFMO plus spermidine analogues such as homospermidine, N^4^-methylspermidine, or N^4^-ethylspermidine, growth was sustained at more than 80% of control rates and the spermidine analogues were not converted to spermine analogues. This finding suggests that spermidine, but not spermine, was necessary to sustain the growth, and that spermidine analogues could substitute for the spermidine.

It is not clear why cadaverine, which was not found in *Acanthamoeba*, could reverse the inhibitory effect of OrnDC inhibitors. In *Neurospora*, the arginase deficient *aga* mutant which could not make ornithine from arginine, grew normally in minimal medium with normal levels of polyamines (Paulus et al., 1982). Cadaverine was not produced. The addition of arginine to the medium blocked ornithine production by feedback inhibition of acetylglutamate kinase. Putrescine and spermidine also were depleted.
However, the OrnDC activity of the mutant was highly derepressed, having increased 75-fold. The addition of arginine to the aga mutant also led to an increase of lysine decarboxylase activity and the production of cadaverine from lysine. The mold now grew indefinitely. In addition, externally added cadaverine stimulated growth of the mutant. Thus, cadaverine can substitute for the missing polyamines. The finding that cadaverine only appeared in cells having high OrnDC activity and lacking ornithine, suggested that OrnDC was responsible for the lysine decarboxylation (Paulus et al., 1982). It seems likely that cadaverine also could serve as a substitute for normal polyamines in the growth of Acanthamoeba in the presence of OrnDC inhibitors.

OrnDC activity was reduced 50% in vivo by 1 mM DFMO with minimal effects on growth (Table 8) and was reduced 63% at 5 mM without totally blocking multiplication. I was not able to measure putrescine, but DFMO caused spermidine levels to increase and diaminopropane levels to decrease. In T. vaginalis, 4 mM DFMO blocked OrnDC activity over 99% with little effect on parasite growth (North et al., 1986). The level of putrescine was reduced but spermidine and spermine levels were not altered. These findings and my results suggest that measured OrnDC activities can greatly exceed that necessary for maintenance of growth. The results also are consistent with other observations.
discussed above that cell multiplication is correlated with normal or elevated levels of spermidine (and/or spermine in *T. vaginalis*).

The possibility that polyamine depletion was important for encystment was suggested by the drastic decreases in OrnDC (Table 6), spermidine and diaminopropane (Table 5) during encystment in NEM. Thus, it was possible that inhibition of polyamine synthesis could induce encystment. High concentrations of DFMO that completely blocked multiplication did not induce encystment in *Acanthamoeba* unless supplemented with divalent cations in OGM cultures (Fig. 24). It is possible that the increased ionic concentration worked synergistically with DFMO to induce differentiation. The observation that high levels of DFMO caused cell lysis suggests another possibility—that the inorganic cations permitted encystment because they stabilized the plasma membrane. Another possibility is suggested by the observation that the membrane of *Acanthamoeba* contains a large number of Ca++-rich structures of unknown function (Sobota and Przelecka, 1981; Sobota et al., 1977). It has been suggested that they might play a role in transmembrane or membrane-cytosol communication and this might be important for encystment. Calcium, at least, is an important second messenger in other cells (Cox, 1984; Kruskal et al., 1984). In another laboratory (Chagla and Griffiths, 1978), MgCl₂ induced
encystment in *Acanthamoeba*, by itself, but I have never been able to repeat that result. Divalent cations also affect certain enzymes of polyamine metabolism (Boonekamp, 1985) and this could be another mechanism that would promote encystment. I did not determine whether the divalent cations would promote encystment with \(\text{DFMO} \) or \(\text{R,R-MAP}\) because these inhibitors were in such short supply. It would be interesting to know this, especially if lower concentrations of cations were effective. An important question is whether DFMO, \(\text{\Delta-MFMO}\) and \(\text{R,R-MAP}\) fail to induce encystment by themselves because they fail to completely inhibit polyamine synthesis, or because polyamine synthesis is not important for differentiation.

Differentiation was achieved by treatment of cultures with several "structural analogues" of spermidine. All spermidine analogues tested inhibited cell multiplication. Amebas were 100 to 10,000-fold more sensitive to spermidine analogues than to inhibitors of OrnDC (Fig. 30). MGBG was the least effective inhibitor and the inhibition was fully (in OGM) or partially (in DGM-11) reversed by addition of polyamines. In contrast, inhibition of multiplication by Berenil, hydroxystilbamidine, pentamidine, amicarbalide, Antrycide, or ethidium bromide couldn't be by-passed by the addition of polyamines. The difference between MGBG and the rest might be related to differentiation inducing activity. Amebas treated with all drugs except MGBG
committed to differentiate.

MGBG is a reversible inhibitor of S-adenosylmethionine decarboxylase. It also has several other effects on cells, including severe mitochondrial damage, inhibition of diamine oxidase, and interference with polyamine transport (Pegg, 1986). Berenil and pentamidine are irreversible inhibitors of SAMDC in other organisms (Bitonti et al., 1986; Karvonen et al., 1985). Inhibition of acanthameba SAMDC by Berenil could explain the decreases in levels of spermidine and diaminopropane which were observed during encystment induced by the drug in DGM-11 cultures (Table 10) and OGM cultures (Fig. 33). In both media, the changes in spermidine levels were more abrupt than those of diaminopropane. It is not known whether hydroxystilbamidine, amicarbalide, ethidium bromide, or Antrycide are inhibitors of SAMDC, but their structural similarities to Berenil and pentamidine (Fig. 25) suggest that these "spermidine analogues" also could be irreversible inhibitors. If this is the case, then differences in reversibility of the inhibition of SAMDC could be an explanation for why polyamines only restore multiplication in the MGBG treated cultures. It must be remembered, however, that all of the "spermidine analogues" affect other processes, such as inhibiting nucleic acid and protein synthesis, aggregating isolated ribosomes, releasing polyamines and cations from isolated ribosomes,
and interacting with other enzymes (Bacchi et al., 1981). This is another possible explanation for why some of their inhibition of multiplication is irreversible.

The most interesting aspect of the "spermidine analogues" is their ability to induce differentiation and the question of whether this is related to polyamine metabolism. The inhibition of diamidine-induced encystment by exogenous polyamines (Fig. 32) is consistent with the proposal that the diamidines depress polyamine pools, that this condition triggers differentiation, but that exogenous polyamines refill pools and block completion of encystment. I have shown that Berenil does block the doubling of the spermidine pool that occurs during culture aging (Table 10), but it has little effect on diaminopropane levels. This, of course, would support the proposal that the spermidine pool has special relevance to regulation of differentiation as suggested for mammalian cells (Casero et al., 1984; Oka et al., 1981). One observation seemingly inconsistent with a role of polyamine pool size in encystment is the fact that polyamines did not block differentiation during starvation even though spermidine and other polyamine pools were depressed (Fig. 14). Unfortunately, no data are available on the effect of exogenous polyamines on endogenous pool sizes. Possibly, however, polyamine depletion is only one form of starvation that can trigger differentiation. Total starvation may
continue to promote encystment even when polyamine pools
have been refilled.

Another polyamine-related explanation for the
induction of encystment by diamidines, ethidium bromide and
Antryclide is related to changes of S-adenosylmethionine and
decarboxylated S-adenosylmethionine pools. Inhibitors of
SAMDC increase the intracellular concentration of SAM,
which is known for its role as a donor in transmethylation
reactions, and depress dcSAM concentrations.
Decarboxylated SAM is the source of adenine and
subsequently de novo synthesis of nucleotides (Porter and
Sufrin, 1986). An imbalance between SAM and dcSAM might
stop cell multiplication and eventually lead to
differentiation. Heby and his colleagues who worked with
mammalian embryonal carcinoma cells (Holm et al., 1985;
Oredsson et al., 1986) found that DFMO treatment caused a
1,500-fold increase in dcSAM partly due to the absence of
the aminopropyl acceptors putrescine and spermidine, and
partly due to the activation of SAMDC. The excess
accumulation of dcSAM caused growth inhibition, probably
blocking the recycling of adenine, because it caused an
increase in the intracellular levels of ATP, ADP, and some
other nucleotides (Oredsson et al., 1986). These authors
suggested that DFMO might induce differentiation because
depletion of the aminopropyl acceptor pools led to this
SAM/dcSAM imbalance. If it is true that the imbalance
induces differentiation, it is interesting that an imbalance in the opposite direction might correlate with differentiation in *Acanthamoeba*. This is suggested because the diamidines, which induce encystment probably block SAMDC and cause accumulation of SAM rather than dcSAM. In contrast, inhibitors of OrnDC, which should cause dcSAM to accumulate, do not induce encystment. Clearly, measurements of SAMDC, SAM and dcSAM are needed to answer this question.

The possibility that diamidines, ethidium bromide and Antrycide affect growth and differentiation at a different intracellular location from the site where OrnDC inhibitors are effective must be considered seriously. Most of the OrnDC probably is in the cytosol, but a role for mitochondria in the diamidine effect is suggested by previous studies. Treatment of amebas growing in OGM with Berenil, ethidium bromide, erythromycin, or chloramphenicol, all known inhibitors of mitochondrial macromolecular synthesis, resulted in the appearance in the medium of an encystment enhancing activity (EEA) (Akins and Byers, 1980; Akins et al., 1985). EEA did not inhibit cell multiplication nor induce encystment by itself, but was essential for maximal encystment. Isolated *Acanthamoeba* mitochondria released EEA in response to Berenil treatment (Akins, 1981; Akins and Byers, 1980). EEA also was important for encystment caused by glucose starvation.
(Akins et al., 1985). These findings suggest the involvement of the mitochondria in differentiation in Acanthamoeba. It is unknown whether the other inhibitors used in the present study affect EEA synthesis. MGBG and pentamidine cause damage to mitochondrial structure and function (Byczkowski et al., 1981; Croft and Brazil, 1982; Diala et al., 1980; Porter et al., 1979). Pentamidine may bind preferentially to mitochondrial DNA (Sands et al., 1985). It is not known whether the diamidines or the distantly related amicarbalide and Antrycide influence mitochondria. Antrycide and pentamidine both release polyamines or polycations from ribosomes (Newton, 1966; Wallis, 1966) and this might occur either in the mitochondria or in the cytoplasm. In Neurospora crassa, most available polyamines are sequestered in vacuoles, and are not freely mixed with cytoplasmic polyamines. The localization of polyamines in Acanthamoeba has not been studied. If there is sequestration in mitochondria, the encystment-inducing activity of the antagonists could be associated with a functional modification of mitochondrial ribosomes and, possibly, production of EEA.
5. SUMMARY OF RESULTS

1. *Acanthamoeba castellanii* grown in either OGM or DGM-11 had putrescine, diaminopropane, spermidine, and norspermidine. Spermine was found only in OGM cultures. Among those polyamines, diaminopropane was the most abundant one in both OGM and DGM-11.

2. Arginase, urease, citrulline hydrolase (or citrullinase), and ornithine decarboxylase were found in the 38,000 g homogenate supernatants of *A. castellanii* in vitro. Arginine deiminase or diaminopropane synthetase activities were not found in vitro but the latter was detected in vivo. The presence of a deiminase was not ruled out. Arginine decarboxylase could not be detected either in vivo or in vitro.

3. The levels of polyamine decreased when cell numbers stopped increasing at the stationary phase. During starvation-induced encystment in OGM cultures, the levels of putrescine, spermidine, and diaminopropane dropped to 50% of the uninduced control within one day. The levels of
spermine and norspermidine slowly decreased. In DGM-11 cultures, levels of polyamines decreased some during starvation-induced encystment but Ornithine decarboxylase activity dropped 91% within two days.

4. Ornithine decarboxylase activity was inhibited by its irreversible inhibitors: DFMO, Δ-MFM0me, and R,R-MAP. R,R-MAP was the most effective in vitro, and Δ-MFM0me was the most potent in vivo. The growth inhibition induced by OrnDC inhibitors could be reversed by addition of polyamine but not by inorganic cations.

5. MGBG, a reversible inhibitor of S-adenosylmethionine decarboxylase in other organisms, blocked cell multiplication. Diamidines (Berenil, hydroxystilbamidine, pentamidine, and amicarbalide), Antrycide, and ethidium bromide also inhibited cell growth in both OGM and DGM-11 cultures. These spermidine "structural analogues" were 100 to 10,000 times more effective than OrnDC inhibitors in the inhibition of multiplication. The growth inhibition could not be by-passed with putrescine or spermidine except for MGBG where spermidine could reverse the inhibitory effect. It is not known whether the diamidines inhibit SAMDC in Acanthamoeba, but the levels of spermidine and diaminopropane decreased during the Berenil-induced encystment in both OGM and DGM-11 cultures. OrnDC activity
dropped to 30% of the untreated control.

6. None of the three OrnDC inhibitors induced encystment, but DFMO could induce differentiation in the presence of inorganic ions. All of the spermidine "structural analogues" except MGBG induced encystment in both OGM and DGM-11 cultures. The differentiation-inducing effects of the "structural analogues" could be blocked by the addition of polyamines without restoring multiplication; spermidine was more effective than putrescine.
Table 13. Components of culture medium

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>OGM</th>
<th>DGM-11</th>
</tr>
</thead>
<tbody>
<tr>
<td>(in g/liter, except as indicated)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteose peptone</td>
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<tr>
<td>Yeast extract</td>
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<td>Glucose</td>
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<td>Vitamins (mg/liter)</td>
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